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SYNTHESIS OF FLUORESCENT OR BIOTINYLATED NUCLEOSIDE COMPOUNDS

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Abstract: The synthesis of two types of modified nucleosides derivatives of 2'-deoxycytidine and 2'-deoxyadenosine, useful for the specific attachment of non-radioactive labelling reagents such as fluorescent or biotinyl group is reported. The dATP analogue is converted into the biotinylated derivative which is a substrate for DNA polymerase.

The most common technique to label oligodeoxyribonucleotides is the enzymatic incorporation of the radioisotope ^{32}P at the 5'-end. Although this method affords high sensitivity of detection, the use of the radiolabelled probes is precluded in many clinical and diagnostic applications. In the last few years, several labelling procedures for the detection of specific nucleic acid sequences in situ using non-radioactive tags as biotin or fluorophores have been developped. The hapten is introduced either chemically or enzymatically before hybridization of the probe. The strong and specific non covalent interaction of biotin for egg white avidin or bacterially derivatived streptavidin forms the basis of enzyme-linked detection kits that are commercially available[1]. In the case of oligodeoxynucleotides tagged with fluorophores, they can be detected in gel by means of an argon ion laser and a fluorescence detection system[2].

The enzymatic incorporation of biotin into DNA was accomplished by reaction of DNA polymerase using TTP and UTP analogues which contain a biotin molecule at the C-5 position of the pyrimidine rings[1,3-5]. This technique suffers from some disadvantages, as the preparation of large amounts of probe that is expensive in terms of enzymes and substrates. Thus, chemical methods for the labelling of DNA with biotin of fluorophores are more suitable.

A possible approach for the chemical synthesis of fluorescent probes consists in the photochemical labelling with a photoactivable analogue of biotin (photobiotin), which forms stable linkage with nucleic acids[6]. In another approach a biotin derivative attached to amide groups of basic proteins is linked by formaldehyde or glutaraldehyde to the DNA probe[7,8]. Another method consists in the chemical incorporation of a fluorescent pyridopyrimidine nucleoside into an oligomer[9]. Recently, efficient methods for the attachment of nonradioactive labels to the 5'-ends of synthetic oligodeoxynucleotides has been reported. The biotinyl or fluorescent group is linked to the 5'-terminal hydroxyl[10], amino[2] or phosphate[11-16] end of the synthetic oligomer directly or via a spacer arm (2,6,12 C). This approach offers a possible further chain extension by polymerase from the 3'-end. The synthesis of biotinylated and fluorescent probes by chemical incorporation of modified pyrimidine at the C-5 position with a functionalized linker arm into oligomers has been recently reported[17-19].

We report in this paper our own investigations on the preparation of non-radioactive probes. We propose another approach for the chemical labelling of DNA which consists in the synthesis of modified purine and pyrimidine nucleosides having a chain at the ring moiety. This base modification let free the 3'- and 5'-hydroxyles. Therefore, these modified nucleosides could be further incorporated either enzymatically or chemically into polynucleotides. This last approach offers total control over number and location of labelling sites.

We attempted the synthesis of 2'-deoxycytidine bearing a twelve atoms linker arm at the C-4 position (Scheme I) and an analogue of dATP substitued at C-8 by the same spacer arm (Scheme II).

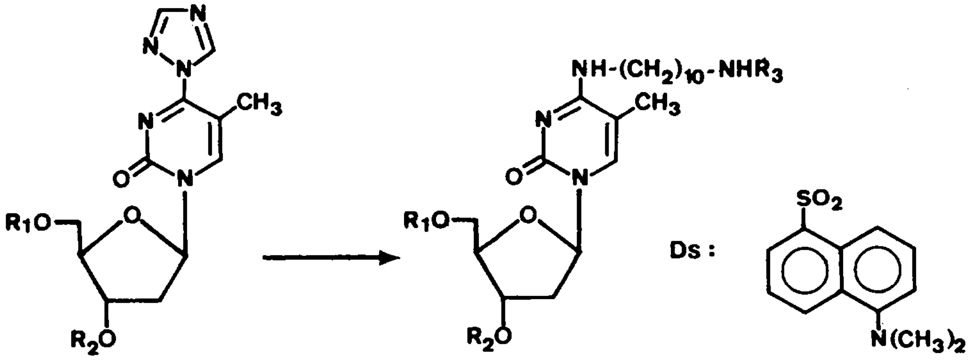
This spacer chain possesses a primary amino group suitable for covalent attachment of labelling reagents. For example, the amino group could easily react with the activated ester of biotin or dansyl chlorid to give a labelled product. We describe the synthesis of a fluorescent pyrimidine nucleoside and a biotinylated 5'-triphosphate adenosine as possible labelled derivatives.

Synthesis of the spacer arm of 2'-deoxycytidine

Sung et al have demonstrated the possible conversion of 4-triazolothymidine into 5-methylcytidine on reaction with ammonia. However, the 4-triazolo group was found to be stable towards more sterically hindered amines such as diethylamine, diisopropylamine and triethylamine; no nucleophilic displacement of triazole by these amines was observed[20].

We have extended the Sung's methodology to prepare 5-methylcytidine having an alkylamino linker chain at position 4. Thus, the reaction of 5'-O-dimethoxytrityl-3'-O-benzoyl 4-triazolothymidine (1) with 1,10-diaminodecane and 1-N-dansylamino-10-aminodecane (2) in dioxane afforded the corresponding 4-alkylamino derivatives 3 (52% yield) and 4 (88% yield) respectively (scheme I).

Subsequent deprotection of dimethoxytrityl group with a 2% solution of benzenesulfonic acid in CH_2Cl_2 at 0°C followed by treatment with 1.56 N NaOH gave the completely deprotected 4-functionalized 5-methylcytidine 7 and 8 (10% and 47% yield in two steps, respectively).



1
R_1 = DMT
R_2 = Bz

	3	4	5	6	7	8
R_1	DMT	DMT	H	H	H	H
R_2	Bz	Bz	Bz	Bz	H	H
R_3	H	Ds	H	Ds	H	Ds

Scheme I

Synthesis of the spacer arm of 2'-deoxyadenosine

Bromination[21] of 2'-deoxyadenosine with bromine water at pH 5 in acetate buffer afforded 8-bromo-2'-deoxyadenosine 9 (Scheme II).

Nucleophilic displacement of bromine by 1,10-diaminodecane in refluxing ethanol gave 8-[10-aminodecyl]-amino-2'-deoxyadenosine 10 with 55% yield.

Synthesis of the modified 2'-deoxyadenosine 5'-triphosphate

Before phosphorylation of the 5'-hydroxyl, the free primary amino group of the linker chain was protected with a benzyloxycarbonyl group[22] by reaction with 1-benzyloxycarbonyl-3-methylimidazolium chloride in a mixture of water and ethanol to give 11 (72% yield).

Various methods to prepare adenosine 5'-triphosphate directly from adenosine have been recently described[23,24]. However, in our hands, these reported procedures proved to be unsuitable for transforming 11 into a corresponding triphosphate derivative. Alternatively, we prepared the phosphomonoester 14 from 11. Thus, perbenzoylation of 11 with benzoyl chloride in pyridine followed, by saponification with 8N NaOH gave 12. Selective protection of 5'-OH with dimethoxytrityl chloride benzoylation of 3'-OH with benzoyl chloride and final removal of DMT group by treatment with 2% benzene sulfonic acid afforded compound 13. The free 5'-hydroxyl group of 13 was phosphorylated[25] by condensation with cyanoethylphosphate in presence of DCC in anhydrous pyridine in 49% yield. By treatment with concentrated ammonia at 60°C for 24 h, 13 underwent simultaneous decyanoethylation and debenzoylation to afford the phosphomonoester 14.

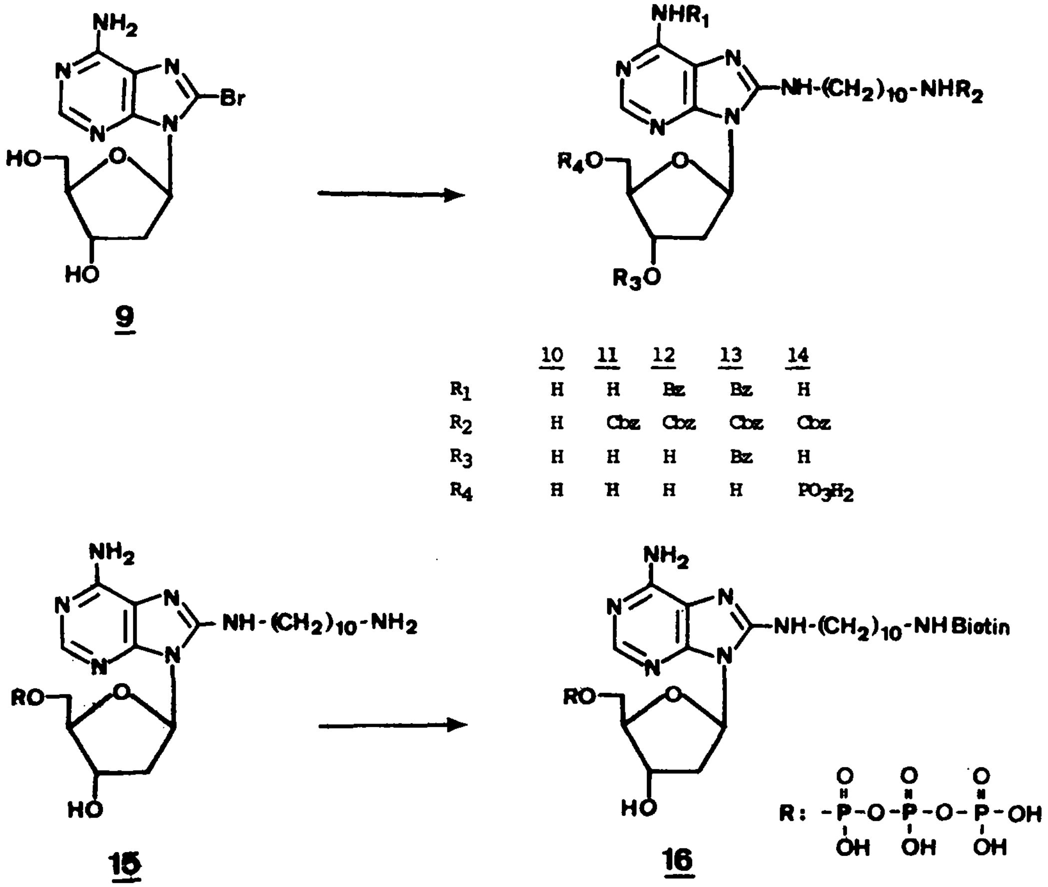
The phosphomonoester 14 was converted into 2'-deoxyribonucleotide 5'-phosphomorpholidate in quantitative yield by refluxing with morpholine in presence of DCC using a mixture of t-butanol and water as solvent[26]. The benzyloxycarbonyl protecting group of the latter was removed by hydrogenolysis with 10% Pd-C as catalyst. Treatment with tetrabutylammonium pyrophosphate in dry DMSO for two days yielded triphosphate derivative 15, which was purified by ion exchange chromatography on a DEAE cellulose column in the bicarbonate form using a linear gradient of triethylammonium bicarbonate (0 to 0.35 M) as eluent.

The triphosphate derivative 15 has been characterized by mass and n.m.r. spectroscopy. ^{1}H-NMR and ^{31}P-NMR spectra of compound 15 are presented on figure 1 and figure 2, respectively.

The primary amino group of 15 was biotinylated by reaction of biotinyl-N-hydroxy succinimide ester (BHSE) in a sodium bicarbonate buffer (pH = 9) at 25° to afford 16. Compound 16 was purified by gel filtration on Sephadex G-10 using water as eluent, followed by reverse phase HPLC on a Nucleosil HS5 C18 column.

Use

The biotinylated derivative replaced adenine triphosphate (ATP) in a standard nick translation catalysed by E. coli polymerase I; it was incorporated into a plasmid DNA (10.7 Kb) and it was visualized with avidin enzyme conjugates.



	10	11	12	13	14
R_1	H	H	Bz	Bz	H
R_2	H	Cbz	Cbz	Cbz	Cbz
R_3	H	H	H	Bz	H
R_4	H	H	H	H	PO_3H_2

Scheme II

EXPERIMENTAL

General methods. ^1H-NMR spectra were measured at 400 MHz with a Brucker AM 400 spectrometer, operating in FT mode under the control of a ASPECT computer (AM 400/ASPECT 3000); chemical shifts are given in ppm [δ] relative to tetramethylsilane (TMS) as internal standard or 0.05 mM internal DSS (sodium 2,2-dimethyl-2-silapentane sulfonate).

^{31}P-NMR spectra were measured at 169 MHz; ^{31}P-chemical shifts in ppm [δ] relative to 85% H_3PO_4 as external standard.

Mass spectra were recorded on a VG 70-250 double focusing instrument (VG instruments, Le Chesnay, France) equipped with a fast atom bombardment gun (Ion Tech., UK). The gun was operated with Xenon at 8 kv and 1 mA. Cesium iodide or glycerol were used for calibration, glycerol or thioglycerol as the matrix.

Column chromatography was carried out on silica gel Merck Kieselgel 7734. TLC were performed on silica gel (Merck TLC aluminium sheets silica gel $60F_{254}$) using as eluent solvent A: BuOH-AcOH-H_2O (4:1:1, v/v/v). Compounds were visualized by UV light or by spraying TLC plates with the appropriate reagents. Thus compounds containing sugar moieties were visualized by spraying TLC plates with anisaldehyde-H_2SO_4-EtOH (1:1:18, v/v/v) and charring at 110°C for few minutes, amino compounds with ninhydrine (0.2 g) in ethanol (100 ml). Phosphorus containing compounds were revealed by spraying with the reagent of Ditmer and Lester[27]. Biotinylated compound gives a strong positive reaction (an orange-red color) when treated with p-dimethylamino cinnamaldehyde in 2% ethanolic sulfuric acid[28].

Pyridine was dried by refluxing over CaH_2 for 2 h and distilled; it was redistilled over p-toluenesulfonyl chloride. Dimethyl sulfoxide was distilled under reduced pressure. All solvents were stored over molecular sieves 3 Å or 4 Å.

Biotinyl-N-hydroxysuccinimide ester was prepared from biotin (Sigma) as described[29].

5'-O-Dimethoxytrityl-3'-O-benzoyl-5-methyl-4-N-[10-aminodecyl] cytidine (3). To a solution of 5'-O-dimethoxytrityl-3'-O-benzoyl-4-triazolyl thymidine (1) (1 g, 1.43 mmol)[20] in dioxane (18 ml) was added 1,10-diaminodecane (0.37 g, 2.15 mmol). After stirring for 4 h at room temperature (reaction monitored by TLC in methanol), the resulting mixture was evaporated to dryness. The residue was dissolved in dichloromethane and washed with water. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. After a chromatographic purification on a column of silica gel using a gradient of methanol in dichloromethane (0-10%), 3 was isolated (0.6 g, 52% yield). m.p. 84-90°C; Rf (solvent A) = 0.65; Anal. Calcd for $C_{48}H_{58}N_4O_7$ + 1/2 H_2O (811.32): C, 70.99; H, 7.27; N, 6.90. Found: C, 70.63; H, 6.83; N, 6.67.

3'-O-Benzoyl-5-methyl-4-N-[10-aminodecyl] cytidine (5). Compound 3 (200 mg, 0.25 mmol) was treated with 2% benzenesulfonic acid in dichloromethane-methanol (7:3) (7ml) at 0°C. The reaction was monitored by TLC. After 20 min., dichloromethane (50 ml) was added to the reaction mixture. The organic layer was washed with 10% $NaHCO_3$ and water, dried over sodium sulfate and evaporated to dryness. The residue was dissolved in a minimum amount of dichloromethane. Addition of light petroleum ether gave a white precipitate. Further chromatographic purification afforded 5 (70 mg, 56% yield). Rf (solvent A) = 0.52.

5-Methyl-4-N-[10-aminodecyl] cytidine (7). To a solution of 5 (70 mg, 0.14 mmol) in THF-MeOH (1:1) (2 ml) at 0°C was added slowly 1.56 N NaOH (1.3 ml) under vigorous stirring. After 45 min., the resulting mixture was neutralized by the addition of Dowex-50 W (H^+). The resin was filtered off, further washed with ethanol and the filtrate was evaporated to dryness. Purification by HPLC on Zorbax ODS 9.3 mm (acetonitrile-10^{-2}M triethylammonium acetate buffer) gave 7 (10 mg, 18% yield). m.p. 92-96°C; Rf (solvent A) = 0.48; Anal. Calc for $C_{20}H_{36}O_4N_4$ (396.53): C, 60.58; H, 9.15; N, 14.13. Found: C, 60.03; H, 9.50; N, 14.89; M.S. (C.I., NH_3): 397 (M); 307 (B+28); 281 (B+1); 173 (NH_2-$(CH_2)_{10}$-NH_2).

N-[5-Dimethylamino-1-naphthalenesulfonamido]-1,10-diaminodecane (N-dansyl-1,10-diaminodecane) (2). To a solution of 1,10-diaminodecane (0.77 g, 4.5 mmol) in dioxane (60 ml) was added 5-dimethylamino-1-naphthalene sulfonamide chloride (dansyl chloride) (0.4 g, 1.5 mmol) under vigorous stirring. After 1 h at room temperature, the resulting gum was heated at 90°C for 30 min. After evaporation to dryness, the residue was purified on a column of silica gel to give 2 (0.42 g, 72% yield). Rf (solvent A) = 0.27; Anal. Calcd for $C_{22}H_{35}O_2N_3S$ + 1/2 H_2O (414.60): C, 65.14; H, 8.70; N, 10.36. Found: C, 64.24; H, 8.71; N, 9.76.

5'-O-Dimethoxytrityl-3'-O-benzoyl-5-methyl-4-N-[10-N-dansylaminodecyl] cytidine (4). To a solution of 1 (0.52 g, 0.75 mmol) in dioxane (15 l) was added N-dansyl-1,10-diaminodecane (0.3 g, 0.75 mmol). After stirring for 48 h, the resulting mixture was evaporated to dryness. After chromatographic purification, 4 was isolated (0.68 g, 88% yield). m.p. 98-100°C; TLC (CH_2Cl_2-MeOH: 93-7) Rf = 0.70; Anal. Calcd for $C_{60}H_{69}O_9N_5S$ (1035,30): C, 69.54; H, 6.71; N, 6.75. Found: C, 69.21; H, 6.39; N, 6.34.

3'-O-benzoyl 5-methyl-4-N-[10-N-dansylaminodecyl] cytidine (6). Compound 4 (0.40 g, 0.44 mmol) was treated with a solution of 2% benzenefulfonic acid (14 ml) at 0°C. After 5 min., the mixture was submitted to a work-up similar to compound 5. After chromatographic purification, compound 6 was isolated with 94% yield (0.31 g). m.m. 88-90°C TLC (CH_2Cl_2-MeOH: 93-7) Rf = 0.42; Anal. Calcd for $C_{39}H_{51}O_7N_5S$ (733.90) C, 63.82; H, 7.00; N, 9.54; S, 4.37. Found: C, 63.76; H, 7.00; N, 9.15; S, 4.62.

5-Methyl-4-N-[10-N-dansylaminodecyl] cytidine (8). To a solution of 6 (260 mg, 0.35 mmol) in THF-MeOH (1:1) (1 ml) at 0°C was added 1.56 N NaOH (5 ml). After 10 min., the resulting mixture was neutralized by addition of Dowex-50W (H^+ form). After the work-up similar to compound 7, 8 was isolated (110 mg, 50% yield). m.p. 98-100°C; Rf (CH_2Cl_2-MeOH: 85-15) = 0.46; Anal. Calcd for $C_{32}H_{47}N_5O_6S$ (629.80): C, 61.02; H, 7.52; N, 11.12; S 5.09. Found: C, 59.83; H, 7.33; N, 10.87; S, 5.26; M.S. (C.I. NH_3): 630 (M), 514 (B+1), 406 ($NH_2-(CH_2)_{10}$-NHdS).

8-[10-aminodecyl]-amino-2'-deoxyadenosine (10). A mixture of 8-bromo-2'-deoxyadenosine (9) (15.7 g, 47 mmol) and 1,10-diaminodecane (32 g, 186 mmol) in ethanol (350 ml) was refluxed for 24 hours. The solution was concentrated to dryness and the residual material was washed with anhydrous diethyl ether until the product was free from 1,10-diaminodecane. The residue was chromatographed on a column of silica gel using isopropanol-ammonia-water (6:1:1). Solvents were evaporated to dryness and the residue was crystallized from ethanol-water give 10 (10.8 g, 55% yield). m.p. 190°C; Anal. Calcd for $C_{20}H_{35}N_7O_3$ (421): C, 56.98; H, 8.37; N, 23.25. Found: C, 56.83; H, 8.59; N, 23.65.

8-[10-benzyloxycarbonyl)aminodecyl)]-amino-2'-deoxyadenosine (11). To a solution of 10 (3.7 g; 8.5 mmol) in a mixture of water (30 ml) and ethanol (40 ml), 1-benzyloxycarbonyl-3-methylimidazolium chloride (4.5 g; 17.7 mmol) was added and the reaction mixture was stirred at room temperature overnight. The solvents were evaporated to dryness. The N-benzyloxycarbonyl compound 11 (3.3 g; 72%) was isolated by chromatographic purification on silica gel using (CH_2Cl_2-MeOH: 10-1) as eluent.

6-N-benzoyl-8[10-benzyloxycarbonyl aminodecyl]-amino-2'-deoxyadenosine (12). Benzoyl chloride (3.7 ml) was added to dried N-benzyloxycarbonyl compound 11 (2.38 g; 4.28 mmol) in anhydrous pyridine (15 ml) at 0° and the mixture was then left overnight at room temperature. The reaction mixuture was quenched with methanol (5 ml) and the solvent was evaporated to dryness. The residue was dissolved in CH_2Cl_2 (100 ml), washed with a saturated solution of $NaHCO_3$ and then with water. Drying and evaporation left a residue; the latter was dissolved in a mixture of pyridine (12.6 ml) and ethanol (6.4 ml) at 0°C; 8 N NaOH (9.5 ml) was added. After 30 min. the reaction mixture was neutralized with Dowex-50W (H^+ form). The resin was filtered off and washed with methanol and the combined filtrate was evaporated to dryness. The monobenzoyl derivative 12 (2.15 g; 76%) recrystallized from ethanol. m.p. 87-88°C; ^1H-NMR 400 MHz: ($CDCl_3$ + TMS): δ = 1.20 (m, 12H, CH_2(3-8)); 1.38 (m, 2H, CH_2(9)); 1.55 (m, 2H, CH_2(2)); 2.22 (m, 1H, H"-2); 2.67 (m, 1H, H'-2); 3.10 (m, 2H, CH_2(10)); 3.35 (m, 2H, CH_2(1)); 3.87 (m, 2H, H-5'H-5"); 4.08 (m, 1H, H'-4); 4.69 (m, 1H, H'-3); 4.76 (s,1H, NH); 5.06 (s 3H, $CH_2\Phi$ + NH); 6.50 (q, 1H, H'-1); 7.37 (s, 5H, Φ); 7.41-7.49 (m, 3H, HΦ'p,HΦ'mm'); 7.98-8.00 (s+s, 2H, HΦ'OO'); 8.4 (s, 1H, H-2).

6-N-benzoyl-8-[10-benzyloxycarbonyl aminodecyl]-3'-O-benzoyl-2'-deoxyadenosine (13). Dimethoxytrityl chloride (1.13 g; 3.3 mmol) was added to the monobenzoylated compound 12 (2 g, 3 mmol) in anhydrous pyridine (6.5 ml); after 2 1/2 h at room temperature, the reaction was quenched with methanol (2 ml) and the solvent was evaporated to dryness. The residue was dissolved in CH_2Cl_2 and washed with a saturated $NaHCO_3$ solution and water, and the CH_2Cl_2 solution was dried over anhydrous Na_2SO_4 and evaporated. After washing twice with light petroleum ether, the resulting material (1.85 g; 1.9 mmol) was dissolved in pyridine (25 ml), benzoyl chloride (2.6 g; 11.4 mmol) and dimethylamino pyridine (DMAP) (115 mg) were added. After 1 1/2 h at room temperature the solution was evaporated, the residual syrup was dissolved in CH_2Cl_2, washed with saturated $NaHCO_3$ solution, water and finally precipitated with light petroleum ether. The latter was treated with 2% benzenesulfonic acid in CH_2Cl_2-MeOH (7:3), (30 ml) for 10 min. Washing, drying and evaporating left a syrup which was chromatagraphed on a column of silica gel (CH_2Cl_2-MeOH, 93:7). ^1H-NMR 400 MHz: ($CDCl_3$ + TMS): δ = 1.25 (m, 12H, CH_2 (3-8)); 1.46 (m, 2H, CH_2 (9)); 1.63 (m, 2H, CH_2(2)); 2.47 (dd; 1H, H-2"); 2.97 (m, 1H, H-2'); 3.17 (q, 2H, CH_2 (10)), 3.47 (q 2H, CH_2 (1)); 4.03 (d, 1H, H"-5); 4.16 (d, 1H, H'-5); 4.31 (m, 1H, H'-4); 5.08 (s, 2H, $CH_2\Phi$); 5.71 (d, 1H, H'-3); 6.66 (q, 1H, H'-1); 7.33 (s, 5H, Φ); 7.45-7.65 (m, 2H,HΦ'mm'); 7.82 (d, 1H, HΦ'p); 8.00-8.12 (2d, 2H, HΦ'oo'); 8.57 (s, 1H, H-2); 9.10 (s, 1H, NHCOΦ').

8-[10-aminodecyl]-amino-5'-dATP (15). A solution of 13 (0.327 g; 0.48 mmol) and 1 mmol (1 ml of stock solution) of 2-cyanoethyl phosphate in pyridine (5 ml) was concentrated in vacuo at 30°C to a syrup. The syrup was then taken up in anhydrous pyridine (10 ml) and the solution was evaporated to dryness. This process was repeated. Anhydrous pyridine (5 ml) and dicyclohexyl carbodiimide (DCC) (0.5 g) (6 mmol) was added, the reaction flask tightly stoppered and set aside for 48 hours at room temperature. Water (3 ml) was added and the solution left half an hour at room temperature; pyridine was evaporated under reduced pressure and the residue taken up in water (10 ml) and filtered to remove dicyclohexylurea. The urea was washed well with water (10 ml). Concentrated ammonium hydroxide (15 ml) was added and the mixture heated at 60°C for 24 h. The solution was concentrated to dryness in vacuo and applied to a column of Sephadex G-10 gel (100 x 25 cm) eluted with 50% ethanol with a flow rate of 40 ml per h. Fractions of 10 ml were collected. The appropriate fractions (as monitored by UV and phosphorus determination) were pooled, concentrated to a small volume and lyophilized, to afford 178 mg (49%) of pure monophosphate (14). The phosphomonoester (0.2 g) was dissolved in a mixture of methanol and water (1:1) and passed through a column of Dowex-50W (morpholinium form) (1 x 3 cm). After washing the column free of the nucleoside with the mixture of methanol and water, the total eluent was evaporated to dryness and the residue dissolved in a mixture of water (3 ml) and t-butanol (3 ml) containing morpholine (0.105 ml; 4 eq.); dicyclohexylcarbodiimide (0.259 g; 1.25 mmol) dissolved in t-butanol was added dropwise. After refluxing the mixture for 4 h, TLC on silica gel (isopropanol-NH_4OH-H_2O:7-2-1) showed a single product moving faster than the phosphomonoester. The solution was cooled at room temperature, an insoluble material was removed by filtration and the filtrate was evaporated to dryness. The residual syrup was dissolved in water. After three extractions with diethyl ether (15 ml), the solvent was removed in vacuo and the residual sirup was dried by evaporation. The residual material was dissolved in a mixture (5 ml) of water and ethanol (1/1), palladium on charcoal (10 mg) at 10% was added and the solution was hydrogenated at room temperature and atmospheric pressure for 30 min. After filtration the solution was concentrated to dryness by four coevaporations with pyridine (10 ml). Residual pyridine was removed by evaporation twice with toluene (5 ml). A mixture of pyropoephoric acid (178 g; 1 mmol), water (5 ml), pyridine (10 ml) and tri-n-butylamine (1.0 ml; 4.2 mmol) was evaporated to a syrup and dried by 4 evaporations with 10 ml of pyridine. The residual pyridine was then removed by 2 evaporations with 5 ml portions of toluene.

The pyrophosphate was dissolved in fresly distilled dimethylsulfoxide (5 ml) and added to the dry morpholidate. The solution was kept at room temperature for 24 h, pyrophosphate (0.5 mmol) was added as before and after three days, water (5 ml) was added and the resulting solution is applied to a column (2.5 x 30 cm) of DEAE cellulose in the bicarbonate form. The column is then washed with water until the optical density of the effluent falls to zero and then eluted with a linear gradient of 0–0.35 M triethylammonium bicarbonate. The appropriate fractions, monitored by UV and phosphorus content were pooled, concentrated to dryness with a bath temperature of 30°C. All the residual triethylammonium bicarbonate was removed by three evaporations in methanol, dissolution in water followed by lyophillisation. These operations led to 63 mg of a pure triphosphate derivative (25% yield). M.S. (FAB): 662.16 ($M+H^+$); ^1H-NMR 400 MHz: (D_2O): δ = 1.8 (m, 14H, decyl protons); 1.49 (t, 2H, decyl protons); 1.61 (m, 2H, decyl protons); 2.18 (dd, 2H, H"-2); 2.68 (m, 1H, H'-2); 2.84 (t, 2H, decyl protons); 3.38 (m, 2H, H-5' H-5"); 4.14 (dd, 1H, H'-4); 4.30 (t, 1H, H'-3); 4.65 (HDO); 6.35 (q, 1H, H'-1); 7.98 (s, 1H, H-2); ^{31}P-NMR 162 MHz: (D_2O): δ (PO_4H_3 as reference) = - 8.209 (d, 1P, P_γ, $Jp_\gamma\text{-}p_\beta$ = 19.45 Hz); - 9.866 (d, 1P, P_α, $Jp_\alpha\text{-}p_\beta$ = 19.45 Hz, $J p_\alpha\text{-}p_\gamma$ = 0 Hz); - 20.987 (t, 1P, P_β, $J p_\beta\text{-}p_\alpha$ = $J p_\beta\text{-}p_\gamma$ = 19.45 Hz).

8-[10-biotinylaminodecyl]-amino-5'-dATP (16). To triphosphate 15 (9 mg; 13.6 mmol) in 1 M sodium bicarbonate pH 9 (0.2 ml), was added biotinyl-N-hydroxysuccinimide ester (5 mg; 14.6 mmol) in dimethylformamide (0.4 ml). The reaction mixture was left at 4°C for 18 hours, diluted with water (1 ml) and then loaded directly on a column (100 x 2.5 cm) Sephadex G-10 and eluted with deionised water. Fractions containing biotinyl-dATP-chain were pooled and lyophillised. The resulting material was purified by HPLC on Nucleosil 5C 18 (4.6 x 250 mm) using a linear gradient ranging from 5 to 50% acetonitrile against 10^{-2} M trietylammonium acetate over 20 min. at 1 ml/min (1.8 mg, 15% yield). M.S. (FAB): 888.63 ($M+H^+$).

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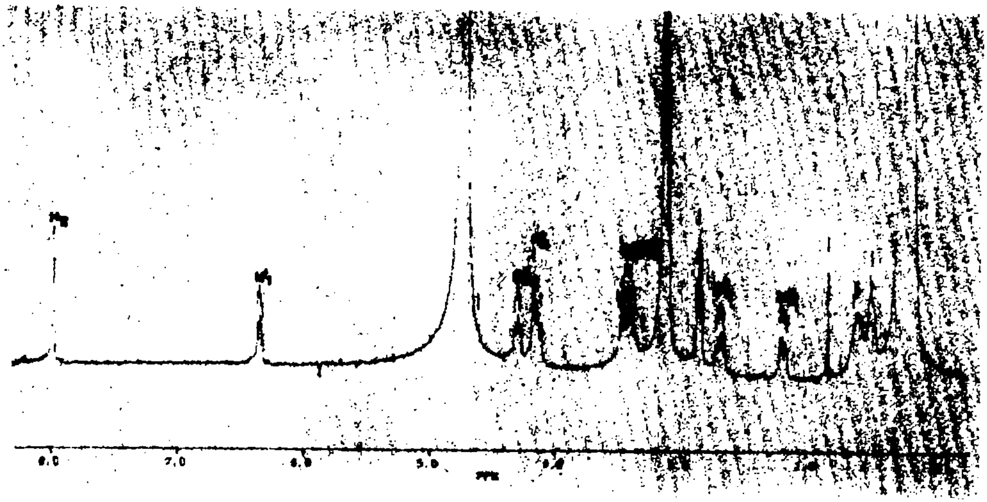

Figure 1: ^{1}H-NMR spectra of dATP derivative 15 measured at 400 MHz

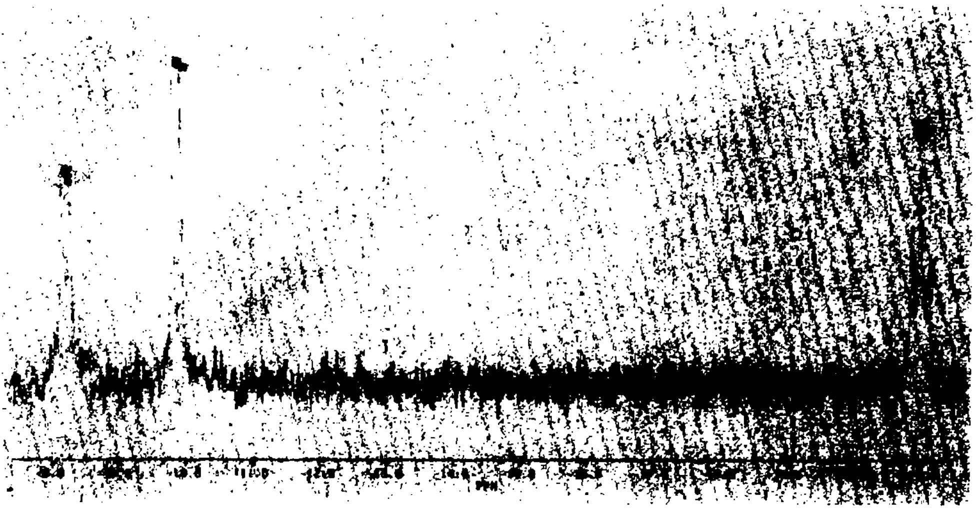

Figure 2: ^{31}P-NMR spectra of dATP derivative 15