

Norbert Sewald and Hans-Dieter Jakubke
Peptides: Chemistry and Biology

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 **WILEY-VCH**

Prof. Dr. Norbert Sewald

Universität Bielefeld
Fakultät für Chemie
Organ. und Bioorgan. Chemie
PO Box 100131
33501 Bielefeld, Germany

Prof. em. Dr. Hans-Dieter Jakubke

Universität Leipzig
Institut für Biochemie
Private address:
Albert-Richter-Straße 12
01465 Dresden-Langebrück, Germany

Cover

The cover picture shows the TPR1 domain of Hop in complex with -Gly-Pro-Thr-Ile-Glu-Glu-Val-Asp-OH (GPTIEEVD). TPR domains participate in the ordered assembly of Hsp70-Hsp90 multichaperone complexes.

The TPR1 domain of the adaptor protein Hop specifically recognizes the C-terminal heptapeptide -Pro-Thr-Ile-Glu-Glu-Val-Asp-OH (PTIEEVD) of the chaperone Hsp70 while the TPR2A domain of Hop binds the C-terminal pentapeptide -Met-Glu-Glu-Val-Asp-OH (MEEVD) of the chaperone Hsp90. The EEVD motif is conserved in all soluble forms of eukaryotic Hsp70 and Hsp90 proteins.

Peptide binding is mediated with the EEVD motif. Both carboxy groups of the C-terminal aspartate anchor the peptide by electrostatic interactions. The hydrophobic residues located N-terminally within the peptide are critical for specificity.

[C. Scheufler, A. Brinker, G. Bourenkov, S. Pegoraro, L. Moroder, H. Bartunik, F. U. Hartl, I. Moarefi, Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine, *Cell* 2000, 101, 199; PDB entry 1ELW (<http://www.rcsb.org/pdb/>)]

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Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library.

Die Deutsche Bibliothek – CIP-Cataloguing-in-Publication Data

A catalogue record for this publication is available from Die Deutsche Bibliothek.

© WILEY-VCH Verlag GmbH
D-69469 Weinheim, 2002

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Printed in the Federal Republic of Germany
Printed on acid-free paper

Typesetting K+V Fotosatz GmbH, Beerfelden

Printing betz-druck gmbH, Darmstadt

Bookbinding J. Schäffer GmbH & Co.KG, Grünstadt

ISBN 3-527-30405-3

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Preface

The past decades have witnessed an enormous development in peptide chemistry with regard not only to the isolation, synthesis, structure identification, and elucidation of the mode of action of peptides, but also to their application as tools within the life sciences. Peptides have proved to be of interest not only in biochemistry, but also in chemistry, biology, pharmacology, medicinal chemistry, biotechnology, and gene technology.

These important natural products span a broad range with respect to their complexity. As the different amino acids are connected via peptide bonds to produce a peptide or a protein, then many different sequences are possible – depending on the number of different building blocks and on the length of the peptide. As all peptides display a high degree of conformational diversity, it follows that many diverse and highly specific structures can be observed.

Whilst many previously published monographs have dealt exclusively with the synthetic aspects of peptide chemistry, this new book also covers its biological aspects, as well as related areas of peptidomimetics and combinatorial chemistry. The book is based on a monograph which was produced in the German language by Hans-Dieter Jakubke: *Peptide, Chemie und Biologie* (Spektrum Akademischer Verlag, Heidelberg, Berlin, Oxford), and first published in 1996. In this new publication, much of the material has been completely reorganized and many very recently investigated aspects and topics have been added. We have made every effort to produce a practically new book, in a modern format, in order to provide the reader with profound and detailed knowledge of this field of research. The glossary, which takes the form of a concise encyclopedia, contains data on more than 500 physiologically active peptides and proteins, and comprises about 20% of the book's content.

Our book covers many different issues of peptide chemistry and biology, and is devoted to those students and scientists from many different disciplines who might seek quick reference to an essential point. In this way it provides the reader with concise, up-to-date information, as well as including many new references for those who wish to obtain a deeper insight into any particular issue. In this book, the “virtual barrier” between peptides and proteins has been eliminated because, from the viewpoint of the synthesis or biological function of these compounds, such a barrier does not exist.

This monograph represents a personal view of the authors on peptide chemistry and biology. We are aware however that, despite all our efforts, it is impossible to include all aspects of peptide research in one volume. We are not under the illusion that the text, although carefully prepared, is completely free of errors. Indeed, some colleagues and readers might feel that the choice of priorities, the treatment of different aspects of peptide research, or the depth of presentation may not always be as expected. In any case, comments, criticisms and suggestions are appreciated and highly welcome for further editions.

Several people have contributed considerably to the manuscript. All the graphical material was prepared by Dr. Katherina Stembera, who also typed large sections of the manuscript, provided valuable comments, and carried out all the formatting. We appreciate the kindness of Professor Robert Bruce Merrifield, Dr. Bernhard Streb and Dr. Rainer Obermeier for providing photographic material for our book. Margot Müller and Helga Niermann typed parts of the text. Dr. Frank Schumann and Dr. Jörg Schröder contributed Figures 2.19 and 2.25, respectively. We also thank Dirk Bächle, Kai Jenssen, Micha Jost, Dr. Jörg Schröder and Ulf Strijowski for comments and proofreading parts of the manuscript.

Dr. Gudrun Walter, Maike Petersen, Dr. Bill Down, and Hans-Jörg Maier took care that the manuscript was converted into this book in a rather short period of time, without complications.

Bielefeld
and
Dresden-Langebrück
April 2002

Norbert Sewald
Hans-Dieter Jakubke

Abbreviations

aa	amino acid
AA	antamanide (anti-amanita peptide)
Aad	2-aminoadipic acid
β Aad	3-aminoadipic acid
AAP	antimicrobial animal peptides
aatRS	amino-acyl tRNA synthetase
Ab	antibody
Abu	aminobutyric acid
A ₂ bu	2,4-diaminobutyric acid
Abz	aminobenzoic acid
Ac	acetyl
ACE	angiotensin-converting enzyme
AChR	acetylcholine receptor
Acm	acetamidomethyl
ACP	acyl carrier protein
ACTH	corticotropin
AD	Alzheimer's disease
Ada	adamantyl
Adoc	adamantylloxycarbonyl
ADP	adenosine diphosphate
Aet	aminoethyl
Ag	antigen
AGaloc	tetra-O-acetyl- β -D-galactopyranosyloxycarbonyl
AGE	advanced glycation end products
AGloc	tetra-O-acetyl-D-glucopyranosyloxycarbonyl
AGRP	agouti-related protein
Ahx	2-aminohexanoic acid (norleucine)
ϵ Ahx	6-aminohexanoic acid
AHZ	alanyl-histidinato zinc (β)
Aib	α -aminoisobutyric acid
AIDS	acquired immune deficiency syndrome
Alle (alle)	alloisoleucine
AKH	adipokinetic hormone

Al	allyl (used only in 3-letter code names)
Ala	alanine
βAla	β-alanine
Aloc	allyloxycarbonyl
Alom	allyloxymethyl
AMP	adenosine monophosphate
AM-PS	aminomethyl polystyrene
ANF	atrial natriuretic factor
ANP	atrial natriuretic peptide
Ans	anthracene-9-sulfonyl
Aoc	1-azabicyclo[3.3.0]octane-2-carboxylic acid
AOE	(S)-2-amino-8-oxo-(S)-9,10-epoxidecanoic acid
AOP	7-azabenzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate ¹⁾
Apa	6-aminopenicillanic acid
APM	aspartame
Apm	2-aminopimelic acid
AQP	aquaporin
Ar	aryl
Arg	arginine
Asn	asparagine
Asp	aspartic acid
Aβ	amyloid-β
Asu	aminosuberic acid
At	azabenzotriazolyl
AT	angiotensin
ATP	adenosine triphosphate
AVP	8-arginine-vasopressin
Bac5	bactenecin
BAL	backbone amide linker
BBB	blood brain barrier
Bet	α-betainyl
BGloc	tetrabenzylglucosyloxycarbonyl
BGP	bone Gla protein
BHA	benzhydramine
Bip	biphenyl-4-sulfonyl
BK	bradykinin
BLP	bombinin-like peptide
BMP	brain morphogenetic protein
BNP	brain natriuretic peptide
Boc	<i>tert</i> -butoxycarbonyl
BOI	2-[(1 <i>H</i> -benzotriazol-1-yl)oxy]-1,3-dimethylimidazolidinium hexafluorophosphate
Bom	benzyloxymethyl

BOP	benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
Bpoc	2-(biphenyl-4-yl)prop-2-yloxy carbonyl
BPTI	basic pancreatic trypsin inhibitor
BroP	bromotris(dimethylamino)phosphonium hexafluorophosphate
BSA	bovine serum albumin
Bsmoc	1,1-dioxobenzo[b]thiophen-2-ylmethoxycarbonyl
Bspoc	2-(<i>tert</i> -butylsulfonyl)-2-propenyloxy carbonyl
Bt	(benzylsulfanylmethyl)
Btb	1- <i>tert</i> -butoxycarbonyl-2,3,4,5-tetrachlorobenzoyl
Btm	benzylsulfanylmethyl (benzylthiomethyl)
Bu	butyl
tBu	<i>tert</i> -butyl
Bum	<i>tert</i> -butoxymethyl
Bz	benzoyl
Bzl	benzyl (Bn in contemporary organic synthesis)
Bzl(4-Me)	4-methylbenzyl
CADD	computer-aided drug design
CaM	calmodulin
Cam	carboxamidomethyl (carbamoylmethyl, aminocarbonylmethyl)
CAMD	computer-aided molecular design
CAMM	computer-assisted molecular modeling
cAMP	cyclic AMP
CBD	chitin binding domain
CCAP	crustacean cardioactive peptide
CCK	cholecystokinin
CD	circular dichroism
CD4	cell surface glycoprotein 4
CD8	cell surface glycoprotein 8
cDNA	complementary DNA
CDR	complementary determining region
CE	capillary electrophoresis
CF ₃ -BOP	6-(trifluoromethyl)benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate
CF ₃ -HBTU	2-[6-(trifluoromethyl)benzotriazol-1-yl]-1,1,3,3-tetramethyluronium hexafluorophosphate ²⁾
CF ₃ -PyBOP	6-(trifluoromethyl)benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
CG	chorionic gonadotropin
Cg	chromogranin
cGMP	cyclic guanosine monophosphate
CGRP	calcitonin gene related peptide
Cha	cyclohexylalanine
Cho	choline

cHp	cycloheptyl
CID	chemically ionized desorption
CIEF	capillary isoelectric focusing
Cit	citrulline
CJD	Creutzfeldt Jakob disease
2,6-Cl ₂ Bzl	2,6-dichlorobenzyl
CLIP	corticotropin-like intermediate lobe peptide
Clt-Cl	2-chlorotritylchloride
CLTR	2-chlorotrityl resin
Cm	carboxymethyl
CM	casomorphin or chorionic mammotropin
CN	calcineurin
CNBr	cyanogen bromide
Cne	2-cyanoethyl
CNP	C-type natriuretic peptide
CNS	central nervous system
cOc	cyclooctyl
COSY	correlated NMR spectroscopy
Cpa	4-chlorophenylalanine
CP	carboxypeptidase
cPe	cyclopentyl
CPP	cell-penetrating peptide
CPS	convergent peptide synthesis
CRF	corticotropin releasing factor
CRIF	corticotropin release-inhibiting factor
CRL	cerulein
CsA	cyclosporin A
CSF	colony stimulating factor
CSPPS	convergent solid-phase peptide synthesis
CST	cortistatin
CT	calcitonin or charge-transfer
Cy	cyclohexyl
Cya	cysteic acid
Cyp	cyclophilin
Cys	cysteine
(Cys) ₂	cystine
CZE	capillary zone electrophoresis
Dab	α,γ -diaminobutyric acid
DABCO	1,4-diazabicyclo[2.2.2]octane
DABSCL	4-(dimethylamino)azobenzene-4'-sulfonyl chloride
DABITC	4-(dimethylamino)azobenzene-4'-isothiocyanate
DAG	diacylglycerol
DAST	N,N-diethylaminosulfur trifluoride
DBF	dibenzofulvene

DBIP	diazepam-binding inhibitor peptide
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
Dcb	dichlorobenzyl
DCC	N,N'-dicyclohexylcarbodiimide
DCHA	dicyclohexylamine
DCM	dichloromethane
DCME	dichloromethyl methyl ether
Dcp	α,α -dicyclopropylglycine
DCU	N,N'-dicyclohexylurea
DDAVP	[1-deamino,D-Arg ⁸]vasopressin
Dde	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl
Ddz	α,α -dimethyl-3,5-dimethoxybenzyloxycarbonyl
DEA	diethylamine
DEAE	diethylaminoethanol
Deg	C ^{α,α} -diethylglycine
DEPBT	3-(diethoxyphosphoryloxy)-1,2,3-benzotriazine-4(3 <i>H</i>)-one
DEPC	diethyl pyrocarbonate
DFIH	2-fluoro-4,5-dihydro-1,3-dimethyl-1 <i>H</i> -imidazolium hexafluoro-phosphate
Dha	α,β -didehydroalanine (more commonly, α,β -dehydroalanine)
Dhbt	3,4-dihydro-4-oxobenzotriazin-3-yl
DIC	N,N'-diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DKP	diketopiperazine
DMA	dimethylacetamide
Dmab	4-[[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino]benzyl
DMAE	2-(dimethylamino)ethanol
DMAP	4-(dimethylamino)pyridine
2,4-Dmb	2,4-dimethoxybenzyl
3,4-Dmb	3,4-dimethoxybenzyl
DMBHA	dimethoxybenzhydramine
DMF	N,N-dimethylformamide
Dmh	2,6-dimethylhept-4-yl
Dmp	2,4-dimethoxyphenyl
DMS	dimethyl sulfide
DMSO	dimethyl sulfoxide
Dmt	2',6'-dimethyltyrosine
DNA	deoxyribonucleic acid
Dnp	dinitrophenyl
Dns	5-(dimethylamino)naphthalene-1-sulfonyl (dansyl)
Doc	2,4-dimethylpent-3-yloxycarbonyl
DOPA	3,4-dihydroxyphenylalanine
Dpa	3,3-diphenylalanine
Dpg	C ^{α,α} -diphenylglycine

Dpm	diphenylmethyl (benzhydryl)
DPPA	diphenyl phosphorazidate
Dpr	2,3-diaminopropionic acid
DPTU	N,N'-diphenylthiourea
DSC	di(N-succinimidyl)carbonate
DSIP	delta sleep-inducing peptide
DSK	drosulfakinin
Dsu	(2S,7S)-2,7-diaminosuberic acid
Dts	dithiasuccinyl
DTT	dithiothreitol
DVB	divinylbenzene
Dyn	dynorphin
E ⁺	electrophile (or E-X)
<E	pyroglutamic acid
EC	ethylcarbamoyl
ECD	extracellular domain
ECE	endothelin converting enzyme
ECEPP	Empirical Conformational Energy Program for Peptides
ECGF	endothelial cell growth factor
ECM	extracellular matrix
ECP	ecdysteroid carrier protein
ED ₅₀	median effective dosis
EDC	N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDF	epidermal growth factor or erythrocyte differentiation factor
EDFR	epidermal growth factor receptor
EDT	ethanedithiol
EDTA	ethylenediaminetetraacetic acid
EEDQ	ethyl 2-ethoxy-1,2-dihydroquinoline-1-carboxylate
EF	elongation factor
EGF	epidermal growth factor
EH	eclosion hormone
ELAM	endothelial leukocyte adhesion molecule
ELH	egg laying hormone
ELISA	enzyme linked immunosorbent assay
EM	electron microscopy
EMIT	enzyme multiplied immunoassay technology
EMSA	electrophoretic mobility shift assay
ENK	enkephalin
EPL	expressed protein ligation
EPO	erythropoietin
EPR	electron paramagnetic resonance
ER	endoplasmatic reticulum
ES	electrospray
ESI-MS	electrospray ionisation mass spectrometry

ES-MS	electrospray mass spectrometry
ESR	electron spin resonance
ET	endothelin
Et	ethyl
ETH	ecdysis-triggering hormone
Etm	ethoxymethyl
Fa	3-(2-furyl)acryloyl
Fab	antigen binding Ig fragment
FAB-MS	fast atom bombardment mass spectrometry
FACS	fluorescence-activated cell sorter
FAD	flavin adenine dinucleotide, oxidized form
FADH ₂	flavin adenine dinucleotide, reduced form
Farn	farnesyl
FaRP	FMRFamide-related peptide
Fbg	fibrinogen
Fc	ferrocenyl
Fd	ferredoxin
FGF	fibroblast growth factor
FITC	fluoresceinyl isothiocyanate
FKBP	FK506 binding protein
Fm	9-fluorenylmethyl
FMDV	foot-and-mouth disease virus
Fmoc	9-fluorenylmethoxycarbonyl
FN	fibronectin
For	formyl
FP	fluorescence polarisation
Fpa	4-fluorophenylalanine
FPLC	fast protein liquid chromatography
FPP	farnesyl pyrophosphate
FRET	fluorescence resonance energy transfer
FRL	formin-related peptide
FS	follistatin
FSF	fibrin-stabilizing factor
FSH	follicle-stimulating hormone
FTase	farnesyltransferase
FTIR	Fourier-transform infrared
GABA	γ -aminobutyric acid
Gal	galanin, galactose
GalT	galactosyl transferase
GC	gas chromatography
gCSF	granulocyte colony stimulating factor
GFC	gel filtration chromatography
GFP	green fluorescent protein

GGTase	geranylgeranyltransferase
GH	growth hormone
GHRH	growth hormone releasing hormone
GHRP	growth hormone releasing peptide
GHS	growth hormone secretagogue
GHS-R	growth hormone secretagogue receptor
GIP	gastric inhibitory polypeptide
Gla	4-carboxyglutamic acid
GLC	gas liquid chromatography
Glc	glucosyl, glucose
GlcNAc	N-acetyl-D-glucosamine
Gln	glutamine
GLP	glucagon-like peptide
Glu	glutamic acid
Gly	glycine
GMP	guanosine monophosphate
GnRH	gonadotropin releasing hormone
GPC	gel permeation chromatography
GPCR	G-protein coupled receptor
GPI	glycosylphosphatidylinositol or guinea pig ileum
GRF	growth hormone releasing factor
GRP	gastrin-releasing peptide
GRPP	glicentin-related pancreatic peptide
GSF	glutathion-S-transferase
GSH	glutathione reduced
GSSG	glutathione oxidized
GT	gastrin
GTP	guanosine triphosphate
Gva	δ -guanidinovaleric acid
hXaa	homoamino acid
h	human
HA	head activator
HAL	5-(4-hydroxymethyl-3,5-dimethoxy)-valeric acid (derived hypersensitive acid-labile linker)
HAMDU	O-(7-azabenzotriazol-1-yl)-1,3-dimethylimidazolidinium hexa- fluorophosphate ¹⁾
HAMTU	O-(7-azabenzotriazol-1-yl)-1,3-dimethyl-1,3-trimethylenonium hexafluorophosphate ^{1,2)}
HAPipU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate ^{1,2)}
HAPyTU	S-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)thiuronium hexafluorophosphate ^{1,2)}
HAPyU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate ^{1,2)}

hArg	homoarginine
HATTU	S-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethylthiuronium hexafluorophosphate
HATU	O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; correct IUPAC name: 1-[bis-(dimethylamino)methylumyl]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridin-3-oxide hexafluorophosphate or 1-[(dimethylamino)-(dimethyliminium)methyl]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridin-3-oxide hexafluorophosphate
HAV	hepatitis A virus
Hb	hemoglobin
HBMDU	O-(benzotriazol-1-yl)-1,3-dimethyl-1,3-dimethyleuronium hexafluorophosphate ²⁾
HBP <u>Y</u>	O-(benzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate ²⁾
HBsAg	hepatitis B virus surface antigen
HBTU	2-(1 <i>H</i> -benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate ²⁾ ; correct IUPAC name: 3-[Bis(dimethylamino)methyliumyl]-3 <i>H</i> -benzotriazol-1-oxide hexafluorophosphate
HBV	hepatitis B virus
Hbz	2-hydroxybenzyl
Hci	homocitrulline
HCRT	hypocretin
HCV	hepatitis C virus
hCys	homocysteine
HDCOTU	O-(dicyanomethylenamino)-1,1,3,3-tetramethyluronium hexafluorophosphate
HDL	high density lipoprotein
HDTU	O-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hep	heptyl
Hepes	N-(2-hydroxyethyl)piperazine- <i>N'</i> -2-ethanesulfonic acid
HEPP	human IgE pentapeptide
HF	hydrogen fluoride
HFBA	heptafluorobutyric acid
HFIP	hexafluoroisopropanol
HG	human little-gastrin
hGH	human growth hormone
HIC	hydrophobic interaction chromatography
Hip	hippuryl, hippuric acid
His	histidine
HIV	human immunodeficiency virus
HMB	hydroxymethylbenzoic acid
Hmb	2-hydroxy-4-methoxybenzyl

HMFS	N-[9-(hydroxymethyl)-2-fluorenyl]succinamic acid
HMK	high molecular weight kininogen
HMP	4-(hydroxymethyl)phenoxy
HMPA	hexamethylphosphoric triamide
HMPAA	4-(hydroxymethyl)phenoxyacetic acid
HMPB	4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid
HMPPA	3-[4-(hydroxymethyl)phenoxy]propanoic acid
HMPT	hexamethylphosphorous triamide
HMQC	heteronuclear multiple quantum coherence spectroscopy
Hnb	2-hydroxy-6-nitrobenzyl
HOAt	1-hydroxy-7-aza-1 <i>H</i> -benzotriazole
HOBT	1-hydroxy-1 <i>H</i> -benzotriazole
Hoc	cyclohexyloxycarbonyl
HOCT	ethyl 1-hydroxy-1 <i>H</i> -1,2,3-triazole-4-carboxylate
HODhbt	3-hydroxy-1,2,3-benzotriazin-4(3 <i>H</i>)-one
HOPIP	N-hydroxypiperidine
HOPPipU	2-[2-oxo-1(2 <i>H</i>)pyridyl]-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate
HOSu	N-hydroxysuccinimide
HOTU	O-[(ethoxycarbonyl)cyanomethyleneamino]-1,1,3,3-tetramethyluronium hexafluorophosphate
HPCE	high performance capillary electrophoresis
HPLC	high performance liquid chromatography
Hpp	5-hydroxy-1-(4'-nitrophenyl)pyrazole
HppTU	2-[1-(4'-nitrophenyl)-1 <i>H</i> -pyrazol-5-yl]-1,1,3,3-tetramethyluronium tetrafluoroborate
HPSEC	high performance size exclusion chromatography
hPTH	human parathyroid hormone
HpyClU	chloro-1,1,3,3-bis(tetramethylene)-uronium hexafluorophosphate
HRMS	high resolution mass spectrometry
HS	hymenstatin
HSA	human serum albumin
HSAB	hard and soft acids and bases
Hse	homoserine
Hsp	heat shock protein
HSPTS	high speed peptide synthesis
HSV	herpes simplex virus
HTS	high-throughput screening
HVE	high voltage electrophoresis
Hyp	hydroxyproline
Hyv	α -hydroxyisovaleric acid
iBu	isobutyl
IC	inhibitory concentration
iC	isocaproic

ICAM	intracellular adhesion molecule
IEC	ion-exchange chromatography
IF	initiation factor
IFN	interferon
Ig	immunoglobulin
IGF	insulin-like growth factor
IHB	inhibin
IIDQ	1-isobutoxycarbonyl-2-isobutoxy-1,2-dihydroquinoline
IL	interleukin
Ile	isoleucine
im	imidazole
IMAC	immobilized metal ion affinity chromatography
iMds	2-methoxy-4,6-dimethylbenzenesulfonyl
in	indole
iNoc/iNOC	isonicotinyloxycarbonyl (4-pyridylmethoxycarbonyl)
Ioa	isooctanoic acid
IP	inositol phosphate
Ipc	isopinocampheyl
IPL	intein-mediated protein ligation
IPNS	isopenicillin N synthase
iPr	isopropyl
IRaa	internal reference amino acid
IRMA	immunoradiometric assay
IS-MS	ion spray mass spectrometry
IU	international unit
Iva	isovaline
IvDde	1-(4,4-dimethyl-2,6-dioxocyclohexyliden)-3-methylbutyl
kB	kilo base pair
kDa	kilodalton
KGF	keratinocyte growth factor
K _M	Michaelis constant
Lac	lactic acid
Lan	lanthionine
LAP	leucine aminopeptidase
Lau	lauroyl
LD ₅₀	lethal dose 50%
LDL	low density lipoprotein
LD-MS	laser desorption mass spectrometry
LDToF	laser desorption time-of-flight
LEC	ligand-exchange chromatography
Leu	leucine
LFA-1	leukocyte function-associated antigen-1
LH	luteinizing hormone

LHRH	luteinizing hormone releasing hormone
LIF	leukemia inhibitory factor
LPH	lipotropic hormone
LPS	lipopolysaccharide
LSF	lung surfactant factor
LSI-MS	liquid secondary ion mass spectrometry
LVP	8-lysine vasopressin
Lys	lysine
MA	mixed anhydride
MAB	monoclonal antibody
Mal	maleoyl
MALDI-MS	matrix-assisted laser desorption/ionization mass spectrometry
MALDI-ToF	matrix-assisted laser desorption/ionization time-of-flight
MAP	membrane-anchored protein or multiple antigen peptides
MARS	multiple automatic robotic synthesizer
Mba	2-mercaptobenzoic acid
Mbc	4'-methyl-2,2'-bipyridine-4-carboxylic acid
Mbh	dimethoxybenzhydryl
MBHA	methoxybenzhydrylamine
MBHAR	methoxybenzhydrylamine resin
Mbom	4-methoxybenzyloxymethyl
MBP	maltose binding protein or myelin basic protein
Mbs	4-methoxybenzenesulfonyl
Mca	(7-methoxycoumarin-4-yl)acetyl
MCDP	mast cell degranulating peptide
MCH	melanin-concentrating hormone
MCPBA	3-chloroperoxybenzoic acid
MCPS	multiple constrained peptide synthesis
MD	molecular dynamics
Mdc	4-methoxy-2,6-dimethylbenzenesulfonyl
MDP	maduropeptin
Me	methyl
β -ME	β -mercaptoethanol
MeBHA	methylbenzhydrylamine
Mee	methoxyethoxyethyl
Mem	(2-methoxyethoxy)methyl
Men	menthyl
Menpoc	1-(6-nitro-1,3-benzodioxol-5-yl)ethoxycarbonyl
Menvoc	1-(4,5-dimethoxy-2-nitrophenyl)ethoxycarbonyl
MeO	methoxy
MeOSu	methoxysuccinyl
Mes	mesityl
Met	methionine
MGP	matrix Gla protein

MHC	major histocompatibility complex
MIC	minimum inhibitory concentration
MIF	melanotropin release-inhibiting factor
MIH	melanotropin release-inhibiting hormone
MK	midkine
MMA	metamorphisin A
mMIF	macrophage migration inhibitory factor
Mmt	4-methoxytrityl
Mnp	2-methyl-2-(2'-nitrophenoxy)propionyl
Moa	6-methyloctanoic acid
Mob	4-methoxybenzyl
Moc	methoxycarbonyl
MoEt	2-(N-morpholino)ethyl
Mom	methoxymethyl
Mot	motilin
Mp	4-methoxyphenyl
MP	myelopeptides
Mpa	3-sulfanylpropanoyl (3-mercaptopropionyl)
Mpe	3-methylpent-3-yl
Mpg	3-methoxypropylglycine
MPGF	major proglucagon fragment
MPLC	medium pressure liquid chromatography
Mpr	3-mercaptopropionic acid
MPS	multiple peptide synthesis
MPTA	dimethylphosphinothioyl azide
MRF	melanotropin-releasing factor
MRH	melanotropin-releasing hormone
MRIH	melanotropin release-inhibiting hormone
mRNA	messenger ribonucleic acid
MS	mass spectrometry
Ms	mesyl(methanesulfonyl)
Msbs	4-(methanesulfonyl)benzenesulfonyl
Msc	2-(methylsulfonyl)ethoxycarbonyl
MSH	melanocyte stimulating hormone (melanotropin)
MSNT	1-(mesitylenesulfonyl)-3-nitro-1 <i>H</i> -1,2,4-triazole
Msob	4-(methylsulfinyl)benzyl
MsOH	methanesulfonic acid
Mspoc	2-(methylsulfonyl)-3-phenyl-2-propenyloxycarbonyl
Msz	4-(methylsulfinyl)benzyloxycarbonyl
Mtb	2,4,6-trimethoxybenzenesulfonyl
MTBE	methyl <i>tert</i> -butyl ether
Mtbs	3,5-di- <i>tert</i> -butyl-4-methoxybenzenesulfonyl
Mte	4-methoxy-2,3,5,6-tetramethylbenzenesulfonyl
Mthp	4-methoxytetrahydropyran-4-yl-methylthiomethyl
MTLRP	motilin-related peptide

Mtr	4-methoxy-2,3,6-trimethylbenzenesulfonyl
Mts	2,4,6-trimethylbenzenesulfonyl (mesitylsulfonyl)
Mtt	4-methyltrityl
Mtz	4-(methylthio)benzyl
Mur	muramic acid
MVD	mouse vas deferens
Mwt	molecular weight
Mz	4-(methoxyphenylazo)benzyloxycarbonyl
β NA	β -naphthylamide
pNA	4-nitroanilide
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
Nal	2-naphthylalanine
Nbb	nitrobenzamidobenzyl
Nboc	2-nitrobenzyloxycarbonyl
NBS	N-bromosuccinimide
Nbs	nitrobenzenesulfonyl
Nbz	4-nitrobenzyl
2Nbz	2-nitrobenzyl
NC	nociceptin
NCA	α -amino acid N-carboxyanhydride
NCL	native chemical ligation
Nde	1-(4-nitro-1,3-dioxindan-2-ylidene)ethyl
NEM	N-ethylmaleimide
Neu	neuraminic acid
NeuNAc	N-acetylneuraminic acid
¹⁵ N-HSQC	¹⁵ N heteronuclear single quantum correlation
NGF	nerve growth factor
Nic	nicotinoyl
NIDDM	noninsulin-dependent (type II) diabetes mellitus
NK	neurokinin
Nle	norleucine
NM	neuromedin
NMA	N-methylaniline
NMDA	N-methyl-D-aspartate
NMM	N-methylmorpholin
NMP	N-methyl-2-pyrrolidinone
NMR	nuclear magnetic resonance
NN	neuromedin N
Noc	4-nitrocinnamyloxycarbonyl
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser enhanced spectroscopy
NO ₃ ⁻	6-nitrobenzotriazol-1-ylxytris(dimethylamino)phosphonium hexafluorophosphate

Np	4-nitrophenyl
NP	neurophysin
Npa	nitrophenoxyacetyl
Npe	2-(4-nitrophenyl)ethyl
NPF	neuropeptide F
NPFF	neuropeptide FF
NPK	neuropeptide K
Npoc	(6-nitro-1,3-benzodioxol-5-yl)-methoxyxycarbonyl
Nps	2-nitrophenylsulfenyl
NPY	neuropeptide Y
Npys	3-nitro-2-pyridylsulfanyl
NP _γ	neuropeptide γ
Nsc	2-[(4-nitrophenyl)sulfonyl]ethoxyxycarbonyl
NT	neurotensin
3-NT	3-nitrotyrosine
NTA	N-thiocarboxy anhydride or nitrilotriacetic acid
Nu/Nuc ⁻	nucleophile
Nva	norvaline
Nvoc	4,5-dimethoxy-2-nitrobenzyloxycarbonyl
OAI	allyloxy
OBt	1 <i>H</i> -1,2,3-benzotriazol-1-yloxy
OBzl	benzyloxy
OCM	oncostatin M
Oct	octanoyl
OEt	ethoxy
OGP	osteogenic growth peptide
OGp	4-guanidinophenylloxy
OMe	methoxy
ONbz	4-nitrobenzyloxy
O2Nbz	2-nitrobenzyloxy
ONC	onconase
ONdc	5-norbornene-2,3-dicarboximidooxy
ONp	4-nitrophenylloxy
O2Np	2-nitrophenylloxy
ONs	2-nitro-4-sulfophenoxy
OPA	2-phthalaldehyde
OPcp	pentachlorophenylloxy
OPfp	pentafluorophenylloxy
OPip	1-piperidinoxy
O2Py	2-pyridylloxy
Orn	ornithine
OSu	succinimidooxy
OtBu	<i>tert</i> -butoxy
OTcp	2,4,5-trichlorophenylloxy

Ox	1,3-oxazolidine
OXT/OT	oxytocin
Pa	palmitic acid
Pab	4-alkoxybenzyl
Pac	phenacyl
PACAP	pituitary adenylate cyclase activating polypeptide
PAGE	polyacrylamide gel electrophoresis
Pal	3-pyridylalanine
PAL	4-aminomethyl-3,5-(dimethoxy)phenoxy
PAM	4-(hydroxymethyl)phenylacetic acid (resin linker) or peptidylglycine α -amidating monooxygenase
PAOB	4-phenylacetoxybenzyl
PAPS	3'-phosphoadenosine-5'-phosphosulfate
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-yl-sulfonyl
PD-ECGF	platelet-derived endothelial cell growth factor
PDGF	platelet-derived growth factor
PDH	pigment dispersing hormone
PDI	protein disulfide isomerase
PD-MS	plasma desorption mass spectrometry
Pdpm	pyridyldiphenylmethyl
PEG	poly(ethylene glycol)
PEGA	poly(ethylene glycol)-dimethylacrylamide copolymer
Pen	penicillamine (3-mercaptopalvaline)
PEt	polyethylene
Pfp	pentafluorophenyl
PfPyU	O-pentafluorophenyl-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate
PfTU	O-pentafluorophenyl-1,1,3,3-tetramethyluronium hexafluorophosphate
PG	protecting group
PGLa	peptidyl-glycyl-leucine carboxamide
pGlu	pyroglutamic acid
Ph	phenyl
Pha	phenoxyacetyl
Phac	phenylacetyl
Phacm	phenylacetamidomethyl
Phe	phenylalanine
Phen	phenanthrene-3-sulfonyl
PhFl	9-phenylfluoren-9-yl
Phg	phenylglycine
PHI	peptide histidine isoleucine amide
Phlac	phenyllactic acid
Phpa	3-phenylpropanoic acid
Phth	phthaloyl

pI	isoelectric point
PI3K	phosphatidylinositol-3-kinase
Pic	4-picolyl
PicCO	picolinoyl
Picoc	4-picolylloxycarbonyl
PIH	prolactin-release-inhibiting hormone
Pip	pipecolic acid (homoproline)
PITC	phenyl isothiocyanate
Piv	pivaloyl
PKA	proteinkinase A
PKS	phytosulfokinin
PL	placenta lactogen
PLC	phospholipase C
Plm	palmitoyl
PLR	peptide leucine arginine amide
Pmc	2,2,5,7,8-pentamethylchroman-6-yl-sulfonyl
Pme	2,3,4,5,6-pentamethylbenzenesulfonyl
3-Pn	pent-3-yl
pNA	4-nitroaniline
PNA	peptide nucleic acid
Poc	cyclopentylloxycarbonyl
Pom	pivaloyloxymethyl
POMC	proopiomelanocortin
PP	pancreatic polypeptide
PPIase	peptidyl prolyl <i>cis/trans</i> isomerase
PPOA	(4-propionylphenoxy)acetic acid
PPST	tyrosyl protein sulfotransferase
Ppt	diphenylphosphorothionyl
Pr	propyl
Pra	propargylglycine
PRH	prolactin-releasing hormone
PRL	prolactin
Pro	proline
PrRP	prolactin-releasing peptide
PS	polystyrene
PSA	preformed symmetrical anhydride
Psec	2-(phenylsulfonyl)ethoxycarbonyl
PS-POE	polystyrenepolyoxyethylene
PS-SCL	positional scanning synthetic combinatorial libraries
PST	pancreastatin
PTB	phosphotyrosine binding domain
PTC	phenylthiocarbamyl
PTH	phenylthiohydantoin or parathyroid hormone
PTHrP	parathyroid hormone-related hormone
PTK	protein-tyrosine kinase

PTP	protein-tyrosine phosphatase
PVA	polyvinyl alcohol
Py	pyridine
PyAOP	7-azabenzotriazol-1-yloxytripyrrolidinophosphonium hexafluoro-phosphate
PyBOP	benzotriazol-1-yloxytripyrrolidinophosphonium hexafluoro-phosphate
PyBroP	bromotripyrrolidinophosphonium hexafluorophosphate
PyCloP	chlorotripyrrolidinophosphonium hexafluorophosphate
PyFOP	6-fluorobenzotriazol-1-yloxytripyrrolidinophosphonium hexa-fluorophosphate
PyNOP	6-nitrobenzotriazol-1-yloxytripyrrolidinophosphonium hexa-fluorophosphate
Pyoc	pyridylethoxycarbonyl
Pyr	pyrrolidide
PYY	peptide tyrosine tyrosine
Pz	4-(phenyldiazenyl)benzyloxycarbonyl
QCl	5-chloro-8-quinolyl
RAFT	regioselectively addressable functionalized template
RAMP	receptor activity modifying protein or (<i>R</i>)-1-amino-2-(methoxy-methyl)-pyrrolidine
rDNA	recombinant DNA
RER	rough endoplasmatic reticulum
RET	resonance energy transfer
R _f	retention factor (TLC)
RGD	fibrinogen binding sequence (-Arg-Gly-Asp-)
RIA	radioimmunoassay
RLX	relaxin
RNA	ribonucleic acid
RNase	ribonuclease
ROE	rotating frame nuclear Overhauser effect
ROESY	rotating frame nuclear Overhauser enhanced spectroscopy
RP	reversed phase
RPCH	red pigment concentrating hormone
RP-HPLC	reversed phase high performance liquid chromatography
SA	symmetrical anhydride
Saa	sugar amino acid
SABR	Structure Activity Bioavailability Relationships
Sar	sarcosine
SAR	Structure Activity Relationship
SASRIN	super acid sensitive resin
sBu	<i>sec</i> -butyl

SBzl	thiobenzyl
SCAL	safety catch acid-labile linker or safety catch amide linker
Scg-MT	schistomyotropin
SCL	synthetic combinatorial libraries
SDB	styrene divinylbenzene
SDS	sodium dodecylsulfate
Sec	selenocysteine (one-letter symbol U)
SEC	size-exclusion chromatography
SEM	2-(trimethylsilyl)ethoxymethyl
Ser	serine
SES	2-(trimethylsilyl)ethanesulfonyl
SH2	Src homology 2 domain
Shk	stichodactyla toxin
SIH	somatotropin release-inhibiting hormone
SLC	sublethal concentration
Sle ^x	sialyl-Lewis ^x
SM	somatomedin
Smc	S-methylcysteine
SMPS	simultaneous multiple peptide synthesis
SN	secretoneurin
SP	substance P
SP5	splenopentin
SPCL	synthetic peptide combinatorial library
SPOCC	solid phase organic combinatorial chemistry
SPPS	solid-phase peptide synthesis
SR	sarcoplasmic reticulum
SRH	somatotropin releasing hormone
SRTX	sarafotoxin
ssDNA	single stranded DNA
SST	somatostatin
Sta	statine, (3S,4S)-4-amino-3-hydroxy-6-methylhexanoic acid
StBu	<i>tert</i> -butylsulfanyl
STH	somatotropin
STI	soybean trypsin inhibitor
Su	succinimide
Suc	succinoyl
Sulfmoc	2-sulfo-9-fluorenylmethoxycarbonyl
SVG	sauvagine
Tac	2-toluidinocarbonyl
Tacm	trimethylacetamidomethyl
TASP	template-assembled synthetic protein
TbFmoc	tetrabenzo-9-fluorenylmethoxycarbonyl
TBAF	tetrabutylammonium fluoride

TBPipU	2-(benzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium tetrafluoroborate
TBPyU	O-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
Tbs	3- <i>tert</i> -butyl-4-methoxybenzenesulfonyl
Tbtr	4,4',4''-tris(benzoyloxy)trityl
TBTA	<i>tert</i> -butyl trichloroacetimidate
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
tBu	<i>tert</i> -butyl
t-Bumeoc	1-(3,5-di- <i>tert</i> -butylphenyl)-1-methylethoxycarbonyl
TCE	2,2,2-trichloroethyl
TCL	thin layer chromatography
Tcp	trichlorophenyl
TDBTU	O-(3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TEA	triethylamine
TEMPO	2,2,6,6-tetramethylpiperidin-1-yloxy
Teoc	2-(trimethylsilyl)ethoxycarbonyl
TES	triethylsilyl
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride
TFE	trifluoroethanol
TFFH	tetramethylfluoroformamidinium hexafluorophosphate
Tfm	trifluoromethyl
TFMSA	trifluoromethane sulfonic acid
TfOH	trifluoromethanesulfonic acid
TGF	transforming growth factor
TH	thymus hormone
thexyl	1,1,2-trimethylpropyl
THF	tetrahydrofuran
Thi	thienylalanine
Thr	threonine
Thx	thyroxine
Thz	thiazolidine-4-carboxylic acid
Tic/TIC	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
Tip	2,4,6-triisopropylbenzenesulfonyl
TIS	Triisopropylsilane
TLE	thin layer electrophoresis
TM	thrombomodulin
Tmb	trimethoxybenzyl
TMBHA	4-(benzyloxy)-2',4'-dimethoxybenzhydramine
TMS	trimethylsilyl
TMSBr	trimethylsilyl bromide

TMSE	2-(trimethylsilyl)ethyl
TMSOTf	trimethylsilyl trifluoromethanesulfonate
Tmtr	tris(4-methoxyphenyl)methyl
Tn	troponin
TNBS	2,4,6-trinitrobenzenesulfonic acid
TNF	tumor necrosis factor
TNTU	2-(5-norbornene-2,3-dicarboximido)-1,1,3,3-tetramethyluronium tetrafluoroborate
TOAC	4-amino-4-carboxy-2,2,6,6-tetramethylpiperidiny-1-oxyl
TOCSY	total correlation spectroscopy
Tol	tolyl
TOPPIP _U	2-[2-oxo-1,2-dihydropyridyl]-1,1,3,3-bis-(pentamethylene)uronium tetrafluoroborate
Tos	tosyl
TOTU	O-[(ethoxycarbonyl)cyanomethyleneamino]-1,1,3,3-tetramethyluronium tetrafluoroborate
TP	thymopietin
TPST	tyrosine protein sulfotransferase
TP5	thymopentin
tPA	tissue plasminogen activator
TPTU	O-(1,2-dihydro-2-oxo-1-pyridyl)-N,N,N',N'-tetramethyluronium tetrafluoroborate
t _R	retention time
TRCOSY	transferred rotational correlated NMR spectroscopy
TRF	time-resolved fluorescence
TRH	thyrotropin releasing hormone
tRNA	transfer RNA
Tris	tris(hydroxymethyl)aminomethane
TRNOE	transferred nuclear Overhauser effect
Troc	2,2,2-trichloroethoxycarbonyl
Trp	tryptophan
Trt	triphenylmethyl (trityl)
TS	thrombospondin
Tse	2-(4-toluenesulfonyl)ethyl
TSH	thyroid stimulating hormone (thyrotropin)
Tsoc	2-(4-toluenesulfonyl)ethoxycarbonyl
TSTU	2-(succinimido)-1,1,3,3-tetramethyluronium tetrafluoroborate
Tyr	tyrosine
UCN	urocortin
UF	ultrafiltration
uHTS	ultra-high throughput screening
UK	urokinase
UNCA	urethane protected α -amino acid N-carboxy anhydride
uPA	urokinase-type plasminogen activator

XXXIV | Abbreviations

UT	urotensin
UV	ultraviolet
Val	valine
VIC	vasoactive intestinal contractor
VIP	vasoactive intestinal peptide
VLDL	very low density lipoprotein
Vn	vitronectin
VP	vasopressin
WSCl	water-soluble carbodiimide, e.g. [1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide hydrochloride]
Xaa	any amino acid
Xan	9 <i>H</i> -xanthen-9-yl
XRP	xenopsin-related peptide
Z	benzyloxycarbonyl
Z(2-Br)	2-bromobenzyloxycarbonyl
Z(2-Cl)	2-chlorobenzyloxycarbonyl
Z(Br)	4-bromobenzyloxycarbonyl
Z(NO ₂)	4-nitrobenzyloxycarbonyl
Z(OAc)	acetoxybenzyloxycarbonyl
Z(OAcPh)	phenylacetoxybenzyloxycarbonyl
Z(OMe)	4-methoxybenzyloxycarbonyl
Z(SMe)	4-(methylsulfonyl)benzyloxycarbonyl
Zte	1-benzyloxycarbonylamino-2,2,2-trichloroethyl

1) The fragment name 7-azabenzotriazole is used for simplicity, despite the fact that the correct IUPAC nomenclature requires it to be named as triazolopyridine (cf. HATU)

2) Many benzotriazole and 7-azabenzotriazole-based uronium salts have been shown to exist as guanidium salts in solution. For simplicity, they still are named as uronium salts (cf. HBTU)

1

Introduction and Background

Peptide research has experienced considerable development during the past few decades. The progress in this important discipline of natural product chemistry is reflected in a flood of scientific data. The number of scientific publications per year increased from about 10000 in the year 1980 to presently more than 20000 papers. The introduction of new international scientific journals in this research area reflects this remarkable development.

A very useful bibliography on peptide research was published by John H. Jones [1]. The Houben-Weyl sampler volume E 22 "Synthesis of Peptides and Peptidomimetics" edited by Murray Goodman (Editor-in-Chief), Arthur Felix, Luis Moroder and Claudio Toniolo [2] represents the most actual and exhaustive general treatise in this field. This work is a tribute to the 100th anniversary of Emil Fischer's first synthesis of peptides and is the successor of two Houben-Weyl volumes in German language edited by Erich Wünsch in 1974 [3].

A number of very important physiological and biochemical functions of life are influenced by peptides. Peptides are involved as neurotransmitters, neuromodulators, and hormones in receptor-mediated signal transduction. More than 100 peptides with functions in the central and peripheral nervous systems, in immunological processes, in the cardiovascular system, and in the intestine are known. Peptides influence cell-cell communication upon interaction with receptors, and are involved in a number of biochemical processes, for example metabolism, pain, reproduction, and immune response.

The increasing knowledge of the manifold modes of action of bioactive peptides led to an increased interest of pharmacology and medical sciences in this class of compounds. The isolation and targeted application of these endogenous substances as potential intrinsic drugs is gaining importance for the treatment of pathologic processes. New therapeutic methods based on peptides for a series of diseases give rise to the hope that diseases, where peptides play a functional role, can be amenable to therapy.

Peptide chemistry considerably contributes to research in the life science area. Synthetic peptides serve as antigens to raise antibodies, as enzyme substrates to map the active site requirements of an enzyme under investigation, or as enzyme inhibitors to influence signaling pathways in biochemical research or pathologic processes in medical research. Peptide ligands immobilized to a solid matrix may

facilitate specific protein purification. Protein-protein interaction can be manipulated by small synthetic peptides. The “peptide dissection approach” uses relatively short peptide fragments that are part of a protein sequence. The synthetic peptides are investigated for their ability to fold independently, with the aim to improve the knowledge on protein folding.

The isolation of peptides from natural sources often is problematic, however. In many cases, the concentration of peptide mediators ranges from 10^{-15} to 10^{-12} mol per mg fresh weight of tissue. Therefore, only highly sensitive assay methods such as immunohistochemical techniques render cellular localization possible. Although not all relevant bioactive peptides occur in such low concentrations, isolation methods generally suffer from disadvantages, such as the limited availability of human tissue sources. Complicated logistics during collection or storage of the corresponding organs, e.g., porcine or bovine pancreas for insulin production, additionally imposes difficulties on the utilization of natural sources. Possible contamination of tissue used for the isolation of therapeutic peptides and proteins with pathogenic viruses is an enormous health hazard. Factor VIII preparations for treatment of hemophilia patients isolated from natural sources have been contaminated with human immunodeficiency virus (HIV), while impure growth hormone preparations isolated from human hypophyses after autopsy have led to the transmission of central nerve system diseases (Creutzfeld-Jacob disease). Nowadays, many therapeutic peptides and proteins are produced by recombinant techniques. Immunological incompatibilities of peptide drugs obtained from animal sources have also been observed. Consequently, the development of processes for the synthesis of peptide drugs must be pursued with high priority.

Chemical peptide synthesis is the classical method which has been mainly developed during the past four decades, although the foundations were laid in the early 20th century by Theodor Curtius and Emil Fischer. Synthesis has often been the final structural proof of many peptides isolated only in minute amounts from natural sources.

The production of polypeptides and proteins by recombinant techniques has also contributed important progress in terms of methodology. Genetically engineered pharmaproteins verify the concept of therapy with endogenous protein drugs (endopharmaceuticals). Cardiovascular diseases, tumors, auto-immune diseases and infectious diseases are the most important indications. Classical peptide synthesis has, however, not been questioned by the emergence of these techniques. Small peptides, like the artificial sweetener aspartame (which has an annual production of more than 5000 tons) and peptides of medium size remain the objectives of classical synthesis, not to mention derivatives with non-proteinogenic amino acids or selectively labeled (^{13}C , ^{15}N) amino acid residues for structural investigations using nuclear magnetic resonance (NMR).

The demand for synthetic peptides in biological applications is steadily increasing. The new targets do not allow for an isolated position of peptide chemistry exclusively oriented toward synthesis. Modern interdisciplinary science and research require synthesis, analysis, isolation, structure determination, conformational analysis and molecular modeling as integrated components of a cooperation be-

tween biologists, biochemists, pharmacologists, medical scientists, biophysicists, and bioinformaticians. Studies on structure-activity relationships involve a large number of synthetic peptide analogues with sequence variation and the introduction of nonproteinogenic building blocks. The ingenious concept of solid-phase peptide synthesis has exerted considerable impact on the life sciences, whilst methods of combinatorial peptide synthesis allow for the simultaneous creation of peptide libraries which contain at least several hundreds of different peptides. The high yields and purities enable both *in-vitro* and *in-vivo* screening of biological activity to be carried out. Special techniques enable the creation of peptide libraries that contain several hundred thousands of peptides; these techniques offer an interesting approach in the screening of new lead structures in pharmaceutical developments.

Peptide drugs, however, can be applied therapeutically only to a limited extent because of their chemical and enzymatic labilities. Many peptides are inactive when applied orally, and even parenteral application (intravenous or subcutaneous injection) is often not efficient because proteolytic degradation occurs on the locus of the application. Application via mucous membranes (e.g., nasal) is promising. Despite the utilization of special depot formulations and new applications systems (computer-programmed minipump implants, iontophoretic methods, etc.) a major strategy in peptide chemistry is directed towards chemical modification in order to increase its chemical and enzymatic stability, to prolong the time of action, and to increase activity and selectivity towards the receptor.

The synthesis of analogues of bioactive peptides with unusual amino acid building blocks, linker or spacer molecules and modified peptide bonds is directed towards the development of potent agonists and antagonists of endogenous peptides. Once the amino acids of a protein that are essential for the specific biological mode of action have been revealed, these pharmacophoric groups may be incorporated into a small peptide. The development of orally active drugs is an important target. Rational drug design has contributed extensively in the development of protease-resistant structural variants of endogenous peptides, and in this context the incorporation of D-amino acids, the modification of covalent bonds, and the formation of ring structures (cyclopeptides) must be mentioned.

Peptidomimetics imitate bioactive peptides. The original peptide structure can hardly be recognized in these molecules, which induce a physiological effect by specific interaction with the corresponding receptor. Hence, a peptide structure may be transformed into a nonpeptide drug. This task is another timely challenge for peptide chemists, because only sufficient knowledge of the biologically active conformation of a peptide drug and of the interaction with the specific receptor enable the rational design of such peptide mimetics.

The variety of the tasks described herein renders peptide research an important and attractive discipline of modern life sciences. Despite the development of gene technology, peptide chemistry will have excellent future prospects because gene technology and peptide chemistry are complementary approaches.

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4

Peptide Synthesis

4.1

Principles and Objectives

As the biosynthesis even of complicated proteins *in vivo* occurs within seconds or minutes, the relatively tedious classical chemical synthesis of peptides and proteins in the laboratory appears to be a somewhat hopeless enterprise, especially with regard to speed. The first chemical synthesis of insulin during the early 1960s took over two years to complete. Today, peptide and protein synthesis have become much more efficient, not only by the use of recombinant DNA techniques but also with the advent of automated solid-phase peptide synthesis and multiple peptide synthesis.

Peptides and proteins can be considered as encoded biomolecules. The translation products are determined genetically and synthesized for highly specific functions. The above-mentioned seemingly hopeless competition between a peptide chemist and nature takes on a completely new aspect when peptides and proteins can be synthesized which are not available by using biosynthetic mechanisms. Hence, the initial quest is to identify the main targets of peptide synthesis.

4.1.1

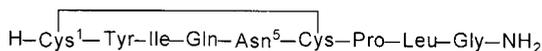
Main Targets of Peptide Synthesis

4.1.1.1 Confirmation of Suggested Primary Structures

There is a general chemical principle that total synthesis is the most convincing structural proof. Despite application of the latest techniques, misinterpretations in the sequence determination of peptides have in the past led to incorrect suggestions of primary structures, most notably for corticotropin, human growth hormone, and motilin. Erroneous sequences have been identified upon comparison of products obtained by total syntheses and by isolation from natural sources. Moreover, in some cases the final structural proof has been obtained only by chemical syntheses.

4.1.1.2 Design of Bioactive Peptide Drugs

Numerous analogues of peptides have been synthesized in order to identify the structural parameters for biological activity. In the case of oxytocin **1**, early efforts succeeded in obtaining an analogue by the exchange of glutamine in position 4 with threonine, the analogue subsequently displaying much higher biological activity compared with the native hormone. [Thr⁴]oxytocin is thought to undergo a much more efficient interaction with the receptor compared with the native hormone, and hence this phenomenon has been considered a “chemical mutation” which would have succeeded in evolution, but probably only in the distant future.



1

A systematic amino acid exchange is hampered by the variety of possible combinations of proteinogenic amino acids in bioactive peptides; however, combinatorial chemistry has today opened a series of completely new perspectives. In addition to the exchange of amino acids by other proteinogenic building blocks, the modification by isofunctional or isosteric amino acids is also a viable approach. An isofunctional amino acid exchange, for example of lysine by ornithine or diaminobutyric acid, of arginine by homoarginine, or the replacement of hydroxy acids (serine by threonine) and amino dicarboxylic acids (aspartic acid by glutamic acid) and *vice versa* has revealed information concerning the influence of side-chain length on biological activity. The role of amino acid side-chain structure, or of its chemical functionality, can be examined by using directed exchange with isosteric amino acids, for instance, Val/Thr, Leu/Asp, Met/Nle, Cys/Ser, Arg/Cit, Asn/Asp, Gln/Glu or His/ β -(pyrazolyl)alanine. Extensive investigations on the influence of the side chain on biological activity have been performed, and it has been observed that peptide drugs with a biologically active N-terminal (e.g., corticotropin, parathyrin) or C-terminal partial sequence (e.g., eledoisin, angiotensin, secretin) occur in nature. Nonetheless, some peptides exist in which the complete sequence is required for biological activity (e.g., calcitonin, bradykinin, oxytocin, vasopressin). Modifications of the C- and N-terminal amino and carboxy groups, of functional groups in side-chain positions, and of the configuration of the peptide backbone are of central interest in structure-activity relationships. All such chemical modifications of a bioactive peptide drug have a major influence on the conformation, so that conformational studies of native drugs in comparison with their modified analogues are central targets of timely research.

In general, peptides are much more soluble and flexible than proteins. Usually, small acyclic peptides do not prefer certain conformations in solution. Longer peptides that are able to form stable secondary structures often occur in equilibrium with partially folded structures. Structural concepts and theoretical methods have considerably influenced peptide research during the past years, and only the broad methodological spectrum of peptide synthesis has made such concepts evident. The conformational analysis of linear peptides and analogues with modified structure is not

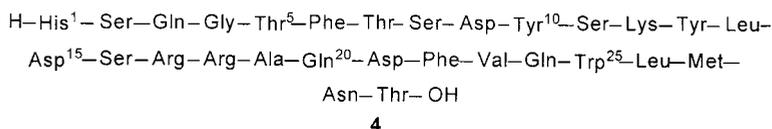
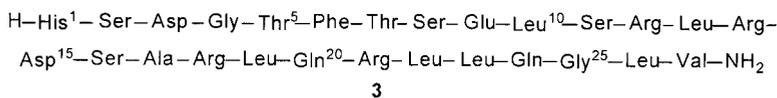
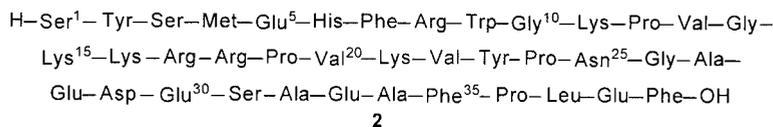
only very difficult but also is often ambiguous, because linear peptides are rather flexible unless stable secondary structures such as helices, β -sheets, turns, and other folds are present. As a result, conformational studies are usually performed with cyclic oligopeptides (often using NMR spectroscopy) because these materials display a greater degree of conformational homogeneity. Most chemical modifications of bioactive compounds are directed towards the design of new peptide drugs. In principle, peptides would be ideal drugs as they often interact in a highly specific manner with the corresponding receptor. However, peptides are rapidly degraded *in vivo* by the action of luminal, pancreatic, cytosolic, or lysosomal proteases, and are then processed and excreted by the liver. In addition, many body barriers demonstrate a high diffusional resistance towards the uptake of larger molecules, and suitable carrier systems rarely exist. Consequently, the main disadvantages of using a peptide drug are the low bioavailability and short half-life, especially after oral administration. Possibilities exist by which peptide degradation by proteases can be reduced, and uptake enhanced. For example, chemical modification (e.g., cyclization, D-amino acid substitution, or the incorporation of peptide mimetics) leads to the production of compounds with higher stability *in vivo*. Other strategies aimed at stabilizing peptides *in vivo* in order to increase their half-life include the coadministration of protease inhibitors, or the use of specific formulation such as emulsions, liposomes, or nanoparticles. Another alternative is to use a pro-drug to alter the peptide's physico-chemical properties.

Peptides may be used as lead structures in order to create nonpeptidic bioactive compounds, the process often being supported by conformational studies and molecular modeling. In the course of this structure-based design, an amino acid sequence of a bioactive peptide is transformed into a nonpeptide lead compound. Here, functional and structural information is combined and the peptide backbone topography with respect to the pharmacophoric groups is reproduced in the small nonpeptide lead compound. These substances must display pharmacological activity, especially after oral administration, and must imitate the peptide in terms of its receptor interaction (agonist activity); alternately, it may block this interaction (antagonist activity). Peptide mimetics may also act as enzyme inhibitors.

4.1.1.3 Preparation of Pharmacologically Active Peptides and Proteins

Based on economic considerations, pharmacologically active peptides, such as oxytocin 1, corticotropin 2, and secretin 3, have been synthesized chemically. Synthetic secretin costs about 10% less than the natural product isolated from porcine intestine, and a similar situation applies to many other peptide drugs. In addition to economic reasons, the better accessibility via a synthetic approach is important because many peptide drugs occur naturally only in nanogram quantities. Moreover, chemical synthesis is often preferred in the case of peptides and proteins that occur specifically in humans, as isolation following autopsy is generally not a viable route.

In the case of corticotropin 2, secretin 3, and glucagon 4, the products synthesized chemically are characterized by a higher purity than those isolated from natural sources. Often, the separation of peptides with similar sequences and antagonist or other activity is not possible using standard isolation and purification



methods. With the development of recombinant techniques, the industrial production of pharmacologically active proteins using biotechnological methods has become a viable route on a preparative scale. In particular, immune modulators and tissue proteins represent the focal point of promising new concepts in tumor therapy. In addition, diagnostics are being directed increasingly towards enzymes and monoclonal antibodies, while vaccines based on specialized proteins and peptides are also under development. Today, pharmacological research and medicine stand on the brink of a series of completely new developments, with the most important therapeutic areas likely to be those of cardiovascular conditions, tumors, and autoimmune or infectious diseases.

4.1.1.4 Synthesis of Model Peptides

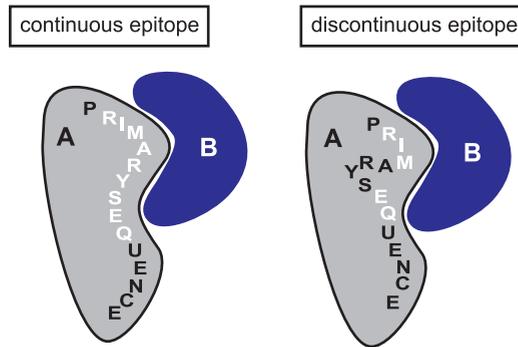
Tailor-made model peptides serve to study conformational behavior, using a variety of physico-chemical methods. Peptide substrates may also be used in studies of enzymology, while investigations into the antigenicity of larger proteins can be made using specialized multiple peptide synthesis techniques (see Chapter 8). Peptide epitope mapping especially is directed towards diagnostic targets and vaccine development. Protein-protein interactions often rely on the specific recognition of surface-exposed amino acid residues, which may be presented in either of two ways (Fig. 4.1):

- as a continuous epitope, where the amino acids involved occur consecutively in the primary structure;
- as a discontinuous epitope, where the crucial amino acids are located in different positions within the protein sequence.

An overlapping series of short peptide sequences (6–15 amino acids) is synthesized on the basis of the protein primary structure. Binding studies then reveal sequences necessary for protein-protein recognition.

In future, model peptides will serve as valuable tools in investigations of the tertiary structure of polypeptide chains. The question of whether the general rules of protein folding derived from the primary structure (amino acid sequence) can be

Fig. 4.1 Schematic view of the interaction between two proteins A and B via continuous or discontinuous epitopes.



maintained remains a controversial issue. Synthetic model peptides imitating protein surfaces can also be synthesized, and the flexibility of the peptide backbone can be reduced by conformational restrictions.

4.1.2

Basic Principles of Peptide Bond Formation

The formation of a peptide bond – resulting in a dipeptide – is seemingly a very simple chemical process. The two component amino acids are connected by a peptide (amide) bond with the elimination of water (Fig. 4.2).

The synthesis of a peptide bond under mild reaction conditions can only be achieved after activation of the carboxy component (A) of one amino acid. The second amino acid (as the amino component, B) attacks the activated carboxy component in a nucleophilic attack, with formation of the dipeptide (A-B, Fig. 4.3). If the amino function of the carboxy component (A) is unprotected, then formation of the peptide bond occurs in an uncontrolled manner (Fig. 4.3, lower part). Linear and cyclic peptides are formed as undesired by-products, together with the target compound A-B. Consequently, during the course of peptide synthesis all functional groups not involved in peptide bond formation must be blocked, both temporarily and reversibly.

Peptide synthesis – the formation of each peptide bond – is therefore a three-step procedure (Fig. 4.4).

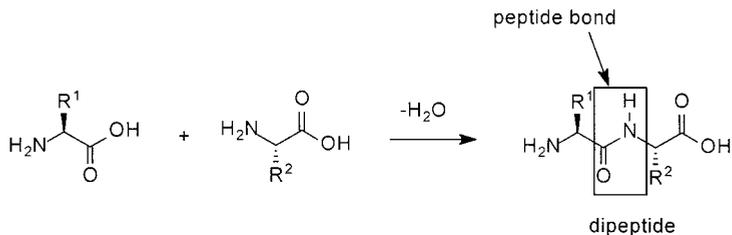


Fig. 4.2 Simplified scheme of peptide bond formation.

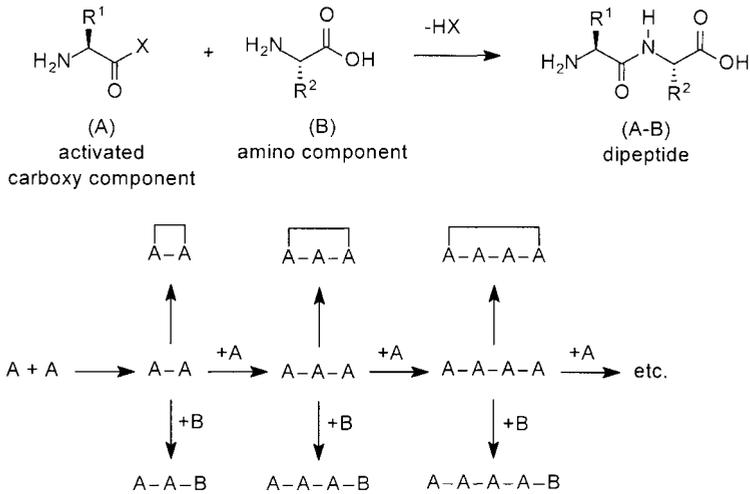


Fig. 4.3 Schematic view of possible side reactions of a peptide synthesis involving an activated N^{α} -unprotected carboxy component (A). Linear and cyclic oligomers are formed besides the desired dipeptide A-B. X=activating group; R^1 , R^2 =amino acid side chains.

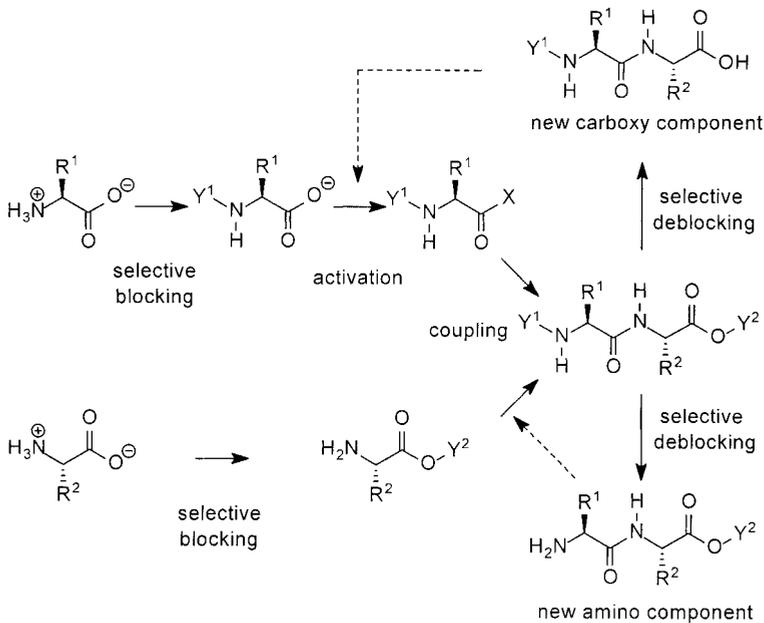


Fig. 4.4 The multi-step process of peptide synthesis. Y^1 =amino-protecting group; Y^2 =carboxy protecting group; R^1 , R^2 =amino acid side chains.

1. In the first step, the preparation of a partially protected amino acid is required. After this protection, the zwitterionic structure of the amino acids is no longer present.
2. The second step, the formation of the peptide bond, occurs in two partial steps. The N-protected amino acid must be activated at the carboxy function in order to be converted into a reactive intermediate. Subsequently, the formation of the peptide bond occurs. This coupling reaction can be performed either as a one-pot reaction or in two separate, consecutive steps.
3. In the third reaction step, selective or total cleavage of the protecting groups is carried out. Although total deprotection is required only when the peptide chain has been fully assembled, selective cleavage of the protecting groups is usually necessary in order to continue the peptide synthesis.

Peptide synthesis becomes further complicated by the fact that ten of the proteinogenic amino acids (Ser, Thr, Tyr, Asp, Glu, Lys, Arg, His, Sec, and Cys) have functional groups in the side chain which need to be selectively protected. Because of the different requirements with respect to selectivity, a distinction must be made between the intermediary (temporary, or transient) and semipermanent protecting groups. The intermediary groups are used for temporary protection of the amino or carboxy function involved in subsequent bond formations. These groups must be cleaved selectively under conditions that do not interfere with the stability of peptide bonds already present, or that of semipermanent protecting groups at amino acid side chains. The semipermanent protecting groups are usually cleaved only at the end of the peptide synthesis or, occasionally, also at an intermediate stage. The different types of protecting groups will be described in Section 4.2.

Activation of the carboxy component and subsequent bond formation (the coupling reaction) should, under ideal conditions, occur at a high reaction rate and without racemization or formation of by-products. High yields are required upon application of equimolar amounts of both components, but unfortunately no such chemical coupling method yet exists which meets these requirements. The number of appropriate methods applicable to practical synthesis is rather small compared to the high number of coupling methods described. Further details will be provided in Section 4.3.

During the course of peptide synthesis, a number of reactions occur which involve functional groups that are usually connected to a chiral center (glycine is the only exception), and consequently there is a potential risk of racemization (see Section 4.4).

Cleavage of the protecting groups is the last step in the peptide synthesis cycle. Except for the synthesis of dipeptides, where total deblocking is required, the selective cleavage of protecting groups in order to extend the peptide chain is of major importance. The synthetic strategy requires thoughtful planning, which is described as the "strategy and tactics of peptide synthesis" (see Chapter 5). Depending on the strategy chosen, either the protecting group of the N^α-amino function is cleaved selectively, or the protecting group of the carboxy function is cleaved. The term "strategy" refers to the sequence of condensation reactions between sin-

gle amino acids to produce a peptide. Usually, a distinction is made between stepwise synthesis and segment condensation (fragment condensation). When peptide synthesis is performed in solution (also referred to as “conventional synthesis”), for example with difficult sequences, the stepwise peptide chain elongation is limited in most cases to the assembly of shorter fragments. When the synthesis of a longer peptide is planned, the target molecule must be divided into appropriate segments. Suitable fragments have to be identified, perhaps where only minimal C-terminal epimerization occurs during fragment condensation. Following stepwise assembly of the single segments, the latter are connected to produce the target compound. The tactics of peptide synthesis also include selection of the most suitable combination of protecting groups, together with application of the best coupling methods for each segment condensation.

Solid-phase peptide synthesis (SPPS) is, in its original version, a variant of the stepwise synthesis of peptides and proteins. It is a synthetic concept where the growing peptide chain is attached to an insoluble polymeric support, and was first described in 1963 by Robert Bruce Merrifield. Today, this method is referred to as the Merrifield synthesis in honor of the 1984 Nobel laureate (for details, see Section 4.5). Nowadays, segment condensations may also be performed on polymeric support (see Chapter 5, Section 3).

4.2

Protection of Functional Groups

Temporary and semipermanent protecting groups are distinguished with respect to cleavage selectivity. A temporary protecting group must fulfil the following requirements:

1. Introduction of the protecting group leads to an amino acid derivative that is no longer present in a zwitterionic structure.
2. Cleavage must occur without hampering the stability of semipermanent protecting groups or peptide bonds.
3. Racemization must not occur during all necessary operations.
4. Stability and characterization of protected intermediates must be favorable.
5. Solubility of the protected amino acid components must be favorable.

According to the tactical requirements, semipermanent protecting groups are usually cleaved at the end of the peptide synthesis. In this reaction, only those de-blocking reagents that do not damage the final product can be used. The requirements with respect to cleavage selectivity are less stringent because temporary protecting groups may also be cleaved in the final deblocking step. Criteria (3), (4), and (5) must also be applied to the semipermanent protecting groups.

The selection of protecting groups must be carried out in accordance with the strategy envisaged for the synthesis of a particular peptide (see Chapter 5). Protecting group orthogonality is a criterion that is especially important for the success of a peptide synthesis.

Protection schemes rely on either quantitative rate differences of deprotection reactions, or on mechanistically different deprotection reactions in the case of orthogonal protecting groups [1]. Orthogonality means that subsets of protecting groups present in a molecule can be cleaved selectively under certain reaction conditions, while all other protecting groups remain intact. Often, orthogonality refers to a mechanism-based distinction, for example between semipermanent and temporary protecting groups. It generally provides the opportunity of substantially milder overall reaction conditions.

In the following section, the most important protecting groups are discussed in the context of the functional group to be blocked.

4.2.1

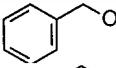
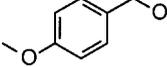
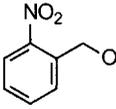
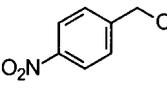
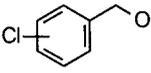
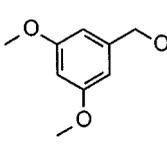
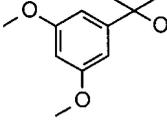
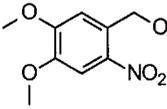
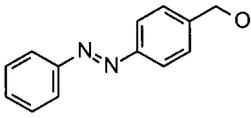
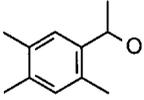
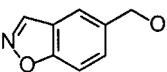
N^α-amino Protection

Protecting groups of this type are used for the N^α-amino group of all proteinogenic amino acids (including the imino group of proline), for N^ω-amino groups (e.g., lysine, ornithine) and also, if necessary, for the temporary blocking of N-acyl amino acid hydrazides and other functional groups. In principle, an amino group can be blocked reversibly by acylation, alkylation, and alkyl-acylation. Protecting groups based on sulfur and phosphorus derivatives have also been developed. Salt formation at the amino group does not offer a genuine protection during the operations of peptide synthesis. Several hundred different amino-protecting groups have been developed during the past decades – which confirms the non-existence of a universal, ideal amino-protecting group. A classification can be made based on the structure of the protecting group, or on the cleavage conditions: acidolysis, base cleavage, reduction/oxidation, nucleophilic substitution, and photolysis. Because most peptides are sufficiently stable under moderately acidic conditions, amino-protecting groups with different labilities towards acidic deblocking agents are preferred. Alternately, the base-labile compound 9-fluorenylmethoxycarbonyl group (Fmoc) has found widespread application. It has been estimated that, in practical terms, over 80% of all syntheses use these two deblocking methods. Some protecting groups may be cleaved by hydrogenolysis. Protection of the amino group can also be achieved easily by acylation and by alkylation. The disadvantage of structurally and chemically simple acyl residues (e.g., acetyl-, benzoyl-, monochloroacetyl-) is that the carboxamide group of the protecting function is chemically very similar to the peptide bond, which renders selective cleavage very difficult or even impossible, and this eventually led to the development of urethane-type amino-protecting groups.

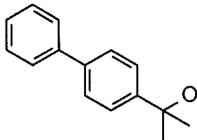
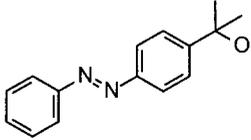
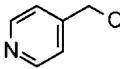
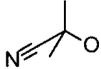
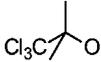
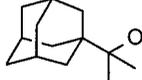
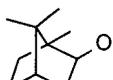
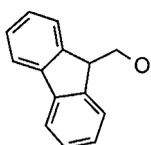
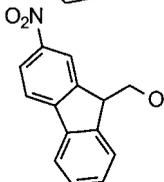
4.2.1.1 Alkoxy-carbonyl-Type (Urethane-Type) Protecting Groups

A variety of urethane-type amino-protecting groups, which are cleavable under different reaction conditions, is available. Selected representatives are listed in Tab. 4.1; the most prominent groups are discussed in the following section.

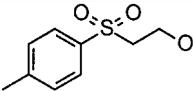
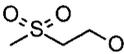
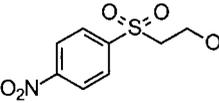
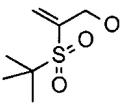
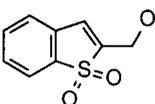
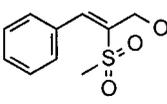
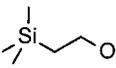
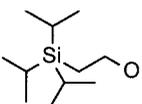
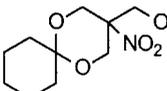
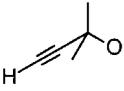
Tab. 4.1 Selected amino-protecting groups of the urethane type Y-O-(C=O)-NH-R¹.

Group	Symbol	Y-O	Cleavage conditions	Reference(s)
Benzyloxycarbonyl	Z		H ₂ /Pd, HBr/AcOH, Na/liq. NH ₃	2
4-Methoxybenzyloxy-carbonyl	Z(OMe)		TFA; H ₂ /Pd; Na/liq. NH ₃	3, 4
2-Nitrobenzyloxy-carbonyl	Z(2-NO ₂)		H ₂ /Pd (more labile than Z) HBr/AcOH (more stable than Z) photolysis	5
4-Nitrobenzyloxy-carbonyl	Z(NO ₂)		Like Z, but more stable to HBr/HOAc	6, 7
Chlorobenzyloxy-carbonyl	Z(Cl) Z(3-Cl) Z(2-Cl) Z(2,4-Cl)		Like Z, but more stable to H ₂ /Pd or HBr/AcOH	8–12
3,5-Dimethoxybenzyloxy-carbonyl	Z(3,5-OMe)		TFA/CH ₂ Cl ₂ Photolysis	13
α,α -Dimethyl-3,5-dimethoxybenzyloxy-carbonyl	Ddz		Photolysis, 5% TFA in CH ₂ Cl ₂	14
6-Nitroveratryloxy-carbonyl (4,5-Dimethoxy-2-nitrobenzyloxy-carbonyl)	Nvoc		Like Z Photolysis	5
4-(Phenyldiazenyl)-benzyloxy-carbonyl	Pz		H ₂ /Pd, HBr/AcOH, Na/liq. NH ₃	15
α -Methyl-2,4,5-trimethylbenzyloxy-carbonyl	Tmz		3% TFA in CHCl ₃	16
Benzisoxazol-5-yloxy-carbonyl	Bic		Isomerization with triethylamine in DMF and solvolysis in aq. buffer (pH 7)	17

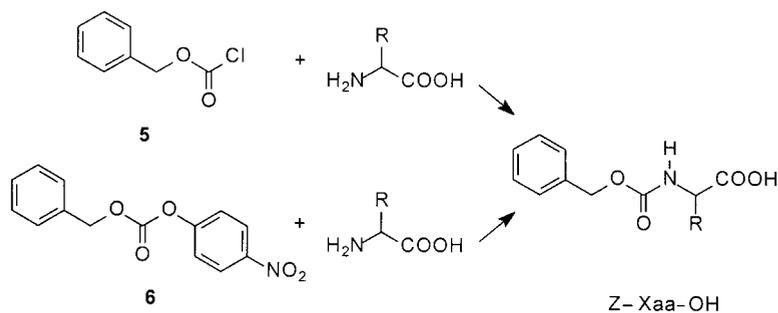
Tab. 4.1 (continued)

Group	Symbol	Y-O	Cleavage conditions	Reference(s)
2-(Biphenyl-4-yl)-2-propoxycarbonyl	Bpoc		80% AcOH MgClO ₄	18, 19
(4-Phenylazophenyl)-isopropoxycarbonyl	Azoc		Like Bpoc	20
Isonicotinyl-oxycarbonyl	iNoc		Zn/AcOH, H ₂ /Pd, acid stable	21
<i>tert</i> -Butoxycarbonyl	Boc		TFA, TFA/CH ₂ Cl ₂ , HCl/organic solvent	3, 22
2-Cyano- <i>tert</i> -butoxycarbonyl	Cyoc		Weak base (aq. K ₂ CO ₃ , triethyl- amine)	23
2,2,2-Trichloro- <i>tert</i> -butoxycarbonyl	Tcboc		Co(I)-phthalocyanine Zn/AcOH	24
Adamantyl-1-oxycarbonyl	Adoc		TFA	25
1-(1-Adamantyl)-1-methylethoxycarbonyl	Adpoc		3% TFA in CH ₂ Cl ₂ , labile to H ₂ /Pd	26
Isobornyl-oxycarbonyl	Iboc		TFA, stable to H ₂ /Pd and bases	27
Fluorenyl-9-methoxycarbonyl	Fmoc		Piperidine, DBU, 2- aminoethanol, Morpholine, Liq. NH ₃	28, 29
(2-Nitrofluoren-9-yl)methoxycarbonyl	Fmoc(NO ₂)		Photolysis	30

Tab. 4.1 (continued)

Group	Symbol	Y-O	Cleavage conditions	Reference(s)
2-(4-Toluenesulfonyl)-ethoxycarbonyl	Tsoc		NaOC ₂ H ₅ /ethanol	31
Methylsulfonyl-ethoxycarbonyl	Msc		Base-catalyzed β-elimination	32
2-(4-Nitrophenylsulfonyl)ethoxycarbonyl	Nsc		Like Fmoc	33–35
2-(tert-Butylsulfonyl)-2-propenyloxy-carbonyl	Bspoc		Nucleophile	36
1,1-Dioxobenzo[b]-thien-2-ylmethoxy-carbonyl	Bsmoc		Nucleophile, e.g., 2% tris(2-aminoethyl)amine	37, 38
2-(Methylsulfonyl)-3-phenyl-2-propenyl-oxycarbonyl	Mspoc		Nucleophile	39
Allyloxycarbonyl	Aloc		Pd ⁰ /nucleophile	40–42
2-(Trimethylsilyl)-ethoxycarbonyl	Teoc		F ⁻ , e.g. TBAF, TFA	43
Triisopropylsilyl-ethoxycarbonyl	Tipseoc		F ⁻ , e.g. TBAF	44
Piperidinyloxy-carbonyl	Pipoc		Electrolysis H ₂ /Pd	45
Cyclopentyloxy-carbonyl	Poc		HBr/AcOH, Na/liq. NH ₃ , stable to H ₂ /Pd	3
3-Nitro-1,5-dioxaspiro[5.5]undec-3-ylmethoxycarbonyl	P ^T nm		"Relay deprotection": H ₃ O ⁺ , then pH 8.5	46
2-Ethynyl-2-propyl-oxycarbonyl	Epoc		H ₂ , Lindlar-catalyst	47

Benzyloxycarbonyl group The introduction of the benzyloxycarbonyl group into peptide synthesis provided a major breakthrough in the development of modern peptide chemistry. Today, this amino-protecting group is among those used most frequently, and is abbreviated as Cbz (or “Z”, in honor of its inventor, Leonidas Zervas). In older references, Cbz is also referred to as the carbobenzyoxy (Cbo) group. Introduction of the Z group in amino acids is usually performed by reaction with benzylchloroformate **5** (the correct names benzyloxycarbonyl chloride or benzylchlorocarbonate are seldom used) under Schotten-Baumann conditions in the presence of NaOH, NaHCO₃ or MgO, mostly in aqueous organic solvents under vigorous stirring. Amino acid esters can be converted into the Z-protected derivatives in chloroform. Selective protection in the case of amino acids with functional groups in the side chain often requires special reaction conditions. Arginine may be selectively N^α-monoprotected using benzyl chloroformate in a buffered NaHCO₃-NaOH solution at pH 9–11 [48]. Usually, Z-amino acids are obtained in high yields in crystalline form, but products obtained as an oil can be crystallized as salts with dicyclohexylamine.



Benzyl-4-nitrophenylcarbonate **6** and similarly activated benzyl derivatives can also be used to introduce this group. Cleavage of the Z group is achieved by acidolysis using hydrogen bromide in acetic acid, liquid HBr, boiling trifluoroacetic acid (TFA), HBr/TFA, liquid hydrogen fluoride (HF), or by catalytic hydrogenation using different catalysts under a broad scope of reaction conditions (Fig. 4.5). The catalytic hydrogenation proceeds smoothly in organic solvents (e.g., acetic acid, alcohols, dimethylformamide (DMF)) or in aqueous organic solution with palladium black, palladium on charcoal, or palladium on barium sulfate. Only toluene and carbon dioxide are formed in addition to the desired free peptide. Cessation of CO₂ evolution or H₂ consumption are used to monitor the cleavage reaction.

Although hydrogenolytic cleavage of Z groups fails when cysteine/cystine is present in the peptide, the reaction is successful in the presence of BF₃·OEt₂. Acidolysis is performed preferably using 2 N hydrogen bromide in acetic acid, although several other variants (e.g., hydrogen chloride, hydrogen iodide) and solvents (e.g., dioxan, nitromethane, TFA, tetrachloromethane) have been suggested. Side reactions can also occur for this standard method; for example, O-acetylations have been observed at threonine or serine, while S-transetherifications may occur in the presence of methionine. Tryptophan or nitroarginine building blocks

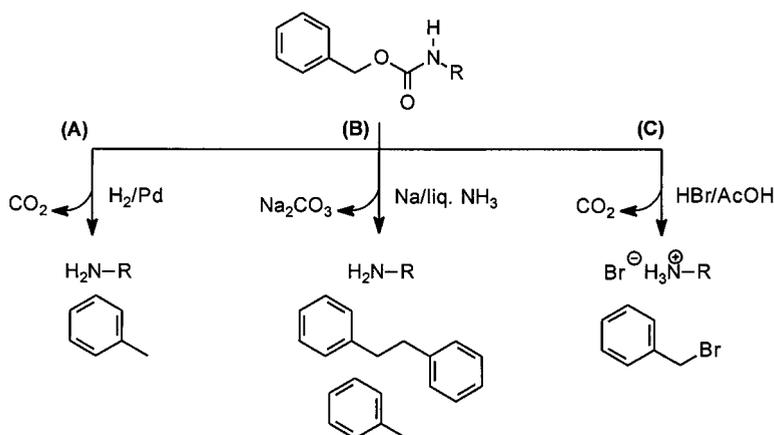


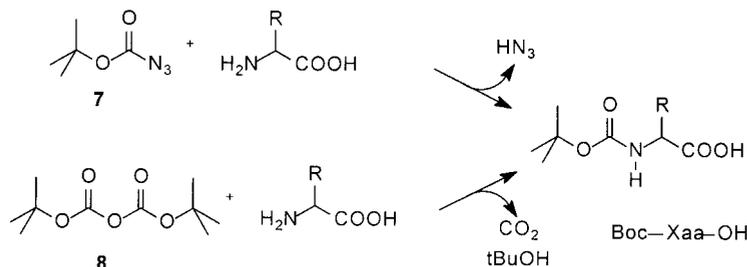
Fig. 4.5 Cleavage conditions of the benzyloxycarbonyl group. $\text{H}_2\text{N}-\text{R}$ =amino acid.

may be destroyed, and benzyl esters or carboxamide groups are sometimes cleaved under these conditions. However, these side reactions may be minimized under certain reaction conditions. The Z group can also be cleaved with anhydrous liquid hydrogen fluoride (HF method).

More than 20 different benzyloxycarbonyl type groups with a variety of substituents have been described, mainly with regard to tuned sensitivity towards acidolytic cleavage reagents. The 4-methoxybenzyloxycarbonyl residue provides a methodological advantage because the protected amino acids are crystalline. It can be cleaved in the presence of the Z group with anhydrous TFA at temperatures below 0°C . Z groups with halogen substituents in the 3- or 4-position of the phenyl ring are also of interest because they lead to higher stability of the modified Z group towards acidic reagents. This can be used to better differentiate between protecting groups of the Z type and Boc type (see below). The (3,5-dimethoxybenzyl)oxycarbonyl, (2-nitrobenzyl)oxycarbonyl, and (6-nitroveratryl)oxycarbonyl groups can be cleaved photochemically.

tert-Butoxycarbonyl group The *tert*-butoxycarbonyl group (Boc) is, like the Z and the Fmoc group, one of the most important amino-protecting groups. It should also be mentioned that amino-protecting groups of the urethane type safeguard racemization-free peptide bond formations during the stepwise assembly of a peptide chain starting from the C-terminus.

The *tert*-butoxycarbonyl azide **7** is an excellent acylation reagent. It reacts with the salts of amino acids in water/dioxan mixtures in the presence of tertiary amines or magnesium oxides or under pH control with 2 N to 4 N NaOH to give the Boc-protected amino acids Boc-Xaa-OH, but also with amino acid esters in pyridine.



pH-Stat-controlled reaction conditions are advantageous. Although this acylation protocol has been proved to be very efficient, further methods for the introduction of the Boc group have been described, mainly because of the explosive nature of azides. The *tert*-butoxycarbonyl fluoride obtained from chlorocarbonyl fluoride and *tert*-butanol at -25°C has attracted special attention because it gives Boc-protected amino acid in high yields under pH-stat conditions. *tert*-Butyl-*S*-4,6-dimethylpyrimidyl-2-thiocarbonate, 2-*tert*-butoxycarbonyloximino-2-phenylacetonitrile and, preferably, di-*tert*-butyldicarbonate (Boc)₂O **8** are also used as reagents. Amino acid salts can be used in aqueous solution with solubilizing additives such as dioxan or tetrahydrofuran.

The Boc protecting group is compatible with most coupling methods for peptide synthesis. It can be cleaved under mild acidolytic conditions, and is resistant towards catalytic hydrogenation, alkaline hydrolysis and reduction with Na/liquid ammonia. Hydrogen chloride in acetic acid, dioxan, ether, and ethyl acetate are important cleavage reagents. A cleavage method frequently used is treatment with non-aqueous TFA at temperatures around 0°C , which smoothly cleaves Boc groups (Fig. 4.6).

Undesired *tert*-butylations may occur during acidolysis at nucleophilic sites of the peptide chain. For instance, the indole system of tryptophan or the thioether group of methionine can be *tert*-butylated. The intermediate carbenium ions responsible for these side reactions are usually trapped using scavengers such as anisole or thioanisole. Further deblocking reagents are $\text{BF}_3 \cdot \text{OEt}_2$, 2-mercaptoethanesulfonic acid, aqueous TFA, or 98% formic acid. However, a smooth reaction has not been observed in all cases. This variety of reagents can be used when other protected functional groups and the Z group are present.

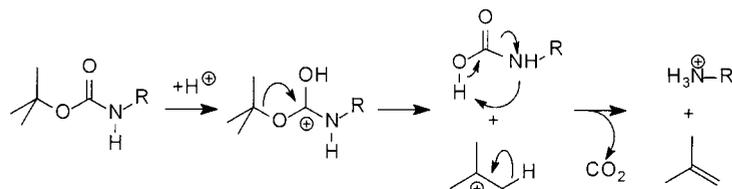


Fig. 4.6 Mechanism of acidolytic cleavage of the *tert*-butoxycarbonyl group. $\text{H}_2\text{N-R}$ =amino acid or peptide.

Modified analogues of the *tert*-butoxycarbonyl group have been described on many occasions, and some representatives are discussed in the following section. The (α,α -dimethyl-3,5-dimethoxybenzyl)oxycarbonyl group (Ddz) can be cleaved both acidolytically with 5% TFA in methylene chloride, and also photolytically. Therefore, it provides an interesting variant for special applications. The 2-(4-biphenyl)-2-propoxycarbonyl residue (Bpoc) can be cleaved with 80% acetic acid, and is therefore often combined with protecting groups for ω -amino-, hydroxy-, and carboxy functionalities based on the *tert*-butyl type. The 2-ethynyl-2-propyloxycarbonyl group is cleaved by catalytic hydrogenation (with Lindlar catalyst), even in the presence of methionine-containing peptides. Alternately, it can be cleaved by acidic hydrolysis under the formation of a cyclic carbonate. A different variant is the 2-cyano-*tert*-butoxycarbonyl residue (Cyoc) which can be cleaved by β -elimination under weakly basic conditions.

9-Fluorenylmethoxycarbonyl group The 9-fluorenylmethoxycarbonyl group (Fmoc) is the only amino-protecting group of the urethane type of widespread practical importance that can be cleaved under mildly basic conditions. The sensitivity of Fmoc-protected amino acids (Fig. 4.7; **9**), especially towards secondary amines, enables deblocking of the amino group with dilute solutions of piperidine or diethylamine in DMF under mild conditions. Usually, 20% piperidine in DMF is applied

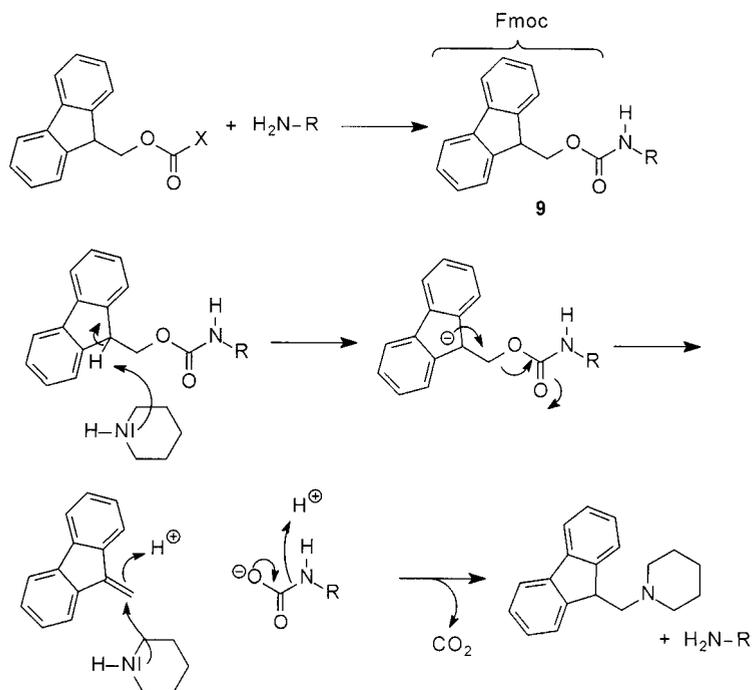


Fig. 4.7 Mechanism of Fmoc cleavage. H₂N-R=amino acid or peptide.

routinely where the cleavage of the Fmoc group occurs within seconds at room temperature. Other reagents, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) or fluoride ion may be used as an alternative. The reaction proceeds according to a E1cB mechanism, with initial proton abstraction to give the stabilized dibenzocyclopentadienyl anion. The dibenzofulvene formed reacts with piperidine to give a stable adduct as a by-product of the cleavage reaction (Fig. 4.7). In cases where no primary or secondary amine is used, the addition of 2% piperidine is recommended to trap the dibenzofulvene.

Under these conditions, neither Z nor Boc groups or other modern amino-protecting groups usually applied are attacked. In order to prevent premature cleavage under peptide-coupling conditions, addition of the weakly acidic additive 1-hydroxybenzotriazole has been recommended. CaCl₂ addition has been reported to increase Fmoc lifetime, and hence may be used for the synthesis of Fmoc-protected peptide segments by saponification of C-terminal esters or linkers [49]. Interestingly, the Fmoc group is not totally inert towards standard hydrogenolytic cleavage conditions.

The reagents Fmoc-Cl and, alternately, Fmoc-OSu (9-fluorenylmethyl-N-succinimidyl carbonate) have been recommended for the formation of Fmoc-protected amino acids. Although, the Fmoc group was introduced into peptide chemistry in 1970, its application in the Merrifield solid-phase peptide synthesis was reported much later. Nowadays, the Fmoc tactics is one of the most preferred synthetic concepts in solid-phase peptide synthesis. The acid stability, combined with the mildly basic cleavage conditions, allows for the application of acid-labile semipermanent protecting groups of the *tert*-butyl type for side-chain functionalities in solid-phase peptide synthesis.

The purification of synthetic peptides and proteins may be facilitated when a polyaromatic analogue of the Fmoc group is attached to the N-terminus [50].

New base-labile N^α-protecting groups The 2-(4-nitrophenylsulfonyl)ethoxycarbonyl group Nsc (see Tab. 4.1) has recently been considered as a valuable alternative to the Fmoc group, because it is more base-stable and, therefore, less sensitive towards weakly basic media [51–53]. Nsc is more hydrophilic than Fmoc, and less prone to aspartimide formation [51]. Like Fmoc, Nsc forms in the deprotection reaction an olefin that subsequently is trapped by the secondary base piperidine. In the Fmoc case, the formation of the dibenzofulvene piperidine adduct is reversible, while the corresponding adduct in the Nsc case is formed irreversibly.

Carpino et al. developed the new base-labile amino-protecting groups Bspoc, Bsmoc, and Mspoc (see Tab. 4.1) [36–39]. Cleavage relies on the conjugate addition of a nucleophile (e.g., secondary amine) with concomitant liberation of the carbamate. Bsmoc is stable towards acids and tertiary amines, but is cleaved readily with secondary amines. As the deprotection step simultaneously is the scavenging step, fewer side reactions should occur.

Protecting groups that are orthogonal to Boc and Fmoc The N^α-allyloxycarbonyl (Aloc) group [40–42] may be used for the synthesis of labile derivatives (see Sec-

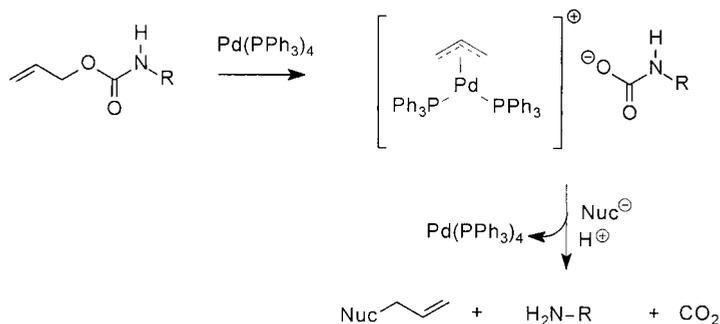
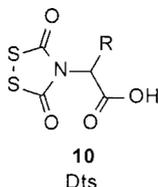


Fig. 4.8 Mechanism of Aloc cleavage. H₂N-R=amino acid or peptide.

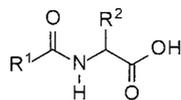
tions 6.3–6.5). It is completely orthogonal to Boc and Fmoc and, therefore, finds application in the synthesis of cyclic peptides (see Chapter 6.1). The deprotection step involves palladium(0)-catalyzed allyl transfer (Fig. 4.8) to various nucleophiles (e.g., amines, amine-borane complexes, carboxylates, organosilanes, organostannanes) [54]. Amine-borane complexes are especially suited as allyl scavengers under neutral conditions without undesired N-allylation of the deprotected product [54]. Tandem deprotection-acylation of N^α-Aloc derivatives has also been reported [55].

The dithiasuccinoyl group Dts **10** [56–58] may formally be regarded as a urethane thio analogue that can be cleaved by treatment with thiols. It is stable to acids and bases and, consequently, orthogonal to Boc or Fmoc.



4.2.1.2 Carboxamide-Type Protecting Groups

Representatives of the carboxamide-type protecting groups are the formyl group for **11** (R=H) which can be cleaved by solvolysis, oxidation, or hydrazinolysis, and the trifluoroacetyl group Tfa **11** (R=CF₃), which is labile towards alkaline conditions.

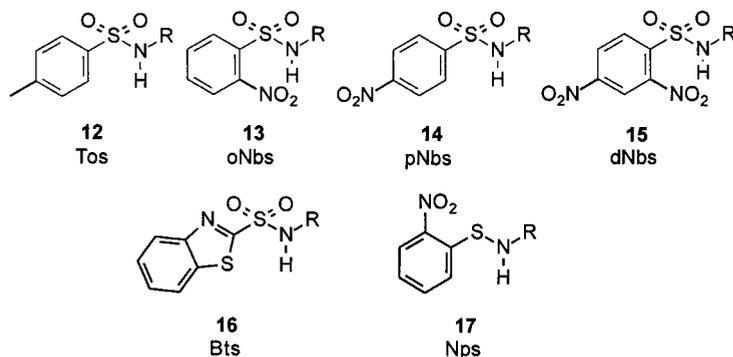


11 For: R¹ = H
Tfa: R¹ = CF₃

4.2.1.3 Sulfonamide and Sulfenamide-Type Protecting Groups

Arenesulfonyl groups feature some properties that render them potential amino-protecting groups. In the past, mainly the 4-toluolsulfonyl (tosyl, Tos) group **12**

has been used, though this suffers from the disadvantage that it is only cleavable by reduction with Na/NH₃. In principle, N^α-sulfonamide protecting groups allow for N-alkylation of amino acids [59, 60] and do not suffer from racemization via the oxazolone mechanism (cf. Section 4.4.2) [61]. N^α-Arenesulfonyl amino acid chlorides are highly reactive species that are well suited for peptide synthesis [62]. Consequently, N^α-arenesulfonamide-type protecting groups experienced a renaissance with the introduction of the *o*- and *p*-nitrobenzenesulfonyl groups (*o*Nbs **13**, *p*Nbs **14**) [59, 63] and of *d*Nbs **15** [64] and Bts **16** [60]. These protecting groups can be removed by treatment with thiophenol or alkanethiols, and hence are orthogonal to *tert*-butyl and 9-fluorenylmethyl-type protecting groups.



The 2-nitrophenylsulfanyl (Nps) group **17**, which has been used in solution-phase peptide synthesis, may be removed by HCl in inert solvents or with the use of thiol reagents [65–67].

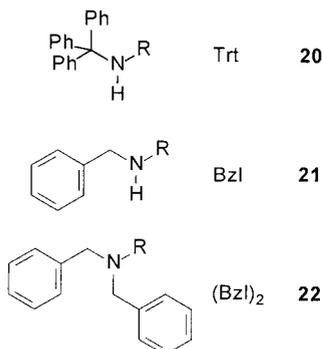
4.2.1.4 Alkyl-Type Protecting Groups

The protecting groups 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl Dde **18** (R² = CH₃) [68, 69], 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl *iv*Dde **18** (R² = *i*Bu) [70], and 1-(4-nitro-1,3-dioxoindan-2-ylidene)ethyl Nde [71] are alkyl-type protecting groups that are orthogonal to Boc and Fmoc, because they are cleaved by hydrazinolysis.



The most popular protecting group of the alkyl type is the triphenylmethyl (Trt) group **20** that can be cleaved acidolytically under mild conditions. The activation

of Trt-protected amino acids is very complicated however because of the steric hindrance. Mono- or disubstituted *N*-benzyl protecting groups **21**, **22** have received very little attention in peptide synthesis, but they can be cleaved by catalytic hydrogenation in the presence of palladium black at elevated temperatures. This fact probably led to the finding that the exchange of an ethyl by a benzyl residue in the ethoxycarbonyl group, as described by Fischer, could result in an amino-protecting group being cleavable by hydrogenolysis. This paved the way for the protecting groups of the urethane type (as the protecting groups based on carbamates are referred to).



4.2.2

C^α-Carboxy Protection

There are few exceptions (e.g., [72–74], cf. Section 5.1.1) from the usual synthetic strategy where a peptide chain is assembled from the C-terminus to the N-terminus (C → N direction). Consequently, C-terminal protection has not been investigated as extensively as N-terminal protection. C^α-carboxy-protecting groups in solution-phase synthesis, and also as linker moieties in solid-phase synthesis, must be orthogonal to the temporary N^α-protecting group.

In principle, it is possible to perform peptide synthesis without complete protection of the carboxy group of amino components. Removal of the zwitterionic structure of the corresponding amino acid or the peptide is achieved by salt formation. Here, alkali or earth alkali metal salts of amino acids can usually be reacted in water or aqueous dioxan with the corresponding activated carboxy components. The corresponding carboxylate salts of tertiary bases (*N*-methyl morpholine, *N*-ethyl piperidine, triethylamine, 1,1,3,3-tetramethylguanidine, etc.) have been recognized as important for peptide synthesis in organic solvents, especially in DMF. The mixed anhydride and azide methods are, besides active esters, best suited for the so-called salt coupling. A major advantage is that, after the coupling reaction, the carboxy group is liberated by acidification. This protocol is not universally applicable, as side reactions or solubility problems may occur without genuine protection of the carboxy group of the amino component used in the reaction.

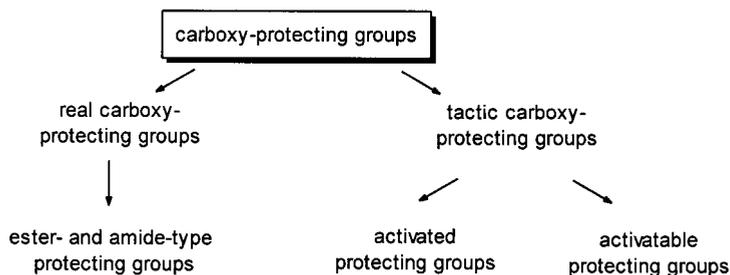


Fig. 4.9 Classification of carboxy-protecting groups.

Esters of the methyl, ethyl, benzyl, *tert*-butyl, or aryl type are appropriate for the reversible blocking of the carboxy group.

Carboxy-protecting groups may be divided into two categories on the basis of strategic-tactic criteria (Fig. 4.9):

- The first group contains protecting groups which can be deblocked normally after the end of the synthesis by regeneration of the free carboxy group.
- The second group contains derivatives that are activated towards aminolysis at the end of a synthesis, either immediately or after special chemical transformation.

Although these two different types have been classified as genuine and nongenuine carboxy-protecting groups, a better discrimination between them may be to refer to “real” and “tactic” carboxy-protecting groups. The expression “tactic” protecting groups emphasizes the application of these functionalities in special synthetic strategies where the mere role of a protecting group is exceeded.

4.2.2.1 Esters

Activated carboxy-protecting groups can be used only under special conditions for peptide synthesis. In order to safeguard the desired reaction pathway, the activation method used for the coupling must have a higher acylation potential compared with the activated carboxy-protecting group of the amino component. This so-called “backing-off” strategy preferentially makes use of active esters as carboxy-protecting groups, and the mixed anhydride method for coupling. In this case, the N-protected carboxy component is transformed into an active ester. The N^α-protecting group is then cleaved and subsequently acylated with another N-protected amino acid to form the protected dipeptide with the active ester moiety located at the C-terminus. The mixed anhydride method is mainly applied in these cases. Successive repetition of these steps allows the formation of segments in a stepwise manner, starting from the C-terminus and without practical risk of racemization. These segments can then be connected with other segments via the activated C-terminus to give larger peptides. Carboxy-protecting groups that potentially can be activated behave like real protecting groups during the coupling reac-

tion, and the risk of undesired side reactions by uncontrolled self-condensation can be neglected in practical cases. One example of an ester that is transformed into the reactive form immediately before the envisaged coupling step is the 4-(methylthio)phenyl ester, which can be transformed oxidatively into the 4-(methylsulfonyl)phenyl ester.

Most of the currently known carboxylic protecting groups are esters derived from either primary, secondary, or tertiary alcohols (Tab. 4.2).

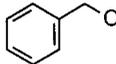
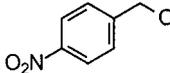
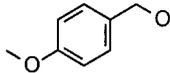
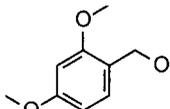
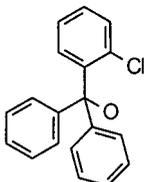
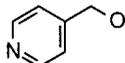
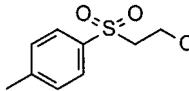
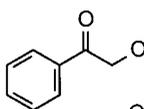
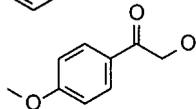
Methyl and ethyl esters have been used previously for peptide synthesis by Curtius and Fischer, and can be obtained easily on reaction of the amino acid with the corresponding alcohol and thionyl chloride at low temperature. Presumably, the alkyl chlorosulfinate (AlkO-SO-Cl) is formed as a reactive intermediate from SOCl_2 and the alcohol with cleavage of HCl . This intermediate reacts with the amino acid, liberating SO_2 and HCl to give the amino acid ester hydrochloride. Benzyl esters can be prepared in a similar manner. Using a different approach, cesium or tertiary ammonium salts of N^{α} -protected amino acids can be esterified with an alkyl halide. Methyl esters are also easily obtained with diazomethane.

Cleavage of these protecting groups on completion of a peptide synthesis proceeds by mild alkaline hydrolysis in mixtures of water and an organic solvent such as dioxan, methanol, ethanol, acetone, or DMF to solubilize the starting materials. The alkyl esters are usually applied only for the synthesis of short-chain peptides, as hydrolytic cleavage becomes very difficult with increasing chain length, and extreme saponification conditions (excess of alkali and long reaction times) are necessary. Consequently, racemization and other side reactions are observed. Enzyme-catalyzed deprotections often provide ingenious alternatives. Both methyl and ethyl ester resist hydrogenolysis and mild acidolysis. Transformation of these esters into the corresponding hydrazides (the starting materials for azide synthesis) using hydrazine is possible, as is the transformation into amides by aminolysis.

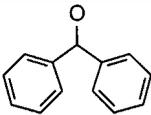
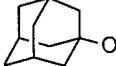
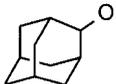
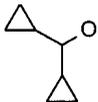
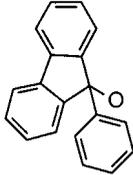
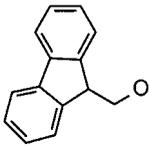
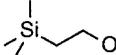
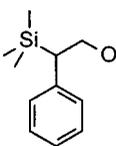
Benzyl esters of free amino acids are obtained by direct esterification with benzyl alcohol in the presence of acidic catalysts (e.g., 4-toluenesulfonic acid, hydrogen chloride, benzene sulfonic acid, polyphosphoric acid). The water formed during the course of the esterification is usually removed by azeotropic distillation (with benzene, toluene, or tetrachloromethane). Benzyl esters of N -protected amino acids are obtained after activation of the carboxylic group with N,N -dicyclohexyl carbodiimide (DCC) or thionyl chloride and reaction with benzyl alcohol, but also by transesterification reactions. Benzyl ester derivatives can be cleaved by alkaline hydrolysis, catalytic hydrogenation, treatment with saturated HBr /acetic acid (12 h at room temperature or 1–2 h at 50–60°C), by treatment with sodium in liquid ammonia, or with liquid hydrogen fluoride.

Free amino acids are transformed into the *tert*-butyl esters by addition of isobutene under acidic condition or by transesterification with *tert*-butyl acetate. The hydroxyl groups of serine or threonine are transformed under these conditions into the *tert*-butyl ethers, likewise. N -protected amino acids such as Z -amino acids can be reacted with isobutene in the presence of catalytic amounts of sulfuric acid or with *tert*-butyl acetate in the presence of perchloric acid to give the corresponding *tert*-butyl ester. The *tert*-butyl ester group, which is easily cleavable by acidolysis

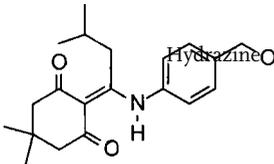
Tab. 4.2 Selected carboxy-protecting groups of the ester type Y-O-(C=O)-R.

Group	Symbol	Y-O	Cleavage conditions	Reference(s) ^{a)}
Methyl	Me		Alkaline or enzymatic hydrolysis	
Ethyl	Et		Alkaline or enzymatic hydrolysis	
Benzyl	Bzl		H ₂ /Pd, HBr/AcOH, Liq. HF, Alkaline hydrolysis	
4-Nitrobenzyl	Nbz		H ₂ /Pd, alkaline hydrolysis, stable to HBr/AcOH	
4-Methoxybenzyl	Mob		TFA/anisole, HCl/nitromethane, H ₂ /Pd, liq. HF, alkaline hydrolysis	
2,4-Dimethoxybenzyl	2,4-Dmb		1% TFA/CH ₂ Cl ₂	
o-Chlorotrityl	Trt(2-Cl)		1% TFA/CH ₂ Cl ₂	
Pyridyl-4-methyl-(4-picolyl)	Pic		H ₂ /Pd, alkaline hydrolysis, electrolytic reduction	
2-(Toluene-4-sulfonyl)-ethyl	Tse		β-elimination in H ₂ O/dioxan and Na ₂ CO ₃	
Phenacyl	Pac		Sodium thiophenolate, H ₂ /Pd, Zn/AcOH	
4-Methoxyphenacyl	Pac(OMe)		UV-photolysis at 20 °C	

Tab. 4.2 (continued)

Group	Symbol	Y-O	Cleavage conditions	Reference(s) ^{a)}
Diphenylmethyl (Benzhydryl)	Dpm		H ₂ /Pd, TFA at 0 °C, HCl/AcOH, BF ₃ ·OEt ₂ /AcOH at 25 °C	
<i>tert</i> -Butyl	tBu		TFA, HCl/AcOH, HBr/AcOH, BF ₃ ·OEt ₂ /AcOH	
Cyclohexyl	Cy		Strong acids	
1-Adamantyl	1-Ada		TFA	
2-Adamantyl	2-Ada		Strong acids	
Dicyclopropylmethyl	Dcpm		1% TFA/CH ₂ Cl ₂	82
9-Phenylfluoren-9-yl	Pf		>1% TFA/ scavenger/CH ₂ Cl ₂	75
9-Fluorenylmethyl	Fm		Piperidine, TBAF	76, 77
2-Trimethylsilylethyl	TMSE		TBAF	78, 79
2-Phenyl-2-trimethyl- silylethyl	PTMSE		TBAF	80

Tab. 4.2 (continued)

Group	Symbol	Y-O	Cleavage conditions	Reference(s) ^{a)}
Allyl	Al		Pd(0), nucleophile	41
4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]-amino}benzyl	Dmab			81

a) For additional information see [83–86].

(TFA, HCl/AcOH, $\text{BF}_3 \cdot \text{OEt}_2/\text{AcOH}$), is resistant towards hydrogenolysis and quite stable towards alkaline hydrolysis, hydrazinolysis, and ammonolysis.

A new carboxy-protecting group, which was introduced by Carpino [82], was the dicyclopropylmethyl group (Dcpm). This can be cleaved with 1% TFA in dichloromethane, even in the presence of side chain-protecting groups of the *tert*-butyl type.

The above-mentioned representatives have been used in many practical applications. Polymeric esters in either soluble or insoluble form represent the prototype of anchoring groups in peptide synthesis on solid supports. Consequently, this type of carboxy-protecting group is of crucial importance from a methodological point of view. The carboxy-protecting groups mentioned can be used both as α - and as ω -carboxy-protecting groups in the case of amino dicarboxylic acids. Peptides with a C-terminal amino dicarboxylic acid can be assembled very easily, because in this case both carboxy groups can be blocked for solution synthesis with the same protecting group. Peptides with an internal or N-terminal amino dicarboxylic acid cannot be assembled without taking differential blocking measures [83–86].

In certain specialized cases, for example the head-to-tail cyclization of peptides (cf. Section 6.1), the use is required of special carboxy-protecting groups with a third dimension in orthogonality. This may rely on differences in cleavage kinetics, as are present in the combination of super acid-sensitive esters such as 2,4-dimethoxybenzyl ester (2,4-Dmb), 2-chlorotrityl [Trt(2-Cl)] or the corresponding linkers with Fmoc/tBu chemistry. Different cleavage mechanisms lead to complete orthogonality, as for example in the application of allyl esters or the Dmab group [81] in Fmoc/tBu chemistry.

4.2.2.2 Amides and Hydrazides

Although hydrazides may also be used for C ^{α} and side-chain carboxy group protection, further azide couplings are usually obligatory under such circumstances. N'-substituted hydrazides are also of great importance in this context. The prepa-

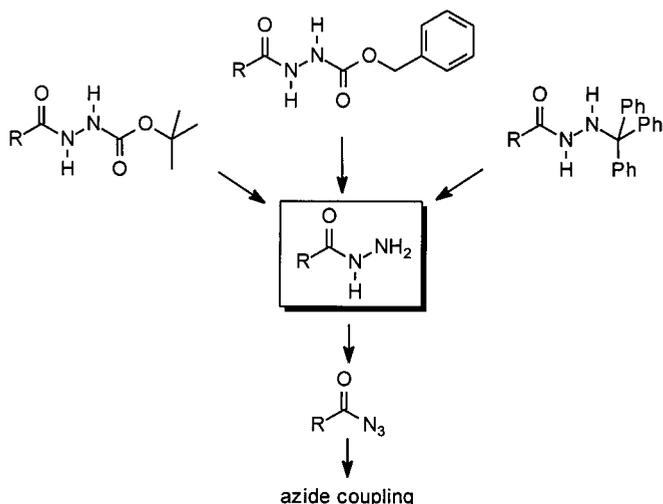


Fig. 4.10 Differently protected hydrazide groups as potentially activatable carboxy-protecting groups. R-CO=peptidyl residue.

ration is achieved by reaction of the N-protected amino acid with a partially blocked hydrazine derivative using the carbodiimide or mixed anhydride method. After cleavage of the amino-protecting group, the corresponding derivative is used for a segment synthesis and selective deblocking of the hydrazide protecting group is performed on completion of the synthesis. The resulting N-terminally protected, C-terminal hydrazide can then be transformed into the azide and used as an activated carboxy component (Fig. 4.10).

Phenylhydrazides of protected amino acids or peptides are cleaved oxidatively by a tyrosinase isolated from mushrooms to provide the free acid [87].

Carboxamides principally are suited as carboxy-protected amino acid or peptide derivatives. Selective chemical methods for cleavage have been lacking before highly specific peptide amidases became available. The carboxamide group is more easily hydrolyzed than a peptide bond, although because of the rather small differences selective cleavage is seldom successful.

4.2.3

C-Terminal and Backbone N^α-carboxamide Protection

Additional protection of backbone amide groups may be necessary to avoid prominent side reactions such as aspartimide formation (cf. Sections 4.2.4.3 and 4.2.4.9) or interchain aggregation of the resin-bound peptides containing “difficult sequences” (cf. Section 4.5.4.3). This modification appears to prevent or disrupt unwanted hydrogen-bonding networks, both by removal of the native amide hydrogen and by alteration of the backbone conformation based on the tertiary amide bond that is formed. Weygand et al. [88] used the 2,4-dimethoxybenzyl group for

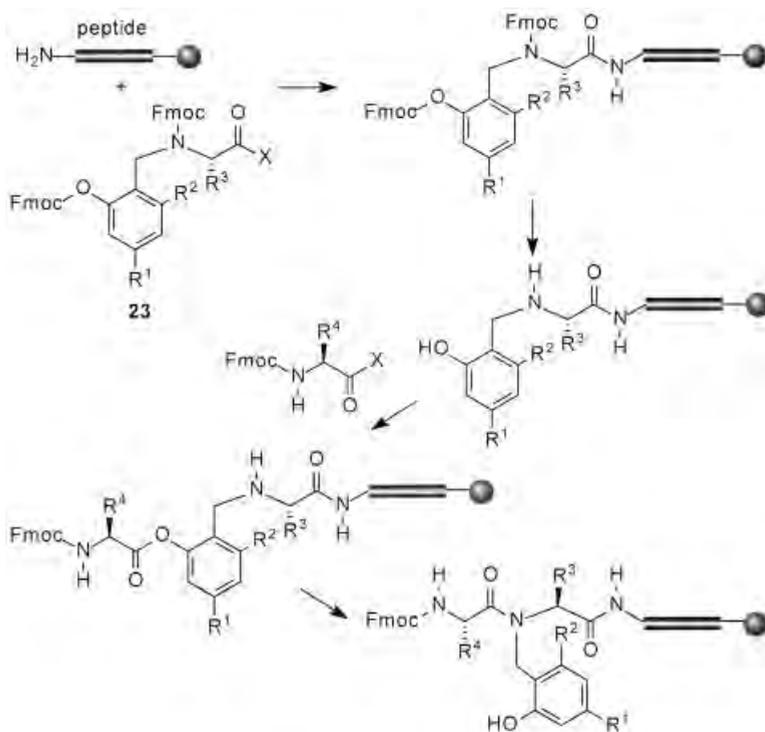
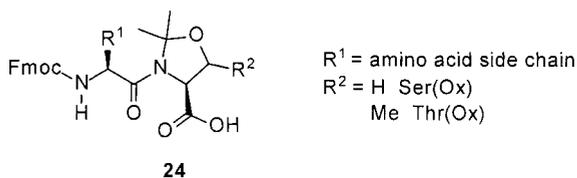


Fig. 4.11 Acyl capture and O \rightarrow N acyl transfer involving Hmb (R^1 =OMe; R^2 =H) or Hnb (R^1 =H; R^2 =NO₂). R^3 , R^4 =amino acid side chains; X=activating group.

this purpose. Based on the reversible N^{α} -substitution effect studied by Weygand et al. [88], Narita et al. [89], and Bartl et al. [90], an elegant solution to this problem of difficult sequences was developed by Sheppard et al. These authors introduced the 2-hydroxy-4-methoxybenzyl group (Hmb) that has proven very useful for this purpose [91, 92]. It comprises an acid-labile N-benzyl-type protecting group with an additional 2-hydroxy substituent. Therefore, acyl capture of an activated amino acid by this substituent is followed by peptide bond-forming intramolecular O \rightarrow N acyl transfer (Fig. 4.11). Improved acyl transfer kinetics is observed in the case of the photolabile 2-hydroxy-6-nitrobenzyl group (Hnb) [93]. The incorporation of either Hmb or Hnb or 3-methylsulfinyl-4-methoxy-6-hydroxybenzyl (SiMb)-protected derivatives [94] at every fifth to seventh sequence position during SPPS using preformed *N*, *O*-bis-(Fmoc)-*N*-(Hmb/Hnb) amino acid derivatives **23** seems to be sufficient in order to suppress aggregation.

Alternatively, pseudoproline dipeptide building blocks Fmoc-Xaa-Ser(Ox)-OH or Fmoc-Xaa-Thr(Ox)-OH **24**, as developed by Mutter et al. [95–97], may be employed. This approach has been reported to be superior to Hmb protection, provided that the sequence is compatible with the incorporation of the pseudoproline dipeptides analogues [98].



4.2.4

Side-chain Protection

As the side chain functionality of an amino acid exerts a crucial influence on the reactivity of the α -amino or α -carboxy group, it is especially important that those side-chain functionalities which give rise to undesired side reactions be protected in any situation. In particular, ω -amino or ω -carboxy groups of diamino carboxylic acids or amino dicarboxylic acids must be mentioned in this context. Selective blocking represents a crucial problem in peptide chemistry, and selection of the protecting group combination is a question that must be addressed by synthesis tactics. The ω -protecting groups of trifunctional amino acids are termed “semi-permanent protecting groups” (see Section 4.1.2) because they are usually only cleaved on completion of peptide synthesis. The thiol group of cysteine, and usually also the guanidino group of arginine, also require semipermanent masking. In other cases, side reactions arising from side-chain functional groups can be suppressed or minimized to a certain extent by using special reaction conditions. Despite this possibility of minimal protection, maximum protection is preferred in a practical sense, and this applies especially to SPPS.

4.2.4.1 **Guanidino Protection**

Although the guanidino group of arginine is usually protected by protonation under normal reaction conditions due to its strongly basic character, the low solubility of the corresponding derivatives in organic solvents hampers peptide synthesis. Partial acylation of the guanidino group may occur under these conditions, and represents a crucial disadvantage.

Derivatives of arginine hydrobromide can be used in principle as amino components in peptide synthesis. If required, N^α -benzyloxycarbonyl-protected arginine hydrobromide can be obtained very easily by treatment of Z-Arg-OH with 1.4 N HBr in methanol and subsequent precipitation with absolute ether.

Ideally, all three side-chain nitrogen atoms should be protected, but most strategies rely on N^ω - or N^α -, N^δ -protection, respectively. Despite the existence of a variety of blocking possibilities described for the strongly basic guanidino group, no ideal protecting group exists until now. The different protecting groups can be divided into four classes: nitro; urethane (acyl); arenesulfonyl; and trityl types. However, the latter derivatives are poorly soluble in organic solvents and hence are not widely used.

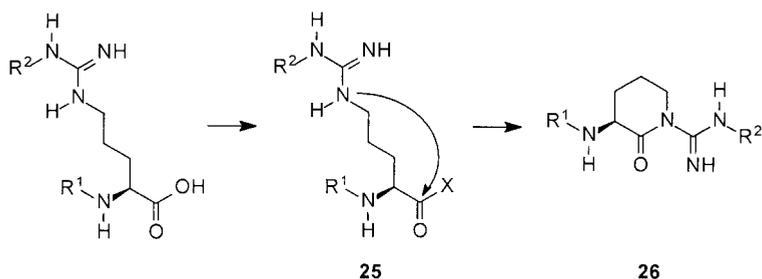


Fig. 4.12 δ -Lactam formation in carboxy-activated arginine derivatives.

The ω -nitro group is stable toward TFA, HBr/AcOH, and alkali. Treatment with liquid HF and several reductive methods (Zn/AcOH, electrolysis) or catalytic hydrogenation with Pd or Raney-nickel are suitable methods for cleavage. ω -Nitroarginine is prone to other side reactions during acylation and cleavage. Lactam formation (Fig. 4.12; **25** \rightarrow **26**) and consecutive aminolysis have been observed upon activation of protected ω -nitro arginine derivatives ($\text{R}^2 = \text{NO}_2$).

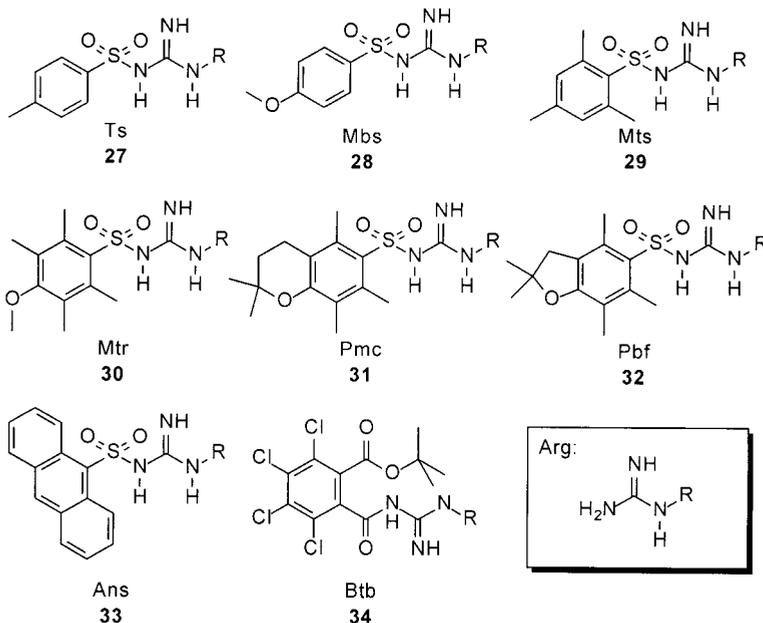
Protecting groups of the urethane type have been successfully applied to achieve N^ω -mono-acylation. In this case, the synthesis of disubstituted $\text{N}^\alpha, \text{N}^\omega$ -diacyl compounds can be performed most easily.

Z-Arg(Z)-OH can only be coupled with hydrochlorides of the amino component (additional protonation of the guanidino group) to give peptide derivatives because in other cases lactam formation by attack of the N^δ to the activated carboxy group has been observed.

Differently substituted $\text{N}^\alpha, \text{N}^\omega$ -diacyl derivatives such as Z-Arg[Z(4- NO_2)]-OH or Z-Arg(Boc)-OH [99] show very little tendency towards lactam formation. The differences in deblocking selectivity – the 4-nitrobenzyloxycarbonyl group is stable towards HBr/AcOH – permit a variety of peptide synthetic applications. Derivatives on the basis of Z-Arg(ω, δ -Z₂)-OH, and especially the corresponding 4-nitrophenyl- or N-hydroxysuccinimidyl esters, are excellent building blocks for arginine-containing peptides [83]. The $\text{N}^\omega, \text{N}^\delta$ -di(1-adamantylloxycarbonyl) derivative Adoc is obtained from Z-Arg-OH and 1-adamantyl chloroformate [100]. Subsequently, different N^α -protecting groups may be introduced after hydrogenolytic cleavage of the Z group. However, the guanidino group is in this protection scheme and in the case of the N^ω -Boc derivative still sufficiently nucleophilic to be acylated. Consequently, substantial conversion of arginine residues to ornithine residues via acylation of the unprotected ω -nitrogen during coupling and subsequent intramolecular decomposition during deprotection has been observed [101]. $\text{N}^\omega, \text{N}^\delta$ -Bis-Boc protection is not in all cases a viable alternative because of the rather poor coupling efficiency.

A single arene sulfonyl moiety offers complete protection of the guanidino group. Consequently, members of this class belong to the most commonly used guanidino protecting groups. Albeit undesired lactam formation upon activation of the carboxy group cannot be excluded completely by N^ω -monoprotection using

arene sulfonyl groups, it may be suppressed sufficiently by the application of additives such as 1-hydroxybenzotriazole. The 4-toluenesulfonyl group **27** can only be removed by acidolysis with liquid HF or by reduction with Na/liquid NH₃. Arene sulfonyl-type protecting groups for the guanidino function have been modified with respect to acid lability by the introduction of electron-donating substituents on the aryl residue. Groups such as 4-methoxybenzenesulfonyl **28** (Mbs) [102], 2,4,6-trimethylbenzenesulfonyl **29** (Mts) [103], 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) **30** [104], 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) **31** [105], and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) **32** [106] have become very popular in SPPS.

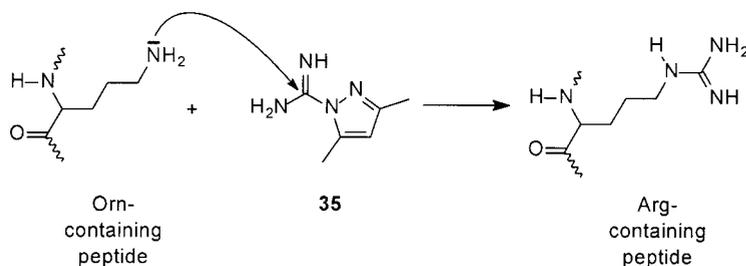


Acid lability increases in the series Tos < Mbs < Mts < Mtr < Pmc < Pbf, with Pbf displaying the best deprotection kinetics.

The Mts group is highly compatible with Boc as the temporary protecting group and can (unlike Tos) be removed with trifluoromethane sulfonic acid (TFMSA)/TFA/thioanisole. Mtr has often been used in the Fmoc tactics, but requires prolonged cleavage time with TFA/thioanisole. The acid lability of the Pmc group is similar to that of the Boc group; therefore, Boc (e.g., for Lys) and Pmc or Pbf (for Arg) may be advantageously combined as side chain-protecting groups. The risk of tryptophan, serine, or threonine sulfonation has been shown to be minimized by the addition of suitable scavengers [107].

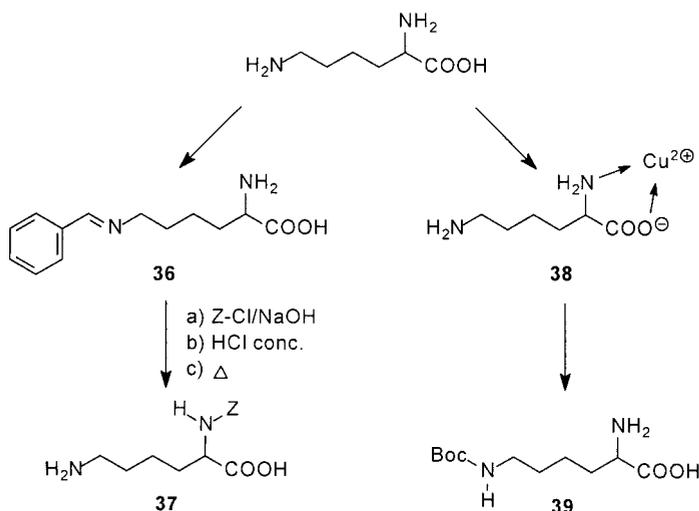
The 9-anthracenesulfonyl (Ans) group **33**, which is cleaved by mild reducing agents [108], and the 1-*tert*-butoxycarbonyl-2,3,4,5-tetrachlorobenzoyl (Btb) group **34** [109] have also been successfully applied in both liquid-phase and solid-phase peptide synthesis.

Alternatively, the guanidino group can be introduced by selective guanylation of ornithine-containing peptides on completion of peptide synthesis. Besides other guanylation reagents, 1-amidino-3,5-dimethylpyrazole **35** is well suited for this purpose.



4.2.4.2 ω -Amino Protection

Diaminocarboxylic acids such as lysine and ornithine often require orthogonal protection schemes for the α - and ω -amino groups, as the protection of the ϵ -amino group is mandatory. Appropriate protecting groups are presented in Tab. 4.1. The ϵ -amino group of lysine is more basic and more nucleophilic than the α -amino group, and can be converted into the ϵ -imine **36** with one equivalent of benzaldehyde in the presence of one equivalent of LiOH. Under basic reaction conditions the free α -amino group can then be selectively acylated. Subsequent cleavage of the imine to form, e.g., Z-Lys(H)-OH **37** allows for orthogonal protection of the ϵ -amino group, for example with Boc₂O to give Z-Lys(Boc)-OH. Alternatively, the α -amino and α -carboxy groups may be simultaneously deactivated by the formation of a chelate complex **38** with copper(II) ions **38**. Subsequently, the ϵ -amino group can then be selectively acylated to give, for example, H-Lys(Boc)-OH **39**.



In Boc tactics, the N^ε-amino group of lysine usually is protected with the 2-chlorobenzoyloxycarbonyl group [Z(2-Cl)], which displays 60-fold less acid lability compared to the Z group. This prevents premature deprotection during acidolytic Boc cleavage in the course of peptide chain assembly.

In the Fmoc approach, lysine side-chain protection as the Boc derivative can be regarded as the optimum combination.

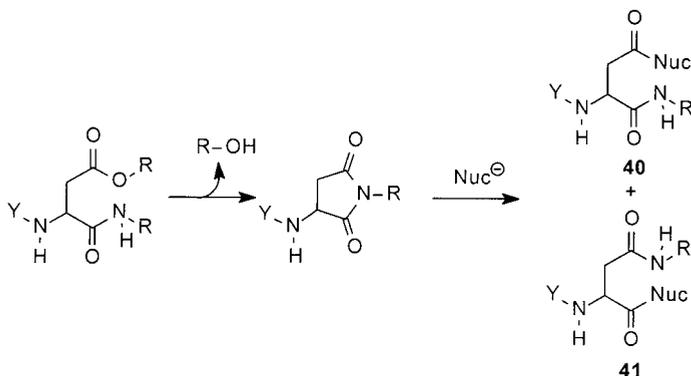
On occasion, completely orthogonal protection of the N^ε-amino group is necessary, and in these special cases the trifluoroacetyl group (Tfa), the 3-nitro-2-pyridinesulfonyl group (Npys), the 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) **18** [68, 69], or the allyloxycarbonyl (Aloc) [40, 42] groups are available. The Tfa and Npys groups are very compatible with Boc chemistry, as cleavage occurs in the first case with piperidine and in the latter case with triphenylphosphine or 2-pyridinethiol-1-oxide. Dde **18** (R²=CH₃) and the related ivDde **18** (R²=iBu) [70] groups are orthogonal to Boc and Fmoc, because they are removed with dilute hydrazine solutions. Cleavage results in the formation of a pyrazole derivative, and this can be monitored spectroscopically at 300 nm.

Aloc is an orthogonal protecting group to be applied both in Boc and Fmoc chemistry, as deprotection occurs upon treatment with a suitable nucleophile in the presence of a palladium catalyst.

A new relay deprotection strategy using the PTnm group (Tab. 4.1) for N^ε-amino protection of lysine has been developed by Ramage et al. [46].

4.2.4.3 ω-Carboxy Protection

Orthogonal protection is required in most cases for the aminodicarboxylic acids aspartic acid and glutamic acid. Formation of isoaspartyl peptides via the corresponding succinimide intermediate is the most prominent side reaction of Asp side-chain esters. Here, the nitrogen atom of the amino acid located C-terminally with respect to the Asp residue attacks the Asp side-chain ester moiety. Subsequent ring opening with a nucleophile (e.g., water or amines such as piperidine) yields a mixture of α-aspartyl **40** and β-aspartyl peptides **41** [110], a phenomenon that is also observed upon aging of Asp-containing proteins in biological systems.

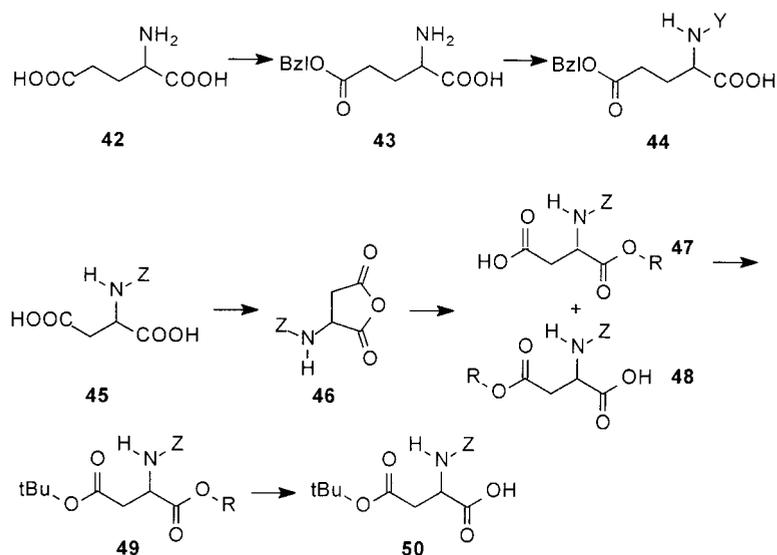


This problem may in most cases be circumvented in Fmoc chemistry by application of the β -*tert*-butyl ester of Asp. However, several publications have reported that, under certain circumstances, aspartimide formation and subsequent side reactions occur in Fmoc protocols, even with *tert*-butyl-protected derivatives. Aspartimide formation appears to be sequence-dependent [111, 112]. The main factors influencing this side reaction are the base employed, the β -carboxy protecting group, and the residue located C-terminally next to the Asp residue. Sequences containing Asp(OtBu)-Gly, Asp(OtBu)-Thr(*t*Bu), Asp(OtBu)-Cys(Acm), Asp(OtBu)-Asn(*Trt*), and Asp(OtBu)-Asp(OtBu), respectively, seem to be most problematic [113]. The base deprotonates the Asp-Xaa amide group, which then attacks the side-chain ester. Aspartimides are prone to base-catalyzed epimerization and to nucleophilic ring opening (e.g., by piperidine), giving mixtures of α - and β -piperidines, aspartyl- and isoaspartyl peptides together with the corresponding diastereomers formed by epimerization. Addition of HOBT has been shown to minimize these side reactions, but if they cannot be completely suppressed then additional protection (e.g., with Hmb, Hnb) of the amide nitrogen becomes obligatory (cf. Section 4.2.3).

In Boc chemistry the β -cyclohexyl ester Asp(OCy) that is nowadays favored over the benzyl ester efficiently prevents aspartimide formation, even in sensitive sequences. Prolonged treatment with strong acids during protecting group cleavage may, however, be accompanied by cyclization to the aspartimide.

Glutamic acid usually is protected at the side chain with the same groups as used for aspartic acid. Glu derivatives are less prone to undergo this type of cyclization; however, during HF cleavage in Boc chemistry dehydration to the acylium ion and further side reactions are sometimes observed.

Regioselective acid-catalyzed esterification at the ω -carboxy group (e.g., with benzyl alcohol) can be achieved using H_2SO_4 as the acid, and yields approximately 75% of the desired side-chain ester derivative **43** of glutamic acid **42**. This mono-



ester can then be converted into the N^α-urethane-protected derivative **44**. The protocol may also be applied to derivatives of aspartic acid.

Alternatively, Z-protected aspartic acid is converted into the cyclic anhydride **46** upon treatment with acetic acid. After alcoholysis and separation of the two regioisomers **47/48**, the α ester Z-Asp(OH)-OR **47** is converted to the *tert*-butyl derivative Z-Asp(OtBu)-OR **49** and subsequently saponified by alkali to give Z-Asp(OtBu)-OH **50**. The Z group may then be exchanged by other amino-protecting groups such as Fmoc [114].

The β-2,4-dimethyl-3-pentyl ester has been suggested as novel side chain-protecting group for aspartic acid, and is supposed to prevent undesired side reactions – especially during solid-phase synthesis [115]. If orthogonal protection of the side chains of Asp and Glu is required, viable alternatives are available with the base-labile 9-fluorenylmethyl ester (OFm) [77], the allyl ester (OAl) [40], which is cleaved by palladium-catalyzed transfer to a suitable nucleophile, and Dmab, which is cleaved by hydrazinolysis [81].

4.2.4.4 Thiol Protection [116]

The structural requirements of cysteine-containing peptides further complicate peptide synthesis. In some cases, the Cys residue is needed in its free thiol form, whilst other peptide products require regioselective intra- or intermolecular disulfide bridge formation. Solutions to this problem will be addressed in Section 6.2.

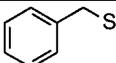
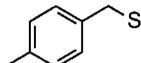
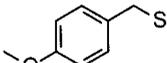
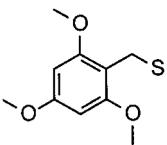
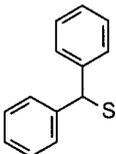
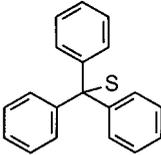
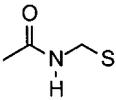
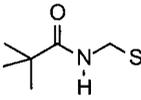
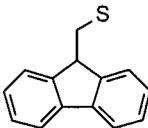
The high nucleophilicity, the ease of oxidation, and the acidic character of the cysteine thiol group require selective, semipermanent blocking of this functional group during all synthetic operations (Tab. 4.3).

The S-benzyl group was introduced in 1930 by du Vigneaud in peptide chemistry for the synthesis of oxytocin, and proved to be a major breakthrough for further synthetic projects involving peptides containing either cysteine or cystine. As this protecting group can be cleaved only by reduction with sodium in liquid ammonia, significant damage to longer peptides may occur because of the rather drastic reaction conditions, as was observed in the first total synthesis of insulin.

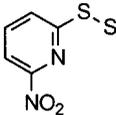
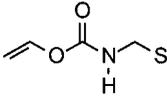
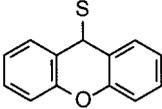
S-benzyl-protected cysteine residues are especially prone to racemization, however. Epimerization has been observed for resin attachment of protected (Acm, tBu, Trt, StBu, Tacm, Bzl(4-Me)) Cys residues and even for internal Cys residues during repetitive cleavage of the N^α-protecting group in Fmoc tactics [138–140].

Several substituted benzyl derivatives have been introduced into peptide chemistry in order to facilitate cleavage of S-benzyl-type protecting groups. The 4-methylbenzyl residue [Bzl(4-Me)] [118] is cleaved by strong acids and thallium trifluoroacetate, respectively. Higher acid lability is displayed by the 4-methoxybenzyl derivative Mob, which is stable toward TFA and iodine, respectively, but is cleaved by boiling TFA, HF, Na/liq. NH₃, mercury(II) salts, TFMSA, etc. The 4-methylbenzyl and the 4-methoxybenzyl derivatives require treatment with liquid HF for cleavage and, therefore, often are employed in Boc chemistry. The 2,4,6-trimethylbenzyl (Tmb) group and the 2,4,6-trimethoxybenzyl group are cleaved by acidolysis with TFA and consequently are compatible with Fmoc chemistry [120]. Further poten-

Tab. 4.3 Thiol-protecting groups Y-SR.

Group	Symbol	Y-S	Cleavage conditions	Reference(s)
Benzyl	Bzl		Na/liq. NH ₃	117
4-Methylbenzyl	Bzl(4-Me)		Strong acids TI(TFA) ₃	118
4-Methoxybenzyl	Mob		HF TFA, TFMSA Hg ^{II}	119
2,4,6-Trimethoxybenzyl	Tmb		TFA	120
Diphenylmethyl	Dpm		TFA HBr/AcOH HF Nps-Cl followed by reduction (B/C) ^a	121, 122
Trityl	Trt		HF, TFA, TFA/AcOH I ₂ , Hg ^{II} AgNO ₃ Nps-Cl, then reduction (A/B/C) ^a	123– 126
<i>tert</i> -Butyl	tBu		Nps-Cl	127
Acetamidomethyl	Acm		Hg ^{II} I ₂ , Tl ^{III} Nps-Cl followed by reduction (A/B/C) ^a	128, 129
Trimethylacetamidomethyl	Tacm		Hg ^{II} I ₂ , AgBF ₄	130
9-Fluorenylmethyl	Fm		Piperidine	77

Tab. 4.3 (continued)

Group	Symbol	Y-S	Cleavage conditions	Refer- ence(s)
<i>tert</i> -Butylsulfanyl	StBu		Thiolysis Phosphines	131– 134
3-Nitro-2-pyridyl- sulfanyl	Npys		Thiolysis "low" HF	135
Allyloxycarbonyl- aminomethyl	Alocam		Pd ⁰ /Bu ₃ SnH	136
9 <i>H</i> -Xanthen-9-yl	Xan		TFA/CH ₂ Cl ₂ /Et ₃ SiH I ₂	137

a) A=iodolysis; B=rhodanolysis; C=Kamber method.

tial representatives of this class of compounds have been developed as acid-labile thiol-protecting groups. However, many of them were found to be too resistant toward acids and so were not used in any practical application.

Thiol-protecting groups of the S-acyl type do not have any practical relevance, because acyl migrations from sulfur to nitrogen are energetically favored and represent prominent side reactions.

The triphenylmethyl group (trityl group, Trt) has received much attention, as it may be cleaved by silver salts, mercury(II) salts, iodine, or TFA in the presence of ethanethiol, alkyl sulfenyl chlorides, etc. Deprotection with TFA provides the free thiol, which is in particular useful in the synthesis of peptide antigens for conjugation to carrier proteins. Considerable base-catalyzed racemization has been observed upon in-situ activation of Fmoc-Cys(Trt)-OH with uronium reagents such as TBTU [141, 142].

The *tert*-butyl group has also been used for thiol protection [127, 143] as it is labile towards both TFMSA and mercury(II) salts.

A different type of thiol-protecting group is found in the N-acyl N,S-acetals, most of which are compatible with both Boc and Fmoc chemistry. The acetamidomethyl (Acm) group is completely stable towards acidolysis, and is removed by treatment with mercury(II) salts at pH 4, thallium(III) trifluoroacetate, or iodine. Oxidizing agents such as iodine simultaneously induce disulfide formation. Structural analogues of the Acm group are the chloroacetamidomethyl group, the isobutyrylamidomethyl group, and the 2,2-dimethylpropionamidomethyl group (trimethylacetamidomethyl group, Tacm) [130]. The combination of thiol-protecting

groups with incongruous cleavage conditions enables differential disulfide formation in peptides containing more than two cysteine residues.

In particular, Trt, Acn, and Tacn represent thiol-protecting groups of eminent importance, as they allow for a simultaneous oxidative coupling to give cystine derivatives. Iodolysis (treatment with iodine, method A, Tab. 4.3 [144]), rhodanolysis (treatment with rhodanides, method B, Tab. 4.3 [145]), and the Kamber method, method C, Tab. 4.3 [146] are suitable for the simultaneous conversion of a thiol-protected peptide to the corresponding cystine (disulfide) derivative without separate deprotection. Oxidative cleavage by iodine, however, may be accompanied by methionine S-oxidation, iodination of Tyr residues [147], and the formation of tryptophan thioesters [148]. Additionally, iodine or thallium(III) oxidation may cause Acn migration from Cys to Ser, Thr, or Gln side chains [149].

Disulfide derivatives also serve as protecting groups for thiols. The ethylsulfanyl (R=EtS), the *tert*-butylsulfanyl (R=tBuS), and the 3-nitro-2-pyridylsulfanyl groups belong to this class of unsymmetrical disulfides, and can be selectively reduced by thiols such as 2-mercaptoethanol or by phosphines. In the former case of a thiol-disulfide exchange reaction, the excess of a free thiol acting as a deblocking reagent shifts the equilibrium in the desired direction. Removal of the S-alkylthio group occurs selectively, as many other protecting groups (including several thiol-protecting groups) are stable toward thiolysis.

The general protection scheme of thiol groups by an S-sulfo moiety (S-SO₃⁻) should also be mentioned in this context [150], because the corresponding cysteine S-sulfo derivatives obtained from oxidative sulfolysis from S-alkylthio or S-arylthio cysteine derivatives possess sufficient stability for some peptide synthetic procedures. This temporary blocking is removed by reduction with thioglycolic acid at pH 5.

Protected cysteine residues are prone to several side reactions: β-elimination leading to the formation of dehydroalanine derivatives is favored under basic reaction conditions where subsequent conjugate addition of nucleophiles such as piperidine takes place. Oxidation and alkylation may also occur.

More than 70 variants for the protection of the cysteine thiol group have been described, and further details may be found in several monographs [83, 116]. An interesting thiol-protecting scheme for the formation of intrachain disulfide bridges has been demonstrated in an endothelin synthesis [151].

4.2.4.5 Imidazole Protection

Histidine is one of the most problematic building blocks in peptide synthesis [152]. Side reactions may occur when protection of the imidazole ring of histidine is omitted, because these derivatives are easily N-acylated. Some histidine derivatives are also relatively insoluble in the solvents normally used in peptide synthesis. Undesired side reactions such as racemization, formation of cyclic imidazolides, and N-guanylations of the imidazole ring have been observed, especially upon activation of C-terminal histidine residues. These problems may be avoided by reversible masking of the imidazole function (Tab. 4.4).

Tab. 4.4 Imidazole protecting groups Y-N^{im}.

Group	Symbol	Y-N ^{im}	Cleavage conditions	Reference(s)
Benzyl	Bzl		Na/liq. NH ₃	153
2,4-Dinitrophenyl	Dnp		2-mercaptoethanol, thiophenol stable to acids	154
Benzyloxymethyl	Bom		strong acids	155
<i>tert</i> -Butoxycarbonyl	Boc		TFA, HBr/AcOH HF stable to HCl/dioxan	156
Adamantyl-1-oxycarbonyl	Adoc		TFA	25, 157
Triphenylmethyl	Trt		TFA	158, 159
Diphenylmethyl	Dpm		6 N HBr/AcOH (3 h) HCOOH (10 min) TFA (1 h)	160
Pyridyldiphenylmethyl	Pdpm		catalytic or electrolytic reduction Zn/AcOH	77
4-Toluenesulfonyl (Tosyl)	Tos		strong acids	161
4-Methoxybenzenesulfonyl	Mbs		TFA/(CH ₃) ₂ S	162
<i>tert</i> -Butoxymethyl	Bum		TFA stable to piperidine	163
Allyl	Al		Pd ⁰ , nucleophile	164
Allyloxymethyl	Alom		Pd ⁰ , nucleophile	165

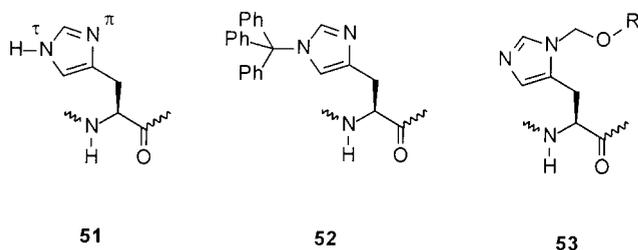
In the past, the imidazole group was protected mainly as the N-benzyl derivative – a method that suffers from a very limited scope of cleavage conditions. This problem was overcome by the development of other protecting groups such as the 2,4-dinitrophenyl group, which is acid stable and can be cleaved by thiolysis (2-mercaptoethanol at pH 8 for 1 h; thiophenol) or by hydrazinolysis.

Imidazole-protecting groups of the urethane type (Z, Boc, Adoc) are stable towards HCl in organic solvents, but as N-acyl imidazolides they may give rise to undesired acyl transfer reactions. The pronounced lability towards aminolysis and hydrolysis leads to problems with these permanent protecting groups during other deblocking reactions.

The N^{imm} Boc group is stable toward piperidine and is, therefore, suited for the application in SPPS according to Fmoc tactics. It is cleaved by HBr/HOAc, TFA, or liquid HF, respectively. The structurally related Adoc group is removed by treatment with TFA.

Arylsulfonyl groups have increasingly received attention as imidazole-protecting groups. The N^{imm}-(4-toluenesulfonyl) group often is used in combination with Boc as the temporary protecting group. It is cleaved under the general deprotection conditions with liquid HF or TFMSA, but it has been reported to be susceptible to cleavage by HOBt. Modification of the aryl residue with electron-donating groups increases the acid lability of the arylsulfonyl protecting group, as has already been discussed in connection with the arylsulfonyl-based guanidino protecting groups. The 4-methoxybenzenesulfonyl (Mbs) group and the 2,4,5-trimethylbenzenesulfonyl group, respectively, are labile toward TFA in the presence of dimethylsulfide.

Some confusion exists in the literature concerning protection of the two heterocyclic nitrogen atoms (N^π/N^τ, 51). While the two regioisomeric derivatives are formed during alkylation in a ratio of 3:1, steric hindrance of the alkylation reagent favors N^τ-alkylation. Trityl chloride reacts regioselectively at the N^τ position, and N^τ-trityl-protected histidine 52 belongs to the most suitable acid-labile protecting groups that are very compatible with Fmoc tactics.



The trityl residue [158, 159] is stable to the conditions of coupling and deprotection during Fmoc synthesis. It can be cleaved with dilute acetic acid at slightly elevated temperatures or with TFA at room temperature, but not with 1 N HCl at room temperature. Surprisingly, the lack of N^π-protection in the N^τ-trityl derivatives does not favor racemization at C^α of the corresponding carboxy-activated histidine derivatives, although usually only N^π-protection is regarded to be devoid of racemization.

Although the N^{τ} -trityl group prevents racemization by its steric bulk to a certain extent [166], epimerization has nevertheless been observed, for example upon condensation of Fmoc-His(Trt)-OH to a 4-benzyloxybenzylalcohol linker [167].

In this respect, the N^{π} -benzyloxymethyl (Bom) group **53** (R=benzyl) is often used in combination with N^{α} -Boc protection [168]. This N^{π} protection of the imidazole significantly decreases the risk of racemization [169]. The Bom group is resistant toward TFA, but it can be easily cleaved with HF, TFMSA, or HBr/HOAc – which renders it especially suited for Boc tactics. Formaldehyde is liberated during HF deprotection, and may damage residues such as N-terminally located cysteine.

The N^{π} -*tert*-butoxymethyl (Bum) group **53** (R=*tert*-butyl) that may be applied in N^{α} -Fmoc tactics is characterized by higher acid lability, though the preparation of the protected derivative is less straightforward. Application of the N^{π} -allyl [164] and N^{π} -allyloxymethyl [165] groups has also been suggested.

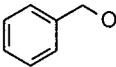
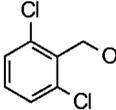
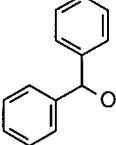
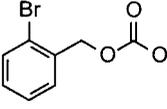
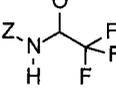
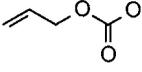
4.2.4.6 Hydroxy Protection

In principle, protection of the primary and secondary hydroxy function of serine and threonine (and of the aromatic hydroxy group of tyrosine) is not necessary, especially when these residues are located in the amino component. However, a substantial excess of the acyl component, as is generally used for example in SPPS, may lead to partial O-acylation (especially of tyrosine residues) as the basic reaction conditions generate the highly nucleophilic phenolate anion. The azide method was regarded as the most useful variant for the activation of hydroxy-substituted amino acids and of peptides with C-terminal hydroxy-substituted amino acids. However, aromatic nitrosations in the case of tyrosine or side reactions based on Curtius rearrangements have been observed during acyl azide synthesis. Tyrosine is also prone to electrophilic aromatic substitution during deprotection procedures. An increased risk of racemization has been reported for carboxy components containing C-terminal tyrosine.

The application of active esters originally failed with these derivatives, as N-protected active esters with unprotected hydroxyl side-chain functionalities could not be obtained in sufficient purity. However, nowadays the corresponding 2,4-dinitrophenylesters and other active ester derivatives (e.g., Z-Ser-OPcp, Boc-Thr-OSu) can be obtained satisfactorily. On occasion, the syntheses applying components with free hydroxy groups suffer from further undesired side reactions, such as N \rightarrow O aminoacyl migrations, O-acylations upon deprotection (e.g., with HBr/HOAc), and racemization under the basic conditions of hydrazinolysis. In any case, the advantages of a global protection scheme with the imminent risk of incomplete deprotection reactions should be compared individually to the above-mentioned strategy with partial protection.

The blocking of hydroxy groups with benzyl or *tert*-butyl type groups is of eminent practical importance (Tab. 4.5). O-Benzyl threonine is obtained from threonine by reaction with benzyl alcohol in the presence of 4-toluenesulfonic acid and subsequent alkaline saponification of the concomitantly formed benzyl ester [180]. O-Benzyl tyrosine is formed by direct alkylation of the tyrosine-copper complex with benzyl

Tab. 4.5 Hydroxy-protecting groups Y-OR.

Group	Symbol	Y-O	Cleavage conditions	Reference(s)
Benzyl	Bzl		H ₂ /Pd HF Na/liq. NH ₃ HBr/dioxan	170
2,6-Dichlorobenzyl	Dcb		Strong acids, stable to TFA	171
Diphenylmethyl (benzhydryl)	Dpm		H ₂ /Pd TFA (reflux)	172
Cyclohexyl	Cy		TFMSA/thioanisole	173
2-Bromobenzyloxy- carbonyl	Z(2-Br)		Strong acids, stable to TFA	174, 175
<i>tert</i> -Butyl	tBu		TFA HCl/TFA conc. HCl (0 °C, 10 min)	176
1-Benzyloxycarbonyl- amino-2,2,2-trifluoro- ethyl	Zte		H ₂ /Pd Acidolysis	177
Methylthiomethyl	Mtm		CH ₃ I in wet acetone (NaHCO ₃)	178
Allyl	Al		Pd ⁰ , nucleophile	41
Allyloxycarbonyl	Aloc		Pd ⁰ , nucleophile	41, 179

bromide [181]. Interestingly, cleavage of the O-benzyl group in Z-Tyr(Bzl)-MeO during hydrogenolysis can be efficiently suppressed by the addition of 2,2'-dipyridyl [182].

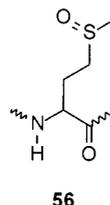
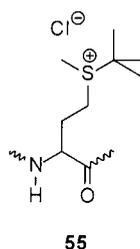
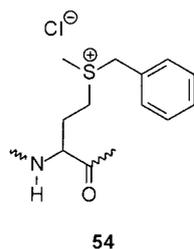
tert-Butyl ethers of hydroxy amino acids are very easily accessible by acid-catalyzed *tert*-butylation of amino acid benzyl esters with isobutene and subsequent hydrogenolysis [183].

O-Benzyl groups can be cleaved by hydrogenolysis or with liquid HF, and hence are appropriate for Boc chemistry. During acidolysis, highly reactive benzyl cations are formed that often give rise to side reactions, but this can be suppressed by the addition of cation scavengers such as anisole or resorcinol. Furthermore, acidolytic deprotection reactions sometimes may be incomplete. Tyrosine residues to be used for Boc chemistry are normally protected using either the 2,6-dichlorobenzyl (Dcb, Tab. 4.5) or the 2-bromobenzyloxycarbonyl (Z(2-Br), Tab. 4.5) groups. The 2-bromobenzyloxycarbonyl group [174, 175] is stable towards nucleophiles and TFA, and can be removed together with other side chain-protecting groups under strongly acidolytic conditions on completion of the synthesis.

The *tert*-butyl ether of Ser, Thr, or Tyr residues is very compatible with Fmoc chemistry. It is removed with TFA (1–2 h at room temperature), with HCl/TFA, and with concentrated HCl at 0°C under an inert gas atmosphere within 10 min. Fmoc-Ser(*t*Bu)-OH has been reported to undergo racemization under continuous-flow solid-phase synthesis involving preactivation. The correct choice of coupling reagent and the base employed (collidine) reduces racemization to an acceptable extent, however [184].

4.2.4.7 Thioether Protection

The methionine thioether group usually does not cause severe complications during peptide synthesis, and the introduction of amino- or carboxy-protecting groups generally succeeds without difficulties. Side reactions that occur during deprotection reactions with methionine-containing peptides are much more problematic. Partial S-demethylation producing homocysteine derivatives has been reported during deblocking reactions with sodium in liquid ammonia, though this method is rarely used nowadays. S-Alkylations may occur on acidolytic cleavage of benzyl- or *tert*-butyl-type protecting groups. The reaction of the benzyl or *tert*-butyl cations, respectively, with the thioether group gives rise to the formation of sulfonium salts, and this sometimes cannot be avoided completely, even by the addition of scavengers such as anisole or ethyl methylsulfide. An exchange of S-alkyl groups has also been reported in this context. The sulfonium salt **54** may react to give S-benzyl homocysteine. The sulfonium salt **55** has been unambiguously identified. S-Methylation of methionine to give the sulfonium salt has been recommended as a protecting measure [185].



The oxidation sensitivity of the methionine thioether group represents another problem. Oxidizing agents such as peroxides or oxygen (or even dimethylsulfoxide (DMSO), a solvent that is frequently used in peptide chemistry), may lead to methionine S-oxide (**56**) formation. There are reports that biologically active peptides become devoid of their activity upon oxidation of a methionine residue. The sulfoxide formation may be reduced or even completely avoided when oxidants are strictly excluded or special cleavage cocktails are used [186].

On the other hand, methionine sulfoxide formation has been suggested as a protecting measure for the thioether moiety. S-Oxidation leads to the creation of a new stereogenic center, and diastereomeric peptides containing methionine S-oxides have been separated by crystallization of the picrates. The sulfoxide group can be reduced, for example with thioglycolic acid, *N*-methylmercaptoacetamide, or ammonium fluoride/2-mercaptoethanol (the latter method does not affect disulfide bonds).

Hydrogenolytic cleavage of protecting groups is possible for methionine-containing peptides despite the presence of the methionine sulfur, when a base or $\text{BF}_3 \cdot \text{OEt}_2$ is added or by hydrogen transfer reaction using formic acid as the hydrogen donor.

Methionine is normally used both in Boc and in Fmoc chemistry without further side-chain protection. Oxidation is avoided in Fmoc chemistry by adding ethyl methylsulfide or thioanisole during cleavage and deprotection.

4.2.4.8 Indole Protection

The indole ring of tryptophan usually does not require any protecting measures. A broad variety of synthetic conditions permit the incorporation of N^{in} -unprotected tryptophan both as the amino or as the carboxy component. However, when the azide method is used for peptide coupling, N^{in} -nitrosation may occur during the synthesis of the acyl azide from the hydrazide, especially in the case of incorrect stoichiometry. The indole system is prone to oxidation by either oxygen or peroxides, and therefore an inert gas atmosphere and absolutely peroxide-free solvents are required. Side reactions at the indole ring (e.g., electrophilic aromatic substitutions) have been observed during some deprotection reactions: *tert*-butylation may occur both at N^{in} and at different positions of the indole ring upon acidolysis of *tert*-butyl-type protecting groups. This also applies to other reactions involving carbenium ions [187]. In most cases, these side reactions are suppressed by the addition of scavengers.

There are few possibilities available for indole ring protection. N^{in} -Formylation increases the stability against alkylation and oxidation [188, 189] and can be removed with dilute aqueous piperidine solution, TFMSA, hydrazine, or hydroxylamine. Consequently, the formyl derivatives are applicable in Boc chemistry. Under basic reaction conditions the formyl group may migrate to free α -amino groups.

In principle, acylation of the indole nitrogen with the Z-group is also a viable alternative. This derivative is stable towards some acids, but is cleaved slowly by TFA and more rapidly by HF. Deprotection by hydrogenolysis and hydrolysis, respectively, is possible.

Although tryptophan may be used without side-chain protection in Fmoc protocols, problems may arise during final deprotection. While indole *tert*-butylation usually is suppressed by the addition of scavengers, sulfonation by the cleavage products of arenesulfonyl-type guanidino-protecting groups may be deleterious. In these cases, Fmoc-Trp(Boc)-OH provides a viable alternative of preventing Trp sulfonation, even in the case of peptides containing multiple arginine residues [190]. Indole Boc protection also diminishes the propensity of trialkylsilane scavengers to reduce the indole ring to an indoline ring.

Further possibilities for indole protection are available [191–193].

4.2.4.9 ω -Amide Protection

From a chemical point of view, the ω -carboxamide groups of glutamine and asparagine, as well as the C-terminal amides of amino acids and peptides, are rather unreactive functionalities that do not require further protection. While this is usually true for the C-terminal carboxamides, side reactions sometimes occur at the side-chain carboxamides of Asn and Gln during peptide synthesis. Especially under the conditions of carbodiimide couplings, undesired nitrile formation from the carboxamide is very prominent (Fig. 4.13). Activating reagents such as BOP, PyBOP, and HBTU are also reported to favor this dehydration, which can be suppressed by the addition of HOBT or HOAt to the coupling reaction. As this side reaction occurs only during activation of Asn or Gln residues because of mechanistic considerations, it should not cause any problem once these residues have been incorporated into the peptide chain.

Hydrazinolytic conditions may convert the primary amides into the corresponding hydrazides, and the conversion of carboxamides into esters during solvolytic deprotection reactions has also been observed.

Cyclization reactions of asparagine in a peptide chain involving the β -carboxamide group with release of ammonia have also been reported. The tendency towards deamidation depends both on the primary and secondary structure, as well as on the solvent [194–196]. The aspartimide derivative **57** may subsequently undergo hydrolysis to give a mixture of α -aspartyl and β -aspartyl peptides (**58**, **59**).

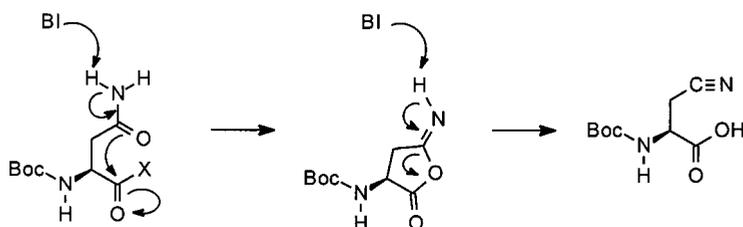
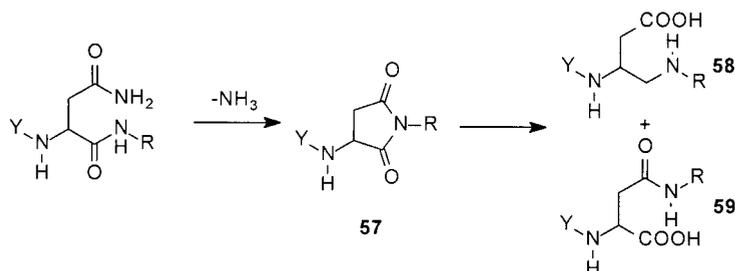
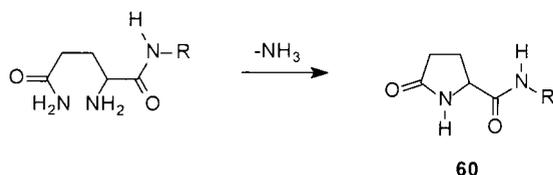


Fig. 4.13 Mechanism for the dehydration of carboxy-activated asparagine. Glutamine dehydration follows an analogous mechanism. B=base; X=electron-withdrawing activating group.



Peptides containing glutamine in the N-terminal position are easily converted into pyroglutamyl peptides **60**. This side reaction is catalyzed by acids and is, therefore, especially problematic in Boc tactics. It may be avoided by the application of high TFA concentrations for Boc cleavage. Base-catalyzed pyroglutamate formation in Fmoc chemistry is less prominent.



Most of the side reactions described above can be suppressed by reversible blocking of the side-chain carboxamide group, which also confers better solubility in organic solvents on the peptides – which is indispensable for normal peptide synthetic operations. Unprotected amide groups tend to form intra- or intermolecular hydrogen bonds, and this results in lower solubility in organic solvents and higher solubility in aqueous solvents. Substituted N-benzyl derivatives that can be cleaved under acidolytic conditions are well suited for this purpose. N-Methoxybenzyl residues or the N-diphenylmethyl protecting group can be introduced directly by the reaction of N^α -protected asparagine or glutamine, respectively, with the benzyl or diphenylmethyl alcohol (Fig. 4.14A). Alternately, the corresponding $\text{N}^\alpha/\text{C}^\alpha$ -protected aminodicarboxylic acids (aspartic acid or glutamic acid) are transformed into the side chain-protected carboxamides with benzyl amine or diphenylamine derivatives and DCC as coupling reagent (Fig. 4.14B).

All amide-protecting groups are cleaved with liquid HF. Cleavage with TFA at ambient temperature is also possible for some derivatives, except for the 4-methoxybenzyl and the diphenylmethyl protecting groups. The addition of anisole as a scavenger favors clean cleavage reactions, as the intermediate benzyl-type cations are trapped.

The N-trimethoxybenzyl (Tmb) and the N-triphenylmethyl (Trt) groups are stable towards piperidine and can be removed by treatment with TFA [197], though Trp alkylation may be detrimental with Tmb [198]. The trityl cation usually does not alkylate Trp or other sensitive side chains. Hence, Trt and Tmb are very appropriate carboxamide-protecting groups for solid-phase synthesis in combination with Fmoc as the temporary protecting group.

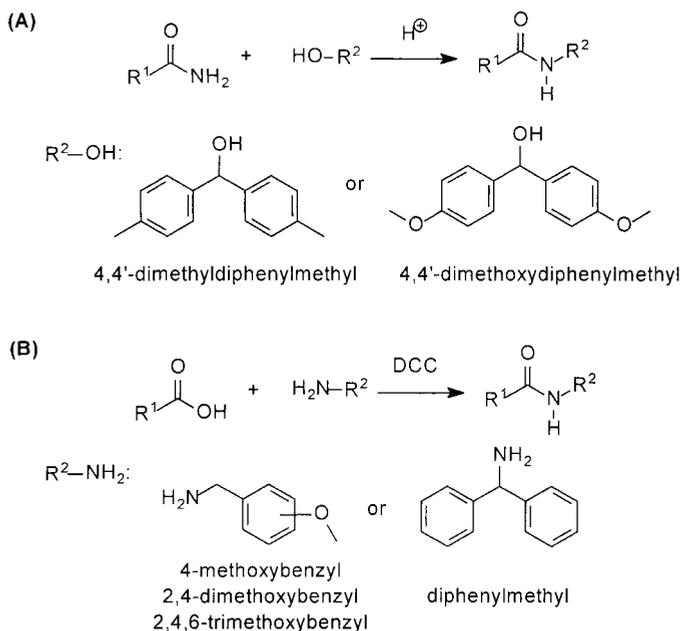


Fig. 4.14 Introduction of ω -amide-protecting groups into amino acid derivatives via N-alkylation of a carboxamide (A) or amide formation (B).

The base-stable 9-xanthenyl (Xan) or the 4,4'-dimethoxydiphenylmethyl (Mbh) groups have found some application for amide protection in Boc chemistry, as they can be removed with strong acids. However, they display somewhat limited acid stability, and this results in progressive cleavage during the consecutive operations of peptide synthesis according to the Boc protocol.

4.2.5

Enzyme-labile Protecting Groups

The synthesis of acid- and base-labile peptide derivatives and peptide conjugates requires the application of protecting groups that can be cleaved under very mild or even neutral reaction conditions. In particular, glyco-, phospho-, and lipopeptides as well as sulfated peptides (cf. Sections 6.3–6.6) belong to this class of sensitive compounds. The treatment of such derivatives with bases often leads to β -elimination reactions in the case of serine and threonine derivatives, or to other undesired side reactions. Treatment with acids in several cases causes degradation of the nonpeptide moiety of the conjugate.

Therefore, these targets may require the application of enzyme-labile protecting groups.

The first peptide synthesis using the Bz-Phe residue as an enzyme-labile protecting group to be cleaved with chymotrypsin was described by Holley in 1955 [199]. Subsequently, the development of enzyme-labile blocking groups has experi-

enced a logical progression from the early specialized application to most sophisticated synthetic strategies [200–202]. Enzymatic manipulation of protecting groups comprises more than simple deprotection, as regioselectivity and stereospecificity are favorable features of enzyme reactions. In addition, because of the mild reaction conditions enzymes can compete successfully with chemical reagents in several cases of protecting group manipulation. The specificity of the biocatalyst safeguards that neither the peptide bonds nor other protecting groups present are attacked. Consequently, enzymatic protecting group techniques represent a particularly efficient methodology for this type of sensitive compound [203–207].

4.2.5.1 Enzyme-labile N^α-amino Protection

The first enzyme-labile urethane type protecting groups were developed by Waldmann et al. [205]. The overall strategy utilized in many of these derivatives employs an enzyme-labile bond, where cleavage may additionally result in a fragmentation reaction of the urethane, thus liberating the free amino group (Fig. 4.15).

The tetrabenzylglucosyloxycarbonyl group (BGloc) [208] comprises O-benzyl-protected glucose as the carbohydrate moiety which is attached to the urethane oxygen. After removal of the benzyl groups, a mixture of α -glucosidase from baker's yeast and β -glucosidase from almonds results in cleavage of the glycosidic bond, thus liberating the carbamic acid (Fig. 4.16). Based on this concept, a whole set of enzyme-labile carbohydrate-based protecting groups can be generated that may be selectively removed using a glycosidase. Furthermore, tetra-O-acetyl- β -D-glucopyranosyl-oxycarbonyl (AGloc) and tetra-O-acetyl- β -D-galactopyranosyl-oxycarbonyl (AGaloc) have been developed as urethane-type protecting groups. These are regarded as second-generation enzyme-labile urethane-type protecting groups which are readily attached to the desired amino acid and, after further synthetic steps, may be cleaved in two separate or sequential enzymatic steps [209].

The acetoxybenzyloxycarbonyl group [Z(OAc)] [210, 211] and the chemically related phenylacetyloxybenzyloxycarbonyl group [Z(OAcPh)] [212] are cleaved by an esterase or lipase [Z(OAc), Fig. 4.17] or penicillin G acylase [Z(OAcPh)]. These enzymes recognize the enzyme-labile ester bond and selectively cleave it. Conse-

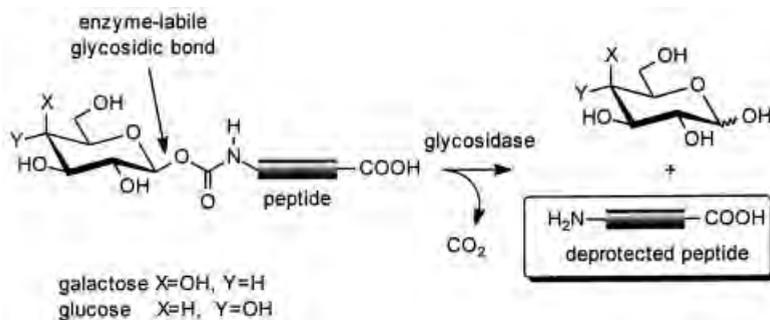


Fig. 4.15 Enzyme-labile carbohydrate-derived protecting groups.

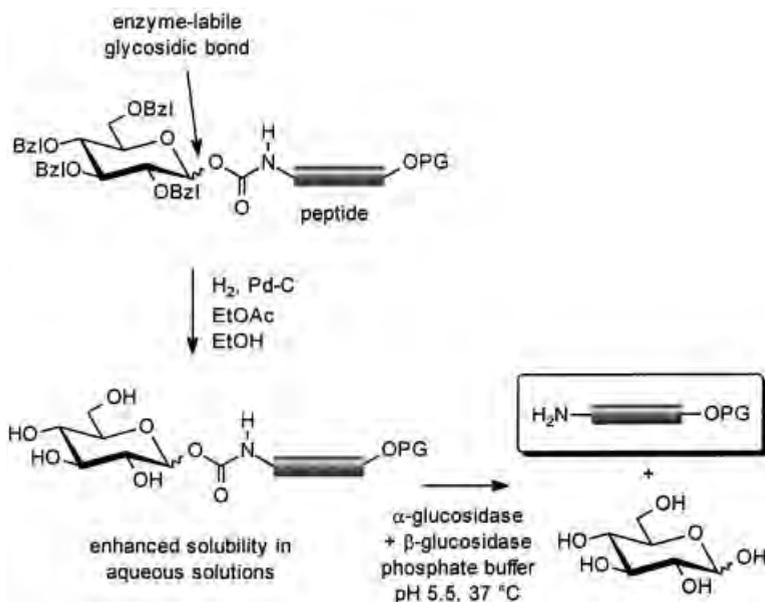


Fig. 4.16 Enzymatic cleavage of the BGloc protecting group (PG: protecting group).

quently, a *p*-hydroxybenzylurethane is formed that spontaneously undergoes fragmentation with release of the desired amino group. Depending on the acyl residue selected (acetate or phenylacetate), enzymes with different selectivity may be used.

4.2.5.2 Enzyme-labile C^α-carboxy Protection and Enzyme-labile Linker Moieties

Esters form one class of carboxy-protecting groups that are labile towards cleavage by enzymes, and can be removed by lipases. The lipase from *Mucor javanicus* selectively deblocks heptyl esters of the C-terminus in sensitive amino acid derivatives, without any undesired side reactions [213, 214]. However, some problems are associated with the use of the very hydrophobic heptyl ester, these being mainly due to the low solubility of the substrate and, hence, the low turnover rate, especially in the presence of further hydrophobic amino acids. The more hydrophilic 2-(*N*-morpholino)-ethyl ester (MoEt) and the methoxyethoxyethyl ester (Mee) circumvent this problem. The Mee ester may be cleaved by lipase-catalyzed hydrolysis or even papain-catalyzed hydrolysis [215, 216].

Waldmann's group was able to transfer the principle of deprotection by enzyme-induced fragmentation of enzyme-labile urethane-type protecting groups also to an enzyme-labile ester group [217]. Cleavage of the 4-phenylacetoxymethyl (PAOB) ester is mechanistically related to Z(OAcPh) cleavage (Fig. 4.17), and is initiated by the action of penicillin G acylase at pH 7 and room temperature, with liberation of phenylacetate. The phenolate formed concomitantly undergoes fragmenta-

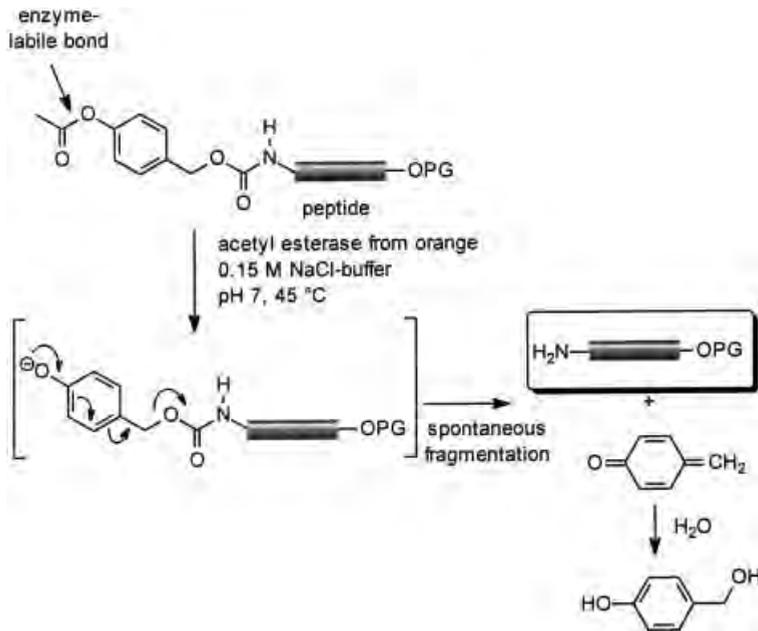


Fig. 4.17 Mechanism of enzymatic Z(OAc) protecting group cleavage (PG: protecting group).

tion to give the desired C-terminally deblocked dipeptide and the quinone methide, which is efficiently trapped by water, forming 4-hydroxybenzyl alcohol.

Another strategy uses amino acid choline esters (Fig. 4.18) that may be cleaved by the enzyme butyryl choline esterase. This commercially available biocatalyst recognizes positively charged choline esters and cleaves them under very mild conditions [218, 219]. Ester groups of sensitive peptide conjugates, such as phosphorylated and glycosylated peptides [220], lipidated peptides [221, 222] and nucleopeptides [223] can be hydrolyzed under very mild conditions with choline esterases, such as acetylcholine esterase (AChE) and butyrylcholine esterase (BChE).

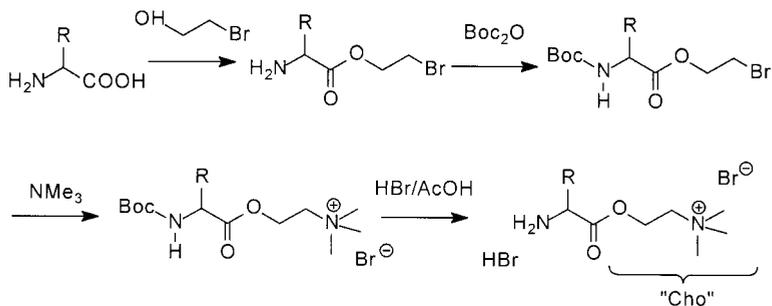


Fig. 4.18 Synthesis of amino acid choline esters.

4.2.6

Protecting Group Compatibility

Protecting group orthogonality [1] is required for the different types of protecting groups that are used for a particular synthesis. Orthogonality means that each class of protecting group (temporary, semipermanent) is cleaved independently from the other. Therefore, the chemical cleavage mechanisms should be different for an optimum degree of orthogonality. However, in most cases strict orthogonality cannot be achieved easily and the protecting schemes rely on differences in cleavage kinetics.

The protecting group schemes most frequently used in solid-phase synthesis, and which often are also suitable for solution-phase syntheses, are detailed briefly in Section 4.5.3.

For example, the application of Boc as the temporary protecting group excludes the use of semipermanent protecting groups at the side chains and at the C-terminus with comparable acid-lability. Consequently, semipermanent protection in Boc tactics must employ functional groups that resist the conditions necessary for the iterative Boc cleavages, such as benzyl-type groups. This tactic is, therefore, called the Boc/Bzl tactic or protection scheme.

Fmoc as the temporary protecting group is cleaved after each coupling step by treatment with bases. In such cases, side-chain and C-terminal protection may employ *tert*-butyl-type groups. Hence, this is referred to as the Fmoc/*t*Bu tactic or protection scheme.

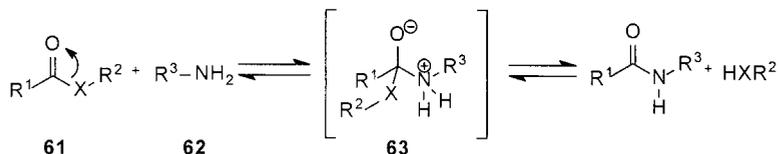
Special protection schemes may also be required, for example in the synthesis of fully side chain-protected peptides, or for subsequent cyclization reactions.

4.3**Peptide Bond Formation**

Peptide bond formation is a nucleophilic substitution reaction of an amino group (nucleophile) at a carboxy group involving a tetrahedral intermediate. However, carboxylic acids react at room temperature with ammonia or amines to give an ammonium salt instead of a carboxamide. Therefore, the carboxy component **61** ($X-R^2=OH$) must be activated prior to peptide bond formation. Furthermore, the peptide coupling reaction must be performed under mild conditions, and at room temperature.

Activation of the carboxy component (an increase of the electrophilicity) is achieved by the introduction of electron-accepting moieties. Groups which exert either an inductive (-I) effect or mesomeric (-M) effect (or both) decrease the electron density at the C=O group, thereby favoring the nucleophilic attack of the amino component (**62**). The latter attacks with its nitrogen lonepair the electrophilic position of the carboxy group to give the tetrahedral zwitterionic intermediate **63**. Peptide bond formation is then completed by dissociation of the leaving group (nucleofuge R^2X) from **63**. The leaving group capacity (nucleofugicity) is another factor which influences the reaction rate.

The variation of the leaving group XR^2 provides a broad spectrum of methods for peptide bond formation, and this methodological arsenal has been summarized in several monographs [83–86].



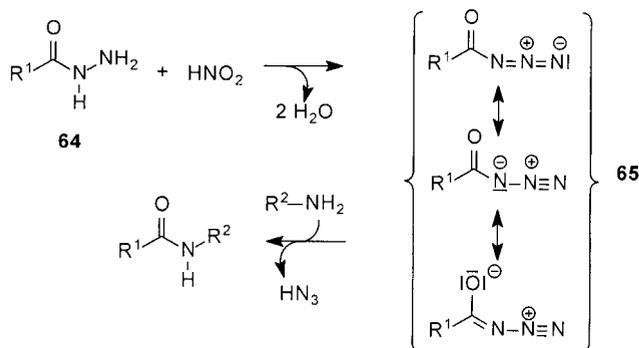
It is beyond the scope of this book to discuss all the available methods in detail. Rather, attention will be focused on the reagents that are generally accepted in practical applications. Among these are the acyl azides, anhydrides, carbodiimides, active esters, and acyl halides, together with phosphonium and uronium reagents.

4.3.1

Acyl Azides

The acyl azide method [224] was introduced into peptide chemistry as early as 1902 by Theodor Curtius, and hence is one of the oldest coupling methods. Besides free amino acids and peptides, which are coupled with an acyl azide in alkaline aqueous solution, amino acid esters can be used in organic solvents. In contrast to many other coupling methods, the addition of an auxiliary base or a second equivalent of the amino component in order to trap the hydrazoic acid is not necessary.

For a long time the azide method was considered to be the only racemization-free coupling method, and it experienced a tremendous renaissance following the introduction of selectively cleavable amino-protecting groups. The starting materials for this method are the crystalline amino acid or peptide hydrazides **64**, respectively, that are easily accessible from the corresponding esters by hydrazinolysis. These hydrazides are transformed into the azides by N-nitrosation with one equivalent of sodium nitrite in hydrochloric acid at -10°C and simultaneous water elimination. Alternately, mixtures of acetic acid, tetrahydrofuran, or DMF



with hydrochloric acid have been used. The azide **65** is extracted from the aqueous layer with ethyl acetate, washed, dried, and reacted with the corresponding amino component. Some azides can be precipitated upon dilution with ice water.

Diphenylphosphoryl azide (DPPA) may also be used for the synthesis of acyl azides [225]. The Honzl-Rudinger method uses *tert*-butyl nitrite for the N-nitrosation and allows for the application of organic solvents in azide couplings [226]. The coupling reaction should be performed at low temperature because of the inherent thermal instability of acyl azides. At higher temperature, the Curtius rearrangement, i.e., conversion of the acyl azide to an isocyanate, becomes a prominent side reaction and eventually leads to the undesired formation of ureas. As a consequence of the low reaction temperature (e.g., 4 °C), the reaction rate is rather low and the peptide coupling reactions often take several days to reach completion.

Hydrazinolysis of ester groups in longer N-terminally protected peptides is often difficult, and consequently the application of orthogonally N'-protected hydrazine derivatives is the method of choice. The peptide segments assembled according to this backing-off strategy can be used for azide couplings after selective de-blocking of the hydrazide group.

Although, as mentioned previously, the azide method has been considered as the method with the lowest tendency towards racemization, an excess of base in the reaction is accompanied by considerable racemization. Hence, any contact with bases during the coupling reaction must be avoided; for example, ammonium salts of the amino component should be neutralized with *N,N*-diisopropylamine or *N*-alkylmorpholine instead of triethylamine.

Despite all the limitations mentioned, the azide method is still important, especially for segment condensations, because of its low tendency towards racemization, the applicability with O-unprotected serine or threonine components, and the highly variable use of N'-protected hydrazides.

4.3.2

Anhydrides

Basic considerations towards the application of anhydrides in peptide synthesis date back to the early investigations of Theodor Curtius in 1881 in the synthesis of hippuric acid. Curtius obtained Bz-Gly_n-OH (n=2–6) besides hippuric acid upon reaction of silver glycinate with benzoyl chloride. In those early days it was argued that N-benzoyl amino acids or N-benzoyl peptides formed unsymmetric anhydrides with benzoic acid as reactive intermediates when treated with benzoyl chloride.

About 70 years later, Theodor Wieland took advantage of these findings when his group made the mixed anhydride method available for modern peptide synthesis. Nowadays, besides this method, symmetrical anhydrides and N-carboxy anhydrides (NCA, Leuchs anhydrides) formed intramolecularly from the carboxy groups of the amino acid and a carbamic acid are used in peptide couplings. Last,

but not least, it should be mentioned that unsymmetrical anhydrides are often involved in biochemical acylation reactions.

4.3.2.1 Mixed Anhydrides

Carboxylic acids and inorganic acids, respectively, are used for the formation of mixed anhydrides [227]. However, only a few representatives of the variety of synthetic variants and acids used have found widespread practical application, and in most cases alkyl chloroformates (or correctly, alkyl chlorocarbonates) are used. Ethyl chloroformate (ethyl chlorocarbonate), which in the past was used very frequently, has nowadays mainly been replaced by isobutyl chloroformate (isobutyl chlorocarbonate). The mechanism of the mixed anhydride method is shown in Fig. 4.19.

The regiochemistry of the aminolysis reaction of the mixed anhydride, which is initially formed from the carboxy component and the chloroformate, depends on the electrophilicity and/or the steric situation of the two competing carboxy groups. In the case of mixed anhydrides formed from a N-protected amino acid carboxylate (carboxy component) and an alkyl chlorocarbonate (activating component, e.g., from alkyl chloroformate), the amine nucleophile predominantly at-

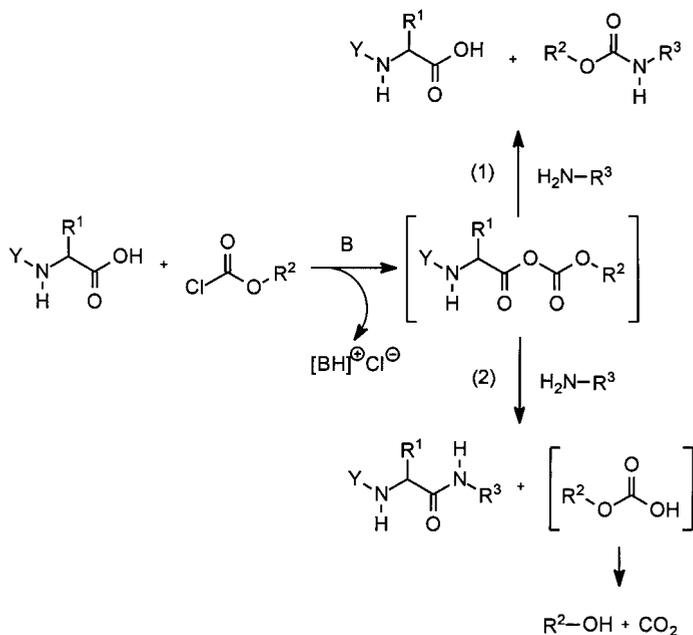


Fig. 4.19 Mechanism of mixed anhydride formation and regiochemistry of aminolysis. Y=amino-protecting group; R¹ = side chain of the carboxy component; R²=substituent of the chlorocarbonate; H₂N-R³=amino component; B=tertiary base.

tacks the carboxy group of the amino acid component with formation of the desired peptide derivative and release of the activating component as free acid (Fig. 4.19, reaction 2). On application of an alkyl chloroformate (R^1 =isobutyl, ethyl, etc.), the free monoalkyl carbonic acid formed is unstable and immediately decomposes into carbon dioxide and the corresponding alcohol. However, there are some reports of the opposite regiochemistry (Fig. 4.19, reaction 1) of the nucleophilic attack, with formation of a urethane and regeneration of the N-protected amino acid component.

In order to form a mixed anhydride, N-protected amino acids or peptides are dissolved in dichloromethane, tetrahydrofuran, dioxan, acetonitrile, ethyl acetate, or DMF, respectively, and treated with one equivalent of a tertiary base (*N*-methylpiperidine, *N*-methylmorpholine, *N*-ethylmorpholine, etc.). The required alkyl chloroformate is then added at temperatures between -15 and -5 °C under vigorous stirring to form the unsymmetrical anhydride (activation). After a short activation period, the nucleophilic amino acid component is added. If it is used as an ammonium salt (which requires more base), then overstoichiometric application of base must be avoided. The mixed anhydride method can be performed very easily and is one of the most powerful coupling methods, provided that the reaction protocol described above is followed strictly.

Profound studies on the stability of mixed anhydrides, on the minimization of the undesired urethane formation and racemization which were carried out by Benoiton and coworkers eventually led to an improved mechanistic understanding and to an increase in efficiency of this coupling method, which now finds widespread application. An investigation into the reasons for excess urethane formation (Fig. 4.19, reaction 1), especially after the activation of isoleucyl or valinyl residues, led to the conclusion that this prominent side reaction can be prevented by using dichloromethane as the solvent and *N*-methylpiperidine as the tertiary base. The relatively high stability of mixed anhydrides towards hydrolysis allows for their purification by washing the organic phase containing the mixed anhydride with water [228–230]. The stability of mixed anhydrides obtained with alkyl chloroformates also depends on the alkyl group present. Mixed anhydrides from Boc-, Z-, and Fmoc-protected amino acids and isopropyl chloroformate can be purified and isolated. These are much more stable compared to the derivatives obtained with ethyl or isobutyl chloroformate [231, 232]. In the absence of a suitable nucleophile, the decomposition of a mixed anhydride in organic solvents starts with cyclization to produce 2-alkoxy-5(4*H*)-oxazolones, with liberation of carbon dioxide and the alcohol R^2 -OH [233, 234]. Symmetrical anhydrides and esters are formed as by-products.

Several interesting aspects with respect to the practical realization of a mixed anhydride coupling should be noted: Although aqueous DMF is a good solvent both for the formation of the mixed anhydride and for the subsequent coupling reaction, it promotes racemization to a much greater extent than either tetrahydrofuran or halogenated solvents, as has been shown in the reaction of Z-Gly-Xaa-OH (Xaa=Ala, Leu, Val, Phe) with H-Val-OEt [231]. Isopropyl chloroformates are superior to ethyl or isobutyl chloroformates. Interestingly, activation with ethyl

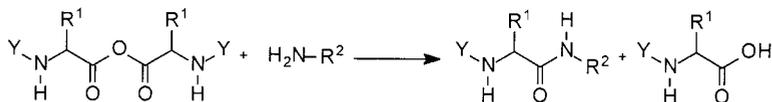
chloroformate in DMF or *N*-methylpyrrolidone leads to less racemization than activation with isobutyl chloroformate. Nevertheless, mixed anhydride formation with ethyl chloroformate, and also the application of triethylamine as a tertiary base, currently are of very little practical importance. Side reactions during mixed anhydride couplings have been observed initially upon activation of N^α -tosyl-, N^α -trityl, and N^α -trifluoroacetyl-protected amino acids, respectively.

Mixed anhydride formation with pivalic acid (2,2-dimethylpropionic acid) as the activation reagent is sometimes recommended, especially for N^α -acyl-protected asparagine. These unsymmetrical anhydrides are prepared analogously from N^α -acyl amino acids and pivaloyl chloride, and react in high yields with amino nucleophiles. The strong +I effect of the *tert*-butyl group in pivalic acid reduces the electrophilicity at this carbonyl group and leads, together with the steric hindrance, to the desired regioselective attack of the nucleophile on the activated amino acid.

For mechanistic reasons, the application of acylphosphonium salts as reactive intermediates in peptide couplings must be mentioned in this context (cf. Section 4.3.6) [235, 236].

4.3.2.2 Symmetrical Anhydrides

Symmetrical anhydrides of N^α -acyl amino acids **66** are highly reactive intermediates for peptide bond formation. Ambiguous regioselectivity upon reaction with amine nucleophiles is not possible, in contrast to the mixed anhydride method. However, a maximum peptide yield of 50% (with reference to the carboxy component) can be obtained.



66

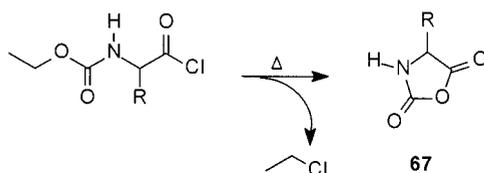
Although the free N^α -acyl amino acid formed on aminolysis of a symmetrical anhydride together with the desired peptide can be recovered by extraction with saturated sodium bicarbonate solution, the practical importance of this method initially was very small. Symmetrical anhydrides can be obtained from N^α -protected amino acids with phosgene or, more conveniently, with carbodiimides. The reaction of two equivalents of N^α -acyl amino acid with one equivalent of carbodiimide favors formation of the corresponding symmetrical anhydride that may be isolated, but can also be used in the subsequent coupling reaction without further purification. Symmetrical anhydrides based on N^α -alkoxycarbonyl amino acids are stable towards hydrolysis. This permits purification analogously, as described above for the mixed anhydrides.

The general interest in symmetrical anhydrides for stepwise peptide chain elongation increased as Boc-protected amino acid derivatives became commercially available at reasonable prices. Although crystalline symmetrical anhydrides can be obtained commercially, in-situ preparation remains the variant of choice.

4.3.2.3 N-Carboxy Anhydrides

Simultaneous activation of the carboxy group and acyl protection of an amino acid is found in the N-carboxy anhydrides (NCA), which were discovered in 1906 by Hermann Leuchs. In the German literature they are, therefore, also often called Leuchs-anhydrides. In principle, these derivatives should possess ideal preconditions for application in peptide synthesis.

The first N-carboxy anhydrides (1,3-oxazolidine-2,5-diones) **67** were obtained by thermal elimination of chloroethane from N-(ethoxycarbonyl) amino acid chloride.



An elegant procedure for the preparation of these derivatives is the reaction of free amino acids with phosgene via the corresponding carbamoyl chloride as an intermediate. However, even traces of water polymerize N-carboxy anhydrides, because the carbamic acid initially formed spontaneously decarboxylates to give a free amine that acts as a nucleophile in further ring-opening reactions. Therefore, the practical application of the NCA method in peptide synthesis was seriously limited until 1966, when correct reaction conditions were published which allowed for controlled peptide synthesis with N-carboxy anhydrides in an aqueous medium [237]. As shown in Fig. 4.20, amino acids and peptides are rapidly acylated by N-carboxy anhydrides at low temperature and at pH 10.2. The intermediate peptide carbamates are decarboxylated by a decrease in the pH to values between 3 and 5. The next coupling cycle starts with an increase of the pH to 10.2, and proceeds by addition of the next N-carboxy anhydride. Vigorous stirring of the reaction mixture is necessary in order to minimize the risk of undesired carboxylate exchange between the intermediary peptide carbamate and the amino component. Exact control of the pH value (pH 10.2–10.5 for amino acids, and pH 10.2 for peptides) is another precondition, as hydantoins are formed as by-products at pH > 10.5.

The sulfur analogues of the N-carboxy anhydrides, N-thiocarboxy anhydrides (NTA), can be favorably applied in peptide syntheses, because of the higher stability of the thiocarbamate salts. The acylations can be performed at pH values as low as 9–9.5, which prevents the possible hydrolytic conversion to the hydantoins. The NCA/NTA method is especially suited for segment syntheses without isolation of reaction intermediates. Trifunctional amino acids (except lysine and cysteine) do not require side-chain protection. This method has been used to assemble several segments of the ribonuclease S-protein that were then combined, using the azide method to produce the full-length S-protein [238].

Recently, the NCA methodology has again attracted significant interest, as urethane-protected N-carboxy anhydrides (UNCA) have been synthesized and applied to peptide syntheses [239]. NCA can be acylated at the ring nitrogen with suitable

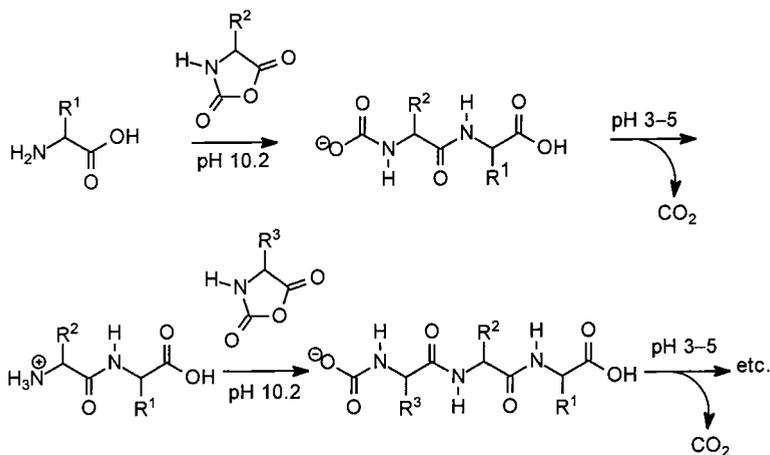
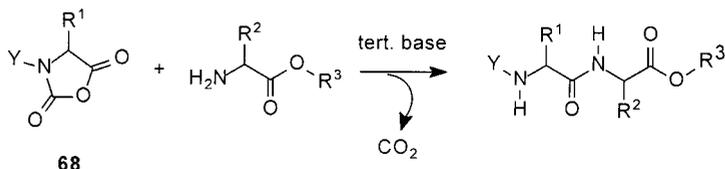


Fig. 4.20 Controlled peptide synthesis with N-carboxy anhydrides in aqueous medium.

reagents for the introduction of a Boc, Z, or Fmoc group in an aprotic solvent in the presence of a tertiary base to give the corresponding UNCA 68 (Y=Boc, Z, Fmoc). UNCA can be obtained for most amino acids as crystalline compounds, and are stable in the absence of water. They display high reactivity towards nucleophiles, and form the required peptide bonds at high rates in most anhydrous solvents (with the exception of alcohols) normally used in peptide synthesis, both in solution or on a polymeric support. Carbon dioxide is the only by-product, and there is no risk of either oligo- or polymerization, because the amino terminus of the growing peptide chain is still urethane-protected after the coupling reaction.



Likewise, N-trityl- and N-phenylfluorenyl-protected NCA have been recently reported [240].

4.3.3

Carbodiimides

Carbodiimides can be used for the condensation of a carboxy group and an amino group [241, 242]. Initially *N,N'*-dicyclohexyl carbodiimide (DCC) was used and is now well established, because it is relatively cheap and soluble in the solvents used in peptides synthesis. During peptide bond formation the carbodiimide is converted into the corresponding urea derivative, which in the case of *N,N'*-dicyclohexyl urea precipitates from the reaction solution. Clearly, the different reaction

rates of aminolysis and hydrolysis of the reactive intermediate after carbodiimide activation enable peptide couplings in aqueous media. The reaction mechanism for carbodiimide-mediated peptide couplings has been established in extensive studies performed by several groups, and is shown in Fig. 4.21. The carboxylate anion adds to the protonated carbodiimide with formation of a highly reactive *O*-acylisourea **69**; this has not yet been isolated, but its existence is postulated by close analogy to stable compounds of this class. The *O*-acylisourea reacts with the amino component to produce the protected peptide and the urea derivative (Fig. 4.21, reaction A). Alternatively, the *O*-acylisourea **69** – in equilibrium with the *N*-protonated form **70** – is attacked by a second carboxylate as the nucleophile forming the symmetrical amino acid anhydride **72** together with *N,N'*-disubstituted urea (Fig. 4.21, reaction C). The former reacts with the amino acid component to give the peptide derivative and the free amino acid (Fig. 4.21, reaction D). An undesired side reaction is the base-catalyzed acyl migration from the isourea oxygen to nitrogen; this results in an *N*-acylurea **71** that does not undergo further aminolysis (Fig. 4.21, reaction B). This *O* → *N* acyl migration is catalyzed not only by an excess of base, but also by the basic amino component or the carbodiimide. Polar solvents additionally favor this reaction pathway.

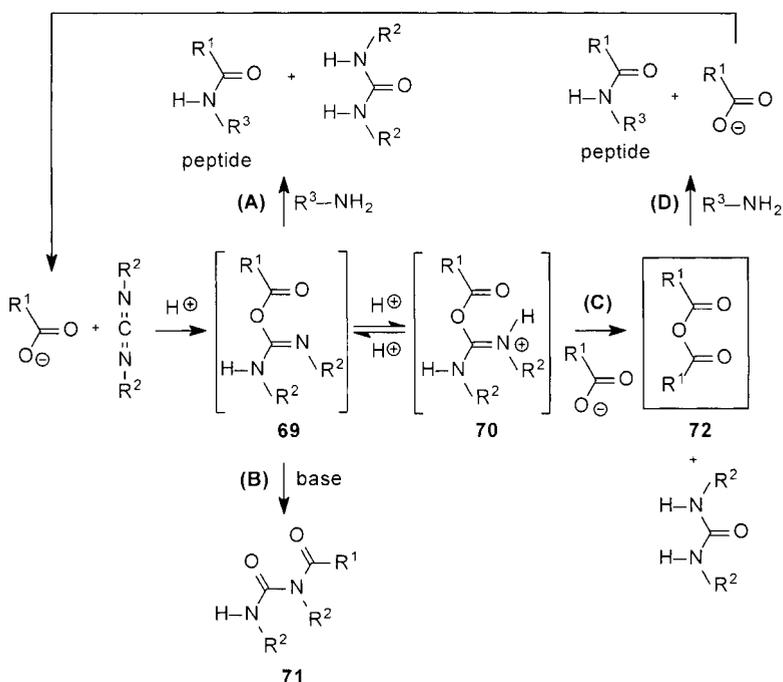
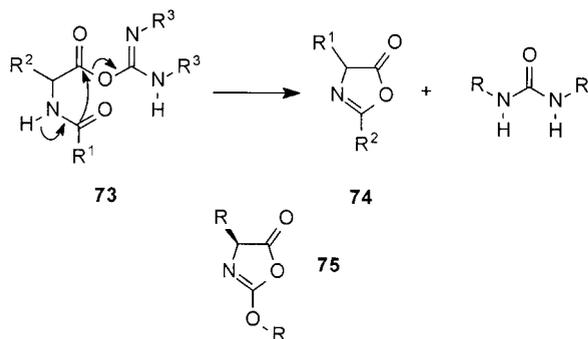


Fig. 4.21 Mechanism of carbodiimide-mediated peptide couplings. R^1COOH =carboxy component (amino acid or peptide); R^2 =e.g. cyclohexyl (DCC); R^3-NH_2 =amino component (amino acid or peptide).

Furthermore, the *O*-acylisourea may undergo cyclization to give the 5(4*H*)-oxazolone **74**, again with liberation of the *N,N'*-dialkylurea as a good leaving group. This intramolecular side reaction explains the high propensity of the carbodiimide method towards racemization, when applied to *N*-acyl-protected amino acids and peptides. 5(4*H*)-Oxazolones are themselves acylating agents, but are prone to racemization (cf. Section 4.4.2).



Derivatives with *N*-protecting groups of the urethane type are the only exception. Contrary to earlier speculations, 2-alkoxy-substituted 5(4*H*)-oxazolones **75** are real intermediates that are formed during this reaction from the *O*-acylisourea, but are converted immediately with free carboxy component to give the symmetrical anhydride **72** [243]. All three postulated intermediates are attacked by nucleophiles such as amino groups, with the reactivity decreasing in the series *O*-acyl isourea > 2-alkoxy-5(4*H*)-oxazolone > symmetrical anhydride. Which of the three activated carboxy derivatives is involved in the peptide bond-forming step depends on the presence, availability, and reactivity of the nucleophilic amino component. In cases where no amino component is present (e.g., in ester syntheses) or where the activated carboxy component is added in excess (in SPPS), the symmetrical anhydride is formed predominantly.

The oxazolone-forming cyclization is favored especially upon application of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, WSCI). Aminolysis of β -methyl-substituted amino acid derivatives of type **69** is not favored. Consequently, cyclization and rearrangements may compete efficiently with aminolysis after activation of these derivatives. Different reactivities of 2-alkoxy-5(4*H*)-oxazolones, depending on the amino acid side chain R^1 have been observed, especially with respect to hydrolysis.

The DCC method is a reliable variant for the stepwise assembly of peptides using urethane-protected amino acids (*Z*, Boc, Fmoc) in both solid-phase and solution-phase peptide synthesis, as well as for segment condensations. However, only peptide segments containing C-terminal glycine or proline residues should be used as acyl components, because of the risk of epimerization at the C-terminal position. The *N*-acyl urea formation mentioned above may be a prominent side reaction that can be suppressed by keeping the reaction at low temperatures,

or by using nonpolar solvents in which the dicyclohexyl urea should, nevertheless, dissolve. Free dimethylamine, which is present in low concentrations even in highly pure DMF or *N,N*-dimethylacetamide also favors the $O \rightarrow N$ acyl migration to produce *N*-acyl urea. When activated with DCC, unprotected glutamine or asparagine side chains may undergo dehydration of the carboxamide group to yield the nitrile. Therefore, a global (maximum) protection strategy for the side-chain functionalities should be applied when DCC couplings are involved.

Besides *N*-acyl urea formation, a serious problem in peptide synthesis when using the carbodiimide method – and especially in the synthesis of difficult sequences – is imposed by incomplete separation of the *N,N'*-dialkyl urea, and especially of *N,N'*-dicyclohexyl urea. Several carbodiimide derivatives which have been substituted with different tertiary amino or quaternary ammonium groups have been introduced. The ureas formed during peptide synthesis with these modified reagents are easily separated from the reaction mixture because of their increased solubility in aqueous or acidic aqueous solutions. Reagents of this type, for example EDC (WSCl) or 1-cyclohexyl-3-(3-trimethylammonio)propyl carbodiimide iodide, are appropriate for peptide couplings even in alcohol [244] (or aqueous solution as required) for coupling reactions involving proteins or peptide cyclizations. In addition, polymeric carbodiimides have been developed which allowing for straightforward separation of the urea formed during the reaction. Unsymmetrically substituted carbodiimides have been reported to be suitable for suppression of the $O \rightarrow N$ acyl migration forming the undesired *N*-acyl urea, as was shown for 1-benzyl-3-ethylcarbodiimide [245]. Simultaneously, a lower tendency towards racemization has been observed.

The application of appropriate additives was investigated in a systematic manner in order to suppress or diminish both *N*-acyl urea formation and racemization. Additives intercept highly reactive intermediates, such as *O*-acylisourea, thereby forming an active ester which has lower reactivity but is still sufficiently potent to allow for rapid amide bond formation. The DCC-additive protocol was introduced into peptide chemistry in 1966 during the course of the syntheses of glucagon and calcitonin [246, 247], whereby one equivalent of DCC and two equivalents of *N*-hydroxysuccinimide (HOSu) were applied simultaneously (Wünsch-Weygand protocol). Subsequently, the search for further possible additives continued because of the internally competing reactions between DCC and HOSu [248] that were observed during the coupling of sterically hindered peptides. The side reaction mentioned does not occur upon addition of *N*-hydroxy-5-norbornene-2,3-dicarboxamide (HONdc), which proved to be advantageous in challenging syntheses.

Doubtlessly, the König-Geiger protocol belongs to the DCC-based coupling methods that have found the most widespread application [249]. In this variant, 1-hydroxybenzotriazole (HOBt, Tab. 4.6) is mainly preferred as the additive. 3,4-Dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine (HODhbt) is more efficient, but is also prone to side reactions [250]. Like all additives of this type, 1-hydroxybenzotriazole (HOBt) reacts rapidly with the *O*-acylisourea initially formed to produce the corresponding active ester derivative. Benzotriazolyl esters (Fig. 4.22) undergo aminolysis up to three orders of magnitude faster than succinimidyl or related esters.

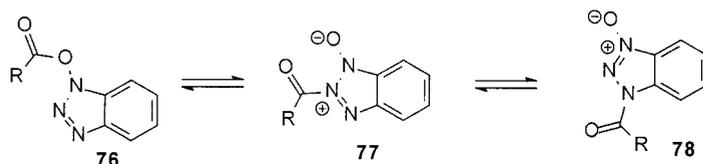


Fig. 4.22 Amino acid benzotriazolyl esters.

Thus, rearrangements, cyclization, and the formation of symmetrical anhydrides are efficiently suppressed. Dehydration of asparagine or glutamine residues is also not observed. Likewise, 2-alkoxy-5(4*H*)-oxazolones react immediately with HOBT or HOSu, respectively, to produce the active esters that undergo peptide coupling with amino components present in the reaction mixture [251].

The N^{α} -protected amino acid HOBT esters **76** are thought to exist in solution in an equilibrium with the N-oxides **77** and **78** [249]. In the crystalline state, structure **78** has been observed using X-ray analysis [252].

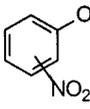
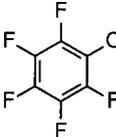
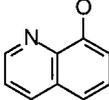
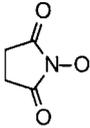
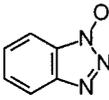
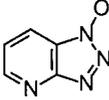
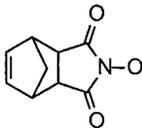
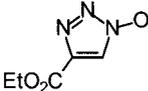
Many other N-hydroxy compounds besides HOSu and HOBT have been suggested as potential additives for the DCC method, for example ethyl 1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (HOCT) [253] or other hydroxy-substituted triazole and tetrazole derivatives [254]. Interestingly, zinc halogenides ($ZnCl_2$, ZnF_2 [255]) have also been applied successfully as additives. $CuCl_2$ was found to be even more superior in terms of suppressing racemization, and led (especially when applied in combination with HOBT) to a virtually complete retention of the configuration, as was shown in several highly sensitive test systems [256]. Liquid crystal-forming compounds have also been reported as being efficient additives [257–259]. The most efficient additive is currently the 7-aza analogue of HOBT, commonly referred to as 1-hydroxy-7-azabenzotriazole [260–263]. This combines the features of HOBT and a tertiary base in the same molecule, and is reported to accelerate reaction rates significantly as well as provide concomitant optimal coupling yields and minimal racemization [262]. Nowadays, phosphonium and uranium salts derived from this compound (cf. Sections 4.3.6 and 4.3.7) are also available as excellent coupling reagents.

4.3.4

Active Esters

Peptide bond formation by ester aminolysis proceeds analogously to ester saponification ($X=O, S, Se$) according to a $B_{AC}2$ mechanism. Nucleophilic attack of the amino component **79** to the carboxy group of **80** leads, in the rate-determining step of this reaction, to the tetrahedral intermediate **81**, the formation of which is favored by electron-withdrawing groups XR^3 . The rate of peptide bond formation correlates with the leaving group capacity of XR^3 . For example, a good leaving group is the conjugate base of a relatively strong acid, and this is the case when the leaving group anion $^-XR^3$ is well-stabilized by inductive or mesomeric effects which facilitate cleavage of the $(C=O)-XR^3$ bond.

Tab. 4.6 Active esters of the type X-(C=O)-R.

Group	Symbol	X
Thiophenyl	SPh	
Cyanomethyl	OCm	
Nitrophenyl		
2-Nitrophenyl	O2Np	
4-Nitrophenyl	ONp	
2,4-Dinitrophenyl	O2,4Np	
Chlorphenyl		
2,4,5-Trichlorophenyl	OTcp	
Pentachlorophenyl	OPcp	
Pentafluorophenyl	OPfp	
N-Hydroxypiperidinyl	OPip	
8-Quinolyl	OQ	
N-Hydroxysuccinimidyl	OSu	
1-Hydroxybenzotriazolyl	OBt	
7-Aza-1-hydroxybenzotriazolyl	OAt	
N-Norbornene-2,3-dicarboximidooxy	ONdc	
Ethyl-1-hydroxy-1H-1,2,3-triazole-4-carboxylate	OCt	

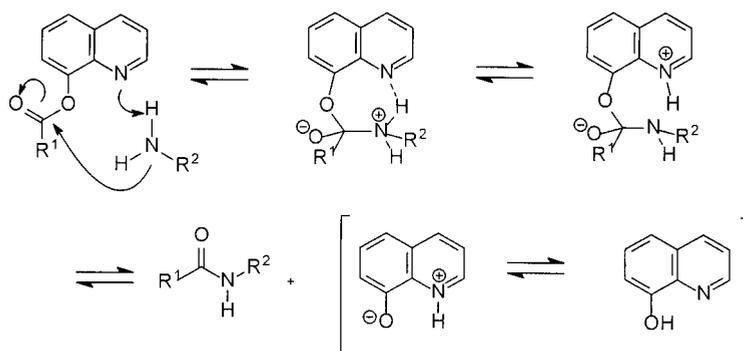
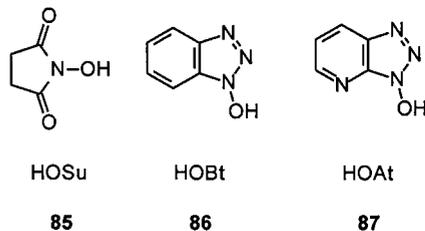


Fig. 4.23 Intramolecular base catalysis during aminolysis of acylamino 8-quinolyl esters. $R^1\text{COOH}$ =carboxy component (protected amino acid or peptide); $R^2\text{NH}_2$ =amino component (amino acid or peptide).

lo[5,4-b]pyridine), has been recommended as a highly efficient additive for DCC coupling reactions [260]. As mentioned above for the hydroxyquinoline derivatives, 7-aza-1-hydroxybenzotriazole is most efficient [269]. Its active esters are characterized by very little racemization, this being explained by mechanistic considerations analogously as described for 8-quinolyl esters. HOAt as an additive is superior to HOBT for stepwise peptide couplings and segment condensations, especially with respect to reaction rate and the degree of racemization.

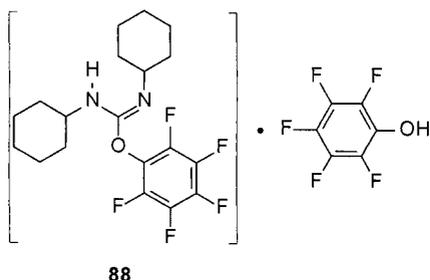


Active esters of protected amino acids can be synthesized by the mixed anhydride method, the carbodiimide method, the carbonate method, and other more specialized variants. Protected amino acid active esters can also be obtained starting from amino acid salts in a “one-pot” reaction, provided that the carbonate component has been carefully chosen. Treatment of amino acid salts with benzyl (4-nitrophenyl)carbonate or *tert*-butyl (4-nitrophenyl)carbonate, respectively, gives the corresponding Z- or Boc-protected derivatives that, upon acidification and further addition of a carbodiimide, yield the amino-protected (4-nitrophenyl)esters. These are crystalline compounds that may be stored in the dark at room temperature for longer periods of time and which display high reactivity in aminolysis reactions. Aminolysis may even be further enhanced after the addition of catalysts such as azoles or N-hydroxy compounds. Separation of the 4-nitrophenol formed during aminolysis may sometimes be tedious, though precipitation with DMF/water or DMF/ether, adsorp-

tion on neutral alumina, or complexation with pyridine at pH 6.5 may be successful. Incomplete separation often disturbs subsequent reactions such as hydrolysis.

Likewise, the crystalline *N*-hydroxysuccinimidyl esters are characterized by high reactivity with amines and low sensitivity towards hydrolysis in aqueous/organic solvent mixtures (water/ethanol, water/dioxan, water/tetrahydrofuran, etc.) that makes peptide syntheses feasible in such media. The water-solubility of the *N*-hydroxysuccinimide formed upon aminolysis is a further advantage of this type of active ester.

Halogen-substituted phenyl esters usually are crystalline compounds with high aminolysis activity. Separation of the halogenated phenols liberated during reaction of these active esters with amines often is less problematic compared to the corresponding 4-nitrophenols. The “backing off” protocol is a suitable method for the synthesis of optically pure *N*-protected activated peptidyl esters. Moreover, a halogenated phenol ($X=\text{Cl}$, F) may be reacted with dicyclohexyl carbodiimide to give a crystalline *O*-arylisourea/phenol complex. For instance, the *O*-pentafluorophenyl-*N,N'*-dicyclohexylisourea/pentafluorophenol complex **88** possesses high acylation potential and may be used directly for segment condensations.



For mechanistic reasons, the isoxazolium method [270] (Fig. 4.24) can be discussed in the context of this chapter, because the *N*-ethyl-5-phenylisoxazolium-3'-

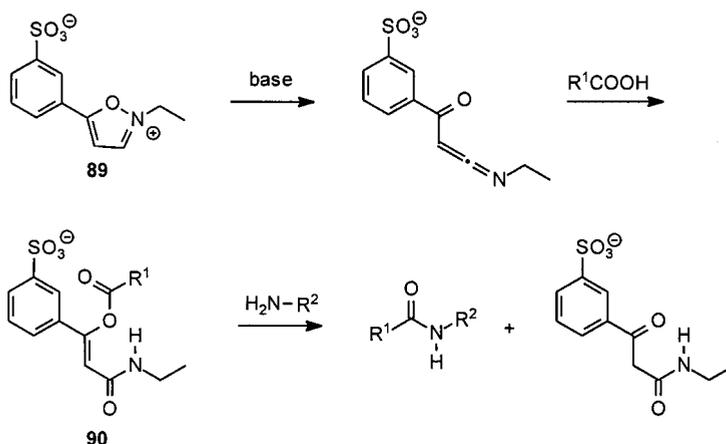


Fig. 4.24 Mechanism of the isoxazolium method. R^1COOH =carboxy component (amino acid or peptide); $\text{H}_2\text{N-R}^2$ =amino component (amino acid or peptide).

sulfonate **89** (Woodward reagent K) forms an N-protected enol ester **90** with N-protected amino acids and peptides under mildly basic conditions. This enol ester couples *in situ* with amino components and forms, besides the desired product, a water-soluble aryl sulfonate that is easy to separate.

4.3.5

Acyl Halides [271]

By analogy to organic chemistry, an obvious method of activating a carboxy group for amide formation at room temperature or below would utilize the acid chloride. However, amino acid chlorides have until recently [62, 272] rarely been used as they are reputed to be “overactivated” and prone to numerous side reactions, including racemization.

Despite this, because the Fmoc group is highly stable under acidic conditions and does not readily undergo S_N1 or S_N2 displacement reactions at the 9-fluorenylmethyl residue, the Fmoc-protected amino acids have emerged as ideal substrates for conversion into the corresponding acid chlorides. Consequently, these derivatives have experienced a renaissance in peptide synthesis. All common amino acids lacking polar side chains, as well as a number of those bearing benzyl- or allyl-based side-chain protection, can be converted to stable, crystalline acid chlorides. For chemical reasons, amino acids bearing *tert*-butyl-protected side chains cannot generally be accommodated. A hindered base such as 2,6-di-*tert*-butylpyridine is used routinely as hydrogen chloride acceptor, as conversion to oxazolone is slow with such bases. Ammonium or potassium salts of *N*-hydroxybenzotriazole (HOBt) have also been applied as bases in solid-phase syntheses involving Fmoc amino acid chlorides.

Fmoc amino acid chlorides have been used as stable, easily accessible derivatives for rapid coupling reactions, without any racemization. Their general application is, however, somewhat limited as not all Fmoc-protected amino acid derivatives are accessible. This holds true especially for trifunctional amino acids with side chain-protecting groups of the *tert*-butyl type (Boc, *tert*-butyl ester, *tert*-butyl ether).

In contrast, the corresponding acid fluorides do not suffer from such limitations. Urethane-protected amino acid fluorides of trifunctional amino acids with a side-chain protection of the *tert*-butyl or trityl type can be obtained and are sufficiently stable.

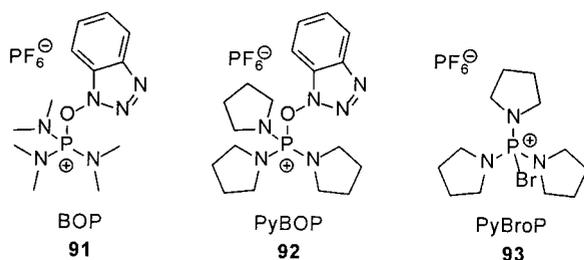
Further advantages of the acid fluorides relative to the chlorides include their greater stability toward water, including moisture in the air, and their relative lack of conversion to the corresponding oxazolones on treatment with tertiary organic bases. Fmoc-protected amino acid fluorides were found to be efficient carboxy-activated derivatives suited both for solution syntheses and for SPPS [273–275]. The highly reactive Fmoc amino acid fluorides are especially recommended for SPPS of complicated longer peptides, and also mainly for the coupling of sterically hindered amino acid building blocks [276]. The advantage of amino acid fluorides in

the coupling of sterically hindered amino acid components, compared to other coupling methods, is clearly based on an efficient stabilization of tetrahedral transition state by the highly polarized C–F dipole and the relatively small size of the fluoride leaving group. Acylations with Fmoc amino acid fluorides also occur in the absence of a tertiary base – a fact which is important with respect to minimal racemization [277]. Fmoc amino acid fluorides are usually stable crystalline derivatives and can be synthesized from the N-protected amino acid with 2-diethylamino sulfur trifluoride (DAST), cyanuric fluoride, or tetramethyl fluoroformamidinium hexafluorophosphate $[(\text{Me}_2\text{N})_2\text{CF}]^+ \text{PF}_6^-$ (TFFH). The latter reagent is a non-hygroscopic, stable salt that is also suitable for generating the acyl fluoride *in situ* on treatment with ethyl diisopropyl amine and is, therefore, also regarded as a coupling reagent for solution- or solid-phase syntheses [278].

4.3.6

Phosphonium Reagents

Coupling reagents allowing for the in-situ generation of active esters are of considerable importance in peptide chemistry. The HOBt-derived benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) **91** developed by Castro et al. [279] is a very efficient reagent which has also been applied successfully in SPPS. The main disadvantage of this reagent however is that the highly toxic and carcinogenic HMPA is formed during the course of the reaction, whilst an unacceptably high risk of racemization has also been observed [280]. The PyBOP coupling reagent **92** [281] and its HOAt analogue PyAOP [282], where the dimethylamino groups are replaced by pyrrolidine substituents, represent viable alternatives to BOP that do not produce the hazardous by-product. Sterically hindered amino acids often can be coupled successfully using bromo-tris(pyrrolidino)phosphonium hexafluorophosphate PyBroP **93** [283]. These reagents do not react with α -amino groups, and so may be added directly to a mixture of the amino and carboxy component to be coupled. A tertiary amine is usually added in order to form the anion of the carboxy component. Nucleophilic attack of the carboxylate leads to a highly reactive acylphosphonium species that immediately is transformed into the HOBt ester in the presence of HOBt.

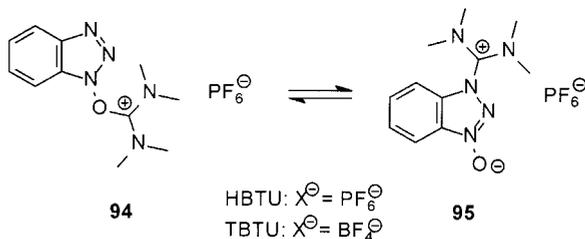


Recently, the application of 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT) as a coupling reagent with superior performance has been published by the group of Murray Goodman [284].

4.3.7

Uronium Reagents

Several uronium reagents have found widespread application as coupling reagents in SPPS, for segment condensations, and other purposes of peptide and protein chemistry. Among them are TBTU (**94**, $X = \text{BF}_4^-$) and HBTU (**94**, $X = \text{PF}_6^-$) [285, 286], which are based on *N*-hydroxybenzotriazole HOBt **86** [249] as well as the 7-azabenzotriazole HOAt **87** [260, 261] (Tab. 4.7). Considering the fact that HBTU and HATU are synthesized from HOBt or HOAt, respectively, they have been erroneously classified – by structural analogy to BOP – as uronium reagents. However, X-ray crystallographic studies revealed that they exist, at least in the solid state, as zwitterions **95** comprising a triazole-*N*-oxide and a guanidinium moiety. It is very likely that other uronium reagents display the same behavior. According to the IUPAC nomenclature, the forms of HATU must be named as 1-[Bis-(dimethylamino)methyliumyl]-1*H*-1,2,3-triazolo[4,5-*b*]pyridine-3-oxide hexafluorophosphate or as 1-[(dimethylamino)-(dimethyliminium)methyl]-1*H*-1,2,3-triazolo[4,5-*b*]pyridine-3-oxide hexafluorophosphate. It is plausible that the HBTU/TBTU forms **94** and **95** exist in an equilibrium in solution. The same applies to other derivatives.

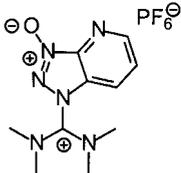
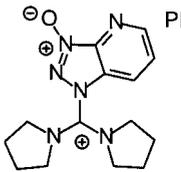
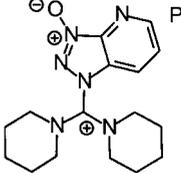
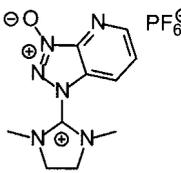
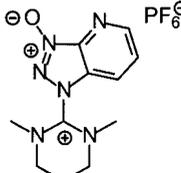


Mechanistically, the uronium reagents function in a similar way as the phosphonium reagents mentioned above. Nucleophilic attack of the carboxylate results in an *O*-acyluronium species that further reacts to give the HOBt (or HOAt) active ester. If a reaction of the guanidinium form of HOBt (or HOAt) is taken into consideration, it may be attacked by the carboxylate ion and then undergo a further acyl migration to give the HOBt/HOAt active ester.

Unlike carbodiimides or phosphonium salt reagents, they may undergo a reaction with free amino groups present. Consequently, the formation of tetramethylguanidinium derivatives of free amines (e.g., *N*-terminal amino groups) has been observed [287, 288]. The replacement of the dimethylamino moieties by pyrrolidino groups has also been suggested [289], and other active ester types, such as in PfPyU, have been considered [290].

All phosphonium and uronium salt reagents based on HOBt (or even better, on HOAt) serve as direct coupling reagents. A comparative study [288] and theoretic-

Tab. 4.7 HOAt-derived "uronium-type" coupling reagents.

Group	Structure
HATU	
HAPyU	
HAPipU	
HAMDU	
HAMTU	

cal investigations [291] on the mechanism have been published. BOP 91, HATU, HAPyU, and HAPipU (Tab. 4.7) are especially suited for peptide cyclizations [292]. 2-Trifluoroacetylthiopyridine-HOBT as a highly efficient reagent for segment couplings must also be mentioned in this context [293].

In addition, HOBT- or HOAt-derived iminium salts have been proposed [294, 295].

4.3.8

Further Special Methods

Comparison of biochemical peptide and protein synthesis with the chemical methods justifies the application of phosphorus compounds for peptide synthesis, as adenosine triphosphate serves as an activating reagent in amino acid and protein biosynthesis. The elimination of pyrophosphate leads to an enzyme-bound mixed anhydride involving the amino acid and the phosphate group of the nucleotide which are able to transfer the aminoacyl component to the accepting group of the transfer RNA (tRNA).

In the classical phosphorazo-method [296] and other related protocols, phosphorus compounds – in many cases mixed anhydrides of phosphate derivatives and the amino acid – are used for peptide bond formation.

Mechanistically, the Mukaiyama reaction is an oxidation-reduction condensation reaction where a disulfide reduction combined with a phosphine oxidation provides the driving force for the formal dehydration, and also accepts the water hydrogen and oxygen atoms [297]. The combination of triphenylphosphine and 2,2'-dipyridyldisulfide was found to be highly favorable as the pyridine-2-thiol formed initially tautomerizes spontaneously into the stable pyridine-2-thione; hence the addition of a thiol capture reagent is unnecessary (Fig. 4.25). This method may also be applied in solid-phase synthesis.

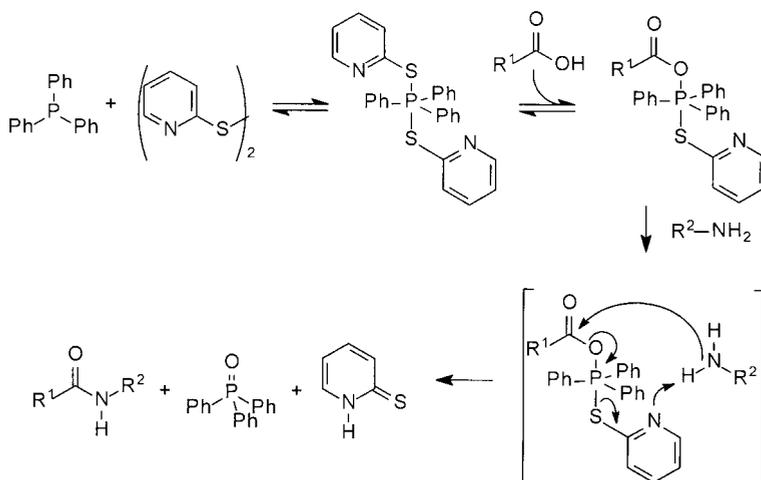


Fig. 4.25 Redox condensation according to Mukaiyama. R^1COOH =carboxy component (amino acid), $\text{R}^2\text{-NH}_2$ =amino component (amino acid or peptide).

4.4

Racemization During Synthesis

An inherent risk of racemization is imposed on all reactions involving functional groups directly connected to the stereogenic center of an α -amino acid. This may lead to a partial or total loss of the stereochemical information. While the expression “racemization” in organic chemistry is used for the complete conversion of a single enantiomer into the racemate, it is often used in peptide chemistry also for partial or total epimerization at one chiral center [298], irrespective of whether a mixture of diastereomers or enantiomers is formed in the course of the process. The optical purity and stereochemical integrity of a synthetic peptide consequently depends very much on the degree of partial epimerization during coupling steps or other types of reactions sensitive toward racemization. If only 1% epimerization occurs on each amino acid residue during peptide synthesis of a 10-peptide, the mixture of diastereomers contains only 90.4% of the peptide with the correct stereochemistry. In the case of a 50-peptide, only 60.5% of the desired diastereomer are obtained in theory. As the biological activity of a peptide or a protein depends strongly on the configuration of the chiral centers of the building blocks, racemization must be minimized during peptide synthesis [298, 299].

4.4.1

Direct Enolization

Although free amino acids are usually configurationally stable, the situation may be different when carboxy-activated derivatives **96** are involved. In an equilibrium, reversible carbanion formation **97** leading to a partial loss of the stereochemical integrity may be observed, because re-protonation occurs without facial selectivity.

The acidity of the proton H^α of an amino acid depends on the nature of the substituents. Only a base-catalyzed enolization mechanism (Fig. 4.26A) plays a significant role under peptide coupling conditions. The rate of racemization depends on the electron-withdrawing effect of the groups R, Y, and X, on the solvent, temperature, and on the chemical properties of the base B used. Base-catalyzed racemization has been observed under different coupling conditions, during ester saponification, etc., while an acid-catalyzed enolization (Fig. 4.26B) results in the racemization of N-substituted N-methyl amino acids upon reaction with HBr/HOAc.

4.4.2

5(4H)-Oxazolone Mechanism

Racemization during coupling reactions is mainly caused during amino acid activation by the formation of stereochemically labile 5(4H)-oxazolones (2-oxazolin-5-ones, azlactones) **D/L-100** as reaction intermediates (Fig. 4.27).

The propensity toward 5(4H)-oxazolone formation strongly correlates with the activation potential of the activating group X in **98**, and with the electronic proper-

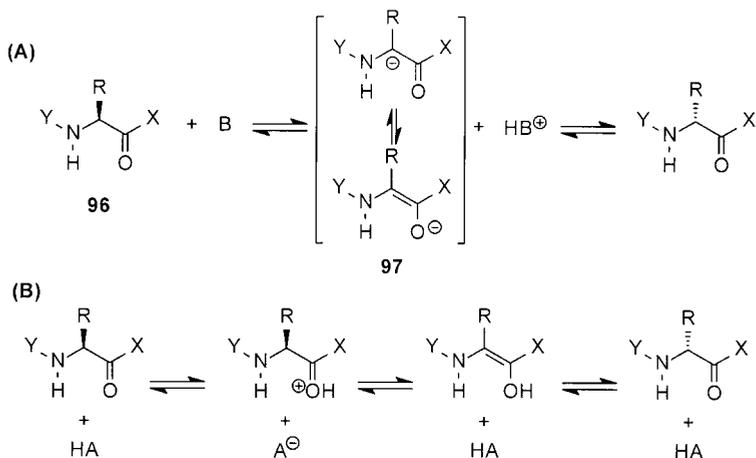


Fig. 4.26 Mechanisms of base-catalyzed (A) and acid-catalyzed epimerization (B) of activated amino acid and peptide derivatives. X=activating group; Y=N^α-protecting group of peptide chain; B=base; HA=acid; R=amino acid side chain.

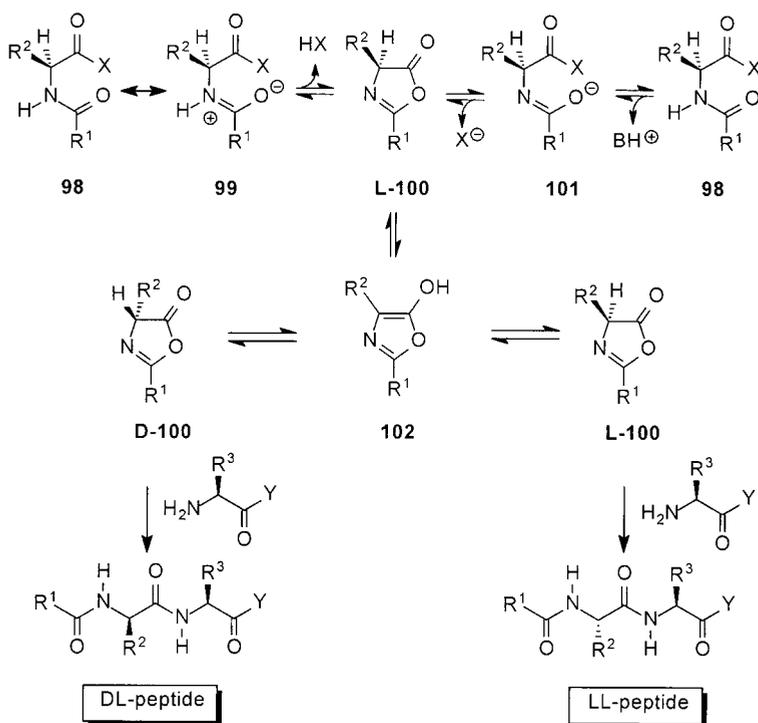
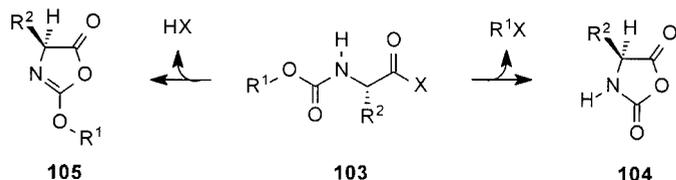


Fig. 4.27 Racemization via 5(4H)-oxazolone formation. R¹=alkyl, aryl or alkoxy residue; R², R³=amino acid side chain; B=base; X=activating group.

ties of the N-acyl residue $R^1\text{-CO-}$. Acyl residues such as an acetyl, benzoyl, or trifluoroacetyl favor oxazolone formation due to the nucleophilic potential of the carbonyl oxygen. The mesomeric electron donation by the amino group enhances the nucleophilic attack of the oxygen atom to the activated carboxy group (Fig. 4.27). The oxazolone **1-100** formed by this process from an L-configured amino acid, is prone to epimerization in the tautomeric equilibrium between **1-100**, **102**, and **D-100**. An oxazolone of type **100** may easily racemize under coupling conditions in a base-catalyzed reaction via the aromatic intermediate **102**, giving rise to a mixture of both enantiomers **1-100** and **D-100**. In the past, oxazolone formation was not supposed to occur with N-alkoxycarbonyl-protected amino acid derivatives. If at all, activated N-alkoxycarbonyl amino acids **103** were believed to undergo cyclization giving N-carboxy anhydrides **104**, but not to suffer from 2-alkoxy-5(4*H*)-oxazolone (**105**) formation. The synthetic tactics of peptide chain assembly starting from the C-terminus using urethane-type protecting groups, which is regarded to be devoid of racemization, relies on this assumption. However, it was shown in 1977 that 2-alkoxy-5(4*H*)-oxazolones may in fact be formed upon activation of the carboxy group [233, 234, 251].



These derivatives seem to be much less prone to racemization than the 2-alkyl or 2-aryl counterparts, and also undergo aminolysis much more rapidly [243, 299]. Although the reasons for that behavior have not yet been fully clarified, the decreased acidity of a urethane-protected NH group ($R\text{-O-CO-NH}$) compared to an amide group ($R\text{-CO-NH}$) seems to be a crucial factor. As depicted in Fig. 4.27, the base abstracts the amide proton in a rapid reaction step. The rate-determining ring closure is initiated by nucleophilic attack of the oxygen atom of the anionic amide group to the activated carboxy group (**101**). Hence, a decrease in the NH acidity – as is the case in the urethane-protected derivatives – lowers the rate of oxazolone formation. Preactivation of amino acids with coupling reagents is sometimes accompanied by racemization, and hence should be avoided when, for example, racemization-prone building blocks are to be incorporated.

Despite their tendency to racemize, the oxazolones still represent carboxy-activated amino acid derivatives, and so are incorporated into the peptide. The degree of racemization observed after peptide coupling reactions depends on both the propensity for oxazolone formation and the rate of oxazolone aminolysis. The nucleophilicity/basicity relationship of the amino component is also a crucial parameter.

Asymmetric induction may also be involved in this kind of epimerization process. In 1966, Weygand et al. reported that so-called positive diastereomers (L,L-peptides) are predominantly formed upon reaction of racemic 5(4*H*)-oxazolones with L-configured amino acid esters [300]. This phenomenon relies on the reac-

tion of a configurationally labile molecule present in two enantiomeric forms **L-100**, **D-100** with a chiral compound and may, in a positive sense suppress or, in a negative sense enhance racemization in peptide synthesis. Consequently, the degree of epimerized product observed after a peptide synthesis is the result of both the racemization according to the oxazolone mechanism and asymmetric induction.

4.4.3

Racemization Tests: Stereochemical Product Analysis

Determination of the degree of racemization occurring in one discrete coupling step, and evaluation of the diastereomeric purity of peptide products, is a necessary measure because of the imminent risk of racemization. Subsequent to the proof of sequence homogeneity of the product using different analytical techniques (e.g., high-performance liquid chromatography (HPLC), capillary electrophoresis, mass spectrometry), the absence of undesired diastereomers in the final product has to be proven. This may be done by complete proteolytic degradation, as reported by Finn and Hofmann [301]. Total hydrolysis of the peptide, followed by chiral resolution of all amino acid components with analytical techniques is a viable alternative, but is impeded by the uncertainty of partial epimerization during hydrolysis. The application of isotope-labeled hydrolytic agents (deuterium- or tritium-labeled HCl) circumvents this disadvantage [298, 302]. The configurational integrity of amino acids may be determined by incubation with L-amino acid oxidase, the transformation into diastereomeric dipeptides, or gas-liquid chromatography (GLC) on enantiomerically pure stationary phases.

Information on the degree of racemization during a coupling reaction may be obtained by the synthesis of model peptides, applying either an activated N-acyl amino acid or an activated N-protected peptide. Sometimes, the additional chiral center of isoleucine is utilized in a simple test method, as the D-allo-isoleucine residue formed by racemization at the isoleucine C^α is a diastereomer of L-isoleucine and hence can easily be separated. The variety of methods described has been summarized in review articles [298, 299]. Despite the value of such model systems [261, 303–305] in the development of new coupling methods, a generalization of the results is commonly not possible, because racemization is also sequence-dependent. A HPLC-based racemization test utilizing fluorescence detection has been published by Griehl et al. (Fig. 4.28) [304]. In this investigation, the coupling of Z-Ala-Trp-OH to H-Val-OMe was examined using different coupling methods. The diastereomeric tripeptides L,L,L-Z-Ala-Trp-Val-OMe and L,D,L-Z-Ala-Trp-Val-OMe were separated by reversed-phase (RP) HPLC. Fluorimetric detection provides 100 times higher sensitivity compared to the UV-detection commonly used.

In summary, the problem of racemization still impedes the chemical synthesis of peptides.

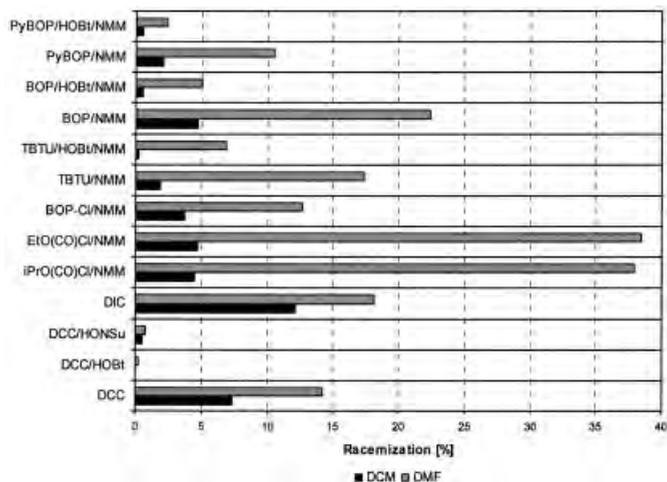


Fig. 4.28 Percentage of racemization upon coupling of Z-Ala-Trp-OH to H-Val-OMe with different coupling reactions.

4.5

Solid-Phase Peptide Synthesis (SPPS)

Ever since the early days of peptide chemistry, reactions involving peptide synthesis have been traditionally performed in solution, and many impressive results – including the total synthesis of small proteins – have been achieved using this conventional technique. In 1981, ribonuclease A – which consists of 124 amino acids – was synthesized in crystalline form by Yajima and Fujii [306] and shown to have a high specific activity. Unfortunately, peptide synthesis in solution is highly labor-intensive and requires extensive knowledge with regard to the strategy and tactics in choosing protecting groups and coupling methods, as well as solving problems of solubility.

One major advantage of solution-based synthesis is the high purity of the final product, though this is clearly dependent upon the purification of intermediate compounds. Despite these restrictions, highly specialized and experienced research teams have successfully completed many ambitious syntheses following this technique.

The ingenious concept of peptide synthesis on a solid support – which is now known as SPPS – was developed by Robert Bruce Merrifield in 1963 [307], and provided a major breakthrough in peptide chemistry. Merrifield was awarded the Nobel prize in 1984 for this unique invention that has revolutionized organic chemistry during the past 20 years. Today, the concept has been extended and generalized to organic synthesis on polymeric supports, which includes not only heterogeneous reactions involving an insoluble polymer, but also the application of soluble polymeric materials which allow homogeneous reactions (liquid-phase peptide synthesis) to be conducted. A combination of these two variants is also possible, being referred to as alternating solid-liquid-phase peptide synthesis.

In SPPS the peptide chain is assembled in the usual manner, starting from the C-terminus. The amazingly simple concept is that the first amino acid of the peptide to be synthesized is connected via its carboxy group to an insoluble polymer that may be easily separated from either reagents or dissolved products by the use of filtration. The general principle is shown in Fig. 4.29.

A necessary prerequisite is that anchoring groups (linkers) are introduced into the polymeric material (Fig. 4.29, step 1). An amino acid which is protected at N^α is then reacted with the functional group of the linker (step 2). Subsequently, the temporary protecting group is removed (step 3) and the next amino acid compo-

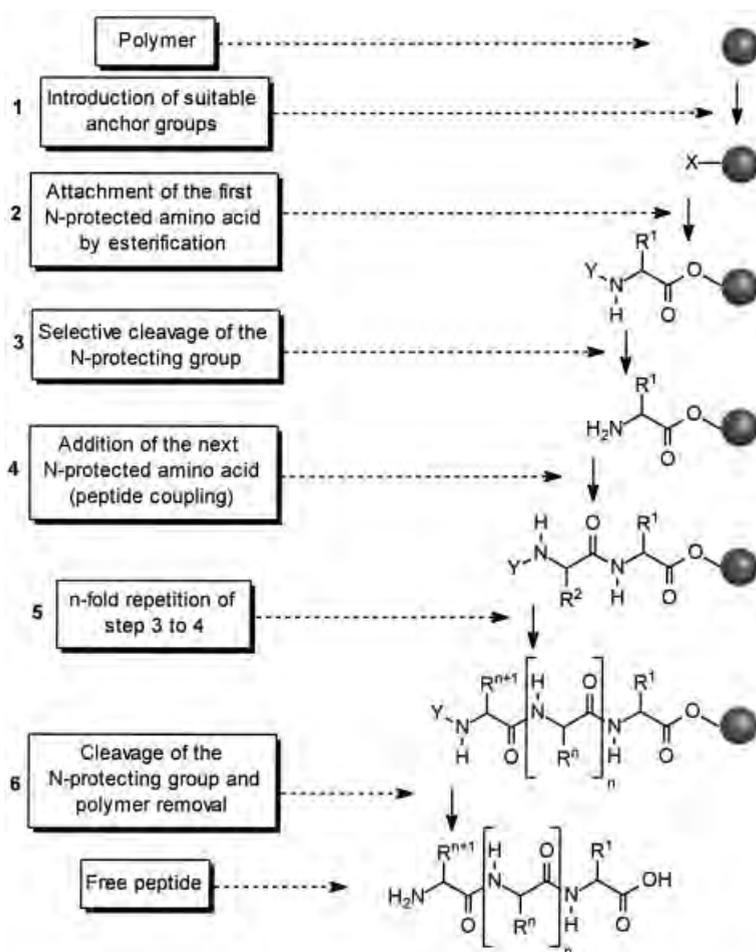


Fig. 4.29 Process of solid-phase peptide synthesis. Y=temporary protecting group; R^1 , R^2 , R^n , R^{n+1} =amino acid side chains, if necessary, protected with semipermanent protecting group.

ment is coupled (step 4). Steps 3 and 4 are then repeated (step 5) until the required peptide sequence has been assembled. Finally, the covalent bond between the linker moiety and the peptide chain is cleaved. In many cases the semipermanent side chain-protecting groups may be simultaneously removed (step 6). The insoluble polymeric support is then separated from the dissolved product by filtration.

When syntheses are carried out on a polymeric support there is no need to perform the tedious and time-consuming isolation and purification of all intermediates, as would be necessary in solution synthesis. The product of all the reactions (the growing peptide chain) remains bound to the support during steps 3 to 5, and excess reagents and by-products are removed by filtration. The simple technical operation and the potential for automation initially led to the euphoric conclusion that the chemical synthesis of polypeptides and proteins had, in principle, been solved by the solid-phase concept. Clearly, the Merrifield synthesis had a major influence on chemical peptide and protein synthesis as well as on solid-phase organic synthesis. The concept of chemical synthesis on a polymeric support also promoted the chemical synthesis of many other biomolecules, including as oligo- and polynucleotides.

Unfortunately, despite the high expectations that were initially imposed on the Merrifield method, it does suffer from several limitations:

- The final product of a synthesis carried out on a polymeric support is only a homogeneous compound, if all deprotection and coupling steps proceed quantitatively.
- A large excess of each amino acid component is required in the corresponding coupling reaction in order to achieve complete conversion.
- There is a permanent risk of undesirable side reactions during activation, coupling, and deprotection.
- Monitoring the reaction progress and analysis of complete conversion are difficult to perform in heterogeneous reaction systems, and are hampered by experimental error.
- Swelling properties of the polymeric resin and diffusion of the reagents are important parameters for the success of a solid-phase synthesis.
- Aggregation phenomena of the growing peptide chain may complicate the synthesis.
- On occasion, drastic conditions required to cleave the peptide from the polymer may also damage the final product.

Although all methods described for synthesis of peptides and organic molecules on polymeric support rely on the basic principle introduced by Merrifield, the expression “Merrifield synthesis” has mainly been coined for SPPS. More specialized information is available in a series of review articles and monographs [308–318].

4.5.1

Solid Supports and Linker Systems

The polymeric support must inevitably be chemically inert, mechanically stable, completely insoluble in the solvents used, and easily separated by filtration. It must contain a sufficient number of reactive sites where the first amino acid of the peptide chain to be synthesized can be attached. Interactions between the peptide chains bound to the resin should be minimal. As with a carboxy-protecting group, the resin must be stable under the chemical conditions of the synthetic procedures, and cleavage under mild conditions must be possible. Furthermore, it has to display mechanical stability in order for the necessary separation procedures to be carried out.

Initially, a copolymer of polystyrene with 1–2% divinyl benzene as cross-linker was used in SPPS. The dry resin beads (Fig. 4.30) are normally 20–80 μm in diameter, and are able to swell to five- or six-fold volume in the different organic solvents used for peptide synthesis (e.g., dichloromethane; Tab. 4.8). Consequently, the polymeric support – when suspended in these solvents – is not a static solid matrix but a well-solvated gel with mobile polymeric chains. This facilitates diffusional access of the reagents to all reaction sites. Polystyrene with a low-

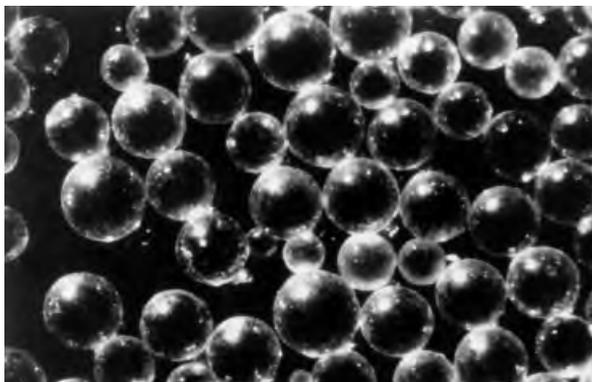


Fig. 4.30 Polystyrene/divinylbenzene cross-linked resin.

Tab. 4.8 Swelling properties (based on dry volume) of polystyrene/1% divinylbenzene resin beads in different solvents.

<i>Solvent</i>	<i>Swelling factor</i>	<i>Solvent</i>	<i>Swelling factor</i>
Tetrahydrofuran	5.5	N,N-Dimethylacetamide	3.4
Dichloromethane	5.1	Diethylether	2.5
Dioxan	4.6	Acetonitrile	2.0
Toluene	4.5	Ethanol	1.05
N,N-Dimethylformamide	3.5	Methanol	0.95

er degree of cross-linking (1% divinylbenzene rather than 2%) was found to have a superior performance, mainly because of the improved swelling properties.

By using autoradiography with tritium-labeled building blocks, it has been shown that the peptides are distributed uniformly within the gel matrix (Fig. 4.31). This finding contradicts the previous assumption that the reactions proceeded only on the surface of the resin beads. Approximately 10^{12} polypeptide chains can locate on one polystyrene/divinylbenzene bead of 50 μm diameter, and with a resin loading of 0.3 mmol peptide per gram resin.

The heterogeneous reactions used in SPPS are usually two- or three-fold slower than homogeneous reactions. The reaction sites are uniformly distributed within the polymeric matrix, and the reaction rates of the early coupling and deprotection steps are usually comparable with those at the end of a synthesis. An optimum degree of resin loading with the first amino acid ranges from 0.2 to 0.5 mmol per gram resin. On the basis of this assumption, it can be calculated that the peptide, after coupling of 15 amino acids, contributes more than 50% of the total mass. The growing mass ratio of peptide/resin material does not usually reduce the efficiency of further chain elongations, although the swelling behavior in nonpolar solvents is significantly reduced. The addition of higher percentages of DMF or urea is reported to be advantageous in such cases. Support materials that are more polar (e.g., polyamide-based resins) have also been recommended for the synthesis of longer peptides.

The classical Merrifield resin complies well with the requirements for suitable polymeric support materials, but it is not optimal with respect to mechanical stability, loading capacity, diffusional problems, and differences in solvation between the polymer and the peptide.

Cross-linked poly(dimethylacrylamide) was introduced as a more hydrophilic support by Atherton and Sheppard [312]. This forms a gel and is highly solvated

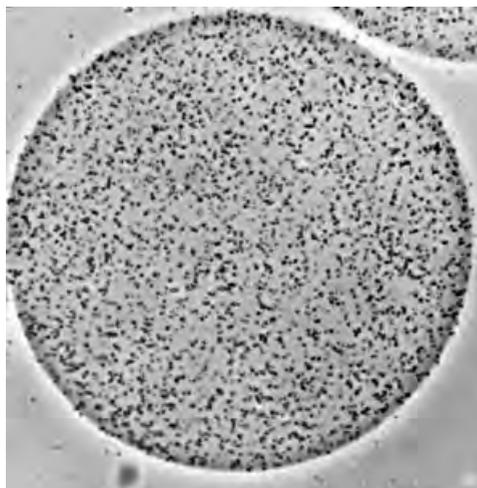


Fig. 4.31 Distribution of ^3H -labeled peptides in a single polystyrene/divinylbenzene bead, as revealed by autoradiography.

by solvents appropriate for peptide synthesis (e.g., DMF) which favors peptide synthetic reactions. The swelling properties of this polymer are superior to those of polystyrene-based resins, and this allows improved accessibility of the functional groups present on the polymer matrix, even in the micropores. This type of support has also been used in further developments such as polymerization in the pores of a macroporous rigid silica (kieselguhr) matrix, providing improved mechanical stability. Hence, syntheses can be performed under moderate pressure in a column, while the reaction progress can be monitored continuously by spectrophotometry (see Fig. 4.35).

Hydrophilic tentacle polymers are obtained by grafting polyethylene glycol (PEG) chains with an arbitrary degree of polymerization onto polystyrene beads. Grafted copolymers using porous polystyrene are suited best for this purpose [313], with the high solvation of the PEG chains conferring high mobility on the peptides bound to the copolymer. The term "tentacle polymer gel" (TentaGel) was coined for these materials, which allow solid-state NMR techniques such as magic angle spinning (MAS) $^1\text{H-NMR}$ or even solution-phase $^{13}\text{C-NMR}$ techniques to be used to monitor reaction progress. Different types of conformation have been found in relaxation time measurements that distinguish between solvated tentacles and nonsolvated random coils. While the latter are characterized by broad lines, the former give rise to sharp NMR absorptions, even though they are present in the solid state. The swelling factors in different solvents are quite similar; therefore, these polymers are stable to moderate pressure and may be used in columns for continuous-flow peptide synthesis.

Other types of polymers have been tested for applications in synthesis. The polystyrene resin itself has been chemically modified, but structurally different polymers were also studied. Nonswelling, highly cross-linked polystyrene/divinylbenzene matrices are rigid and have a high inner surface area; pellicular and brush-type resins have also been developed. While the former are obtained by the polymerization of a thin polymeric layer on the surface of inert glass beads, the latter consist of a linear polystyrene brush polymer grafted onto a polyethylene film surface. These present the anchoring linker moieties in the form of a brush on the surface. Polymers in the shape of strips, films, and fibers have also been tested in automated syntheses.

Sintered polyethylene, cellulose, silica, controlled pore glass (CPG), and chitin have also been used as planar support materials.

Nowadays, in line with the growing development of combinatorial chemistry and solid-phase bioassays [319], further requirements must be imposed on resin materials for synthetic purposes. Among these are biocompatibility, good swelling properties in either aqueous or buffered aqueous solutions, and a reduced nonspecific binding affinity to biomolecules.

The introduction of anchoring groups (linkers) on a polymeric support is the precondition for application in peptide synthesis, and the many different linker systems currently available for SPPS and solid-phase organic synthesis have been recently reviewed [320]. Additional handles in the form of bifunctional linker moieties may be attached to the polymer matrix. One of the functional groups fulfils

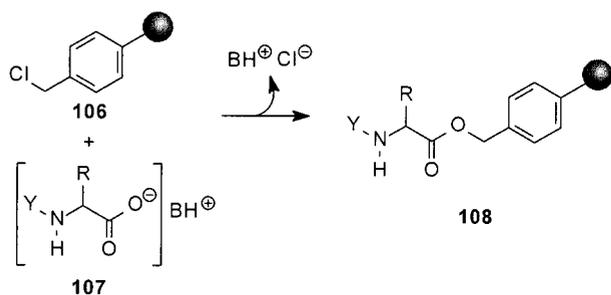
the requirements for a protecting group allowing for cleavage under mild conditions, while the other is used for attachment to the resin. This concept provides improved control of the level of resin loading, and also allows greater freedom in the choice of polymer matrix type (e.g., polystyrene, polyacrylamides, CPG, chitin, cellulose). Handles often contain internal reference amino acids (IRAA); these improve the monitoring of reaction progress, provide an exact yield determination, and help to control the integrity of the resin-bound peptide chain [312].

Because linkers must be considered as protecting groups for the C-terminal carboxy group of the peptide to be synthesized, their design and implementation must consider especially their chemical properties. Depending on whether the C-terminus of the desired peptide is required as a carboxylate, a carboxamide, a hydrazide (for backing-off strategy), an ester, a thioester [321], or an alcohol, the linker system must be chosen appropriately. In most cases, the first amino acid is connected to the resin via an ester bond, though amide or hydrazide bonds may also be involved.

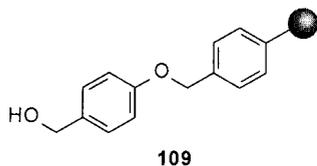
Correct selection of the handle allows yield optimization with respect to the attachment and cleavage steps, as well as suppression of racemization during attachment of the first amino acid and diketopiperazine formation on the dipeptide stage. For certain applications (e.g., peptide backbone cyclizations) the linker must behave orthogonally to the other semipermanent and temporary protecting groups.

Meanwhile, many different variants for the chemical functionalization of polymers have been identified, and the number is steadily increasing as the solid-phase synthesis of peptides and organic molecules undergoes rapid development.

Chloromethyl resin (Merrifield resin) [322, 323] The chloromethyl group in **106** is the classical anchoring moiety present in the Merrifield resin, and it is introduced into polystyrene/divinylbenzene resins by Friedel-Crafts-type chloromethylation with an alkoxy-substituted chloromethane in the presence of tin(IV)chloride. Attachment of the first amino acid to the resin to give **108** is performed as a nucleophilic substitution reaction of chloride by the amino acid carboxylate **107** under reflux conditions in an organic solvent such as ethanol, tetrahydrofuran, or dioxan. Often, cesium salts or alkyl ammonium salts are used. Other bases such as tertiary amines or anhydrous potassium fluoride may also be applied.



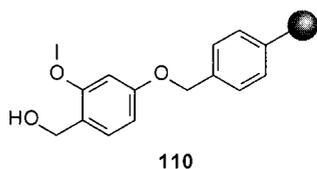
This protocol prevents racemization that might otherwise occur with an activated amino acid derivative. Therefore, the chloromethyl resin is preferred over the corresponding hydroxymethyl resin. The resulting benzyl-type peptidyl ester can be cleaved on completion of the peptide chain assembly only with very strong acids such as liquid HF, TFMSA, or HBr/TFA. Consequently, this type of resin is used in combination with Boc as a temporary protecting group and semipermanent TFA-stable side chain protection of the benzyl or cyclohexyl type to obtain peptides with a free carboxy group at the C-terminus (peptide acids). One disadvantage of the Merrifield resin is that ~1–2% of the growing peptide is cleaved from the resin during each of the repetitive acidolytic deprotection steps.



4-Benzyloxybenzyl alcohol resin (Wang resin) [324] The Merrifield resin can easily be converted into the Wang resin **109** by etherification with methyl 4-hydroxybenzoate, followed by reduction of the methyl ester with LiAlH_4 .

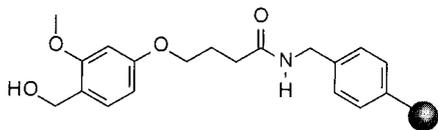
This anchoring group comprises a 4-alkoxy-substituted benzyl alcohol moiety, which confers increased acid-lability onto the linker. Hence, the Wang resin is used routinely in batch Fmoc chemistry.

Active esters (e.g., pentafluorophenyl esters) or carbodiimides (e.g., DCC or carbonyldiimidazol) are used for the direct attachment of the first amino acid on the resin. 4-(Dimethylamino)pyridine often is added as an acylation catalyst in carbodiimide couplings, but in these cases racemization may occur and the addition of HOBT is recommended. Cleavage of the final product proceeds smoothly with 95% TFA, and yields peptide acids with concomitant removal of *tert*-butyl-type side chain-protecting groups.



2,4-Dialkoxybenzyl alcohol resin (super acid-sensitive resin, SASRIN®) [325] The introduction of one more methoxy group in the 2-position of the benzyl alcohol moiety (**110**) results in a further increased sensitivity towards acid, because the benzyl cation intermediate of acidolysis is highly stabilized by both electron-donating alkoxy groups in 2- and 4-positions. Cleavage occurs even in the presence of 0.5–1% TFA in dichloromethane, or by treatment with the acidic alcohol 1,1,1,3,3,3-hexafluoroisopropanol in dichloromethane. This property makes the resin very well suited for the synthesis of peptide acids that are fully protected at

the side chains. Attachment of the first amino acid component is performed analogously, as discussed for the Wang resin. Racemization during this esterification may be a severe problem.



111

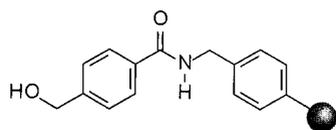
4-Hydroxymethyl-3-methoxyphenoxybutyric acid resin (HMPB resin) The HMPB handle type 111 is chemically related to the 2,4-dialkoxybenzyl alcohol resin, and displays similar cleavage characteristics. Detachment of the final product, even in the form of fully protected peptides, is achieved with dilute acid (1% TFA/dichloromethane) to yield peptide acids.

4-(Hydroxymethyl)phenylacetamidomethyl resin (PAM resin) [326, 327] The PAM resin (Fig. 4.32) was developed as an alternative to the Merrifield resin, where loss of peptides during acidolytic Boc deprotections and insufficient reactivity in the nucleophilic displacement of the linker benzyl chloride by the first amino acid carboxylate is sometimes observed.

Aminomethyl groups can also be attached to the aromatic moieties of polystyrene. The electron-withdrawing carboxamidomethyl group renders the PAM resin about 100 times more stable towards treatment with TFA. It is introduced by acylation of aminomethyl polystyrene with (4-hydroxymethylphenyl)acetic acid. The first amino acid is introduced by carbodiimide or active ester chemistry forming a resin-bound ester.

Alternatively, a PAM anchoring group, where the first amino acid residue is already attached to it, may be introduced by the reaction sequence displayed in Fig. 4.32 (pre-formed handle strategy). The bromide substituent in 4-(bromomethyl)phenylacetic acid phenacyl ester undergoes nucleophilic displacement upon treatment with the cesium carboxylate of the corresponding amino acid. Subsequently, the phenacyl ester is cleaved by reduction with zinc in acetic acid and the loaded PAM anchoring group is attached to aminomethyl polystyrene.

The PAM resin is fully compatible with the Boc/Bzl protection scheme, and is cleaved with strong acids such as liquid HF, TFMSA, or HBr/TFA to give peptides with a free acid located C-terminally.



112

Hydroxymethylbenzoic acid resin (HMBA resin) [328] The hydroxymethylbenzoic acid resin 112 is closely related to the PAM resin. The only difference is the miss-

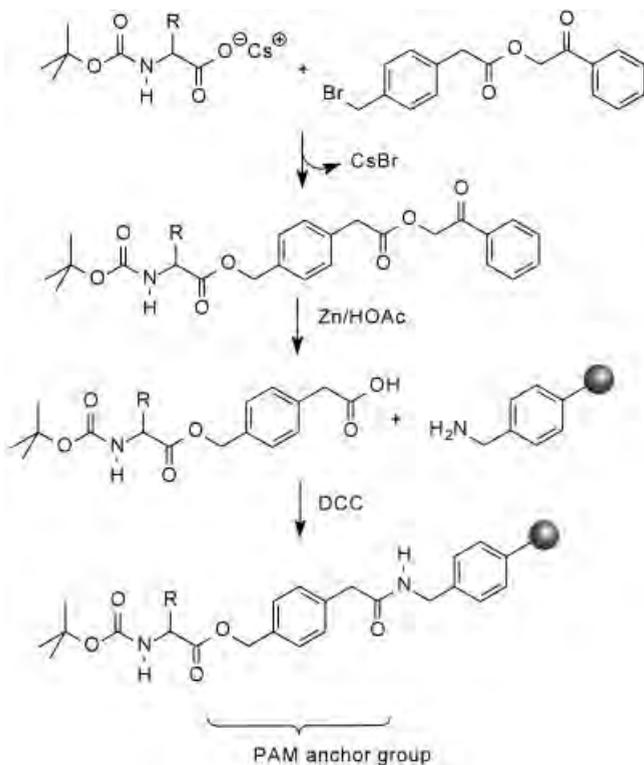
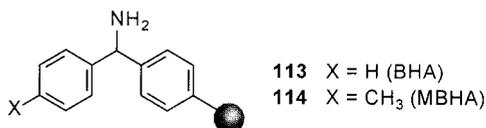


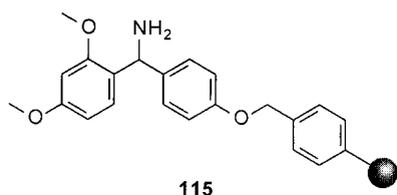
Fig. 4.32 Coupling of the PAM linker, loaded with the first Boc-protected amino acid, to aminomethylated polystyrene.
PAM = phenylacetamidomethyl.

ing benzylic CH_2 group, but this results in a completely altered chemical reactivity. It is obtained by acylation of aminomethyl polystyrene with 4-(hydroxymethyl)benzoic acid and is completely resistant towards treatment with acids (even liquid HF). Consequently, on-resin side-chain deprotection can be accomplished. The first amino acid is introduced using carbodiimide or active ester chemistry. The highly versatile HMBA linker is cleaved by a variety of nucleophiles such as hydroxide ions (to give peptide acids), alcohols (to give peptide esters), ammonia or amines (to give peptide amides), hydrazine (to give peptide hydrazides), or LiBH_4 (to give peptide alcohols).

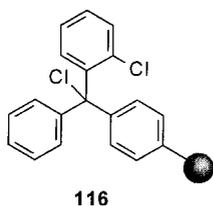


Benzhydrylamine resin (BHA resin) and 4-methylbenzhydrylamine resin (MBHA resin) [329, 330] The acid lability of a benzyl ester can be increased not only by the in-

roduction of additional electron-donating groups on the aromatic ring, but also by the attachment of one (BHA resin **113**, MBHA resin **114**, Rink amide resin **115**) or two additional aromatic rings (Barlos resin **116**) to the benzylic carbon atom. The benzhydrylamine resin (BHA resin) was designed accordingly. Acidolysis with HF, TFMSA, or HBF₄/TFA yields the peptide amide. This fact renders the BHA resin suitable for the Boc/Bzl protection scheme. Increased acid lability is observed after introduction of an electron-donating methyl substituent in the 4-position of the second aromatic ring (MBHA resin **114**).

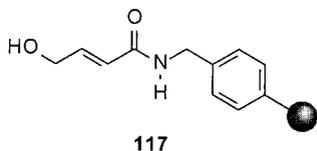


(2,4-Dimethoxy)benzhydrylamine resin (Rink amide resin) [331] The combination of a benzhydrylamine group with two additional methoxy substituents on the second aromatic ring renders the (2,4-dimethoxy)benzhydrylamine resin **115** [(4-(4'-dimethoxyphenyl)aminomethyl)phenoxy]methylpolystyrene even more labile towards acidolysis, and allows cleavage of peptide amides with 95% TFA.



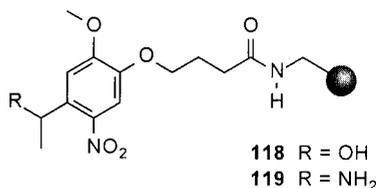
***o*-Chlorotritylchloride resin (Barlos resin)** [332]

Attachment of the first amino acid to the *o*-chlorotritylchloride resin **116** is accomplished upon reaction with an amino acid carboxylate salt (e.g., diisopropylethylammonium or triethylammonium salts). This procedure does not suffer from racemization, as the amino acid acts as the nucleophile and not as an electrophilic species. The steric constraints of the *o*-chlorotrityl group impedes diketopiperazine formation on the dipeptide stage [333]. The trityl group has already been discussed in the context of side chain-protecting groups (cf. Section 4.2.4). *o*-Chloro substitution slightly increases stability towards acids, but the *o*-chlorotrityl linker remains very sensitive to acid. Cleavage occurs upon treatment with 0.5% TFA in dichloromethane or with 1,1,1,3,3,3-hexafluoroisopropanol in dichloromethane. The *o*-chlorotrityl handle is also suitable for the attachment of C-terminal alcohols, thiols, and amines.



Hydroxycrotonoyl aminomethyl resin (Hycram resin) [334] Allyl esters are completely orthogonal to other types of protecting groups, because they are completely stable towards acids or bases usually employed in peptide synthesis. Consequently, an allyl-based handle represents a useful alternative to other linker types. The Hycram resin **117**, loaded with the first amino acid component is obtained upon acylation of aminomethyl polystyrene with esters of, for example, Boc- or Fmoc-protected 4-hydroxycrotonyl derivatives. Cleavage of the final product from the resin is accomplished under neutral conditions by treatment with a Pd(0) catalyst that effects allyl transfer to a suitable nucleophile. Fully protected peptides or very sensitive derivatives (e.g., glycopeptides) can be synthesized according to this concept.

Photolabile linkers Photocleavage provides an additional dimension in orthogonality. Similarly to photolabile protecting groups, linker moieties (e.g., **118**, **119**) are available that allow for cleavage by photolysis [335].



4.5.2

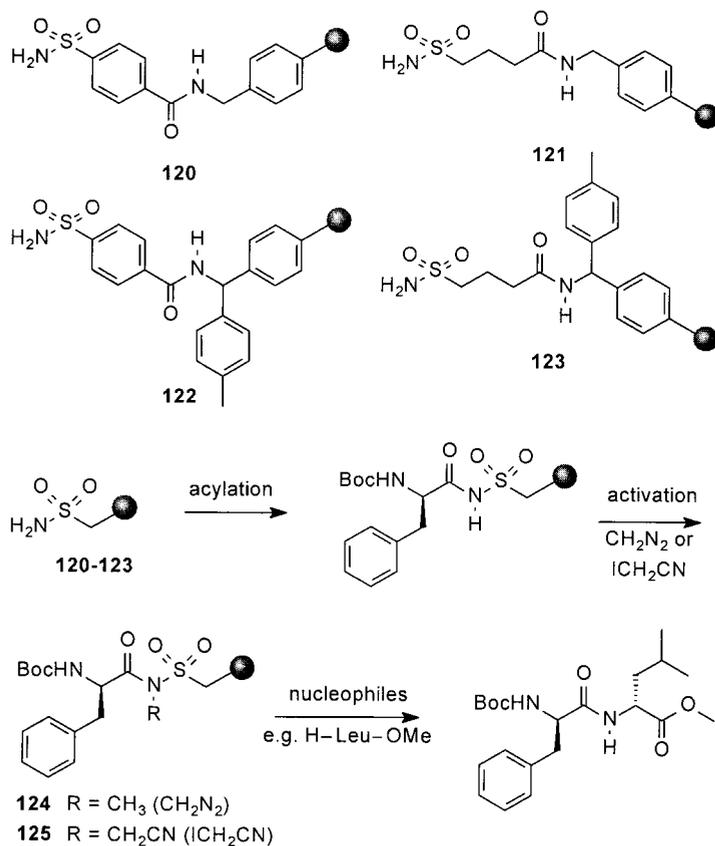
Safety-Catch Linkers

Safety-catch linker strategies [320, 336] rely on a special linker moiety that connects the polymeric support and the growing peptide chain. This linker is stable under the conditions of peptide synthesis but is finally activated for cleavage by a discrete chemical modification. In addition, the activation step usually provides an activated C-terminus of the peptide which may react with diverse nucleophiles. Intramolecular reaction of this activated position with the free N-terminus of the peptide leads to cyclic peptides and simultaneously liberates the target compound from the solid support. Two major types of safety-catch linkers may be distinguished:

- Those characterized by a hidden orthogonality of the stable and labile form, which means that cleavage mechanisms of the two forms are different.
- Those which rely on different kinetics of the cleavage reaction in both forms.

Kenner's sulfonamide safety-catch linker tethers a carboxylic acid to a solid support (e.g., aminomethyl polystyrene, **120**, **121** or MHBA, **122**, **123**), and is stable towards acidic and basic reaction conditions. After activation by treatment with diazomethane, the secondary sulfonamide **124** can be cleaved by aminolysis, peptidolysis, hydrazinolysis, or saponification to provide the primary amides, peptides, hydrazides, and carboxylic acids, respectively [337]. However, relatively poor load-

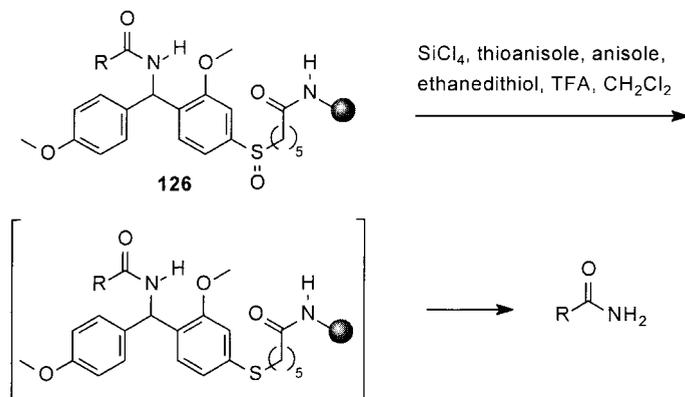
ing capacity, racemization during resin loading, and poor reactivity of the *N*-methylated peptidyl sulfonamide obtained upon diazomethane activation are major limitations of this method. The application of iodoacetone nitrile as alkylating agent led to a significant improvement because the *N*-cyanomethyl *N*-acylsulfonamide **125** is extremely reactive in nucleophilic displacement reactions under mild conditions. Alkanesulfonamide-type linkers (**121**, **123**) are superior compared to arenesulfonamide linkers (**120**, **122**) because of the greater nucleophilicity of the alkanesulfonamide nitrogen [338, 339]. Coupling conditions have been developed to load Boc- or Fmoc-protected amino acids with high efficiency and minimal racemization. Near-racemization-free displacement of the activated linker moiety with various nucleophiles has been demonstrated.



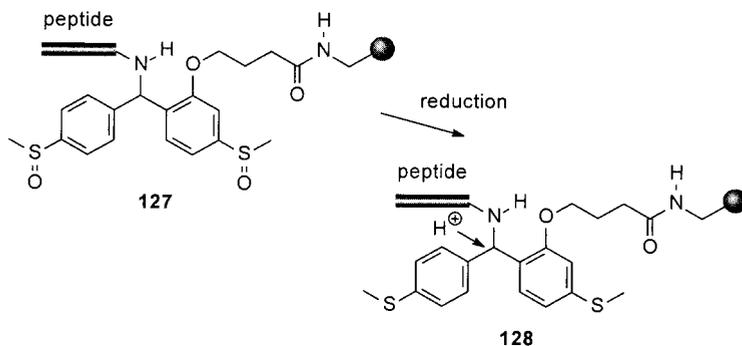
Even C-terminal thioesters, that may be subsequently used for ligation purposes, can be obtained on thiolysis of peptides bound to a safety-catch linker [340].

Safety-catch linkers relying on oxidative activation have been employed in the synthesis of linear and cyclic peptides [341, 342], but their application is limited to the synthesis of peptides that do not contain any oxidation-sensitive amino acid. The sulfoxide-containing safety-catch linker DSA **126** resists treatment with acid

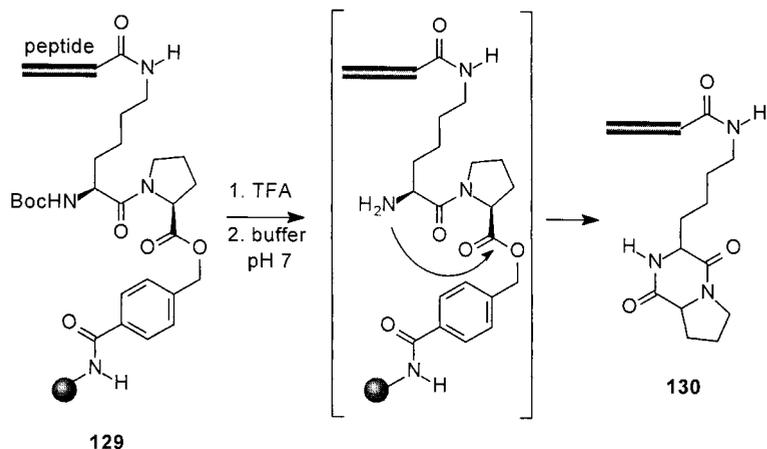
but undergoes acidolysis after reduction of the sulfoxide group; this generates a third electron-releasing group on the aromatic system [343].



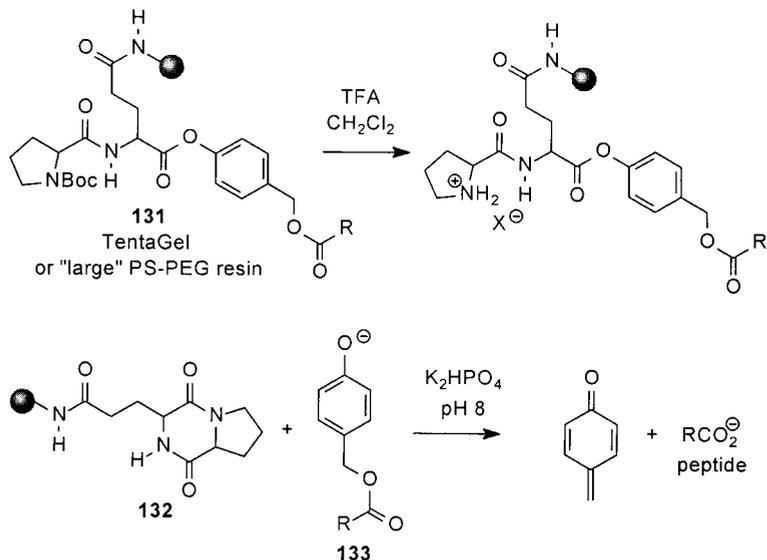
The DSA linker is structurally related to the safety-catch amide linker (SCAL) developed by Lebl [344]. In the oxidized form **127**, SCAL is stable to a wide range of chemical conditions (TFA, HF, thiophenol, reducing agents, basic conditions, and Hg^{2+}). Upon reduction of the two aryl sulfoxide groups to give the corresponding aryl sulfide **128** by $\text{PPh}_3/\text{Me}_3\text{SiCl}/\text{CH}_2\text{Cl}_2$ or $(\text{EtO})_2\text{P}(\text{S})\text{SH}/\text{DMPU}$, SCAL becomes labile towards treatment with TFA. SCAL has also been used for SPPS by chemical ligation [345].



Another concept for a safety-catch linker **129**, developed by Geysen et al. [346–348] relies on the triggered formation of peptide with a C-terminal diketopiperazine **130** that is accompanied by cleavage from the resin.



A similar linker for TentaGel or “large” PS-PEG resin has been designed by Atrash and Bradley; this combines diketopiperazine formation with subsequent fragmentation. Cleavage of the Boc group at proline in **131** triggers diketopiperazine **132** formation, which in turn releases a peptidylloxymethylphenolate **133** that spontaneously undergoes fragmentation, liberating the peptide assembled on the resin as the free carboxylate [349]. These linkers have been shown to be suitable for a direct biological evaluation of a peptide released from the resin *in situ*.



A concept involving linker cleavage by neighboring group participation has been presented by Frank et al. [350, 351].

4.5.3

Protection Schemes

Each of the repetitive synthesis cycles (deblocking – coupling) is initiated by selective cleavage of the N^α-protecting group. The anchoring group (linker) and the semipermanent amino acid side chain-protecting groups must be correctly chosen with respect to the chemical properties of the temporary N^α-protecting group, which is usually held constant for all amino acids used throughout the synthesis. The selection and optimization of protecting group chemistry doubtlessly holds a key position in the success of a peptide synthesis.

In SPPS, as in liquid-phase synthesis, either the acid-labile *tert*-butyloxycarbonyl group or the base-labile fluorenyl-9-methyloxycarbonyl group are applied preferably as temporary protecting groups (cf. Section 4.2.1.1).

4.5.3.1 **Boc/Bzl-protecting Groups Scheme (Merrifield Tactics)**

The standard Merrifield system is based on Boc-protecting group tactics, which rely on selective acidolytic cleavage. The *tert*-butyloxycarbonyl group is used as the temporary N^α-protecting group in combination with benzyl-type semipermanent side-chain protection, as Boc is usually cleaved with TFA (20–50%). Fig. 4.33 shows an example of the protecting group combination for a tripeptide amide synthesis (H-Asp-Gly-Tyr-NH₂) on MBHA resin.

The semipermanent benzyl-type groups must be stable under the conditions of repetitive Boc cleavage. In order to safeguard the stability of the side-chain protection, benzyl-type groups with electron acceptors may be applied (e.g., 2,6-dichlorobenzyl, 2,6-Dcb). Cyclohexyl esters often are applied preferentially for the protection of side-chain carboxy groups. The semipermanent protecting groups and the MBHA

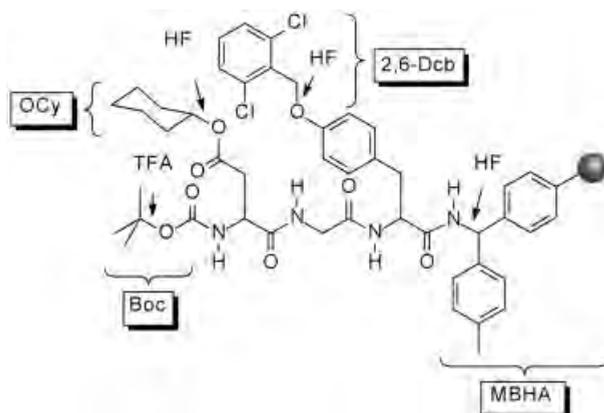


Fig. 4.33 Boc/Bzl protecting group tactics in solid-phase peptide synthesis according to Merrifield. MBHA=4-methylbenzhydrylamine resin.

linker group are cleaved simultaneously by treatment with liquid HF on completion of the synthesis, as shown in Fig. 4.33. Peptide assembly on the PAM resin would result in the peptide with C-terminal acid under similar cleavage conditions.

As discussed previously, scavengers such as anisole, thioanisole, dimethyl sulfide, or triisopropylsilane must be added to the deprotection reaction in order to avoid side reactions of the intermediate carbenium ions. Other scavengers have especially been recommended in the context of the two-stage, low–high HF cleavage method that usually provides improved product purity [352, 353].

Absolute stability of the side chain-protecting groups is generally required for the success of a peptide synthesis. In cases where N^α-deprotection relies on acidolysis, this is especially critical because cleavage of the temporary and semipermanent protecting groups as well as of the linker proceed mechanistically in a very similar manner. Fine-tuning of the cleavage kinetics is required.

Special attention must be paid to selection of the side-chain protection. As any protected amino acid residue employed in the synthesis must be strictly compatible with the protecting group tactics chosen for the synthesis of a particular peptide, some protecting group schemes are more appropriate than others. The following semipermanent protecting groups are very compatible with Boc/Bzl tactics (final cleavage with liquid HF):

- Arg(Tos), Arg(Mts)
- Asp(OBzl), Asp(OCy), Glu(OBzl), Glu(OCy)
- Cys(Acm), Cys[Bzl(4-Me)], Cys(Mob)
- His(Bom), His(Dnp) – pre-cleavage treatment is necessary, His(Z), His(Tos)
- Lys[Z(2-Cl)]
- Ser(Bzl), Thr(Bzl)
- Trp(For) – pre-cleavage treatment is necessary
- Tyr[Z(2-Br)].

4.5.3.2 Fmoc/tBu-protecting Groups Scheme (Sheppard Tactics)

The Fmoc-protecting group tactics makes use of the base lability of the fluorenyl-9-methyloxycarbonyl group (Fmoc). It is a widely applied alternative to the Boc/Bzl scheme with two-dimensional orthogonality (Fig. 4.34) [312]. Fmoc is cleaved by base-catalyzed elimination where the secondary amine (piperidine) also traps the dibenzofulvene initially formed in the reaction. The semipermanent side chain-protecting groups are mostly of the *tert*-butyl type, and can be cleaved under relatively mild reaction conditions with TFA. Linker moieties displaying comparable acid lability are mainly used.

Although introduced into peptide chemistry as early as 1970, the Fmoc/tBu scheme has been widely applied in SPPS only since 1978. Many different coupling methods are compatible with the Fmoc group, among which are the highly reactive amino acid chlorides and amino acid fluorides [277]. Fig. 4.35 shows a schematic view of a semi-automatic continuous-flow peptide synthesizer developed by Atherton and Sheppard [312]. Pressure-stable resin material is required for application in a column reactor.

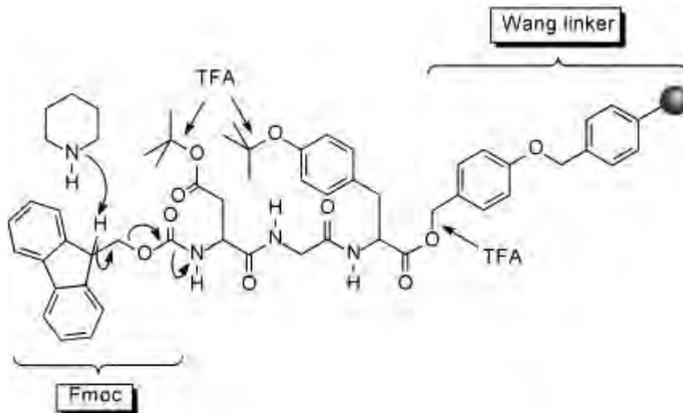


Fig. 4.34 Fmoc/tBu protecting group tactics in solid-phase peptide synthesis according to Sheppard.

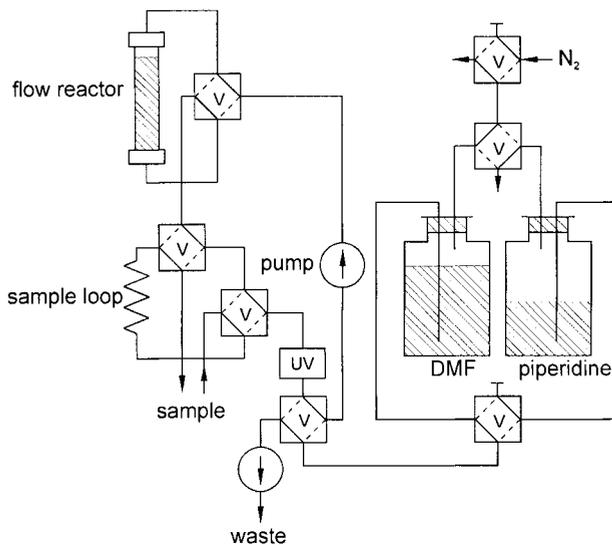


Fig. 4.35 Schematic view of a semiautomatic continuous-flow peptide synthesizer according to Atherton and Sheppard [312]. DMF=dimethylformamide; UV=ultraviolet detection; V=valve.

The following side chain-protecting groups have been used preferentially in Fmoc tactics:

- Arg(Pmc), Arg(Pbf)
- Asp(OtBu), Glu(OtBu)
- Asn(Trt), Asn(Tmb), Gln(Trt), Gln(Tmb)
- Cys(Trt)

- His(Trt)
- Lys(Boc)
- Ser(tBu), Thr(tBu)
- Trp(Boc)
- Tyr(tBu).

4.5.3.3 Three- and More-Dimensional Orthogonality

As alternatives, completely orthogonal protection schemes are available [1, 308]. These are characterized by a set of protecting groups where deprotection reactions are chemically independent. The third dimension of orthogonality relies on the application of protecting groups or linkers labile to, for example, photolysis, thiolysis (Dts), hydrazinolysis, or cleavage with transition metal catalysts (Aloc) [41, 42].

The acid-stable N^α-dithiasuccinoyl group (Dts) represents an elegant example of three-dimensional orthogonality in protection schemes of SPPS [308], and can be cleaved by thiolysis under neutral reaction conditions. A possible protecting group combination is shown in Fig. 4.36. While free thiols cleave the intramolecular disulfide bond of the temporary protecting group Dts, the semipermanent side-chain protection is removed by acidolysis. The linker containing a photolabile 2-nitrobenzyl moiety can be cleaved photolytically at 350 nm. The sequence of cleavage reactions can be chosen arbitrarily, and as a consequence fully or partially protected peptide acids can be obtained.

4.5.4

Chain Elongation

4.5.4.1 Coupling Methods

The coupling reaction is a process of eminent importance for the success of a SPPS, because complete conversion is the basic precondition for the formation of a homogeneous final product. Coupling reagents are, therefore, applied in excess

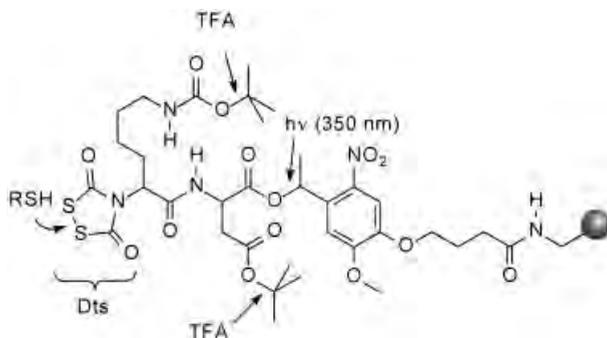


Fig. 4.36 Completely orthogonal protecting group tactics (three-dimensional orthogonality) in solid-phase peptide synthesis [309].

(usually three-fold); this increases the conversion but may also give rise to several undesired side reactions. Initially carbodiimides – especially DCC, but also DIC, because of the higher solubility of the corresponding urea – were used preferentially as coupling reagents. Peptide couplings in solid-phase syntheses are usually performed in dichloromethane, sometimes with addition of DMF, and at ambient temperature. The carbodiimide/HOBt method again prevails due to its major advantages, and not only with respect to the detrimental dehydration of Asn or Gln carboxamide side chains. Symmetrical anhydrides likewise proved to be efficient acylating agents. Active esters, especially of the pentafluorophenyl type, have also become very popular. The rather new coupling reagents of the uronium or phosphonium type, such as TBTU, HBTU, BOP, and PyBOP, UNCA derivatives, and Fmoc-protected amino acid fluorides encompass high coupling yields in nonpolar solvents and minimize or even exclude racemization and other side reactions.

4.5.4.2 Undesired Problems During Elongation

Incomplete conversion in solid-phase synthesis leads to a mixture of mismatch and core sequences, as shown in Fig. 4.37.

Considering the synthesis of a pentapeptide A-B-C-D-E, incomplete coupling may lead to four core sequences and three mismatch sequences. The latter arise when acylation or deprotection are incomplete and one or more amino acid components are skipped in the chain elongation. Mismatch sequences may also be formed when the amino acid sequence is correct, but acylation of nucleophilic side-chain functionalities, for example, occurs after partial deblocking.

The separation of undesired side products from the target peptide on completion of the synthesis is very tedious, and often impossible on a preparative scale. Hence, all possible preventative measures must be considered to safeguard any quantitative heterogeneous reaction. Double coupling steps represent one of these measures.

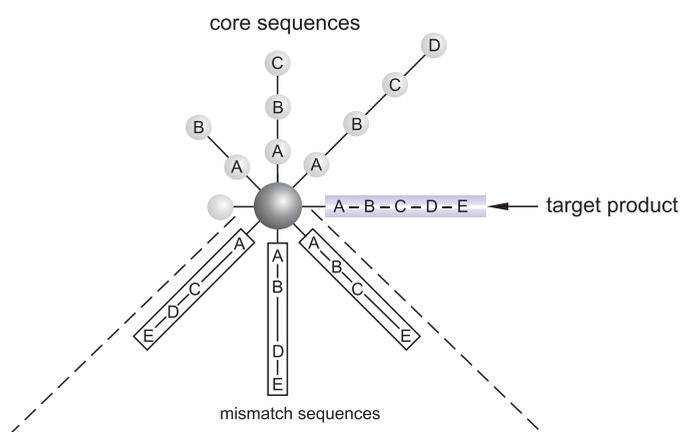


Fig. 4.37 Schematic view of side-chain reactions in solid-phase peptide synthesis.

The application of large excesses of reagents used may, however, result in other side reactions, such as aminoacyl insertions. If an acylation reaction is found to remain incomplete even after multiple repetition of a single coupling step, capping is a viable alternative. Capping means acylation of the unreacted amino groups with acetic anhydride, *N*-acetyl imidazole, or other acylating agents in order to avoid further chain elongation of the mismatch sequence at a later stage. These capped core sequences can usually easily be separated from the final product.

Peptides with a chain-terminating pyroglutamyl residue may be formed during N^{α} -deprotection of glutamine or glutamate residues located at the N-terminus.

The permanent and irreparable risk of side reactions on all synthetic steps is, despite all advantages, one of the peculiarities of SPPS. Mismatch and incomplete core sequences are caused by incomplete conversions in each of the repetitive steps of the synthesis cycle. If the yield of each cycle is hypothetically assumed to be 99%, the desired product is formed after 10 cycles in a maximum total yield of 90%, while after 100 cycles only 37% of the product is present in the mixture. An individual yield of 95% per cycle causes the total product yield after 10 or 100 cycles to decrease to 60% or 0.6%, respectively. Hence, the desired product may easily be contaminated by a series of structurally and chemically very similar compounds such as diastereomers, formed by epimerization, or mismatch and incomplete core sequences. Isolation and purification of one product from such a microheterogeneous mixture is a very difficult, though not unsolvable, problem. Despite all attempts that have been undertaken during past years to investigate most of the possible side reactions and to elucidate the mechanisms, single-cycle yields exceeding 99.5% do not appear attainable. Consequently, the remaining 0.5% adds up to a cumulative product contamination.

Mismatch and incomplete core sequences may be identified and quantified by a modified Edman degradation, also known as preview analysis [354]. In a successful synthesis, these are present in an amount below 0.3%. Mismatch or even wrong sequences are often the result of harmful or sometimes destructive reaction conditions during the repetitive deblocking steps or the final deprotection reaction.

Furthermore, N^{α} -Boc derivatives from commercial sources, for example, might in the past have been contaminated with ~0.3% of the corresponding N^{α} -*sec*-butyloxycarbonyl derivative. Once incorporated into the growing peptide chain, these derivatives resist acidolytic deprotection and so terminate the peptide chain.

Fmoc-protected amino acids formerly obtained by the classical methods might contain 1–4% of Fmoc-protected dipeptides as a consequence of competing mixed anhydride activation of the amino acid during the protection step. Incorporation of one or more of these contaminating dipeptides leads to mismatched elongated peptides. Nowadays, most *N*-protected amino acid derivatives are commercially available with chemical and optical purities >99.5%. Indeed, it is essential that all reagents and solvents, as well as the amino acid building blocks, be of high purity when used for peptide synthesis.

The application of N^{α} -urethane-protected amino acids usually maintains the degree of racemization below the analytical limits of the test systems employed

(<0.03%). Consequently, in normal cases this process does not significantly impede the outcome of a synthesis. Nevertheless, the esterification of amino acids, for example in the course of an attachment to a hydroxymethyl linker moiety or the activation of the C-terminus of a peptide, may lead to racemization of between 0.2 and 10%.

Diketopiperazine formation may efficiently compete with the coupling of the third amino acid to a resin-bound dipeptide. The diketopiperazine is cleaved from the resin during the attack of the free amino terminus to the C-terminal ester bond. Some amino acids such as glycine, proline, D-amino acids, and N-alkyl amino acids in the second position especially favor this process.

4.5.4.3 Difficult Sequences

One major precondition for complete peptide couplings is diffusion of the acyl component through the matrix, which only occurs to a satisfactory extent if the peptidyl-resin is sufficiently solvated. The most serious problem in SPPS is associated with sequence-related incomplete acylation reactions. In this context, random and nonrandom “difficult sequences” may be distinguished. Both types are characterized by reproducible incomplete acylation, limited improvement by repetitive coupling or the introduction of capping steps, and even inferior results when sterically hindered amino acids or higher resin loading are applied. Random difficult aminoacylation is associated with the incorporation of sterically hindered amino acids, for example β -branched derivatives or building blocks with bulky side chain-protecting groups. Nonrandom problems occur due to the sequence-specific formation of stable β -sheets that tend to aggregate and prevent further acylation [355, 356]. Secondary structures stabilized by intermolecular hydrogen bonds impede the diffusion and accessibility of the reactive N-terminus.

Merrifield et al. reported that a SPPS proceeded well up to the eleventh amino acid, but provided low yields at residues 12 to 17 [357]. According to Kent [355], such sequences may occur at a distance of 5–15 residues from the C-terminal residue at the resin. Despite the considerable research carried out in order to understand this phenomenon [358–360], it remains a major obstacle in peptide and protein synthesis.

Nonrandom difficult sequences may be predicted to a certain extent [361]. The propensity of the amino acids to occur in a random coil structure, facilitating synthesis, or in ordered secondary structures, hampering acylation, has been expressed by P_c^* values. The inherent tendency of a peptide chain to form a random coil conformation is then expressed by the average of the single P_c^* values of the n amino acids:

$$\langle c^* \rangle = \left(\sum P_c^* \right) / n$$

Sequences with $\langle P_c^* \rangle$ values >1.0 are associated with near-quantitative acylation reactions, while sequences with $\langle P_c^* \rangle$ values of 0.9–1.0 require longer reaction times or multiple couplings. $\langle P_c^* \rangle$ values <0.9 indicate that persistent acylation problems

Tab. 4.9 Conformational parameters of proteinogenic amino acids.

<i>Amino acid</i>	<i>P_c</i>	<i>Amino acid</i>	<i>P_c</i>
Ala	0.75	Leu	0.75
Arg	0.96	Lys	0.90
Asn	1.26	Met	0.70
Asp	1.07	Phe	0.76
Cys	1.08	Pro	1.64
Gln	0.83	Ser	1.21
Glu	0.80	Thr	0.98
Gly	1.47	Trp	0.79
His	0.96	Tyr	0.98
Ile	0.74	Val	0.73

may occur (“nonrandom difficult sequences”). β -Sheet formation can be monitored using FT-IR spectroscopy [362].

Aggregation is not a severe problem in Boc chemistry, because it is abolished by the repetitive treatment with TFA during cleavage of the temporary protecting group.

Fmoc chemistry does not encompass this advantage, but sustains β -sheet formation. This usually leads to prolonged reaction times, and either an increase in the reaction temperature or solvent changes often becomes necessary; this in turn may favor racemization [229, 363] and premature Fmoc cleavage [364, 365].

The β -sheet aggregates can be disintegrated by the application of:

- solvent additives that break up stable secondary structures, such as 2,2,2-trifluoroethanol [366], 1,1,1,3,3,3-hexafluoroisopropanol [366, 367], DMSO [368], or ethylene carbonate and Triton X [369];
- chaotropic salts in aprotic solvents [370, 371];
- temporary backbone amide-protecting groups (cf. Section 4.2.3) such as *N*-phenylthiomethyl [372], Hmb [91, 92, 373], Hnb [93], SiMb [94], or serine or threonine-derived pseudoproline dipeptide building blocks Fmoc-Xaa-Ser(Ox)-OH and Fmoc-Xaa-Thr(Ox)-OH [95–98];
- HOAt-based coupling reagents;
- polymeric supports based on polyoxyethylene-polystyrene copolymers [374], that diminish interaction between growing peptide chains;
- in-situ neutralization in the context of a modified Boc/Bzl synthesis scheme [375, 376];
- introduction of a pre-sequence [377];
- sonication [378];
- reduced resin loading; and
- coupling at elevated temperature [379].

4.5.4.4 On-Resin Monitoring

Stringent control of the coupling reactions is another indispensable prerequisite in SPPS. The ninhydrin test is one of the simplest and most frequently used methods for this purpose [380]. A positive color reaction, performed with a small aliquot of the resin material, indicates unconverted amino groups. This so-called Kaiser test is simple, reliable (in most cases), and requires only minutes to perform. Titrimetric methods are also quite easy to perform. Further monitoring methods that rely on color reactions include the TNBS test [381], the acetaldehyde/chloroanil test [382], and the bromophenolblue test [383]. Dorman developed a protocol involving the determination of chloride ions after protonation of unconverted amino groups of the peptide with pyridinium chloride and subsequent elution of bound chloride with triethylamine [384]. Free amino groups may also be directly titrated with 0.1 N HClO₄, for example. Although these methods could be integrated in an automated process, they are too time-consuming. A similar situation applies to gel-phase NMR spectroscopy, which allows a direct determination of resin-bound groups.

In the Fmoc synthesis scheme the coupling of the N^α-Fmoc-protected amino acids can be monitored directly and quantitatively by the decrease in absorbance of the reaction solution at 300 nm. Similarly, cleavage of the Fmoc group with piperidine can be monitored by the increase in absorbance at the same wavelength.

Cleavage and total hydrolysis of an aliquot of resin-bound peptide after a certain number of synthetic steps is also an important and sensible tool to check the integrity of the product. Analysis may also be performed on each synthetic step by mass spectroscopy (MALDI-ToF MS) [385]. Exact yield determination and control of the integrity of the peptides bound to the resin can reliably be performed using the IRAA technique [312, 386, 387]. Dual linker analytical constructs comprising additional analytical components for analysis have also been developed [388].

4.5.5

Automation of the Process

The first automated peptide synthesizer was developed by Merrifield in 1966 for batchwise peptide synthesis (Fig. 4.38). The original machine, which is now in the Smithsonian Museum, consists of a reactor unit and a controlling unit. The former comprises the reaction vessel and valve systems for solvents, reagents, and amino acid derivatives as well as reservoirs for all these components. Nowadays, different types of synthesizers are commercially available.

Solid-phase peptide syntheses may also be performed in a continuous-flow mode using resin-filled columns. In some instances this method has proven to be superior compared to the batchwise synthesis developed by Merrifield. Major advantages lie in the reduced reagent and solvent consumption, and in the very short coupling cycles (1–2 min for TentaGel polymers of size 8 μm). The reaction progress may be monitored on a real-time basis by recording the conductivity of the solution [389]. However, the usual types of polymeric support material cannot be used, because of their insufficient pressure stability and the solvent-dependent



Fig. 4.38 Robert Bruce Merrifield and the first-generation solid-phase peptide synthesizer.

swelling properties. Hence, chemically modified silica gel [390] and kieselguhr/polydimethylacrylamide hybrid materials have been successfully applied to automated continuous-flow synthesis [391, 392].

TentaGel-type polymers are also appropriate solid support materials for continuous-flow syntheses [313] as they are chemically and physically inert, and also stable towards moderate pressure. Their uniform spherical shape and homogeneous swelling behavior are further advantages.

4.5.6

Special Methods

The application of soluble polymeric support materials for peptide synthesis is characterized by the advantage of homogeneous reaction conditions. This may

overcome some disadvantages of SPPS. Although the use of non-cross-linked soluble polystyrene ($M_r \sim 200$ kDa) as a solid support allows synthesis to be carried out in homogeneous phase [393], the excess reagents present after each coupling step can only be separated by quite tedious precipitation operations.

The use of polyethyleneglycol (PEG) as a polymeric support and C-terminal-protecting group for the growing peptide chain led to the important improvement of liquid-phase peptide synthesis [394]. In this method, excess low-molecular weight coupling reagents can be separated by ultrafiltration. Further progress was achieved in the development of the so-called crystallization method of Bayer and Mutter [395]. The addition of a suitable organic solvent (diethylether) rapidly induces a spontaneous and quantitative crystallization of the peptidyl-PEG by the formation of helical structures. Low-molecular weight starting materials and reagents are usually not co-precipitated, and may easily be separated. The principle of liquid-phase peptide synthesis is shown in Fig. 4.39. Although coupling times correspond to those of conventional synthesis, an excess of acylating agents and double or multiple couplings are necessary in order to obtain a maximum conversion. The growing polypeptide chain influences the solubility, and viscous solutions may result even when DMF is used as solvent. The preferred area for the application of liquid-phase peptide synthesis is for polypeptides containing fewer

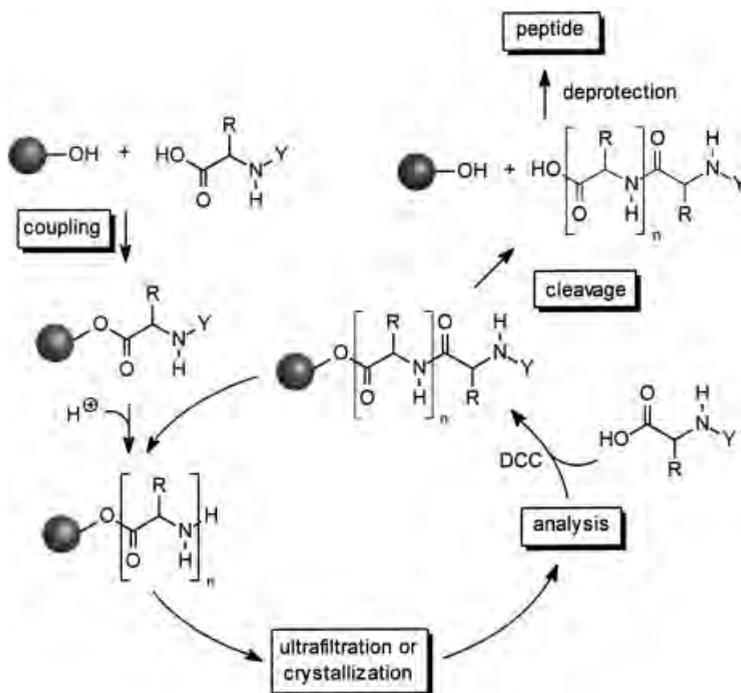


Fig. 4.39 Scheme of liquid-phase peptide synthesis on a soluble polymer. Y = amino-protecting group.

than 30 amino acid building blocks. The clear disadvantages of liquid-phase peptide synthesis are the long time taken for the operation, and the difficulties in its automation.

An alternating solid-phase/liquid-phase peptide synthesis [396] combines the strategic aspects of both methods, and of the polymer reagent peptide synthesis. The basic idea comprises a physical modification of the growing polypeptide chain during the coupling reaction in order to facilitate separation of the starting material from the product. This interesting concept has not yet received very much practical application, however.

The polymer reagent peptide synthesis does not utilize synthesis on polymeric support, because the growing polypeptide chain is retained permanently in solution. Instead, the polymer-bound reagents – and especially the activated carboxy components – are reacted with amino components with liberation of the polymer and of the protected peptide derivative. The polymer-bound reagents can be applied in excess, and the peptide formed may be separated – usually without difficulty. Wieland and Birr [397] developed a continuous process for peptide synthesis by applying polymer-bound active esters. The columns contain a polymer cross-linked with *p,p'*-dihydroxydiphenylsulfone where the active esters of N-protected amino acids are attached. However, the repetitive acidolytic deblocking reagents with subsequent neutralization steadily increase the salt concentration of the reaction solution. Photolabile N^α-amino-protecting groups have also been recommended for this column operation. Following synthesis, these are cleaved simply by photolysis, after which the resulting peptide with the free amino-terminus may be subjected to the next synthetic cycle.

4.5.7

Peptide Cleavage from the Resin

Final cleavage of the peptide from the polymeric support usually represents the last step of SPPS. As the anchoring group (linker) usually is chosen to be compatible with the peptide synthetic operations, selective cleavage between the C-terminus of the peptide and the solid support occurs upon treatment with reagents that may concomitantly effect partial or complete deprotection of the peptide side chains. In most cases, acidolytic methods are used, though other methods (transition metal catalysis for allyl-based linkers, photolysis [398, 399]) are available for the synthesis of sensitive compounds or special applications.

4.5.7.1 Acidolytic Methods

The Boc/Bzl scheme often relies on final cleavage by anhydrous liquid HF at 0°C. This reaction must be carried out in a special apparatus, because HF decomposes glassware. Scavengers such as anisole or others must be added. Both the anchoring group and all nitrogen- or oxygen-protecting groups based on the *tert*-butyl type or benzyl type are cleaved. The nitro group of nitroarginine and the methoxybenzyl group of cysteine for example are also labile towards these conditions.

Other functionalities, such as N^{im}-benzyl groups, N^{im}-2,4-dinitrophenyl groups (His), S-alkylthio groups (Cys), and 4-nitrobenzylesters (Asp, Glu) are resistant to these cleavage conditions. The two-step, high–low HF protocol often provides the final product at greater purity.

Other acidolytic cleavage reactions do not require a special apparatus and may be performed in the reactor that is used for solid-phase synthesis. Cleavage reactions with TFMSA in TFA as the solvent can be improved considerably by the addition of scavenging sulfur compounds such as thioanisole or methionine [400, 401]. Several trialkylsilyl compounds have been selected as cleavage reagents on the basis of the HSAB (*hard and soft acids and bases*) concept developed by Pearson. Hard Lewis acids such as trimethylsilyl trifluoromethane sulfonate (TMSOTf) or trimethylsilyl bromide (TMSBr) are combined in TFA as the solvent with soft bases (e.g., thioanisole) as a highly efficient deblocking system both for solution-phase and for solid-phase purposes [402]. Cleavage with this “cocktail” (e.g., 1 M TMSOTf/thioanisole in TFA; 0 °C for 60 min) is much faster than the conventional method applying 1 M TFMSA/thioanisole in TFA. The different methods for acidolytic sulfide-assisted cleavage in nonaqueous solvents have been reviewed with respect to mechanism and applications [403].

The Fmoc/tBu scheme allows for much milder final deprotection conditions, for example with TFA or acetic acid in dichloromethane as the solvent (cf. Section 4.5.1).

In the case of special linker systems (e.g., HMBA), cleavage with different nucleophiles is possible, and results in the formation of C-terminal peptide acids (cleavage with aqueous or alcoholic hydroxide solutions, thiophenolate in DMF, or solvated cyanide), peptide esters (alcohols in the presence of tertiary amines), peptide amides (ammonia or amines), peptide hydrazides (hydrazine), or even peptide aldehydes and alcohols (reductive cleavage with LiBH₄).

Without doubt, the final cleavage reaction is the most critical step of a SPPS, though on occasion even the separation of the two steps, linker cleavage and side-chain deprotection, may be advantageous.

4.5.7.2 Side Reactions

Sensitive peptide bonds such as Asp-Pro or Aib-Pro may be cleaved under the strongly acidic cleavage conditions. Asp-Gly sequences are often prone to succinimide formation and concomitant isoaspartyl peptide ($\alpha \rightarrow \beta$ shift) formation, especially when treated with strong acids such as HF or TFMSA. Similar reaction conditions facilitate an N \rightarrow O acyl shift involving serine or threonine residues. The products formed are thermodynamically labile, and the process may be reversed in aqueous bicarbonate solution (pH 7.5).

Tryptophan or tyrosine residues may, under certain circumstances, suffer from oxidative degradation. Tyrosine is prone to electrophilic C-alkylation, which may be prevented by the 2-bromobenzyl protecting group in Boc tactics. Arginine residues are reported to undergo deguanylation to give ornithine residues. As mentioned previously, methionine and cysteine residues are susceptible to oxidation

and alkylation. In Fmoc tactics, most side reactions (e.g., side-chain alkylation or lactam formation) and others are sufficiently reduced by appropriately protected amino acid building blocks, for example Fmoc-Arg(Pbf)-OH [404], Fmoc-Arg(Pmc)-OH [405], Fmoc-Trp(Boc)-OH [406], and Fmoc-Asn(Trt)-OH [407].

4.5.7.3 Advantages and Disadvantages of the Boc/Bzl and Fmoc/tBu Schemes

Although, as mentioned previously, one advantage of the Boc tactics lies in the termination of β -sheet structures during repetitive acidolysis of the temporary Boc protection, the major drawbacks are to be seen in the harsh reaction conditions for the final deprotections that give rise to different side reactions (e.g., side-chain alkylation, cleavage of sensitive peptide bonds, rearrangements, cf. Section 4.5.7.2). Moreover, special equipment is needed for the final HF cleavage reaction.

Fmoc tactics are characterized by the possibility of synthesizing side chain-protected fragments for segment condensations and peptide cyclizations, the relatively mild cleavage conditions of the side chain-protecting groups and of the linker, and by UV-spectroscopic monitoring of the reaction progress. A detrimental feature is the favored formation of β -sheets and the propensity for diketopiperazine formation on the dipeptide stage. Spontaneous cleavage of Fmoc has also been observed [364, 365].

Systematic investigations slightly favor the Fmoc/tBu scheme over the Boc/Bzl scheme [408]. However, this conclusion does not in the main rely on the synthetic performance, but rather on the greater practicability and easy technical application. Rapid continuous-flow protocols [409] and optimized support materials [374] are regarded as beneficial.

Nonetheless, a general decision regarding which protecting group tactics would be preferred cannot be made. All significant parameters in the synthesis of longer peptides or peptides containing difficult sequences should be carefully analyzed, and the selected synthesis scheme should first be investigated in a small-scale synthesis (preview synthesis) combined with appropriate analytical methods in order to identify potential weak points in the synthetic plan.

4.5.8

Examples of Syntheses by Linear SPPS

Since its invention in 1963, the Merrifield concept has been applied to the synthesis of thousands of biologically active peptides and peptide analogues, and in this respect, a large number of improved variants have been used besides the original protocol. It must be mentioned that this synthetic concept has had an enormous impact on the development of peptide chemistry and organic chemistry during the past few decades. Indeed, the manifold approaches to combinatorial chemistry would not have been developed without the foundations having been set by Bruce Merrifield.

SPPS underwent extreme activity between 1968 and 1972, when the commercialization of automated synthesizers fostered its widespread application. In those early

days, the chemical synthesis of small proteins had its limitations, and an attempted synthesis of lysozyme resulted in a complex mixture with a specific activity of only 0.5–1%. The total synthesis of ribonuclease A [410] proved to be a benchmark in that it was the first synthesis of an enzyme with a satisfactory activity of 3.5%. The application of HPLC methods in the purification of synthetic peptides during the early 1980s again led to an enormous increase in solid-phase synthesis activities that continues to this day. Initially, the solid-phase syntheses of molecules such as conotoxin G1, thymosin α , glucagon, β -endorphin, parathyrin, β -lipotropin, interleukin-3, HIV protease, and green fluorescent protein provided an impressive announcement of the vast potential of this synthetic method. Today, the commercial large-scale production of peptides includes the preparation of compounds such as corticotropin, atriopeptin, parathyrin, and calcitonin on a scale up to 200 g [310].

4.6

Biochemical Synthesis

Cells are capable of assembling amino acids and modifying peptides and proteins in a highly regulated and chemically efficient manner (cf. Section 3.2). These *in vivo* reactions occur under mild conditions, and the catalyzing enzymes are the keys to the specificity and efficiency of the process. Before the ribosomal synthesis pathway had been elucidated, general peptide and protein biosynthesis was believed to be performed by a number of simple enzymatic processes. Moreover, despite the fact that the ribosomal cycle of protein biosynthesis (cf. Section 3.2.1) is now known to comprise complex machinery, the formation of a peptide bond was originally thought to be catalyzed by an enzyme that was tentatively named peptidyl transferase (nowadays known to be a ribozyme).

Regiospecificity and stereospecificity are two major advantages of biocatalysts. In chemical peptide synthesis, the obligatory protection and deprotection of the α amino function, the carboxy group and the various side-chain functionalities before and after a synthetic step are the most fundamental time-consuming operations. Furthermore, as many of the synthetic operations take place at groups adjacent to a center of chirality, a permanent risk of racemization must be taken into consideration. Therefore, the chemical synthesis of peptides continues to be a formidable chemical effort.

Although there are more than 150 chemical methods for peptide bond formation, an ideal coupling method should allow for a rapid reaction, without racemization or other side reactions, and result in quantitative coupling when the carboxy and amino components are applied stoichiometrically.

Recombinant DNA techniques, genetic engineering, genome sequencing, biochemical protein ligation (cf. Section 5.3.4), and the application of enzymes in chemical synthesis to control stereo- and regiospecificity have led to the so-called “biotechnological revolution”. For this reason, new biochemically inclined methodologies are required at the synthetic front, both for the functional analysis of peptides and proteins, and the discovery of new therapeutic agents.

4.6.1

Recombinant DNA Techniques

The nucleotide sequence of the human genome has now been elucidated. Despite the existence in human cells of about 150 000 different genes each containing approximately 5×10^9 base pairs, the isolation of gene sequences is a realistic approach. The methods used for sequence analysis of DNA according to Maxam and Gilbert (1977) or Sanger (1977) can be applied reliably. Today, the sequence analysis of an isolated gene is more efficient than protein sequence analysis, and consequently protein sequences are often derived from DNA sequences.

Only a limited number of specialized peptides (cyclic peptides, peptide antibiotics) are synthesized nonribosomally by multi-enzyme complexes. A preparative procedure for the ribosomal synthesis of polypeptides and proteins is based on the principles of gene technology. Proteins and peptides can be expressed on a large scale by the recombination and expression of genetic material, for example in bacteria, and pharmacologically active peptides and proteins form a special focal point of interest in this context [411]. Indeed, based on their endogenous functions in the human body, these compounds may find application as therapeutic drugs, as well as influencing both pathophysiology and healing processes (cf. Chapter 9).

4.6.1.1 Principles of DNA Technology

Since the elucidation of the molecular structure of DNA by Watson and Crick, genetics and molecular biology have experienced almost unbelievable development. There is no species barrier for DNA, which serves as the “blueprint” for protein biosynthesis. For example, bacteria such as *Escherichia coli* may not only accept genetic material from other microorganisms and stably transmit it to their successors, but may also may serve as recipients of genetic material from either plants or animals.

Gene expression comprises synthesis of the corresponding mRNA (transcription) and synthesis of the protein (translation). The biosynthesis of a foreign gene product (protein) in an organism relies on a recombination of the genetic material of the microorganism with the DNA fragment encoding for the desired protein.

Eukaryotic genes contain noncoding elements (introns) in addition to the sequence information encoding for a protein. The precursor messenger RNA (mRNA) formed after transcription is subsequently processed into mature mRNA by splicing, at which point the introns are removed from the sequence.

Consequently, the genomic DNA of eukaryotes is not appropriate for direct transfection; instead, DNA which is complementary to the mature mRNA (cDNA) must be synthesized and used for transfection. The formation of cDNA is a prerequisite in order to obtain the correct protein sequence derived from a human gene, for example. Mature mRNA isolated from the donor cells is used as a template to synthesize cDNA *in vitro* with the enzyme reverse transcriptase, which oc-

curs naturally in retroviruses. This enzyme requires a synthetic primer (of about 12 nucleotides) that is complementary to the sequence of the 3'-end of mRNA. In eukaryotic cells, this nucleotide sequence is often a poly-adenosine tail, but it may also encode for the C-terminal amino acid sequence of the protein. The hybrid composed of cDNA and mRNA is then subjected to alkaline hydrolysis, which destroys the mRNA. The cDNA is subsequently replicated and amplified by polymerase I. Blunt ends are formed by S1-nuclease, and synthetic oligonucleotide sequences containing cleavage points for restriction endonucleases are attached using DNA ligase for insertion into the vector. Alternatively, homopolymeric sequences are added using terminal transferase. The procedure is shown schematically in Fig. 4.40. Finally, the encoding DNA may be sequenced for verification purposes and subsequently be inserted into expression vectors of suitable host cells.

The process comprises the following steps (Fig. 4.41):

1. Isolation of the encoding DNA fragment from the donor organism.
2. Insertion of the DNA into a vector.
3. Transfection of the vector into the host organism.
4. Cultivation of the host organism (cloning), which leads to gene amplification, mRNA synthesis, and protein synthesis.
5. Isolation of the recombinant protein.

Originally, the term “cloning” meant the amplification of cells, with formation of identical daughter cells, but nowadays the term is also used for the amplification of DNA. Initially, minute amounts of the protein are identified using biological as-

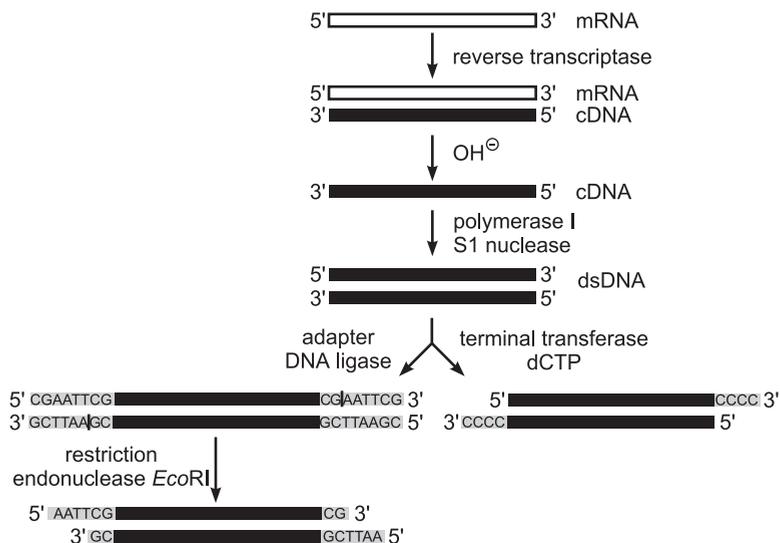


Fig. 4.40 Reverse transcription of mRNA into cDNA, followed by attachment of synthetic oligonucleotides.

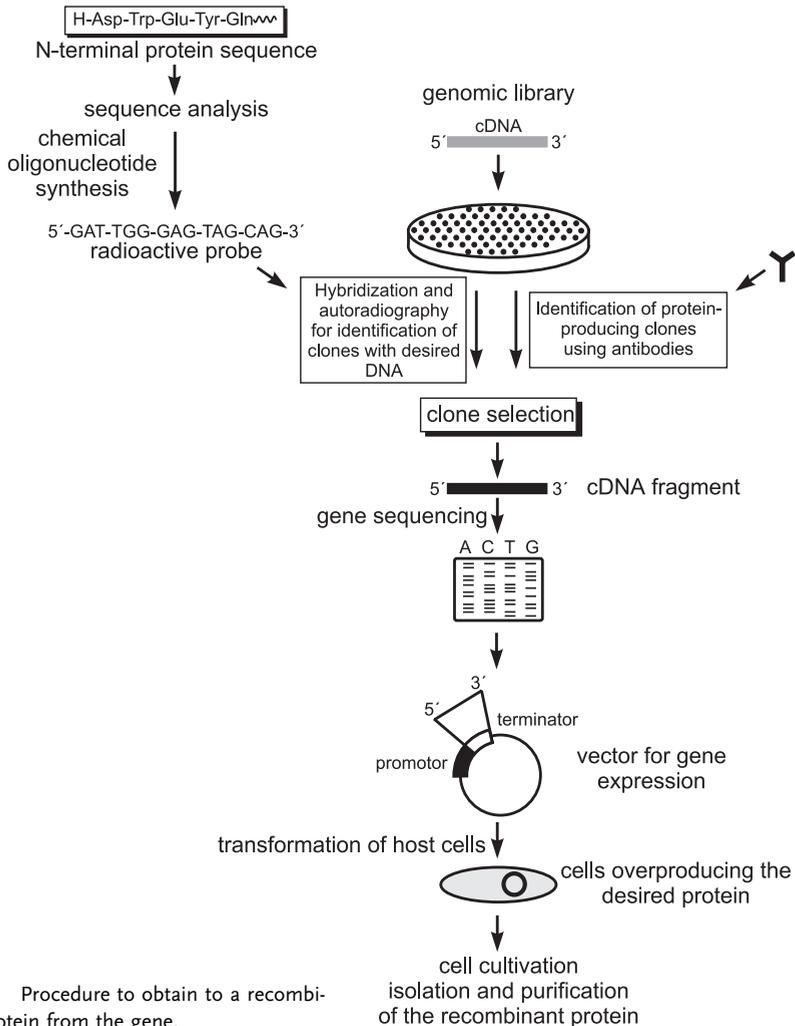


Fig. 4.41 Procedure to obtain to a recombinant protein from the gene.

says. Often, even microgram amounts are sufficient in order to raise antibodies or to perform partial sequencing, starting from the N-terminus. By using this sequence information, the corresponding genes can be identified and either isolated or obtained from gene banks.

The DNA fragments containing the genetic material to be transfected can be introduced into bacteria or other host organisms using so-called vectors. Usually, either plasmids (circular double-stranded DNA molecules that exist extrachromosomally in bacteria) or phages (viruses that infect bacteria) are used as vectors. The discovery and isolation of restriction endonucleases capable of cleaving double-stranded DNA at well-defined positions was the major precondition in the development of gene technology.

The DNA encoding for a protein can be obtained by isolation of the corresponding gene, by enzymatic synthesis from mRNA, or, in special cases by chemical synthesis. Chemical DNA synthesis [412] can be performed on commercial automatic synthesizers more efficiently compared to peptide synthesis. Genes of different proteins (e.g., the genes of human interferons) that contain more than 500 base pairs, or the DNA sequences encoding for the two insulin chains, have been synthesized chemically. The chemical synthesis of oligonucleotides containing special cleavage sequences for restriction endonucleases is also of eminent importance. Such synthetic linker groups are attached to the ends of a DNA molecule (Fig. 4.40) in order to allow the insertion of this DNA fragment into the vector. The DNA to be amplified and expressed, as well as the vector DNA, are cut by an appropriate restriction endonuclease, and the desired DNA fragment is inserted. The phosphate linkage between the DNA portions is formed by ligases to give the intact recombinant vector DNA molecule, which then is introduced into the host organism. Successful transfection can be monitored with labeled oligonucleotides (in-situ hybridization), or on the basis of the protein product using antibodies against this specific protein.

The successful expression of a recombinant protein in a host system requires not only the DNA sequence that encodes for the corresponding protein, but also additional regulatory sequences on the DNA inserted into a plasmid or another vector. Usually, highly efficient regulatory DNA domains are inserted preceding the DNA fragment encoding for the gene product in order to provide a high protein yield.

The selection of new host organisms for recombinant DNA is a major task, because *E. coli* as a host suffers from several disadvantages. Very often, eukaryotic proteins are modified post-translationally (e.g., by glycosylation), and hence contain carbohydrate chains of different lengths. Microorganisms do not modify proteins post-translationally, and usually do not export proteins into the cell culture medium. Furthermore, *E. coli* produces endotoxins that must be separated from the desired protein before its use as a pharmacologically active compound.

In the meantime, recombinant techniques for *Bacillus*, *Rhizobium* and *Streptomyces* have been described. Eukaryotic cells (from yeasts, insects, or mammals) can also be used as host organisms. Baker's yeast (*Saccharomyces cerevisiae*) or other yeasts are ideal expression systems that also allow for post-translational glycosylation. Stable plasmids are known for baker's yeast. Yeast applied in biotechnological processes usually is allowed to reproduce asexually, which safeguards stable transmission of the genetic material. The genes for interferon and insulin have been expressed in yeast.

Complex post-translational modifications such as glycosylation patterns specific for humans, or γ -carboxylations on glutamate residues, require mammalian cells as expression host systems.

An interesting concept to expand the genetic code has been introduced by Schultz et al. [413]. This uses chemically synthesized 3'-aminoacyl suppressor tRNA that allows for the incorporation of nonproteinogenic amino acids of any type into a protein. The codon UAG on mRNA usually associates with nonacylated suppressor

tRNA, and leads to protein chain termination. If the suppressor tRNA has been either chemically or enzymatically [414, 415] loaded with a nonproteinogenic aminoacyl residue, this residue is incorporated into the peptide chain.

4.6.1.2 Examples of Synthesis by Genetic Engineering

As outlined in the preceding section, microorganisms may be genetically programmed to produce human hormones (e.g., insulin, somatotropin), enzymes, or vaccines. The potential of this area of applied gene technology has quickly been recognized, and many new applications are currently under investigation in medicinal chemistry, biotechnology, and agriculture. Currently, many therapeutic peptides and proteins are obtained by the fermentation of recombinant cells (cf. Chapter 9).

Somatostatin was the first peptide to be obtained by the use of recombinant DNA techniques [416]. The DNA encoding for the amino acid sequence of somatostatin was synthesized chemically using the amino acid triplet codons used most frequently in *E. coli*, after which the DNA sequence was inserted into an expression plasmid. The relatively small peptide was protected against proteolysis by fusion to the protein β -galactosidase. Cleavage of the 14-peptide from the hybrid protein with bromocyan was only possible because somatostatin does not contain methionine.

Interestingly, the genes encoding for the interferons from human leukocytes do not contain any introns, and so interferons can be produced in a straightforward manner in bacteria.

The production of human growth hormone using recombinant DNA techniques represents a major milestone in the substitution therapy of dwarfism, as before 1985 this hormone could be obtained only from human hypophyses at autopsy. All analogues produced natively by other mammalian organisms have different amino acid sequences, and the potential for viral contamination has also been a point of criticism. The gene family of the human growth hormone was discovered during molecular biology studies. The gene contains four introns, and the regulatory N-terminal pre-sequence is composed of 26 amino acids, which is typical for an export protein. This sequence is cleaved during secretion by a signal peptidase. Expression of the cDNA in *E. coli* originally provided the human growth hormone at a yield of 2.4 mg L^{-1} , which is far from optimum [417]. Protropin[®] (Genentech), which was approved for therapeutic use in 1985, additionally contains one nonsequence-specific N-terminal methionine residue, while the peptide with the original sequence was marketed as Humatrop[®] (Eli Lilly) [418].

Two alternative procedures are described for the overexpression of insulin. Eli Lilly expressed human insulin by separate overexpression of the A and B chains in the form of fusion proteins in *E. coli* (Fig. 4.42A) [419]. Following the isolation of both chains, they were connected by reductive thiolysis and subsequent oxidation (in air) to produce the active hormone. In the second variant (Fig. 4.42B), proinsulin was synthesized analogously to biosynthesis as a fusion protein in *E. coli* and transformed enzymatically into active insulin [420].

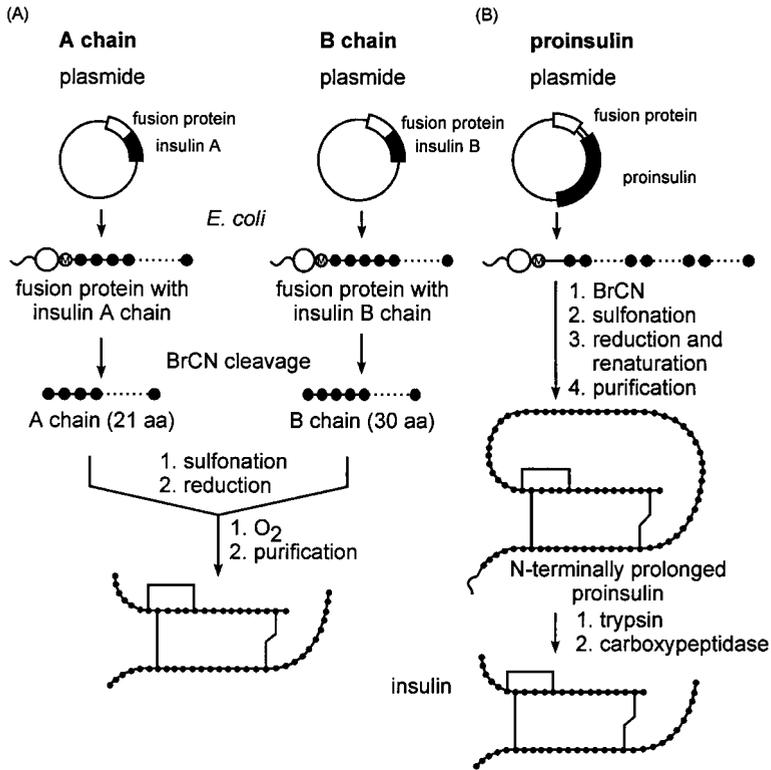


Fig. 4.42 Biotechnological insulin synthesis. (A) Separate synthesis of A and B chain, followed by oxidation to give active insulin. (B) Synthesis of proinsulin with subsequent enzymatic processing.

Currently, insulin analogues designed to improve the therapeutic properties of the hormone have been produced by recombinant DNA technology [421]. The first clinically available insulin analogue, lispro, was marketed in the U.S. in 1996 [422].

Insulin glargine (HOE 901, Lantus®) is produced using a nonpathogenic genetically engineered strain of *E. coli* and has found application as a long-acting agent for the treatment of type 1 and 2 diabetes mellitus (cf. Section 9.3.1). A typical DNA technology production plant shows Fig. 4.43.

4.6.1.3 Cell-free Translation Systems

As shown above, the biotechnological synthesis of natural products is mainly based on cellular systems. Methods for the expression of foreign genetic material in living cells often suffers from limitations however, and this is especially true for the production of polypeptides of a comparatively short length (up to 50–60 amino acid residues). Since most bioactive peptides and polypeptides are released

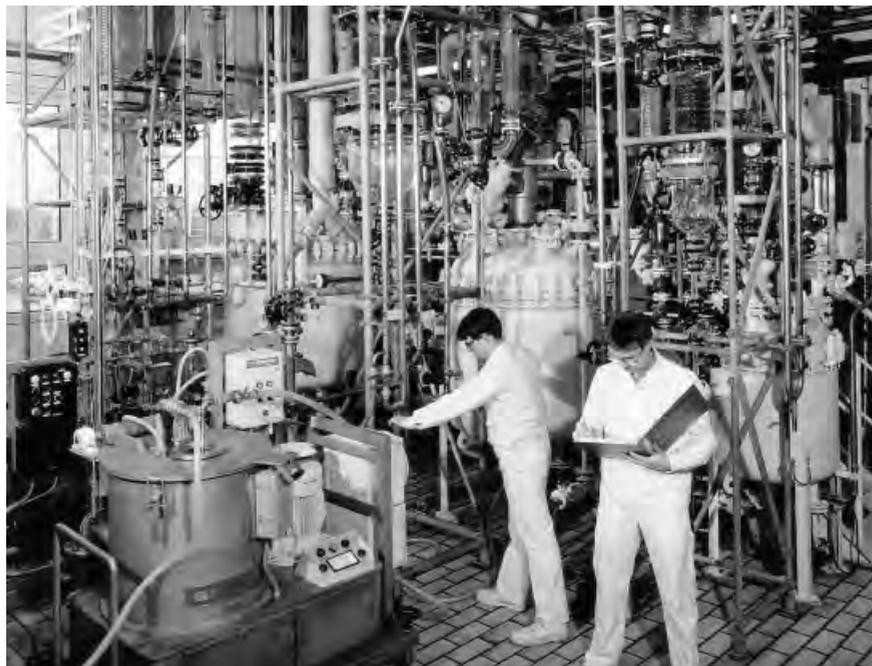


Fig. 4.43 Production plant for industrial gene technological insulin synthesis (Photo: Walter Kloos/Hoechst AG).

after the complex processing of biosynthetic precursors, it is difficult to create vectors that result in the formation of active peptide products when expressed in either prokaryotic or eukaryotic cells. Even when an expression system has been successfully created, the peptide products may be unstable or toxic to the cells. Other problems also result from insoluble peptide products forming aggregates and inclusion bodies. These general limitations may be avoided when translation is performed in cell-free systems.

Zamechnik et al. [423] described the first systems for cell-free protein synthesis, and demonstrated that peptide synthesis proceeds on ribosomes, and requires ATP, GTP, and tRNA. The coat protein of coliphage F2 was the first protein synthesized in a cell-free translation system using an extract from *E. coli* [424]. The main disadvantage of cell-free translation systems is the low peptide yield, as under these conditions only two to three polypeptide chains are formed per mRNA molecule used [425, 426].

A continuous cell-free translation system capable of producing polypeptides in high yield was described by Spirin et al. [427]. This continuous-flow, cell-free (CFCF) system allows for a continuous feeding of amino acids, ATP, and GTP to the reaction mixture containing the cell extract and mRNA, and a continuous removal of the polypeptide product (product removal proved to be essential to maintain prolonged ribosomal activity). The simplest device for performing a continu-

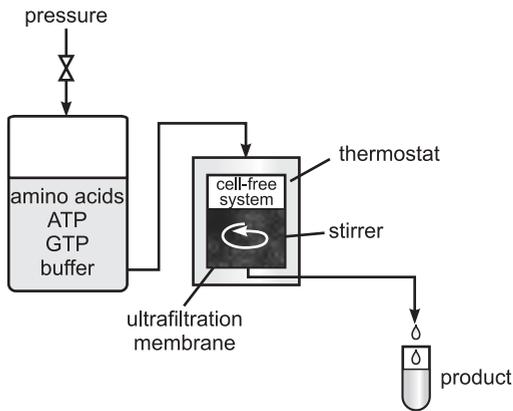


Fig. 4.44 Schematic diagram of a bioreactor for continuous-flow translation, fitted with an ultrafiltration membrane.

ow-flow translation contained an Amicon 8 MC microfiltration system which was installed in a thermostatically controlled chamber (Fig. 4.44).

Later, a thermostatically controlled chromatography column supplied with an ultrafiltration membrane at the output and a standard column adapter at the input was used. These continuous-flow bioreactors have been applied to cell-free translation using both prokaryotic (*E. coli* lysate) and eukaryotic (wheat embryos, *Triticum* sp.) systems. The mRNA of human [Val⁸]calcitonin was obtained by in-vitro SP6 phage polymerase transcription of the synthetic calcitonin gene inserted into a plasmid under SP6 promoter, and was used for human [Val⁸]calcitonin synthesis. Between 150 and 300 copies of calcitonin were synthesized per mRNA in both types of translation systems during 40 h. Pulsed bioreactors fitted with a hollow fiber ultrafiltration membrane and with a flat ultrafiltration membrane, respectively, have also been used. The inclusion of the translation mixture in granules (e.g., alginate) or polysaccharide vesicles was found to be advantageous. In this configuration, the translation of calcitonin mRNA proceeded for 100 h and produced 250 µg calcitonin from 5 mL of the incubation mixture. The CFCF system enables the production of rather pure polypeptides based on the fact that the ultrafiltration membrane prevents leakage of most proteins present in the reaction mixture. The CFCF system has doubtlessly opened new fields of application, and many of the limitations originally imposed on cellular systems have now been overcome using cell-free gene expression. An ongoing problem is that the productivity and efficiency of cell-free systems are still low, and newer bioreactors that incorporate efficient systems to monitor the translation course are required. Moreover, strict maintenance of ATP and GTP concentration, and the development of efficient regeneration systems for both cofactors, are indispensable. Several promising approaches to increase the efficiency of the CFCF system have been suggested, however [428–430]. A coupled replication-translation system might be developed which would solve the problems of RNA template stability and the efficiency of its translation.

4.6.2

Enzymatic Peptide Synthesis4.6.2.1 **Introduction**

Although enzymes have in general become valuable tools in medium- to large-scale synthetic organic chemistry [431], a universal C–N ligase with high catalytic efficiency for all possible combinations of the 21 proteinogenic amino acids to be coupled has yet to be developed. Indeed, the demands on specificity are so severe that not even nature could solve this problem! Hence, protein biosynthesis (cf. Section 3.2.1) has been developed as a stepwise strategy, starting from the N-terminus of the growing peptide chain. The process is catalyzed by the ribosomal peptidyl transferase, and followed by limited proteolysis and other post-translational modifications of the precursor molecules (cf. Section 3.2.2). Despite intensive investigations during the past years, details of the nature and the basic mechanism of the peptidyl transferase reaction within the ribosome remain largely unknown. According to studies by the Nobel laureate Thomas R. Cech et al. [432], an in-vitro-selected ribozyme is capable of catalyzing the same type of peptide bond formation as a ribosome, and its sequence and secondary structure appear strikingly similar to the “helical wheel” portion of 23S rRNA involved in the activity of ribosomal peptidyl transferase. These findings provided evidence for the feasibility of the “RNA world” hypothesis, by demonstrating that RNA itself is capable of catalyzing peptide bond formation. In comparison to the specificity requirements of proteases, peptidyl transferase appears to be an old, unspecific ribozyme, which is in accordance with its function in evolution.

Even if it were possible to separate ribozyme activity from the ribosome, or to isolate an in-vitro-selected ribozyme that catalyzes peptide bond formation like a ribosome, such a biocatalyst does not seem suitable for simple practical use in peptide synthesis. In principle, this conclusion is also valid for the nonribosomal poly- or multienzymes involved in the biosynthesis of peptide antibiotics (cf. Section 3.2.3).

Consequently, only those enzymes which usually act as hydrolases, catalyzing the cleavage of peptide bonds, should be considered as peptide ligases. The reaction of proteolysis is irreversible, as peptide bond hydrolysis is exergonic. Although the ionic hydrolysis products are thermodynamically more stable, the fundamental suitability of proteases to catalyze the formation of peptide bonds is based on the principle of microscopic reversibility that was predicted by J. H. van't Hoff in 1898. Despite this, about 40 years elapsed before the first experimental proof of van't Hoff's prediction was produced, this being based on the clear-cut protease-catalyzed synthesis of an amide bond [433]. Unfortunately, another 40 years elapsed before this approach gained any industrial importance, though during the past few decades considerable effort has been made to determine the optimum conditions for protease-catalyzed peptide synthesis (for reviews, see [434–438]).

4.6.2.2 Approaches to Enzymatic Synthesis

According to the short notation of protease-substrate interactions [439], proteases have binding grooves ($S_n \dots S_3-S_2-S_1-S'_1-S'_2-S'_3 \dots S'_n$) on both sides of the scissile bond $-P_1-P'_1-$ of the substrate, whereas substrate binding ($P_n \dots P_3-P_2-P_1-P'_1-P'_2-P'_3 \dots P'_n$) in proteolysis is mostly reduced to the P_1 amino acid residue. Peptide bond formation is, in contrast to proteolysis, a two-substrate reaction that not only requires a specificity-dependent insertion of the carboxy component into the S subsites of the active site, but also optimal binding of the amino component in the S' region. Therefore, the S'-P' interactions essentially determine the outcome of the C-N ligation reaction.

As mentioned above, the equilibrium of a protease-catalyzed reaction normally lies on the side of the thermodynamically more stable cleavage products. Several mechanistically different manipulations are possible to shift the equilibrium in favor of peptide bond formation. Approaches towards protease-catalyzed peptide bond formation are generally classified according to the type of carboxy component used (Fig. 4.45).

In the equilibrium-controlled approach (Fig. 4.45A), the carboxy component contains a free carboxy group, while in the kinetically controlled approach (Fig. 4.43B) the carboxy component is employed in activated form, mainly as an alkyl ester. Both strategies are fundamentally different due to the energy required for the conversion of the starting components into the peptide products.

Equilibrium-controlled synthesis This equilibrium-controlled or thermodynamic approach (Fig. 4.45A) represents the direct reversal of proteolysis. Consequently, all proteases can be used – independent of their mechanisms. Besides this advantage, the large amount of enzyme required and the low reaction rate are draw-

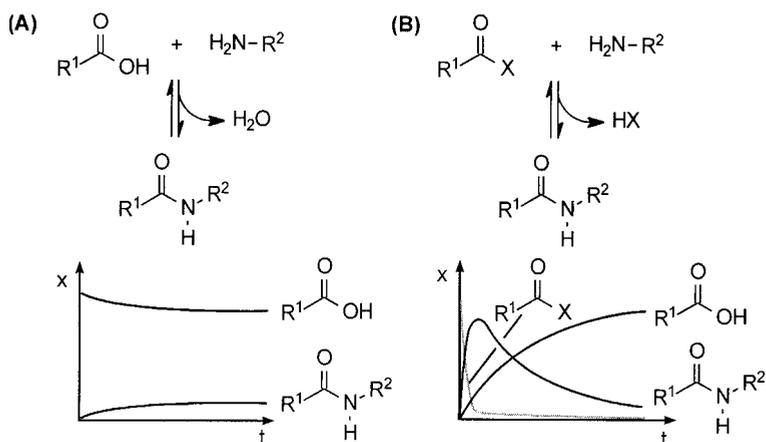


Fig. 4.45 Comparison of the equilibrium- (A) and the kinetically controlled approach (B) of protease-catalyzed peptide synthesis. R^1-COOH =carboxy component; R^1-CO-X =slightly activated carboxy component; H_2N-R^2 =amino component; HX =leaving group.

backs of this approach. The ionization equilibrium must be manipulated to shift the equilibrium towards peptide bond formation. Addition of water-miscible organic solvents to the aqueous reaction mixture is a viable approach. However, the catalytic activity of the enzyme may decrease under such conditions. The lower dielectric constant of the medium reduces the acidity of the carboxy group and, to a smaller extent, the basicity of the amino group of the nucleophilic amino component. In addition to the manipulations mentioned above, reverse micelles, anhydrous media containing minimal water concentrations, water mimics, and reaction conditions promoting product precipitation are often employed.

Kinetically controlled synthesis In contrast to the equilibrium-controlled approach, the kinetically controlled protease-catalyzed peptide synthesis [440] requires much less enzyme, the reaction time to obtain maximum product yield is significantly shorter, and the product yield depends both on the properties of the enzyme used and its substrate specificity. Whereas the equilibrium-controlled approach ends up in equilibrium (Fig. 4.45A), the concentration of the product formed in the kinetic approach passes a maximum before the slower hydrolysis of the product becomes important. Amidase activity of most proteases is lower than esterase activity. The product inevitably is hydrolyzed, if the reaction is not stopped once the acyl donor ester is consumed. Fig. 4.46 indicates the kinetic model of a protease-catalyzed acyl transfer reaction.

The kinetic approach requires an acyl donor ester (Ac-X) as carboxy component, and is limited to proteases that rapidly form an acyl enzyme intermediate (Ac-E), for example serine and cysteine proteases. The enzyme (EH) acts as a transferase catalyzing the transfer of the acyl moiety to the nucleophilic amino component (HN). The amino component reacts in competition with water with the acyl enzyme to form the peptide (Ac-N). The ratio between aminolysis and hydrolysis is of decisive importance for successful preparative peptide synthesis. However, serine and cysteine proteases are not perfect acyltransferases, and undesired reactions may take place due to their limited specificity (e.g., hydrolysis of the acyl en-

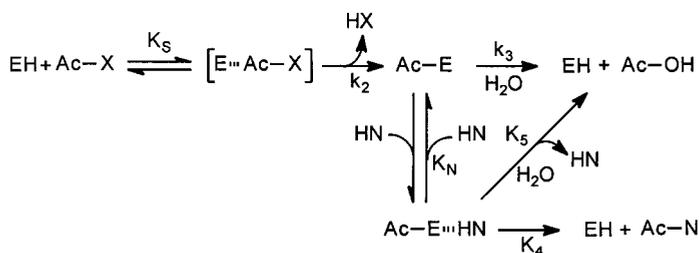


Fig. 4.46 Kinetic model for protease-catalyzed acyl transfer reaction. EH=free enzyme; Ac-X=acyl donor (carboxy component); HX=leaving group; Ac-E=acyl enzyme complex; Ac-OH=hydrolysis product; HN=acyl acceptor (amino component); Ac-N=aminolysis product (synthesized peptide).

zyme, secondary hydrolysis of the ligation product and proteolytic cleavage of other protease-labile peptide bonds present in the segments to be ligated). An understanding of the molecular interactions between the acyl enzyme and the attacking nucleophilic amino component allows for an optimization of the acyl transfer efficiency. Efficient nucleophilic attack of the amino component essentially depends on optimal binding within the active site by S'-P' interactions. More information on the specificity of the S' subsites of serine and cysteine proteases has been obtained by systematic acyltransfer studies with libraries of nucleophilic amino components, known as S' subsite mapping [440].

Esterase activity can be further positively manipulated by varying the kind of leaving group. For preparative peptide synthesis, such manipulation is very important as it allows complete conversion of the acyl donor ester before the product will be hydrolyzed. There is no doubt that the course of kinetically controlled protease-catalyzed peptide synthesis can be more efficiently influenced than the equilibrium approach.

In contrast to the chemical approach, peptide synthesis using stepwise enzymatic chain assembly may start either from the N-terminus (cf. Fig. 5.2) or the C-terminus, because enzyme-catalyzed couplings are devoid of racemization. However, stepwise protease-catalyzed assembly should not be directly compared to automatic solid-phase technique (cf. Section 4.5). Selected di- and tripeptides (cf. Section 9.2), which are important intermediates in many pharmaceutical products, can be synthesized enzymatically on a large scale even in a continuous process, using solubilizing protecting groups [441–443].

In addition, the application of cross-linked enzyme crystals (CLECs) of thermolysin [444] and subtilisin [445] has been recommended for the synthesis of small peptides and peptide alkylamides. As will be shown below, the solid-to-solid conversion has been proven as a very useful method for the synthesis of selected short peptides that comply with the requirements for this special synthetic procedure. Methodological support for practical application of protease-catalyzed peptide synthesis is available [446].

Although the kinetic approach should be preferred, the final decision depends on the overall synthesis concept. The largest industrial-scale application of the equilibrium approach is most likely the enzymatic synthesis of Z-Asp-Phe-OMe, the precursor of the peptide sweetener aspartame [447]. Transpeptidation technology on a large industrial scale is well documented for the conversion of porcine insulin into human insulin by trypsin [448] – a process that had widely been used before the gene technology production of insulin.

4.6.2.3 Manipulations to Suppress Competitive Reactions

The most important factors limiting the widespread routine application of proteases in kinetically controlled peptide synthesis are hydrolysis of the acyl donor ester and proteolysis within the starting fragments to be coupled, or the final condensation product. These undesired reactions can be minimized by various manipulations of the reaction medium, the enzyme, the substrate, and on mechanistic features of the process.

Medium engineering is based on the replacement of water by organic solvents, and uses mono- or biphasic aqueous-organic and organic monophasic mixtures or microaqueous systems [449]. Competing hydrolysis can be minimized by decreasing the water concentration in the reaction medium of enzymatic synthesis in microaqueous systems that do not employ organic solvents, termed solid-to-solid conversion [450–452], and in frozen aqueous systems [453].

Enzyme engineering describes a range of techniques, from deliberate chemical modification to genetic remodeling of a wild-type enzyme [454]. A mutant of subtilisin BNP', termed "subtiligase", was obtained by Jackson et al. [455] by protein design and used in a further synthesis of RNase A (cf. Section 5.2.2.2, Fig. 5.9).

4.6.2.4 Irreversible C–N Ligations by Mimicking Enzyme Specificity

The substrate-mimetic approach represents a very specific and efficient form of substrate engineering. Despite the development of various possibilities to suppress competing reactions, proteolytic cleavage of the peptide bond formed cannot be completely suppressed. In this context, the fact must be considered that the newly formed peptide can be cleaved during the course of the catalytic process by the same enzyme. The peptide bond-forming step and the proteolytic cleavage step do not differ in substrate specificity requirements. Since the specificity of proteases is manifested by the side chain of the P₁ amino acid residue, an irreversible peptide bond formation does not seem possible.

In ribosomal peptide synthesis (cf. Section 3.2.1), the selection of the amino acid residues to be coupled is performed as a specific recognition process prior to the acyl transfer reaction, catalyzed by the unspecific ribozyme peptidyl transferase. An irreversible C–N ligation in a biomimetic fashion would be possible also in enzymatic peptide synthesis if the specificity selection could be separated from the actual peptide bond-forming step. This proved to be feasible by transferring the moiety that determines the specificity from the P₁ amino acid side chain to the leaving group of the acyl donor ester. Hence, the enzyme is capable of recognizing, in a kinetically controlled acyl transfer reaction, the acyl donor ester by the specificity determinant present in the leaving group. The specificity-bearing leaving group is released upon formation of the acyl enzyme followed by peptide bond-forming aminolysis. Consequently, the newly formed peptide bond can no longer be cleaved by the enzyme, because the moiety responsible for recognition by the protease is then lacking.

This irreversible synthesis concept was for the first time confirmed in a model peptide synthesis catalyzed by trypsin involving various nonspecific N^α-protected amino acid 4-guanidinophenyl esters (OGp) as acyl donors, and various amino acid and peptide derivatives as nucleophilic acyl acceptors. Trypsin accepts the guanidino group of the 4-guanidinophenyl ester as a substitute for the arginine residue, that is usually recognized by the S₁ subsite [456]. This concept was exploited in further examples by another group, who named this type of acyl donor "ester inverse substrate" [457]. The more correct term "substrate mimetic" was introduced by Bordusa et al. [458], together with an extension of this approach to

irreversible peptide segment condensations with other proteases. The mechanism of the substrate mimetic concept has been elucidated [459–461]. As shown in Fig. 4.47, a common acyl donor **A** binds its acyl residue to the S-binding site, where the leaving group is present at the S'₁ subsite.

The scissile ester bond may be attacked by Ser¹⁹⁵ in the case of trypsin, followed by acylation of the enzyme. However, applying the same binding principle, a substrate mimetic with the leaving group located either in S'₁ (**B**) or in S₁ (**C**) leads to catalytically unproductive binding. Recognition of the specificity determinant in the leaving group by the S₁ subsite fails in binding arrangement **B**, whereas in the binding position **C** the scissile ester bond would be far away from the active Ser¹⁹⁵, and neither hydrolysis nor acylation would occur. Hence, the binding of a substrate mimetic and the subsequent acylation leads to an acyl enzyme having the acyl residues at the S'-subsites (**D**). Prior to the deacylation step, the acyl residue has to flip to the S-subsite of the enzyme. The common kinetic model has to be extended by a rearrangement step between the two acyl enzyme species E-Ac, with the acyl moiety in the S' subsite and the common acyl enzyme Ac-E. Peptide nucleophiles that bind specifically to the S' subsite should be able to remove the acyl moiety from the S' region more efficiently than water, which decreases unwanted hydrolysis of the acyl enzyme.

In summary, cationic, anionic, and hydrophobic types of substrate mimetics have been designed, characterized and applied for various peptides synthesis, including segment condensations [462]. Consequently, the substrate mimetic strategy should be useful for combining the enzymatic ligation of segments with suitable SPPS methods, especially for the preparation of acyl component peptide segments in the form of appropriate esters (cf. Section 5.2.2.2, Fig. 5.10).

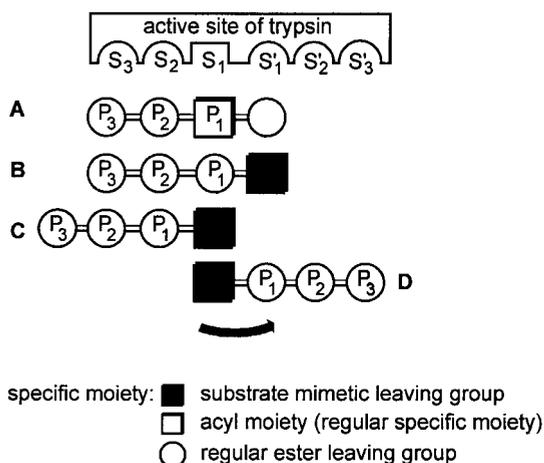


Fig. 4.47 Comparison of the binding of a common acyl donor ester (**A**) and a substrate mimetic in different binding positions (**B–C**) to the active site of trypsin with the hypothetical binding mode of a substrate mimetic (**D**).

Enzymatic methods have several advantages over chemical procedures, but at present more peptides are synthesized by chemical synthesis than in protease-catalyzed processes. The use of peptide synthesizers, in addition to recent new developments in the field of chemical ligation procedures (cf. Section 5.4), still favors chemical methods over the enzymatic approach. However, the enzymatic methods have undoubted advantages, such as the prevention of racemization, no requirement for time-consuming and expensive protection/deprotection procedures of side-chain functions, a reduced use of problematic (toxic) solvents and reagents, and possible re-use of the biocatalysts. Hence, the question should not be whether to use chemical or enzymatic approaches in peptide synthesis. Rather, an ingenious combination of chemical and enzymatic steps should promote general progress in peptide synthesis. Indeed, it might be demonstrated that once the synthesis conditions have been optimized, kilogram quantities of biologically active peptides and analogues could be obtained using enzymatic coupling methods. The semisynthetic synthesis of human insulin and the large-scale production (hundreds to thousands of tons) of the aspartame precursor underline the industrial importance of the enzymatic approach. Ultimately, even proteins may be synthesized via this approach, as shown for the synthesis of RNase A.

The C–N ligation strategy based on the substrate mimetic concept allows, for the first time, an irreversible peptide bond formation catalyzed by proteases. When combined with frozen-state enzymology (as demonstrated in model reactions [463]) or with protease mutants lacking amidase activity, the substrate mimetics C–N ligation approach will undoubtedly contribute to significant progress in enzymatic peptide synthesis. Recombinant polypeptide thioesters as substrate mimetics may be coupled in fragment condensations with chemically synthesized or recombinant fragments. This specific programming of enzyme specificity by molecular mimicry corresponds in practice to a conversion of a protease into a C–N ligase, and results in a biocatalyst which has not been developed evolutionarily by nature.

4.6.3

Antibody-catalyzed Peptide Bond Formation

According to Linus Pauling [464], the action of an enzyme depends to a large extent on the complementarity between the active site of the enzyme and the transition state of the enzyme-catalyzed reaction, as shown schematically for the peptide bond-forming step (Fig. 4.48).

In 1969, it was proposed by Jencks [465] that antibodies could be produced to function as catalysts. According to this idea, structures that mimic the stereoelectronic properties of transition states might be capable of eliciting antibodies that accelerate the corresponding chemical reaction. Consequently, if an antibody could bind a substrate already in the transition state conformation, it might act as an enzyme catalyzing the reaction to which the transition state conformation is predisposed. For this reason, catalytic antibodies have been also termed “abzymes”. Investigations concerning requirements for the catalysis of peptide bond

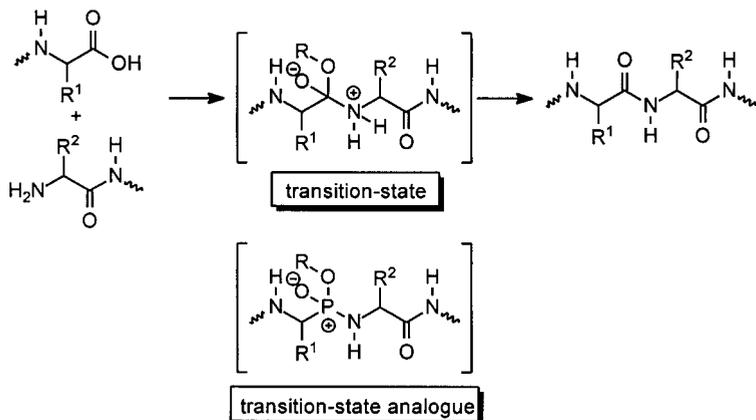


Fig. 4.48 Comparison of the transition-state structure of peptide synthesis by ester aminolysis with the structure of a designed transition-state analogue.

formation using catalytic antibodies was the consequence of progress in antibody catalysis for organic transformations [466, 467].

The normal approach to generate catalytic antibodies requires the design of haptens that are topologically and structurally near-identical to the transition state of the appropriate reaction, as shown in Fig. 4.48. In particular, peptide synthesis should require a large number of catalytic antibodies in order to consider the specificity requirements of the different amino acid residues in the C-terminal position of the carboxy component, and the N-terminal position of the amino component to be coupled.

As shown in Fig. 4.49, Hirschmann et al. [468] synthesized the phosphonamide shown in Fig. 4.49A as a transition-state analogue (cf. Section 7.2.5) which was used to generate antibodies for catalyzing the model peptide synthesis (Fig. 4.49B). The cyclohexyl moiety of the transition-state analogue should create a binding site in the antibody that would accommodate various hydrophobic side-chain moieties of the carboxy component. The *p*-nitrobenzyl residue in the phosphonate was designed in order to facilitate the dissociation of both the leaving group and peptide from the antibody. The authors described antibody-catalyzed dipeptide syntheses starting from all possible stereoisomeric combinations of the ester and amide substrates in yields between 44% and 94%. Two monoclonal antibodies (16G3 and 18C10) were found to cause a ~220-fold acceleration of the reaction. Further studies with the so-called antibody ligase 16G3 have demonstrated that this abzyme catalyzes not only the synthesis of dipeptides but also the coupling of an activated amino acid with a dipeptide to form a tripeptide, as well as a simple (2+2) segment condensation with average yields of 80% within a reaction time of 20 min [469].

Jacobsen and Schultz [470] created an antibody (Fig. 4.50) against a neutral phosphonate diester transition-state analogue (A) that was capable of catalyzing

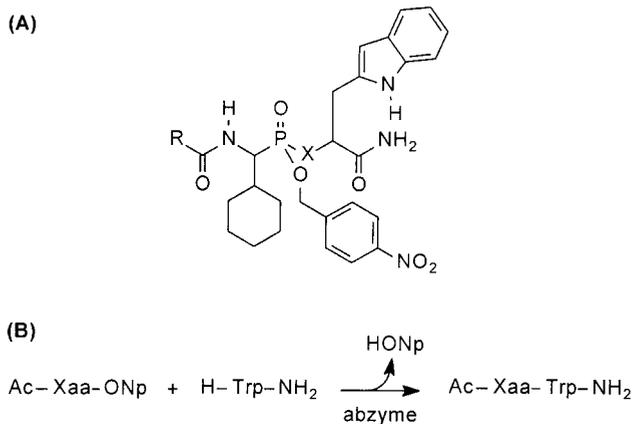


Fig. 4.49 Structural variants of the transition-state analogue (A) for an abzyme-catalyzed model peptide synthesis (B). Xaa=Val, Leu, Phe; X=NH; R=-(CH₂)₃COOH/X=O; R=-(CH₂)₃COOH/X=O; R=CH₃.

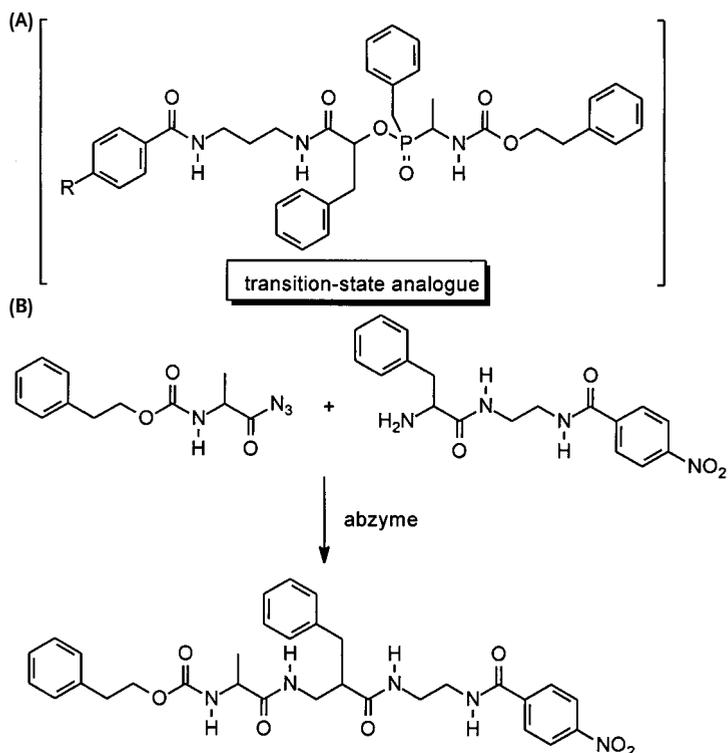


Fig. 4.50 Transition-state analogue (A) and model peptide synthesis reaction (B) catalyzed by a catalytic antibody. R=NH₂ or NO₂.

the reaction (B) between *N*-phenylethoxycarbonyl-L-alanyl azide with *N*-(4-nitrobenzoyl)-*N'*-L-phenylalanyl-1,3-diaminopropane at a 10,000-fold rate relative to the uncatalyzed reaction, and in 65% yield.

Interestingly, in both cases the catalytic antibodies do not catalyze the hydrolysis of both the active ester and the azide to an appreciable degree. Product hydrolysis and racemization have not been observed. As mentioned earlier, the main disadvantage of the catalytic antibody approach is the requirement of a large number of antibody catalysts to accommodate the wide specificity pattern of amino acids in coupling reactions. On the other hand, these results provide an impetus for producing further generations of antibodies capable of catalyzing the ligation of longer unprotected fragments, combined with a general strategy for the development of sequence-specific antibody ligases.

4.7

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7

Peptide and Protein Design, Pseudopeptides, and Peptidomimetics

Native peptides can be directly applied as pharmacologically active compounds only to a very limited extent. The major disadvantages of the application of a peptide in a biological system – for example, rapid degradation by proteases, hepatic clearance, undesired side effects by interaction of conformationally flexible peptides with different receptors [1–7], and low membrane permeability due to their hydrophilic character – are in most cases detrimental to oral application. Furthermore, most peptides are not able to pass body barriers such as the blood-brain barrier, and also suffer from rapid processing and excretion. The high structural flexibility, especially of linear peptides, has the consequence that the receptor-bound, biologically active conformation represents only one member of a large ensemble of conformers.

Peptide chemistry however may contribute considerably to drug development. The interaction of a peptide or a protein epitope with a receptor or an enzyme is the initial event based on molecular recognition [8], and generally elicits a biological response. Likewise, ligand or substrate binding to a protein relies on the surface complementarity of the binding partners.

Chemically modified peptides with improved bioavailability and metabolic stability may be directly used as drugs (cf. Chapter 9). Alternately, peptidic receptor ligands or enzyme substrates (peptide leads) may serve as starting points for the development of nonpeptide drugs [9]. The first step of any such investigation is to identify the amino acid side-chain residues responsible for receptor-ligand interaction. Subsequently, the topography of these functional (pharmacophoric) groups is reproduced by similar nonpeptidic functionalities on a rigid scaffold [10]. Many efforts have been made to develop peptide-based, pharmacologically active compounds, including peptide modification and the design of peptidomimetics. Whilst modified peptides (by definition) contain nonproteinogenic or modified amino acid building blocks, peptidomimetics (cf. Section 7.3) are nonpeptidic compounds that imitate the structure of a peptide in its receptor-bound conformation and – in the case of agonists – also the biological mode of action on the receptor level.

According to the definition by Ripka and Rich [11], three different types of peptidomimetics may be distinguished:

- Type I: these are peptides modified by amide bond isosteres (cf. Section 7.2.2) and secondary structure mimetics (cf. Section 7.2.4). These derivatives are usually designed to closely match the peptide backbone.
- Type II: these are small nonpeptide molecules that bind to a receptor or enzyme (functional mimetics, cf. Section 7.3). However, despite being often presumed to serve as structural analogues of native peptide ligands, these nonpeptide antagonists often bind to a different receptor subsite and, hence, do not necessarily mimic the parent peptide.
- Type III: these may be regarded as ideal mimetics, because they are nonpeptide compounds and contain the functional groups necessary for the interaction of the native peptide with the corresponding protein (pharmacophoric groups) grafted onto a rigid scaffold.

The design of all three types of peptidomimetics may be assisted by X-ray crystallographic or nuclear magnetic resonance (NMR) data [12], computational de-novo design (“in-silico screening”) [13], and combinatorial chemistry (cf. Chapter 8) [14].

Furthermore, the construction of novel bioactive polypeptides and artificial protein structures or miniaturized enzymes currently form the focal point of intensive investigations [15]. Although the secondary structure elements (cf. Section 2.5) of a polypeptide chain with known primary structure can be predicted to limited extent, our knowledge on protein folding is still incomplete. Therefore, reliable predictions on the composition of secondary structure elements to produce a three-dimensionally folded biologically active peptide or protein are, at present, beyond reach.

7.1

Peptide Design

The development of a drug (peptide drug) may start with the identification and design of the peptide sequence essential for biological activity. Such structure-activity relationships for example rely in the first instance on a systematic substitution of each amino acid residue of a native peptide by a simple amino acid such as alanine (“alanine scan”). Studies on the receptor selectivity or on the agonism or antagonism of a peptide hormone require structure-activity relationships to be performed, using many synthetic peptide analogues. In the past, this approach was highly labor-intensive, but today it is much more straightforward following the application of combinatorial chemistry and automation (cf. Chapter 8).

A viable design procedure is outlined in Fig. 7.1. A peptide sequence 1 is identified as being responsible for interaction with a receptor. In the example shown, the peptide contains the sequence Arg-Gly-Asp (RGD) that is known as a universal recognition motif for cell-cell and cell-matrix interactions. Some peptides which contain the RGD sequence in a well-defined conformation efficiently inhibit the binding of extracellular proteins (e.g., fibrinogen, vitronectin) to cellular receptors (integrins). The corresponding protein-receptor interaction is involved, for

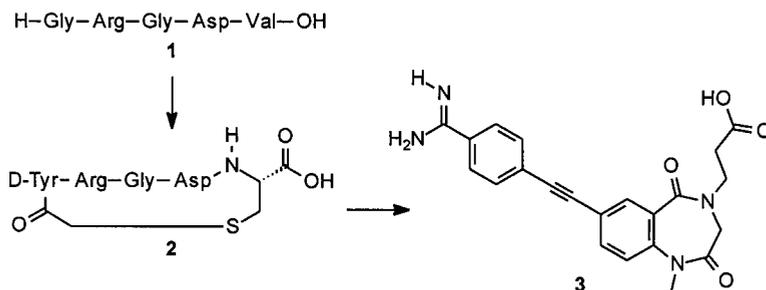


Fig. 7.1 Conversion of a peptide sequence into a nonpeptide lead.

example, in blood platelet aggregation, tumor cell adhesion, angiogenesis, and osteoporosis [16]. The peptide conformation subsequently is constrained, perhaps by cyclization (2) or by sterically hindered amino acids (type I peptidomimetics). If the biological activity is retained, it is likely that the receptor-bound conformation is still accessible [17]. In the next step, the three-dimensional array of the pharmacophoric groups can be deduced and a nonpeptide lead compound 3 (type III peptidomimetic) may be constructed, where the pharmacophoric groups are attached to a rigid scaffold and are presented in the appropriate three-dimensional array [18, 19].

In-vitro or in-vivo biological activity is not sufficient for a drug candidate, however, and many further investigations of toxicology, pharmacokinetics (ADME: absorption, distribution, metabolism, excretion), pharmacology, and clinical trials are required before a drug candidate is approved by national authorities for the treatment of patients. Considerable effort is needed to develop a biologically active compound (lead compound) as a drug candidate, and finally to register it as a pharmacologically active drug that can be marketed. Vast research expense, long development times, and high risks are imposed on innovative drugs where, in contrast to the so-called “me-too” products, no other substances with a similar mode of action are being marketed concurrently. In most cases, the development pathway is aimed towards a specific drug, and starts with a pathological phenomenon (biological target). Extensive biochemical and medical knowledge is required to understand the physiological and pathological events involved, and in this respect the recent advances made in biochemistry, molecular biology, gene technology, protein purification, protein crystallography, protein NMR, genomics, proteomics, and computational methods will provide the necessary platform for drug development.

Originally, the term “drug design” was coined for the rational design of a molecule that interacts appropriately on a structural or mechanistic basis with the target (enzyme, receptor, or DNA sequence) in a complementary manner, and that ultimately exerts the desired pharmacological effect by activation or blocking of the corresponding target. The complex interactions in drug design are shown schematically in Fig. 7.2 [20].

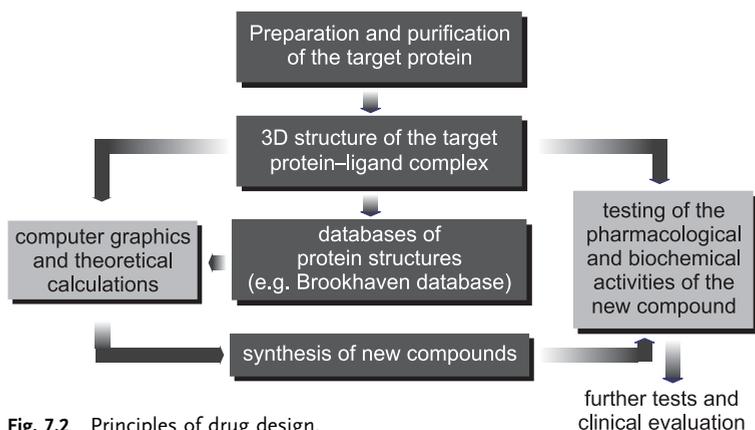


Fig. 7.2 Principles of drug design.

Determination of the three-dimensional (3D) structure of the target protein is a crucial precondition for the rational design of small molecule agonists, antagonists or enzyme inhibitors [21]. In many cases, protein engineering facilitates structure determination because the corresponding protein can be obtained in larger amounts by overexpression (cf. Section 4.6.1). Methods of gene technology may also provide single domains of a protein for structure determination by X-ray crystallography or NMR methods. The 3D structure often results in suggestions for site-directed mutagenesis, and/or the exchange of single or multiple amino acid residues using molecular biology methods in order for example to optimize the stability, substrate specificity, or pH-optimum of an enzyme-catalyzed reaction.

Methods of molecular modeling are used to visualize the structures of receptors and peptide drugs on a computer, and then to simulate their interaction. However, as the torsion angles of the peptide backbone and of the side-chain residues may adopt different values, numerous conformations usually result for an unbound peptide ligand that are not biased for the most efficient conformation with respect to ligand-receptor interaction. Force-field calculations have been developed as a strategy to predict conformations by comparison of their relative potential energy [22]. The single atoms of a protein are considered as hard spheres, and classical mechanics calculations are used to simulate their time-dependent motion. Even solvent molecules may be explicitly included.

Statistical methods (Monte Carlo methods) may also be used to produce a huge number of conformations and to calculate subsequently their potential energy in the local minima [23]. Finally, the conformation with the lowest energy or a family of low-energy conformations are further investigated. This procedure, which initially is based on statistics, can be supplemented by molecular dynamics calculations and may result in an evaluation of the conformational flexibility of molecular structures by force-field methods [22, 24]. In computer-aided molecular design (CAMD) [25], the complementary fitting of a peptide or nonpeptide drug to a receptor plays a crucial role, though sensible application of this concept requires

knowledge of the 3D structure of the receptor. During recent years much effort has been imposed on developing new methods and algorithms (e.g., neuronal network methods, genetic algorithms, machine learning, and graph theoretical methods) in order to predict molecular structures [25–28].

If the site of action of a biologically active compound is known, then direct computer-aided drug design is used [29]. Tailor-made molecular structures are constructed to optimally fulfil the requirements for binding to the receptor or the active site of an enzyme [30]. In this context, protein–ligand docking is an important method, and many docking programs based on a series of searching algorithms such as surface complementary matching [31, 32], fragment growing [33, 34], random sampling (Monte Carlo), simulated annealing [35–37], and genetic algorithms [38, 39] have been developed.

One NMR-based method has been described to identify and optimize small organic molecules binding to proximal subsites of a single protein in order to produce from these moieties ligands with high affinity to the protein. Di- or multivalent protein-ligand interactions are obtained by covalently linking these pharmacophoric groups. This approach experimentally provides structure-activity relationship data, as obtained from NMR [40,41]. The method relies on ^{15}N -labeled proteins for instance, because changes of the ^{15}N or ^1H amide chemical shift are observed in ^{15}N heteronuclear single quantum correlation spectra (^{15}N HSQC) upon addition of a ligand to the labeled protein (Fig. 7.3).

If no structural data of a target protein are available, the known 3D structure of another, sequence-related protein may be used as the starting point for the so-called homology modeling.

Indirect computer-aided drug design (indirect CADD) is a very useful protocol in the process of rational drug design when the molecular structure of the target is unknown. It allows for the evaluation of structural changes upon modification of a lead structure. Systematic substituent variation of a lead structure is correlated with biological data, and this results in the identification of crucial structural elements of the ligand that are required for high-affinity receptor binding (receptor mapping). This finally allows for the creation of a hypothetical receptor model, and is intensively applied in modern drug design.

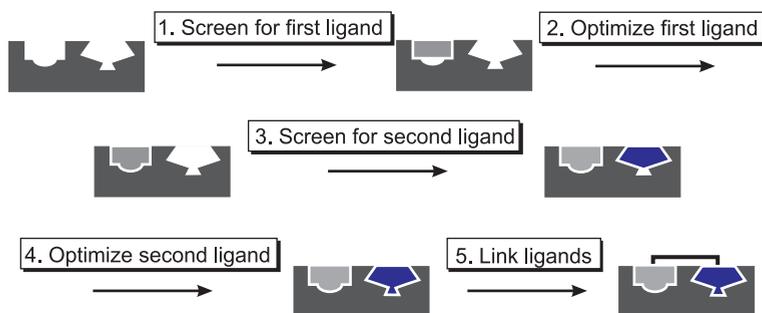


Fig. 7.3 Optimization process in establishing structure-activity relationships (SAR) by NMR.

The synthesis of chemically modified peptides often employs the incorporation of conformationally constrained amino acids (proline, proline analogues, N-methyl amino acids, C^α-dialkyl amino acids, etc.). This type of modification results in rather rigid peptide analogues where the conformational freedom is reduced. A further viable route for the design of peptides with reduced conformational freedom relies on peptide cyclization (cf. Section 6.1).

The design of a peptide depends primarily not only on the targeted application, but also on synthetic considerations [42, 43]. In general, several steps are necessary to obtain a peptide suitable for biochemical or biomedical applications, and several optimization cycles are often required. There is no such thing as a general routes for the design, although the procedure is guided by the desired function.

Conformational analysis is a valuable tool in the design of new peptide drugs. Linear peptide chains are usually characterized by high flexibility which renders determination of the 3D structure nontrivial. The basics of conformational analysis were established by Ramachandran et al. [44], Scheraga et al. [45], and Blout et al. [46]. These authors established basic concepts for the classification and description of peptide structures (cf. Section 2.5), and contributed extensively to an improved understanding of peptide and protein conformation and of protein-folding processes. Conformational transitions in polypeptides have been studied using several different methods, for example by Goodman et al. [47]. Cyclic peptides offer the advantage of limited conformational freedom, and this makes them particularly suited for conformational studies. Furthermore, many cyclic peptides (Section 6.1) display interesting biological profiles of activity. Peptide conformations can be very efficiently analyzed using NMR spectroscopy (Section 2.5.3) as reviewed in [48, 49].

Sequence-based peptide design relies not only on the deletion, the exchange, or the modification of amino acid-building blocks, but also on structural elements of the peptide backbone. Besides the 21 proteinogenic amino acids (including selenocysteine), at present more than 1000 nonproteinogenic amino acids are known. Some of these occur naturally in the free form, or are bound in peptides.

The truncation of a sequence by deletion of amino acid-building blocks that practically do not contribute to biological activity is an important approach in the design of pharmacologically active peptides starting from longer bioactive peptides. Many peptide hormones and also other bioactive peptides very often contain a minimum sequence responsible for binding to the receptor and the biological activity. Of course, it is sometimes not very easy to identify the essential partial sequence. Often, the spatial arrangement of those residues responsible for activity depends on the 3D peptide structure where non-neighboring amino acids, with respect to the primary structure, come into close vicinity by peptide folding. By contrast, many peptides are known where the residues essential for the biological activity are localized in well-conserved partial sequences. Messenger segments, responsible for the triggering of physiological events, are located C-terminally in the tachykinins. The C-terminal N^α-acetylated 8-peptide sequence Ac-His-Trp-Ala-Val-Gly-His-Leu-Met-NH₂ of gastrin-releasing peptide displays higher relative activity compared to the native sequence. Furthermore, this C-terminal sequence is highly

homologous to the 14-peptide bombesin isolated from frog skin. Melanin-concentrating hormone (MCH) is a 17-peptide with a disulfide bridge between Cys⁵ and Cys¹⁴; only the partial sequence 5–15 is necessary for full biological activity, with Trp¹⁵ being of eminent importance for interaction with the MCH receptor.

7.2

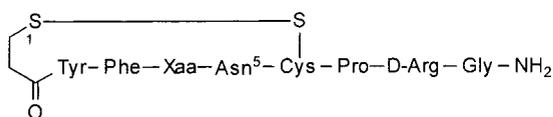
Modified Peptides

7.2.1

Side-Chain Modification

The most straightforward approach for peptide modification is to introduce changes into the side chains of single amino acids. On this level, a multitude of possibilities for the synthesis of nonproteinogenic amino acids already exists, and useful preparative routes for the asymmetric synthesis of many derivatives have been developed. This strategy allows for the incorporation of amino acids with side chains that do not occur naturally in peptides or proteins, with the aim being to introduce special functional groups, to restrict the conformational flexibility of a peptide, or to enhance its metabolic stability. Furthermore, *D*-configured amino acids, *N*^α-alkylated amino acids, or *C*^α-dialkylated amino acids may be employed.

Stereochemical substitutions provide information about the steric requirements in the formation of distinct secondary structures and their influence on peptide-receptor interaction. Furthermore, the incorporation of *D*-amino acids usually confers higher metabolic stability on the peptide. [1-deamino,*D*-Arg⁸]Vasopressin (DDAVP) **4** is a modified peptide applied as a selective antidiuretic agent with long-term activity suitable for the treatment of diabetes insipidus. DDAVP acts as a V₂-receptor agonist in the kidney, with the missing α-amino group increasing proteolytic stability. According to investigations of Manning et al. [50], the exchange of Gln⁴ by Val results in the potent V₂-receptor agonist [Val⁴]vasopressin **5** that simultaneously is a weak V₂-receptor antagonist.



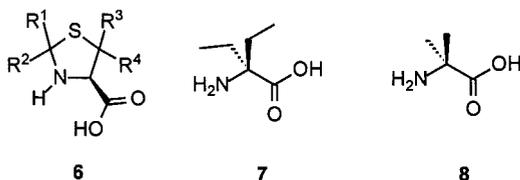
4 Xaa = Gln

5 Xaa = Val

Isosteric and other substitutions similarly provide valuable information on peptide-receptor interactions. Often, methionine is exchanged in this context by norleucine (Nle); indeed, such a substitution in the 14-peptide α-MSH resulted in [Nle⁴]α-MSH displaying two-fold increased activity compared to the native peptide. Oxytocin modification through an exchange of Tyr² by Phe resulted in only weak agonist activity, while [4-MePhe²]oxytocin is an antagonist.

Many substitutions have been performed in gonadoliberin (gonadotropin-releasing hormone, GnRH) that can be used either for diagnostic purposes or as a drug against infertility. Interestingly, GnRH superagonist analogues result in receptor down-regulation. Such long-term activity reduces LH and FSH liberation, and in males this provides therapy for prostate carcinoma.

Conformational and topographical restrictions are particularly suited as manipulations for peptide design targeted towards an increase of receptor selectivity, metabolic stability, and the development of highly potent agonists or antagonists. The incorporation of N-methyl amino acids influences the *cis/trans* ratio of the peptide bond by lowering the relative energy for the *cis*-isomer. Consequently, the torsion angle ω can also be influenced by the incorporation of special amino acids. Pseudoprolines **6** are synthetic proline analogues that can be obtained by a cyclocondensation reaction of the amino acids cysteine, threonine or serine with aldehydes or ketones [51]. These derivatives, which have been mentioned in the context of the synthesis of difficult sequences (cf. Section 4.5.4.3), also influence the *cis/trans* peptide bond ratio [52, 53]. The introduction of different substituents at C² of the pseudoprolines can be used to predetermine the *cis/trans* peptide bond ratio. In particular, the C²-disubstituted analogues **6** ($R^3=R^4=CH_3$) induce up to 100% *cis*-configured peptide bonds in di- or tripeptides [52–54].



The influence of certain naturally occurring amino acids on the secondary structure of proteins and peptides has been discussed in Chapter 2 (Section 4). This information on the conformational bias of certain amino acids can be utilized to guide the design of peptide analogues. Besides their ability to influence the *cis/trans* peptide bond ratio, N-alkyl-substituted amino acids can favor the formation of turn structures because they very often occur in position *i*+2 of a β -turn [55]. Similarly, D-amino acids may also be used for the rational design of peptides with defined secondary structure because they stabilize a β II'-turn where they occur in position *i*+1. The same position *i*+1 is often favored by proline in β I- or β II-turns. In these cases, a *trans*-peptide bond is involved. Proline with a *cis*-peptide bond usually occupies position *i*+2 in a β VIa- or β VIb-turn; this situation is also often observed for N-alkyl amino acids.

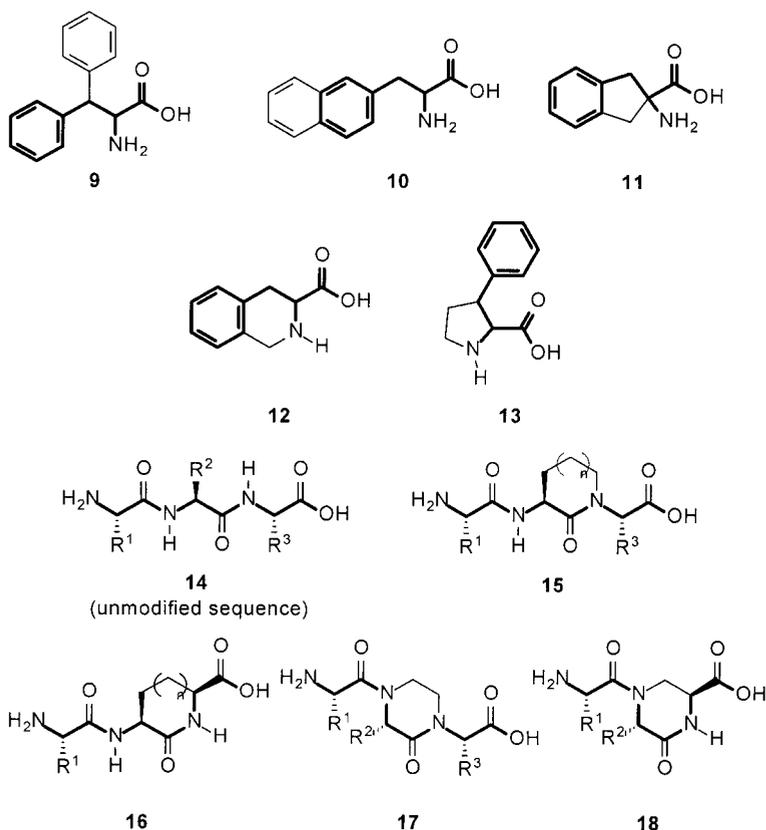
The dipeptide sequence D-Pro-Gly especially favors a β -turn, and also promotes hairpin nucleation [56]. Strategies for the stabilization of peptide conformations by secondary structure mimetics will be discussed in Section 7.2.4.

The [N-MeNle³]CCK-8 analogue displays higher specificity for the CCK-B receptor in comparison to the CCK-A receptor. This preference is due to a *cis*-peptide

bond present in the [Nle³]CCK-8 analogue, but not in the [N-Me-Nle³]CCK-8 derivative as has been shown by NMR spectroscopy [57].

Some C^α-dialkyl amino acids, such as diethylglycine (Deg) 7, α-aminoisobutyric acid (Aib) 8, and isovaline (Iva, 2-amino-2-methylbutyric acid) occur naturally. These derivatives are often incorporated into peptides to investigate the conformational requirements of receptors [58]. They also play an important role as building blocks for the stabilization of short peptides in a well-defined conformation, depending on the nature of the two substituents attached to C^α-carbon. 3₁₀-Helices are stabilized by the incorporation of Aib and other C^α-dialkyl-substituted amino acids [59, 60].

Compounds 9–13 are constrained derivatives of phenylalanine. The introduction of a sterically demanding functional group at C^β, as in 9, mainly constrains conformations around the side-chain dihedral angle χ^1 . This angle, in combination with the backbone dihedrals ϕ , ψ , and ω , determines the 3D positioning of side-chain functional groups (e.g., as pharmacophores). Consequently, amino acids with additional substituents at C^α, C^β, or cyclic amino acids are suitable building blocks for the introduction of a restriction in “ χ -space” [9, 61, 62].



2-Naphthylalanine **10** is a sterically demanding derivative that may also lead to improved interaction of the aromatic system with hydrophobic areas of a protein receptor. Compound **11** is a representative of C^α-dialkyl amino acids. Tetrahydroisoquinoline carboxylic acid **12** (Tic) is a phenylalanine analogue where the torsion angle χ^1 is limited to very small range of values. 3-Phenylproline **13** may be regarded as a chimera containing both proline and phenylalanine substructures.

The introduction of bridges between two adjacent amino acid residues leads to the formation of dipeptide mimetics.

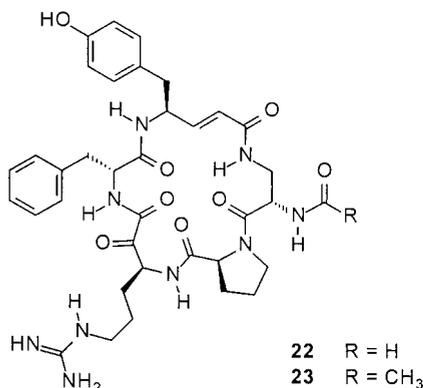
Bridges can, for example, be formed between C^α and N^α (**15**, **18**), between two C^α (**16**), or between two N^α (**17**). This type of modification also makes the peptide more rigid.

7.2.2

Backbone Modification

The modification of the peptide chain (backbone) comprises, for instance, the exchange of a peptide bond by amide analogues (e.g., ketomethylene, vinyl, ketodifluoromethylene, amine, cyclopropene). Some basic types of peptide backbone modifications are displayed in Fig. 7.4 [63].

The NH group of one or more amino acids within a peptide chain may be alkylated [64] or exchanged by an oxygen atom (depsipeptide), a sulfur atom (thioester), or a CH₂ group (ketomethylene isostere) [65]. However, peptidylthioesters are quite unstable. The CH moiety (C^α) may be exchanged in a similar manner by a nitrogen atom (azapeptide) [66, 67], by a C-alkyl group (C^α-disubstituted amino acid) [58], or by a BH⁻ group (borapeptide). The carbonyl groups may be replaced by thiocarbonyl groups (endothiopeptide) [68], CH₂ groups (reduced amide bond) [69], SO_n groups (sulfinamides, n=1; sulfonamides, n=2), POOH groups (phosphonamides), or B-OH groups. The peptide bond of one or more amino acid residues in **19** (cf. Fig. 7.5) may be inverted (NH-CO), giving retro peptides. In order to maintain the original side-chain orientation, the retro modification (**20**, **21**) has to be accompanied by an appropriate stereochemical compen-



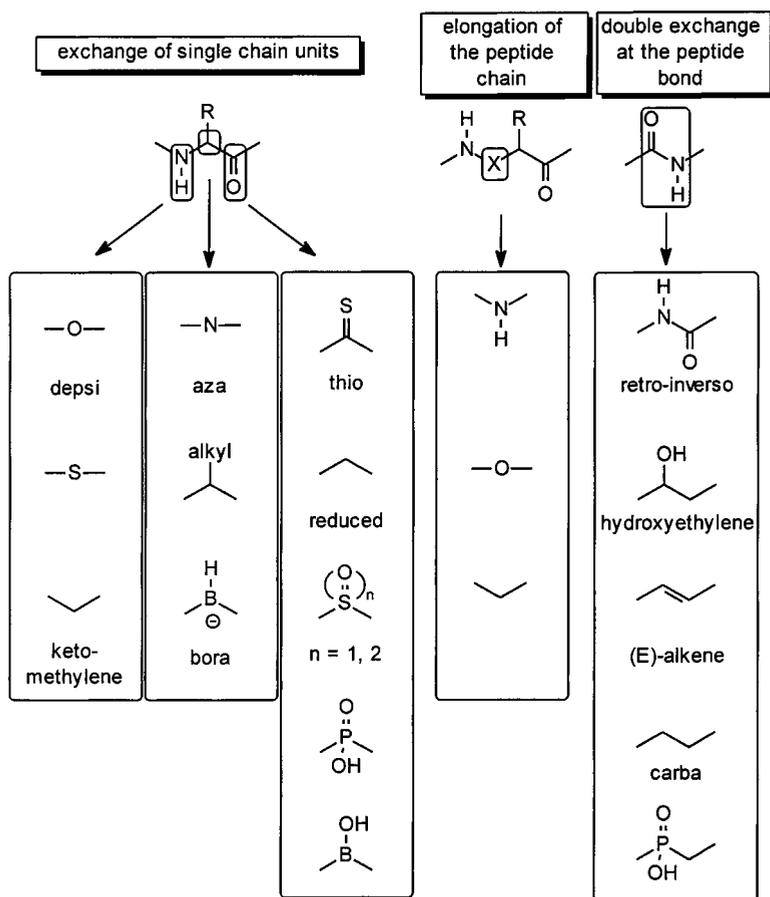


Fig. 7.4 Selected types of peptide backbone modification.

sation (inversion of the configuration at C^α). The consequence is formation of the so-called retro-inverso peptides (Fig. 7.5) [70].

Peptides, where an amide bond has been replaced to give hydroxyethylene [71], E-alkene [72, 73], or alkane structures (carbapeptides), have also been described. Besides these isosteric replacements, the peptide chain may be extended by one atom (O: aminoxy acid [74]; NH: hydrazino acid [75, 76]; CH_2 : β -amino acid [77–79]; Section 7.4).

Vinylogous peptides contain amino acids extended by a vinyl group as building blocks, which is also not an isosteric replacement (Section 7.4). Such peptide analogues have been found in nature, as in the compounds cyclotheonamide A 22 ($R=H$) and B 23 ($R=Me$) that occur in the sponge *Theonella*. Besides vinylogous tyrosine, these peptides further contain the nonproteinogenic amino acid β -amino- α -oxohomoarginine [80]. They act as thrombin inhibitors and are of interest in medicinal chemistry as potential antithrombotic agents.

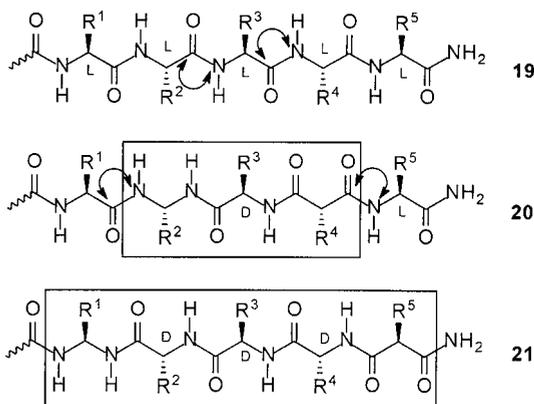


Fig. 7.5 Retro-inverso peptide modification.

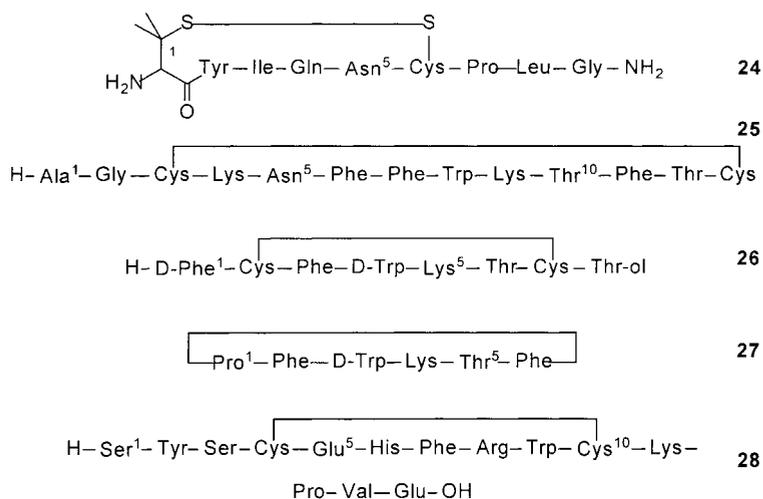
7.2.3

Combined Modification (Global Restriction) Approaches

The introduction of global restrictions in the conformation of a peptide is achieved by limiting the flexibility of the peptide through cyclization (Section 6.1). This may eventually lead to a stabilization of peptidic secondary structures, and consequently this is an important approach toward drug development. Turns (or loops) are important conformational motifs of peptides and proteins, besides α -helix or β -sheet structures. Reverse turns comprise a diverse group of structures with a well-defined 3D orientation of amino acid side chains. β -Turns constitute the most important subgroup, and are formed by four amino acids (see Section 2.5.1.3). Turns lead to a reversion of the peptide chain, and may be mimicked by cyclic peptides with well-defined conformation. Depending on the type of cyclization (Sections 6.1 and 6.2) and on the ring size, the angles ψ , ϕ , ω , and χ will have limited flexibility. This reduction in the decrease of freedom may eventually lead to receptor binding with high affinity because of entropic reasons, provided that the receptor-bound conformation is still accessible to the modified peptide.

In heterodetic cyclic peptides where the cyclization is obtained by cysteine disulfide bridges, cysteine residues may also be replaced by sterically hindered cysteine analogues such as penicillamine (3-mercapto-valine, Pen). Such an exchange may lead to a differentiation between agonistic and antagonistic analogues, as has been shown by Du Vigneaud et al. for [Pen¹]oxytocin 24 and by Hruby et al. for cyclic enkephalin analogues [61]. [D-Pen¹,D-Pen⁵]enkephalin (DPDPE), a cyclic disulfide, is a highly potent and selective δ -opioid receptor antagonist.

Ring size and the formation of stable conformations play important roles in the design of cyclic peptide analogues. This has been intensively investigated in the oxytocin and vasopressin series. In these cases cyclization to produce a 20-membered ring is essential for biological activity. However, this cyclization does not necessarily have to occur by disulfide formation, because the carba analogues,



where the sulfur atoms are replaced by CH_2 groups, also display biological activity.

Somatostatin **25**, which is a regulatory peptide with a broad spectrum of activity, likewise contains an intrachain disulfide bridge. Potential applications have been envisaged in the treatment of acromegaly or of retinopathy in diabetes because **25** suppresses the liberation of growth hormone and glucagon, an increased level of which contributes especially to organ damage in diabetes. Native somatostatin is rapidly metabolized and therefore is not suited to the treatment of juvenile diabetes; hence, metabolically stable analogues have to be developed. The excision of six amino acid residues from the ring in **25** produced the 20-membered somatostatin analogue **26** developed by Bauer et al. [81]. This peptide drug, named octreotide, has been approved for the treatment of acromegaly and of patients with metastasizing carcinoid and vasoactive tumors. Finally, the ring size may be further reduced to produce an 18-membered cyclic hexapeptide **27** that induces the liberation of growth hormone, insulin and glucagon with the same potency as somatostatin [82].

Peptides that natively are linear sequences can also be modified by the incorporation of cyclic structural elements in order to improve receptor interaction. In this context, the superagonist **28** of the α -melanocyte-stimulating hormone should be mentioned, where a β -turn predicted for the linear peptide may be stabilized by the replacement of Met^4 and Gly^{10} with two cysteine residues and disulfide bridge formation [83].

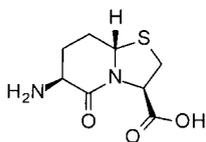
Kessler et al. [16] first established the concept of “spatial screening”, whereby small libraries of stereoisomeric peptides with conformational constraints (e.g., cyclic peptides) are used for different 3D presentations of pharmacophoric side-chain groups (Section 6.1). The correlation of biological activity with peptide conformation provides useful information about the peptide conformation with the best fit to the corresponding target receptor [16].

7.2.4

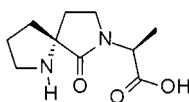
Modification by Secondary Structure Mimetics

It is desirable to have a repertoire of building blocks that reliably induce a desired conformation in a peptide. Consequently, numerous efforts have been undertaken to synthesize secondary structure mimetics that induce a well-defined secondary structure [84]. The desired conformation should be imitated as closely as possible, and the synthetic route for the secondary structure mimetic should permit the introduction of appropriate side chains onto the mimetic scaffold. While certain α -amino acids also exert conformational bias on the peptide chain (see Sections 2.5.1.3 and 6.1), this section focuses on synthetic nonproteinogenic modules that induce and stabilize secondary structure. A large variety of conformationally constrained dipeptide mimetics that may be introduced into a strategic position in a peptide, using methods of peptide synthesis, in order to induce a defined secondary structure has been compiled in reviews [85–87]. Derivatives of this type may also be used as scaffolds for peptidomimetics (Section 7.3).

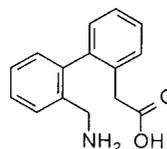
The β -turn is a structural motif that is often postulated to occur in the biologically active form of linear peptides [88]. Consequently, most secondary structure mimetics imitate a β -turn. Compounds 29–37 are examples of β -turn mimetics (29 [89], 31 [90], 32 [91], 33 [92], 34 [93], 35 [94], 36 [95], 37 [96]).



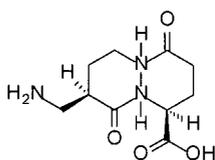
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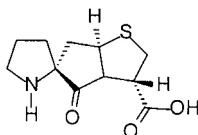
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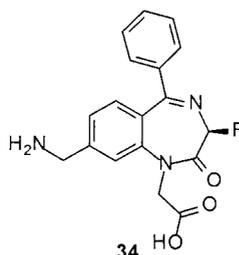
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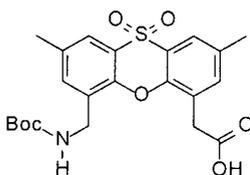
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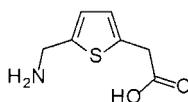
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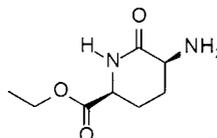
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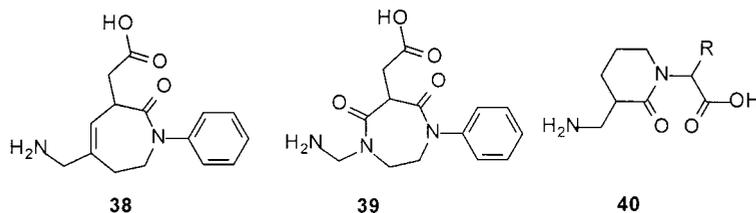
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37

However, as has been shown by both experimental [97] and theoretical methods [98], not all scaffolds designed as β -turn mimetics actually display the desired properties.

γ -Turns, which occur less frequently than β -turns in proteins and peptides, may also be mimicked by a series of compounds. β -Amino acids are most likely the simplest γ -turn mimetics; they induce stable conformations, for example in cyclic tetra- and pentapeptides composed of four α -amino acids and one β -amino acid [79]. Other γ -turn mimetics are compounds **38** [99], **39** [100], and **40** [101].



α -Helix initiators [102, 103] and ω -loop mimetics have also been described [104]. ω -Loops are larger reverse turns in a peptide chain comprising 6–16 amino acid residues.

7.2.5

Transition State Inhibitors

Structural complementarity is a key issue in enzyme-substrate interactions, and both substrates and substrate analogues compete for binding to the active site and formation of the enzyme-substrate complex. It was first recognized in the 1920s that the affinity of a substrate towards a catalyst increases concomitantly with the distortion of the reactant towards its structure in the transition state. The transition state of an enzyme reaction can be assumed to differ significantly from the substrate ground state conformation. For example, a transition state with a tetrahedral configuration is formed in nucleophilic displacement reactions starting from a sp^2 -hybridized carboxy group, as is present in peptide bond hydrolysis. Pauling [105] suggested that a potent enzyme antagonist might be developed that mimics the enzyme-substrate transition state despite being unreactive (transition state analogue, Fig. 7.6). Substrate and substrate analogues are relatively weakly bound in their ground state, because tight binding would be counterproductive to efficient catalysis, while the substrate in its transition state is bound much more closely [106].

Based on this concept, transition state inhibitors of enzyme reactions (e.g., protein or peptide cleavage by a protease) have been developed (Fig. 7.6) [5, 107, 108]. Nature once more provided a prototype for this kind of inhibitors with pepstatin **41**, an inhibitor of aspartyl proteases. This peptide (isovaleryl-Val-Val-Sta-Ala-Sta-OH), which is isolated from culture filtrates of *Streptomyces* species, contains – besides the N-terminal acyl moiety – the unusual amino acid statine (Sta), which is

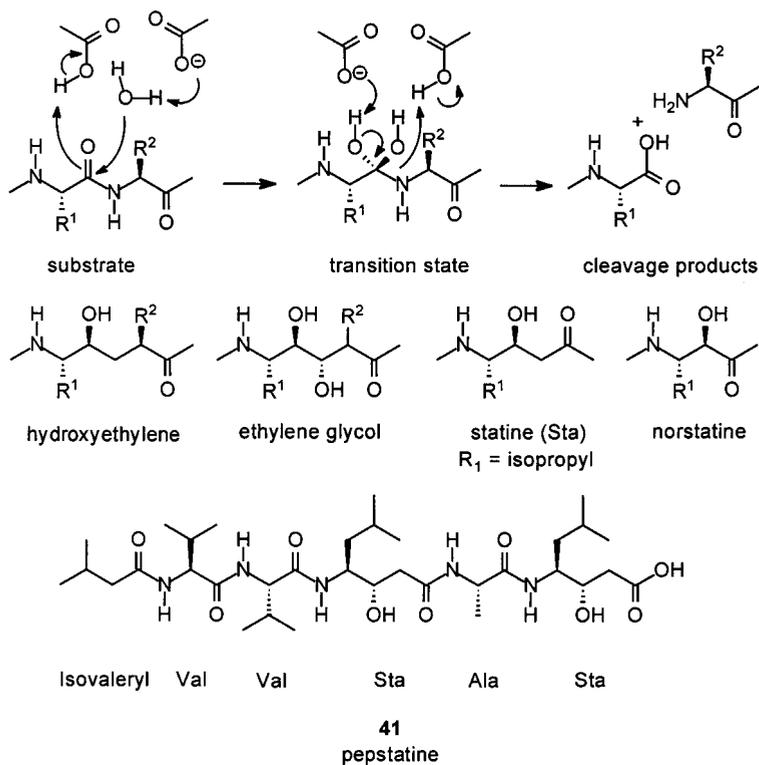


Fig. 7.6 Mechanism of proteolysis by an aspartyl protease and structures of some transition state inhibitors.

regarded as an amino acid analogue mimicking the transition state of peptide bond cleavage. Hydroxyethylene, ethylene glycol, or norstatine represent other building blocks for transition state inhibitors. This amino acid and its analogues have been applied in the development of many mechanism-based inhibitors of proteases and other enzymes [5]. The concept has also been utilized for the design of suitable haptens in the creation of catalytic antibodies (see Section 4.6.3) [109, 110].

7.3

Peptidomimetics

As the therapeutic application of peptides is limited because of certain disadvantages (see above), small organic molecules remain in most cases the most viable approach for the identification and optimization of potential drugs [111]. A major goal of modern medicinal chemistry is to find rational first principles for systematically transforming the information present in a natural peptide ligand into a

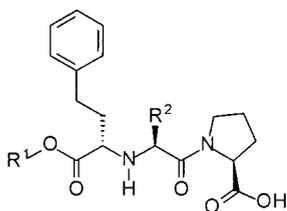
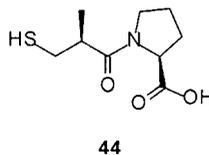
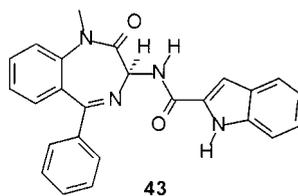
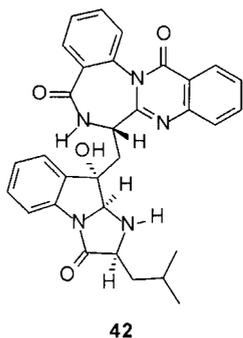
nonpeptide compound of low molecular weight (type II and type III peptidomimetics). Chemical structures designed to convert the topographical information present in a peptide into small nonpeptide structures are referred to as peptidomimetics. Today, these compounds – which combine bioavailability and stability superior to that of bioactive peptides with increased receptor selectivity – are the subject of major interest by pharmaceutical companies. Peptidomimetics range from peptide-isosteric molecules to compounds where similarities to peptides can barely be identified. Information obtained from the structure-activity relationships and conformational properties of peptide structures can enhance the design of nonpeptide compounds [112]. The design of a peptidomimetic relies primarily on knowledge of the conformational, topochemical, and electronic properties of the native peptide and its corresponding receptor. Structural effects, such as a favorable fit to the binding site, the stabilization of a conformation by introducing rigid elements, and the placement of structural elements (functional groups, polar or hydrophobic regions) into strategic positions favoring the required interactions (hydrogen bonds, electrostatic bonds, hydrophobic interactions) must be taken into account.

The major objective in the development of small molecules displaying pharmacological activity is to mimic the complex molecular interactions between natural proteins and their ligands. Often, the mode of action of a biologically active peptide on the receptor level can be imitated by a small molecule (agonism), or can be blocked (antagonism) [1, 4, 9, 113]. A peptidomimetic may also be designed as an enzyme inhibitor.

The design of peptidomimetics exceeds the mere application of peptide modifications, and is targeted towards nonpeptidic compounds, characterized by a high degree of structural variation. Both random screening and design using molecular modeling have proved to be helpful in drug development. Peptidomimetic receptor antagonists or enzyme inhibitors usually imitate the binding of nonadjacent peptide substructures (pharmacophoric groups) to a protein, with this interaction often also relying on hydrophobic protein-ligand interactions. However, small organic molecules containing hydrophobic moieties may undergo a conformational transition in aqueous solutions because of an undesired intramolecular hydrophobic interaction (hydrophobic collapse). Consequently, the pharmacophoric groups should be presented on a rather rigid scaffold in order to avoid hydrophobic collapse of the bioactive conformation in an aqueous environment [5].

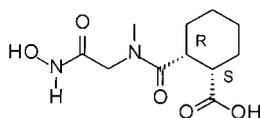
Peptidomimetics may be identified or discovered by extensive screening of natural or synthetic product collections (compound libraries). The combinatorial synthesis (Chapter 8) of a multitude of different peptidic or nonpeptidic compounds, combined with careful evaluation of receptor binding, are promising approaches for the discovery of new lead structures that subsequently must be further optimized with respect to their pharmacological properties.

Asperlicin **42** was discovered during a screening of fungal metabolites, and was found to be a lead structure as a cholecystokinin A (CCK-A) antagonist. **42** is structurally very similar to diazepam, which consequently was combined with a D-tryptophan structural motif; this eventually led to the design and synthesis of a se-



45 $R^1 = \text{H}$, $R^2 = (\text{CH}_2)_4\text{NH}_2$

46 $R^1 = \text{CH}_2\text{CH}_3$, $R^2 = \text{CH}_3$

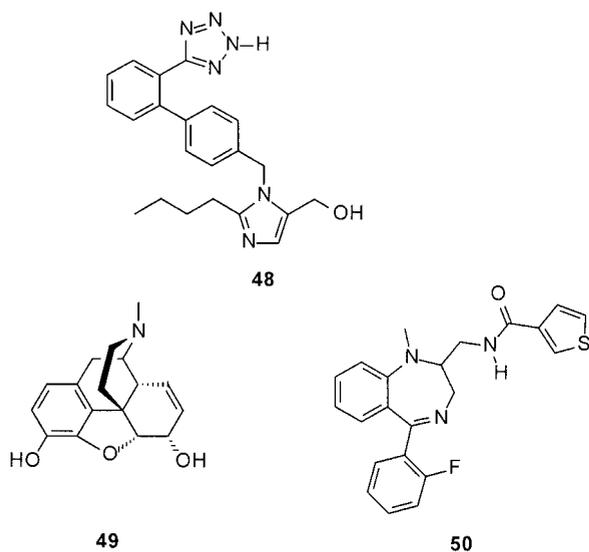


lective orally administered peptidomimetic antagonist of the peptide hormone cholecystokinin 43 [114].

The angiotensin-converting enzyme (ACE) inhibitor captopril 44 [115] was the first peptidomimetic compound to have been developed by rational design. Wolfenden introduced (*R*)-2-benzylsuccinic acid as an inhibitor for carboxypeptidase A (CPA), the enzyme mechanism of which is closely related to that of ACE [116, 117]. (*R*)-2-Benzylsuccinic acid belongs to the class of transition state analogues (Section 7.2.5).

The 9-peptide teprotide Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro-OH isolated from snake venoms possesses bradykinin-potentiating activity that is based on the inhibition of ACE. While the modified C-terminal dipeptide sequence Ala-Pro displayed only weak inhibition of ACE, exchange of the N-terminal amino group by a carboxy group resulted in a more potent ACE inhibitor. This modification was based on the finding by Wolfenden concerning the inhibitory activity of benzylsuccinic acid on CPA. Replacement of the carboxy group by a thiol function, which strongly coordinates metal ions (e.g., Zn^{2+}), resulted in captopril, which is an inhibitor of the Zn-dependent metalloprotease ACE, and has been approved as an orally administered drug. The highly potent analogues lisinopril 45 and enalapril 46 have been synthesized by variation of different regions of captopril [118]. The hydroxamate 47 displays extremely low toxicity and an activity for ACE inhibition which is comparable to that of 44 ($\text{IC}_{50} = 7 \text{ nM}$) [119].

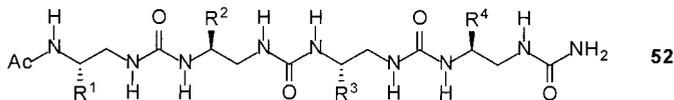
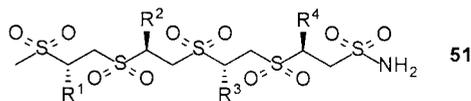
Losartan 48 is highly active angiotensin II antagonist that was optimized by molecular design. It is the first nonpeptide angiotensin II antagonist, and is currently used for the treatment of hypertension.



Morphine **49**, as a representative of the opioid alkaloids, is the classic example of a nonpeptidic compound that was found to be a mimetic of endogenous peptides. Morphine imitates the biological effect of β -endorphin on the corresponding receptor. The endogenous opioids resemble morphine very much from a pharmacological point of view, even in the side effects such as addictive potential and respiratory depression. Important developments following the discovery of opioid peptides included evidence of the existence of three different opioid receptor classes (μ , δ , κ), each with various subclasses (see Section 3.3.2.1). High expectations were imposed on this field of research, which was aimed towards the development of an opioid compound with high analgesic potency but without any adverse side effects, and as a result the field of opioid peptide and peptidomimetics became popular with regard to the application of new design principles. Concepts such as conformational restriction, peptide bond replacements, the incorporation of turn mimetics, and library screening for the identification of novel ligands were extensively tested in the area of opioids. However, despite many interesting developments, the original aim has not (yet) been achieved. For example, the benzodiazepine derivative tipludom **50** is an agonist of the κ -opioid receptor and an antagonist of the CCK-A receptor. Although animal experiments have shown to be devoid of addictive potential and respiratory depression [120], its administration is associated with locomotor incoordination [121].

7.4 Pseudobiopolymers

Antisense oligonucleotides form one of the first classes of non-natural biopolymers [122]. Several other types of pseudobiopolymers have been described, and these are composed of nonpeptidic molecules in a similar, repetitive manner as peptides. Moreover, some pseudobiopolymers fold to give stable, reproducible sec-

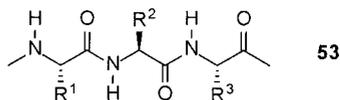


ondary structures that mimic protein secondary structures such as helices, sheets, or turns [84, 123–125]. They often are characterized by metabolic stability, resemble peptidic structures, and some of them may be used therapeutically. The peptoids (Section 7.4.1) [126], peptide nucleic acids (Section 7.4.2) [127], and β -peptides (Section 7.4.3) [128–133] are important representatives of this class of compounds. Oligomers of aza-amino acids (hydrazine carboxylates) have been named azatides [134, 135]. Oligosulfonamides [136–138], hydrazinopeptides, composed of α -hydrazino acids [75, 139] and aminoxypeptides, composed of α -aminoxycarboxylic acids [74], may be regarded as β -peptide analogues with respect to the backbone atom pattern (Section 7.4.3). Oligocarbamates (Section 7.4.4) [140], oligopyrrolinones (Section 7.4.5) [141], oligosulfones **51**, γ -peptides [142, 143], oligoureas **52** [125], vinylogous peptides [144], vinylogous oligosulfonamides [145], and carbopeptides (oligomers of carbohydrate-derived amino acids) [146, 147] form further classes of pseudobiopolymers. Gellman first coined the term foldamers for any polymer that reproducibly adopts a specific ordered conformation [123]. Some representatives of these classes will be briefly introduced in the following sections.

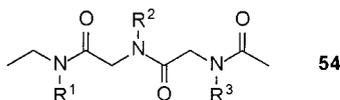
7.4.1

Peptoids

Peptoids are oligomers composed of N-substituted glycine building blocks [126, 148, 149]. The side chain of each amino acid in the peptides formally is shifted in the peptoids by one position from C^α to the amino group nitrogen. Comparison of the peptide chain **53** with the peptoid chain **54** shows that the direction of the peptide bond in **54** should be reversed (retro-sequence) in order to provide the



peptide sequence



peptoid sequence



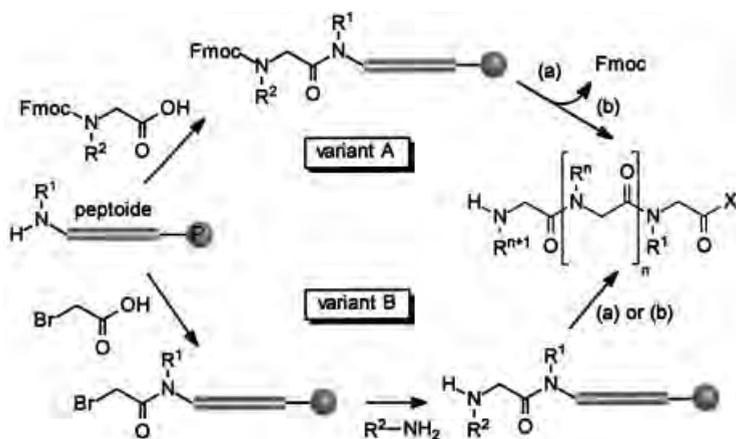


Fig. 7.7 Solid-phase synthesis of peptoids. Variant A, building block approach; variant B, submonomer approach. (a)=chain elongation (n repetitions); (b)=deprotection and cleavage from solid support; $R^1 \dots R^n$ =side-chain residues; $X = \text{NH}_2, \text{OH}$.

same relative arrangement of side-chain residues R and carbonyl groups. Peptoids are achiral, and resemble the structures of the retro-inverso-peptidomimetics [70]. Helical peptoid structures are favored when bulky N -alkyl side chains are present [150]. Interestingly, these helices are not stabilized by hydrogen bonds, and the helicity depends on side-chain chirality [151] and oligomer length. N -Substituted glycine derivatives can be synthesized easily. The principle of solid-phase peptoid synthesis is shown in Fig. 7.7 [7].

Among the different types of polymeric support which may be used, the Rink amide resin has provided good results. Variant A utilizes the Fmoc/tBu tactics where the suitably protected N -substituted glycine derivatives are coupled to polymer-bound NHR groups using PyBOP or PyBroP [126]. The submonomer solid-phase synthesis (variant B) [148] does not apply the N -substituted glycine monomer. Instead, bromoacetyl building blocks are coupled to the growing peptoid chain and then subjected to a nucleophilic substitution reaction of the bromide substituent by a primary amine carrying the side-chain substituent. Additional protection of the amino group is not necessary, and no different monomeric building blocks have to be synthesized.

Conformational studies revealed that peptoids display higher conformational flexibility compared to peptides. They resist proteolysis and are, therefore, metabolically stable. Preliminary studies show that peptoids with structural resemblance to biologically active peptides have for example a similar activity as enzyme inhibitors compared to the native peptide sequence. The peptoid analogue of the hepatitis A 3C-protease inhibitor Ac-Glu-Leu-Ala-Thr-Gln-Ser-Phe-Ser- NH_2 displays, in the retro-sequence, a similar inhibition to the original peptide. An additional important finding is that peptoid analogues of peptide sequences from HIV-Tat protein bind to the corresponding RNA. This class of peptoids represents a promising concept in the

search for new lead structures to influence pathological events. Peptoid libraries may be synthesized in a similar manner to peptide libraries, and this facilitates high-throughput screening for the identification of novel nonpeptide drugs.

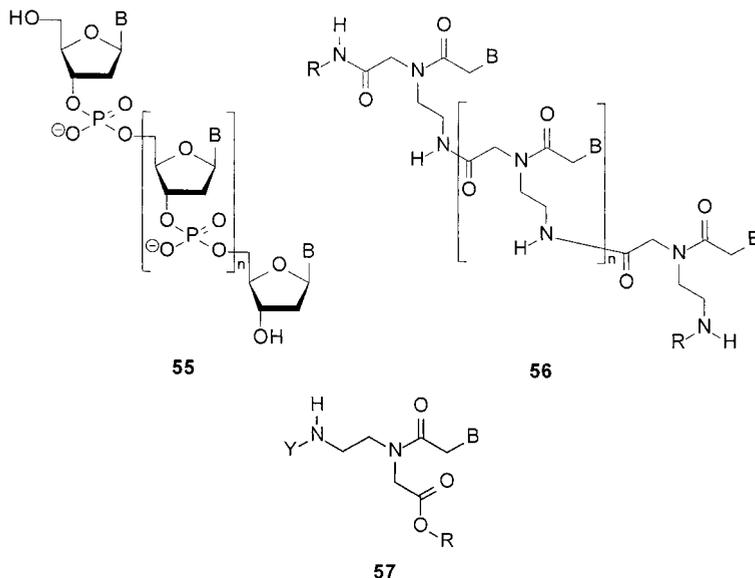
7.4.2

Peptide Nucleic Acids (PNA)

Peptide nucleic acids are nonionic analogues of oligonucleotides containing a peptide backbone [127, 152–154]. They are mimetics of DNA that can be employed as antisense nucleotides. PNA originally were designed to mimic an oligonucleotide binding to double-stranded DNA in the major groove, but they have eventually been shown as good structural analogues of nucleic acids. The antisense concept is based on the application of oligonucleotides to hybridize with DNA or RNA in order to prevent transcription by the formation of a triple helix, or to bind mRNA by duplex formation. However, the first examples – which were described as DNA/RNA backbone analogues [155, 156] – did not display any ability to hybridize with oligonucleotides. Today, this concept is of therapeutic importance in cases where diseases can be treated on the DNA or mRNA level. The nonionic oligonucleotides of the PNA type are totally inert towards degradation by nucleases, and can also be synthesized in larger amounts by solid-phase synthesis.

The desoxyribose-phosphate backbone of an oligodesoxynucleotide **55** is replaced in the PNA **56** by a pseudopeptidic 2-(aminoethyl)glycine unit. Besides the 2-(aminoethyl)glycine moiety, other monomers may be used for PNA synthesis [127].

The N-protected monomer unit **57** (Y = Boc or Fmoc) comprises an amino acid [2-(aminoethyl)glycine] that is connected via a methylenecarbonyl spacer to the corresponding nucleobase B (thymine, cytosine, adenine, or guanine).



In principle, most of the coupling reagents used in peptide chemistry can be applied to the oligomerization of PNA. The active ester **57** ($R = \text{Pfp}$) may be used. The building blocks of type **57** can be obtained by alkylation of the corresponding base (B) with methyl-2-bromoacetate in DMF in the presence of K_2CO_3 . After saponification of the methyl ester and conversion of the free carboxylic acid to the pentafluorophenylester, the nucleobase derivative is reacted with N -(N' -Boc-aminoethyl)glycine (N' -Boc-Aeg), which again is subsequently converted into the pentafluorophenylester **57** using DCC. An alternative synthesis of Fmoc-PNA-OPfp **57** ($Y = \text{Fmoc}$) has been described [157].

7.4.3

 β -Peptides, Hydrazino Peptides, Aminoxy Peptides, and Oligosulfonamides

One characteristic feature of β -amino acids and β -homoamino acids (where an additional C_1 unit is inserted between the carboxy group and C^α of a natural amino acid) is the additional carbon atom between the amino group and the carboxy group. Side chains may be attached either to C^α (β^2 -homoamino acid, **59**) or to C^β (β^3 -homoamino acid, **58**), or to both of them. This fact considerably influences the secondary structure of the corresponding oligomer. The first structural investigations on homo-oligomers of β -amino acids were performed over 30 years ago.

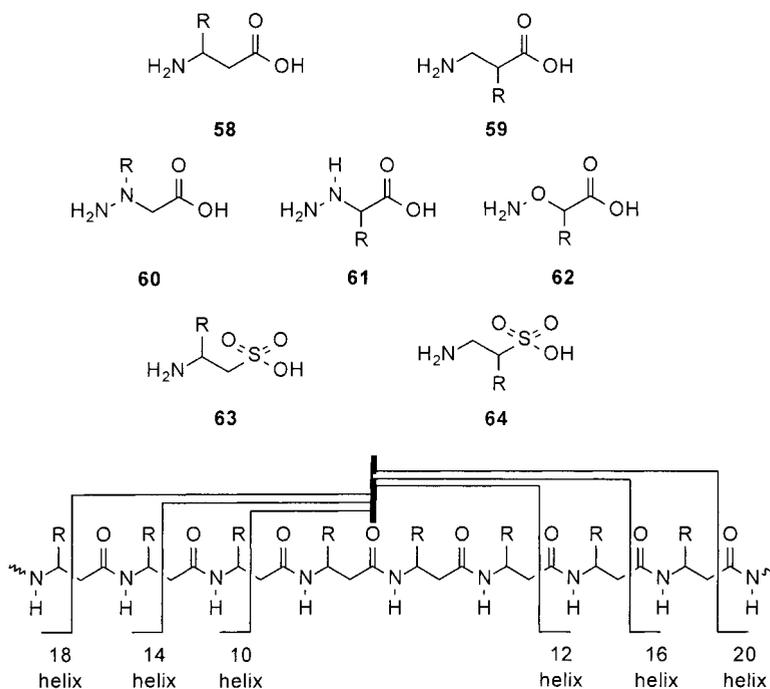


Fig. 7.8 β -Amino acids, β -amino acid analogues and hydrogen bond patterns in β -peptide helices.

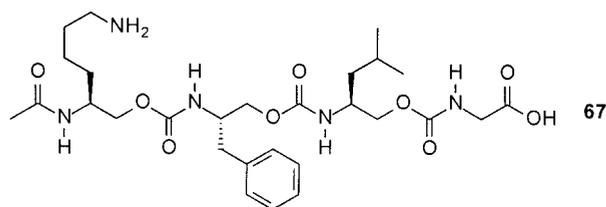
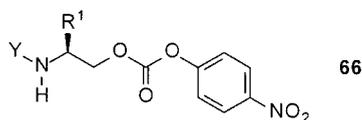
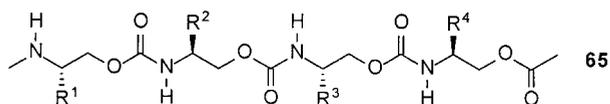
Kovacs et al. postulated in 1965 that poly-(L-β-aspartic acid) forms a 3.4₁₄-P-helix [right-handed helix with 3.4 amino acid residues per turn and a 14-membered hydrogen bonded “ring” from NH (i) to CO (i+2)] [158]. Homopolymers of poly-(L-β-aspartic acid) have been investigated by Muñoz-Guerra et al. [128, 159], while Gellman et al. [123, 129, 130] and Seebach et al. [131, 132] profoundly examined the conformational behavior of a series of different well-defined β-peptides. All these compounds have been found to adopt predictable and reproducible helical conformations with hydrogen bond patterns depending on the substituent position (Fig. 7.8). Indeed, many β-peptides prefer 3₁₄-helices even in aqueous solutions [132, 160–162]. Helix formation occurs already in β-peptide hexamers, while in the case of α-amino acids usually more than 10–12 amino acid residues are required to form a stable helix. In molecular dynamics simulations, β-peptide helices have been shown to refold spontaneously after thermal unfolding [163]. An alternating combination of β²- (59) and β³- (58) amino acids leads to an irregular helix with 10- and 12-membered hydrogen bond “rings” [164]. Both Boc- [165, 166] and Fmoc-protected β-amino acids [77] are available for peptide synthesis.

Hydrazino peptides [75, 139] and aminoxy peptides [74], composed of α-hydrazino acids (60, 61) or α-aminoxy acids (62) are structurally related to β-peptides, because they also contain an additional skeleton atom between amino and carboxy group. Aminoxy peptides form helices stabilized by hydrogen bonds in eight-membered “rings” [74, 136–138].

7.4.4

Oligocarbamates

Oligocarbamates 65, which form another class of pseudobiopolymers (biopolymer mimetics), contain a carbamate moiety instead of a peptide bond [140]. The oligocarbamates may be considered as γ-peptide analogues, with three skeleton atoms



Ac-Lys^C-Phe^C-Leu^C-Gly-OH

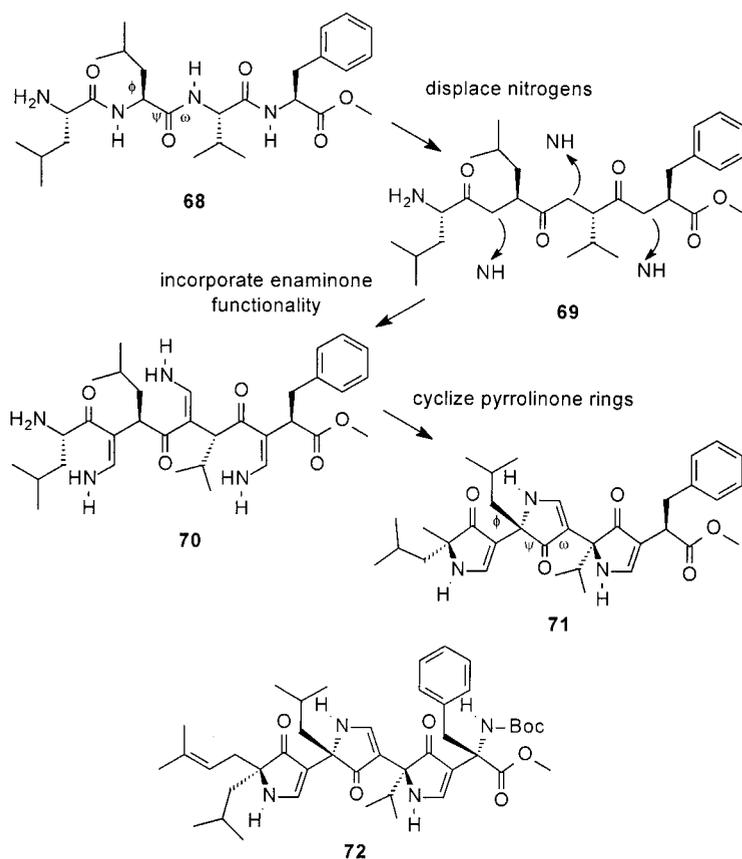
being found between the amino group and the carboxy group of one monomer. Oligocarbamates are stable towards proteolysis and are more hydrophobic compared to the peptides; hence, they may be able to permeate body barriers such as the blood-brain barrier.

The monomeric N-protected aminoalkylcarbonates are accessible starting from the corresponding amino alcohols that are converted into a carbamate active ester (e.g., *p*-nitrophenylester **66**). The oligocarbamates may be obtained in solid-phase synthesis using either the base-labile Fmoc group (**66**, Y=Fmoc) or the photolabile nitroveratryloxycarbonyl group (**66**, Y=Nvoc). Cho et al. [140] succeeded in synthesizing a library containing 256 different oligocarbamates, with **67** being one representative. The superscript "c" indicates the carbamate.

7.4.5

Oligopyrrolinones

Oligopyrrolinones contain a novel peptidomimetic principle where a strongly modified peptide backbone is integrated in cyclic structures also containing vinylogous amino acids [141]. A structural comparison between the oligopyrrolinone **71**



and the tetrapeptide **68** with a parallel β -sheet structure clearly shows that **71** comprises comparable dihedral angles ϕ , ψ , and ω as well as a similar orientation of the side chains. Formally, the amide nitrogen is displaced (**69**), incorporated in an enamionone (**70**), and then cyclized (**71**). Preconditions for the formation of hydrogen bonds, as in a β -sheet, are fulfilled. The 3D similarity of both the side chains and carbonyl groups in **68** and **72** has been proven by X-ray crystallography. **72** is present in an antiparallel β -sheet structure.

7.5

Macropeptides and De-novo Design of Peptides and Proteins

The de-novo design of peptides and proteins has emerged as a challenging approach to study the relationship between the structure and function of a protein. Solving the protein folding problem remains as the “Holy Grail” for computational structural biology, and this is still out of reach. De-novo design can be regarded as an alternate route to tackle the problem of protein folding, the term having been coined for approaches that involve the construction of a protein with a well-defined 3D structure that does not consist of a sequence directly related to that of any natural protein.

7.5.1

Protein Design

Proteins are characterized by an impaired variety of shape and function. Consequently, protein design is not easy to realize compared to the design of smaller model compounds, for example the active sites of enzymes. Protein design represents a major challenge, and provides valuable information on the complex interplay between the structure and function of proteins. The principles and methods for the design of proteins has been the topic of several reviews [167–172].

Most de-novo design strategies rely on knowledge obtained from an increasing amount of crystallographic protein structure data. These data provide basic information on the propensity of a single amino acid (or of a short amino acid sequence) to form a particular secondary structure. The assembly of supersecondary and tertiary folds relies on further hydrophobic or electrostatic interactions. Recent success in protein design is not merely the result of a simple hierarchic procedure, but has relied heavily on computer-based strategies [173–175].

Although the assembly of proteins by use of chemical synthesis, chemoenzymatic strategies and gene technological approaches is feasible (see also Chapters 4 and 5), it is not a straightforward task. Different concepts have been developed that mainly follow two important strategies [176]:

- The assembly of a larger peptide with known secondary structure motifs that forms a well-defined tertiary structure.
- The design of smaller peptide moieties of known secondary structure followed by assembly on a template.

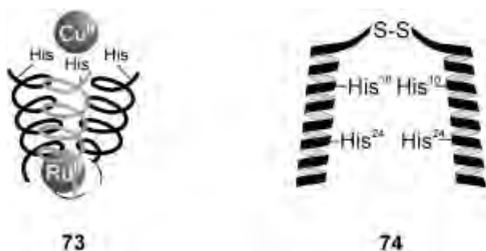
De-novo protein design comprises the arrangement of simple secondary structures (β -turn, α -helix, β -sheet) to tertiary structures (folds) of increasing complexity (β -hairpin [177, 178], helix-turn-helix [179], coiled coils, helix bundles; Section 2.4) [167, 168, 180–182].

The design of non-natural redox proteins according to the former strategy has been realized by a sensible combination of complex chemistry and protein chemistry [168].

An interesting concept utilizes metal ion-induced self-association for the difficult organization of small amphiphilic peptide subunits in aqueous solution to give topologically well-determined protein structures. Such a de-novo design of functional protein requires the assembly of molecular scaffolds with predetermined structure, where the desired functional groups of an active site of an enzyme can be introduced. Using this approach, Ghadiri et al. [183] obtained a Ru^{II} metalloprotein with an exactly defined metal coordination site. Within this metalloprotein, three histidine residues were located proximal to the C-terminus of the three-helix protein bundle and formed a coordinating site for a metal, thereby favoring the incorporation of Cu^{II} to give the $\text{Ru}^{\text{II}}\text{Cu}^{\text{II}}$ protein 73.

Two different metal ions and three peptide subunits assemble in a highly selective process of self-aggregation to a heterodinuclear metalloprotein with three parallel helices. This achievement is one of the preconditions for the design of potentially redox-active metalloproteins and of artificial light-collecting metalloproteins.

DeGrado et al. [184] synthesized helical 31-peptides that assembled to give dimeric bundles of four helices that are connected pairwise via disulfide bridges. The model peptide 74 contains two histidine building blocks in each subunit (His^{10} , His^{24}), dimerizes upon addition of Fe^{II} -heme to give four redox centers at the putative binding sites, and also serves as a mimetic for the cytochrome b subunit of cytochrome bc_1 . Furthermore, miniaturized metalloproteins have been designed and synthesized *de novo* [168, 169, 185].



The Ghadiri group has also rationally designed tubular peptide nanostructures (peptide nanotubes) and transmembrane ion channels (Fig. 7.9). This concept is based on the assumption that cyclopeptides with alternating D- and L-configured amino acid building blocks form a flat ring where the planes of the amide groups in the scaffold are arranged perpendicularly to the ring plane [186].

Furthermore, it was postulated that this special arrangement is energetically favored under certain conditions, leading to the formation of tubular associates that

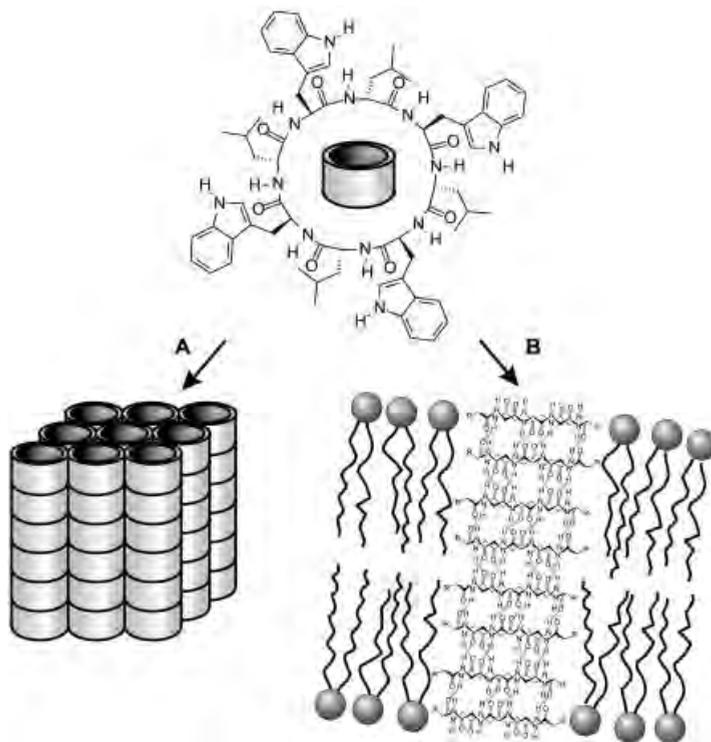


Fig. 7.9 Assembly of peptide nanotubes (A) and transmembrane ion channels (B) starting from cyclic peptides with alternating D- and L-amino acids.

are open at both ends. This process is favored by intermolecular hydrogen bonds together with interactions leading to a stacking of the cyclopeptide rings. Eventually, a selectively N-methylated cyclic peptide, cyclo-[(Phe-D-N²-Me-Ala)₄] could be synthesized that assembles in apolar organic solvents by self-organization to produce a discrete, soluble, cylinder-shaped associate. In the solid state, the peptide forms by self-organization a crystal that is characterized by a pore structure in the shape of an ordered parallel arrangement of water-filled channels with a diameter of 7–8 Å.

The rational design of such structures is of considerable interest in the investigation into molecular transport processes, for the chemistry of inclusion compounds, and for catalytic processes, as well as the manufacture of new optical and electronic units.

The preparation of new artificial proteins and enzymes with predetermined 3D structure and tailor-made chemical, biological, and catalytic properties represents the ultimate goal for peptide and protein chemists.

The second strategy referred to at the beginning of this chapter is the template-associated synthetic proteins (TASP) concept, developed by Mutter et al. [187, 188]. These TASP with predetermined 3D structure are obtained by synthesis. The de-

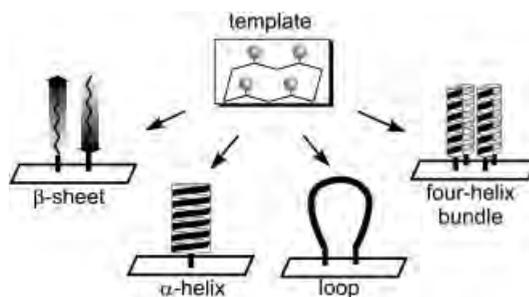


Fig. 7.10 Structural motifs of a template-assembled synthetic protein (TASP).

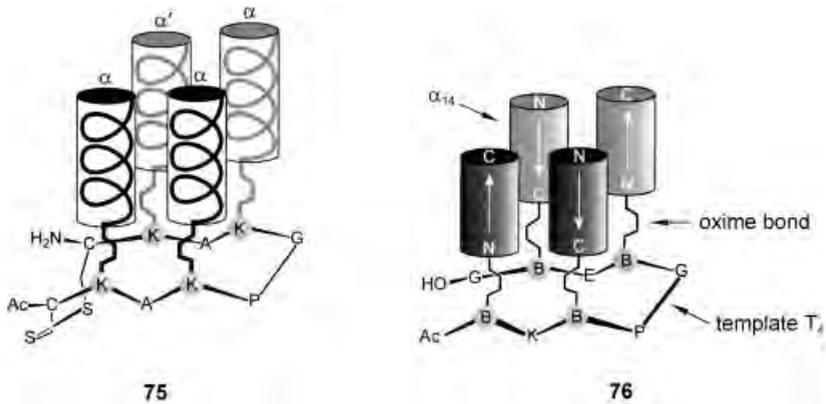
novo design of polypeptide sequences is limited by the protein folding problem, though this can be avoided by constructing proteins of non-natural chain architecture with predetermined intramolecular folding. TASP molecules combine structural features of natural proteins such as peptide moieties with predetermined secondary structure and synthetic elements such as topological templates; the result is branched structures with different possible folding topologies [170–172] (Fig. 7.10).

By selecting the correct template, TASP with for example $\beta\alpha\beta$, α -helix bundle, or β -barrel tertiary structures can be obtained, whilst protein-like folding motifs may be constructed from a “molecular kit”. The individual building blocks (α -helix, β -sheet, loops, turns) are assembled in a regioselective manner by chemoselectively addressable groups at both ends of each building block [171, 189]. The schematic view of a TASP molecule **75** shows that the peptide chains attached to a cyclic octapeptide template via the lysine side chains form α -helical structures, and also associate intramolecularly to a four α -helix bundle that closely resembles protein structures.

The computational design of a TASP helical bundle, for example, requires the following steps:

- The design of amphiphilic helical peptide blocks based on the principles for secondary structure formation.
- Self-associations of the peptide blocks by optimal intramolecular interaction.
- The design of tailor-made templates and functional attachment of the peptide building blocks.
- Stabilization of hypothetical TASP structure by minimization of the conformational energy.

Solid-phase synthesis is used for the assembly of the peptides. The nonconsecutive mode of connection of the subunits excludes the application of synthetic methods by recombinant DNA techniques. Regioselectively addressable functionalized templates (RAFT) are used for chemoselective ligation [170, 190], and allow for the attachment of four different peptide building blocks or for the assembly of binding loops as receptor mimetics. The antiparallel four-helix bundle TASP T_4 -($2\alpha_{14}\downarrow, \alpha_{14}\uparrow$) **76** was designed as a mimetic for the lysozyme epitope mAb HyHel-



10 [191]. The TASP concept has also been used for the de-novo synthesis of four-helix bundle metalloproteins in a combinatorial approach [192].

The α -helical coiled coil is another simple but highly versatile protein folding motif. Approximately 2–3% of all protein residues form coiled coils [193], the latter consisting of two to five amphipathic α -helices that are wrapped around each other in a left-handed twist with a seven-residue periodicity. The stability of this fold is mainly achieved by a packing of apolar side chains into a hydrophobic core (“knobs-into-holes”) [194]. In the seven-residue repeat the first and fourth positions are occupied by hydrophobic uncharged (U) residues forming a hydrophobic core at the interhelical interface. The fifth and seventh positions of the heptad repeat are often occupied by charged residues (C) (Fig. 7.11). The residues 2,3,6 in the outer boxes (X) do not contribute to interhelical contacts (Fig. 7.11).

This α -helical coiled coil motif is often regarded as ideal model system for investigations into the principles of protein stability and for de-novo design [195–199]. Many studies have elucidated the influence of sequence variations on structure, orientation and aggregation state of coiled coils [200–203]. The design principle of coiled coil proteins has also been used for the concept of a de-novo-designed peptide ligase [204].

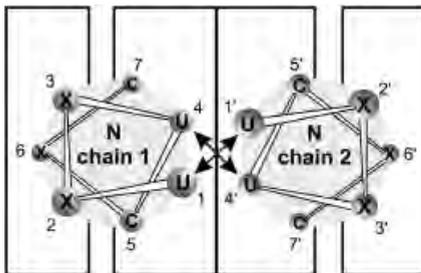


Fig. 7.11 Interactions in coiled coils. U=uncharged amino acid; C=charged amino acid; X=any amino acid.

7.5.2

Peptide Dendrimers

Dendrimers are highly ordered, highly branched polymers with a wide variety of potential chemical applications. Dendrimers bearing peptide moieties with predetermined secondary structure are useful tools in the de-novo design of proteins that may be applied as antigens and immunogens, for serodiagnosis, and for drug delivery [205]. Dendrimers are formed by successive reactions of polyfunctional monomers around a core, and consequently have many terminal groups.

Dendrimeric macromolecules differ from normal polymers as they are constructed from AB_n monomers in an iterative fashion. They may be synthesized either by a divergent or by a convergent strategy (Fig. 7.12). The divergent strategy makes use of the assembly starting from the initiator core to the periphery. Each new layer of monomer units attached to the growing molecule is called a “generation”. In the convergent strategy, the assembly of dendrimers starts from the periphery and continues towards the central core [206].

Tam et al. [207] utilized branched oligolysine cores as synthetic carriers of fully synthetic antigens. The multiple antigen peptides (MAP) developed by Tam et al. comprise a simple amino acid such as glycine or β -alanine as an internal standard, an inner oligolysine core, and multiple copies of the synthetic peptide antigen (Fig. 7.13).

One advantage of MAP is that they have a well-defined and high antigen:carrier ratio. The derivatives are stable, and no conjugation to an antigenic protein is necessary; hence, undesired epitopes are not present.

Dendrimers may be synthesized either in solution or on solid phase. While the former approach is often very challenging and requires long reaction times and nontrivial purification steps, the application of solid-phase strategies provides the possibility of driving the reactions to completion by use of a large excess of reagents. Furthermore, a distinction may be made between direct and indirect approaches for the synthesis (Fig. 7.14).

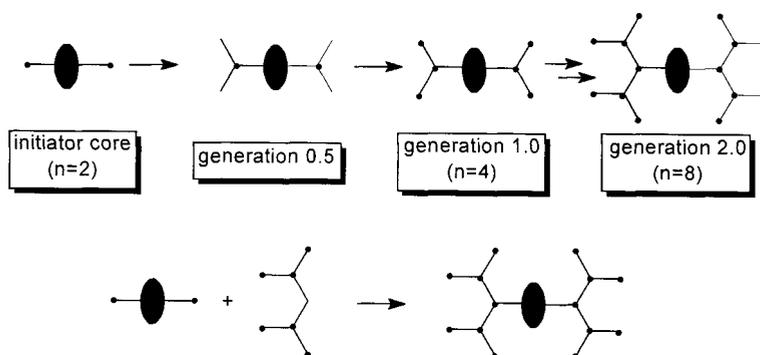


Fig. 7.12 Divergent and convergent synthesis of dendrimers.

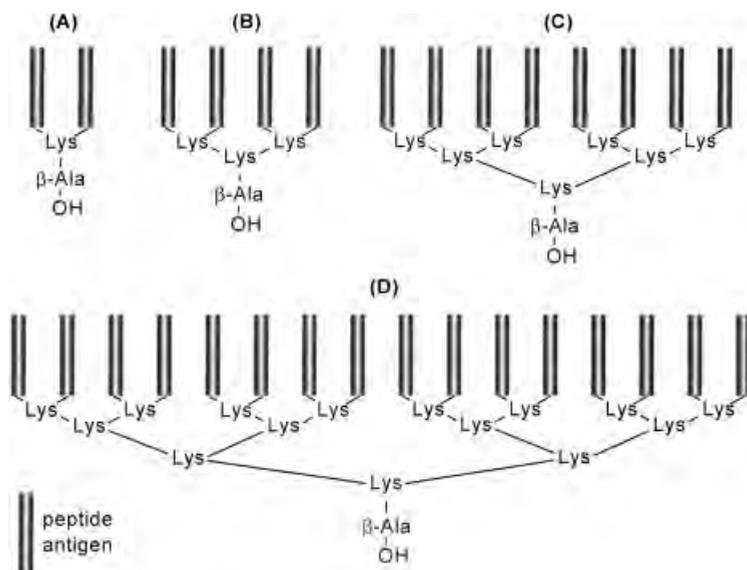


Fig. 7.13 Schematic structure of (A) di-, (B) tetra-, (C) octa-, and (D) hexadeca- valent multiple antigen peptides (MAP).

In the direct approach, multiple copies of the peptide antigen are synthesized by stepwise solid-phase peptide synthesis (SPPS) on a solid-phase-bound dendrimer core. Both Fmoc and Boc tactics have been successfully applied [208, 209]. Low resin loading is necessary in the synthesis of peptide dendrimers in order to minimize interchain interaction that may limit coupling efficiency [210]. The efficiency must be monitored, and when necessary repetitive couplings should be performed using different activating agents. Similarly, galactose has been used as a core moiety, where four identical peptide strands were assembled by solid-phase synthesis [211].

The indirect approach (Fig. 7.14) is characterized by the separate synthesis of a suitably functionalized core to where the full-length peptide is ligated in the final step. A classical fragment condensation in solution using fully protected peptides may be applied, but this often suffers from low yield, slow coupling reactions, poor solubility, and problems of racemization. On the other hand, the possibility of purifying the peptide segments before ligation excludes the occurrence of mismatched sequences. Alternately, unprotected or partially protected peptide epitopes, prepared by SPPS, may be conjugated to the dendrimer core in aqueous solution under mild conditions. In this approach, either thiol chemistry, hydrazone chemistry, or oxime chemistry [212] may be applied (for further information, see [205, 213]).

Using this approach, either lipidated or glycosylated peptide dendrimers may also be synthesized [205, 214, 215]. Cationic dendrimers may also be used for gene delivery because they form stable complexes with DNA. Polylysine dendri-

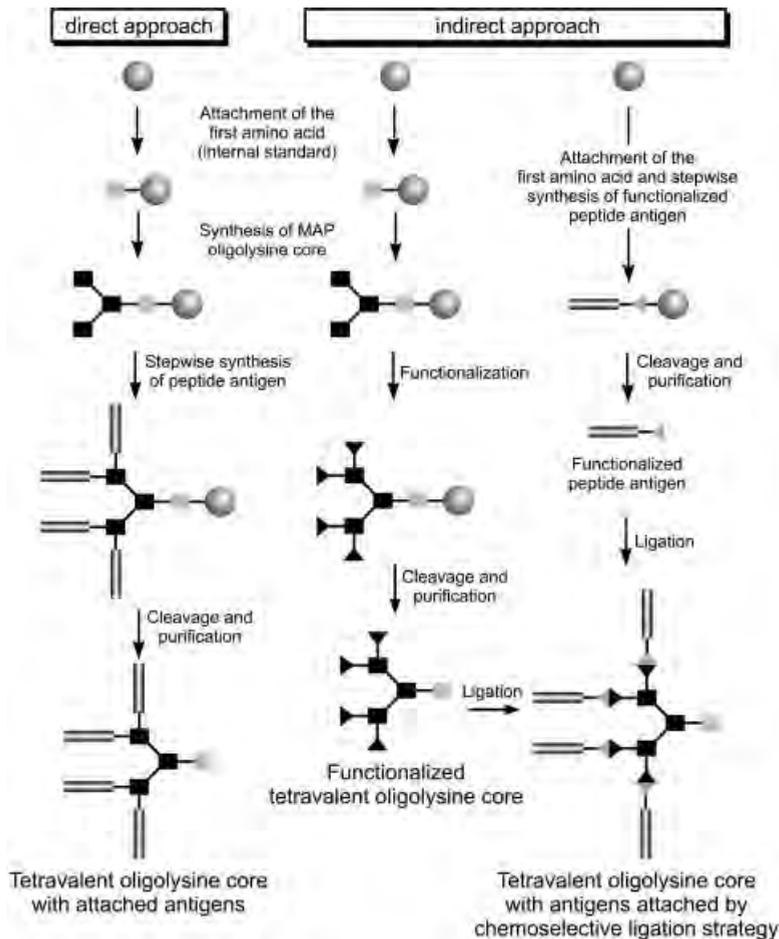


Fig. 7.14 Methods for the preparation of multiple antigen peptides (MAP).

mers containing a polyethylene glycol moiety have been reported to form a spherical water-soluble complex with DNA [216].

Peptide dendrimers containing a porphyrin core form another important group of functional dendrimers that may be used as either chemosensors or catalysts [217–219].

7.5.3

Peptide Polymers

As oligo- or polypeptides are polymers by definition, three other types of polymers will be discussed in the following section. The first of these comprises homopolymers composed of only one type of amino acid. In particular, polylysine or polyar-

ginine, both of which are present under physiological conditions as polycations, have a unique ability to cross the plasma membrane of cells [220]. Consequently, they may be used to transport a variety of biopolymers and small molecules into cells [221]. For example, polylysine interacts electrostatically with the negatively charged phosphate backbone of DNA and may, therefore, be used for gene transfer [222].

Polylysine is synthesized by polymerization of the N-carboxy-anhydride of lysine, and this is subsequently fractionated and characterized with respect to the average molecular weight [223]. The heterogeneity of commercially available polylysine in terms of degree of polymerization is a major obstacle in the preparation of reproducible, stable formulations however. Therefore, rationally designed synthetic peptides have been suggested as DNA delivery systems.

A second type of peptide-based polymer (also called protein-based polymers or sequential polypeptides) is formed by compounds composed of repeating peptide sequences. In contrast to other polymers, these can be prepared either by chemical synthesis (solution or solid phase) or alternately by recombinant technology.

Peptide-based polymers can be transformable hydrogels, elastomers, regular thermoplasts, or inverse thermoplasts that are postulated to be molecular machines [224].

The third type of peptide polymers comprises peptide drugs that are chemically conjugated to nanoscale polymer particles such as hydroxypropylmethacrylamide or polyethyleneglycol; this may be carried out using a formulation approach in order to enhance drug stability and targeting possibilities. It has been shown that a vinylacetate derivative of luteinizing hormone-releasing hormone (LHRH) could be co-polymerized with butylcyanoacrylate to form particles of a mean size of 100 nm that are stable *in vitro*. In this way, the average half-life of LHRH in blood could be increased from 2–8 min (peptide) to up to 12 h [225].

Co-polymers of peptides and polyethyleneglycol or polyacrylates have an intermediate position between the second and third types referred to above [226, 227].

7.6

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