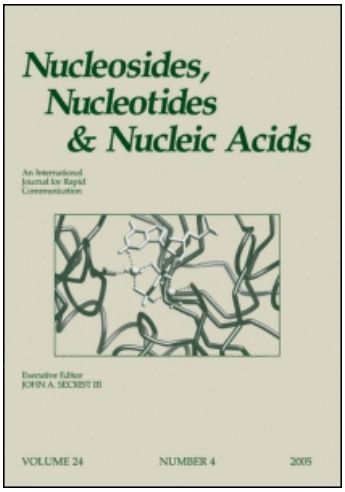

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Labelling of DNA with a Non-Radioactive Analogue of dGTP

I. C. Gillam[a]; G. M. Tener[a]
[a] Department of Biochemistry, 2146 Health Sciences Mall, University of British Columbia, Vancouver, B. C., Canada

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LABELLING OF DNA WITH A NON-RADIOACTIVE ANALOGUE OF dGTP

I.C. Gillam* and G.M. Tener
Department of Biochemistry, 2146 Health Sciences Mall, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

Abstract 8-Bromo-2'-deoxyguanosine-5' phosphate reacts with 2-mercaptoethylamine. Oxidation of the reaction mixture generates a disulphide with free aliphatic amino group. Biotinylation yields an analogue of dGMP, Bio-15-dGMP. The triphosphate, Bio-15-dGTP.may be incorporated into DNA by DNA polymerase I of Escherischia coli and detected by reagents conjugated to avidin.

INTRODUCTION

Development of methods of gene analysis has depended upon the use of radioactively labelled nucleotides both for sequence determination and in hybridization studies to localize specific nucleotide sequences. The disadvantages of radioactive labels, short lifetime, hazards and costs in handling and disposal, have led to the development of alternative, non-radioactive labels without these limitations[1] Methods of labelling generally depend on the introduction of an easily modified aliphatic amino group onto the base moiety of a nucleotide. This serves as the point of attachment for the label, often a biotinyl residue, that is later detected via a multiplying chain linking numerous molecules of a signal-generating enzyme to a specific binding protein such as avidin (or streptavidin). The classical procedure of Ward and his co-workers[2] incorporates biotinylated pdU residues into DNA or oligonucleotide probes in place of pdT. Since it is desirable to have a range of alternative substrates, we have investigated the preparation of a derivatized dGTP.

An early description of non-radioactive probes[3] utilized commercially available 8-(6-aminohexylamino)ATP. This may be prepared[4] in three stages by bromination of AMP under mild conditions, reaction of the resultant 8-bromoAMP with 1,6-diaminohexane followed by subsequent phosphorylation. Similar reactions directly on ATP have been reported[5]. Studies on the chemistry of guanine nucleotides are often hampered by their notorious insolubility though analogous reactions have been described for GMP[6,7].

dGMP is less stable than GMP and 8-bromoguanosine is much less reactive than 8-bromoadenosine[8]. Under conditions similar to those used to prepare 8-(6-aminohexylamino)AMP and 8-(6-aminohexylamino)GMP we were unable to obtain the corresponding dGMP compound (details not presented). Reaction of 8-bromo-dGMP (8BrdGMP) with the more strongly nucleophilic thiol group of 2-mercaptoethylamine led to isolation of a product suitable for attachment of biotinyl groups.

The product isolated had the desired feature of an aliphatic amino group attached via C8 of guanine and allowed the preparation of an active biotinylated dGTP analogue. Its structure was more complex than anticipated, containing a disulphide linkage rather than a simple thioether and is described in detail below.

MATERIALS AND METHODS

Solutions of nucleotides were evaporated under reduced pressure with a bath temperature of 30° C or less and with repeated additions of water. Brij 58 is a non-ionic detergent, polyoxyethylene 20 cetyl ether, purchased from Sigma Chemical Co., as was 2-mercaptoethylamine. Cellulose nitrate filters were BA85 from Schleicher and Schuell. Nylon filters were Hybond N from Amersham Corp.

Preparation of 8BrdGMP (after Kapmeyer et al.[7])

dGMP NH_4^+ salt (206 mg, 565 µmole) was dissolved in 20 mL of 0.67 M acetic acid, pH 3.0 (NaOH) and stirred at 20° C. Water (4.3 mL) freshly saturated with bromine (2 equiv) was added dropwise and the solution stirred for 10 min. The colour was carefully discharged by addition of bisulphite solution and the faintly yellow solution adjusted to pH 8 with ammonia and diluted to 270 mL. It was loaded onto a column (2 X 13 cm) of QAE-Sephadex A25 (Pharmacia), HCO_3^- form, equilbrated with 10 mM NH_4HCO_3, pH 8 (NH_4OH) and the column washed with the same until negligible UV-absorbing material eluted, when it was further eluted with a gradient of concentration of NH_4HCO_3, pH 8 (NH_4OH) from 10 mM to 400 mM (total of 2 L). Fractions of 10 mL were collected every 6.5 min. The main peak of UV-absorbing material, eluting about the middle of the gradient, was pooled and evaporated to dryness. Yield of 8BrdGMP, 4500 A_{260} units, 288 µmole.

Reaction of 8BrdGMP with 2-mercaptoethylamine

8BrdGMP (3500 A_{260} units, 224 µmole) was transferred in water to a small, glass-stoppered flask and dried. To the sample was added 7 mL of 0.5 M 2-mercaptoethylamine, pH 9.44 (HCl). The flask was stoppered and incubated at 63° C for 3 days. The resulting solution was cooled, diluted to 1 L with water and subjected to oxidation by bubbling air overnight. The solution was adjusted to pH 8 by addition of CO_2 and applied to a column (2.0 X 14.5 cm) of QAE-Sephadex A-25 equilibrated with 10 mM NH_4HCO_3, pH 8.0. The column was washed with the same solution and then briefly

with water. It was eluted with a linear gradient of acetic acid from 1 L of water to 1 L of 0.1 M acetic acid (pH unadjusted). Fractions of 10 mL were collected every 6.2 min. UV absorbance of fractions was recorded and the broad peak of material eluting around tube 85 (nominal 40 mM acetic acid) was pooled and recovered (total of 1970 A_{260} units). The product (IIIa) showed;

λ_{max1}=261 nm, λ_{max2}=294 nm, λ_{min1}=228 nm, λ_{min2}=288 nm at pH 7;

λ_{max1}=253.5 nm, λ_{max2}=287 nm, λ_{min1}=227 nm, λ_{min2}=276 nm at pH 2;

λ_{max1}=262.5 nm, shoulder 279 nm, λ_{min}=230.5 nm at pH 12.

Preparation of Bio-15-dGMP (IVa)

Nucleotide IIIa (540 A_{260} units, approx. 40 µmole) was dissolved in 13.3 mL of water plus 26.7 mL of 0.1 M H_3BO_3, pH 9.0 (NaOH). To the solution at room temperature was added the sulpho-N-hydroxysuccinimide ester of N-biotinyl-6-aminohexanoic acid (Pierce Chemical Co.; 56 mg, 100 µmole). The solution was incubated in the dark with occasional shaking for two and a half hours, when it was diluted to 230 mL with water and applied to a column (2 X 19 cm) of QAE-Sephadex A25 equilibrated with 10 mM NH_4HCO_3, pH 8.0 (NH_4OH), washed in, and eluted with a linear gradient of concentration of NH_4HCO_3, pH 8.0 from 10 to 400 mM (total of 1.5 L). Fractions of 7.5 mL were collected every 9.2 min. The first major peak of UV-absorbing material eluting around tube 100 was pooled and evaporated to dryness. Yield 329 A_{260} units (60%).

Synthesis of Bio-15-dGTP (method of Smith and Khorana [9])

Bio-15-dGMP (IVa, 165 A_{260} units) was dissolved in a mixture of pyridine (305 mL), water (30 mL), tributylamine (38 mL) and 85 % phosphoric acid (5 mL). N,N'-Dicyclohexylcarbodiimide (DCC, 79 mg) was added and the solution incubated at room temperature. After two days, further pyridine (305 mL) and DCC (79 mg) were added. After a total of four days, water was introduced, the solution incubated about an hour and extracted with ether. The crystals (N,N'-dicyclohexylurea) were filtered off from the aqueous layer and washed with water. The clear filtrate was evaporated and the residue dissolved in 10 mM NH_4HCO_3, pH 8 (NH_4OH) and applied to a column (2 X 11 cm) of QAE-Sephadex A25 equilibrated with the same. The column was eluted with a linear gradient of concentration of NH_4HCO_3, pH 8 from 10 to 700 mM (total of 1 L). Fractions of 5 mL were collected every 6.1 min. Material in the major UV-absorbing peak around tube 110 (nominal 390 mM NH_4HCO_3) was pooled and evaporated. Yield 37 A_{260} units (23% of original). UV absorption spectra of the product at various pH values were the same as those of Bio-15-dGMP (above).

Determination of phosphorus and acid-labile phosphate content

Total phosphorus was determined spectrophotometrically after combustion with magnesium nitrate as described by Ames[10]. Acid-labile phosphate was released by

hydrolysis in 0.5 M HCl at 100° C. Liberated phosphate was determined[10] on samples hydrolysed for various times to 30 min to record the time course of release.

Conversion of nucleotide IIIa to the nucleoside IIIb

Nucleotide IIIa (980 A_{260} units) was dissolved in 20 mL of 50 mM NH_4HCO_3, pH 8.0 (NH_4OH) and treated with alkaline phosphatase of Escherichia coli at 37° C. Samples were examined by paper electrophoresis at pH 8 to determine when hydrolysis was complete. The reaction mixture was diluted with water to 200 mL and loaded onto a column (2 X 22 cm) of CM-cellulose equilibrated with 10 mM NH_4HCO_3 and washed with the same. The column was eluted with a linear gradient of concentration of NH_4HCO_3, pH 8.0 from 10 to 50 mM (total of 2 l). Fractions of 10 mL were collected every 6.1 min. A_{260} of eluted fractions was determined against water. A single peak of UV-absorbing material eluting around tube 48 (nominal 20 mM NH_4HCO_3) contained 644 A_{260} units (66% of original). Pooled material was evaporated to dryness as a glassy film. The product crystallized from water as a fine, white precipitate. On drying it left a faintly brown residue.

Mass spectroscopy of nucleoside IIIb

A sample was subjected to fast atom bombardment mass spectrometry in a matrix of 4-nitrobenzyl alcohol (British Columbia Regional Mass Spectrometry Centre, Department of Chemistry, University of B.C.).

Incorporation of nucleotides into DNA by nick translation

The incorporation of radioactivity from [α^{32}P]dATP into acid-insoluble material was observed essentially as described[11]. Solutions containing [α^{32}P]dATP and the three other natural deoxynucleoside triphosphates or with Bio-15-dGTP substituting for dGTP were incubated with DNA polymerase I of E. coli (Pharmacia) at 16° C. Samples were removed at intervals onto filter paper discs and washed in acid. Radioactivity remaining insoluble was determined by Cerenkov radiation detected in a scintillation counter.

Labelling of DNA by nick translational incorporation of Bio-15-dGTP

Reaction mixes contained the following components in a total volume of 50 mL; 50 mM Tris HCl, pH 7.4; 10 mM 2-mercaptoethanol; 5mM $MgSO_4$; 0.5 mM $MnSO_4$; 0.1 % w/v Brij 58; DNA 3μg; 10 μM Bio-15-dGTP; 0.2 μM dGTP (optional); 10 μM each dATP,dCTP, dTTP; 3.5 units DNA polymerase I of E. coli; 25 pg DNase I. Solutions were assembled at 0° C, mixed well and incubated at 16° C for 2 h. DNA was recovered by gel filtration as described earlier[11] on centrifugal columns (1 X 6 cm) of BioGel P6 (Bio-Rad) equilibrated with 14.5 mM NaCl/1.45 mM sodium citrate, pH 7/0.1% sodium dodecyl sulphate, then washed with 50 mL of the same. Recovery was assumed to be complete and the volume of eluate was measured with an adjustable micropipette.

Dot blotting, hybridization and detection of DNA

Appropriate serial dilutions of biotinylated DNA or homologous unlabelled DNA were prepared in 1.5 M NaCl/0.5 M NaOH. Samples of 2 mL were applied as dots to discs of cellulose nitrate or nylon, neutralized and fixed according to the supplier's instructions. Dots of unmodified DNA were hybridized with Bio-15-dGTP-labelled homologous DNA following standard procedures [12 and instructions in detection kit]. Biotinylated DNA bound to filters either directly or through hybridization was detected with a streptavidin-phosphatase complex (BluGene detection kit, BRL or Detek I-acp, Enzo Biochem) according to the supplier's directions.

Paper electrophoresis

Samples spotted onto 3MM paper (Whatman) wetted with 50 mM NH_4HCO_3/0.1% (w/v) EDTA Na_2, pH 8.0 (NH_4OH) were run in a Shandon water-cooled apparatus at 2kV (27 V/cm) for 2 h. Papers were dried and UV-absorbing spots marked before dipping into ninhydrin [13] or other reagent[15].

RESULTS AND DISCUSSION

Bromination of dGMP under the conditions described by Kapmeyer et al.[7] for the preparation and isolation of 8-BrGMP proceeded easily and rapidly. Displacement of the bromine in 8-bromoAMP by one of the nitrogen atoms of 1,6-diaminohexane in aqueous solution required prolonged heating at high temperature[7]. The less stable 8BrdGMP failed to yield detectable 2'-deoxy-8-(6-aminohexylamino)-GMP under similar conditions (data not presented). Attempts to reduce decomposition using non-aqueous solutions were unsuccessful, perhaps because of the poor solubility of guanine nucleotides. As a thiol group is expected to be a more effective nucleophile than an amino group we tried the use of 2-mercaptoethylamine to displace the bromine and introduce a potentially reactive amino group into the nucleotide. Reaction proceeded under relatively mild conditions. Its progress could be followed by paper electrophoresis at pH 8. Introduction of an aliphatic amino group reduces the charge on the nucleotide and hence its electrophoretic mobility. The expected change in charge and mobility of the product was observed (Table 1). This product had properties expected of 8(2-aminoethylthio)dGMP (I) including an absorption spectrum related to that of guanine nucleotides, reduced negative charge, positive ninhydrin reaction and positive test for divalent sulphur[14].

The product isolated was, however, not the anticipated 8-(2-aminoethylthio)dGMP (I) but a mixed disulphide (IIIa) of the isomeric 8-(2-mercaptoethylamino)dGMP (II).

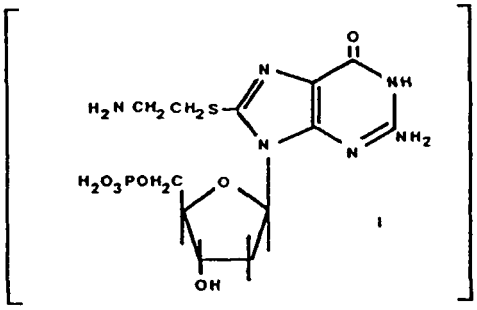
Presumably the intermediate I (not characterized) rearranges into the more stable form II during heating. As evidence for structure IIIa spots of the product separated by paper electrophoresis gave a positive nitroprusside test for the thiol

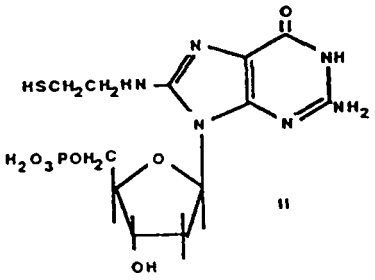
TABLE 1
Electrophoretic Mobility of Some Nucleotides
(pH 8, 2 h at 27 V/cm on paper)

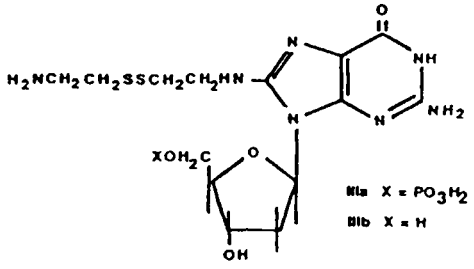
Compound	Distance migrated (cm)
dGMP	19
dGTP	22
II	16
IIIa	5
Bio-15-dGTP (IVb)	12.5

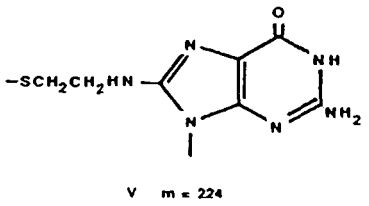
group[15] only after reduction with sodium cyanide (data not presented). After reduction with dithiothreitol the nucleotide ran on electrophoresis almost as far as dGMP (Table 1), was ninhydrin negative and gave a positive nitroprusside test without reduction by cyanide, in agreement with structure II. Material dephosphorylated to the nucleoside (IIIb) was recovered by chromatography on CM-cellulose as an apparently crystalline solid. On purification this gave a mass spectrum having parent ion at 418, corresponding to m+1 (calculated for IIIa, m=417.125 on C = 12.000 scale). A major peak at mass 225 corresponds to the fragment V (m = 224) +1.

As the stable nucleotide product (II) contains a thiol group it is necessary to oxidize it in the presence of 2-mercaptoethylamine to form the disulphide IIIa with



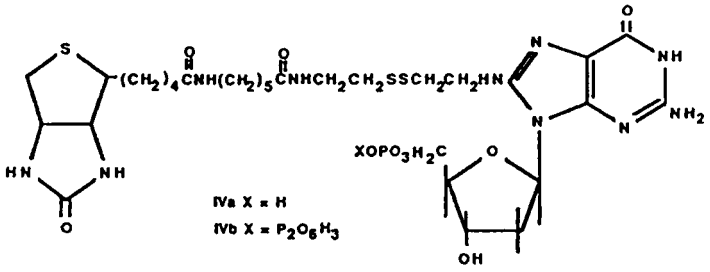






added primary amino group, allowing chromatographic isolation on the basis of reduced charge. Oxidation of the reaction mixture, for example by air, is an essential step in obtaining the required nucleotide IIIa in good yield. Nucleotide IIIa carries a derivatizable aliphatic amino group attached to C8 of the purine by a chain containing seven atoms and including a disulphide bond. Such bonds are stable under non-reducing conditions and on the acid side of neutrality. A commercially available biotinylation reagent[16] contains a similar disulphide link, allowing ready dissociation of complexes between molecules labelled with it and avidin, by mild reduction. (Avidin complexes so strongly with biotin that dissociation by other means is sometimes difficult.) While not utilized in this study this cleavage by reduction makes the biotinylated nucleotide of possible use in the isolation of, for example, specific DNA-binding proteins.

The mild conditions of bromination allowed direct preparation of 8BrdGTP from dGTP but the polyphosphate was not stable enough to withstand reaction under similar conditions with 2-mercaptoethylamine (data not presented). In order to prepare the required triphosphate from the monophosphate the introduced aliphatic amino group needed protection. For this study acylation with the desired biotinyl group provided it. Brigati et al.[17] have shown that in the case of DNA labelled with dUTP derivatives bearing a biotinyl group at the 5 position, sensitivity of detection is increased as longer connecting chains are introduced between the nucleotide and the biotinyl group. Here we used a commercially available long chain biotinyl ester of the readily water-soluble N-hydroxysulphosuccinimide. This reacted smoothly with IVa to form a deoxyguanylate linked at C8 to a biotinyl group through a spacer arm fifteen atoms long, Bio-15-dGMP (IVa). Its UV absorption spectrum was similar to that of IIIa (data not presented). Reaction of IIIa with other types of non-radioactive labels might be useful in generating DNA probes of different kinds.



The phosphorylation procedure of Smith and Khorana[9] is extremely simple, requiring reaction of otherwise unprotected 5'-nucleotide with inorganic phosphate and carbodiimide in partly aqueous medium. The last is particularly expedient due to the difficulty of solution of guanine nucleotides in non-aqueous solvents. With simple

nucleotides a principal product is the triphosphate, isolable by ion-exchange. While yields are not as high as with more recently developed reagents the simplicity of the system recommended itself. Ion-exchange chromatography (not shown) separated a series of products formed from Bio-15-dGMP, as expected. The major peak was considered likely to be Bio-15-dGTP (IVb) and was recovered for further examination. Paper electrophoresis (Table 1) showed that the material ran with a mobility only 60% of that of dGTP. While its charge should be the same as that of dGTP, Bio-15-dGTP is of more than twice the molecular weight, explaining the relative rate of migration.

A critical test of the identity of a polyphosphate is the proportion of its phosphate content that is released by hydrolysis in dilute acid. Studies of the time course of hydrolysis (data not presented) showed that release of phosphate from this nucleotide levelled off after 30 min with 86% of the total being hydrolysed. This is significantly more than the 67% expected for a nucleoside triphosphate. However, comparison with authentic dGTP showed that its hydrolysis followed the same course, releasing 87% its phosphate in 30 min (data not presented). Thus we conclude that the nucleotide isolated is indeed a triphosphate, Bio-15-dGTP. The unexpectedly high release of phosphate in acid is interpreted as being due to the ready depurination of deoxyguanosine derivatives and the instability of the resulting 2-deoxyribose-5 phosphate. Total phosphorus determination allowed calculation for Bio-15-dGTP of $\epsilon = 11.7 \times 10^3\ M^{-1}cm^{-1}$ at pH 7.

Further confirmation of the structure and utility of Bio-15-dGTP was sought in determining whether it is a substrate for DNA polymerase I of E. coli. This enzyme catalyzes extensive nick translational resynthesis of double-stranded DNA only when supplied with all four deoxynucleoside triphosphates or accepted analogues. The reaction is readily followed by incorporation of radioactive label into the DNA. Initial results in a standard nick translation system[12] were negative. Bio-15-dGTP could not replace dGTP in supporting synthesis (data not presented). Only when the system contained low levels of Mn^{2+} as well as 5 mM Mg^{2+} was synthesis observed with Bio-15-dGTP. The optimum level was around 0.5 mM Mn^{2+} (data not presented).

Thus Bio-15-dGTP is a substrate for DNA polymerase, though an inefficient one. The rate of incorporation is low compared with that in the presence of dGTP. After one hour at 16° C it reached 40% of the control (results not shown). As it is neither necessary nor desirable for detection with streptavidin to have every dGp residue in a stretch of DNA replaced by Bio-15-dGp[1], a mixture of dGTP and the analogue may be used. With Bio-15-dGTP and dGTP in a 50:1 molar ratio incorporation was more rapid, reaching 60% of the control in one hour at 16° C (data not presented).

The final test of the utility of Bio-15-dGTP is whether DNA labelled with it may be detected by biotin-specific reagents. DNA of E. coli was labelled , recovered and fixed to

filters, then subjected to detection by the colour-forming capacity of conjugates of streptavidin and phosphatase. The BluGene system (BRL) allowed the detection of 6 pg of DNA as spots 4 mm in diameter. The presence of dGTP at one fiftieth of the concentration of Bio-15-dGTP during nick translation did not affect the sensitivity of detection (data not presented).

When unlabelled DNA of E. coli was immobilized on filters and hybridized with a probe of homologous DNA labelled with Bio-15-dGTP and dGTP (50:1 ratio) the same system subsequently detected dots containing 60 pg of DNA. Sensitivity was the same in parallel experiments using probes labelled with other substrates, N^4-(6-biotinylaminohexyl)-2'-deoxycytidine-5' triphosphate (Bio-ahdCTP)[11] plus dCTP and Bio-11-dUTP plus dTTP (data not presented).

Bio-15-dGTP may be used as a label for DNA probes. The corresponding dATP analogue is likely to be easier to prepare in good yield by the same method. Another dATP analogue is commercially available in biotinylated form and a third has been described[18]. Several biotinylated analogues of dTTP are commercially available[2,17] Bio-ahdCTP[11,18] is simple to prepare but apparently not at present in commerce. Each of these analogues is prepared through biotinylation of deoxynucleotides substituted to bear an aliphatic amino group. Other simple derivatization of these amino groups could yield nucleotides with other appropriate labels, haptens, fluorescent or metal-binding groups. The addition of a similar analogue of dGTP now provides non-radioactively labelled analogues of all four deoxynucleoside triphosphates that are acceptable substrates for DNA polymerase. Availability of all four raises interesting possibilities in developing new methods of gene analysis and sequence determination.

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