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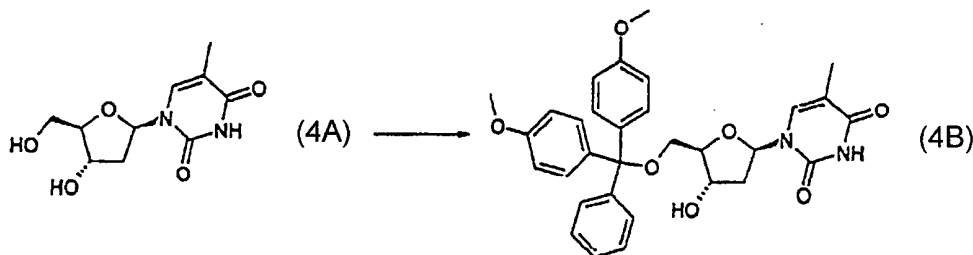
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(54) Title: PROTECTED NUCLEOSIDES

Preparation of 5'-O-Dimethoxytritylthymidine



(57) Abstract: Processes for the synthesis and purification of protected nucleosides using a solvent system without the use of pyridine as a solvent, and separation processes without the use of chromatography.

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PROTECTED NUCLEOSIDES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/137,639 filed June 4, 1999.

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FIELD OF THE INVENTION

The invention relates to the synthesis and purification of protected nucleosides, and more particularly to methods for the synthesis and purification of protected nucleosides without the use of pyridine as a solvent.

BACKGROUND OF THE INVENTION

10 Nucleosides are compounds of importance in physiological and medical research, obtained during partial decomposition, i.e., hydrolysis, of nucleic acids, and containing a purine or pyrimidine base linked to either D-ribose (forming ribonucleosides) or D-deoxyribose (forming deoxyribonucleosides). They are nucleotides minus the phosphate group. Well-known nucleosides include adenosine
15 (A), cytidine (C), uridine (U), and guanosine (G), as well as the deoxynucleosides deoxyadenosine (dA), deoxycytidine (dC), deoxyguanosine (dG), and deoxythymidine (dT). It should be noted that thymidine is actually a deoxynucleoside, and may be referred to in the literature as either thymidine (T) or deoxythymidine (dT).

20 Structural representations of the four deoxynucleosides dA, dC, dG and dT are shown in Figures 1A, 2A, 3A and 4A, respectively. The "deoxy" site in each compound is at the 2' position of the furan ring. The active hydroxy sites are at the 3' and 5' positions. In each of dA, dC and dG there is an exocyclic NH₂ group which is protected, preferably by acylation, as discussed below.

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Nucleosides are multi-functional compounds, having both amino and hydroxy functional groups. They may be used in automatic synthesizers to produce oligonucleotides as well as synthetic genes. Oligonucleotides and genes are formed by stringing together nucleosides in a predetermined sequence through phosphate ester linkages between the 3'-hydroxyl group of one nucleoside and the 5'-hydroxyl group of the next.

In order to conduct syntheses selectively and efficiently, it is necessary to block or "protect" specific functional groups in order to achieve reaction at the desired sites. The "protecting" groups are designed to be removed under specific carefully controlled conditions, usually under relatively mild and typically acidic conditions. To be useful as precursors in the synthesis of high value pharmaceuticals, it is necessary that protected nucleosides be of very high purity (i.e., greater than about 99%, preferably greater than about 99.5%) Unless otherwise indicated, purity percentages herein are expressed as percent area as measured by HPLC, but may be expressed as percent by weight, where indicated.

Typically, the protection of nucleosides involves the derivatization of both amino and hydroxy functional groups, except for thymidine, which requires only the protection of hydroxyl groups. Various schemes are employed to achieve these protected nucleosides, but usually the N-protected derivatives (most often N-acylated) are isolated and purified before protecting the hydroxyl groups. For example, the 2-amino group of dC, and the 6-amino group of dA are protected by benzoyl groups, while the 2-amino group of dG is protected by an isobutyryl group. The hydroxyl group, typically a 5'-hydroxyl group in all of the nucleosides, is generally protected by a 4,4'-dimethoxytrityl (DMT) group.

When phosphorylated, a protected nucleoside will react at the 3'-hydroxyl group. A phosphorylated protected nucleoside will then react with a second nucleoside at the 5' position after the 5' position has been deblocked by removal of the DMT group. The phosphorylation thus occurs between the 3'-hydroxyl group of the first nucleoside and the 5'-hydroxyl group of the second nucleoside to form a

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dinucleotide. By repeating the phosphorylation procedure, the synthesizer can produce oligonucleotides containing a predetermined sequence of nucleosides.

Discussions of the synthesis and protection of nucleosides by derivatization may be found in many references, including the following, all of which are

- 5 incorporated herein by reference. One method of protecting nucleosides is described in Ti, et al., "Transient Protection: Efficient One-flask Syntheses of Protected Deoxynucleosides", J. Am. Chem. Soc., Vol. 104, 1316-1319 (1982), which is discussed in more detail below in regard to the examples. Other methods of synthesizing protected nucleosides are set forth in Charubala, et al., "Nucleotides
- 10 XXIII: Synthesis of Protected 2'-Deoxyribonucleoside-3'-phosphotriesters Containing the p-Nitrophenylethyl Phosphate Blocking Group", Synthesis, 965 (1984). Still other methods for synthesizing such protected nucleosides are set forth in Kierzek, "The Synthesis of 5'-O-dimethoxytrityl-N-acyl-2'-deoxynucleosides, Improved 'Transient Protection' Approach", Nucleosides & Nucleotides, 4(5), 641-649 (1985).
- 15 In all of these references, protection by N-acylation is effected with benzoyl chloride on adenosine and cytidine derivatives, and with isobutyric anhydride on guanosine derivatives, as is well-known in the art. The compounds are then further protected by the introduction of methoxytrityl or dimethoxytrityl groups, also as is well-known in the art. An earlier article on the protection of such nucleosides may be found in
- 20 Schaller, et al., J. Amer. Chem. Soc., Vol. 85, 3821-3827 (1963). Another article on protected nucleosides is McGee, et al., "A Simple High Yield Synthesis of N²-(2-Methylpropanoyl)-2'-deoxyguanosine", Synthesis, 540 (1983). In all of the reported syntheses, the protected nucleosides must be subjected to purification prior to their use in pharmaceutical syntheses.

- 25 A detailed description of the preparation of protected deoxynucleosides is provided in Jones, "Preparation of Protected Deoxyribonucleosides", Oligonucleotide Synthesis: A Practical Approach, IRL Press, 23-34 (1984), incorporated herein by reference. This reference describes various processes for protecting such nucleosides, protecting the 5'-hydroxyl group as a trityl ether and the 3'-hydroxyl group with
- 30 benzoyl chloride or a levulinic anhydride. For ribonucleosides, protection of the 2'-

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hydroxyl group is also needed, most commonly provided by tert-butyldimethylsilyl (TBDMS) group. The exocyclic amino groups are protected by acylation with a benzoyl moiety (Bz) or an isobutyryl group (iB), as discussed above. Acyl protection is also discussed in Köster, et al., "N-Acyl Protecting Groups for Deoxynucleosides", Tetrahedron, 37(2), 363-369 (1981). TBDMS is discussed for protection of any hydroxyl group, using 4-(dimethylamino)pyridine (DMAP) as a catalyst, in Chaudhary, et al., "4-Dimethylaminopyridine: An Efficient and Selective Catalyst for the Silylation of Alcohols", Tetrahedron Letters, No. 2, 99-102 (1979).

All of these reported preparation procedures use pyridine as a solvent in the synthesis processes. Pyridine has been found to be chemically compatible with the nucleosides, the protected nucleosides, and with the various reactants used to form such products. However, pyridine is highly toxic and its ability to dissolve nucleosides is limited. Large amounts of pyridine have to be used in the reaction to maintain the nucleosides in solution. These large amounts of pyridine are difficult to remove from the protected nucleoside products. Thus, the pyridine solvent processes have very low productivity, and the processing costs are high. It would, therefore, be desirable to have a process for preparing protected nucleosides that does not require the use of pyridine as a solvent.

As discussed in Chaudhary, et al., cited above, 4-(dimethylamino)pyridine (DMAP) is often used as a catalyst in processes in which pyridine is the solvent. In fact, DMAP has become a standard catalyst for use in such processes. However, DMAP is also considered highly toxic, is very expensive, and is difficult to separate from the desired product. It is, therefore, also desirable to provide a process which does not require the use of DMAP as a catalyst.

Another reason for the use of pyridine is that it is a mild base, and can neutralize acids formed in the reaction mixture. In this capacity it acts as an "acid scavenger". Many of the chemical reactions in the protection process produce acid byproducts, such as HCl. For example, benzoyl chloride or isobutyryl chloride are used to acylate the amino group. In the acylation reaction, these acid chloride compounds react with the amino group to produce HCl as a byproduct. Similarly, in

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the tritylation reaction, dimethoxytrityl chloride (DMT-Cl) or other trityl chlorides react with hydroxy groups to generate HCl as a byproduct of this reaction. It is necessary to neutralize the acid produced by these reactions to prevent the acid from undesirably reacting with the nucleosides, and therefore an acid scavenger is needed in such processes.

The automatic synthesis of oligonucleotides as well as synthetic genes has previously been carried out on a milligram scale for research purposes. More recently, oligonucleotides are being used in larger quantities in the formation of commercial products such as anti-sense drugs that prevent the synthesis of disease-causing proteins in the human body. The very sensitive nature of the protecting groups together with the variety of polar and non-polar impurities generated during the syntheses of these derivatives makes their purifications complicated, expensive, and difficult to scale-up to industrial-scale production. Therefore, procedures are now needed to manufacture nucleosides in relatively large industrial quantities.

Column chromatography, especially flash silica gel chromatography, has been used extensively to purify protected nucleosides on a small to medium scale. This method requires the use of large volumes of high purity solvents in proportion to the amount of material purified. The method is also labor-intensive, requiring precise monitoring to make the fraction cuts at the appropriate times to maximize yield of desired product. For these reasons, large-scale use of this method of purification can be very costly.

The equipment required to conduct flash silica gel chromatography on a multi-kilogram scale is expensive to purchase and operate. For example, one commercially available production scale chromatography unit is capable of separating up to about 4 kg of material per run. Run times can vary from 18 to 36 minutes, at an elution rate of 7 liters per minute. The basic unit investment is very expensive, coupled with the cost (and subsequent disposal cost) of 125 to 250 liters of expensive high purity solvent per run. In addition, some products need to be purified multiple times by

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chromatography to reach the desired purity, each time at a significant yield loss. The high costs associated with chromatographic purification make it unattractive to industrial-scale operations.

For the above reasons, it is desirable to provide a process for protecting
5 nucleosides which does not required the use of pyridine as a solvent, which does not require the use of DMAP as a catalyst, and which does not require the use of separative chromatography to purify the product. The present invention meets these needs and offers additional advantages as discussed in the following detailed description.

10 The process of acylation for protection of exocyclic amino groups is well known in the art and is described, for example, in Jones, "Preparation of Protected Deoxyribonucleosides," op. cit. As discussed in that reference, a key problem in the preparation of protected deoxyribonucleosides is chemically differentiating between hydroxyl and amino groups. Only in the case of dC has it been possible to selectively
15 acylate the amino group (N-acylation) without acylating the hydroxyl group. The dA and dG amino groups have been found to be too weakly basic for such a selective reaction. However, it is possible to selectively de-acylate, that is, to differentiate by making use of the more rapid hydrolysis, at pH greater than 10, of the esters versus the amides. Thus the chemical procedure which has been used is to per-acylate the
20 nucleoside, acylating both the hydroxyl groups and the amino group, and then to selectively hydrolyze the esters to leave the desired N-acylated nucleoside. Such a per-acylation process, however, has been found to be difficult to use, because it requires isolation of the per-acylated intermediate. An alternative procedure is discussed in the Jones reference for selective N-acylation based on temporary
25 protection of the hydroxyl groups as trimethylsilyl ethers, and is referred to as "TMS-transient protection." Unlike ester groups, the trimethylsilyl ethers can be hydrolyzed in solution without the need for isolation. For these reasons, the TMS-transient protection method of N-acylation is the preferred method for use with dA and dG. However, the acylation procedures described in the Jones reference require the use of
30 pyridine as a solvent.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A, 1B, 1C and 1D present a schematic representation of the preparation of protected N⁶-benzoyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxyadenosine from deoxyadenosine free-base.

5 FIGURES 2A, 2B and 2C present a schematic representation of the preparation of protected N⁴-benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine from deoxycytidine free-base.

FIGURES 3A, 3B, 3C and 3D present a schematic representation of the preparation of protected 5'-O-dimethoxytrityl N²-isobutyryl-2'-deoxyguanosine from
10 deoxyguanosine free-base.

FIGURES 4A and 4B present a schematic representation of the preparation of protected 5'-O-dimethoxytritylthymidine from thymidine free-base.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention protected nucleosides are synthesized
15 in polar organic solvents, purified by liquid-liquid extraction and/or selective adsorption, and then solidified by precipitation or crystallization by adding a solvent in which the product is insoluble. The solidification process is considered precipitation in the case of a product which is amorphous in solid form, and crystallization in the case of a product which is crystalline in solid form. By the use
20 of this process, and repeating the process as needed, essentially pure protected nucleosides are obtained without the use of chromatography and without the use of pyridine as a solvent or DMAP as a catalyst. In addition, the process can be scaled up readily to any desirable scale. For purposes of this application, an essentially pure nucleoside is one of greater than about 98% purity, and preferably greater than about
25 99% purity, and most preferably greater than about 99.5% pure, all by weight.

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The process of the present invention is applicable to the preparation of protected deoxyribonucleosides. As discussed above, the deoxynucleosides dA, dC and dG, each of which have an exocyclic amino (NH_2), are first protected by acylation of that amino group. This step is necessary, because the highly reactive amino groups would otherwise react with the compounds being used to protect the hydroxyl group. As discussed above, the amino groups of dA and dC preferably are protected by benzoyl groups, while the amino group of dG preferably is protected by an isobutyryl group. These acylated compounds will be referred to herein as Bz-dA, Bz-dC and iB-dG, respectively. This step is not used for dT, because it does not have an exocyclic amino group.

In accordance with the present invention, there is provided also a process for selectively protecting the exocyclic amino group of a starting deoxyribonucleoside which has an exocyclic amino group which is to be protected by acylation and at least one hydroxyl group which is to be left unprotected, the process comprising:

- a) dispersing the starting deoxyribonucleoside in a polar solvent which is substantially free of pyridine and which is a solvent for the protected product formed in step b); and
- b) selectively acylating said exocyclic amino group of said starting deoxyribonucleoside to form a protected product in which said hydroxyl group(s) are unprotected.

This method for the N-acylation of deoxyribonucleosides does not use pyridine as a solvent or DMAP as a catalyst. This eliminates the need for the difficult step of separating product from pyridine solvent and/or DMAP catalyst, as generally required in prior processes. This also provides a pyridine-free amino-protected product for subsequent protection of hydroxyl groups, as discussed below.

The polar solvent should be one in which the N-acylated deoxynucleoside produced in step b) is soluble. The starting deoxynucleoside of step a) need not be fully soluble in the solvent provided it can be dispersed to carry on the acylation process of step b). The preferred polar solvent to use depends on the particular deoxynucleoside being acylated, as discussed further below.

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The preferred acylation procedures for protecting exocyclic amino groups in accordance with the present invention vary for each of the three deoxyribonucleosides which require acylation, dA, dC and dG. As indicated above, dC can be acylated directly by selective acylation, while dA and dG preferably are acylated indirectly via
5 TMS-transient protection.

The direct acylation of dC will be discussed first. In this acylation procedure, a starting material of dC free-base preferably is used as the starting deoxynucleoside. The dC free-base may be derived from commercially available dC salts, such as dC-HCl salt. A preferred process for producing dC free-base from dC-HCl salt is to first
10 dissolve the dC-HCl in a suitable polar solvent, preferably a protic polar solvent such as a C₁ to C₃ alcohol, with methanol particularly preferred. A non-aqueous organic base, such as the acid-scavengers discussed below, is then added to neutralize the dC-HCl. A preferred organic base is a tertiary amine such as triethylamine (TEA). The mixture should be refluxed until the dC-HCl converts to dC free-base. Then the dC
15 free-base is precipitated out of solution by adding a sufficient amount of a second solvent which is miscible with the first polar solvent, but in which the free-base is insoluble. Good results were obtained with CH₂Cl₂ as the second solvent.

The dC free-base is dispersed in a polar solvent which is free of pyridine. The solvent is preferably as anhydrous as possible, because any water present must be
20 removed or otherwise tied up prior to acylation. Preferred polar solvents for use in the direct acylation of dC are protic solvents such as straight chain or branched C₁ to C₃ alcohols, particularly methanol, ethanol, propanol or butanol. Relatively low boiling point secondary alcohols, particularly isopropanol, are preferred. Ethanol is also suitable, but is difficult to use commercially because of regulatory problems.
25 Methanol is less desirable because it may cause side reactions under certain conditions. Furthermore, isopropanol was found to provide high selectivity of acylation, and the product was found to crystallize cleanly out of the isopropanol. An acylating reagent, preferably a benzoylating reagent, is then added to the dC solution. Benzoic anhydride is a suitable reagent for acylation of dC by benzoylation. Benzoic
30 acid, which requires a base catalyst, and benzoyl chloride are not as suitable for use in

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this process. A preferred molar ratio of benzoic anhydride to dC was found to be about 1.2 to 1.3. An excess of benzoic anhydride results in the formation of the undesirable bisacylation by-product, and too little results in incomplete conversion of the dC to benzoylated dC (Bz-dC). The reaction temperature should be close to, preferably within about 10°C of, or at the boiling point of the polar solvent. The reaction is slower at lower temperatures, which may result in an incomplete reaction which may contaminate the product with unreacted starting materials. For isopropanol, which has a boiling point of 82.4°C, the reaction temperature preferably is from about 70°C to about 82.4°C, more preferably from about 75°C to about 80°C.

As discussed above, the acylating agent which is added to the starting deoxyribonucleoside solution is often in the form of a chloride or other material which produces acid byproduct upon reacting with the nucleosides. In such cases, the acid byproduct must be neutralized, or it may cause decomposition of the nucleoside. Therefore, an acid scavenger preferably is included in the reaction mixture. A preferred acid scavenger is a tertiary amine, which forms a salt with the acid, with a particularly preferred tertiary amine being triethylamine (TEA). Other suitable tertiary amines include any tri-alkyl amine of the general formula $N(R)_3$, wherein R represents the same or different C_1 to C_6 alkyl groups. In addition to TEA, such tri-alkyl amines include trimethylamine, tripropylamine and di-isopropyl-monoethyl amine (DIPEA). Other suitable tertiary amines include triethanolamine.

As indicated above, the acylation of dA preferably is achieved by transient TMS protection of both the amino and hydroxyl sites, followed by benzoylation, and then selective deprotection of the hydroxyl sites to leave just the amino sites benzoylated, thus forming benzoylated dA (Bz-dA). The polar solvent in which the starting dA is dispersed is preferably an aprotic polar solvent, preferably as anhydrous as possible. Suitable aprotic polar solvents include tetrahydrofuran (THF), acetonitrile, amides, such as dimethylformamide (DMF), dimethylacetamide and N-methylpyrrolidone, as well as dimethylsulfoxide (DMSO), dimethylsulfone and hexamethylphosphate (HMPA). In a preferred embodiment of this process, dA monohydrate ($dA \cdot H_2O$) is suspended in a polar solvent such as tetrahydrofuran (THF)

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with about 10 equivalents of triethylamine (TEA). THF is a preferred solvent for the TMS protection and subsequent benzylation steps because it provides solubility for the intermediates and is inert to the reagents. Of the 10 eq of TEA, 7 eq is to neutralize HCl produced from the chemical reactions and 3 eq to buffer the reaction mixture to ensure a slightly basic environment. About 5 equivalents of trimethylchlorosilane (TMSCl) is added slowly while maintaining the reaction at a relatively low temperature of about 0 to 10°C, preferably about 2 to 5°C. The TMSCl reacts at active OH and NH₂ sites, and also quenches the water of hydration from the dA monohydrate. The reaction should be monitored, as by TLC and/or HPLC, until all of the dA is consumed. About 2 equivalents of benzoyl chloride (BzCl) are added and the reaction is continued until no intermediate from the first step is observable. At this point, the exocyclic amino groups and the hydroxyl groups are all benzylated.

The benzylated hydroxyl groups are then restored to their original hydroxyl form by hydrolysis. The hydrolysis reaction selectively deprotects the hydroxyl sites, while leaving the amino sites benzylated. A preferred hydrolysis method is a two-phase step-wise hydrolysis with NaOH. After the hydrolysis proceeds half way, the aqueous phase is separated and neutralized immediately with HCl solution to reduce the pH to about 8 to 9. The remaining organic phase is hydrolyzed with another portion of NaOH until the hydrolysis is complete. Then the aqueous phase is separated, combined with the first portion of aqueous phase, and again neutralized with HCl solution to a pH of about 8 to 9. A miscible organic solvent in which the Bz-dA product is insoluble is then added to cause the product to precipitate out of solution. The precipitated product may then readily be collected, as by filtration. The strategy of this process is to utilize the phase separation between THF and H₂O to provide a means for isolating the product. Two-step hydrolysis, rather than one-step, provides better control of the reaction to avoid extensive over-hydrolysis which was encountered using a conventional one-step method.

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The acylation of dG preferably is achieved also via TMS-transient protection. In this case the acylation is by isobutyryl protection of the exocyclic amino group to form isobutyryl dG (iB-dG). A preferred acylation agent for this reaction is isobutyryl chloride (iBCl). As with the benzylation of dA, in a preferred embodiment of this process dG monohydrate ($\text{dG}\cdot\text{H}_2\text{O}$) is suspended in a polar solvent such as tetrahydrofuran (THF) with about 10 equivalents of triethylamine (TEA). THF was found to be the best solvent for the TMS protection dG and the subsequent acylation steps because it provides solubility for the intermediates and is inert to the reagents. As with the acylation of dA, of the 10 eq of TEA, 7 eq is to neutralize HCl produced from the chemical reactions, and 3 eq to buffer the reaction mixture to ensure a slightly basic environment. About 5 equivalents of trimethylchlorosilane (TMSCl) is added slowly while maintaining the reaction at a relatively low temperature of about 0 to 10°C, preferably about 2 to 5°C. The reaction should be monitored, as by TLC and/or HPLC, until all of the dG is consumed. About 2 equivalents of iBCl is added and the reaction is continued until no intermediate from the first step is observable. At this point, the exocyclic amino group and the hydroxyl groups have all been acylated by the iBCl.

The acylated hydroxyl groups are now restored to their original hydroxyl form by hydrolysis. The hydrolysis reaction selectively deprotects the hydroxyl sites, while leaving the amino sites isobutyryl protected. A preferred deprotection method is by hydrolysis with NaOH, which can be a simple one-step hydrolysis. After the hydrolysis is complete, the reaction mixture is neutralized with an acidic material, such as solid NH_4Cl , to reduce the pH to about 8 to 9. The organic solvents are then removed by vacuum distillation. A miscible organic solvent in which the iB-dG product is insoluble is then added to cause the product to precipitate out of solution. The precipitated product may then readily be collected, as by filtration.

The pyridine-free deoxyribonucleosides which have had their exocyclic amino groups protected by acylation may now be used to form 5'-protected deoxyribonucleoside products. In the following process, the 5'-hydroxyl group of the deoxyribonucleoside is protected, and the protected product is purified and separated

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out of solution as a solid. Purification and solidification comprise liquid-liquid extraction steps which take advantage of the fact that the protected nucleoside products are insoluble in water, while soluble in selected polar and non-polar solvents.

In accordance with the present invention, there is provided a process for
5 preparing an essentially pure 5'-protected deoxyribonucleoside comprising:

- a) dissolving a starting deoxyribonucleoside in a polar, aprotic solvent which is inert to the starting deoxyribonucleoside, to the 5'-protected deoxyribonucleoside, and to the other reactants, wherein any exocyclic amino groups of the starting deoxyribonucleoside are protected, preferably by acyl protection;
- 10 b) reacting the starting deoxyribonucleoside in said solution with a protecting reagent to form a 5'-protected deoxyribonucleoside product;
- c) removing polar impurities by one or more liquid-liquid extractions using immiscible polar and non-polar solvent systems in which the product preferentially partitions into the non-polar phase, and the impurities preferentially
15 partition into the polar phase; and
- d) removing non-polar impurities by solidifying the product out of solution while leaving the non-polar impurities in solution.

In step (a), the starting deoxyribonucleoside may be one that already has its exocyclic amino group protected, or dT which does not have an exocyclic amino
20 group. Protection of the exocyclic amino group may be made by the above-described acylation protection process of the present invention, or by any other suitable protection process. The nucleoside to be protected is dissolved in a polar, aprotic organic solvent. Examples of polar, aprotic solvents suitable for use in this step include amides, such as dimethylformamide (DMF), dimethylacetamide and
25 N-methylpyrrolidone, as well as dimethylsulfoxide (DMSO), dimethylsulfone and hexamethylphosphate (HMPA). DMF is particularly preferred. For the reasons discussed above, pyridine is not used as a solvent in the processes of the present invention.

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In step (b), a protecting reagent is added to the starting deoxyribonucleoside solution which selectively protects the 5'-hydroxyl group of the deoxyribonucleoside. The protecting reagent must react with the 5' hydroxyl group preferentially over the 3' hydroxyl group to form a removable protecting group at the 5' position. A preferred method of protecting this hydroxyl group is to form a trityl derivative compound, a process referred to as "tritylation". Preferred protecting groups are trityl, methoxytrityl and dimethoxytrityl groups. Preferred protecting reagents are trityl chloride and substituted trityl chlorides. For example, dimethoxytrityl chloride (DMT-Cl) is used in the examples set forth below. During the tritylation reaction, the 3'-hydroxyl group should be left unreacted. This is necessary to permit selective bonding of the 5' and 3' positions during subsequent oligonucleotide synthesis. The tritylation reaction is preferably conducted at a temperature ranging from about -10°C to about 40°C, more preferably from about 10°C to about 25°C.

Protecting reagents such as trityl chloride or substituted trityl chlorides produce an acid byproduct upon reacting with nucleosides. In such cases, the acid byproduct must be neutralized, or it may cause decomposition of the nucleoside. Therefore, an acid scavenger is preferably included in the reaction mixture. Preferably the acid scavenger is present in a mole ratio to the tritylation agent of about 1:1 to about 3:1. A preferred acid scavenger is a tertiary amine, which forms a salt with the acid. A particularly preferred tertiary amine is triethylamine (TEA). Other suitable tertiary amines include any tri-alkyl amine of the general formula $N(R)_3$, wherein R represents the same or different C_1 to C_6 alkyl groups. In addition to TEA, such tri-alkyl amines include trimethylamine, tripropylamine and di-isopropyl-monoethyl amine (DIPEA). Other suitable tertiary amines include triethanolamine. Although pyridine is not used as a solvent in the process of the present invention, a small amount of pyridine can be used as an acid scavenger. When used in small amounts, the pyridine can be purified out of the product with the other impurities.

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The polar solvents and acid scavengers must be chemically compatible with the starting nucleosides, the reactants, and the nucleoside products to which they are exposed. They must also not interfere adversely with the reaction of the reactants with the nucleosides.

5 Because the DMT-Cl used in tritylation is highly reactive towards water, all materials involved in the tritylation must be anhydrous. In the preferred acylation processes of the present invention, Bz-dA may pick up water from the TMS-transient protection acylation reactions, and therefore need to be dehydrated. It was found that ordinary thermal and vacuum drying techniques were unsatisfactory for removing the
10 water contained in this material, because the water may exist in hydrate form. In accordance with another aspect of the present invention, an azeotropic dehydration process has been developed which is effective to remove water in the Bz-dA.

 The azeotropic dehydration process of the present invention is applied to the solution of the starting deoxyribonucleoside dissolved in the polar aprotic solvent
15 prior to the addition of the protecting reagent. The dehydration process comprises adding a dehydrating solvent to the solution of step (a) that forms an azeotrope with water and the polar aprotic solvent, and distilling the azeotrope from the mixture. For a starting solution of deoxyribonucleoside in the polar aprotic solvent dimethylformamide (DMF) suitable dehydrating solvents include one or more C₅ to
20 C₁₀ hydrocarbons, which may be linear, branched or cyclic, and may be substituted or unsubstituted. Preferred dehydrating solvents include pentane, hexane and heptane, particularly hexane. It was also found desirable to include a small amount of a tertiary amine, such as TEA, to stabilize the acylated nucleosides during the azeotropic dehydration step.

25 The protecting reagent should be added to the solution of the starting deoxyribonucleoside under controlled conditions of temperature and addition rate. The progress of the reaction should be monitored, as by HPLC analysis. The objective of the monitoring is to control and optimize the conversion without generating too much of over-tritylated impurities. The reaction is quenched by the
30 addition of water when the optimal point is reached.

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An additional advantage of tritylating the starting deoxyribonucleoside in the polar aprotic solvent rather than in pyridine is that the reaction does not require the use of a catalyst. As discussed above, tritylation in pyridine generally requires the use of a catalyst, such as 4-(dimethylamino)pyridine (DMAP) which is a toxic material
5 that is very expensive and difficult to separate from the final product.

Step (c) is a purification step that removes polar impurities from the product. The solution of the protected nucleoside inevitably contains polar and non-polar impurities which must be removed from the final nucleoside product. As discussed above, the final product should be at least about 98% pure, preferably at least about
10 99% pure, and more preferably at least about 99.5% pure, by weight. Among the polar impurities which need to be removed are the amino-protected nucleosides which either did not react with the hydroxyl protecting agent, or which may have two acyl protecting groups (identified as bis-acylated products). Among the non-polar impurities which need to be removed are the bis-tritylated materials, which contain
15 two trityl protecting groups, and impurities derived from DMT-Cl. These tend to be the most difficult impurities to remove because their polarity is relatively close to that of the desired protected nucleosides.

Polar impurities are removed from the solution of protected nucleoside product by a liquid-liquid extraction process which takes advantage of the difference
20 in solubility between protected nucleosides and polar impurities in polar solvent system. The protected nucleosides are generally insoluble in water and basic aqueous salt solutions, while many of the polar impurities are soluble in water or such solutions. Therefore water, or preferably an aqueous solution of water and a basic salt such as a soluble carbonate or bicarbonate salt, can be used to extract polar impurities
25 from the protected nucleoside product. By adding water or a basic aqueous solution to the initial product solution in polar, aprotic solvent, followed by extracting with an immiscible non-polar solvent, such as methylene chloride, the protected nucleoside is transferred to the non-polar phase, while the majority of polar impurities are left in the polar phase. Preferred basic salts include sodium and potassium carbonate and
30 bicarbonate, particularly sodium bicarbonate. Preferably, the basic aqueous salt

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solution contains about 1% to about 10%, preferably about 2% to about 5%, by weight salt. Other halogenated solvents, such as chloroform and 1,2-dichloroethane may also be employed as the non-polar solvent. The product solution in the non-polar solvent can be repeatedly extracted in this manner with water or a basic aqueous solution to achieve higher purity level. It has been found that an aqueous solution comprising about 1 to about 10% by weight NaHCO_3 , preferably about 2% to about 5%, and about 0 to about 40% DMF, is suitable for the removal of polar impurities. After the polar solvent system has been mixed with the non-polar phase, the mixture is allowed to settle, and is separated. The desired protected product remains in the non-polar phase, while the undesired polar impurities are carried away in the polar phase. The process may be repeated as many times as necessary to remove the polar impurities and obtain the desired product purity.

This polar solvent system selectively extracts out all or most of the polar impurities. To remove additional polar impurities, the resulting product solution optionally may be treated further with a suitable adsorbent, such as activated carbon. Other suitable adsorbents, such as silica, alumina, and molecular sieves, are well-known to those skilled in the art. When polar impurities are below desirable levels, the adsorbent with adsorbed impurities may be readily removed, as by filtration. This has been found to be a desirable step in the purification of the protected dA and dC products, but unnecessary in the purification of dG and dT.

In step (d), the product is solidified out of solution by a process which further purifies the product. The product may be solidified out of solution by crystallization, in the case of a crystalline product, by precipitation in the case of an amorphous product, or by a combination of crystallization and precipitation in the case of products which form both crystalline and amorphous solids. Thus the term solidification is intended to encompass the processes of crystallization, precipitation and combinations thereof by which a solid product comes out of solution.

Solidification may be effected by the use of either non-polar solvents or polar solvents. In non-polar solvent solidification, a non-polar phase containing the dissolved product is combined with a miscible solvent in which the product is

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insoluble in an amount effective to crystallize a crystalline product, or to precipitate an amorphous product from the non-polar phase. For crystallization, preferably, the miscible solvent in which the product is insoluble is added slowly to the product solution. For precipitation, preferably, the product solution is added slowly to the
5 miscible solvent in which the product is insoluble.

When the solidification is taking place in a polar solvent, the product is transferred or re-dissolved into a polar phase which comprises a polar solvent that is miscible with water. Because the present protected nucleosides are all insoluble in water, water can be used to solidify the product from the polar phase. To solidify the
10 product from the polar phase, the polar phase containing the dissolved product is combined with a suitable amount of water effective to solidify the product out of solution. The water may be added to the polar phase, or the polar phase may be added to the water. When this process was used to solidify dC product out of solution, the polar phase was added slowly to water. The product was found to be a mixture of
15 crystal and amorphous solids.

Many polar solvents are suitable for use in this solidification process. In addition to being miscible with water and a solvent for the product, the polar solvent must also be inert to the product. Preferably, the polar solvent also has a boiling point of less than 100°C, the boiling point of water, to facilitate subsequent removal by
20 evaporation. Suitable solvents include acetonitrile, acetone, and lower (C_1 to C_3) alcohols, particularly methanol.

When the product is solidified in non-polar solvents, crystallization effectively removes most of the non-polar impurities. The non-polar impurities remain dissolved in the non-polar solvents as the product crystallizes out of solution. Such a
25 crystallization process is preferably used with DMT-dT and Bz-DMT-dA. However, when the product is solidified by precipitation to an amorphous form, it was found that additional purification may be needed to remove non-polar impurities. In such a case, an additional step of liquid-liquid extraction is used to remove non-polar impurities. Such a process is preferably used with protected nucleosides which form
30 amorphous solids, as was found to be the case with Bz-DMT-dC and iB-DMT-dG. In

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liquid-liquid extraction to remove non-polar impurities, the crude product is isolated in a polar phase in which it is soluble. A mixture of DMF and water is preferred for use in the liquid-liquid extraction purification of these products. The impurities are then extracted with a non-polar solvent, or a non-polar solvent system. The non-polar solvent should be a solvent for the non-polar impurities, but not for the nucleoside product. In addition, the polar phase and non-polar solvent system must be immiscible with each other. This will cause the non-polar impurities to partition into the non-polar solvent phase, while keeping the majority of the product in the polar solvent phase. The non-polar impurities can then be removed by phase separation.

Mixtures of aromatic hydrocarbons and aliphatic hydrocarbons are suitable non-polar solvent systems, particularly ones containing about 6 to 12 carbon atoms, with or without hetero atoms. A mixture of cumene and hexane is a preferred composition. Preferably, the mixture of cumene and hexane comprises about 10 to about 90 parts cumene to about 90 to about 10 parts hexane, by volume, more preferably about 1 to about 3 parts cumene to 1 part hexane, with a mixture of about 2 parts cumene to 1 part hexane being particularly preferred.

The liquid-liquid extraction to remove non-polar impurities may be performed either before or after the liquid-liquid extraction to remove polar impurities. However, preferably the non-polar extraction is performed before the polar extraction.

This is because the same non-polar solvent which is used to dissolve the product in the polar extraction step can be used in the final crystallization or precipitation step. Furthermore, the non-polar extraction can be performed with the product dissolved in a polar phase comprising the polar aprotic solvent in which the nucleoside is initially provided. Thus, it is simpler and more efficient to perform the non-polar extraction first.

To solidify the product out of solution by precipitation or crystallization, the non-polar phase in which the product has been isolated, and from which the polar impurities have been removed is mixed with an immiscible non-polar solvent in which the product is insoluble. A wide range of solvents can be used for this purpose, so long as the non-polar phase and the non-polar solvent are miscible with each other.

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For crystalline products, the non-polar solvent is preferably added slowly to the non-polar phase comprising the product. This causes the product to crystallize, while keeping non-polar impurities in the mother liquor. For amorphous products, the non-polar phase comprising the product is preferably added slowly to the non-polar solvent. This causes the product to precipitate, while keeping the non-polar impurities in solution.

The process of the present invention offers many advantages over prior processes in which pyridine is used as the process solvent. Because the polar aprotic solvent used in the present process are better solvents for the nucleosides than pyridine, the reactions can be run at much higher nucleoside concentrations. Thus, higher volumetric efficiency is obtained. As discussed above, the present process eliminates, or at least greatly reduces the use of pyridine and DMAP, which are considered highly toxic substances. The purification process steps of the present invention, including extraction, adsorption, and precipitation/crystallization are common operations, and are more efficient and less costly operations than chromatography. Because of the simplicity and other improvements of the present process, the overall product yield is also improved. Further, the combination of these improvements results in a significant lowering of the processing costs for protected nucleosides. Finally, the new processes are scalable to any desirable scale to meet industrial demand.

Figures 1A to 1D present a schematic representation of a preferred method of preparing protected N⁶-Benzoyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxyadenosine from deoxyadenosine free-base, with the acylation being via TMS-transient protection. The deoxyadenosine free-base of Figure 1A is combined with TMSCl to form TMS-protected dA. As shown in Figure 1B, two TMS groups attach to the exocyclic NH₂ group, as well as to the 3' and 5' OH groups. The TMS-protected dA is then combined with benzoyl chloride (BzCl or PhCOCl) to form benzoylated TMS-dA, which is then hydrolyzed to restore the OH groups, resulting in the acyl-protected

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Bz-dA of Figure 1C. This material is then tritylated by the addition of DMT-Cl to form the final protected product N⁶-Benzoyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxyadenosine as shown in Figure 1D.

Figures 2A, 2B and 2C present a schematic representation of a preferred method for the preparation of protected N⁴-benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine from deoxycytidine free-base. In this case direct acylation is used, without the need for a TMS protected intermediate. The deoxycytidine free-base of Figure 2A is reacted with benzoic anhydride (Bz₂O) to form the acyl protected Bz-dA of Figure 2B. This material is then tritylated by the addition of DMT-Cl to form the final protected product N⁴-benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine as shown in Figure 2C.

Figures 3A, 3B, 3C and 3D present a schematic representation of a preferred method for the preparation of protected 5'-O-dimethoxytrityl N²-isobutyryl-2'-deoxyguanosine from deoxyguanosine free-base, with the acylation being via TMS-transient protection. The deoxyguanosine free-base of Figure 3A is combined with TMSCl to form TMS-protected dG. As shown in Figure 3B, two TMS groups attach to the exocyclic NH₂ group, as well as to the 3' and 5' OH groups. The TMS-protected dG is then combined with isobutyryl chloride to form acylated TMS-dG, which is then hydrolyzed to restore the OH groups, resulting in the acyl-protected iB-dG of Figure 3C. This material is then tritylated by the addition of DMT-Cl to form the final protected product 5'-O-dimethoxytrityl N²-isobutyryl-2'-deoxyguanosine as shown in Figure 3D.

Figures 4A and 4B present a schematic representation of a preferred method for the preparation of protected 5'-O-dimethoxytritylthymidine from thymidine free-base. Because thymidine does not have an exocyclic NH₂ group, there is no acylation step. The thymidine free-base of Figure 4A is reacted with DMT-Cl to form the final protected product 5'-O-dimethoxytritylthymidine as shown in Figure 4B.

EXAMPLES

In the following examples protected nucleosides are made in accordance with the methods of the present invention. The examples set forth processes for making protected forms of the nucleosides deoxyadenosine (dA), deoxycytidine (dC),
5 deoxyguanosine (dG) and deoxythymidine (dT). Each of these nucleosides has special properties which affect the manner in which the protection process is performed.

The amino groups of deoxyadenosine and deoxyguanosine are difficult to protect by acylation because the acylating agents tend to react with both the free
10 amino groups and with hydroxyl groups. Therefore, the acylation process is conducted with a transient TMS protection step, which provides the necessary amino selectivity. This is discussed in the Ti, et al. reference, as cited above. The process for the acylation of dA was found to result in water being present in the acylation product. Therefore, a dehydration step is included in the process prior to protection
15 by tritylation. Deoxycytidine does not need to be acylated via TMS protection, because the acylation process is naturally selective between the desired amino sites and the undesired hydroxyl sites.

In the following examples, the solid protected forms of dA and dT were crystalline in structure, and therefore they were solidified out of solution by
20 crystallization. On the other hand, dG was found to be amorphous, and was therefore solidified by precipitation. When dC was solidified out of solution, it was found to be a combination of crystalline and amorphous. The solidification of dC is therefore a combination of crystallization and precipitation. Generally, the crystallization process successfully reduced the level of non-polar impurities to the desired level. On the
25 other hand, it was found that a step of liquid-liquid extraction to remove non-polar impurities was desirable before the precipitation of dC and dG in order to improve the purity of the final product. Other particular steps in the processing of the different nucleosides will be apparent from the following detailed descriptions of examples.

EXAMPLE 1**Preparation of N⁶-Benzoyl-2'-deoxyadenosine via TMS-transient Protection****Example 1A**

N⁶-benzoyl-2'-deoxyadenosine was prepared on a 500 g scale by the following process which makes use of TMS-transient protection. To a 12-L 4-neck flask equipped with an overhead stirrer, thermometer, nitrogen inlet and addition funnel was added 500 g (1.86 mol) 2'-deoxyadenosine monohydrate (dA·H₂O), 5.0 L anhydrous tetrahydrofuran (THF) and 2.69 L anhydrous triethylamine (hereinafter "TEA") (18.6 mol, 10.0 eq). The mixture was stirred under nitrogen in an ice bath (inner temperature about 2°C) to give a turbid dA solution. The addition funnel was charged with 1.18 L trimethylchlorosilane (TMSCl) (9.3 mol, 5 eq), which was added dropwise to the dA solution over one hour, with the temperature maintained at about 2-5°C, to form TMS-protected dA. The mixture was stirred at this temperature for another hour, and 430 mL benzoyl chloride (BzCl, 3.72 mol, 2.0 eq) was then added slowly over one hour, while maintaining the temperature at about 2-5°C. The mixture was continuously stirred at room temperature for another hour, forming benzoylated TMS-dA. The reaction mixture was then cooled, and 2 L of water was added slowly with stirring, maintaining the temperature at less than 10°C. The mixture was then allowed to settle for phase separation, and the aqueous phase was set aside.

While maintaining the organic phase at about 2-5°C, 1.0 L of 4 N NaOH was added slowly over one hour, and stirred for an additional hour. The mixture was allowed to settle, and the aqueous phase removed. An additional 600 mL 4 N NaOH was added to the organic phase, which was then stirred for another hour. The mixture was allowed to settle and the aqueous phase removed.

The separated aqueous phases were then combined, and neutralized by the addition of 4 N HCl at about 2-5°C until reaching a pH of about 8, during which solids were formed.

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To the mixture was added 5 L 1:1 ethyl acetate/t-butyl methyl ether (TBME), and the mixture stirred for about 30 minutes at room temperature. The solids were filtered and washed twice with 0.5 L 1:1 ethyl acetate/TBME. The solids were then vacuum dried at about 40°C for about 24 hours. A typical yield was about 70%.

5 **Example 1B**

An alternative process for making N⁶-Benzoyl-2'-deoxyadenosine used NH₄OH instead of NaOH in the hydrolysis step. This preparation was on a 200 g scale, and also used TMS transient protection. 200 g (0.743 mol) dA·H₂O, 3.0 L anhydrous THF and 1.04 L anhydrous Et₃N (7.4 mol, 10.0 eq) were combined, and
10 the mixture stirred under nitrogen in an ice bath (inner temperature 2°C) to give a turbid dA solution. 592 mL TMSCl (3.715 mol, 5 eq) was added dropwise to the dA solution over one hour, with the temperature maintained at about 2-5°C. The mixture was stirred at this temperature for another hour to convert dA to TMS-protected dA. 173 mL BzCl (1.486 mol, 2.0 eq) was then added slowly over one hour. The mixture
15 was continuously stirred at room temperature for another hour to convert the TMS-protected dA to benzoylated TMS-dA.

The mixture was then cooled to about 2-5°C, and to this 25 mL of H₂O was slowly added to quench any unreacted TMSCl. The mixture was then vacuum filtered to remove the solid TEA·HCl salt, which was further washed with anhydrous 3 L
20 THF, and the filtrate and washes were combined.

While maintaining this mixture at about 2-5°C, 750 mL of water was added slowly over one hour. Then 1.5 L 29% NH₄OH was gradually added over 30 minutes. To make the mixture homogeneous, 1.5 L of methanol was then added. Stirring was continued for 1-3 hours at room temperature until the reaction was about 75%
25 complete.

Volatile solvents were removed by vacuum distillation to leave a gummy residue. To the residue was added 500 mL water and 3 L 1:1 ethyl acetate/TBME, and the mixture stirred for about 30 minutes at room temperature. White solids were

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observed to precipitate out. The solids were filtered and washed with 250 mL water, and vacuum dried at room temperature for about 12 hours. A typical yield was about 70%.

EXAMPLE 2

5 Preparation of N⁶-Benzoyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxyadenosine

This example demonstrates a method for preparing N⁶-benzoyl-5'-(4,4-dimethoxytrityl)-2'-deoxyadenosine from Bz-dA. 142 g of Bz-dA prepared in accordance with Example 1B (containing an unknown amount of H₂O) was combined with 500 mL of dimethylformamide (DMF), 40 mL of TEA, and 500 mL of hexane.

10 The mixture was heated to reflux and stirred under nitrogen in a flask equipped with a Dean-Stark trap. About 17 g of a heavy layer containing water and DMF was collected over 5 hours. The heavy layer was analyzed and found to contain about 12 g of water. The amount of Bz-dA was recalculated to be 130 g, or 0.366 mol, based on the amount of water removed. This step removed the residual water without having to
15 subject the material to a drying step.

The mixture was cooled to ambient temperature and transferred into a separatory funnel to separate the DMF layer containing TEA and Bz-dA from the remaining hexane. The bottom DMF layer was separated and transferred back into the flask, together with an additional 100 g of TEA (0.99 mol, ~3.0 eq based on
20 Bz-dA). A solution of DMT-Cl (164 g, 98%, 0.476 mol, 1.30 eq based on Bz-dA) in 500 mL of CH₂Cl₂ was added dropwise to the above mixture over 1.5 hours. During the addition, the temperature of the mixture was controlled so that it did not exceed 25°C. Upon the completion of the addition, the mixture was stirred at ambient temperature for another 30 minutes.

25 The mixture was then transferred into a jacketed flask, which was equipped with a mechanical stirrer, an addition funnel, and a bottom take-off valve. The mixture was extracted with 2% aqueous NaHCO₃ solution (1000 mL) and 4 times with a mixture of DMF and 2% NaHCO₃ solution (1000 mL each time). The mixture

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was then extracted again with 2% NaHCO₃ solution (1000 mL) to remove residual DMF. The resulting CH₂Cl₂ solution was concentrated to about 500 mL and transferred into an addition funnel.

To the solution was added 4 L of hexane-TBME (t-butyl methyl ether) (2:1) mixture over one hour with vigorous stirring. A homogeneous solution was obtained first, but a white precipitate product started to appear in about 30 minutes. The mixture was cooled to 15°C, stirred for 3 hours, and filtered. The product filter cake was dried under vacuum at 40°C until reaching constant weight, and was found to be over 99% pure, with a yield of about 80%.

10

EXAMPLE 3

Preparation of N²-Benzoyl-2'-deoxycytidine

The following method was used to make N²-benzoyl-2'-deoxycytidine from a starting material of dC-HCl salt. As a preliminary step, the dC-HCl salt was neutralized to dC free-base as follows. 300 g dC-HCl (1.13 moles) and 144 g TEA (1.42 moles) were charged to 300 mL CH₃OH under stirring. The suspension was heated to reflux, forming a homogenous light brown solution, and heating was then discontinued. 1.5 L CH₂Cl₂ was then gradually added to this solution under stirring, whereby a white solid was formed. Stirring at room temperature was continued for one about hour, and then the solid was filtered out. The solid was then washed with an additional 500 mL CH₂Cl₂, and dried under vacuum for 30 minutes. 260 g of dC free-base product having a purity of about 98% was obtained, which was a yield of about 100%.

The dC free-base (2'-deoxycytidine) may then be used to prepare N²-benzoyl-2'-deoxycytidine (Bz-dC) as follows. 250 g dC was mixed with 3.75 L isopropanol (I-POH) and heated to 60°C. 250 g benzoic anhydride (Bz₂O) (1.1 mole), predissolved in 1.5 L I-POH was added into the above mixture over 5 minutes, and another 500 mL I-POH was used to rinse the inlet. This mixture was continuously heated to 75°C and aged at 75°C for 30 minutes. Another 25 g Bz₂O (0.11 mole)

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predissolved in 500 mL I-POH was added to the above mixture over 5 minutes, and the mixture was heated at 75°C for another 30 minutes. Then, an additional 25 g Bz₂O (0.11 mole) predissolved in 500 mL I-POH was added to the above mixture over 5 minutes, and the mixture was heated at 75°C for another 30 minutes. In all, a
5 total 300 g Bz₂O (1.32 moles) was used, for a molar ratio of Bz₂O to dC of about 1.2.

The mixture was then cooled to about 2-5°C over one hour, stirred for an additional 30 minutes at about 5°C, and filtered. In one test run, after vacuum drying at 37°C for 12 hours, 285 g of Bz-dC as a white solid was obtained, which was a yield of about 80%. NMR showed good purity, with only 0.55% I-POH identified in
10 the sample.

EXAMPLE 4

Preparation of N⁴-Benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine

The following procedure was used to prepare N⁴-benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine from Bz-dC. 300 g of Bz-dC (0.904mol) was
15 dissolved in 1.6 L of DMF at 60°C, and the solution cooled to room temperature. This solution was mixed with 238 mL of TEA (1.71 mol, 1.90 eq. based on the Bz-dC), and the mixture stirred under nitrogen until a homogeneous solution was obtained. 413 g of 98% DMT-Cl (1.22mol, 1.35 eq. based on the Bz-dC) was added gradually over 30 minutes, and the charging port rinsed with 800 mL of DMF. The
20 temperature of the reaction mixture was maintained between 25 and 30°C during this period. Upon the completion of the addition, the mixture was stirred at 25°C for an additional 30 minutes.

Then, 1.0 L of DI water was charged to the reaction mixture gradually over 15 minutes. The mixture was stirred until a homogeneous solution was obtained. This
25 solution was then extracted with a mixture of cumene and hexane (3 x 3.0 L, cumene/hexane=2:1) at 40°C to remove non-polar impurities by liquid-liquid extraction. In some cases, a third phase may be generated during these extractions. If

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that happens, the bottom and the middle phases should be taken together as the product layer. Once these extractions were complete, the DMF-water phase was then extracted with 3.0 L of hexane to remove residual cumene.

The DMF phase was then separated and diluted with 2.0 L of CH_2Cl_2 . The
5 resulting solution was extracted with a mixture of DMF and 2% aqueous NaHCO_3 solution (1:3, 2 x 4 L) to remove polar impurities (including Bz-dC and bis-Bz-dC), together with TEA and TEA-HCl. The CH_2Cl_2 phase was then washed with the 2% NaHCO_3 solution (2 x 4 L) to remove residual DMF in the CH_2Cl_2 solution. The
10 CH_2Cl_2 solution was then separated and stirred with activated charcoal (75 g, Darco G-60 powder) for about 1 hour. Then, the charcoal was filtered off and the charcoal cake rinsed with 200 mL of CH_2Cl_2 .

The combined filtrate and rinse was stripped to dryness under vacuum. The residue was re-dissolved in 500 mL of acetonitrile. The solution was added into 9 L of water with vigorous stirring over 30 minutes. The product precipitated out as fine
15 powder during this period. Upon the completion of the addition, the resulting mixture was stirred further for one hour and then was filtered. The filter cake was dried in a vacuum at 50°C until the weight loss was less than 0.2% in 24 hours. A typical yield was about 75%, with a purity of over 99%.

EXAMPLE 5

20 Preparation of N^2 -Isobutyryl-2'-Deoxyguanosine via TMS-transient Protection

N^2 -isobutyryl-2'-deoxyguanosine (iB-dG) was made by charging 40 g (0.14 mol) of 2'-deoxyguanosine monohydrate (dG), 0.4 L anhydrous THF, and 194 mL anhydrous triethylamine (1.4 mol, 10.0 eq) to a flask, and stirring the mixture under nitrogen in ice bath (inner temperature 2°C) to give a turbid solution. To this mixture
25 was added 89 mL chlorotrimethylsilane (TMSCl , 0.7 mol, 5 eq) by dropwise addition over about one hour, while maintaining the mixture at about 2 - 5°C . The mixture was

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then stirred at this temperature for another hour, during which isobutyryl chloride (iB-Cl, 0.28 mol, 2.0 eq) was added slowly. The mixture was stirred continuously at room temperature until the reaction was complete, about 4-5 hours.

Then, 200 mL H₂O was added to the reaction mixture, which was stirred for 5 15 minutes, and allowed to settle. The two phases were then separated. The bottom aqueous phase containing the polar impurities was drained out. Then, 400 mL of 2N NaOH was added slowly to the remaining organic phase, while maintaining a temperature of about 2-5 °C. This reaction was mildly exothermic, and the pH of the resultant mixture was about 12. Stirring was continued for another 2 hours at about 10 2-5 °C. Then, 40 g of NH₄Cl (s) was added to neutralize the solution to a pH of about 8-9.

Solvents were then removed by vacuum until the solution was cloudy. 600 mL 1:1 ethyl acetate/t-butyl methyl ether was added to the residue, and the mixture stirred for about 30 minutes. The solids were then filtered and washed with 100 mL 15 water and 1:1 ethyl acetate/t-butyl methyl ether, and dried by vacuum at room temperature for 12 hours. In one test, the solids, which still retained some solvent, weighed 38.8 g, a yield of 76.8%.

EXAMPLE 6

Preparation of 5'-O-Dimethoxytrityl N²-Isobutyryl-2'-Deoxyguanosine

20 5'-O-dimethoxytrityl N²-isobutyryl-2'-deoxyguanosine was made by charging 12.00 g of N²-isobutyryl-2'-deoxyguanosine (iB-dG, 35.6 mmol), 14.0 mL of triethylamine, and 60 mL of DMF to a flask. The mixture was stirred at ambient temperature until the iB-dG was completely dissolved. Then, a solution of 15.0 g of 98 % DMT-Cl (44.1 mmol, 1.24 eq based on iB-dG) in 60 mL of CH₂Cl₂ was added 25 slowly to the mixture over 45 minutes. The temperature was controlled to 27 °C using a water bath. Upon the completion of the addition, the mixture was stirred at ambient temperature for another 2 hours.

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The reaction mixture was transferred to a separatory funnel and washed with 3% NaHCO₃ solution (2 x 200 mL). The organic layer was dried with Na₂SO₄. The dried solution was added dropwise to 250 mL of toluene with vigorous stirring. Upon completion of the addition, the mixture was stirred for 2 hours and then filtered. The solid was washed with toluene-and hexane. After drying, 16.1 g of material was obtained. HPLC analysis showed the purity of 5'-O-dimethoxytrityl N²-isobutyryl-2'-deoxyguanosine was 99.05%, with a yield of about 70%.

EXAMPLE 7

Preparation of 5'-O-Dimethoxytritylthymidine

5'-O-dimethoxytritylthymidine (DMT-T) may be made by the following procedure. A solution containing 568 g of 2'-deoxythymidine (dT) (99%, 2.32 mol) and 2.0 L of DMF was prepared at a temperature below about 55°C. The solution was charged to a jacketed 12-L flask equipped with a bottom take-off valve, a circulating bath, and an addition funnel. 1.0 L of triethylamine (7.19 mol, 3.10 eq based on dT) was added, and the mixture cooled to below 10°C.

962 g of 98% DMT-Cl (2.78 mol, 1.20 eq based on dT) was mixed with 2.5 L of CH₂Cl₂ to form a slurry, and the slurry was added slowly to the above flask through the addition funnel over about one hour. During the addition period, the temperature of the mixture should be maintained below 20°C. Upon the completion of the addition, the temperature was raised to about 25°C, and the mixture was stirred for an additional hour.

3.0 L of 2% aqueous NaHCO₃ solution was then added to the flask, and the resulting mixture stirred vigorously for about 10 minutes. The mixture was allowed to settle, and the bottom CH₂Cl₂ layer was drained through the take-off valve and collected. After the top aqueous layer was drained, the CH₂Cl₂ layer was transferred back into the flask. This NaHCO₃ solution wash was repeated twice, and the CH₂Cl₂ layer sampled for analysis after each wash.

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The CH_2Cl_2 layer was then transferred back to the 12-L flask, and 6.0 L of cyclohexane was added over 30 minutes at ambient temperature with vigorous stirring. The resulting mass was cooled to 5°C with slow stirring, and filtered. The circulating bath then was switched to its heating cycle with a setpoint of 45°C . The
5 filter cake was washed with cyclohexane (2 x 3.0 L) and dried under vacuum suction for 15 minutes.

The solid material was then mixed with 3.0 L of CH_2Cl_2 and transferred back to the 12-L flask. The mixture was heated to refluxing to allow the solid to dissolve. When a homogeneous solution was obtained, the circulating bath was switched to its
10 cooling cycle (setpoint: -5°C). Then about 6 L of hexane was added to the solution over 30 minutes with vigorous stirring. The mixture was then cooled to about 5°C with slow stirring, during which the product crystallized out of solution. The solid was filtered off and washed with hexane (2 x 3.0 L). The wet cake was dried under vacuum suction for 15 minutes. A typical yield was about 85%, at a purity of over
15 99%.

CLAIMS

We claim:

1. A process for preparing an essentially pure 5'-protected deoxyribonucleoside product comprising the steps of:
 - 5 a) dissolving a starting deoxyribonucleoside in a polar aprotic solvent which is inert to the starting deoxyribonucleoside, to the 5'-protected deoxyribonucleoside, and to the other reactants, wherein any exocyclic amino groups of the deoxyribonucleoside are protected;
 - b) reacting said starting deoxyribonucleoside with a protecting reagent to
10 form a 5'-protected deoxyribonucleoside product;
 - c) removing polar impurities by one or more liquid-liquid extractions using immiscible polar and non-polar solvents which form polar and non-polar phases in which the product preferentially partitions into the non-polar phase and the impurities preferentially partition into the polar phase, and separating the non-polar
15 phase from the polar phase; and
 - d) removing non-polar impurities by solidifying the product out of solution while leaving the non-polar impurities in solution.
2. The process of claim 1 further comprising the step of recovering said 5'-protected deoxyribonucleoside product.
- 20 3. The process of claim 1 wherein the exocyclic amino groups are protected by acyl protection.
4. The process of claim 1 further comprising a step of removing non-polar impurities by one or more liquid-liquid extractions using immiscible polar and non-polar solvents which form polar and non-polar phases in which the product
25 preferentially partitions into the polar phase and the impurities preferentially partition into the non-polar phase, and separating the polar phase from the non-polar phase.

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5. The process of claim 1 further comprising the step of dehydrating the deoxyribonucleoside of step (a) prior to adding the reagent of step (b).
6. The process of claim 5 wherein said dehydrating step comprises adding a dehydrating solvent which forms an azeotrope with water to the solution of step (a),
5 and then distilling the azeotrope out of the solution.
7. The process of claim 6 wherein said dehydrating solvent is one or more C₅ to C₁₀ hydrocarbons, which may be linear, branched or cyclic, and may be substituted or unsubstituted.
8. The process of claim 7 wherein said dehydrating solvent is selected
10 from the group consisting of pentane, hexane, heptane and combinations thereof.
9. The process of claim 1 wherein said reagent is a tritylation agent and said protected deoxyribonucleoside is a 5'-tritylated deoxyribonucleoside.
10. The process of claim 9 wherein said tritylation agent is dimethoxytrityl chloride (DMT-Cl), methoxytrityl chloride or trityl chloride.
11. The process of claim 1, wherein an acid scavenger is added to said
15 solution with said reagent.
12. The process of claim 11 wherein said acid scavenger is one or more tertiary amines.
13. The process of claim 12 wherein said acid scavenger is triethylamine
20 (TEA).

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14. The process of claim 1 wherein step (c) further comprises selectively adsorbing polar impurities with a solid adsorbent.

15. The process of claim 1 wherein said deoxyribonucleoside is selected from the group consisting of deoxyadenosine (dA), deoxycytidine (dC),
5 deoxyguanosine (dG) and deoxythymidine (dT)

16. The process of claim 15 wherein the starting deoxyribonucleoside of step (a) is selected from the group consisting of N⁶-benzoyl-2'-deoxyadenosine (Bz-dA), N²-benzoyl-2'-deoxycytidine (Bz-dC), N²-isobutyryl-2'-deoxyguanosine (iB-dG), and deoxythymidine (dT).

10 17. The process of claim 1 wherein said polar aprotic solvent is selected from the group consisting of dimethylformamide (DMF), dimethylacetamide, N-methylpyrrolidone, dimethylsulfoxide (DMSO), dimethylsulfone, hexamethylphosphate (HMPA) and combinations thereof.

15 18. The process of claim 1 wherein said step (d) comprises solidifying the product out of solution in a non-polar solvent by combining the solution with a miscible solvent in which the product is insoluble in an amount effective to crystallize a crystalline product, or to precipitate an amorphous product from the non-polar phase.

20 19. The process of claim 1 wherein said step (d) comprises transferring the product to a solution in a polar solvent that is miscible with water, and solidifying the product out of the solution by combining the solution with an amount of water effective to solidify the product out of solution.

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20. The process of claim 1 wherein said protected deoxyribonucleoside is N⁶-benzoyl-5'-(4,4-dimethoxytrityl)-2'-deoxyadenosine, said starting deoxyribonucleoside is N⁶-benzoyl-2'-deoxyadenosine (Bz-dA), said polar aprotic solvent is dimethylformamide (DMF), said reagent is dimethoxytrityl chloride (DMT-Cl), and said non-polar solvent of step (c) is CH₂Cl₂.

21. The process of claim 21 wherein said step (d) comprises adding a mixture of hexane and t-butyl methyl ether (TBME) to a solution of product in CH₂Cl₂ to crystallize the product out of solution.

22. The process of claim 21 wherein the hexane-TBME mixture is about 2 parts hexane to 1 part TBME, by volume.

23. The process of claim 20 further comprising the step of dehydrating the Bz-dA prior to adding the reagent of step (b)

24. The process of claim 22 wherein said dehydrating step comprises adding a dehydrating solvent which forms an azeotrope with water to the solution of step (a), and then distilling the azeotrope out of the solution.

25. The process of claim 1 wherein said protected deoxyribonucleoside is N⁴-benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine, said starting deoxyribonucleoside is N²-benzoyl-2'-deoxycytidine (Bz-dC), said polar aprotic solvent is dimethylformamide (DMF), said reagent is dimethoxytrityl chloride (DMT-Cl), and said non-polar solvent of step (c) is CH₂Cl₂.

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26. The process of claim 25 further comprising, after step (b) and before step(c), a step of removing non-polar impurities by first adding water to the solution of product in DMF, and then performing one or more liquid-liquid extractions by adding an immiscible non-polar solvent to the solution, allowing polar and non-polar
5 phases to form in which the product preferentially partitions into the polar phase and the impurities preferentially partition into the non-polar phase, and separating the polar phase containing the product from the non-polar phase.

27. The process of claim 26 wherein immiscible non-polar solvent is a mixture of cumene and hexane.

10 28. The process of claim 27 wherein the cumene-hexane mixture comprises about 10 to about 90 parts cumene to about 90 to about 10 parts hexane, by volume.

29. The process of claim 28 wherein the cumene-hexane mixture comprises about 2 parts cumene to 1 part hexane, by volume.

15 30. The process of claim 26 wherein step (d) comprises transferring the product to a polar solvent that is miscible with water, and solidifying the product out of the solution by combining the solution with an amount of water effective to solidify the product out of solution.

20 31. The process of claim 30 wherein the polar solvent of said step (d) is acetonitrile.

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32. The process of claim 1 wherein said protected deoxyribonucleoside is 5'-O-dimethoxytrityl N²-isobutyryl-2'-deoxyguanosine, said starting deoxyribonucleoside is N²-isobutyryl-2'-deoxyguanosine (iB-dG), said polar aprotic solvent is dimethylformamide (DMF), said reagent is dimethoxytrityl chloride (DMT-Cl), and said non-polar solvent of step (c) is CH₂Cl₂. -

33. The process of claim 32 wherein step (d) comprises adding a solution of the product in CH₂Cl₂ to hexane to precipitate the product out of solution.

34. The process of claim 1 wherein said protected deoxyribonucleoside is 5'-O-dimethoxytritylthymidine, said starting deoxyribonucleoside is 2'-deoxythymidine (dT), said polar aprotic solvent is dimethylformamide (DMF), said reagent is dimethoxytrityl chloride (DMT-Cl), said non-polar solvent of step (c) is CH₂Cl₂.

35. The process of claim 34 wherein said step (d) comprises adding hexane to a solution of product in CH₂Cl₂ to crystallize the product out of solution.

36. A process for selectively protecting the exocyclic amino group of a starting deoxyribonucleoside which has an exocyclic amino group which is to be protected by acylation and at least one hydroxyl group which is to be left unprotected, the process comprising:

- a) dispersing the starting deoxyribonucleoside in a polar solvent which is substantially free of pyridine and which is a solvent for the protected product formed in step b) ; and
- b) selectively acylating said exocyclic amino group of said starting deoxyribonucleoside to form a protected product in which said hydroxyl group(s) are unprotected. .

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37. The process of claim 36 wherein said polar solvent is a straight chain or branched C₁ to C₅ alcohol.

38. The process of claim 37 wherein said polar solvent is isopropanol.

39. The process of claim 36 wherein said deoxyribonucleoside is selected
5 from the group consisting of deoxyadenosine (dA), deoxycytidine (dC) and deoxyguanosine (dG).

40. The process of claim 36 further comprising adding an acid scavenger to said polar solvent solution of deoxyribonucleoside.

41. The process of claim 40 wherein said acid scavenger is one or more
10 tertiary amines.

42. The process of claim 41 wherein said acid scavenger is triethylamine (TEA) or di-isopropyl-monoethyl amine (DIPEA).

43. The process of claim 36 wherein said deoxyribonucleoside is deoxycytidine, which is acylated with benzoic anhydride.

44. The process of claim 43 wherein said starting deoxyribonucleoside is
15 deoxycytidine free-base.

45. The process of claim 44 wherein said deoxycytidine free-base is made by the neutralization of deoxycytidine-HCl.

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46. The process of claim 45 wherein the deoxycytidine free-base is made by dissolving deoxycytidine-HCl in a protic polar solvent, adding enough non-aqueous organic base to neutralize the deoxycytidine-HCl, refluxing the mixture until the deoxycytidine-HCl converts to deoxycytidine free-base, and then adding a second
5 solvent which is miscible with the polar protic solvent, but in which deoxycytidine free-base is insoluble, to the mixture until the deoxycytidine free-base precipitates out of solution.

47. The process of claim 46 wherein said protic polar solvent is methanol, said non-aqueous organic base is triethylamine and said second solvent is CH_2Cl_2 .

10 48. The process of claim 43 wherein the molar ratio of benzoic anhydride to deoxycytidine is about 1.2 to about 1.3.

49. The process of claim 36 wherein step (c) comprises:

- (i) reacting the starting deoxyribonucleoside with a transient protection agent which reacts with both the exocyclic amine group(s) and the hydroxyl group(s);
15 (ii) reacting the transiently protected deoxyribonucleoside with a selective acylation agent which acylates the transiently protected exocyclic amino group(s) but leaves the transiently protected hydroxyl group(s) essentially unreacted; and
(iii) reacting the deoxyribonucleoside with a hydrolyzing agent to return said transiently protected hydroxyl group(s) to hydroxyl form.

20 50. The process of claim 49 wherein said transient protection agent is a trimethylsilane salt.

51. The process of claim 50 wherein said transient protection agent is trimethylchlorosilane (TMSCl).

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52. The process of claim 49 further comprising adding an acid scavenger to the solution.

53. The process of claim 52 wherein said acid scavenger is triethylamine.

54. The process of claim 53 wherein enough triethylamine is added to the
5 solution to neutralize any acid produced during the process.

55. The process of claim 49 wherein said deoxyribonucleoside is deoxyadenosine or deoxyguanosine.

56. The process of claim 49 wherein said deoxyribonucleoside is deoxyadenosine, said transient protection agent is trimethylchlorosilane (TMSCl),
10 said acylation agent is benzoyl chloride, and enough triethylamine is added to the solution to neutralize any acid produced during the process.

57. The process of claim 56 wherein said hydrolyzing agent is NaOH or NH_4OH .

58. The process of claim 49 wherein said deoxyribonucleoside is deoxyguanosine, said transient protection agent is trimethylchlorosilane (TMSCl),
15 said acylation agent is isobutyryl chloride, and enough triethylamine is added to the solution to neutralize any acid produced during the process.

59. The process of claim 58 wherein said hydrolyzing agent is NaOH or NH_4OH .

Preparation of N⁶-Benzoyl-5'-O-(4,4-dimethoxytrityl)-2'-deoxyadenosine

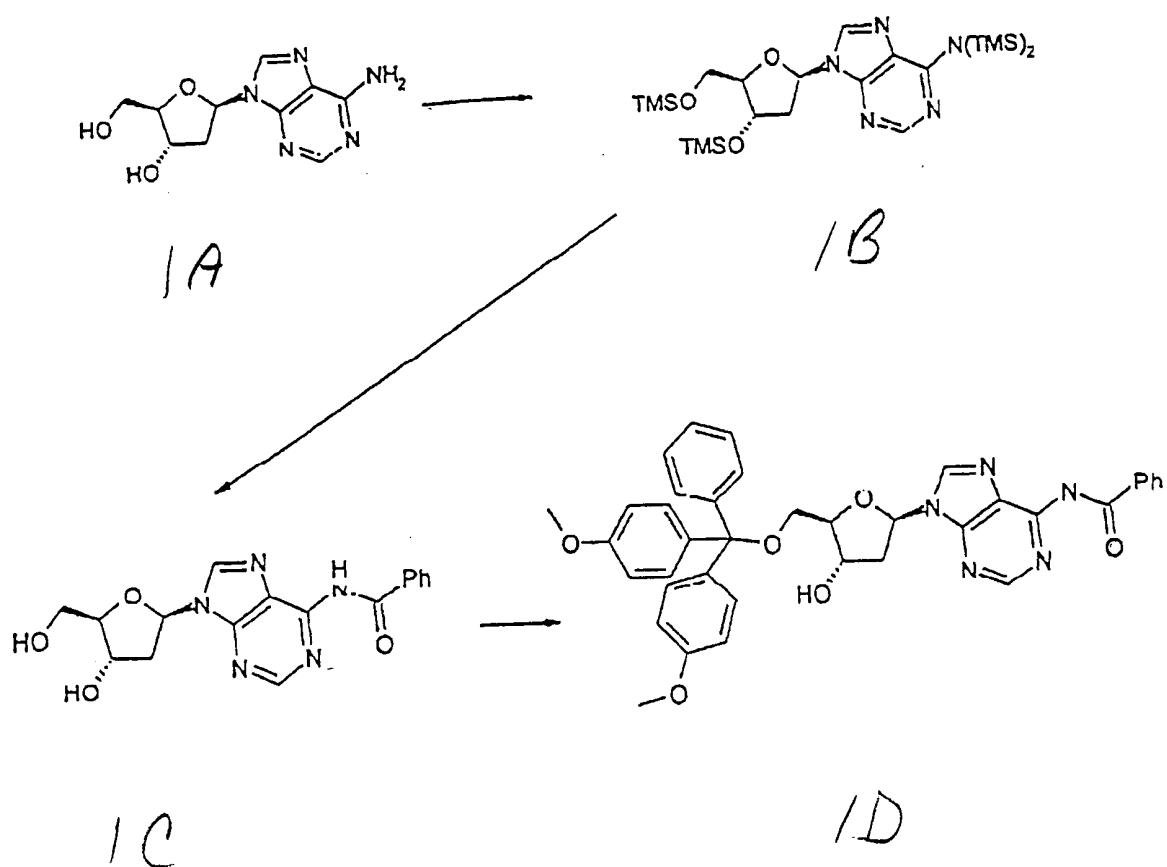


FIG. 1

Preparation of N⁴-Benzoyl-5'-(4,4-dimethoxytrityl) deoxycytidine

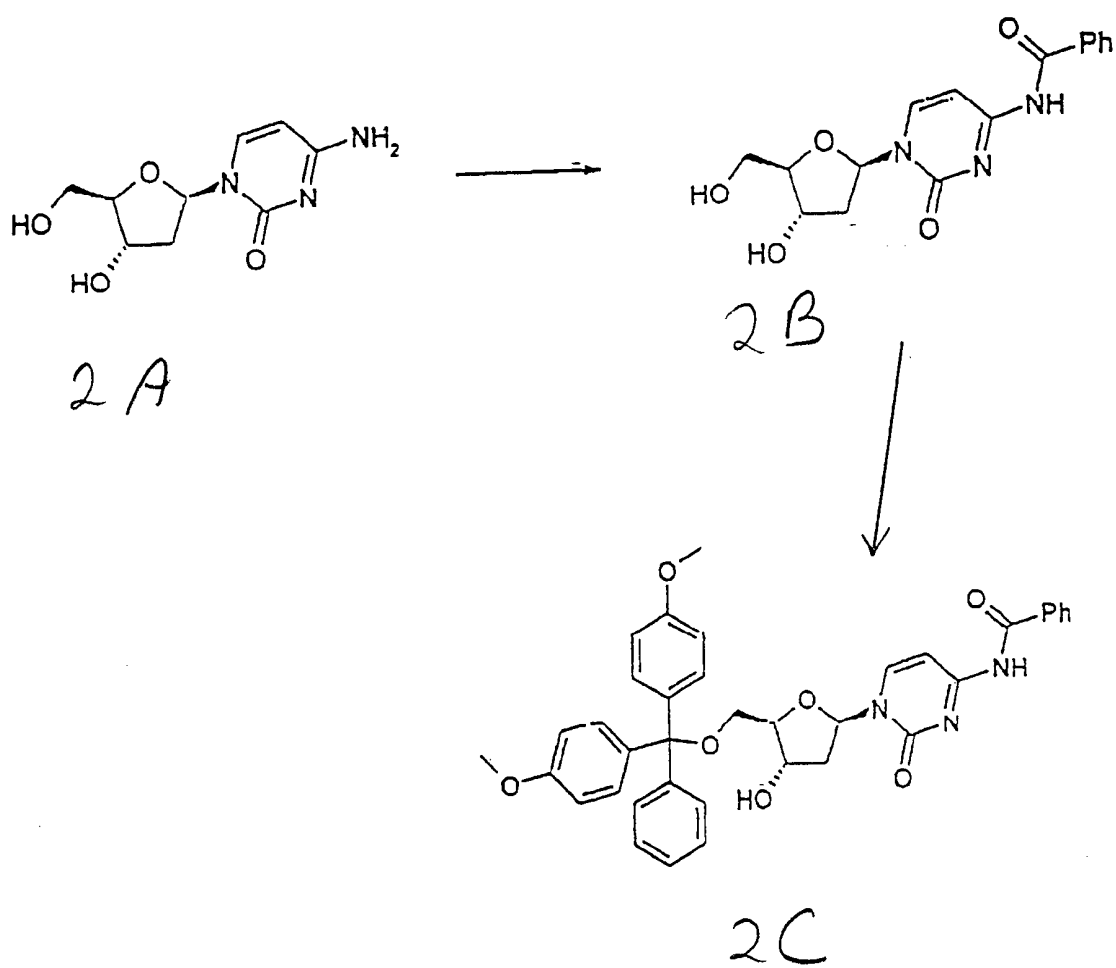


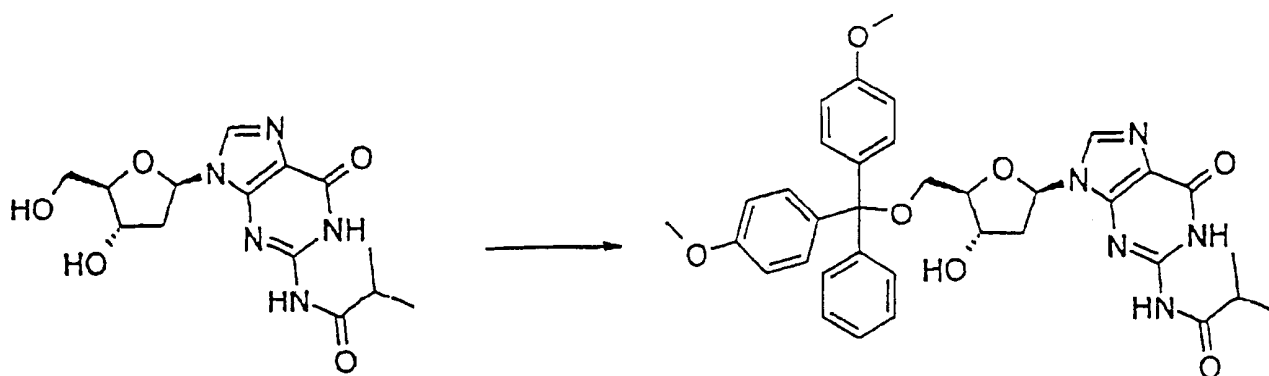
FIG. 2

Preparation of 5'-O-Dimethoxytrityl N²-Isobutyryl-2'-Deoxyguanosine



3A

3B



3C

3D

F16. 3

Preparation of 5'-O-Dimethoxytritylthymidine

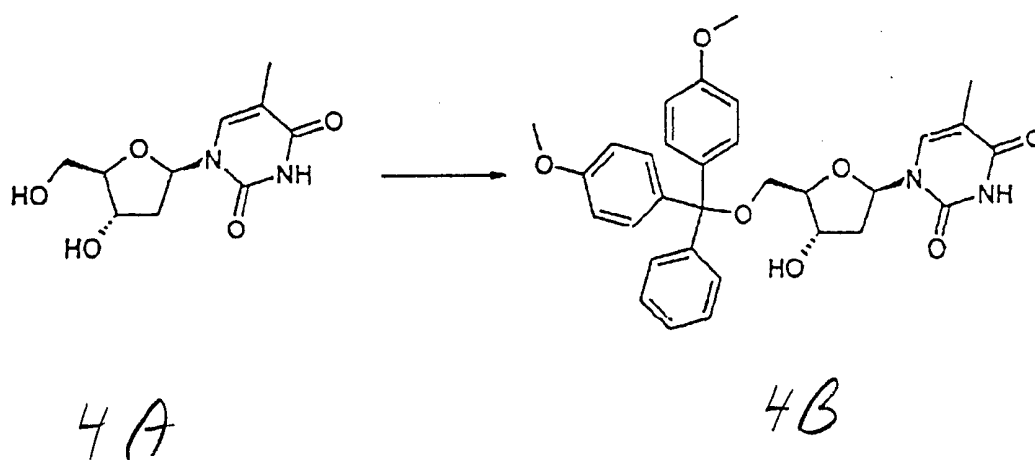


FIG. 4