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Marazza et al.

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[54] SEPARATION TECHNIQUE FOR A MIXTURE OF (6RS)-DIASTEREOISOMERS OF AN AMMONIUM SALT OF N⁵-METHYL-5,6,7,8-TETRAHYDROFOLIC ACID INTO SINGLE (6R) AND (6S)-DIASTEREOISOMERS

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[52] U.S. Cl. 544/258

[58] Field of Search 544/258

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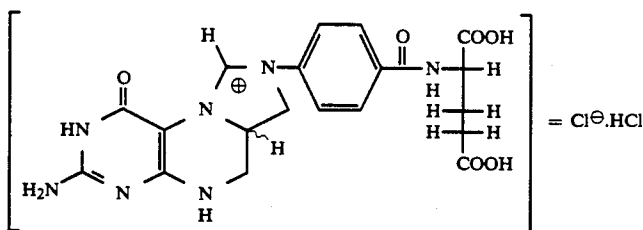
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[57] ABSTRACT

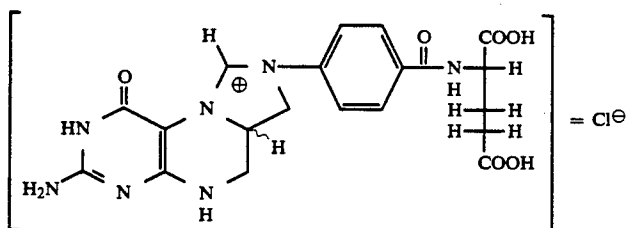
There are described ammonium salts of N⁵-methyl-5,6,7,8-tetrahydrofolic acid as well as processes for their preparation. These ammonium salts may be separated into the single (6R) and (6S)-diastereoisomers. The separated diastereoisomers may be transformed into the corresponding alkali metal or alkaline earth metal salts.

15 Claims, No Drawings



and of N⁵, N¹⁰-methenyl-THF.Cl⁻ of the formula

first inventive process. The other steps of the second



respectively, followed by the chemical transformation of the separated, single (6R) and (6S)-diastereoisomers into N⁵-methyl-THF. Despite the fact that these processes are practicable in an industrial scale, they have the drawback that they involve several chemical steps, which renders them economically unattractive.

SUMMARY OF THE INVENTION

Hence, it is a general object of the present invention to provide a simple, cheap and efficient process, by which a mixture of (6RS)-diastereoisomers of a N⁵-methyl-THF-derivative may be separated into the pure, single (6R) and (6S)-diastereoisomers.

Quite surprisingly it was found, that this object may be solved according to this invention by means of a fractional crystallization of novel ammonium salts of a mixture of (6RS)-diastereoisomers of N⁵-methyl-THF.

In addition to the inventive separation process and the inventive ammonium salts, two inventive processes for the preparation of the ammonium salts are also provided. Still a further inventive process for the preparation of alkali metal or alkaline earth metal salts of (6R) or (6S) N⁵-methyl-THF from a corresponding inventive diastereoisomer pure ammonium salt is described.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the first inventive process for the preparation of the inventive ammonium salts, N⁵-methyl-THF, preferably in the form of the calcium salt, is placed into water. A nitrogen containing base, which then is added, may be a primary or secondary alkyl or aryl amine. Practically, the calcium cation is precipitated by the addition of an oxalic acid as calcium oxalate, which is then filtered off. The aqueous filtrate contains the dissolved ammonium salt. After the evaporation of the water, either under reduced pressure or after lyophilization, the ammonium salt is obtained.

In the second inventive process for the preparation of the inventive ammonium salts, N⁵-methyl-THF, in the form of a carboxylic acid, is preferably placed into water or into a mixture of water and ethanol and/or methanol. In this process the steps of the precipitation of the alkali metal or alkaline earth metal cation and its separation are the same, as has been described in the

inventive process correspond with the corresponding steps of the first inventive process.

The inventive ammonium salts may also be prepared by ion exchange on a corresponding column, on which, for example, a calcium cation is exchanged against the desired ammonium ion.

The inventive ammonium salts, for example prepared according to one of the above described processes, are then subjected to a fractional crystallization, wherein the mixture of the (6RS)-diastereoisomers is separated into the single, pure (6R) and (6S)-diastereoisomers. Thereby preferably one part by weight of an inventive ammonium salt is mixed with 5 parts by weight, referred to the above mentioned part per weight of ammonium salt, of a mixture of 85 vol.-% ethanol and 15 vol.-% water. Depending on the nature of the ammonium salt, heating to a temperature from 50° C. to 60° C. may be necessary in order to obtain a clear solution.

From this clear solution one diastereoisomer is allowed to crystallize, and is then filtered off. The crystallization may be realized at ice water temperature or in a refrigerator or at a temperature from 10° C. to 25° C. If necessary the clear solution may also be treated with seed crystals and then occasionally treated with ultrasonics. The obtained mother liquor is then diluted, preferably with ethanol having a low water content. From this diluted solution the other diastereoisomer is allowed to crystallize, and then also filtered off. This second mother liquor may be evaporated and may be subjected to a new fractional crystallization.

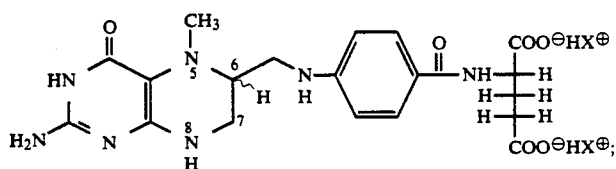
Which diastereoisomer crystallizes first depends on the nature of the ammonium salt to be separated. In the case of the N⁵-methyl-THF-cyclohexyl ammonium salt the (6R)-salt crystallizes first. The (6S)-salt is crystallized from the other liquor according to the above techniques. In the case of the N⁵-methyl-THF-diisopropyl ammonium salt, the (6S)-salt crystallizes first. The (6R)-salt is then crystallized from the mother liquor.

The so separated diastereoisomer pure ammonium salts of N⁵-methyl-THF may be transformed into the alkali metal or alkaline earth metal salts, preferably into the calcium salt or into the magnesium salt. For this a corresponding ammonium salt is dissolved preferably in

one part of volume of water. Then an aqueous solution of the desired alkali metal or alkaline earth metal salt is added, for example calcium chloride. In this mixture the pH-value may be adjusted to a value from 6.0 to 7.0. This solution is then cooled to a temperature from 0° C. to 10° C., and is stirred preferably for about one hour. Then 1.5 to 2.5 parts by volume, referred to the above mentioned part by volume of water, of, for example, ethanol or acetone, are added preferably under stirring during 30 to 90 minutes. Thereby the corresponding alkali metal or alkaline earth metal salt of the N⁵-methyl-THF precipitates and may be filtered off.

The analysis of an isomeric composition may be carried out in a known way on a chiral HPLC column. See, for example, K. E. Choi and R. L. Schilsky, "Resolution of the Stereoisomers of Leucovorin and N⁵-methyl-THF by Chiral HPLC." *Anal. Biochem.* (1988) 168, 398.

Looking now more specifically to the preferred embodiments, one aspect of the invention is ammonium salts of N⁵-methyl-5,6,7,8-tetrahydrofolic acid of the formula (I)



in the form of the mixture of the (6R)-disastereoisomers, or in the form of the single (6R)-or (6S)-disastereoisomers. In the above formula X is a nitrogen containing base, preferably selected from primary, secondary and tertiary amines and basic amino carboxylic acids, especially selected from the group consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine, especially (L)-arginine.

Another aspect of the invention is a process for the preparation of ammonium salts of (6R)-N⁵-methyl-5,6,7,8-tetrahydrofolic acid of formula (I), where X is a nitrogen containing base, preferably selected from primary, secondary and tertiary amines and basic amino carboxylic acids, especially selected from the group consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl dimethyl amino-ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine, especially (L)-arginine. According to the process, (6R)-N⁵-methyl-5,6,7,8-tetrahydrofolic acid, in the form of an alkali metal or alkaline earth metal salt, especially a Ca²⁺ or Mg²⁺ salt, is placed into a solvent, preferably water. Then, a nitrogen containing base is added, preferably in an amount from 2 to 2.5 moleequivalents. The base is preferably selected of from primary, secondary and tertiary amines and basic amino carboxylic acids, especially those selected from the group, consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine, especially (L)-arginine. Then the process involves transforming to a substantially insoluble salt or precipitate of the above mentioned salt of the alkali metal or alkaline earth metal cation by the addition of a suitable acid such as sulfuric acid or oxalic acid. The substantially insoluble salt is then separated by means of filtration, followed by removing the sol-

vent, for example, by means of evaporation under reduced pressure or by means of lyophilization.

Another embodiment of the invention is another process for the preparation of ammonium salts of (6R)-N⁵-methyl-5,6,7,8-tetrahydrofolic acid of formula (I) where X is a nitrogen containing base, preferably selected from primary, secondary and tertiary amines and basic amino carboxylic acids, especially selected from the group consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine. According to the process, (6R)-N⁵-methyl-5,6,7,8-tetrahydrofolic acid is placed into at least one solvent, preferably water. Then, a nitrogen containing base, preferably in an amount from 2 to 2.5 moleequivalents, is added. The base is preferably selected from primary, secondary and tertiary amines as well as basic amino carboxylic acids, especially selected from the group consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-

ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine, especially (L)-arginine. Then, the solvent(s) is (are) removed by, for example, means of evaporation under reduced pressure by means of lyophilization.

Even another embodiment relates to a process for the separation of the mixture of (6R)-disastereoisomers of an ammonium salt of N⁵-methyl-5,6,7,8-tetrahydrofolic acid of formula (I) into the single (6R) and (6S)-disastereoisomers where X is a nitrogen containing base, preferably selected from primary, secondary and tertiary amines as well as basic amino carboxylic acids, especially selected from the group, consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine, especially (L)-arginine. According to the process, a mixture of (6R)-disastereoisomers, especially in solid form, is mixed with a mixture A of solvents, containing water and at least one water miscible solvent, preferably a polar solvent, especially monovalent of polyhydric alcohols with 1 to 5 carbon atoms (e.g., ethanol), and then transforming the prepared mixture into a clear solution, for example, by means of heating to a temperature from 40° C. to 60° C. Then, one diastereoisomer is allowed to crystallize and is separated.

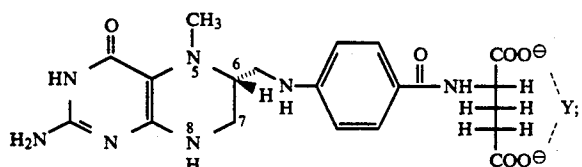
Thereafter, the mother liquor is diluted with the above mentioned water miscible solvent(s) and the other diastereoisomer is allowed to crystallize and is separated from the diluted solution.

The aforementioned mixture A of solvents preferably contains from 50 to 95 vol.-%, preferably from 75 to 95 vol.-%, especially 85 vol.-% of ethanol, and, as residue water. It is most preferred that from 3 to 10 parts by volume, especially about 5 parts by volume, of mixture

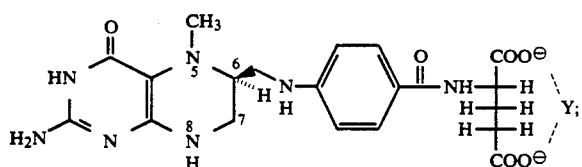
A of solvents are used per 1 part by weight of the mixture of (6RS)-diastereoisomers.

According to the aforementioned process, it is also preferred that the clear solution be treated with seed crystals, and optionally, but also preferred that the solution treated with seed crystals be treated with ultrasonics.

Another embodiment of the invention relates to a process for the preparation of alkali metal or alkaline earth metal salts of (6R) or (6S)-N⁵-methyl-5,6,7,8-tetrahydrofolic acid of the formulae (IIa) or (IIb):



(IIa)

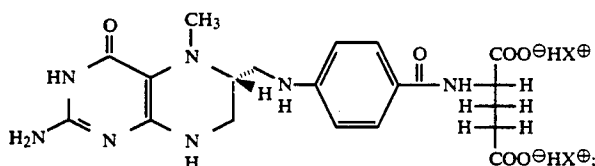


(IIb)

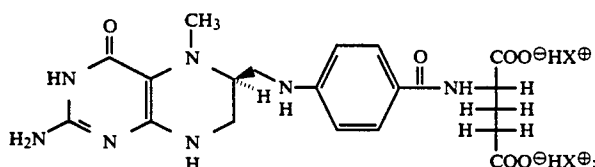
wherein Y is defined as two alkali metal cations, or as one alkaline metal cation, especially Ca²⁺ or Mg²⁺. According to the process, the corresponding diastereoisomer pure ammonium salt of N⁵-methyl-5,6,7,8-tetrahydrofolic acid of the formulae (Ia) or (Ib)

Mixture B is adjusted to a value in the range from 6.0 to 7.0.

In another aspect of the invention there is provided a medicament for the treatment and/or the control of human and/or animal tumors and/or for the synergistic



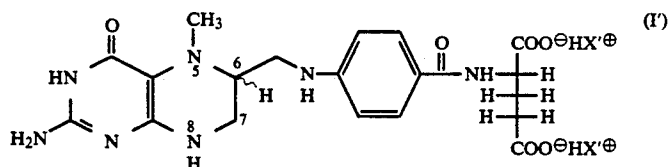
(Ia)



(Ib)

where X is a nitrogen containing base, preferably selected from primary, secondary and tertiary amines and basic amino acids, especially selected from the group consisting of cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-ethanol, tert.-butyl amine, lysine, especially (L)-lysine, and arginine, especially (L)-argi-

exertion of influence of a cancer controlling compound and/or for the reduction of the toxicity of a cancer controlling compound and/or for the protection of human and/or animal cells. The medicament is characterized in that it contains as at least one active compound of at least one ammonium salt of N⁵-methyl-5,6,7,8-tetrahydrofolic acid of the formula (I'):



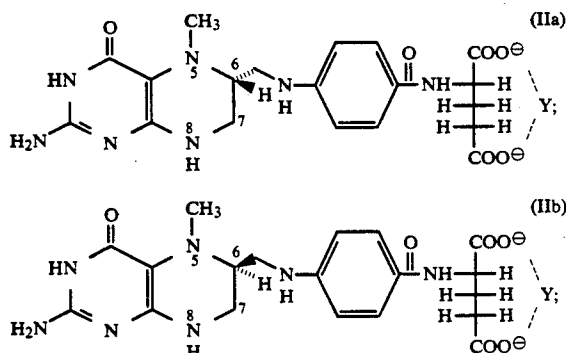
(I')

nine, is optionally, but preferably prepared according to the aforementioned process.

The salt is then dissolved in one part by volume of water, and then, an aqueous solution of an alkali metal or alkaline earth metal salt is added, preferably a corre-

in the form of the mixture of the (6RS)-diastereoisomers or in the form of the single (6R)-or (6S)-diastereoisomers, wherein X' is a pharmacological acceptable, nitrogen containing base, preferably selected from primary, secondary and tertiary amines, and basic amino

carboxylic acids, especially selected from the group consisting of ethanolamine, triethanolamine, 2-dimethylamino-ethanol, tert.-butylamine, lysine, especially (L)-lysine, and arginine, especially (L)-arginine; and/or at least one alkali metal and/or alkaline earth metal salt of (6R) or (6S) N⁵-methyl-5,6,7,8-tetrahydrofolic acid of the formulae (IIa) or (IIb)



wherein Y is defined as two alkali metal cations or as one alkaline earth metal cation, especially Ca²⁺ or Mg²⁺. They are preferably present in an amount from 1 mg to 500 mg, especially from 5 mg to 150 mg, per dosis unit, and the medicament is preferably in the form of a parenteral and/or oral pharmaceutical preparation.

Another aspect of the invention relates to the use of the ammonium salts of N⁵-methyl-5,6,7,8-tetrahydrofolic acid of formula (I'), and/or of the alkali metal and/or alkaline earth metal salts of (6R) or (6S) N⁵-methyl-5,6,7,8-tetrahydrofolic acid of formulae (IIa) or (IIb) in a single form or in any combination for the preparation of a medicament for the treatment and/or the control of human and/or animal tumors and/or for the synergistic exertion of influence of a cancer controlling compound and/or for the reduction of the toxicity of a cancer controlling compound and/or the protection of human and/or animal cells. The salts are preferably present in the medicament in an amount from 1 mg to 500 mg, especially from 5 mg to 150 mg, per dosis unit, and the medicament is preferably in the form of a parenteral and/or oral, pharmaceutical preparation.

The following examples illustrate the present invention.

EXAMPLE 1

Preparation of (6RS)-N⁵-methyl-THF-cyclohexyl ammonium salt

To a suspension of 100 g of (6RS)-N⁵-methyl-THF-calcium salt in 800 ml of water, which was stirred at a temperature of 40° C. under a nitrogen atmosphere, were added to 40 g of cyclohexylamine. The temperature was raised to 60° C., and a clear solution was obtained. Then 24 g of oxalic acid dihydrate in 200 ml of water were added, and this mixture was stirred at a temperature of 0° C. during 30 minutes. After the removal of the formed calcium oxalate by means of centrifugation the supernatant solution was evaporated at a temperature of 50° C. under reduced pressure. There was obtained crude (6RS)-N⁵-methyl-THF-cyclohexyl ammonium salt in the form of a honey-like or glass-like solid. The HPLC and analysis of this produce showed one single peak.

EXAMPLE 2

Separation of the mixture of (6RS)-diastereoisomers of N⁵-methyl-THF-cyclohexyl ammonium salt

A: (6R)-N⁵-Methyl-THF-cyclohexyl ammonium salt

The crude (6RS)-N⁵-methyl-THF-cyclohexyl ammonium salt, prepared according to example 1, was dissolved in 550 ml of a mixture of 85 parts by volume of ethanol and 15 parts by volume of water at a temperature of 60° C. The formed clear solution was cooled to room temperature and treated with seed crystals of crystalline (6R)-N⁵-methyl-THF-cyclohexyl ammonium salt, obtained from a previous batch. After stirring at room temperature over night and at a temperature of 10° C. during 4 hours the obtained crystalline solid was isolated by means of filtration. This solid was washed once with 85% ethanol and dried under reduced pressure at a temperature of 50° C. during 2 hours. There were obtained 30.9 g of (6R)-N⁵-methyl-THF-cyclohexyl ammonium salt.

$$[\alpha]_D = 7.3 + (c = 1 \text{ in } 0.01 \text{ N NaOH})$$

The analysis on a RESOLVOSIL-column showed a ration of diastereoisomers of (6R)/(6S)=96.5/3.5 (see K. E. Choi and R. L. Schilsky, "Resolution of The Stereoisomers of Leucovorin and N⁵-methyl-THF by Chiral HPLC." *Anal Biochem.* (1988) 168, 398).

The HPLC analysis on a "reverse phase"-column showed that this produce was 100% pure.

IR (KBr/cm⁻¹): 3300, 2950, 2860, 1660, 1610, 1390.

elemental analysis	calculated (%) (Dihydrate)	found (%)
C	55.42	55.72
H	7.99	7.86
N	18.46	18.32

B: (6S)-N⁵-Methyl-THF-cyclohexyl ammonium salt

The mother liquor of the filtration described in item A was diluted with 1200 ml absolute ethanol. The obtained solution was treated with authentic seed crystals of (6S)-N⁵-methyl-THF-cyclohexyl ammonium salt.

The temperature was allowed to stand during 24 hours at room temperature. The obtained crystalline solid was isolated by means of filtration. This solid was washed once with 94% ethanol and dried under reduced pressure at a temperature of 50° C. There were obtained 23.7 g of (6S)-N⁵-methyl-THF-cyclohexyl ammonium salt.

$$[\alpha]_D = +30.6^{\circ} (c = 1 \text{ in } 0.01 \text{ N NaOH})$$

The analysis of a RESOLVOSIL-column showed a ration of diastereoisomers of (6R)/(6S)=3/97 (see K. E. Choe and R. L. Schilsky, "Resolution of The Stereoisomers of Leucovorin and N⁵-methyl-THF by Chiral HPLC." *Anal. Biochem.* (1988) 168, 398.).

The HPLC analysis on a "reverse phase"-column showed that this product was 100% pure.

elemental analysis	calculated (%) (Dihydrate)	found (%)
C	55.42	55.72

-continued

elemental analysis	calculated (%) (Dihydrate)	found (%)
H	7.99	7.86
N	18.46	18.32

EXAMPLE 3

Preparation of (6S)-N⁵-methyl-THF-calcium salt

15.0 g of (6S)-N⁵-Methyl-cyclohexyl ammonium salt were dissolved in 120 ml of water and stirred at room temperature under a nitrogen atmosphere. After the addition of 9.9 ml of 2M calcium chloride solution the pH-value was adjusted to a value of 6.8 by the addition of 1 N NaOH. The obtained mixture was stirred at a temperature of 0° C. during 1 hour. Then were added 240 ml of ethanol during 40 minutes. The obtained crystalline solid was isolated by means of filtration. This solid was washed with 85% ethanol and dried under reduced pressure at a temperature of 50° C. There were obtained 10.4 g of (6S)-N⁵-methyl-THF-calcium salt.

The HPLC analysis on a "reverse phase"-column showed that this product was 100% pure.

$$[\alpha]_D = +40^\circ \quad (c = 1/1\% \text{NH}_4\text{OAc, pH 6.0})$$

$$\text{UV (20 mg/l in 1\% NH}_4\text{OAc, pH 6.0):}$$

$$\lambda_{\text{max}} = 290 \text{ nm } (\epsilon = 28600)$$

$$\lambda_{\text{min}} = 245 \text{ nm}$$

$$A_{\text{max}}/A_{\text{min}} = 3.7$$

The analysis on a RESOLVOSIL-column showed a ratio of diastereoisomers of (6R)/(6S)=3/97 (see K. E. Choe and R. L. Schilsky, "Resolution of The Stereoisomers of Leucovorin and N⁵-methyl-THF by Chiral HPLC." *Anal. Biochem.* (1988) 168, 398. reference).

EXAMPLE 4

Preparation of (6R)-N⁵-methyl-THF-calcium salt

Starting from (6R)-N⁵-methyl-THF-cyclohexyl ammonium salt the title product was prepared in an analogous way as described in example 3.

The HPLC analysis on a "reverse phase"-column showed that the product was 100% pure.

$$[\alpha]_D = 4.70 \quad (c = 1/1\% \text{NH}_4\text{OAc, pH 6.0})$$

$$\text{UV (20 mg/l in 1\% NH}_4\text{OAc, pH 6.0):}$$

$$\lambda_{\text{max}} = 290 \text{ nm } (\epsilon = 30200)$$

$$\lambda_{\text{min}} = 245 \text{ nm}$$

$$A_{\text{max}}/A_{\text{min}} = 3.6$$

The analysis on a RESOLVOSIL-column showed a ratio of diastereoisomers of (6R)/(6S)=97/3 (see K. E. Choe and R. L. Schilsky, "Resolution of The Stereoisomers of Leucovorin and N⁵-methyl-THF by Chiral HPLC." *Anal. Biochem.* (1988) 168, 398.).

EXAMPLE 5

Preparation of (6RS)-N⁵-methyl-THF-diisopropyl ammonium salt

To a suspension of 5.0 g of (6RS)-N⁵-methyl-THF-calcium salt in 40 ml of water, which was stirred at a temperature of 40° C. under a nitrogen atmosphere, were added 2.03 g of diisopropylamine. The temperature was increased to about 55° C., and a clear solution was obtained. After the cooling to room temperature a solution of 1.2 g of oxalic acid dihydrate in 10 ml of water was added, and this mixture was stirred at a temperature of 0° C. during 30 minutes. After the removal

of the formed calcium oxalate by means of centrifugation the supernatant solution was evaporated at a temperature of 70° C. under reduced pressure. There was obtained crude (6RS)-N⁵-methyl-THF-diisopropyl ammonium salt in the form of a honey-like or glass-like solid. The HPLC analysis of this product showed a single peak.

EXAMPLE 6

Separation of the mixture of (6RS)-diastereoisomers of N⁵-methyl-THF-diisopropyl ammonium salt; isolation of (6S)-N⁵-methyl-THF-diisopropyl ammonium salt

The crude (6RS)-N⁵-methyl-THF-diisopropyl ammonium salt, prepared according to example 5, was dissolved in 27 ml of a mixture of 85 parts by volume of ethanol and 15 parts by volume of water at a temperature of 60° C. The formed clear solution was cooled to room temperature and stirred at this temperature during 48 hours. The obtained solid was isolated by means of centrifugation. This solid was washed once with 85% ethanol and dried under reduced pressure at a temperature of 50° C. during 2 hours. There were obtained 1.07 g of (6S)-N⁵-methyl-THF-diisopropyl ammonium salt.

The analysis on a RESOLVOSIL-column showed a ratio of diastereoisomers of (6R)/(6S)=90/10 (see K. E. Choe and R. L. Schilsky, "Resolution of The Stereoisomers of Leucovorin and N⁵-methyl-THF by Chiral HPLC." *Anal. Biochem.* (1988) 168, 398.).

EXAMPLE 7

Preparation of (6RS)-N⁵-methyl-THF.2-hydroxyethyl ammonium salt

To a stirred suspension of 15 g of (6RS)-N⁵-methyl-THF in 100 ml of 85% ethanol were added drop by drop 3.9 ml of ethanolamine, whereby an oily suspension was obtained.

This suspension was stirred vigorously during 3 hours at a temperature of 0° C., and the formed crystalline solid was occasionally reduced to small pieces with a spatula.

Then the product was isolated by filtration, washed with absolute ethanol and dried under reduced pressure at a temperature of 50° C. during 90 minutes. There were obtained 16.0 g of (6RS)-N⁵-methyl-THF.2-hydroxy-ethyl ammonium salt.

The HPLC analysis on a "reverse phase"-column showed that this product was 96,5% pure.

$$\text{UV (20 mg/l in 1\% NH}_4\text{OAc, pH 6.0):}$$

$$\lambda_{\text{max}} = 290 \text{ nm}$$

$$\lambda_{\text{min}} = 245 \text{ nm}$$

$$A_{\text{max}}/A_{\text{min}} = 3.55$$

$$\text{IR (kBr/cm}^{-1}\text{): 3360, 2940, 2860, 1610, 1390, 1330.}$$

EXAMPLE 8

Acute intravenous toxicity studies in mice

Compound	LD ₅₀ (mg/kg)
(6RS)-N ⁵ -Methyl-THF.Ca ²⁺	339
(6S)-N ⁵ -Methyl-THF.Ca ²⁺	847
(6R)-N ⁵ -Methyl-THF.Ca ²⁺	460

EXAMPLE 9

Inhibition of the growth of human CCRF-CEM cells

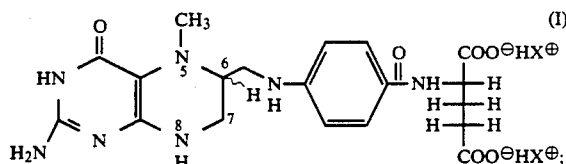
Human leukemia cells (CCRF-CEM) were grown during 72 hours in the presence of increasing concentration of (6R)-N⁵-methyl-THF.Ca³⁺. The following Table shows the percentual cellular growth related to the control experiment.

Concentration of (6R)-N ⁵ -methyl-THF.Ca ²⁺ [μ M]	% growth (72 hrs exp.)
0 (control)	100
1	95
10	99
30	10
100	9.3
250	8.2

While there are shown and described present preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto, but may be otherwise variously embodied and practiced within the scope of the following claims.

We claim:

1. A process for the separation of mixture of (6RS)-diastereoisomers of an ammonium salt of N⁵-methyl-5,6,7,8-tetrahydrofolic acid of the formula (I):



wherein X is a nitrogen containing base selected from primary, secondary and tertiary amines, ammonia and basic amino carboxylic acids,

into single (6R) and (6S)-diastereoisomers,

wherein said process comprises forming a mixture of said mixture of (6RS)-diastereoisomers with a mixture of solvents, the solvent mixture containing water and at least one totally water miscible solvent,

transforming the so prepared mixture into a clear solution,

allowing one diastereoisomer to crystallize and separating the crystallized diastereoisomer from the clear solution,

diluting the mother liquid with the with totally water miscible solvent(s), and allowing the other diastereoisomer to crystallize and separating it from the diluted solution.

2. A process according to claim 1, wherein the mixture of solvents contains water and from 50 to 95 vol.-% ethanol.

3. A process according to claim 1, wherein from 3 to 10 parts by volume of the mixture of solvents per 1 part by weight of the mixture of (6RS)-diastereoisomers are used.

4. A process according to claim 1, wherein the clear solution is treated with crystals.

5. A process according to claim 1, wherein each X is a cyclohexyl amine, diisopropyl amine, benzyl amine, ammonia, ethanol amine, triethanol amine, 2-dimethyl amino-ethanol, tert-butyl amine, lysine, or arginine.

6. A process according to claim 5, wherein said lysine is (L)-lysine and said arginine is (L)-arginine.

7. A process according to claim 1, wherein said mixture of (6RS)-diastereoisomers is in a solid form.

8. A process according to claim 1, wherein the mixture of solvents contains a polar solvent.

9. A process according to claim 8, wherein said solvent is a monovalent or polyhydric solvent with from 1 to 5 carbon atoms.

10. A process according to claim 9, wherein said solvent is ethanol.

11. A process according to claim 1, wherein the mixture is transformed to a clear solution by heating to a temperature of from 40° C. to 60° C.

12. A process according to claim 2, wherein said mixture of solvents contains water and from 75 to 95 vol. % ethanol.

13. A process according to claim 12, said mixture containing about 85 vol. % ethanol.

14. A process according to claim 3, wherein about 5 parts by volume of the mixture of solvents are used.

15. A process according to claim 4, wherein the clear solution treated with seed crystals is further treated with ultrasonics.

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