

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.

Petitioner,

v.

**THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK**

Patent Owner.

Case No. IPR2018-00322

U.S. Patent No. 9,708,358

**DECLARATION OF FLOYD ROMESBERG, PH.D.,
IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF
U.S. PATENT NO. 9,708,358**

(CYTOSINE ANALOGUE)

Illumina Ex. 1067 IPR Petition - USP 9,708,358
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1. I, Floyd Romesberg, Ph.D., have been retained by Knobbe, Martens, Olson & Bear, LLP, counsel for Illumina, Inc. (“Illumina”). I understand that Illumina is petitioning for inter partes review of U.S. Patent No. 9,708,358 (“the ’358 patent,” Ex. 1002) and requests that the United States Patent and Trademark Office cancel Claim 1 of the ’358 patent as unpatentable. The following discussion and analysis provides my opinion as to why Claim 1 would have been obvious to a person of ordinary skill in the art in October of 2000.

**I. BACKGROUND AND QUALIFICATIONS, AND
PREVIOUS TESTIMONY**

A. Background and Qualifications

2. I am currently a professor in the Department of Chemistry at The Scripps Research Institute. I have been a faculty member at The Scripps Research Institute since 1998.

3. I earned a Bachelor of Science in Chemistry from the Ohio State University in 1988.

4. I earned a Master of Science in Chemistry in 1990 and a Doctor of Philosophy in Chemistry in 1994 from Cornell University, where Professor David B. Collum served as my thesis advisor.

5. From 1994 until 1998, I was an NIH postdoctoral research fellow at the University of California, Berkeley, where I studied under Professor Peter G. Schultz.

6. As a principal investigator, I have authored over 120 publications in peer-reviewed journals, including Nature, The Proceedings of the National Academy of Sciences of the United States of America, The Journal of the American Chemical Society, Angewandte Chemie International Edition, Biochemistry, Nucleic Acids Research, and The Journal of Physical Chemistry.

7. I have authored or co-authored at least 20 invited review articles. In addition, I have authored or co-authored at least 17 other publications during my graduate and post-doctoral studies.

8. Over 30 of the publications that I have authored as a principle investigator are related to nucleotide analogues, including nucleotide analogues bearing linkers for attachment of functionalities of interest to polynucleotides.

9. I currently teach several graduate courses in the Department of Chemistry at The Scripps Research Institute, including a Spectroscopy course and a course on Bacteria and Antibiotics.

10. I have mentored numerous graduate students, post-doctoral researchers, interns, and research associates at The Scripps Research Institute.

11. I have been an invited lecturer at multiple universities, symposiums, and conferences throughout the United States and abroad.

12. I have reviewed manuscripts as part of the peer-review process to determine whether they are acceptable for publication for numerous journals,

including the Proceedings of the National Academy of Sciences, Science, Nature, the Journal of the American Chemical Society, Angewandte Chemie, Biochemistry, the Journal of Organic Chemistry, Bioorganic and Medicinal Chemistry Letters, Chemistry & Biology, Nucleic Acids Research, and Nucleosides, Nucleotides, and Nucleic Acids.

13. I am a member of the American Chemical Society and the American Society for Microbiology.

14. I served as a permanent member of the NIH Synthetic and Biological Chemistry (SBCA) study section that handles a significant percentage of the grants dealing with modified nucleotides for four years. Also, I regularly serve on various NSF study sections, and my service on these study sections involves reviewing and determining the merits of numerous grant proposals.

15. I have been awarded numerous research grants from the National Institutes of Health, the National Science Foundation, the Office of Naval Research, Defense Advanced Research Projects Agency, and several other sources, including federal funding for the synthesis and analysis of nucleotide analogues and for the development of DNA polymerases specifically for sequencing DNA.

16. I have been the recipient of multiple awards and honors, including the ACS Nobel Laureate Signature Award for Graduate Education in Chemistry in 2015, Discover Magazine Technology Innovation Award in 2004, the NSF Career

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Award in 2004, the Susan B. Komen Breast Cancer Foundation Award in 2003, the Camille Dreyfus Teacher-Scholar Award in 2003, the Baxter Foundation Fellow Award in 2002, the Mac Nevin Award in 1987, and election to the Defense Science Study Group (DSSG) panel from 2008-2010.

17. I am qualified to render an opinion in the field of nucleotide analogues, DNA polymerases, and DNA sequencing techniques based on my experience in this field. Based on my expertise and qualifications, I am qualified to provide an opinion as to what a person of ordinary skill in the art would have understood, known, or concluded as of 2000. I have been doing research in this field since 1999.

18. Attached as Exhibit 1057 is a copy of my curriculum vitae setting forth my educational experience, employment history, and publications.

B. Previous Testimony

19. I previously testified as an expert witness in:

- *Intelligent Bio-Systems, Inc. v. Illumina Cambridge Ltd.*, IPR2013-00128, regarding U.S. 7,057,026;
- *Intelligent Bio-Systems, Inc. v. Illumina Cambridge Ltd.*, IPR2013-00266, regarding U.S. 8,158,346;
- *Intelligent Bio-Systems, Inc. v. Illumina Cambridge Ltd.*, IPR2013-00517, regarding U.S. 7,566,537;

- *Pacific Oxford Nanopore v. Pacific Biosciences*, ITC-337-TA-1032;
and
- *Illumina, Inc. v. Oxford Nanopore Technologies Ltd.*, ITC-337-TA-0991.

II. MATERIALS CONSIDERED

20. Attached as Exhibit 1058 is a listing of documents that I have considered and reviewed in connection with providing this declaration.

III. LEGAL STANDARD OF NONOBVIOUSNESS

21. I am not a patent attorney nor have I independently researched the law on patentability. I understand that 35 U.S.C. § 103 governs the determination of obviousness. I understand that 35 U.S.C. § 103 states:

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person of ordinary skill in the art to which the subject matter pertains.

22. I further understand that the four factors to be considered in an obviousness inquiry are: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims; (3) the level of ordinary skill in

the pertinent art; and (4) secondary considerations including long-felt need, commercial success, and unexpected results.

23. I also understand that when there is some recognized reason to solve a problem, and there are a finite number of identified, predictable solutions, a person of ordinary skill in the art has good reason to pursue the known options within his or her technical grasp. If such an approach leads to the anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In such a circumstance, when a patent simply arranges old elements with each performing the same function it had been known to perform and yields no more than one would expect from such an arrangement, the combination is obvious.

IV. THE PERSON OF ORDINARY SKILL IN THE ART

24. I understand that obviousness is analyzed from the perspective of a hypothetical person of ordinary skill in the art. I understand that October 2000 is the relevant time frame for analyzing the obviousness of the '358 patent. A person of ordinary skill in the art related to the '358 patent would have been a member of a team of scientists developing nucleotide analogues, researching DNA polymerases, and/or addressing DNA sequencing techniques. Such a person would have held a doctoral degree in chemistry, molecular biology, or a closely related discipline, and had at least five years of practical academic or industrial laboratory experience. Thus, a person of ordinary skill in the art includes a person having a

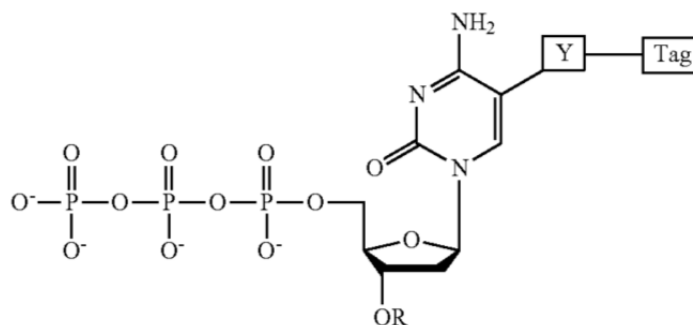
doctoral degree in a field related to chemistry, and at least five years of laboratory experience directed toward the research and development of nucleotide analogues, DNA polymerases, and/or DNA sequencing. My opinions concerning the obviousness of the '358 patent claims, as set forth herein, are from the perspective of a person of ordinary skill in the art, as set forth above.

V. THE '358 PATENT

25. The '358 patent (Ex. 1002) is directed to a “massive parallel method for decoding DNA and RNA.” Ex. 1002 at Title.

26. The '358 patent has one claim, Claim 1:

Claim 1. A cytosine deoxyribonucleotide analogue having the structure:



wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue, (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase, (c) is stable during a DNA polymerase reaction, and (d) does not contain a ketone group;

wherein OR is not a methoxy group or an ester group;

wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction;

wherein tag represents a detectable fluorescent moiety;

wherein Y represents a chemically cleavable, chemical linker which (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase and (b) is stable during a DNA polymerase reaction; and

wherein the cytosine deoxyribonucleotide analogue: i) is recognized as a substrate by a DNA polymerase, ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction, iii) produces a 3'-OH group on the deoxyribose upon cleavage of R, iv) no longer includes a tag on the base upon cleavage of Y, and v) is capable of forming hydrogen bonds with guanine or a guanine nucleotide analogue.

27. In Claim 1, there are six limitations relating to the 3'-O-R group:

- wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue,
- wherein R (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase,
- wherein R (c) is stable during a DNA polymerase reaction,
- wherein R (d) does not contain a ketone group;
- wherein OR is not a methoxy group or an ester group;

- wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction.

28. There is one limitation for “tag”:

- wherein tag represents a detectable fluorescent moiety.

29. There are three limitations for Y:

- wherein Y represents a chemically cleavable, chemical linker;
- wherein Y (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase;
- wherein Y (b) is stable during a DNA polymerase reaction.

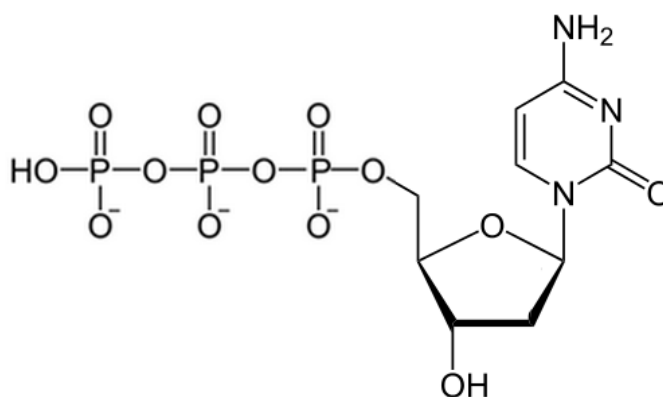
30. There are five limitations for the cytosine deoxyribonucleotide analogue as a whole:

- i) is recognized as a substrate by a DNA polymerase,
- ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction,
- iii) produces a 3'-OH group on the deoxyribose upon cleavage of R,
- iv) no longer includes a tag on the base upon cleavage of Y, and
- v) is capable of forming hydrogen bonds with guanine or a guanine nucleotide analogue.

VI. TECHNICAL BACKGROUND AND STATE OF THE ART

A. Deoxyribonucleotides and DNA Were Known in the Art

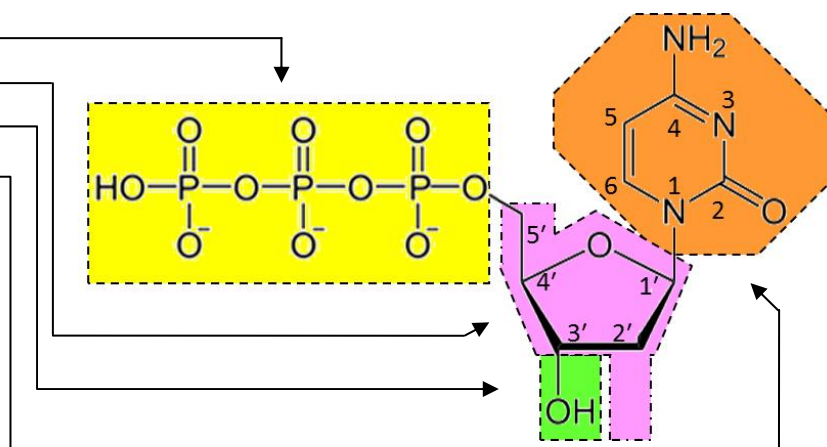
31. Deoxyribonucleotides are found in nature and make up the building blocks of DNA. The chemical formula, nomenclature, and uses of deoxyribonucleotides were firmly established throughout the life sciences and taught in undergraduate textbooks long before October 2000. Ex-1011 (Alberts) at 46-47, 58-60, 98-103. For example, the arrangement of atoms comprising the naturally occurring cytidine deoxyribonucleoside triphosphate were known to be:



Cytidine deoxyribonucleoside triphosphate was commonly referred to by its simple acronym: “dCTP.” This nucleotide has several well-known features: a triphosphate, a deoxyribose (which includes a 3'-OH), and a cytosine base. These features are highlighted in the following diagram, along with the conventional numbering of deoxyribose carbon atoms from 1' to 5' and the numbering of cytosine atoms from 1 to 6:

Well-known features of dCTP

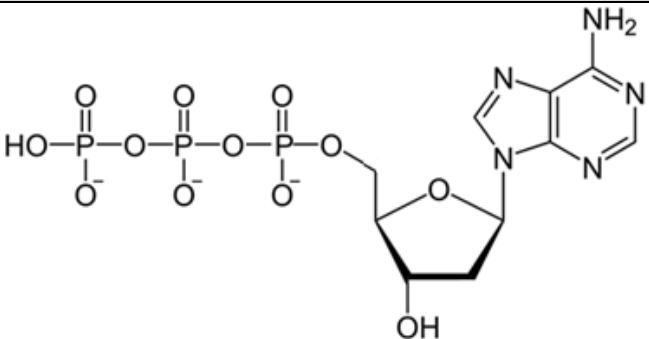
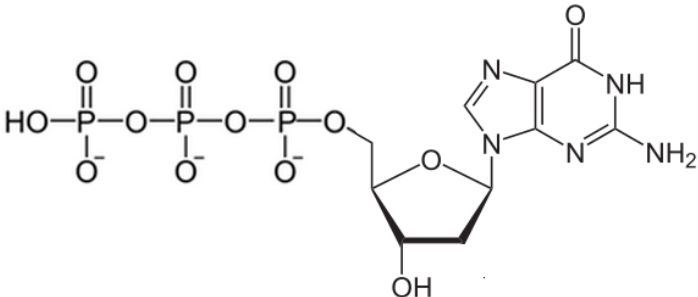
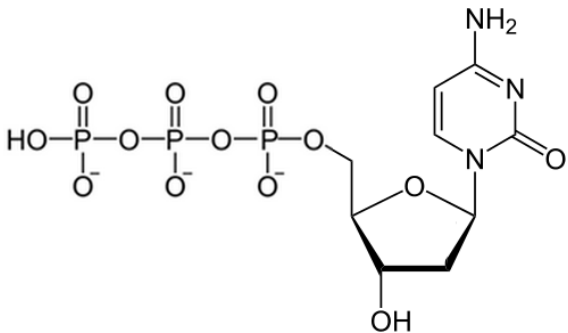
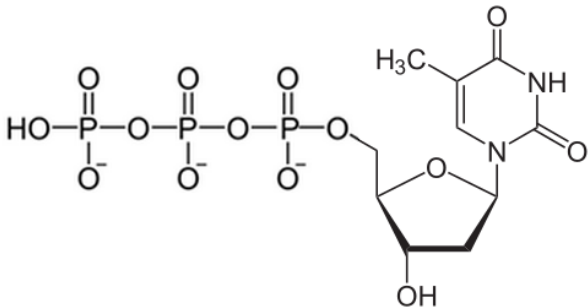
- triphosphate
- deoxyribose
- 3'-OH
- cytosine base



Ex-1011 (Alberts) at 58-59.

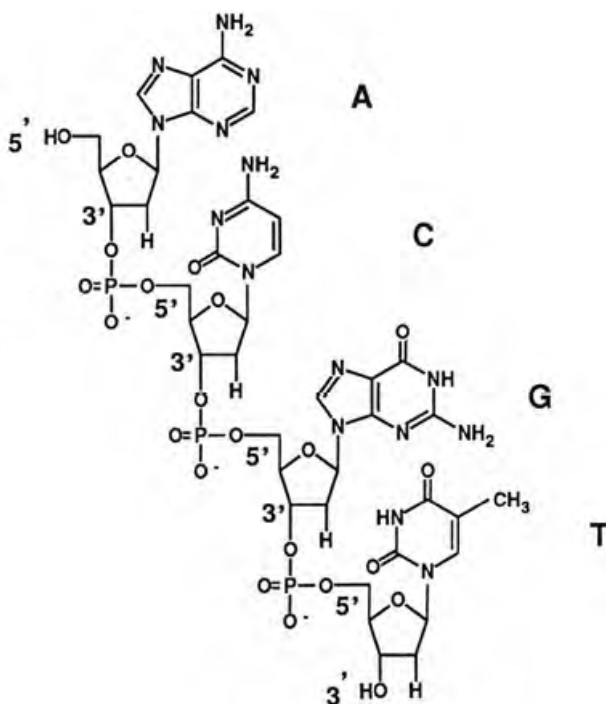
32. A nucleoside is a molecule comprised of a sugar, generally ribose, and a heterocyclic base (such as cytosine). A nucleotide (*e.g.*, a “nucleoside monophosphate”) has the same constituents as a nucleoside, yet it also contains a phosphate moiety, which is commonly attached to its 5' end. A nucleotide may have one phosphate (a “nucleoside monophosphate”), two phosphates (a “nucleoside diphosphate”), or three phosphates (a “nucleoside triphosphate”) bound to the sugar. Ex-1011 (Alberts) at 59.

33. The four deoxyribonucleoside triphosphates that are naturally found in DNA are: adenosine “dATP,” guanosine “dGTP,” cytidine “dCTP,” and thymidine “dTTP.” These nucleotides differ from each other by the base located at the 1'-position of the deoxyribose, as shown below:

deoxyribonucleotide abbreviation	base	chemical formula
dATP	adenine ("A")	
dGTP	guanine ("G")	
dCTP	cytosine ("C")	
dTTP	thymine ("T")	

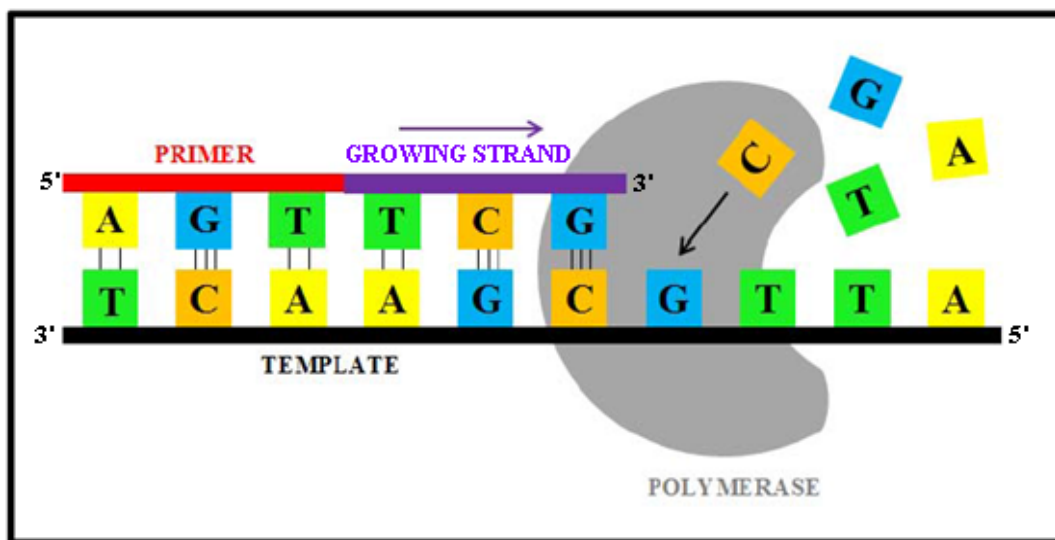
Adenine and guanine are classified as purine bases, while cytosine and thymine are pyrimidines. Ex-1011 (Alberts) at 58.

34. A strand of DNA consists of deoxyribonucleotides in which the 5'-phosphate group of one nucleotide is attached to the 3'-oxygen of the preceding (5') nucleotide:



DNA is typically double-stranded, and the two strands bind to one another through hydrogen bonds between the bases of complementary nucleotides in each strand. In this complementary base pairing, 'A' nucleotides in one strand bind to 'T' nucleotides in the other strand, and 'C' nucleotides in one strand bind to 'G' nucleotides in the other strand. Ex-1011 (Alberts) at 98-101.

35. When DNA is synthesized (or copied), the two strands of DNA are separated. A single strand of DNA serves as a "template" to synthesize the complementary strand. A polymerase enzyme incorporates nucleotides into a new strand of DNA having bases that are complementary to that of the template:



The polymerase enzyme catalyzes the formation of a bond between the 3'-OH group of the preceding nucleotide (which is the terminal nucleotide of the growing strand) and the 5'-phosphate group of the nucleotide being incorporated into the growing strand. Ex-1011 (Alberts) at 98-103. If the 3'-OH group of the last nucleotide is blocked (also known as “capped”) with a protecting group, the polymerase cannot incorporate another nucleotide into the growing strand until the protecting group is removed (also referred to as “deblocked” or “uncapped”) to regenerate or expose a 3'-OH group. Ex-1013 (Tsien) at 12:22-13:29; Ex-1016 (Metzker) at 4259-60; Ex-1002 ('358 patent) at 7:51-58, 3:4-17.

B. Nucleotide Analogues Were Known in the Art

36. The sequence of nucleotides in DNA contains genetic information. Determining the sequence of nucleotides in DNA can help diagnose and treat diseases. There were several prior art methods for determining the sequence of

DNA, including Sanger sequencing (*e.g.*, Ex-1018 (Sanger); Ex-1014 (Prober)), and sequencing-by-synthesis (“SBS”). *E.g.*, Ex-1013 (Tsien); Ex-1015 (Dower); Ex-1016 (Metzker); Ex-1020 (Cheeseman). Sanger sequencing and SBS use a polymerase enzyme to incorporate modified nucleotides (i.e., “nucleotide analogues”) containing a detectable label into a chain of DNA. The label on the incorporated nucleotide analogue is “read” to determine the DNA sequence. Several labeled nucleotide analogues were known in the prior art, including nucleotide analogues containing: (1) removable 3′-OH capping groups to facilitate sequential addition of nucleotides during sequencing and (2) labels attached to the base through a linker.

1. **Nucleotide analogues having a 3′-OH cap and a labeled base were known**

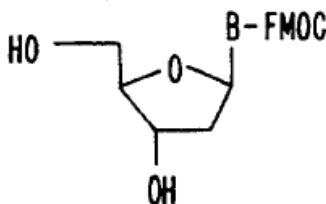
37. Before October of 2000, several prior art references documented nucleotide analogues for DNA sequencing having both a removable 3′-OH cap and a detectable label attached to the base. Exemplary references include Tsien and Dower.

38. Tsien discloses a sequencing method using fluorescently labeled nucleotides having a 3′-OH blocking group. Ex-1013 (Tsien) at 10:17-18:33. Tsien also discloses labeling the nucleotide base: “there are a number of alternatives—particularly the formation of a 3′-blocked dNTP analogue containing

a label such as a fluorescent group coupled to a remote position such as the base.”

Ex-1013 (Tsien) at 27:35-28:6.

39. Likewise, Dower discloses using nucleotide analogues having a removable fluorescent label linked to the base as well as a removable 3'-OH blocking group. Ex-1015 (Dower) at 14:50-53, 15:33-40 (“To prevent elongation by a unit length greater than one monomer, the nucleotide should be blocked at the position of 3' elongation. Usually, the nucleotide will be blocked at the 3' hydroxyl group where successive nucleotides would be attached. In contrast to a dideoxy nucleotide, typically the blocking agent will be a reversible blocking agent thereby allowing for deblocking and subsequent elongation.”); *id.* at Fig. 8, 15:52-56, 18:64-66 (“FIG. 9 schematically illustrates the synthesis of a generic protected nucleotide. A suitable nucleotide is labeled with the FMOC fluorescently detectable label”); *id.* at 18:67-19:10. A portion of Dower's Fig. 9 showing a cleavable FMOC label is shown below:



a. It Was Known that the 3'-capping group should be small

40. Dower disclosed nucleotides having “small blocking groups” on the 3'-OH. Ex-1015 (Dower) at 25:48-51 (“A second, unlabeled and reversible, set of

terminators is also required. Examples of these compounds are deoxynucleoside triphosphates with small blocking groups such as acetyl, tBOC, NBOC and NVOC on the 3'OH. These groups are easily and efficiently removed under conditions of high or low pH, exposure to light or heat, etc.”) (emphasis added). Dower was filed in 1990 and provided blocking groups that were considered small at that time: “acetyl, tBOC, NBOC, and NVOC.”¹ Ex-1015 (Dower) at 25:48-51. The crystal structure of DNA polymerase was published in 1994 by Pelletier. Ex-1021 (Pelletier) at 1897 (Table 3); *id.* at 1903, note 101 (“Full coordinates for both ternary complex structures are available from the Brookhaven Protein Data Bank and are designated 1bpf and 1bpg for the $P6_1$ and $P2_1$ structures, respectively.”).

41. Using the crystal structure published by Pelletier a person of ordinary skill in the art by 2000 would have recognized that the area around the 3'-carbon of the deoxyribose was tightly packed within the polymerase active site. For example, the shortest distance between the 3'-carbon (Atom “C3”) and a protein atom (Atom “O” of “residue Phe²⁷²”) was 3.2 Å. Ex-1021 (Pelletier) at 1897 (Table 3). It was therefore recognized that the space available within the

¹ tBOC stands for t-butoxycarbonyl; NBOC stands for 2-nitrobenzyloxycarbonyl; NVOC stands for 3,4-dimethoxy-6-nitrobenzyloxycarbonyl.

polymerase active site to accommodate a 3'-group on a nucleotide substrate was limited. Thus, a person of ordinary skill in the art would have recognized that a 3'-OH protecting group on a nucleotide should have been small to fit within this constrained space.

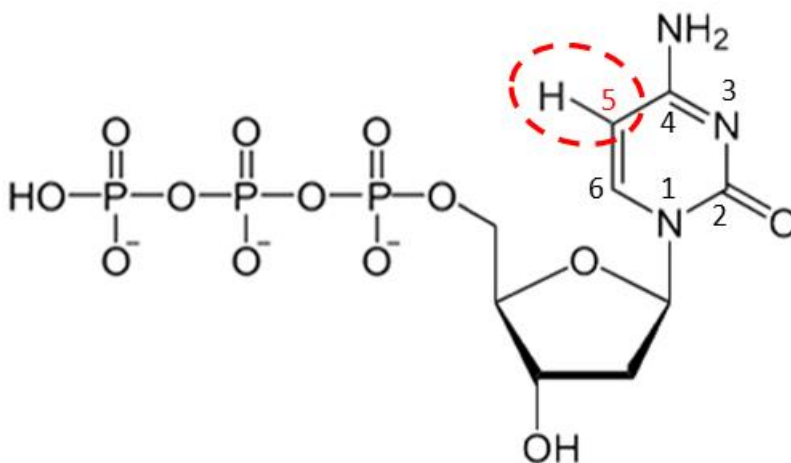
42. By 1999, it had become clear that large fluorophores required to label a nucleotide for detection were too bulky to be accommodated by DNA polymerases when the label was attached at the 3'-position. For example, in 1999 Welch reported that 3'-labeled nucleotide analogues “tend to be too big to fit into the active site of DNA polymerases.” Ex-1023 (Welch) at 956; *see also id.* at 955.²

b. 5-Substituted pyrimidine labels were commonly used

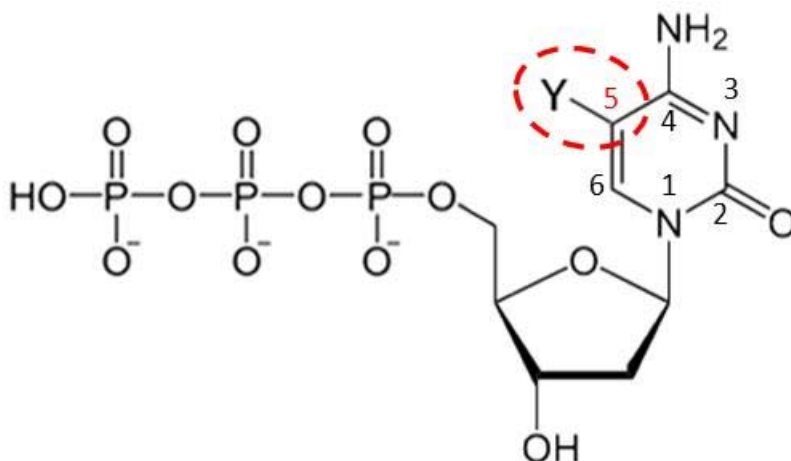
43. Tsien and Dower disclose base-labeled analogues of all the natural nucleotides, dATP, dGTP, dCTP, dTTP. Ex-1013 (Tsien) at 27:33-28:6, 30 (showing Structures A, B, C and D); Ex-1015 (Dower) at 18:64-19:10, Fig. 9. Tsien and Dower point to Prober for base-labeled nucleotides. Ex-1013 (Tsien) at 28:5-18; Ex-1015 (Dower) at 25:4-12, 25:44-47, 20:39-42, 23:16-26, 28:6-17, 17:35-36.

² A Corrigenda to the Welch reference was published correcting the structures of compounds 6, 14a and 14t. *See* Ex-1024 (Welch Corrigenda).

44. Prober discloses the use of a label attached through a linker at the 5-position of cytosine for nucleotide sequencing with a DNA polymerase. Ex-1014 (Prober) at 337 (“The linker is attached to the 5 position in the pyrimidines and to the 7 position in the 7-deazapurines.”); *id.* at 338, Fig. 2A. A 5-substituted cytosine nucleotide analogue has a substituent at the 5-position instead of a hydrogen atom. The structures of cytosine deoxyribonucleoside triphosphate (dCTP) and a 5-substituted cytosine deoxyribonucleoside triphosphate are shown below:



cytosine deoxyribonucleoside triphosphate (dCTP)



5-substituted cytosine deoxyribonucleoside triphosphate;
see e.g., Ex-1014 (Prober) at 337-38, Fig 2A (compound C-519).

45. The 5-substitution of cytosine does not interfere with the hydrogen bonding functionality of the cytosine to its complementary guanine nucleotide, as shown below:

Hydrogen bonding for guanosine to cytidine	
Hydrogen bonding for guanosine to 5-substituted cytidine	

46. By 1999, 5-substituted cytosine nucleotide analogues had become widely and commercially available. Ex-1027 (Rosenblum) at 4501 (disclosing

dye-labeled terminators with d-rhodamine and BigDye labels from PE Applied Biosystems).

47. The preference for 5-substituted pyrimidines stemmed from several known benefits. Attaching a label to the 5-position of pyrimidines minimizes interference with incorporation of nucleotide analogues into a DNA strand by a DNA polymerase. For instance, Hobbs explained “positioning the linker on the 5-position of pyrimidine nucleotides and the 7-position of 7-deazapurine nucleotides eventually places the linker and reporter in the major groove when the nucleotide is incorporated into double-stranded DNA (this will serve to minimize interference with hybridization and other processes, which require that a double-stranded conformation be possible).” Ex-1029 (Hobbs) at 27:52-65.

48. [paragraph not used].

49. [paragraph not used].

50. [paragraph not used].

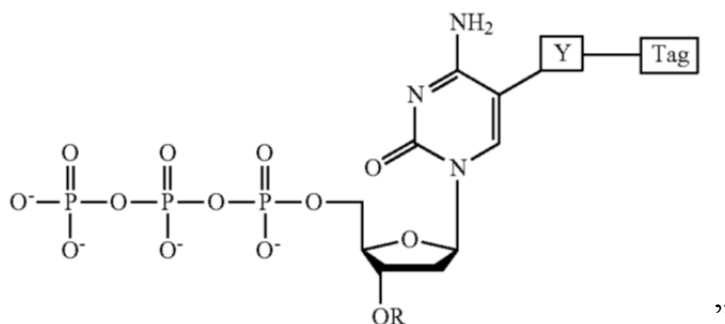
VII. GROUND 1: OBVIOUSNESS OVER TSIEN

A. Claim 1 is Obvious Over Tsien

51. Tsien discloses all limitations of Claim 1 and renders the claim obvious.

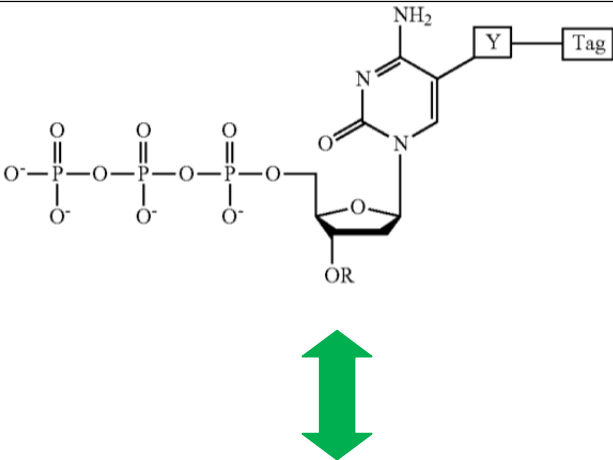
1. The structure depicted in Claim 1 is not new

52. Claim 1 recites: “A cytosine deoxyribonucleotide analogue having the structure:



The atoms drawn in this structure merely depict the well-known arrangement of atoms contained in cytosine deoxyribonucleoside triphosphate. Every atom and group depicted in this structure is fully described using standard nucleotide vocabulary as “a cytosine deoxyribose nucleoside triphosphate having an R group capping the 3'-OH group and a Tag attached through a cleavable linker (Y) to the 5-position of the base.”

53. I understand that, in IPR2012-00007, the Board held unpatentable Claim 21 of Columbia's U.S. 7,790,869 patent that claimed a cytosine deoxyribonucleotide having a 3'-OH capping group and a tag attached through a cleavable linker to the base. The equivalence between the nucleotide of Claim 21 and the present claim is self-evident in the following side-by-side comparison of the claims:

Presently claimed '358 nucleotide depiction with equivalent nomenclature	Unpatentable Claim 21 (along with Claim 12 from which it depends) from IPR2012-00007
 <p>A cytosine deoxyribonucleotide having a 3'-OH capping group (R) and a Tag attached through a cleavable linker (Y) to the base.</p>	<p>21. The nucleotide of claim 12, wherein said nucleotide is an analog of adenosine, guanosine, cytosine, or thymidine.</p> <p>12. A nucleotide having a base that is attached to a detectable label through a cleavable linker, wherein the nucleotide has a deoxyribose comprising a cleavable chemical group capping the 3' OH group, wherein the cleavable linker is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light, and wherein the cleavable chemical group capping the 3' OH group is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.</p>

As shown above, Claim 1 from the '358 patent and Claim 21 from IPR2012-00007 are directed to the same cytosine deoxyribonucleotide having a 3'-OH capping group and a Tag (i.e., label) attached through a cleavable linker to the base.

a. The nucleotide depicted in Claim 1 would have been obvious over Tsien

54. As discussed below, Tsien discloses a cytosine deoxyribonucleotide having a 3'-OH capping group and a tag attached through a cleavable linker at the

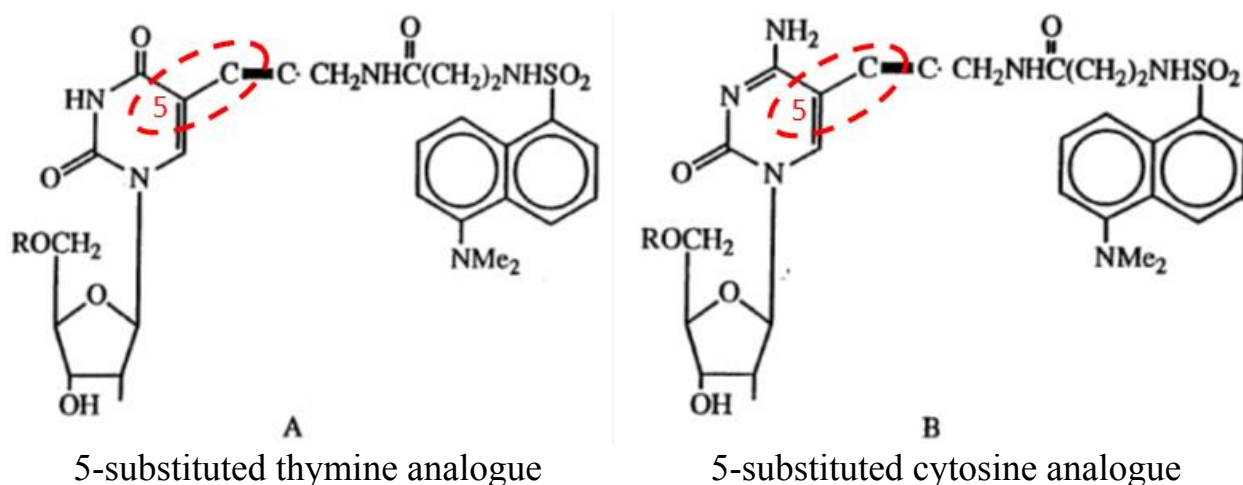
5-position of the base. Such a cytosine deoxyribonucleotide meets the nucleotide analogue depicted in Claim 1.

55. Tsien discloses deoxyribonucleoside triphosphate (dNTP) analogues. An exemplary cytosine analogue is provided in Tsien's Figure 2 as "3'-BLOCKED dC''TP." Ex-1013 (Tsien) at Fig. 2. This cytosine analogue is a deoxyribonucleotide having a 3'-blocking group and a tag attached to the cytosine base as indicated by the apostrophes. Ex-1013 (Tsien) at 9:35-10:14 ("Deoxynucleotide triphosphates 'dNTPs' of these materials are abbreviated as dATP, dCTP, dGTP and dTTP. When these materials are blocked in their 3'-OH position they are shown as 3'blockedddATP, 3'blockedddCTP, 3'blockedddGTP and 3'blockedddTTP. Similarly, when they are each tagged or labeled with a common reporter group, such as a single fluorescent group, they are represented as dA'TP, dC''TP, dG'TP and dT'TP. When they are each tagged or labeled with different reporter groups, such as different fluorescent groups, they are represented as dA'TP, dC''TP, dG'''TP and dT''''TP. As will be explained in more detail below, the fact that the indication of labeling appears associated with the 'nucleoside base part' of these abbreviations does not imply that this is the sole place where labeling can occur."); Ex-1013 (Tsien) at 12:22-29 ("In practice, the polymerase and the four labeled dNTPs are added to the reaction zone 14 under conditions adequate to permit the enzyme to bring about addition of the one, and only the one, of the four

labeled blocked dNTPs which is complementary to the first available template nucleotide following the primer. The blocking group present on the 3'-hydroxyl position of the added dNTP prevents inadvertent multiple additions.”).

56. Tsien discloses a cleavable linker attaching the tag to the base. Ex-1013 (Tsien) at 28:5-10 (“One method involves the use of a fluorescent tag attached to the base moiety. The tag may be chemically cleaved (either separately from or simultaneously with the deblocking step) and measured either in the reaction zone before deblocking or in the reaction eluant after cleavage.”); *see generally* Ex-1013 (Tsien) at 27:35-28:35. Tsien discloses exemplary chemical linkers (such as silyl, allyl, and 2,4-dinitrophenylsulfenyl). Ex-1013 (Tsien) at 28:19-29.

57. Tsien further discloses 5-substituted pyrimidines:



Ex-1013 at 30 (annotated). Tsien’s Structure B is a cytosine deoxyribose nucleotide having a fluorescent label attached through a linker to the 5-position of

the base. Tsien discloses the advantages of labeling pyrimidines at the 5-position as follows:

A number of approaches are possible to produce fluorescent derivatives of thymidine and deoxycytidine. One quite versatile scheme is based on an approach used by Prober et al. (1987) to prepare ddNTPs with fluorescent tags. Structures A, B, C and D below illustrate the type of fluorescent dNTPs that result from these synthetic approaches. The synthetic routes have a great flexibility in that the linker can be varied with respect to length or functionality.

The terminal fluorescent moiety can also be varied according to need.

Ex-1013 at 29:10-19 (emphases added). Thus, Tsien discloses that Structure B is advantageous because it provides great flexibility for the linker length and functionality.

58. Tsien's disclosure is consistent with the known advantages of using 5-substituted pyrimidines. For example, Hobbs explained "positioning the linker on the 5-position of pyrimidine nucleotides and the 7-position of 7-deazapurine nucleotides eventually places the linker and reporter in the major groove when the nucleotide is incorporated into double-stranded DNA (this will serve to minimize interference with hybridization and other processes, which require that a double-stranded conformation be possible)." Ex-1029 (Hobbs) at 27:52-65.

59. In summary, Tsien discloses:

- the desirability of using the 3'-O-allyl capping group in Tsien's sequencing methods,
- the desirability of a cytosine deoxyribose nucleotide having a fluorescent label attached through a linker to the 5-position of the base, and
- the desirability of attaching the label to the base via a cleavable linker,

Thus, a cytosine deoxyribonucleotide having a 3'-OH capping group and a tag attached through a cleavable linker to the 5-position of the base would have been obvious over Tsien.

2. Limitation R(a)

60. Tsien contains disclosures that meet limitation R(a) of Claim 1, which recites: “wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue.”

61. Tsien discloses a “3'-BLOCKED dC"TP,” which is a cytosine deoxyribonucleotide analogue having a blocking group capping the 3'-oxygen of the deoxyribose. Ex-1013 (Tsien) at Fig. 2; *id.* at 12:27-29. Tsien describes 3'-blocking groups, including chemical groups that can be removed using chemical conditions such as acid or base hydrolysis and other removal conditions. Ex-1013

(Tsien) at 20:24-25:34. Tsien specifically discloses the 3'-O-allyl ether blocking group. Ex-1013 (Tsien) at 24:29-30.

62. A person of ordinary skill in the art would have recognized that Tsien's allyl group is advantageous because it was demonstrated to be functionally small enough to fit within the active site of DNA polymerase. A person of ordinary skill in the art knew that the 3'-capping group should be small based on the crystal structure of DNA polymerase published by Pelletier showing that the active site is tightly packed and that the space where the 3'-group was to be attached is very limited. Ex-1021 (Pelletier) at 1897 (Table 3), 1903 (note 101); *see also supra* Paragraphs 40-41. Tsien's 3'-O-allyl capping group has only three carbon atoms ($-\text{CH}_2-\text{CH}=\text{CH}_2$), therefore Tsien's 3'-O-allyl capping group would have been recognized by a person of ordinary skill in the art as being small. Furthermore, it was known that Tsien's 3'-O-allyl capping group was demonstrated to be compatible with the polymerase active site based on Metzker's 1994 publication (which is discussed in the next paragraph and in Paragraph 77).

63. In 1994, Metzker demonstrated that Tsien's 3'-O-allyl group is compatible with DNA polymerase recognition. Ex-1016 (Metzker) at 4263 (Table

2).³ In Table 2, Metzker reported that 3'-O-allyl dATP exhibits “Termination” of Vent_R(exo⁻)[®] DNA polymerase:

Table 2. Activity matrix of RP-HPLC purified 3'-protecting dNTPs challenged against commercially available polymerases

3'-modified-dATP (except compound [8])	AMV-RT	M-MuLV-RT	Klenow fragment	Sequenase [®]	Bst DNA polymerase	AmpliTa [®] DNA polymerase	Vent _R (exo ⁻) [®] DNA polymerase	rTth DNA polymerase
[1] O-methyl	Termination	Termination*	-	-	-	-	Inhibition	Inhibition*
[2] O-acetyl	-	-	-	-	-	-	Inhibition	-
[3] O-allyl	-	-	-	-	-	-	Termination*	-
[4] O-tetrahydropyran	-	-	-	-	-	-	-	-
[5] O-(4-nitrobenzoyl)	-	-	-	-	-	-	-	-
[6] O-(2-aminobenzoyl)	-	-	-	-	-	-	-	-
[7] O-(2-nitrobenzyl)	-	-	-	Inhibition	Termination	Termination*	Termination*	-
[8] 3'-O-methyl-dTTP	-	Inhibition	-	Inhibition	Termination	Termination	Termination	Termination

All compounds were assayed at a final concentration of 250 μM according to the conditions specified in Table 1. ‘-’ means no activity was detected, ‘Termination’ means that the termination bands mimic ddNTP termination bands, and ‘Inhibition’ means the rate of DNA synthesis is reduced in a nonspecific manner. ‘*’ means the activity was incomplete at a final concentration of 250 μM .

The legend of Table 2 states: “‘-’ means no activity was detected, ‘Termination’ means that the termination bands mimic ddNTP termination bands, and ‘Inhibition’ means the rate of DNA synthesis is reduced in a nonspecific manner. ‘*’ means the activity was incomplete at a final concentration of 250 μM.” Ex-1016 (Metzker) at 4263. The “Termination” of Vent_R(exo⁻)[®] DNA polymerase synthesis by 3'-O-allyl dATP indicates that this nucleotide analogue was successfully incorporated by the polymerase. Ex-1016 (Metzker) at 4263, Table 2; *see also id.* at 4265

³ In 2007 Metzker and colleagues corrected the structure of compound 7. Ex-1017 (Wu and Metzker) at 6339-40.

(“First, the protecting groups containing ether linkages at the 3’-position, compounds [1], **[3]** [i.e., 3’-O-allyl dATP], [4], [7], and [8] were incorporated by some of the polymerases.”) (emphasis added). A person of ordinary skill in the art would have understood that this incorporation demonstrates that the polymerase recognized the nucleotide analogue. Ex-1011 (Alberts) at 247 (Fig. 6-36), 251-252. The accommodation by a DNA polymerase of a 3’-O-substituent within dATP is expected to be identical for other dNTPs, including dCTP.

64. Tsien discloses that the allyl group is advantageous because it is “cleaved selectively using chemical procedures other than base hydrolysis ... [which has] some advantages over groups cleavable by base hydrolysis—deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.” Ex-1013 (Tsien) at 24:25-25:3. Tsien discloses that “[a]llyl ethers are cleaved by treatment with Hg(II) in acetone/water (Gigg and Warren, 1968).” Ex-1013 (Tsien) at 24:29-30. It was also known that allyl groups were generally efficiently removed using palladium, including under aqueous conditions. Ex-1035 (Boss) at 559, Table 1; Ex-1036 (Qian) at 2184 (“The O-allyl group was then removed using PdCl₂, providing [the deprotected compound] in quantitative yield.”).

3. Limitation R(b)

65. Tsien contains disclosures that meet limitation R(b) of Claim 1, which recites: “wherein R ... (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase.”

66. Tsien discloses that a “criteria for the successful use of 3'-blocking groups include[s]: (1) the ability of a polymerase enzyme to accurately and effectively incorporate the dNTPs carrying the 3'-blocking groups into the cDNA chain.” Ex-1013 (Tsien) at 20:28-32; *see also id.* at 19:4-18 (“The coupling process employed in this invention to incorporate each of the blocked deoxynucleotide triphosphates into the growing complementary chain is an enzyme moderated process...”); *id.* at 12:11-18 (“Reaction zone 14 is fitted with inlet 16 for the addition of polymerase or another suitable enzyme capable of moderating the template-directing coupling of nucleotides to one another.”); *id.* at 24:1-2. A person of ordinary skill in the art would have known that Tsien’s 3'-O-allyl group does not interfere with recognition by a DNA polymerase because Metzker demonstrated polymerase incorporation (“Termination”) of the 3'-O-allyl group in 1994. Ex-1016 (Metzker) at 4263, Table 2; *supra* Paragraphs 62-63. A person of ordinary skill in the art would have understood that this incorporation demonstrates that the polymerase recognized the nucleotide analogue. Ex-1011 (Alberts) at 247 (Fig. 6-36), 251-252.

4. Limitations R(c) and wherein the 3'-oxygen and R bond is stable

67. Tsien contains disclosures that meet limitation R(c) of Claim 1, which recites: “wherein R ... (c) is stable during a DNA polymerase reaction.” Tsien also contains disclosures that meet the limitation of Claim 1 that recites: “wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction.”

68. The stability of 3'-capping groups during DNA polymerase incorporation is disclosed throughout Tsien. Ex-1013 (Tsien) at 19:4-18 (“The coupling process employed in this invention to incorporate each of the blocked deoxynucleotide triphosphates into the growing complementary chain is an enzyme moderated process...”); *id.* at 12:11-18 (“Reaction zone 14 is fitted with inlet 16 for the addition of polymerase or another suitable enzyme capable of moderating the template-directing coupling of nucleotides to one another.”); *id.* at 23:28-32 (disclosing “successfully incorporating a 3'-blocked nucleotide into the DNA chain”). Tsien discloses that the allyl ether capping group in particular is advantageous because “deblocking only occurs when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.” Ex-1013 (Tsien) at 24:24-25:3. Tsien’s stated “incorporation” refers to DNA polymerase incorporation. Ex-1013 (Tsien) at 20:28-32. Tsien’s discussion of deblocking only occurring by a specific deblocking reagent underscores the overall

stability of the allyl ether capping group during polymerase incorporation. This overall stability of Tsien's 3'-O-allyl group includes the entire allyl ether group, including its linkage to the 3'-oxygen atom. Thus, the overall stability of Tsien's 3'-allyl group includes stability at the bond between the 3'-oxygen and the rest of the blocking group. Tsien therefore discloses limitation R(c) and that the 3'-oxygen to R bond is stable during a polymerase reaction.

5. Limitations R(d) and wherein OR is not methoxy or ester

69. Tsien contains disclosures that meet limitation R(d) of Claim 1, which recites: "wherein R ... (d) does not contain a ketone group." Tsien also contains disclosures that meet the limitation of Claim 1 that recites: "wherein OR is not a methoxy group or an ester group."

70. The 3'-O-allyl capping group disclosed by Tsien (Ex-1013 (Tsien) at 24:24-25:3) does not contain a ketone group, as shown below:

-CH ₂ -CH=CH ₂ allyl Ex-1039 (Vollhardt) at 502-504	-C(=O)- ketone Ex-1039 (Vollhardt) at 43
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and it is not a methoxy or an ester group, as shown below:

-O-CH ₂ -CH=CH ₂ -O-allyl	-O-CH ₃ methoxy	-O-C(=O)-CH ₃ an ester
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6. Limitation “wherein tag represents a detectable fluorescent moiety”

71. Tsien contains disclosures that meet the limitation of Claim 1 that recites: “wherein tag represents a detectable fluorescent moiety.”

72. Tsien discloses an exemplary cytosine analogue having a tag as “3'-BLOCKED dC''TP.” Ex-1013 (Tsien) at Fig. 2; *id.* at 10:4-15. The apostrophes indicate a fluorescent tag. Ex-1013 (Tsien) at 10:7-10 (“When [the dNTPs] are each tagged or labeled with different reporter groups, such as different fluorescent groups, they are represented as dA'TP, dC''TP, dG'''TP and dT''''TP.”). Tsien discloses embodiments where the fluorescent tag is attached to the base moiety and the tag may be chemically cleaved. Ex-1013 (Tsien) at 28:5-8 (“One method involves the use of a fluorescent tag attached to the base moiety. The tag may be chemically cleaved (either separately from or simultaneously with the deblocking step).”) (emphasis added); *see also id.* at 28:19-23, 27:36-28:12, 26:17-36, 13:4-13. Tsien’s fluorescent tags are detected by conventional analytical methods and provide a major advantage of Tsien’s method. Ex-1013 (Tsien) at 31:1-5 (“The labels so incorporated in the growing cDNA chain are detected by conventional analytical methods. In many cases, particularly with fluorescent labels, increased detection sensitivity is a major advantage of the present method.”) (emphases added). Tsien therefore discloses this limitation.

7. **Limitation Y is a “chemically cleavable, chemical linker”**

73. Tsien contains disclosures that meet the limitation of Claim 1 that recites: “wherein Y represents a chemically cleavable, chemical linker.”⁴

74. Tsien discloses “cleavable tethers” (Ex-1013 (Tsien) at 28:23-29) that link the fluorescent tag to the base moiety. *See* Ex-1013 (Tsien) at 28:5-8 (“One method involves the use of a fluorescent tag attached to the base moiety. The tag may be chemically cleaved (either separately from or simultaneously with the deblocking step).”) (emphasis added). Tsien discloses exemplary chemical linkers

⁴ The ’358 patent does not disclose any specific chemical group that meets the recited linker Y. The ’358 patent discloses a 2-nitrobenzyl linker, but the ’358 patent characterizes this linker as a *photocleavable* linker, instead of a *chemically* cleavable linker. Ex-1002 (’358 patent) at 11:16-18 (“In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.”); *id.* at 14:17-23 (“In one embodiment, the linker between the unique label and the nucleotide analogue is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.”).

(such as allyl, silyl, and 2,4-dinitrophenylsulfenyl), along with exemplary chemical cleavage conditions, as follows:

In another type of remote labeling the fluorescent moiety or other innocuous label can be attached to the dNTP through a spacer or tether. The tether can be cleavable if desired to release the fluorophore or other label on demand. There are several cleavable tethers that permit removing the fluorescent group before the next successive nucleotide is added—for example, silyl ethers are suitable tethers which are cleavable by base or fluoride, allyl ethers are cleavable by Hg(II), or 2,4-dinitrophenylsulfenyls are cleavable by thiols or thiosulfate.

Ex-1013 (Tsien) at 28:19-29 (emphases added). Tsien therefore discloses this limitation.

8. Limitation Y(a)

75. Tsien contains disclosures that meet limitation Y(a) of Claim 1, which recites: “wherein Y ... (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase.”

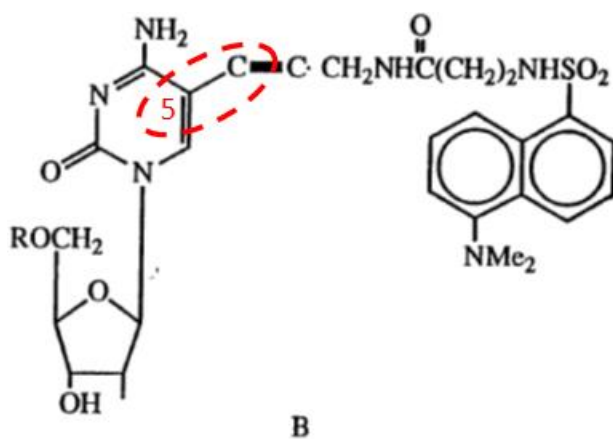
76. Tsien discloses exemplary “cleavable tethers” (Ex-1013 (Tsien) at 28:23-29) that attach the fluorescent tag to the base moiety. Ex-1013 (Tsien) at 28:19-29 (disclosing allyl, silyl, and 2,4-dinitrophenylsulfenyl tethers); *id.* at 28:5-12 (disclosing “a fluorescent tag attached to the base moiety” that “may be chemically cleaved”). Tsien discloses that the cleavable tethers should not interfere with the polymerase. For example, Tsien discloses that long tethers may

be used “so that the large fluorescent groups are spaced sufficiently far away from the base and triphosphate moieties and do not interfere with the binding of the dNTP to the polymerase.” Ex-1013 (Tsien) at 28:31-35. Tsien discloses the labels linked to the nucleotide are “innocuous” as follows:

The labels or markers are "innocuous". An "innocuous marker or label or reporter" refers to a radioactive, fluorescent, or the like marker or reporter which has physical and chemical properties which do not interfere with either the enzymatic addition of the marked nucleotide to the cDNA, or the subsequent deblocking to yield a viable 3'-OH terminus.

Ex-1013 (Tsien) at 26:5-12 (emphases added). These disclosures meet limitation Y(a).

77. Tsien discloses Structure B:



Ex-1013 (Tsien) at 30 (annotated). Structure B is a 5-substituted cytosine analogue. The discussion within Tsien about Structure B cites to Prober. Ex-1013 (Tsien) at 29:10-16 (“A number of approaches are possible to produce fluorescent

derivatives of thymidine and deoxycytidine. One quite versatile scheme is based on an approach used by Prober et al. (1987) to prepare ddNTPs with fluorescent tags. Structures A, B, C and D below illustrate the types of fluorescent dNTPs that result from these synthetic approaches.”). Tsien describes Prober as “show[ing] enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and SequenaseTM.” Ex-1013 (Tsien) at 28:16-18 (“Prober et al. (1987) show enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and SequenaseTM.”) (emphasis added). Tsien is correct that Prober shows polymerase incorporation of 5-substituted cytosine analogues. Ex-1014 (Prober) at 337 (“The fidelity of terminator incorporation is maintained.”); *id.* at 340 (“The chain-terminators reported here are incorporated by both AMV reverse transcriptase and modified T7 DNA polymerase, but they are not accepted by the Klenow fragment of DNA polymerase I from *Escherichia coli*.”). Prober’s 5-substituted cytosine analogues include a fluorescent label and a linker. Ex-1014 (Prober) at Fig. 2A (showing the label and linker of the nucleotide analogues). In addition, other groups demonstrated successful polymerase incorporation of 5-substituted pyrimidines, including successful incorporation using Vent polymerase, which is the same polymerase that Metzker demonstrated incorporates a 3'-O-allyl nucleotide analogue. *See, e.g.*, Ex-1040 (Yu) at 3230 (“Tli (Promega), Pfu (Stratagene), Deep Vent and Vent Exo- (New England Biolabs) were among those that incorporated

most Cy3-dUTP per kb of synthesized DNA under conditions for each of the enzymes as recommended by the manufacturers.”); *id.* at 3228 (showing the structure of Cy3-4-dUTP); Ex-1041 (Livak) at 4833 (disclosing 5-substituted pyrimidine nucleotides, compounds 1a-1d, “were found to be fairly good substrates for Tli DNA polymerase”); *id.* at 4832-33 (disclosing that Tli DNA polymerase is Vent DNA polymerase). A person of ordinary skill in the art would have understood that Vent *exo-* disclosed by Yu and Metzker, and Vent *exo+* disclosed by Livak have identical polymerase domains. The Vent *exo-* is a modification in the proofreading exonuclease domain, and this modification favors incorporation of nucleotides by preventing their removal. Ex-1059 (Sears) at 626 (“*In vitro* mutagenesis was utilized to selectively remove the 3'→5' proofreading exonuclease activity from the Vent_RTM DNA polymerase, creating the modified enzyme, the Vent_R (*exo-*) DNA polymerase. The exonuclease deficient polymerase retains a 2-fold lower error rate than that of *Taq* polymerase and maintains other biochemical properties found with the native Vent DNA polymerase and the cloned unmodified Vent_R DNA polymerase, with half-lives of >1 h at 100°C.”). Thus, while Vent *exo+* has the same polymerase domain as Vent *exo-*, Vent *exo+* is generally more selective against nucleotide analogue incorporation due to exonuclease activity that is not present in Vent *exo-*. Therefore, the nucleotide analogues incorporated by Vent *exo+* in Livak would be

expected to be incorporated at least as well (or even better than) by Vent exo- used by Yu and Metzker.

9. Limitation Y(b)

78. Tsien contains disclosures that meet limitation Y(b) of Claim 1, which recites: “wherein Y ... (b) is stable during a DNA polymerase reaction.”

79. Tsien discloses that “[t]he tether can be cleavable if desired to release the fluorophore or other label on demand.” Ex-1013 (Tsien) at 28:19-23. Tsien discloses exemplary chemical linkers (such as allyl, silyl, and 2,4-dinitrophenylsulfenyl), as follows:

In another type of remote labeling the fluorescent moiety or other innocuous label can be attached to the dNTP through a spacer or tether. The tether can be cleavable if desired to release the fluorophore or other label on demand. There are several cleavable tethers that permit removing the fluorescent group before the next successive nucleotide is added—for example, silyl ethers are suitable tethers which are cleavable by base or fluoride, allyl ethers are cleavable by Hg(II), or 2,4-dinitrophenylsulfenyls are cleavable by thiols or thiosulfate.... Cleavages using acidic conditions are undesirable because DNA is more labile in acid than in base. Long tethers may be used so that the large fluorescent groups are spaced sufficiently far away from the base and triphosphate moieties and do not interfere with the binding of the dNTP to the polymerase or with proper base pairing during complementary chain growth.

Ex-1013 (Tsien) at 28:19-35 (emphases added). By referring to a “next successive nucleotide” addition, Tsien indicates that there are at least two nucleotide additions: the preceding addition and the subsequent (next, successive) nucleotide addition. Tsien’s “next successive nucleotide is added” statement refers to a DNA polymerase reaction that incorporates the subsequent (next, successive) nucleotide analogue. Thus, the removal of the fluorescent group from the nucleotide analogue *after* incorporation and *before* the addition of next nucleotide indicates the linker is stable during the polymerase reaction.

10. Limitation i)

80. Tsien contains disclosures that meet limitation i) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue: i) is recognized as a substrate by a DNA polymerase.”

81. As discussed above in paragraphs 65-66 and 75-77, Tsien discloses polymerase recognition of a nucleotide analogue having a chemically cleavable 3'-capping group (such as Tsien's 3'-O-allyl group, which is small) and a chemically cleavable linker attached to the base.

82. Tsien discloses a “3'-BLOCKED dC"TP” analogue is added to a reaction zone with the three other labeled and blocked dNTPs under conditions to permit polymerase “to bring about addition of the one, and only the one, of the four labeled blocked dNTPs which is complementary to the first available template

nucleotide following the primer.” Ex-1013 (Tsien) at 12:22-27, Fig. 2. Tsien therefore discloses polymerase recognition of a cytosine deoxyribonucleotide having a 3'-OH capping group and a detectable tag attached to the base. *See also* Ex-1013 (Tsien) at 19:4-18 (“The coupling process employed in this invention to incorporate each of the blocked deoxynucleotide triphosphates into the growing complementary chain is an enzyme moderated process...”); Ex-1013 (Tsien) at 23:28-32 (disclosing “successfully incorporating a 3'-blocked nucleotide into the DNA chain”); Ex-1013 (Tsien) at 26:2-12 (“The labels or markers are ‘innocuous’. An ‘innocuous marker or label or reporter’ refers to a radioactive, fluorescent, or the like marker or reporter which has physical and chemical properties which do not interfere with either the enzymatic addition of the marked nucleotide to the cDNA, or the subsequent deblocking to yield a viable 3'-OH terminus.”). Tsien specifically identifies that when the dNTPs are labeled via a cleavable linker, the dNTPs bind to the polymerase with proper base pairing during the polymerase reaction. Ex-1013 (Tsien) at 28:31-35 (“Long tethers may be used so that the large fluorescent groups are spaced sufficiently far away from the base and triphosphate moieties and do not interfere with the binding of the dNTP to the polymerase or with proper base pairing during complementary chain growth”) (emphases added). A person of ordinary skill in the art would have expected a DNA polymerase to incorporate the dNTP analogues disclosed by Tsien. For example, it was known

that Vent polymerase incorporates 3'-O-allyl nucleotide analogues (Ex-1016 (Metzker) at 4263) as well as 5-substituted pyrimidine nucleotide analogues. Ex-1041 (Livak) at 4832-33; Ex-1040 (Yu) at 3228, 3230; *supra* Paragraph 77 (discussing Vent exo- and Vent exo+). The disclosures from Tsien therefore meet limitation i).

11. Limitation ii)

83. Tsien contains disclosures that meet limitation ii) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction.”

84. It was known that DNA polymerases only incorporate nucleotides at the end of a growing DNA strand during a DNA polymerase reaction, and that end is always the 3'-end (i.e., the end containing the 3'-OH). Ex-1011 (Alberts) at 252; Ex-1055 (Stryer) at 89 (“Elongation of the DNA chain proceeds in the 5'→3' direction.”).

85. As discussed above in paragraphs 65-66 and 75-77, Tsien discloses DNA polymerase recognition and incorporation of nucleotide analogues having a small chemically cleavable 3'-capping group and a chemically cleavable linker. This incorporation is depicted in Tsien's Figure 1B:

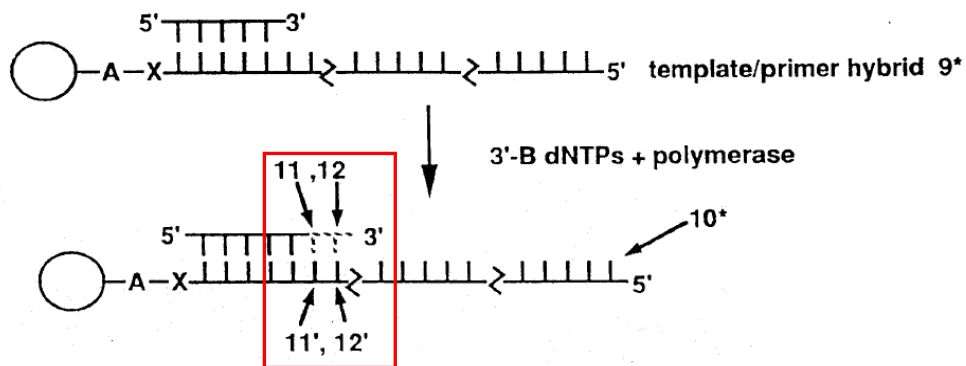


FIG. 1B

Ex-1013 (Tsien) at Figure 1B (annotated). In Figure 1B, individual dNTP analogues are serially added by DNA polymerase, with “11 and 12 represent[ing] the first two such dNTPs incorporated into the growing molecule.” Ex-1013 (Tsien) at 11:5-9. Nucleotide analogues 11 and 12 are added at the 3'-end of the growing chain. Ex-1013 (Tsien) at 12:22-13:29. Tsien therefore discloses limitation ii).

12. Limitation iii)

86. Tsien contains disclosures that meet limitation iii) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... iii) produces a 3'-OH group on the deoxyribose upon cleavage of R.”

87. Tsien discloses that 3'-blocking group removal “generates an active 3' hydroxyl.” Ex-1013 (Tsien) at 13:14-22. Tsien discloses conditions for chemical cleavage of 3'-O-allyl ethers. Ex-1013 (Tsien) at 24:29-30. It was known that the allyl group is cleaved using well-established synthetic procedures to give an -OH group. Ex-1035 (Boss) at 559, Table 1 (demonstrating removal of the allyl

protecting group via cleavage of the allyl ether linkage using aqueous conditions with palladium); Ex-1036 (Qian) at 2184 (“The O-allyl group was then removed using PdCl₂, providing [the deprotected compound] in quantitative yield.”). Therefore, the cleavage of Tsien’s 3’-O-allyl ethers produces a 3’-OH group. Tsien thus discloses limitation iii).

13. Limitation iv)

88. Tsien contains disclosures that meet limitation iv) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... iv) no longer includes a tag on the base upon cleavage of Y.”

89. As discussed above in paragraphs 73-74, Tsien discloses a cleavable linker that removes the tag from the base upon cleavage of the linker. Tsien discloses that “[t]he tether can be cleavable if desired to release the fluorophore or other label on demand.” Ex-1013 (Tsien) at 28:21-23. The fluorophore is removed from the nucleotide via chemical cleavage of the tether. Ex-1013 (Tsien) at 28:23-29 (“There are several cleavable tethers that permit removing the fluorescent group before the next successive nucleotide is added—for example, silyl ethers are suitable tethers which are cleavable by base or fluoride, allyl ethers are cleavable by Hg(II), or 2,4-dinitrophenylsulfenyls are cleavable by thiols or thiosulfate.”) (emphasis added). Tsien therefore discloses limitation iv).

14. Limitation v)

90. Tsien contains disclosures that meet limitation v) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... v) is capable of forming hydrogen bonds with guanine or a guanine nucleotide analogue.”

91. As discussed above in paragraphs 57-59 and 77, Tsien discloses Structure B, which is a cytosine analogue having a label linked to the 5-position. Ex-1013 (Tsien) at 30. Tsien cites to Prober for showing enzymatic incorporation of 5-labeled cytosine analogues. Ex-1013 (Tsien) at 29:10-16 (“A number of approaches are possible to produce fluorescent derivatives of thymidine and deoxycytidine. One quite versatile scheme is based on an approach used by Prober et al. (1987) to prepare ddNTPs with fluorescent tags. Structures A, B, C and D below illustrate the types of fluorescent dNTPs that result from these synthetic approaches.”). Tsien describes Prober as “show[ing] enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and SequenaseTM.” Ex-1013 (Tsien) at 28:16-18 (“Prober et al. (1987) show enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and SequenaseTM.”) (emphasis added). Tsien is correct that Prober shows polymerase incorporation of 5-substituted cytosine analogues. Ex-1014 (Prober) at 337 (“The fidelity of terminator incorporation is maintained.”); *id.* at 340 (“The chain-terminators reported here are incorporated by both AMV reverse transcriptase and modified T7 DNA polymerase, but they are

not accepted by the Klenow fragment of DNA polymerase I from *Escherichia coli*.”). Prober discloses DNA polymerase maintains fidelity during incorporation of 5-labeled pyrimidine analogues. Ex-1014 (Prober) at 337, 340. A person of ordinary skill in the art would recognize that Prober’s statement that DNA polymerase maintains “fidelity” means that during a DNA polymerase reaction Prober’s nucleoside triphosphate analogues base pair with their complementary nucleotide according to standard Watson-Crick base pairing (that is the 5-substituted thymine analogue base pairs like an thymine to an adenine, and the 5-substituted cytosine analogue pairs like a cytosine to a guanine). *Supra* Paragraphs 44-45. Thus, Prober demonstrates that a 5-substituted cytosine nucleotide analogue forms hydrogen bonds with its complementary guanine nucleobase.

92. Tsien discloses that each nucleotide analogue should pair with its complementary nucleotide. A “key” feature of Tsien’s disclosure is “direct identification of the dNTPs as they are incorporated into the growing complementary DNA chain.” Ex-1013 (Tsien) at 7:19-24; *id.* at 28:31-35 (disclosing “proper base pairing during complementary chain growth”). Tsien discloses that four labeled and 3’-blocked dNTP analogues (A, T, G, and C analogues) are added to a reaction zone such that polymerase “bring[s] about addition of the one, and only the one, of the four labeled blocked dNTPs which is complementary to the first available template nucleotide following the primer.”

Ex-1013 (Tsien) at 12:22-27. A cytosine nucleotide analogue forms three hydrogen bonds with its complementary nucleotide, guanine. Ex-1042 (Watson & Crick) at 966; Ex-1011 (Alberts) at 99, Fig. 3-10. Tsien therefore discloses limitation v).

15. There was motivation to combine the disclosures within Tsien

93. This section discusses the motivation for combining the disclosures provided within Tsien.

94. Tsien discloses a nucleotide analogue having a fluorescent label attached via a cleavable linker to a base and a 3'-OH capping group. *Supra* Paragraphs 55-56. Tsien discloses that a 3'-O-allyl is an advantageous capping group as compared to groups cleavable by base hydrolysis (such as esters) because “deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.” Ex-1013 (Tsien) at 24:24-25:3. The full quote from Ex-1013 (Tsien) at 24:24-25:3 is provided below with emphases added:

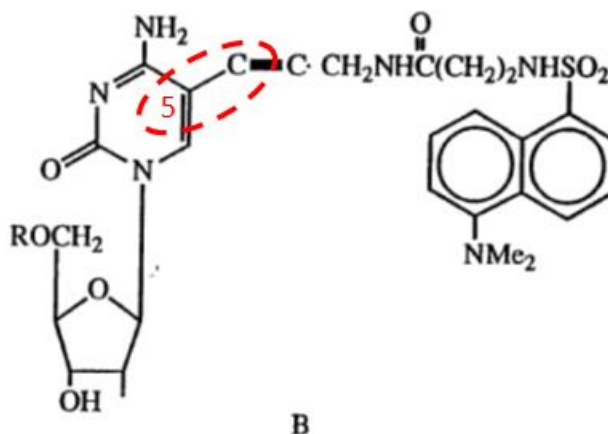
A wide variety of hydroxyl blocking groups are cleaved selectively using chemical procedures other than base hydrolysis. 2,4-Dinitrobenzenesulfonyl groups are cleaved rapidly by treatment with nucleophiles such as thiophenol and thiosulfate (Letsinger et al., 1964). **Allyl ethers are cleaved by treatment with Hg(II) in acetone/water** (Gigg and Warren, 1968). Tetrahydrothiofuranyl ethers are removed under neutral conditions using Ag(I) or Hg(II)

(Cohen and Steele, 1966; Cruse et al., 1978). These protecting groups, which are stable to the conditions used in the synthesis of dNTP analogues and in the sequence incorporation steps, **have some advantages** over groups cleavable by base hydrolysis - *deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.*

Tsien's disclosure of the desirability of the allyl protecting group would have motivated a person of ordinary skill in the art to select and use the 3'-O-allyl capping group on the nucleotide analogues disclosed by Tsien. Thus, Tsien discloses that the 3'-O-allyl group is a desirable protecting group to be used in Tsien's invention. Ex-1013 (Tsien) at 24:29-25:3. This disclosure provides express motivation to use a 3'-O-allyl group in Tsien's nucleotide analogues.

95. Tsien *itself* provides express motivation to use the allyl group, which is different from disclosures *outside of Tsien*, such as the 3'-O-azidomethyl group disclosed by Zavgorodny et al., Tetrahedron Letters, 32:7593-7596 (1991) (Ex-1043), which does not provide motivation for a person of ordinary skill in the art to combine Zavgorodny's disclosure with Tsien's methods for reasons I previously provided in IPR2013-00517, Exhibit 2011. Here, Tsien discloses the desirability of using the 3'-O-allyl group in Tsien's sequencing methods, which provides express motivation to use a 3'-O-allyl group in Tsien's methods.

96. Regarding 5-substituted cytosine, Tsien discloses Structure B, which is a cytosine analogue having a fluorescent label attached through a linker to the 5-position of the base:



Ex-1013 (Tsien) at 30 (annotated). Tsien discloses the advantages of labeling cytosine at the 5-position because it provides flexibility for the linker length and functionality. Ex-1013 (Tsien) at 29:14-19 (“Structures A, B, C and D below illustrate the type of fluorescent dNTPs that result from these synthetic approaches. The synthetic routes have a great flexibility in that the linker can be varied with respect to length or functionality. The terminal fluorescent moiety can also be varied according to need.”) (emphases added). It was known that attaching substituents to the 5-position of cytosine nucleotide analogues was advantageous because this position minimizes interference with incorporation of nucleotide analogues into a DNA strand by a DNA polymerase. For example, Hobbs discloses that alkynylamino linkers at the 5-position of pyrimidine nucleotide analogues are advantageous because “positioning the linker on the 5-position of

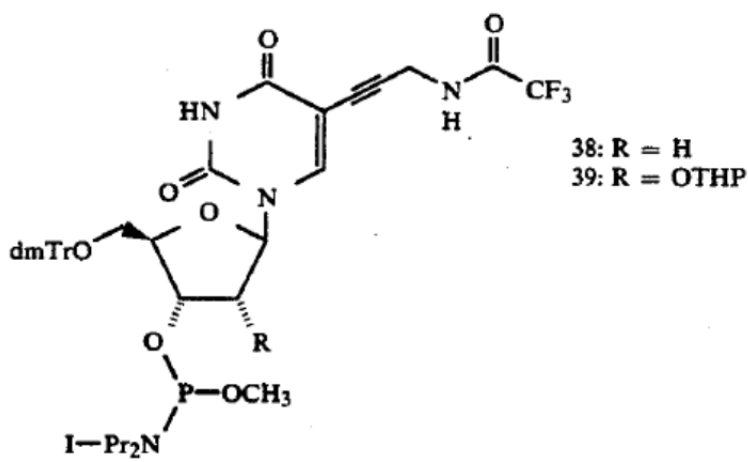
pyrimidine nucleotides and the 7-position of 7-deazapurine nucleotides eventually places the linker and reporter in the major groove when the nucleotide is incorporated into double-stranded DNA (this will serve to minimize interference with hybridization and other processes, which require that a double-stranded conformation be possible).” Ex-1029 (Hobbs) at 27:52-65. In addition, Tsien cites to Prober for “show[ing] enzymatic incorporation” of fluorescently labeled nucleotide analogues (Ex-1013 (Tsien) at 28:16-18), including cytosine analogues having a label attached via a linker of the 5-position of the base. Ex-1013 (Tsien) at 29:12-16 (discussing Structure B and citing Prober). Tsien is correct that Prober shows polymerase incorporation of 5-substituted and labeled cytosine analogues. Ex-1014 at 337, 340; *supra* Paragraphs 77, 91. Therefore a person of ordinary skill in the art would have been motivated to combine Tsien’s disclosures to arrive at a cytosine nucleotide analogue having a 3’-O-allyl group and a tag attached through a cleavable linker at the 5-position of the base.

16. There was a reasonable expectation of success

97. A person of ordinary skill in the art would have had a reasonable expectation of success of making 3’-capped 5-substituted cytosine nucleotide analogues having a label cleavably linked to the base. The synthetic process for preparing the nucleotide analogues disclosed by Tsien were understood to be within the level of ordinary skill. For example:

- **Hobbs** discloses preparing 5-substituted pyrimidine nucleotides with alkynylamino linkers:

The small size of the alkynylamino linkers and their location on the 5-position of pyrimidine nucleotides and the 7-position of 7-deazapurine nucleotides are expected to improve the performance of oligonucleotides containing them. An appropriately protected and activated monomer, (38), similar to one described by Ruth, could be prepared from commercially-available 5-iodo-2'-deoxyuridine by the Pd(0)/Cu(I) catalyzed attachment of an alkynylamino linker, followed by the selective dimethoxytritylation of the 5'-alcohol, and finally conversion to a 3'-phosphoramidite with chloro(diisopropylamino)-methoxyphosphine...



Alternatively, an alkynylamino linker could be used for biotin attachment or, in general, for the attachment of other reporters such as fluorescent dyes.

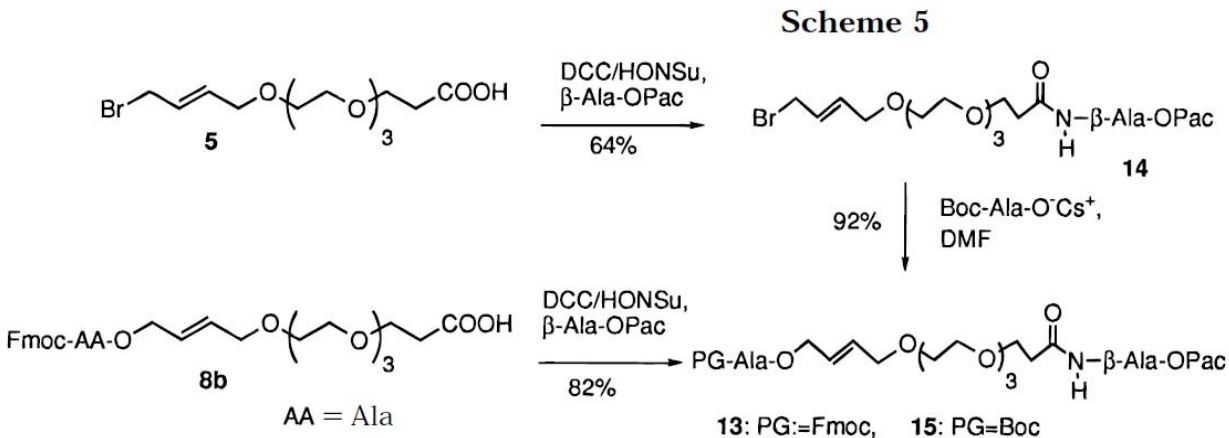
Ex-1029 (Hobbs) at 28:3-29:1.

- **Prober** discloses a general scheme for attaching labels to bases with alkynylamino linkers:

The synthetic scheme devised for these reagents [which include 5-substituted pyrimidines] is highly convergent and general. The preparation of T-526 is illustrative. 5-Iodo-2',3'-dideoxyuridine was coupled to N-trifluoroacetylpropargylamine under palladium(0) catalysis in dimethylformamide. The resulting derivatized nucleoside was converted to its 5-triphosphate and deacylated to afford 5-(3-amino-1-propynyl)-2',3'-dideoxyuridine triphosphate. This amine was coupled with an O-acetyl-protected form of the SF-526-sarcosine conjugate and deprotected to afford T-526.

Ex-1014 (Prober) at 337. A person of ordinary skill in the art would have used the procedures of Hobbs and Prober to make analogous dNTPs by using dNTPs as the starting compound instead of the ddNTPs of Prober as the starting compounds.

- **Seitz** discloses allyl linkers that are cleavable for attaching a fluorescent Fmoc group (compound 8b) to a molecule having an amine group (β -Ala-OPac):



Ex-1052 (Seitz) at 815 (Scheme 5 was modified to show the structure of compounds 5 and 8b). Tsien cites to Prober for its synthetic approach to produce fluorescent derivatives of thymine and cytosine analogues. Ex-1013 (Tsien) at 29:10-16, 28:5-18. Prober discloses a “highly convergent and general” synthetic scheme that produces fluorescent derivatives of thymine analogue T-526 and cytosine analogue C-519. *See* Ex-1014 (Prober) at 337 (“The synthetic scheme devised for these reagents is highly convergent and general. The preparation of T-526 is illustrative. 5-Iodo-2’,3’-dideoxyuridine was coupled to N-trifluoroacetyl**propargylamine** under palladium(0) catalysis in dimethylformamide. The resulting derivatized nucleoside was converted to its 5-triphosphate and deacylated to afford 5-(3-amino-1-propynyl)-2’,3’-dideoxyuridine triphosphate. **This amine** was coupled with an O-acetyl-protected form of the SF-526-sarcosine conjugate and deprotected to afford T-526.”) (emphases added). A person of ordinary skill in the art would have recognized the amine group disclosed by Seitz as being applicable to attach the allyl linker to the propargylamine group used by Prober at the 5-position of cytosine. Furthermore, the use of allyl linkers was common in the prior art. Ex-1051 (Greenberg 1998) at 4062-63 (disclosing allyl linkers and methods for attaching allyl linkers); Ex-1053 (Guillier Review 2000) at 2132-33 (disclosing allyl linkers and methods for attaching allyl linkers).

It was therefore known how to prepare 5-substituted cytosine nucleotide analogues with cleavable linkers.

98. With respect to 3'-O-allyl groups, Tsien cites to Gigg. Ex-1013 (Tsien) at 24:29-30 (“Allyl ethers are cleaved by treatment with Hg(II) in acetone/water (Gigg and Warren, 1968).”). Gigg provides exemplary methods for preparing compounds with allyl ethers, such as Compound I (*see* Ex-1046 (Gigg) at 1907), IV (*id.* at 1908), XIV (*id.* at 1909), XV (*id.* at 1909), XVI (*id.* at 1909), XVIII (*id.* at 1909), XXI (*id.* at 1908), XXIII (*id.* at 1906, n.20a,b), XXXV (*id.* at 1909), XXXVI (*id.* at 1910) and XXXIX (*id.* at 1910). In addition, Metzker describes the synthesis of a 3'-O-allyl nucleotide analogue. Ex-1016 (Metzker) at 4261.

99. In view of the discussion of the prior art provided in the above paragraphs, a person of ordinary skill in the art would have reasonably expected to make a 3'-O-allyl capped 5-substituted cytosine nucleotide analogue with a cleavable linker in accordance with Claim 1. A person of ordinary skill in the art would have expected the cytosine nucleotide analogue disclosed by Tsien would have been modified according to the disclosure of Tsien in such a way that the resulting nucleotide analogue would have met each of the recited limitations recited in Claim 1. *Supra* Paragraphs 60-92.

B. Claim 1 is obvious under a “Lead Compound” Analysis

1. The lead compound analysis

100. I understand that the first step in a “lead compound” analysis is identifying a lead compound. I understand that a lead compound is a compound that a person of ordinary skill in the art would have selected as a starting point for modification and development efforts directed towards obtaining a compound with desired properties. I understand that a lead compound need not be the single best compound in the prior art. I understand that there may be more than one compound that qualifies as a lead compound. I understand that a lead compound need not have been tested nor have demonstrated activity through comparative data.

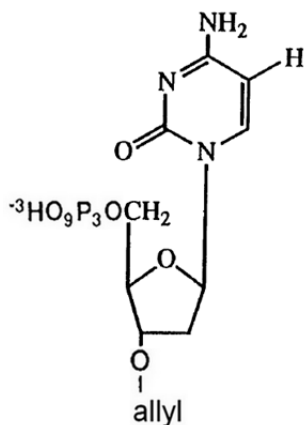
2. Tsien’s 3’-O-allyl dCTP is a Lead Compound

101. Tsien discloses that the 3’-O-allyl ether is one of three advantageous 3’-blocking groups (allyl, 2,4-dinitrobenzenesulfonyl, and tetrahydrothiofuranyl) used in the synthesis of dNTP analogues and in the sequence incorporation steps. Tsien specifically states:

A wide variety of hydroxyl blocking groups are cleaved selectively using chemical procedures other than base hydrolysis. 2,4-Dinitrobenzenesulfonyl groups are cleaved rapidly by treatment with nucleophiles such as thiophenol and thiosulfate (Letsinger et al., 1964). Allyl ethers are cleaved by treatment with Hg(II) in acetone/water (Gigg and Warren, 1968). Tetrahydrothiofuranyl ethers

are removed under neutral conditions using Ag(I) or Hg(II) (Cohen and Steele, 1966; Cruse et al., 1978). These protecting groups, which are stable to the conditions used in the synthesis of dNTP analogues and in the sequence incorporation steps, have some advantages over groups cleavable by base hydrolysis - deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.

Ex-1013 (Tsien) at 24:25-25:3 (emphases added). The “dNTP analogues” recited by Tsien refers to each of the four nucleotide analogues: dATP, dCTP, dGTP, and dTTP. Ex-1013 (Tsien) at 9:35-10:3 (“Deoxynucleotide triphosphates ‘dNTPs’ of these materials are abbreviated as dATP, dCTP, dGTP, and dTTP.”). Based on Tsien’s specific disclosure of 3’-O-allyl dNTP analogues, a person of ordinary skill in the art would recognize that Tsien discloses a 3’-O-allyl dCTP nucleotide analogue with as much clarity as if Tsien had drawn its structural formula or had written its name:



102. A person of ordinary skill in the art would have recognized Tsien’s 3’-

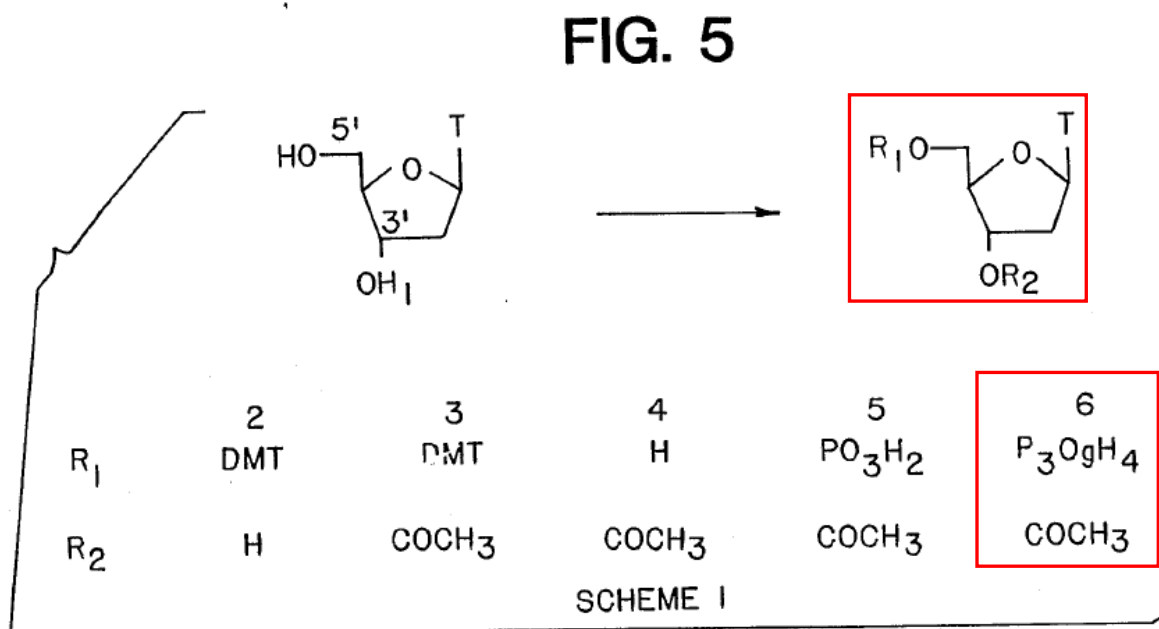
O-allyl dCTP disclosure as a natural starting point for further development efforts because Tsien discloses that the 3'-O-allyl blocking group is cleaved selectively using chemical procedures other than base hydrolysis, which has advantages over groups cleavable by base hydrolysis because deblocking occurs when the specific deblocking reagent is present and premature deblocking during incorporation is minimized. *See* Ex-1013 (Tsien) at 24:25-25:3 (“A wide variety of hydroxyl blocking groups are cleaved selectively using chemical procedures other than base hydrolysis. 2,4-Dinitrobenzenesulfonyl groups are cleaved rapidly by treatment with nucleophiles such as thiophenol and thiosulfate (Letsinger et al., 1964). Allyl ethers are cleaved by treatment with Hg(II) in acetone/water (Gigg and Warren, 1968). Tetrahydrothiofuranyl ethers are removed under neutral conditions using Ag(I) or Hg(II) (Cohen and Steele, 1966; Cruse et al., 1978). These protecting groups, which are stable to the conditions used in the synthesis of dNTP analogues and in the sequence incorporation steps, have some advantages over groups cleavable by base hydrolysis - deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.”) (emphases added). Based on Tsien’s disclosure, a person of ordinary skill in the art would have selected Tsien’s 3'-O-allyl dCTP nucleotide as a lead compound.

103. Other researchers were actually using 3'-O-allyl nucleotides as

promising lead compounds for DNA sequencing before 2000. Metzker used the 3'-O-allyl dATP nucleotide as a lead compound for DNA sequencing development and demonstrated that this nucleotide was incorporated by a DNA polymerase. Ex-1016 (Metzker) at 4263. This experimental validation of Tsien's 3'-O-allyl nucleotide analogues by researchers in the relevant field confirms that such analogues were considered to be lead compounds by those of skill in the art in the 1990s.

3. Tsien's 3'-O-acetyl dTTP also qualifies as a Lead Compound

104. Tsien discloses another lead compound: 3'-O-acetyl dTTP (compound 6). Ex-1013 (Tsien) at 22:1-16 (describing the synthesis of 3'-O-acetyl dTTP); *see also id.* at Fig. 5:



105. Tsien's 3'-O-acetyl dTTP would have been a natural starting point for further development efforts because it is specifically disclosed as a representative 3'-blocked nucleotide for use in Tsien's DNA sequencing methods. Ex-1013 (Tsien) at 21:32-22:3 ("These blocking materials in their fundamental forms have all been described in the literature as has their use as blockers in chemical DNA synthesis settings. Two representative blockers, esters and phosphate, can be incorporated into dNTP's as follows: The general procedure for synthesis of 3'-O-acetyl dNTPs is outlined in Reaction Scheme 1 set forth in Figure 5 for 3'-O-acetyl TTP.") (emphasis added); *see generally id.* at 21:1-20 (for a description of the general synthetic procedure). The 3'-O-acetyl dTTP that is shown in Figure 5 is provided as a "representative labeled nucleotide building block." *See* Ex-1013 (Tsien) at 9:4-7 ("Figures 5 through 8 are pictorial representations of chemical reaction sequences which can be used to synthesize representative labeled nucleotide building blocks for use in the practice of this invention.") (emphasis added). Based on Tsien's disclosure, a person of ordinary skill in the art would have selected Tsien's 3'-O-acetyl dTTP nucleotide as a lead compound.

106. Tsien discloses that the synthetic procedure for synthesis of 3'-O-acetyl dTTP is applicable to the other three bases, including 3'-O-acetyl dCTP. Ex-1013 (Tsien) at 22:17-20 ("Preparation of 3'-O-acetyl derivatives of dATP, dCTP, and dGTP follows the same general scheme [as dTTP], with additional

steps to protect and deprotect the primary amino functions (see below).”).

Therefore, a person of ordinary skill in the art would have been motivated to modify the 3'-O-acetyl dTTP compound to arrive at 3'-O-acetyl dCTP based on Tsien's disclosure.

107. A person of ordinary skill in the art would have been motivated to modify Tsien's 3'-O-acetyl ester group to a 3'-O-allyl ether group because Tsien discloses that the 3'-O-allyl ether is one of three capping groups that are advantageous over ester capping groups because it is “cleaved selectively using chemical procedures other than base hydrolysis ... [which has] some advantages over groups cleavable by base hydrolysis—deblocking occurs only when the specific deblocking reagent is present and premature deblocking during incorporation is minimized.” Ex-1013 (Tsien) at 24:25-25:3; *supra* Paragraph 94. A person of ordinary skill in the art would therefore have been motivated to select Tsien's 3'-O-acetyl nucleotide and modify this nucleotide with a 3'-O-allyl ether group to arrive at a 3'-O-allyl ether dCTP nucleotide analogue.

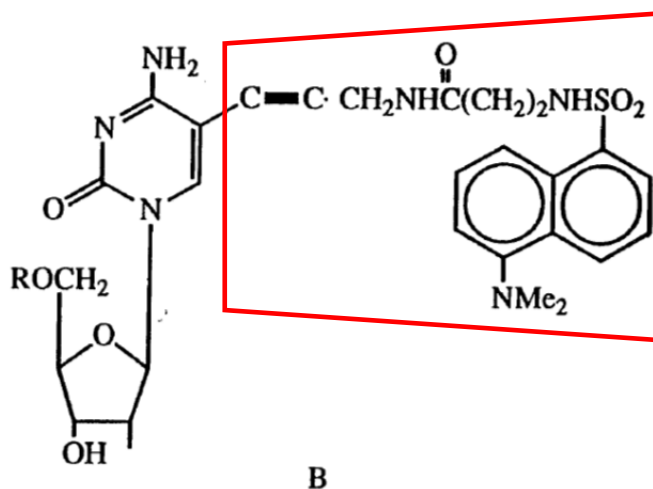
4. Motivation to use Tsien's 5-substituted cytosine base having a linker and label

108. A person of ordinary skill in the art would have been motivated to modify the 3'-O-allyl dCTP by substituting the cytosine base at the 5-position with a fluorescent label attached through a linker. Tsien discloses nucleotide analogues

having a linker attaching a fluorescent label to the base and a 3'-OH capping group.

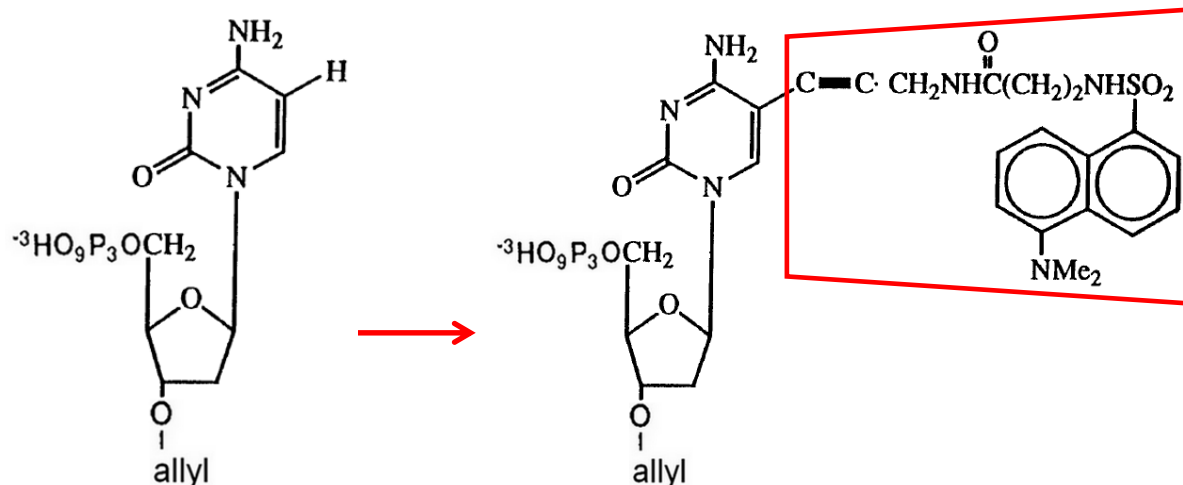
Supra Paragraphs 71-74, 60-64.

109. In addition, Tsien discloses a cytosine nucleotide analogue having a fluorescent label attached through a linker at the 5-position:



Ex-1013 (Tsien) at 30 (Structure B) (annotated). Tsien discloses the desirability of labeling cytosine at the 5-position. Ex-1013 (Tsien) at 29:14-19 (“Structures A, B, C and D below illustrate the type of fluorescent dNTPs that result from these synthetic approaches. The synthetic routes have a great flexibility in that the linker can be varied with respect to length or functionality. The terminal fluorescent moiety can also be varied according to need.”) (emphasis added). The advantages of 5-substituted cytosine bases were known in the art. Ex-1029 (Hobbs) at 27:52-65 (“positioning the linker on the 5-position of pyrimidine nucleotides and the 7-position of 7-deazapurine nucleotides eventually places the linker and reporter in the major groove when the nucleotide is incorporated into double-stranded DNA

(this will serve to minimize interference with hybridization and other processes, which require that a double-stranded conformation be possible)'). Tsien therefore provides encouragement to add a fluorescent label attached through a linker at the 5-position of the cytosine base. A person of ordinary skill in the art would therefore have been motivated to modify the 3'-O-allyl dCTP by substituting the base with Tsien's cytosine base having a fluorescent label attached through a linker at the 5-position as shown below:



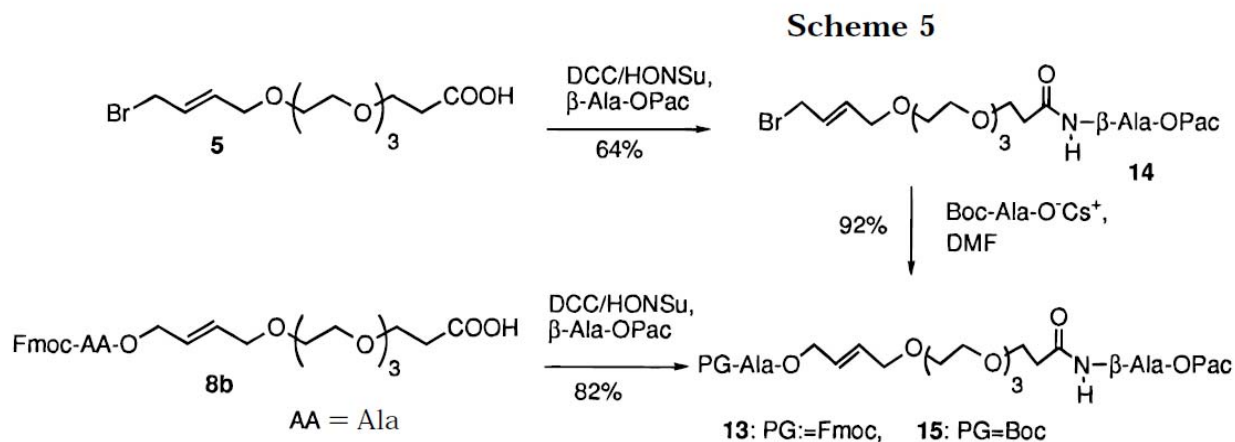
5. Motivation to use a cleavable allyl linker

110. A person of ordinary skill in the art would have been motivated to use a cleavable allyl linker to attach the fluorescent label to the 5-position of a cytosine nucleotide analogue. Tsien discloses a nucleotide having a cleavable linker attaching a fluorescent label to the base and a 3'-OH capping group. *Supra* Paragraphs 71-74, 60-64. Thus, a person of ordinary skill in the art would have

been guided to use the cleavable linker described in Tsien to attach the fluorescent label to the base on the nucleotide.

111. Tsien discloses a cleavable allyl linker as one of three exemplary cleavable linkers for attaching the fluorescent label to the base. Ex-1013 (Tsien) at 28:19-29 (disclosing allyl, silyl, and 2,4-dinitrophenylsulfenyl cleavable linkers); *see generally id.* at 27:35-28:35 (describing cleavable linkers); *supra* Paragraphs 60-64. A person of ordinary skill in the art would have been motivated to place Tsien's cleavable allyl linker on Tsien's 5-substituted cytosine base following Tsien's guidance to use a cleavable allyl linker on the base.

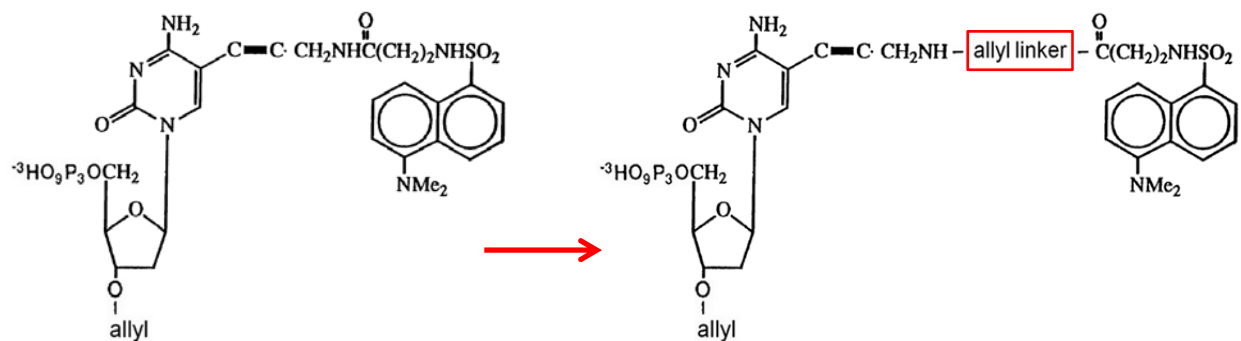
112. Allyl linkers were common in the prior art. For example, Seitz discloses a cleavable allyl linker for attaching a fluorescent Fmoc group (compound 8b) to a molecule having an amine group (β -Ala-OPac):



Ex-1052 (Seitz) at 815 (Scheme 5 was modified to show the structure of compounds 5 and 8b). Tsien cites to Prober for its synthetic approach to produce

fluorescent derivatives of cytosine analogues. Ex-1013 (Tsien) at 29:10-16, 28:5-18. Prober discloses a “highly convergent and general” synthetic scheme that produces fluorescent derivatives of thymine analogue T-526 and cytosine analogue C-519. *See* Ex-1014 (Prober) at 337 (“The synthetic scheme devised for these reagents is highly convergent and general. The preparation of T-526 is illustrative. 5-Iodo-2’,3’-dideoxyuridine was coupled to N-trifluoroacetyl**propargylamine** under palladium(0) catalysis in dimethylformamide. The resulting derivatized nucleoside was converted to its 5-triphosphate and deacylated to afford 5-(3-amino-1-propynyl)-2’,3’-dideoxyuridine triphosphate. **This amine** was coupled with an O-acetyl-protected form of the SF-526-sarcosine conjugate and deprotected to afford T-526.”) (emphases added). A person of ordinary skill in the art would have recognized the amine group disclosed by Seitz as being applicable to attach the allyl linker to the propargylamine group used by Prober at the 5-position of cytosine. Other examples of allyl linkers in the prior art include Greenberg (Ex-1051(Greenberg) at 4062-63) and the allyl linkers described in the Guillier review article. Ex-1053 (Guillier Review 2000) at 2132-33.

113. Following Tsien’s direction to use an allyl linker a person of ordinary skill in the art would have arrived at a cytosine deoxyribonucleotide analogue having a fluorescent group attached through a cleavable allyl linker to the 5-position of the base and a 3’-O-allyl capping group, as shown below:



This compound meets all limitations of Columbia’s claim for the same reasons set forth above in paragraphs 52-92.

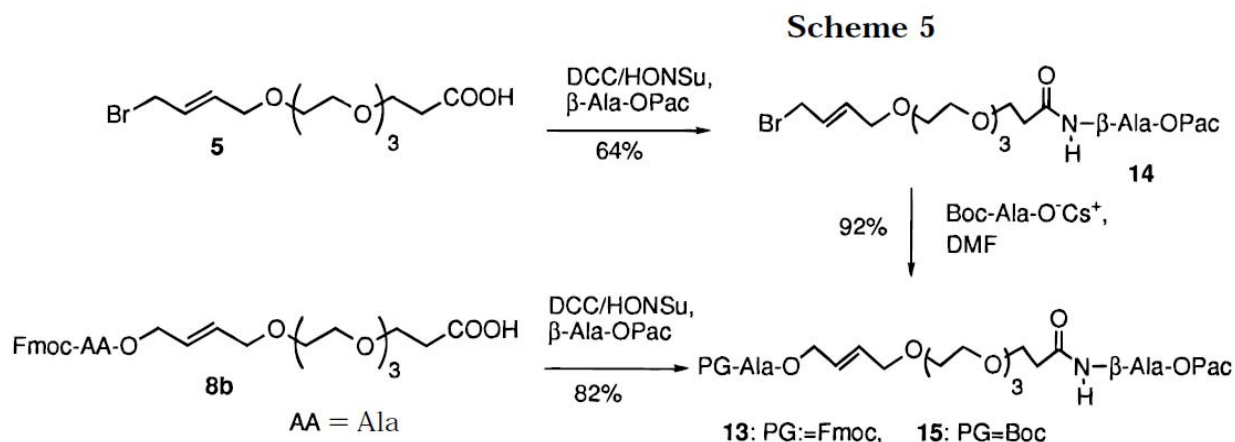
6. There was a reasonable expectation of success

114. A person of ordinary skill in the art would have had a reasonable expectation of success in making 3'-capped cytosine nucleotide analogues with a label cleavably linked to the 5-position of the base. The synthetic process for preparing the nucleotide analogues disclosed by Tsien were understood to be within the level of ordinary skill. *Supra* Paragraphs 97-98 (discussing Hobbs, Prober, and Seitz, Greenberg, and Guillier). It was therefore known how to prepare 5-substituted cytosine nucleotide analogues with cleavable linkers.

115. A person of ordinary skill in the art would have known how to attach a 3'-O-allyl ether based on Tsien’s disclosure. Tsien cites to Gigg for its disclosure of allyl ethers. Ex-1013 (Tsien) at 24:29-30 (“Allyl ethers are cleaved by treatment with Hg(II) in acetone/water (Gigg and Warren, 1968).”). Gigg provides exemplary methods for preparing compounds with allyl ethers, such as Compound I (*see* Ex-1046 (Gigg) at 1907), IV (*id.* at 1908), XIV (*id.* at 1909), XV (*id.* at

1909), XVI (*id.* at 1909), XVIII (*id.* at 1909), XXI (*id.* at 1908), XXIII (*id.* at 1906, n.20a,b), XXXV (*id.* at 1909), XXXVI (*id.* at 1910) and XXXIX (*id.* at 1910). In addition, Metzker describes the synthesis of 3'-O-allyl dATP. Ex-1016 (Metzker) at 4261.

116. Procedures for attaching to a fluorophore to a molecule of interest via a linker containing a cleavable allyl moiety were established in the prior art. For example, Seitz discloses a cleavable allyl linker for attaching a fluorescent Fmoc group (compound 8b) to a molecule having an amine group (β -Ala-OPac):



Ex-1052 (Seitz) at 815 (Scheme 5 was modified to show the structure of compounds 5 and 8b). Tsien cites to Prober for its synthetic approach to produce fluorescent derivatives of cytosine analogues. Ex-1013 (Tsien) at 29:10-16, 28:5-18. Prober discloses a “highly convergent and general” synthetic scheme that produces fluorescent derivatives of thymine analogue T-526 and cytosine analogue C-519. *See* Ex-1014 (Prober) at 337 (“The synthetic scheme devised for these

reagents is highly convergent and general. The preparation of T-526 is illustrative. 5-Iodo-2',3'-dideoxyuridine was coupled to N-trifluoroacetyl**propargylamine** under palladium(0) catalysis in dimethylformamide. The resulting derivatized nucleoside was converted to its 5-triphosphate and deacylated to afford 5-(3-amino-1-propynyl)-2',3'-dideoxyuridine triphosphate. **This amine** was coupled with an O-acetyl-protected form of the SF-526-sarcosine conjugate and deprotected to afford T-526.") (emphases added). A person of ordinary skill in the art would have recognized the amine group disclosed by Seitz as being applicable to attach the allyl linker to the propargylamine group used by Prober at the 5-position of cytosine. Other examples of allyl linkers in the prior art include Greenberg (Ex-1051 (Greenberg) at 4062-63) and the allyl linkers described in the Guillier review article. Ex-1053 (Guillier Review 2000) at 2132-33.

117. In summary, a person of ordinary skill in the art would have expected to successfully make nucleotide analogues using the procedures disclosed in the prior art. Also, a person of ordinary skill in the art would have expected the nucleotide analogue to meet each of the recited claim limitations, as previously discussed. *Supra* Paragraphs 60-92. For all these reasons, a person of ordinary skill in the art would have selected Tsien's 3'-O-allyl dCTP (or Tsien's 3'-O-acetyl dTTP) compound as a lead compound and would have been motivated to modify the compound based on Tsien's disclosures to make 3'-O-allyl 5-substituted

cytosine nucleotide analogues having a fluorophore attached to the base via cleavable allyl linker with a reasonable expectation of success in making and using such nucleotide analogues. Consequently, Claim 1 is obvious over Tsien under a “lead compound” analysis.

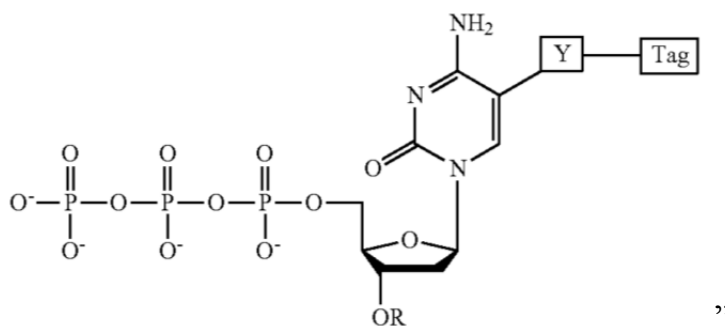
**VIII. GROUND 2: OBVIOUSNESS BASED ON DOWER
IN VIEW OF PROBER AND METZKER**

A. Claim 1 is Obvious Over Dower in view of Prober and Metzker

118. Dower in view of Prober and Metzker disclose all limitations of Claim 1 and render the claim obvious.

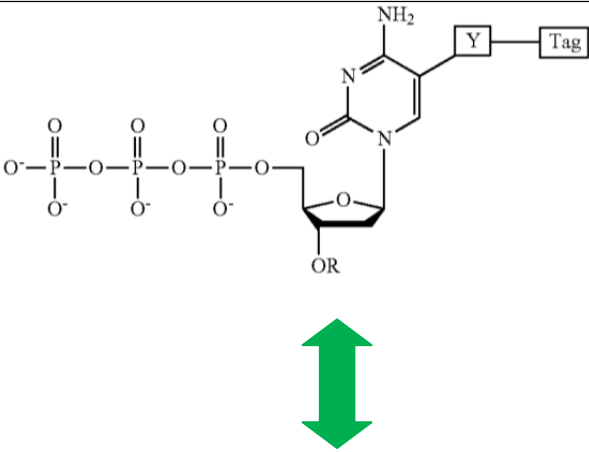
1. The structure depicted in Claim 1 is not new

119. The first part of Claim 1 recites: “A cytosine deoxyribonucleotide analogue having the structure:



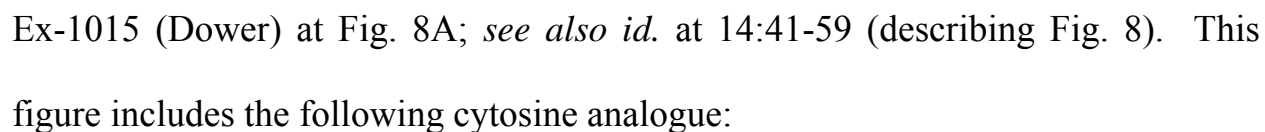
As previously discussed in paragraphs 52-53, this structure is fully described using standard nucleotide vocabulary as “a cytosine deoxyribose nucleoside triphosphate having an R group capping the 3'-OH group and a Tag attached through a linker (Y) at the 5-position of the base.”

120. I understand that, in IPR2012-00007, the Board held unpatentable Claim 21 of Columbia's U.S. 7,790,869 patent that claimed a cytosine deoxyribonucleotide having a 3'-OH capping group and a tag attached through a cleavable linker to the base. The equivalence between the nucleotide of Claim 21 and the present claim is self-evident in the following side-by-side comparison of the claims:

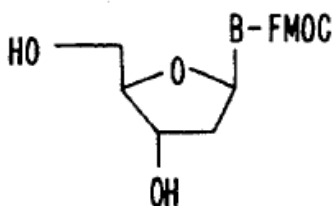
Presently claimed '358 nucleotide depiction with equivalent nomenclature	Unpatentable Claim 21 (along with Claim 12 from which it depends) from IPR2012-00007
 <p data-bbox="191 1333 776 1501">A cytosine deoxyribonucleotide having a 3'-OH capping group (R) and a Tag attached through a cleavable linker (Y) to the base.</p>	<p data-bbox="800 842 1421 976">21. The nucleotide of claim 12, wherein said nucleotide is an analog of adenosine, guanosine, <u>cytosine</u>, or thymidine.</p> <p data-bbox="800 1018 1421 1696">12. A nucleotide having a base that is attached to a detectable label through a cleavable linker, wherein the nucleotide has a <u>deoxyribose</u> comprising a cleavable chemical group capping the 3' OH group, wherein the cleavable linker is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light, and wherein the cleavable chemical group capping the 3' OH group is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.</p>

As shown above, Claim 1 from the '358 patent and Claim 21 from IPR2012-00007 are directed to the same cytosine deoxyribonucleotide having a 3'-OH capping

121. Dower discloses sequencing using deoxyribonucleoside triphosphate (dNTP) analogues. Figure 8A of Dower provides a diagram of its sequencing scheme:



Ex-1015 (Dower) at Fig. 8 (excerpted). This cytosine analogue is a nucleotide having a removable 3'-blocking group (indicated by B) and a label (indicated by the asterisk). Ex-1015 (Dower) at 15:1-3 ("Labeled and blocked monomers 86 are shown, the label depicted by the asterisk and the polymerization blocking groups indicated by B."); *see also id.* at 15:35-40 ("Usually, the nucleotide will be blocked at the 3' hydroxyl group where successive nucleotides would be attached. In contrast to a dideoxy nucleotide, typically the blocking agent will be a reversible blocking agent thereby allowing for deblocking and subsequent elongation."). The label indicated by the asterisk is "a removable moiety." Ex-1015 (Dower) at 14:56-59 ("This analog is also labeled with a removable moiety, e.g., a fluorescent label, so that the scanning system can detect the particular nucleotide incorporated after its addition to the polymerization primer."). Dower discloses a fluorescent label attached to the base. Ex-1015 (Dower) at 18:64-19:12 ("FIG. 9 schematically illustrates the synthesis of a generic protected nucleotide. A suitable nucleotide is labeled with the Fmoc fluorescently detectable label by reaction under the conditions described, e.g., in Ser. No. 624,114 filed Dec. 6, 1990, Fmoc-Cl and H₂O. A protection moiety will be added using conditions also described there."); *id.* at 5:35-37 ("FIG. 9 illustrates the synthesis of a representative nucleotide analog useful in the synthetic scheme. Note that the Fmoc may be attached to adenine, cytosine, or guanine."); *id.* at Fig. 9 (excerpted):



Dower therefore discloses a nucleotide having a removable 3'-capping group as well as a label removably linked to the base.

122. Dower cites to Prober for its disclosure of labeled nucleotide analogues of dATP, dCTP, dGTP, and dTTP, as follows:

Step 2: Fluorescent chain terminators (analogs of dATP, dCTP, dGTP, and [dT]TP, each labeled with fluorophore preferably emitting at a distinguishable wavelength) are added to the reaction at a sufficient concentration and under suitable reaction conditions (time, temperature, pH, ionic species, etc., See Sambrook et al. (1989) *Molecular Cloning*, vols. 1-3, and Prober et al.) to cause essentially all of the chains on the surface to be extended by one base and thereby terminated.

Ex-1015 (Dower) at 25:4-12 (emphases added); *id.* at 17:35-36 (indicating that “Prober et al.” refers to Ex-1014). Dower cites to Prober six times, which would have directed a person of ordinary skill in the art to consider Prober’s disclosure.

Ex-1015 (Dower) at 25:4-12, 25:44-47, 20:39-42, 23:16-26, 28:6-17, 17:35-36.

123. Prober discloses 5-substituted pyrimidines. Prober states:

The set of four fluorescence-tagged chain-terminating reagents we have designed and synthesized is shown in FIG. 2A. These are

ddNTP's to which succinylfluorescein has been attached via a linker to the heterocyclic base. (These reagents are designated N-xxx where N refers to the ddNTP and xxx refers to the SF-xxx portion.) The linker is attached to the 5 position in the pyrimidines and to the 7 position in the 7-deazapurines.

Ex-1014 (Prober) at 337 (emphases added); *see also id.* at 338, Fig. 2A (compounds T-526 and C-519). Prober's compound C-519 is shown in Fig. 2A, reproduced below:

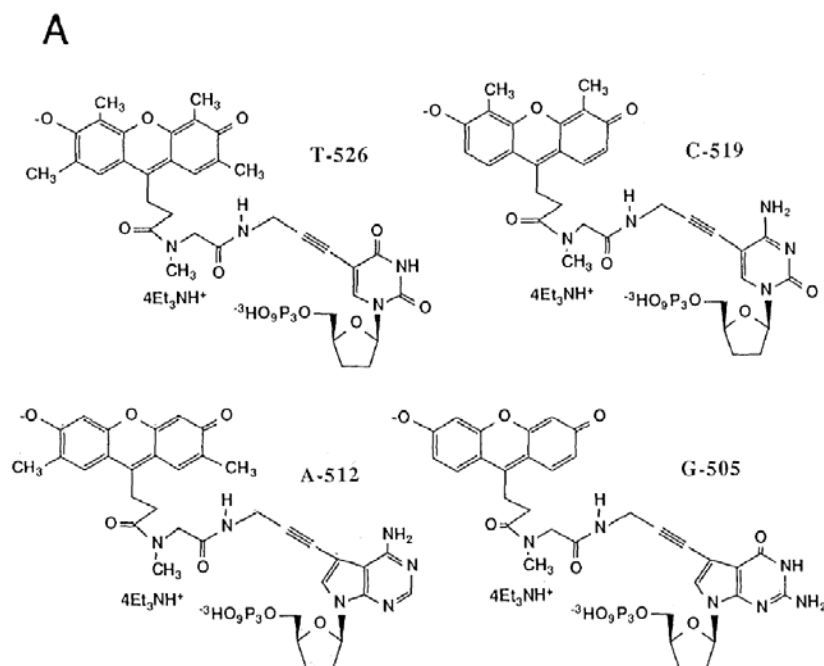


Fig. 2. Fluorescence-tagged chain-terminating reagents. **(A)** Chemical structures of the reagents used in modified dideoxy reactions for DNA sequencing.

124. In view of Dower's citations to Prober for labeling, a person of ordinary skill in the art reasonably would have considered Prober's cytosine nucleobase having a tag attached through a linker at the 5-position of the base would have been desirable for use in Dower's nucleotide analogues. *See*

Paragraphs 144, 146, 168-169 (below). Dower in view of Prober therefore renders obvious a cytosine deoxyribonucleotide having a 3'-OH capping group and a tag attached through a linker at the 5-position, which is the nucleotide analogue depicted in Claim 1.

2. Limitation R(a)

a. Dower discloses R(a)

125. Dower contains disclosures that meet limitation R(a) of Claim 1, which recites: “wherein R (a) represents a small, chemically cleavable, chemical group capping the oxygen at the 3' position of the deoxyribose of the deoxyribonucleotide analogue.”

126. Dower discloses “deoxynucleotide triphosphates with small blocking groups” on the 3'-OH. Ex-1015 (Dower) at 25:48-51 (emphasis added). Dower discloses that such small groups are removed by treatment with pH and other chemical reagents. Ex-1015 (Dower) at 25:15-18 (“The chain termination should be reversible by some means, such as treatment with light, heat, pH, certain other chemical or biological (enzymatic) reagents, or some combination of these.”) (emphases added). Thus, Dower discloses a small chemically cleavable capping group.

127. Dower was filed in 1990 and provided blocking groups that were considered small at that time: “acetyl, tBOC, NBOC, and NVOC.” Ex-1015

(Dower) at 25:48-51. The crystal structure of DNA polymerase was published in 1994 by Pelletier. Ex-1021 (Pelletier) at 1897 (Table 3), 1903 (note 101); *supra* Paragraph 40-41, 62. The crystal structure showed that the polymerase active site is tightly packed where the 3'-group of a nucleotide substrate must be accommodated. The crystal structure thus showed that the 3'-group on a nucleotide substrate should be small to fit within the spatial constraints of the polymerase active site. Thus, the crystal structure confirmed Dower's disclosure that the 3'-capping group should be "small," and Dower's disclosure meets limitation R(a).

b. Metzker also discloses R(a)

128. Metzker discloses 3'-ether capping groups—including the 3'-O-allyl ether group—for sequencing DNA. Ex-1016 (Metzker) at 4261 (compound [3]). Metzker reported that nucleotides having ether capping groups were advantageous because they are incorporated by polymerases. Ex-1016 (Metzker) at 4265 ("First, the protecting groups containing ether linkages at the 3'-position, compounds [1], [3], [4], [7], and [8] were incorporated by some of the polymerases."). Metzker also explored ester capping groups. Ex-1016 (Metzker) at 4260-61. None of the ester-protected nucleotide analogues served as DNA substrates. Ex-1016 (Metzker) at 4265 ("With the exception of inhibition of Vent_R[®] (exo-) DNA synthesis by 3'-O-acyl-dATP [2], those substrates containing ester linkages at the

3'-position, i.e., compounds [2], [5], and [6] were not accepted for any of the polymerases examined here.”); *id.* at 4263, Table 2 (indicating that 3'-O-acyl-dATP [2] exhibits “Inhibition” which reduces DNA synthesis in a nonspecific manner). Based on Metzker’s results, a person of ordinary skill in the art would have been motivated to use Metzker’s ether capping groups, such as the 3'-O-allyl ether, especially because the 3'-O-allyl ether dATP was successfully incorporated by a DNA polymerase.

129. A person of ordinary skill in the art would have keyed into Metzker’s 3'-O-allyl group for two important reasons: (1) it was shown to be incorporated by a polymerase (which indicates that the allyl group is appropriately sized to fit within the active site, and thus functionally fulfills Dower’s disclosure that the 3'-blocking group should be small), and (2) it was known to be chemically cleavable to give a 3'-OH (and thus fulfills Dower’s disclosure that the 3'-blocking group should be cleavable).

130. Metzker demonstrated that a 3'-O-allyl substituted nucleotide was incorporated during a polymerase reaction. Ex-1016 (Metzker) at 4263, Table 2. Thus, Metzker demonstrated that the 3'-O-allyl group is, in fact, appropriately sized to fit within the polymerase active site. Metzker was submitted for publication on June 3, 1994, which was 21 days before Pelletier published the polymerase crystal structure on June 24, 1994. Ex-1016 (Metzker) at 4259; Ex-

1021 (Pelletier) at 1891. The crystal structure published by Pelletier showed that the polymerase active site is tightly packed in the area that binds to the 3'-group of a nucleotide substrate. Metzker and Pelletier therefore provide converging evidence confirming Dower's disclosure that the 3'-O-capping group should be small.

131. Metzker's 3'-O-allyl capping group was known to be advantageous because it is chemically removable to generate a 3'-OH by treatment with palladium. Ex-1035 (Boss) at 559, Table 1; Ex-1036 (Qian) at 2184 ("The O-allyl group was then removed using PdCl₂, providing [the deprotected compound] in quantitative yield."). Metzker's 3'-O-allyl group therefore meets limitation R(a).

3. Limitation R(b)

132. Dower and Metzker contain disclosures that meet limitation R(b) of Claim 1, which recites: "wherein R ... (b) does not interfere with recognition of the analogue as a substrate by a DNA polymerase."

133. Dower discloses primer extension using a DNA polymerase to incorporate a fluorescently labeled dCTP analogue with a 3'-OH blocking group. For example, Dower states:

Step 1: A DNA-dependent, DNA polymerase such as those used for conventional DNA sequencing, for example, Klenow fragment of E.coli DNA Pol, SequenaseTM (modified T7 DNA polymerase), Taq (Thermus aquaticus) DNA polymerase, Bst (Bacillus

stearothermophilus), DNA polymerase, reverse transcriptase (from AMV, MMLV, RSV, etc.) or other DNA polymerases, and the reaction components appropriate to the particular DNA polymerase selected, are placed in the incubation chamber in direct contact with the surface.

Step 2: Fluorescent chain terminators (analogues of dATP, dCTP, dGTP, and [dT]TP, each labeled with fluorophore preferably emitting at a distinguishable wavelength) are added to the reaction at a sufficient concentration and under suitable reaction conditions (time, temperature, pH, ionic species, etc., See Sambrook et al. (1989) Molecular Cloning, vols. 1-3, and Prober et al.) to cause essentially all of the chains on the surface to be extended by one base and thereby terminated. Detection of the specific label thereby incorporated into each chain identifies the last base added at each positional address in the matrix.

Step 3: The chain termination should be reversible by some means, such as treatment with light, heat, pH, certain other chemical or biological (enzymatic) reagents, or some combination of these. Typically the chain termination results from a blocking moiety which is labile to mild treatment. By one of these means, the blocked 3'OH of the terminating base must be made available for chain extension in the next round of polymerization.

Ex-1015 (Dower) at 24:61-25:22 (emphases added).

134. Dower also discloses the label and 3'-blocking group “are selected to be compatible with efficient incorporation into the growing chains by the particular DNA polymerase(s) chosen to catalyze extension.” Ex-1015 (Dower) at 26:6-9; *id.*

at 18:14-16 (nucleotide analogues “should be compatible with the selected polymerase”); *id.* at 15:62-65 (“Nucleotide analogs used as chain-terminating reagents will typically have both a labeling moiety and a blocking agent while remaining compatible with the elongation enzymology.”). A person of ordinary skill in the art would understand that polymerase incorporation of Dower’s dNTP analogues involves recognition by the polymerase. Ex-1011 (Alberts) at 247 (Fig. 6-36), 251-252.

135. In addition, Metzker demonstrated that a 3'-O-allyl substituted nucleotide was incorporated (and thereby recognized) as a substrate by a DNA polymerase. Ex-1016 (Metzker) at 4263, 4265. Dower and Metzker therefore disclose limitation R(b).

4. Limitations R(c) and wherein the 3'-oxygen and R bond is stable

136. Dower and Metzker contain disclosures that meet limitation R(c) of Claim 1, which recites: “wherein R ... (c) is stable during a DNA polymerase reaction.” Dower and Metzker also contain disclosures that meet the limitation of Claim 1 that recites: “wherein the covalent bond between the 3'-oxygen and R is stable during a DNA polymerase reaction.”

137. Dower discloses sequencing using labeled and 3'-blocked nucleotides that are incorporated, detected, and then deblocked so that the 3'-OH is “made available for chain extension in the next round of polymerization.” Ex-1015

(Dower) at 25:4-22. The 3'-blocking group prevents polymerase extension until the 3'-OH is made available by appropriate treatment. Ex-1015 (Dower) at 25:4-22. Dower's discussion of the 3'-blocking group preventing extension until the 3'-OH is made available by appropriate treatment underscores the overall stability of the 3'-blocking group during polymerase incorporation. This overall stability of Dower's 3'-blocking group includes the entire 3'-blocking group, including its linkage to the 3'-oxygen atom. Thus, the overall stability of Dower's 3'-blocking group includes stability at the bond between the 3'-oxygen and the rest of the blocking group (otherwise it would not function as a blocking group). Dower therefore discloses 3'-capping groups that are stable during the polymerase reaction, including stable at the 3'-oxygen and R bond.

138. Metzker states that the capped nucleotides should be "stable during the polymerase phase" of sequencing. Ex-1016 (Metzker) at 4259 ("The complete scheme demands nucleotide analogs that are tolerated by polymerases, spectroscopically distinct for each base, stable during the polymerization phase, and deprotected efficiently under mild conditions in aqueous solution.") (emphasis added). Metzker demonstrated that the 3'-O-allyl group was incorporated and stable during a polymerase reaction. Ex-1016 (Metzker) at 4263, 4265 (demonstrating 3'-O-allyl dATP was incorporated by polymerase and terminated the growing strand). Termination in Metzker's experiment relied upon polymerase

incorporation of the 3'-O-allyl protected nucleotide, and the 3'-O-allyl protecting group was stable because the nucleotide caused “termination.” That is, “termination” in Metzker’s experiment means that the incorporated nucleotide analogue did not degrade and stably blocked further polymerase incorporation throughout the experiment. Ex-1016 (Metzker) at 4263, Table 2 (legend). The stability demonstrated by Metzker included stability at the bond between the 3'-oxygen and the rest of the capping group as reflected by the “termination” of the growing strand. Ex-1016 (Metzker) at 4263.

139. Dower and Metzker therefore disclose limitations R(c) and that the 3'-oxygen to R bond is stable during a polymerase reaction.

5. Limitations R(d) and wherein OR is not methoxy or ester

140. Metzker contains disclosures that meet limitation R(d) of Claim 1, which recites: “wherein R ... (d) does not contain a ketone group.” Metzker also contains disclosures that meet the limitation of Claim 1 that recites: “wherein OR is not a methoxy group or an ester group.”

141. The 3'-O-allyl capping group disclosed by Metzker (Ex-1016 (Metzker) at 4261, compound [3]) does not contain a ketone group, as shown below:

$\text{-CH}_2\text{-CH=CH}_2$ allyl Ex-1039 (Vollhardt) at 502-504	-C(=O)- ketone Ex-1039 (Vollhardt) at 43
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and it is not a methoxy or an ester group, as shown below:

-O-CH ₂ -CH=CH ₂ -O-allyl	-O-CH ₃ methoxy	-O-C(=O)-CH ₃ an ester
--	-------------------------------	--------------------------------------

6. Limitation “wherein tag represents a detectable fluorescent moiety”

142. Dower and Prober contain disclosures that meet the limitation of Claim 1 that recites: “wherein tag represents a detectable fluorescent moiety.”

143. Dower discloses that the cytosine nucleotide analogue shown in Figure 8 is “labeled with a removable moiety, e.g., a fluorescent label, so that the scanning system can detect the particular nucleotide incorporated after its addition to the polymerization primer.” Ex-1015 (Dower) at 14:46-59. Dower further discloses that a fluorescent moiety is the preferred label form. Ex-1015 (Dower) at 20:50-53 (“Although many different labels may be devised including enzyme linked immunosorbent assays (ELISA), spectrophotometric labels, light producing or other-labels, a fluorescent moiety is the preferred form.”) (emphasis added).

144. Dower cites to Prober for labeled nucleotide analogues. Ex-1015 (Dower) at 25:4-12, 25:44-47, 20:39-42, 23:16-26; *supra* Paragraph 122. Prober discloses “[t]he set of four fluorescence-tagged chain-terminating reagents” in which “succinylfluorescein has been attached via a linker to the heterocyclic base ... [t]he linker is attached to the 5-position in the pyrimidines”. Ex-1014 (Prober) at 337-338, Fig. 2A; *supra* Paragraphs 58, 123. Prober therefore discloses

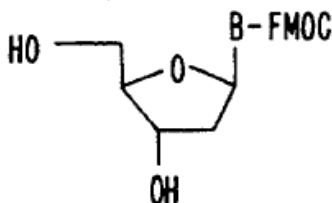
labeling the base of nucleotide analogues, including a cytosine analogue, with a fluorescent label. Dower and Prober therefore disclose this limitation.

7. Limitation Y is a “chemically cleavable, chemical linker”

145. Dower in view of Prober contain disclosures that meet the limitation of Claim 1 that recites: “wherein Y represents a chemically cleavable, chemical linker.”

146. Dower cites to Prober for labeled nucleotide analogues. Ex-1015 (Dower) at 25:4-12, 25:44-47, 20:39-42, 23:16-26; *supra* Paragraphs 122, 144. Prober’s label is attached via a linker to the 5-position of cytosine. Ex-1014 (Prober) at 337-38, Fig. 2A; *supra* Paragraphs 58, 123. A person of ordinary skill in the art following Dower’s citation to Prober would have been motivated to use a base labeled with a fluorescent tag, as disclosed by Prober. Dower discloses “[o]ne important functional property of the [dNTP] monomers is that the label be removable.” Ex-1015 (Dower) at 15:52-53. Thus, one of Dower’s preferred embodiments is a cleavably linked label. Dower specifies that the label is linked to the base and can be cleaved “chemical[ly], using acid, base, or some other, preferably mild, reagent.” Ex-1015 (Dower) at 21:32-40; *see also id.* at 15:52-59 (“One important functional property of the monomers is that the label be removable. The removal reaction will preferably be achieved using mild conditions. Blocking groups sensitive to mild acidic conditions, mild basic

conditions, or light are preferred. The label position may be anywhere on the molecule compatible with appropriate polymerization, i.e., complementary to the template, by the selected polymerase.”); *id.* at 21:35-40 (“Then, a reaction is performed removing the labeled terminal nucleotides from all of the polymers. This removal may be either enzymatic, using a phosphatase, an exonuclease or other similar enzyme, or chemical, using acid, base, or some other, preferably mild, reagent.”); *id.* at 5:35-37 (“FIG. 9 illustrates the synthesis of a representative nucleotide analog useful in the synthetic scheme. Note that the FMOC may be attached to adenine, cytosine, or guanine.”); *id.* at Fig. 9 (excerpted):



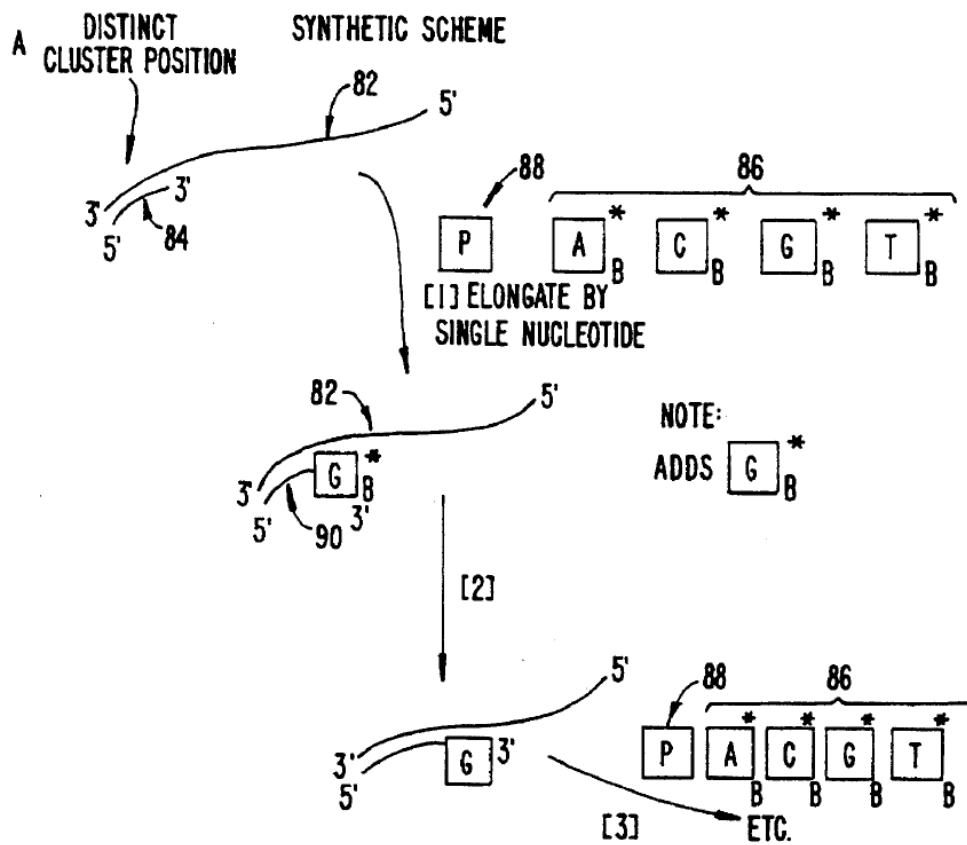
Dower in view of Prober therefore discloses a chemically cleavable linker at the 5-position of cytosine.

8. Limitations Y(a) and Y(b)

147. Dower in view of Prober contain disclosures that meet limitation Y(a) of Claim 1, which recites: “wherein Y ... (a) does not interfere with recognition of the analogue as a substrate by a DNA polymerase.” Dower in view of Prober also contain disclosures that meet limitation Y(b) of Claim 1, which recites: “wherein Y ... (b) is stable during a DNA polymerase reaction.”

148. Dower discloses that the linked label “does not interfere with the nucleotide-specific chemistry or enzymology” (Ex-1015 (Dower) at 20:47-49) and is “compatible with appropriate polymerization” by a DNA polymerase. Ex-1015 (Dower) at 15:56-59 (“The label position may be anywhere on the molecule compatible with appropriate polymerization, i.e., complementary to the template, by the selected polymerase.”). Thus, Dower discloses that the linked label should not interfere with the polymerase enzyme, which includes not interfering with the polymerase recognizing the nucleotide analogue substrate.

149. Dower’s Figure 8 shows that Reaction 1 involves a labeled and blocked monomer 86 that is used to elongate a primer by a compatible polymerase 88. Ex-1015 (Dower) at 15:3-5; *id.* at Fig. 8A:



Then, “Step 2 is a scan, where the signal at the position corresponding to template 82 indicates that the guanosine analog was incorporated.” Ex-1015 (Dower) at 15:11-13. This is followed by Reaction 2, “which removes both the label and blocking group.” Ex-1015 (Dower) at 15:13-14. If the linker attaching the label to the base were unstable, the label would be lost before the scan in Step 2, which would preclude detection of the incorporated nucleotide, the very purpose of the method. Therefore, Dower’s linker must be stable during the polymerase Reaction 1 for Dower’s reaction scheme shown in Fig. 8 to work as intended. Dower therefore discloses that the linker used to attach the label to the base should be stable during the polymerase reaction.

150. Dower cites to Prober for labeled nucleotide analogues. Ex-1015 (Dower) at 25:4-12, 25:44-47, 20:39-42, 23:16-26. Prober discloses a label attached through a linker to the 5-position of cytosine. Ex-1014 (Prober) at 337-38, Fig. 2A; *supra* Paragraphs 58, 123, 146. Prober demonstrated that a DNA polymerase recognized and incorporated a cytosine nucleotide analogue having a linker at the 5-position that is stable during a polymerase reaction. Ex-1014 (Prober) at 337, 340; *supra* Paragraphs 77, 91.

151. Dower in view of Prober therefore disclose limitations Y(a) and Y(b).

9. Limitation i)

152. Dower in view of Metzker and Prober contain disclosures that meet limitation i) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue: i) is recognized as a substrate by a DNA polymerase.”

153. Dower discloses that a fluorescently labeled reversible chain termination dCTP analogue with 3'-OH blocking group is incorporated by a DNA polymerase. Ex-1015 (Dower) at 24:61-25:22; *supra* Paragraphs 133-134. Dower also discloses the label and 3'-OH blocking group “are selected to be compatible with efficient incorporation into the growing chains by the particular DNA polymerase(s) chosen to catalyze extension.” Ex-1015 (Dower) at 26:6-9. Dower’s nucleotide analogues “typically have both a labeling moiety and a blocking agent while remaining compatible with the elongation enzymology” (Ex-

1015 (Dower) at 15:62-65) and “should be compatible with the selected polymerase.” Ex-1015 (Dower) at 18:14-16. A person of ordinary skill in the art would understand that polymerase incorporation and polymerase compatibility of Dower’s nucleotide analogues involves recognition by the polymerase. Ex-1011 (Alberts) at 247 (Fig. 6-36), 251-252.

154. Metzker discloses that a 3'-O-allyl nucleotide analogue was incorporated by a DNA polymerase. Ex-1016 (Metzker) at 4263 (Table 2), 4265.

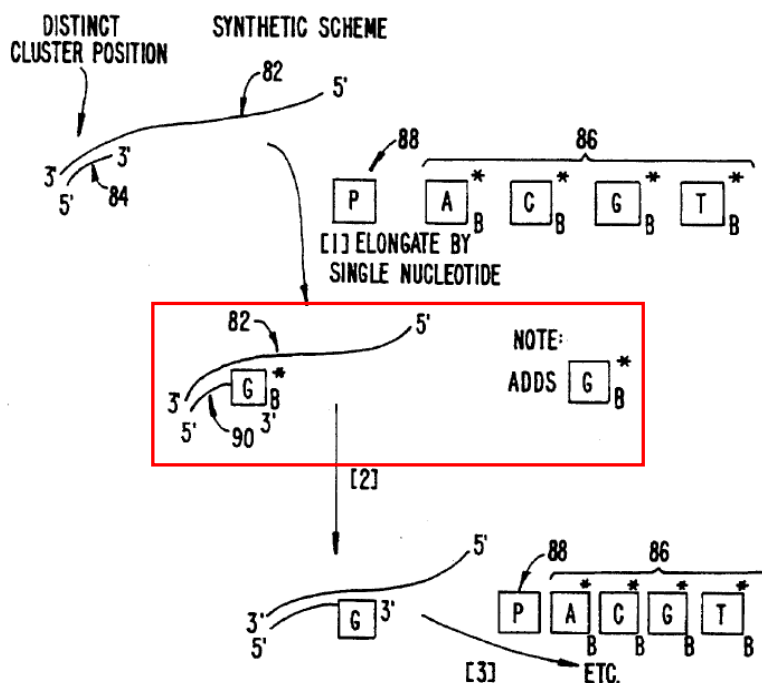
155. Prober discloses that a DNA polymerase incorporates 5-substituted cytosine analogues. Ex-1014 (Prober) at 340 (“The chain-terminators reported here are incorporated by both AMV reverse transcriptase and modified T7 DNA polymerase, but they are not accepted by the Klenow fragment of DNA polymerase I from *Escherichia coli*.”). Fluorescently labeled bases, including 5-labeled pyrimidines and 7-substituted 7-deaza-adenine nucleotides were known to be substrates of numerous polymerases. Vent polymerase was shown to incorporate 5-labeled pyrimidines and 7-substituted 7-deaza-adenine nucleotides. Ex-1041 (Livak) at 4833 (disclosing 5-substituted pyrimidine nucleotides, compounds 1a-1d, “were found to be fairly good substrates for Tli DNA polymerase”); *id.* at 4832-33 (disclosing that Tli DNA polymerase is Vent DNA polymerase). Metzker demonstrated that 3'-O-allyl substituted nucleotides are recognized as substrates for the exonuclease deficient variant of the same Vent

polymerase. Ex-1016 (Metzker) at 4263. The nucleotide analogues incorporated by the Vent exonuclease proficient polymerase in Livak would be expected to be incorporated at least as well (or even better than) by the exonuclease deficient variant of the same enzyme used by Metzker. *Supra* Paragraph 77. Dower in view of Metzker and Prober therefore discloses limitation i).

10. Limitation ii)

156. Dower in view of Metzker and Prober contain disclosures that meet limitation ii) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... ii) is incorporated at the end of a growing strand of DNA during a DNA polymerase reaction.”

157. As discussed *supra* in paragraphs 132-135 and 147-150, Dower discloses DNA polymerase recognition and incorporation of nucleotide analogues having a small chemically cleavable 3'-capping group and a chemically cleavable linker attached to the base. This incorporation is depicted in Dower's Figure 8A:



Ex-1015 (Dower) at Figure 8A (annotated). In Figure 8A, polymerase 88 adds a labeled and blocked dGTP analogue to the 3'-end of the growing strand of DNA, as stated by Dower:

Labeled and blocked monomers 86 are shown, the label depicted by the asterisk and the polymerization blocking groups indicated by B. A compatible polymerase 88 which can elongate the primer with the labeled blocked monomers 86 is used in reaction 1. In the preferred embodiment, the separate labeled monomers can be distinguished from one another by the wavelength of fluorescent emission.

In the example illustrated, a labeled blocked guanosine monomer has been incorporated into the elongated primer 90.

Ex-1015 (Dower) at 15:1-10. Dower also discloses the addition of dCTP analogues, which are likewise incorporated at the 3'-end. Ex-1015 (Dower) at 25:4-11 (Step 2: Fluorescent chain terminators (analogues of dATP, dCTP, dGTP,

and [dT]TP, each labeled with fluorophore preferably emitting at a distinguishable wavelength) are added to the reaction at a sufficient concentration and under suitable reaction conditions (time, temperature, pH, ionic species, etc., See Sambrook et al. (1989) Molecular Cloning, vols. 1-3, and Prober et al.) to cause essentially all of the chains on the surface to be extended by one base and thereby terminated.”) (emphases added); *see also id.* at Fig. 8 (excerpted):



158. Metzker discloses that a 3'-O-allyl nucleotide analogue was incorporated by a DNA polymerase. Ex-1016 (Metzker) at 4263, 4265. Prober discloses DNA polymerase incorporation of 5-substituted cytosine. Ex-1014 (Prober) at 337, 340; *supra* Paragraphs 77, 91. It was known that DNA polymerases only incorporate nucleotides at the end of a growing DNA strand during a DNA polymerase reaction, and that end is always the 3'-end (i.e., the end containing the 3'-OH). Ex-1011 (Alberts) at 252; Ex-1055 (Stryer) at 89 (“Elongation of the DNA chain proceeds in the 5'→3' direction.”). Dower in view of Metzker and Prober therefore disclose limitation ii).

11. Limitation iii)

159. Dower and Metzker contain disclosures that meet limitation iii) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... iii) produces a 3'-OH group on the deoxyribose upon cleavage of R.”

160. Dower discloses that 3'-blocking groups are cleaved by treatment with pH and other chemical reagents. Ex-1015 (Dower) at 25:15-18 (“The chain termination should be reversible by some means, such a treatment with light, heat, pH, certain other chemical or biological (enzymatic) reagents, or some combination of these.”) (emphases added). Dower discloses that the removal conditions make the 3'-OH on the terminating base available for the next round of polymerization. Ex-1015 (Dower) at 25:19-22 (“By one of these means, the blocked 3'OH of the terminating base must be made available for chain extension in the next round of polymerization.”).

161. Metzker's 3'-O-allyl capping group was known to be chemically cleaved to produce a 3'-OH group. Ex-1035 (Boss) at 559, Table 1; Ex-1036 (Qian) at 2184 (“The O-allyl group was then removed using PdCl₂, providing [the deprotected compound] in quantitative yield.”). Therefore, Dower and Metzker disclose limitation iii).

12. Limitation iv)

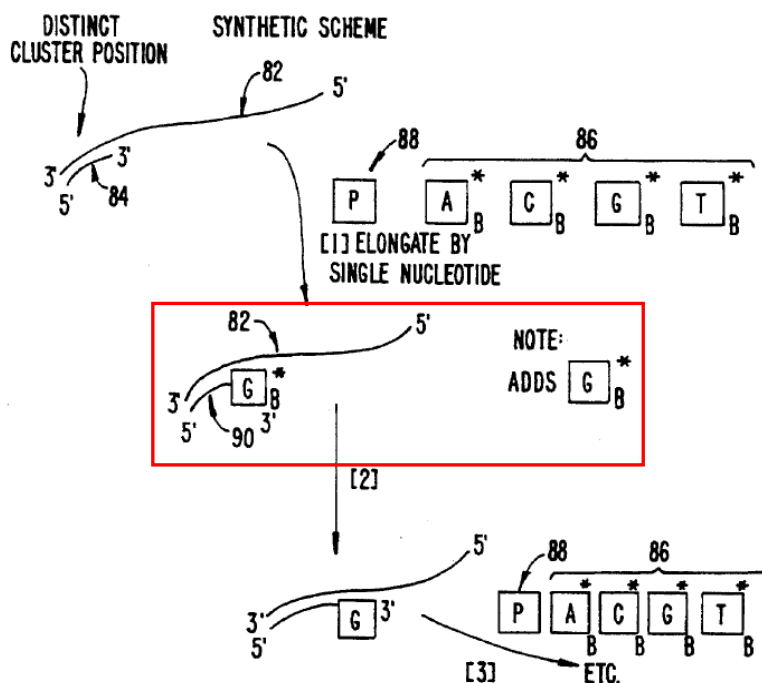
162. Dower contains disclosures that meet limitation iv) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... iv) no longer includes a tag on the base upon cleavage of Y.”

163. As discussed in paragraphs 142-146, Dower discloses a label cleavably linked to the base. Dower discloses “[o]ne important functional property of the [dNTP] monomers is that the label be removable. The removal reaction will preferably be achieved using mild conditions.” Ex-1015 (Dower) at 15:52-56; *id.* at 21:32-40. Dower therefore discloses removal of the label by cleaving the linkage to the base, which meets limitation iv).

13. Limitation v)

164. Dower and Prober contain disclosures that meet limitation v) of Claim 1, which recites: “wherein the cytosine deoxyribonucleotide analogue ... v) is capable of forming hydrogen bonds with guanine or a guanine nucleotide analogue.”

165. Dower discloses that each nucleotide analogue should pair with its complementary nucleotide. For example, Dower uses a polymerase “to extend a primer complementary to a target template” as shown in Figure 8:



Ex-1015 (Dower) at Figure 8A (annotated), 14:44-53 (describing Fig. 8). A person of ordinary skill in the art would understand that Figure 8A shows polymerase 88 adding a labeled and blocked dGTP analogue by forming hydrogen bonds with a cytosine base in the template strand. Ex-1015 (Dower) at 15:1-10. Dower discloses the addition of dCTP analogues. Ex-1015 (Dower) at 25:4-11, Fig. 8. It was firmly established that dCTP analogues form hydrogen bonds with guanine bases in a template strand. Ex-1042 (Watson & Crick) at 966; Ex-1011 (Alberts) at 99, Fig. 3-10.

166. Prober discloses that a DNA polymerase maintains fidelity during incorporation of 5-substituted cytosine analogues. Ex-1014 (Prober) at 337 (“The fidelity of terminator incorporation is maintained.”). A person of ordinary skill in the art would recognize that the hydrogen bonding that mediates fidelity is not

perturbed. Thus, it was understood that 5-substituted cytosine forms the normal hydrogen bonds with the complementary guanine nucleobase. *Supra* Paragraphs 44-45, 94. Dower and Prober therefore disclose limitation v).

14. A person of ordinary skill in the art would have been motivated to combine Dower, Prober and Metzker

167. Dower discloses all features of Claim 1 except (1) a 5-substituted cytosine and (2) that the 3'-capping group should not be a methoxy or an ester/ketone group. Prober discloses 5-substituted cytosine, and Metzker discloses a 3'-O-allyl ether capping group that is not a methoxy or an ester/ketone. A person of ordinary skill in the art would have recognized the benefits of combining Dower, Prober and Metzker.

168. Dower discloses a nucleotide analogue having a cleavably linked fluorescent label and a small 3'-OH capping group. Dower cites to the fluorescently labeled nucleotides described in Prober and this citation provides direction for a person of ordinary skill in the art to turn to Prober for labeling nucleotides. Ex-1015 (Dower) at 25:4-12, 25:4-12, 20:39-42, 23:16-26, 28:6-17, 17:35-36; *supra* Paragraph 122. Dower's discussion of base labeling that refers to Prober reasonably suggests that Dower considered Prober's disclosure beneficial for use in Dower's sequencing method.

169. Prober's disclosure would have been considered beneficial for use in Dower's methods for several reasons. Prober discloses the 5-position of

pyrimidines were reported “to be acceptable for attaching substituents to chain-propagating substrates (that is, deoxynucleotide triphosphates) for DNA polymerases.” Ex-1014 (Prober) at 340. Prober discloses that a DNA polymerase maintains fidelity with 5-substituted cytosine analogues during polymerase-mediated incorporation. Ex-1014 (Prober) at 337 (“The fidelity of terminator incorporation is maintained.”); *id.* at 340 (“The chain-terminators reported here are incorporated by both AMV reverse transcriptase and modified T7 DNA polymerase, but they are not accepted by the Klenow fragment of DNA polymerase I from *Escherichia coli.*”). Furthermore, it was known that linking labels to the 5-position of cytosine was advantageous because this “places the linker and reporter in the major groove when the nucleotide is incorporated into double-stranded DNA” to facilitate hybridization. Ex-1029 (Hobbs) at 27:52-65. For all of these reasons it had become commonplace before 2000 for researchers to use cytosine bases having a fluorescent label linked to position 5 of the base. *Supra* Paragraphs 44-46. Thus, a person of ordinary skill in the art would have been motivated to use Prober’s 5-substituted cytosine as the base in Dower’s fluorescently labeled nucleotide analogue.

170. Dower was filed in 1990 and disclosed that the 3'-capping group should be small. The crystal structure of a typical DNA polymerase was published in 1994 and showed that the polymerase active site is tightly packed where it binds

to the 3'-group of a nucleotide substrate. *Supra* Paragraphs 40-41, 62. In 1994, Metzker demonstrated that nucleotides with the 3'-O-allyl protecting group are incorporated by a polymerase. Ex-1016 (Metzker) at 4265, 4263. The 3'-O-allyl capping group disclosed by Metzker has only three carbon atoms ($-\text{CH}_2-\text{CH}=\text{CH}_2$), therefore the 3'-O-allyl capping group would have been recognized by a person of ordinary skill in the art as being small. A person of ordinary skill in the art would have been motivated to use Metzker's 3'-O-allyl group as Dower's "small" group because Metzker demonstrated that a dNTP with a 3'-O-allyl group is incorporated by a polymerase and therefore appropriately sized to fit within the polymerase active site. A person of ordinary skill in the art would have been further motivated to use Metzker's 3'-O-allyl ether because it was known to be chemically cleavable. Ex-1035 (Boss) at 559; Ex-1036 (Qian) at 2184; *supra* Paragraphs 64, 161.

171. Therefore, a person of ordinary skill in the art would have been motivated to combine Dower, Prober and Metzker to arrive at a cytosine nucleotide analogue having a 3'-O-allyl capping group and a fluorophore attached through a cleavable linker at the 5-position of the base.

15. There would have been a reasonable expectation of success

172. A person of ordinary skill in the art would have had a reasonable expectation of success in combining Dower, Prober and Metzker to make 3'-capped 5-substituted cytosine nucleotide analogues with cleavable linkers.

173. Each step of the synthetic process for preparing the nucleotide analogues disclosed by the combination of Dower, Prober and Metzker were understood to be within the level of ordinary skill. *Supra* Paragraphs 114-117.

174. A person of ordinary skill in the art would have expected the cytosine nucleotide analogue disclosed by Dower, Prober and Metzker to meet each of the recited limitations recited in Claim 1, as previously discussed. *Supra* Paragraphs 128-166.


175. For all these reasons, the nucleotide analogue recited in Claim 1 was disclosed by Dower in view of Prober and Metzker, a person of ordinary skill in the art would have been motivated to make the nucleotide analogue, and a person of ordinary skill in the art had a reasonable expectation of success in arriving at the nucleotide analogue. Consequently, Claim 1 is obvious over Dower in view of Prober and Metzker.

IX. CONCLUSION

176. For the reasons described above, a person of ordinary skill in the art would have found Claim 1 of the '358 patent obvious over (1) Tsien and (2) Dower in view of Metzker and Prober.

177. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Dated: 12/15/17

By: 
Floyd Romesberg, Ph.D.