A new method of synthesis of fluorescently labelled oligonucleotides and their application in DNA sequencing

Wojciech T. Markiewicz*, Gabriele Gröger1, Rudi Rösch1, Anna Zebrowska, Maria Markiewicz, Margit Klotz1, Michael Hinz1, Przemyslaw Godzina and Hartmut Seliger1

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12, PL-61704 Poznan, Poland and 1Universität Ulm, Sektion Polymere, Oberer Eselsberg, D-89069 Ulm, Germany

Received May 14, 1997; Revised and Accepted July 31, 1997

ABSTRACT
A new approach to the chemical synthesis of oligodeoxynucleotides bearing reporter functional groups at base residues of 3′-end nucleosides is reported. Applications of the 3′-end fluorescently labelled primers for automated DNA sequencing are shown.

INTRODUCTION
Many chemical approaches were developed for the introduction of modifications into synthetic oligonucleotides allowing for their use in various different biochemical and biotechnological methods including diagnostics, separation of biomolecules and non-radioactive sequencing of nucleic acids (1–3).

Various published procedures can be divided, in general, according to the synthetic stage at which the modifications are introduced into the oligonucleotide chain. The oligonucleotide is modified either at the final stage of the synthesis or modified nucleotide building blocks carrying the final modifications (in the protected form if necessary) are applied during the oligonucleotide synthesis. The methods described to date fall mostly into the first of above categories, thus allowing us to introduce modifications of different chemical types into the same nucleotide substrate. However, these methods require that the modification reactions should be highly chemoselective and proceed with practically quantitative yields, so that the isolation and purification of final products would be simple, effective and free of ambiguities.

The hybridisation of synthetic oligonucleotides to DNA or RNA and their template dependent enzymatic elongation combined with non-radioactive labelling are the basis for many biomedical applications; examples are: (i) the sequence analysis of nucleic acids by the Sanger technique (4) and (ii) the polymerase chain reaction (PCR) (5,6).

For these applications non-radioactive labelling is now preferred, since problems connected with stability, handling, storage and disposal of radioactive compounds can be avoided. Enzymatic labelling has been used mostly by post-synthetic enzymatic elongation using deoxynucleotidyl terminal transferase applying structurally modified deoxynucleotide triphosphates, e.g. those of 5-bromodeoxyuridine (7), a deoxyuridine modified with biotin (8–12) or digoxigenin (13,14). However, 3′-terminal chain elongation precludes the use of a thus labelled oligonucleotide for template dependent chain elongation. Chemical labelling methods are generally more versatile, however, they must blend with the conditions of solid phase chemistry. Therefore, labelling reactions are usually done after the oligonucleotide synthesis. This includes the risk of side reactions of the unprotected oligonucleotide chain. In the case of the introduction of modified nucleotide building units, one has to deal with decay of sensitive reagents, e.g. labelled phosphoramidites. Whatever positions of the oligonucleotide chain are substituted, hydroxyl termini (15–19) or nucleobases (20–30), the labelling groups must fulfil the following conditions: (i) they should produce a signal detectable at a level as low as possible, (ii) they should not interfere with hybridisation and (iii) they should not interfere with enzymatic chain elongation. Presumably, with respect to condition (iii), the labelling of the 3′-terminal base of synthetic primers, so far, has been avoided.

RESULTS AND DISCUSSION
The concept of labelling supports

It has been mentioned in the literature that template dependent DNA polymerases accept as substrates various 5′-triphosphates of 2′-deoxynucleosides with modified base moieties (31–35). Thus, it seemed to us quite reasonable to expect a similar tolerance of polymerases to modification of the base moiety of a 3′-terminal nucleoside of oligonucleotide primers. To our surprise, the question, whether a primer containing a 3′-end modified nucleoside can be a substrate for DNA polymerases, has so far not been addressed. On the contrary, most of the chemical reactions, which were designed in order to introduce modified nucleosides or labelling modifications in general to oligonucleotide primers implied modifications at the 5′-end, thus somehow indicating a prejudice to avoid disturbance of the enzyme activity to the least extent.

In order to check a hypothesis that DNA polymerases would tolerate a 3′-terminal base modification, we decided to synthesise an oligonucleotide with 4-N-modified deoxyctydine at the 3′-terminal position. For this modification we used a reaction

*To whom correspondence should be addressed. Tel:+48 618 528503; Fax:+48 618 520532; Email: markwt@ibch.posnan.pl
deoxycytidine (dC Ts, dichloromethane as a solvent and was completed in ∼3 h. We have found in the course of our work that this slightly modified procedure of synthesis of nucleoside monosuccinates could be successfully applied for other nucleoside derivatives and leads to pure products in a convenient way. The resultant succinate was attached to the LCAA-CPG support using dicyclohexylcarbodiimide (DCC) as a condensing agent in dichloromethane in overnight reaction. The loading procedure gave DMTdC Ts support with a nucleoside content of 15 µmol/g. Recently several modified procedures for improved succinylation of nucleosides and their coupling to solid supports have been reported as well (36,37).

Then we used the above described DMTdC Ts support to prepare an oligodeoxynucleotide, d-CAGGAACAGCTATGAC Ts, by the phosphoramidite approach (38). The oligonucleotide had the sequence of a primer appropriate for sequencing of a DNA fragment, which had previously been inserted into cloning vectors derived from bacterial phage M13 (39–41). After completing the synthesis using an automated DNA synthesiser the oligonucleotide was released from the support by treatment with concentrated aqueous ammonia at room temperature over ∼1 h. Amino groups of adenine and guanine residues are left in a protected form including the NH2 group of a 3′-terminal deoxycytidine nucleoside which was “protected” as a 4-N-p-toluenesulfonylamine derivative. Then, the crude partially protected oligonucleotide was dissolved in 1,2-bis(2-aminoethoxy)ethane and transformed into an oligonucleotide containing a 3′-end aminooxyacetyl deoxycytidine unit using the same procedure that we described recently (24).

4-N-Benzoylcytosine derivatives were reported to undergo nucleophilic substitution at C-4 in up to 5% yield when treated with primary amines yielding N-substituted derivatives (22,42). However, as proved by the HPLC analysis, 4-N-benzoyl deoxycytidine in oligonucleotides is removed in ∼93% over 1 h of treatment with 32% aqueous ammonia at room temperature (t1/2 = 15 min). Thus, during release from the support, at most 0.35% of 4-N-benzoylcytidine residues might lead to 4-N-substituted cytidine units.

Then, the aminooxyacetyl oligonucleotide was reacted with fluorescein isothiocyanate (FITC) in a borate buffer as described before (24). The fluoresceinated oligonucleotide, d-CAGGAACAGCTATGACAmFl (primer A, Table 1, Fig. 2), was purified by HPLC and polyacrylamide gel electrophoresis (PAGE) and applied in sequencing of M13 DNA. The analysis of sequencing reactions with the ALF DNA sequencer from Pharmacia showed unequivocally that the primer with the 3′-end fluorescein label performed perfectly well in DNA sequencing. The printout diagram of the sequence was clear with well-distinguished bands (peaks) and could easily be read up to 500 bases in length. The performance of 3′-end labelled primer was as good as that of the primer RP (Table 1, Fig. 2) labelled at the 5′-end with the fluoresceinated Aminolink™ residue (44).

3′-End-labelling supports

Encouraged by this result we decided to find an easy route to 3′-end labelled oligonucleotides. Thus, we have undertaken the development of a series of labelling supports, i.e. polymer solid supports loaded with different nucleosides, which are modified with a non-radioactive labelling substituent or a precursor thereof. The use of such supports in solid phase synthesis will generate oligonucleotides, which after completion of synthesis cycles and cleavage from a carrier, retain a label substituent at the 3′-end. Here we describe the preparation and application of such supports and the use of thus synthesised 3′-end labelled primers in sequence analysis.
Table 1. M13 primers used in sequencing of M13mp18 DNA

<table>
<thead>
<tr>
<th>Entry</th>
<th>Sequence (5′–3′)</th>
<th>3′–5′ Base positions</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP</td>
<td>xcaggaaacagctatgac</td>
<td>6220–6204</td>
</tr>
<tr>
<td>A</td>
<td>caggaaacagctatga</td>
<td>6220–6204</td>
</tr>
<tr>
<td>M13F-A</td>
<td>ttgtaaaacgacggcc</td>
<td>6292–6308</td>
</tr>
<tr>
<td>M13F-C</td>
<td>aaacgacggccagtge</td>
<td>6288–6304</td>
</tr>
<tr>
<td>M13F-G</td>
<td>tgtaaacacggcgccttg</td>
<td>6291–6307</td>
</tr>
<tr>
<td>M13F-U</td>
<td>gtaaaacacggcgccta</td>
<td>6290–6306</td>
</tr>
<tr>
<td>M13F-Uni</td>
<td>xgttaaaacacggcgcacg</td>
<td>6290–6306</td>
</tr>
</tbody>
</table>

Fluoresceinated nucleosides in bold; x, fluoresceinated Aminolink™ residue.

Fluorescent deoxycytidine 3′-end labelling support

5′-O-Dimethoxytrityl-4-N-p-toluensulfonyldeoxycytidine (2) was reacted with an excess of 1,2-bis(2-aminoethoxy)ethane in pyridine to give 5′-O-dimethoxytrityl-4-N-(8-amino-3,6-dioxaoctyl)-2′-deoxycytidine (4) in 85% yield. A fluorescein residue was used again as a label. Thus, 4 was reacted with FITC in a mixture of DMF and aqueous borate buffer and gave a fluorescein derivative, 5′-O-dimethoxytrityl-4-N-[fluorescein-4-yl]thioureido-3,6-dioxaoctyl]-2′-deoxycytidine (5) in a practically quantitative yield. Then 5 was reacted with succinic anhydride in the presence of DMAP, triethylamine in a mixture of pyridine and dichloromethane. The reaction of 5 with succinic anhydride led to 3′-OH rather than to 4-N succinylation. The resulting monosuccinate derivative 6 was attached to aminoalkyl controlled pore glass support (LCAA-CPG) to give 3′-end labelling support for deoxycytidine, DMTdC\(^{\text{AmFl}}\) support with a nucleoside content of 31 µmol/g. The loading procedure includes an intensive capping step with acetic anhydride in the presence of DMAP which should accomplish acetylation of unreacted amino groups of nucleoside bases as well as hydroxyl functions of the fluorescein residue.

Then, similarly to before, an oligodeoxynucleotide, d-CAGGAAACAGCTATGAC was prepared by the phosphoramidite approach on the DMTdC\(^{\text{AmFl}}\) support. The support containing the finished oligonucleotide was deprotected under commonly used conditions, i.e. by a treatment with concentrated aqueous ammonia at room temperature over ~1 h and then at 55°C overnight. The crude oligonucleotide d-CAGGAAACAGCTATGAC\(^{\text{AmFl}}\) was purified by polyacrylamide gel electrophoresis under denaturing conditions and then successfully applied in DNA sequencing.

The diagram obtained from printout of the sequencer did not differ from the one observed for a primer obtained on DMTdC\(^{\text{Ts}}\) support after 3′-end fluorescein labelling. This result indicated that fluorescein labelled oligonucleotide primers can effectively be obtained using solid supports loaded with dye labelled nucleosides.

Therefore, a similar approach was used to obtain 3′-end labelling supports for other 2′-deoxynucleosides.
Fluorescent deoxyadenosine 3′-end labelling support

The position 8 in the purine ring system of deoxyadenosine was chosen as a site for attaching a label, because the data available in the literature describe a successful use of 8-modified deoxyadenosine triphosphates as substrates for DNA polymerases (31). Deoxyadenosine was brominated (45) with an aqueous solution of bromine and crude 8-bromodeoxyadenosine (7) was 5′-O-dimethoxytritylated with 4,4′-dimethoxytrityl chloride to give 8 in 56% overall yield. Then 8 was reacted with 1,2-bis(2-aminoethoxy)ethane in pyridine under the same conditions as previously used for the substitution of p-toluenesulfonylamine function in 4-substituted cytosine derivatives, i.e. an overnight treatment with a primary amine at 70°C. We observed a clean transformation into 5′-O-dimethoxytrityl-8-(8-amino-3,6-dioxaoctylamino)-2′-deoxyadenosine (9) which was isolated in 74% yield after silica gel chromatography.

The 8-aminooalkyldideoxyadenosine derivative 9 was reacted with FITC and subsequently with succinic anhydride under the same general conditions as for the deoxyctidine derivative 4 to give 11.

The LCAA-CPG support was then reacted with a monosuccinimide derivative 11 to give the fluoresceinated deoxyadenosine 3′-end labelling support (DMTdaAmFl support) with a loading of 31 µmol/g.

Fluorescent deoxyguanosine 3′-end labelling support

8-Bromodeoxyguanosine (12) was obtained in reaction of 2′-deoxyguanosine with an aqueous solution of bromine (46). Crude 12 was reacted with dimethoxytrityl chloride in pyridine to give 5′-O-dimethoxytrityl-8-bromodeoxyguanosine (13) in 54% overall yield. Then 13 was reacted with 1,2-bis(2-aminoethoxy)ethane in methanol in a sealed tube at 100°C for 20 h. The TLC analysis of the reaction mixture showed the formation of a desired product, 5′-O-dimethoxytrityl-8-(8-amino-3,6-dioxaoctylamino)-2′-deoxyguanosine (14), in 20% yield, only accompanied by unreacted substrate which could be recovered (~50% yield) during the product purification by silica gel column chromatography. Attempts to increase the yield of 14 were unsuccessful. When the aminolysis with 1,2-bis(2-aminoethoxy)ethane was carried in butyl alcohol, pyridine and varying the reaction temperature, and an excess of the amine the increased formation of side products was observed together with a lowering of yield of 14. The identity of 5′-O-dimethoxytrityl-8-(8-amino-3,6-dioxaoctylamino)-2′-deoxyguanosine was corroborated by 1H NMR analysis of its detritylation product, 15. The further treatment of 14 was analogous as for dC and da derivatives (4 and 9) and resulted in preparation of a fluorescent deoxyguanosine 3′-end labelling support (DMTdgAmFl support) with a loading of 28 µmol/g. The treatment of DMTdgAmFl support with concentrated ammonia released DMTdgAmFl practically quantitatively. One can conclude only indirectly that guanine amino group of DMTdgAmFl support was sufficiently protected (acylated) as sequencing primers derived on this support gave the expected nucleosides upon enzymatic digestion.

Fluorescent deoxyuridine 3′-end labelling support

5-Aminodeoxyuridine was obtained in reaction with liquid ammonia (47,48). Thus, in the case of the thymidine analogue we have taken 5-bromodeoxyuridine (18) as a starting compound and in a similar sequence of reactions as described for other nucleosides above we obtained 5′-O-dimethoxytrityl-5-(8-amino-3,6-dioxaoctylamino)-2′-deoxyuridine (20) in ~42% yield. After labelling reactions a 3′-O-succinyl fluorescent deoxyuridine derivative 22 was attached to LCAA-CPG with a loading of 32 µmol/g.

DNA sequencing with 3′-end labelled primers prepared on labelling supports

A series of oligonucleotides carrying at their 3′-end fluorescein modified bases were obtained by the phosphoramidite approach using automated DNA synthesizers. Standard synthetic cycles and deprotection procedures were used. The sequences of primers (17mers) are listed in Table 1.

When the above primers were used for DNA sequencing by the dideoxy approach clear sequence results could be obtained until up to ~500 bp and intensive and relatively sharp bands were observed (Fig. 2A–D). One can observe a slight shift of bands in sequencing with 3′-end labelled primers which might improve reading of sequence (Fig. 2A and C). A comparison of M13 sequencing results obtained with either 5′- (M13F-Uni) or 3′-end labelled primers (M13F-G, M13F-U) is given in Figure 2. The results of sequencing were at least as good as those obtained using primers labelled at their 5′-end.

SUMMARY

We have prepared 3′-end labelling supports based on CPG carriers loaded: (i) with ‘tethered’ nucleosides, convertible to labelled oligonucleotides after solid phase synthesis and (ii) directly with nucleosides substituted with protected fluorescein. Oligonucleotide synthesis could be done conventionally by the phosphoramidite method in yields up to 99% per cycle. In both routes ~90% of oligonucleotide chains were found to contain fluorescein label [incomplete yields of conversion of a ‘tether’ in route (i); slight loss of fluorescein during oligonucleotide work-up in route (ii)]. In cases, where tosylated nucleosides were incorporated as ‘label precursors’, the fraction of labelled oligonucleotides was somewhat lower. 3′-End fluoresceinated primers prepared on ‘labelling supports’ were used for enzymatic DNA sequence analysis according to Sanger and co-workers with the same efficiency as 5′-end fluoresceinated oligonucleotide primers, when Sequenase was used as a DNA polymerase.

MATERIALS AND METHODS

General methods

All the solvents used in the reactions were purified and dried according to procedures published earlier. The organic extracts were dried over anhydrous sodium sulphate. 1H NMR spectra were recorded with 300 MHz Varian Unity 300 NMR spectrometer using tetramethylsilane (TMS) as an internal standard. UV-VIS spectra were measured on a Beckman DU-65 spectrophotometer.

Thin layer chromatography was performed on E. Merck pre-coated plates: (i) silica gel 60 F254 in the following solvent systems: A, dichloromethane/methanol (9:1); B, dichloromethane/methanol (95:5); C, isopropanol/concentrated aqueous ammonia/water (7:1:2); (ii) Merck silica gel 60 F254 silanized (RP-2) in solvent: D, acetone/water (7:3); E, acetone/10% diluted saturated aqueous sodium bicarbonate (7:3). All solvent ratios are by
volume. Short column chromatography was performed on silica gel Merck H 60 in dichloromethane containing methanol or in a mixture of dichloromethane, methanol and triethylamine. 1,2-Bis(2-aminoethoxy)ethane (Fluka) was dried over molecular sieves (4 Å). Fluorescein isothiocyanate isomer 1 (FITC) was obtained from Fluka.

Oligonucleotides were synthesised with the following automated DNA synthesizers: the Gene Assembler Plus from Pharmacia-LKB (Sweden), the Model 392 DNA/RNA Synthesiser from Applied Biosystems and a home built system. NAP-25 columns (Pharmacia LKB) were used to separate oligonucleotides from lower molecular weight compounds (ammonia, salts etc.).

Sequencing with ALF DNA Sequencer™ from Pharmacia LKB was carried out essentially according to instructions of the manufacturer and using the Pharmacia LKB AutoRead™ Sequencing Kit (27-1690-01).

The HPLC analyses were done using Applied Biosystems Model 1783A Absorbance Controller, 1480A Injector/Mixer, 1400A Solvent Delivery System (2×) equipped with Merck D-2000 Chromato-Integrator and DuPont Instruments 850 Liquid Chromatograph. The analyses were performed on Merck C18 4.6 × 100 mm (5 µm) column with linear gradients of acetonitrile in 0.1 M triethylammonium acetate (TEAA) pH 7.0 with flow rate 0.7 ml/min.

**Removal of 4-N-benzoyl protecting group from cytosine residues in oligodeoxynucleotides**

The model oligonucleotide, d-TCBzT, was synthesised and released from the solid support with 32% aqueous ammonia at room temperature. The aliquots were taken after 30 min, 1 and 2 h and immediately were applied to NAP-25 columns to remove ammonia. The oligonucleotide fractions were lyophilised and analysed with the HPLC using acetonitrile gradient in 0.1 M TEAA to 17% (in 30 min) and to 30% (in 10 min). The following retention times (Rt) were observed: 11.2 min (d-TCT, 73 and 94% in 30 and 60 min, respectively) and 27.8 min (d-TCBzT, 27 and 6% in 30 and 60 min, respectively).

**5′-O-Dimethoxytrityl-4-N-p-toluenesulfonyl-2′-deoxyctydite (2)**

2′-Deoxycytidine hydrochloride (1.32 g, 5 mmol) was reacted with trimethylsilyl chloride (2.5 ml, 20 mmol) in anhydrous pyridine (20 ml) for stirring for 1 h. Then the reaction mixture was concentrated under exclusion of moisture to a volume of ~15 ml and p-toluenesulfonyl chloride (1.9 g, 10 mmol) was added. The tightly stoppered reaction vessel was placed in an oven at 60°C and left overnight. The TLC analysis showed that the reaction went to completion. The reaction mixture was partitioned between dichloromethane (50 ml) and aqueous saturated sodium bicarbonate (60 ml). The aqueous layer was extracted twice with dichloromethane (20 ml). The combined extracts were concentrated under reduced pressure (water pump), and pyridine was added to a volume of 15 ml. Concentrated aqueous ammonia (15 ml) was added, and the progress of the desilylation was followed by TLC analysis: Rf(A) 0.35; Rf(D) 0.89. The reaction was completed in 4 h. Then the reaction mixture was concentrated under diminished pressure and the resultant crude 4-N-p-toluenesulfonyl-2′-deoxycytidine was dried by co-evaporation with anhydrous pyridine (3 × 20 ml) and redissolved in anhydrous pyridine (20 ml). 4,4′-Dimethoxytrityl chloride (1.70 g, 5 mmol) was added. The tritylation reaction went to completion in ~2 h: Rf(B) 0.50, Rf(C) 0.70. The mixture was partitioned between dichloromethane (50 ml) and 0.5 M aqueous sodium bicarbonate (50 ml). The aqueous layer was extracted with dichloromethane (2 × 50 ml). Pure 5′-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2′-deoxycytidine (2) was obtained after silica gel column chromatography with dichloromethane/methanol mixture as an eluting solvent. The foam of pure product was dissolved in benzene (20 ml), frozen and lyophilised under oil pump pressure to give the title compound as a white solid: 2.52 g, 73.7% overall yield. 1H NMR (CDCl3): δ (p.p.m.) 8.60 (m, 1H, NH), ~7.8 (m, 3H, H-6 and 2H of Ts), 6.8–7.4 (m, 15H, 2H of Ts and DMT), 6.24 (t, 1H, J 8.8 Hz, H-1′), 5.68 (bs, 1H, H-5), 4.55 (m, 1H, H-3′), 4.03 (m, 1H, H-4′), 4.04 (s, 6H, 2× OCH3), 3.45 (dq, 2H, J 3 and 10 Hz, H-5′ and 5′′), 2.48 and 2.27 (2m, 2H, H-2′ and 2′′), 2.41 (s, 3H, CH3); MS-FAB: M+ 683, calculated for C37H37N3O8S 683.

**Triethylammonium salt of 5′-O-dimethoxytrityl-4-N-p-toluenesulfonyl-2′-deoxycytidine 3′-O-monomuscuccinate (3)**

5′-O-Dimethoxytrityl-4-N-p-toluenesulfonyl-2′-deoxycytidine (128 mg, 0.187 mmol), succinic anhydride (50 mg, 0.5 mmol) and 4-N,N-dimethylaminopyrimidine (DMAP, 3 mg, 0.02 mmol) were stirred with triethylamine (0.14 ml, 1 mmol) in anhydrous dichloromethane under exclusion of moisture. The TLC analysis showed that the reaction was completed in 3 h. The reaction mixture was partitioned between aqueous sodium bicarbonate (10 ml) and dichloromethane (10 ml). The water layer was extracted with dichloromethane (3 × 10 ml). The resultant residue of a crude title compound was dissolved in 1,4-dioxane (3 ml), frozen and lyophilised under oil pump pressure to give the desired product as a white solid: 165 mg, 99% yield.

**Synthesis of oligonucleotides with p-toluenesulfonyl groups and their reaction with 1,2-bis(2-aminooxy)ethane**

Oligonucleotides were synthesised with automated DNA synthe- sisers using appropriate supports (0.2 µmol) and 2-cyanoethyl-N,N-diisopropylphosphoramidites. The oligonucleotides were released from the support into an Eppendorf tube by treatment with concentrated (32%) aqueous ammonia at room temperature for 1 h. Then the collected solution was concentrated to dryness with the Speed-vac evaporator. Aqueous 1,2-bis(2-aminooxy)ethane (40%, 100 µl) was added to the partially deprotected oligonucleotide. The tube was closed and the oligonucleotide was dissolved at room temperature with the help of an ultrasonic bath (5–10 min). The reaction solution was placed in an oven at 70–80°C overnight. The solution was applied on the NAP-25 column and the crude product was washed out with water. Fractions containing a fully deprotected crude oligonucleotide with the substituted p-toluenesulfonyl groups were dried with the Speed-vac evaporator and used in further experiments.

**Reaction of the aminoalkyloligonucleotides with fluorescein isothiocyanate (FITC)**

The crude oligonucleotide (up to 30 A260 OD units) was dissolved in 0.05 M sodium borate buffer pH 9.2 (100 µl), and a freshly prepared solution of fluorescein isothiocyanate isomer I (FITC, ~1 mg) in dimethylsulfoxide (30 µl) was added. The...
reaction was carried out in darkness at room temperature for 4–20 h with essentially the same results. The reaction mixture was then applied to a NAP-25 column and washed with double distilled water (adjusted with aqueous ammonia to pH 8–9.5). The fraction containing oligonucleotide was collected, and the desired labelled oligonucleotide was isolated after polyacrylamide gel electrophoresis (20%, 7 M urea) or HPLC and characterised by its UV-VIS spectrum as well as by digestion with snake venom phosphodiesterase and alkaline bovine phosphatase according to Eadie (49). Analysis of the digestion mixture was performed on a Merck C18 HPLC column using a linear gradient of 90% aqueous methanol in 50 mM potassium phosphate buffer (pH 7.0) containing 1% of methanol. The gradient consisted of the following segments: 0% (for 30 min), to 15% (in 30 min), to 100% (in 20 min) and 100% (for 10 min). The following Rf were observed: 7.1 min (dC), 25.3 min (dG), 27.4 min (dT), 32.7 min (dA) and 71.4 min (dC
Amp).

5′-O-Dimethoxytrityl-4-N-(8-aminoo-3,6-dioxaoctyl)-2′-deoxyctydine (4)

Anhydrous 1.2-bis(2-aminoethoxy)ethane (750 µl, 5.12 mmol) was added to the solution of 5′-O-dimethoxytrityl-4-N-p-toluene-sulfonyl-2′-deoxyctydine (2, 205 mg, 0.30 mmol) in pyridine (750 µl). The flask was tightly closed, placed in an oven and kept overnight at 50°C. The reaction mixture was partitioned between water (15 ml) and dichloromethane (7.5 ml). The reaction went to completion, as shown by the TLC: Rf (A) 0.0, Rf (C) 0.62. Pure 5′-O-dimethoxytrityl-4-N-(8-aminoo-3,6-dioxaoctyl)-2′-deoxyctydine (4) was obtained after silica gel chromatography in dichloromethane/methanol mixture as a white foam: 170 mg, 0.257 mmol, 85.7% yield; 1H NMR (CDCl3): δ (ppm) 7.73 (d, 1H, J 7.2 Hz, H-6), 6.77–7.35 (m, 13H of DMT), 6.39 (t, J 6.6 Hz, H-1′), 6.10 (m, 1H, NH), 5.32 (d, J 6.6 Hz, H-5), 4.42 (q, 1H, J 5.5 Hz, H-3′), 3.96 (q, 1H, J 3 Hz, H-4′), 3.72 (s, 6H, 2×OCH3), 3.65 (m, 1H, 3'-OH), 3.54 (s, 8H, CH2 of amine), 3.45 (t, 2H, J 5.2 Hz, CH2 of amine), 2.83 (t, 2H, J 5.2 Hz, CH2 of amine), 2.55 (m, 2H, H-2′ and H-5′), 2.20 (m, 2H, H-2′′), 13C NMR (CDCl3): δ (ppm) 163.6 (C-4), 156.2 (C-2), 150.1 (C-3), 149.5 (C-4), 119.57 (C-5), 123.7 (C-8), 86.3 (C-1), 85.4 (C-2′), 72.94 (C-3′), 63.78 (C-5′), 36.8 (C-2′′).

5′-O-Dimethoxytrityl-8-bromo-2′-deoxyadenosine (9)

Anhydrous 1.2-bis(2-aminoethoxy)ethane (2.71 ml, 18.35 mmol) was added to the solution of 5′-O-dimethoxytrityl-8-bromo-2′-deoxyadenosine (600 mg, 0.95 mmol) in pyridine (1.0 ml). The flask was tightly closed, placed in the oven and kept overnight at 70°C. Further work-up procedure and purification were the same as for 4 and resulted in 5′-O-dimethoxytrityl-8-bromo-8-aminoo-3,6-dioxaoctyl)-2′-deoxyadenosine (9) as a white foam: 490 mg, yield 74%; Rf (A) 0.0, Rf (C) 0.74; 1H NMR (CDCl3): δ (ppm) 8.09 (s, H-2), 6.39 (t, J 6.6 Hz, H-1′), 4.6 (q, J 5.6 Hz, H-5′), 5.68 (s, 1H, 8-NH2), 5.42 (s, 2H, 6-NH2), 4.68 (m, 1H, H-3′), 4.00 (q, J 4.6 Hz, H-4′), 3.76 (s, 6H, 2×OCH3), 3.55 (m, 4H, CH2 of amine), 3.5 (m, 2H, CH2 of amine), 3.3 (m, 2H, CH2 of amine), 2.95 (m, 1H, H-1′), 2.80 (m, 2H, CH2 of amine), 2.32 (m, 1H, H-2′); 13C NMR (CDCl3): δ (ppm) 158.5, 144.4, 135.6, 130.2, 127.6, 112.9, 111.5, 55.2 (C of DMT), 151.9 (C-6), 150.1 (C-2′), 149.5 (C-4′), 119.5 (C-5′), 123.7 (C-8′), 86.3 (C-1′), 84.9 (C-4′), 70.7 (C-3′), 62.9 (C-5′), 38.4 (C-2′′), 82.6, 72.4, 70.02, 69.1, 42.2, 41.2 (C of amine).

5′-O-Dimethoxytrityl-8-bromo-2′-deoxyguanosine (13)

Bromine water (25 ml) was added in portions to a magnetically stirred suspension of 2′-deoxyguanosine (1.34 g, 5 mmol) in water (25 ml) until yellow colouring was stable. The TLC analysis showed that the reaction was completed: Rf (C) 0.50 → 0.60. The pH of the solution went down to 4 during the reaction course and then was neutralised immediately after completing the reaction by addition of saturated sodium bicarbonate. The reaction product formed during the reaction was filtered off; 1H NMR (δD-DMSO): δ (ppm) 10.81 (s, 1H, NH), 6.50 (s, 2H, NH2), 6.16 (t, 1H, J 6.9
Crude 8-bromo-2'-deoxyguanosine (12) was dried by co-evaporation with anhydrous pyridine (3 x 20 ml) and dissolved under slight heating in anhydrous pyridine (25 ml). The tritylation reaction went to completion in ~1.5 h after addition of 4.4'-dimethoxytrityl chloride (1.70 g, 5 mmol). The reaction mixture was partitioned between dichloromethane (50 ml) and 0.5 M aqueous sodium bicarbonate (50 ml). The aqueous layer was extracted with dichloromethane. Pure 5'-O-dimethoxytrityl-8-bromo-2'-deoxyguanosine (13) was obtained after silica gel column chromatography with dichloromethane/methanol mixture as a foam: 2.16 g, 54% overall yield; Rf (A) 0.30. 1H NMR column chromatography with dichloromethane/methanol (14–18%) mixture as a white foam: 300 mg, 44.3% yield; 1H NMR (CDCl3): δ (p.p.m.) 8.1 (s, 1H, 1-NH), 6.8–7.4 (m, 13H of DMT), 6.20 (m, 1H, H-1'), 5.6 (s, 1H, NH2), 5.5 (s, 1H, 8-OH and NH), 4.05 (m, 1H, H-3'), 3.73 (s, 6H, 2x OCH3), 3.5 (m, 6H, NH2 and CH2 of amine), 3.37 (m, 7H, H-4', H-5' and CH2 of amine), 2.75 (m, 5H, H-2' and CH2 of amine), 2.3 (m, 1H, H-2'). Unreacted substrate (180 mg, ~50%) was recovered during the above purification procedure.

5'-O-Dimethoxytrityl-8-(8-amino-3,6-dioxaoctylamino)-2'-deoxyguanosine (14)

5'-O-Dimethoxytrityl-8-bromo-2'-deoxyguanosine (324 mg, 0.5 mmol), anhydrous methanol (5.5 ml) and anhydrous 1,2-bis(2-aminoethoxy)ethane (2.76 ml, 20 mmol) were sealed in a glass ampoule and kept at 100°C for 20 h. After cooling the ampoule was opened and the reaction mixture was dissolved in dichloromethane (10 ml) and washed with water until aqueous extracts were neutral. The TLC analysis showed that the desired product was partly formed: Rf (A) 0.30 → 0.0; Rf (C) 0.76 → 0.58. The organic layer was dried, concentrated under reduced pressure and the oily residue was purified by silica gel chromatography (20% methanol in dichloromethane) to give 5'-O-dimethoxytrityl-8-(8-amino-3,6-dioxaoctylamino)-2'-deoxyguanosine (14): 80 mg, 20% yield; 1H NMR (CDCl3): δ (p.p.m.) 8.1 (s, 1H, 1-NH), 6.8–7.4 (m, 13H of DMT), 6.20 (m, 1H, H-1'), 5.6 (s, 1H, NH2), 5.5 (s, 1H, 8-OH and NH), 4.05 (m, 1H, H-3'), 3.73 (s, 6H, 2x OCH3), 3.5 (m, 6H, NH2 and CH2 of amine), 3.37 (m, 7H, H-4', H-5' and CH2 of amine), 2.75 (m, 5H, H-2' and CH2 of amine), 2.3 (m, 1H, H-2'). Unreacted substrate (180 mg, ~50%) was recovered during the above purification procedure.

8-(8-Amino-3,6-dioxaoctylamino)-2'-deoxyguanosine (15)

5'-O-Dimethoxytrityl-8-bromo-2'-deoxyguanosine (13) (20 mg, 0.06 mmol) was kept in 80% aqueous acetic acid (0.4 ml) at room temperature. The TLC analysis showed that the reaction was completed in 45 min: Rf (C) 0.58 → 0.20. Then acetic acid was removed under reduced pressure and the residue was dissolved partitioned between dichloromethane and water. The aqueous layer was concentrated under diminished pressure to give 8-(8-amino-3,6-dioxaoctyl)-2'-deoxyguanosine (15): 1H NMR (d6-DMSO): δ (p.p.m.) 6.5 (s, 2H, NH), 6.18 (q, J 5.6 Hz, H-1'), 5.3 (bs, 4H, NH2), 4.35 (d, 1H, J 16 Hz, 3'-OH), 3.83 (d, 1H, J 5.9 Hz, 5'-OH), 3.73 (s, 1H, NH), 3.5 (m, 6H, H-3', H-5' and CH2 of amine), 3.45 (m, 2H, H-5'), 3.4 (m, 4H, CH2 of amine), 2.76 (m, 5H, H-2' and CH2 of amine), 1.9 (dd, 1H, J 6.3 Hz, H-2'). FAB-MS: m.w. calculated for C16H27N2O6: 413.202822, found M+ 413, [M+Na]+ 436.

5'-O-Dimethoxytrityl-5-bromo-2'-deoxyuridine (19)

5-Bromo-2'-deoxyuridine (18) (646.2 mg, 2.0 mmol) was dried by co-evaporation with anhydrous pyridine (2 x 10 ml) and redissolved in anhydrous pyridine (5 ml), 4.4'-Dimethoxytrityl chloride (676 mg, 2 mmol) was added. The tritylation reaction went to completion in ~1.5 h: Rf (A) 0.17 → 0.39. The mixture was partitioned between dichloromethane (50 ml) and 0.5 M aqueous sodium bicarbonate (50 ml). The aqueous layer was extracted with dichloromethane (2 x 50 ml). Pure 5'-O-dimethoxytrityl-5-bromo-2'-deoxyuridine (19) was obtained after silica gel column chromatography with dichloromethane/methanol mixture as a foam: 1.2 g, 95% yield; 1H NMR (d6-DMSO): δ (p.p.m.) 11.89 (s, 1H, NH), 8.01 (s, 1H, H-6), 6.8–7.4 (m, 13H of DMT), 6.12 (t, 1H, J 8.8 Hz, H-1'), 5.35 (d, 1H, J 4.5 Hz, 3'-OH), 4.28 (m, 1H, H-3'), 3.92 (m, 1H, H-4', 3.74 (s, 6H, 2x OCH3), 3.2 (m, 2H, H-5' and 5'), 2.25 (m, 2H, H-2' and 2'); 13C NMR (d6-DMSO): δ (p.p.m.) 159.06 (C-4'), 149.61 (C-2'), 139.52 (C-6), 95.98 (C-5), 85.70 (C-1'), 84.90 (C-4'), 70.28 (C-3'), 63.58 (C-5'), 39.7 (C-2'), 158.03, 144.4, 135.6, 130.2, 127.5, 126.2, 113.17 and 54.95 (C of DMT).

5'-O-Dimethoxytrityl-5-(8-amino-3,6-dioxaoctylamino)-2'-deoxyuridine (20)

Anhydrous 1,2-bis(2-aminoethoxy)ethane (2.76 ml, 20 mmol) was added to the solution of 5'-O-dimethoxytrityl-5-bromo-2'-deoxyuridine (19) (626 mg, 1 mmol) in pyridine (3 ml). The flask was tightly closed, placed in the oven and kept at 50°C for 20 h. The reaction mixture was partitioned between water (15 ml) and dichloromethane (7.5 ml). The aqueous layer was extracted until its pH was neutral. The TLC analysis showed that the reaction went to completion: Rf (A) 0.0, Rf (C) 0.61. Pure 5'-O-dimethoxytrityl-5-(8-amino-3,6-dioxaoctylamino)-2'-deoxyuridine (20) was obtained after a silica gel chromatography in dichloromethane/methanol (14–18%) mixture as a white foam: 300 mg, 0.443 mmol, 44.3% yield; 1H NMR (CDCl3): δ (p.p.m.) 6.85–7.45 (m, 14H, H-6 and DMT), 6.37 (s, 1H, NH), 6.27 (t, 1H, J 7.1 Hz, H-1'), 4.24 (m, 1H, H-3'), 4.0 (m, 4H, H-4', NH2, 3'-OH), 3.73 (s, 7H, 2x OCH3 and NH), 3.5 (m, 9H, 5'-H, H-5' and CH2 of amine and 5-NH), 3.38 (m, 4H, CH2 of amine), 2.4 (m, 2H, H-2' and 2').

General procedure of derivatisation of aminoalkyl 5'-O-dimethoxytrityl-2'-deoxynucleosides with fluorescein isothiocyanate (FITC)

5'-O-Dimethoxytrityl-(8-amino-3,6-dioxacyct)-2'-deoxynucleoside (4, 9, 14 or 20) (0.5 mmol) was dissolved in DMF (5 ml) and aqueous sodium borate buffer (0.1 M, pH 9.2, 0.5 ml) was added followed by fluorescein isothiocyanate (FITC, isomer I) (194.5 mg, 0.5 mmol). The reaction was carried overnight at room temperature and in darkness. Then DMF was evaporated under reduced pressure and chloroform (5 ml) was added to the residue. The resulting sedimentation was filtered off, washed with chloroform and water, dried by co-evaporation several times with anhydrous pyridine and redissolved in pyridine. The pyridine solution (1.5 ml)
was dropped into anhydrous diethyl ether (20 ml) under stirring and the precipitate of fluoresceinythioeuclideanalkyl 5'-O-dimethoxytrityl 2'-deoxynucleoside, was collected and dried in a dessicator over P₂O₅. The TLC analysis showed that the products contained only minor impurities of non-nucleosidic type (from FITC).

5'-O-Dimethoxytrityl-4-N-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyctydine (5). Rp₁(E) 0.69; yield 95.8%; UV-VIS (H₂O): λmax 237 and 490 nm, λah 270 nm, A₂₅₀/A₂₆₀ 1.0, A₂₆₀/A₄₀₀ 0.5.

5'-O-Dimethoxytrityl-8-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyadenosine (10). Rp₁(E) 0.65; yield 90.4%.

5'-O-Dimethoxytrityl-8-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyguanosine (16). Rp₁(E) 0.65; yield 58%.

5'-O-Dimethoxytrityl-5-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyuridine (21). Rp₁(E) 0.67; yield 99%.

General procedure for the preparation of monosuccinate esters of fluorescein derivatised aminoalkyl 2'-deoxynucleosides

Succinic anhydride (450 mg, 4.5 mmol) was added to a stirred solution of fluoresceinthyioeuclideanalkyl-5'-O-dimethoxytrityl 2'-deoxynucleoside (5, 10, 16 or 21, 0.3 mmol), DMAP (30 mg, 0.25 mmol) and triethylamine (1.25 ml, 9 mmol) in a mixture of dichloromethane (7 ml) and pyridine (3 ml). The reaction was completed in 2 h at room temperature. Then dichloromethane (10 ml) was added, and the reaction mixture was extracted with saturated aqueous sodium bicarbonate (3 x 5 ml). The aqueous extracts were neutralised separately with saturated aqueous sodium dihydrogen phosphate (pH 4.5) to deposit the sediment of a succinate derivative. The sediment was collected by centrifugation and dried by several co-evaporations with anhydrous pyridine. Finally, the pyridine solution of the product was dropped into anhydrous diethyl ether to precipitate a chromatographically pure product (TLC in system G).

5'-O-Dimethoxytrityl-4-N-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyctydine 3'-O-succinate (6). Yield 56%.

5'-O-Dimethoxytrityl-8-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyadenosine 3'-O-succinate (11). Yield 80%.

5'-O-Dimethoxytrityl-8-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyguanosine 3'-O-succinate (17). Yield 62%.

5'-O-Dimethoxytrityl-5-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyuridine 3'-O-succinate (22). Yield 75%.

General procedure of derivatization of LCAA-CPG (1000 Å) using 3'-O-monosuccinate derivatives of protected nucleosides

Triethylammonium salt of appropriate 5'-O-dimethoxytrityl-2'-deoxynucleoside 3'-O-monosuccinate derivative (3, 6, 11, 17 or 22, 50 µmol), LCAA-CPG 1000 Å (0.500 g), DCU (300 mg, 1.5 mmol), DMAP (20 mg, 0.16 mmol) and triethylamine (105 µL, 0.75 mmol) in the mixture of anhydrous pyridine (1 ml) and dichloromethane (2 ml) were gently shaken mechanically overnight at room temperature under exclusion of moisture. Then the support was filtered off, washed with pyrid(531,861),(594,884), dichloromethane, methanol, dichloromethane and diethyl ether (50 ml of each) and transferred to the flask for the capping reaction with acetic anhydride (1 ml), pyridine (1 ml) and DMAP (20 mg) at room temperature for 2 h. The support was then filtered off, washed subsequently with dichloromethane, methanol, dichloromethane and diethyl ether (25 ml of each) and dried in the dessicator under oil pump pressure to constant weight. The loading of the support was estimated by measuring spectrophotometrically the dimethoxytrityl cation released from a small sample of the support after acid treatment according to the procedure described in the literature.

The following loadings were obtained:

5'-O-Dimethoxytrityl-4-N-p-toluensulfonyl-2'-deoxyctydine support, DMTdCAmFl support, 15 µmol/g; 5'-O-dimethoxytrityl-4-N-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyctydine support, DMTdAAmFl support, 25.7 µmol/g; 5'-O-dimethoxytrityl-8-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyadenosine support, DMTdCAmFl support, 31.4 µmol/g; 5'-O-dimethoxytrityl-8-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyguanosine support, DMTdCAmFl support, 28 µmol/g; 5'-O-dimethoxytrityl-5-[8-(fluorescein-4-yl)thioeuclidean-3,6-dioxoacycloxy]-2'-deoxyuridine support, DMTdUAmFl support, 32 µmol/g.

The supports were tested prior to their use in oligonucleotide synthesis with concentrated aqueous ammonia over 20 h at room temperature. The attached fluorescinminated nucleosides were released from the supports practically quantitatively.

ACKNOWLEDGEMENTS

The authors thank the State Committee for Scientific Research of the Republic of Poland (project No. 2 P303 008 04) and the European Union Commission (project No. CIPA-CT93-0155) for financial support.

REFERENCES