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An approach to the synthesis of liponucleotides

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Abstract. Phosphorylation of 3′-*O*-levulinoylthymidine*** using 2-chlorophenyl bis-*O*-(1-benzotriazolyl) phosphate, followed by addition of 1-hexadecanol, afforded in high yield a fully protected triester derivative of thymidine.
Similarly, phosphorylation of 1,2-di-*O*-palmitoyl-*sn*-glycerol with the same reagent and subsequent addition of the above thymidine derivative gave a triester derivative of thymidine in excellent yield. Both triester derivatives were efficiently converted into the corresponding liponucleotides by the removal of the 2-chlorophenyl and levulinoyl protective groups.

Introduction

The coenzyme 3-*O*-(ribocytidinyl-5′-diphospho)-1,2-di-*O*-palmitoyl-*sn*-glycerol (CDP-L-dipalmitin) is an example of a naturally occurring liponucleotide. In nature, this liponucleotide functions[1] as a donor of phosphatidyl units in the *de novo* synthesis of acidic phosphoglycerides. For instance[2], in crude homogenates of rat liver, inositol is converted in the presence of the liponucleotide into phosphatidylinositol with the concomitant release of ribocytidine 5′-monophosphate (CMP). The formation of CMP in the above enzymatic reaction has been used to convert arabinocytidine (ara-C), which has proven useful as a chemotherapeutic agent for the treatment of various types of cancer[2], into the liponucleotide ara-CDP-L-dipalmitin[3]. The prodrug thus obtained circumvents several problems[4] which are associated with the use of ara-C as a chemotherapeutic agent.
In this paper we wish to report the synthesis of simple liponucleotides (*i.e.*, compounds **7b** and **8b**) using the bifunctional phosphorylating agent 2-chlorophenyl *O,O*-bis(1-benzotriazolyl) phosphate (**1**).

Results and discussion

An essential feature of liponucleotides is the presence of phosphodiester linkages. Recently, we demonstrated that the phosphorylating agent 2-chlorophenyl bis-*O*-(1-benzotriazolyl) phosphate (**1**) could be used for the introduction of 3′–5′-phosphodiester linkages between properly protected deoxy- or ribo-nucleosides[5]. We also showed[6] that this reagent could be used for the selective formation of a 3′–5′-phosphodiester linkage between a ribonucleoside having a free 3′-hydroxyl and another ribonucleoside which contained two free hydroxyl (3′- and 5′-) groups. Further, the versatility of the phosphorylating agent **1** was amply demonstrated[7] by the preparation of the platelet-activating factor (2-*O*-acetyl-3-*O*-hexadecyl-*sn*-1-glyceryl)phosphorylcholine. We were interested, therefore, in discovering if reagent **1** could also be applied to the synthesis of the liponucleotides **7b** and **8b**, one of which (*i.e.* **7b**) is structurally related to the naturally occurring CDP-L-palmitin.
We initially explored the feasibility of synthesizing the lipophilic phosphodiester derivatives of thymidine **8b** ($R^1 = R^2 = H$). In the first step, we treated 3′-*O*-levulinoylthymidine[8] (**2**) with a slight excess of reagent **1**, which was obtained by the reaction of 2-chlorophenyl phosphorodichloridate with two equivalents of 1-hydroxybenzotriazole and pyridine in dioxane. TLC analysis, after 20 min, showed the formation of intermediate **6** and the absence of a symmetrical product which could have been formed by the reaction of intermediate **6** with starting compound **2**. Intermediate **6** was directly converted into

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*** Levulinoyl = $CH_3COCH_2CH_2CO-$.

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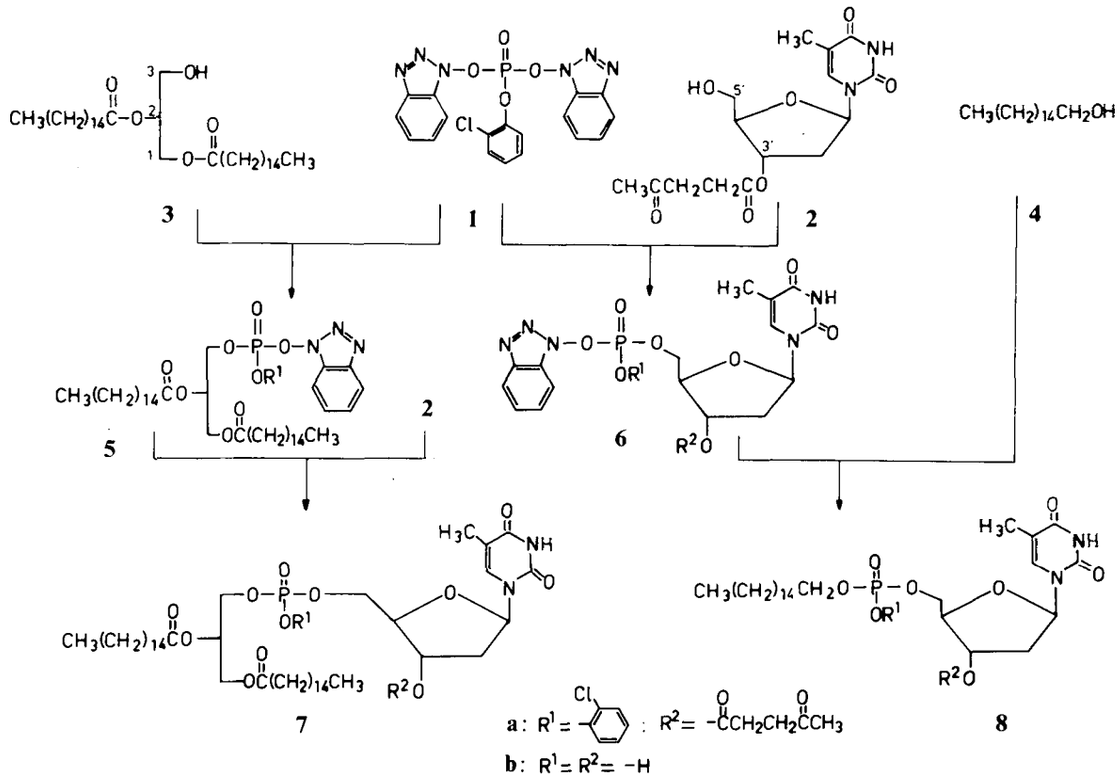

the 5'-triester derivative **8a** (R^1 = 2-chlorophenyl, R^2 = levulinoyl) by the addition of an equimolar amount (based on **2**) of 1-hexadecanol in the presence of an excess of *N*-methylimidazole. Work-up of the reaction mixture, after one hour at 20°C, followed by short-column chromatography, afforded homogeneous **8a** in excellent yield. Thus, having established that the first phosphorylation step (*i.e.* conversion of **2** into **6**) proceeded in the absence of a tertiary base and, further, that the remaining 1-benzotriazolyl function in **6** could be smoothly replaced by a lipophilic alcohol, we turned our attention to the preparation of the diglyceride derivative **7b**.

In the first step in the synthesis of **8a** we phosphorylated compound **2** with reagent **1**. Intermediate **6** thus obtained was then converted by the addition of 1-hexadecanol into the derivative **8a**. However, application of the same sequence of reactions to the preparation of fully protected **7a** could result, due to the presence of the tertiary base *N*-methylimidazole in the second step (*i.e.* reaction of **6** with **3**), in the isomerization of 1,2-di-*O*-palmitoyl-*sn*-glycerol (**3**) into 1,3-di-*O*-palmitoyl-*sn*-glycerol. In order to avoid this unwanted side-reaction, we firstly treated diglyceride **3** with a slight excess of reagent **1**. After 20 min, when TLC analysis indicated complete conversion of **3** into intermediate **5**, an equimolar amount of the nucleoside **2** and a excess of *N*-methylimidazole was added. Work-up of the reaction mixture, after 1 h at 20°C, afforded homogeneous **7a** in excellent yield. The fully protected liponucleotides **7a** and **8a** (R^1 = 2-chlorophenyl; R^2 = levulinoyl) were deblocked as follows. Firstly, the 2--chlorophenyl groups were removed under anhydrous conditions using an excess of N^1,N^1,N^3,N^3-tetramethylguanidinium *syn*-pyridine-2-carboxaldoximate[9], prepared *in situ*, in acetonitrile. The remaining levulinoyl groups were deblocked by a short treatment with hydrazine hydrate in pyridine/acetic acid[10]. Work-up of the crude reaction mixtures, followed by short-column chromatography, afforded pure **7b** and **8b** (R^1 = R^2 = H) in an overall yield (based on **3** and **2**, respectively) of 80%. In conclusion, the results described in this paper clearly demonstrate the effectiveness of the phosphorylating agent **1** in the synthesis of simple liponucleotides. As such it presents, in terms of high overall yields and short reaction times, an attractive alternative to the less easily accessible bifunctional phosphorylating agent bis(2-butene-2,3-diyl) pyrophosphate which has recently[11] been used in the preparation of similar liponucleotides. We are currently studying in detail[12] the introduction of pyrophosphate functions, present in many coenzymes, by using reagent **1** in which the 2-chlorophenyl is replaced by a morpholino group[13].

Experimental

General methods and material

Pyridine, tetrahydrofuran and dioxane were dried by refluxing with CaH_2 for 16 h and then distilled. Pyridine was redistilled from *p*-toluenesulfonyl chloride (60 g per litre) and stored over molecular sieves 4 Å. Tetrahydrofuran and dioxane were redistilled from $LiAlH_4$ (5 g *per* litre) and stored over molecular sieves 5 Å. 1-Methylimidazole was distilled under reduced pressure and stored sieves 4 Å. 1-Hydroxybenzotriazole was purchased from Aldrich and was dried *in vacuo* (P_2O_5) for 70 h at 50°C. Schleicher and Schüll DC Fertigfolien F 1500 LS 254 were used for TLC in solvent systems A (chloroform/methanol, 92/8, v/v) and B (chloroform/methanol, 85/15, v/v). Compounds were visualized

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by UV light (254 nm) or by spraying with molybdatophosphoric acid (25 g) in acetic acid/H_2SO_4 (500/25, ml/ml). Short-column chromatography was performed on Kieselgel 60 (230–400 mesh, ASTM) suspended in chloroform.

^{1}H NMR spectra were measured at 100 MHz using a Jeol JNMPS 100 spectrometer or at 300 MHz using a Brucker WM-300 spectrometer, equipped with an ASPECT-2000 computer operating in the Fourier transform mode. Chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard. ^{13}C NMR and ^{31}P NMR spectra were measured at 25.15 MHz and 40.18 MHz, respectively, using a Jeol JNMPFT 100 spectrometer equipped with an EC-100 computer, operating in the Fourier transform mode. Proton noise decoupling was used. ^{13}C chemical shifts are given in ppm (δ) relative to TMS as internal standard and ^{31}P chemical shifts in ppm (δ) relative to 85% H_3PO_4 as external standard.

*5'-O-(1,2-Di-O-palmitoyl-*sn*-glycero-3-phospho)-2'-deoxythymidine (***7b***: $R^1 = R^2 = H$)*

A solution of phosphorylating agent **1**[8] (0.56 mmole) in dioxane (2.8 ml) was added under anhydrous conditions to lipid **3**[14] (284 mg, 0.5 mmole). The mixture was stirred for 20 min. TLC analysis (system B) of the reaction mixture showed that lipid **3** was completely converted into base-line material. To the solution of intermediate **5** thus obtained were immediately added nucleoside **2**[8] (340 mg, 0.5 mmole) and *N*-methylimidazole (0.25 ml, 3.1 mmole). TLC analysis (system A), after 1 h at 20°C, showed the reaction to be complete. The reaction mixture was diluted with chloroform (75 ml) and washed with triethylammonium bicarbonate (TEAB; 2 × 30 ml, 1 M, pH 7.5) and water (30 ml). The organic layer was dried ($MgSO_4$) and concentrated to an oil. The latter was dissolved in chloroform (2 ml) and applied to a column of Kieselgel (10 g) suspended in chloroform. Elution of the column was effected with chloroform/methanol (100/0 → 98.5/1.5, v/v). The appropriate fractions were concentrated to give a waxy compound. Yield of compound **7a** as a mixture of diastereomers: 0.5 g (90%). R_f 0.65 (system A). ^{1}H NMR ($CDCl_3$): δ 0.9 (t, 6H, 2 × CH_3–, *J* 6 Hz); 1.3–1.6 (m, 48H, 2 × $(CH_2)_{12}$); 1.6–1.7 (m, 4H, $C\underline{H}_2CH_2COO$); 1.8 (s, 3H, CH_3, T); 2.1 (s, 3H, CH_3 lev.); 2.2–2.4 (m, 6H, 2 × CH_2COO, H2', H2''); 2.5–2.8 (m, 4H, CH_2CH_2, lev.); 4.2–4.5 (m, 7H, CH_2OP, CH_2OOCR, H4', H5', H5''); 5.2–5.3 (m, 2H, H3' and HCOOCR); 6.3 (dd, 1H, H1'); 6.9–7.5 ppm (m, 5H, 4H, 2-ClC_6H_4, H6, T). ^{31}P{^{1}H} NMR ($CDCl_3$): δ −6.86, −7.01 (s, 2 × P–O, 2-ClC_6H_4).

The 2-chlorophenyl group was deblocked from compound **7a** (487 mg, 0.45 mmole) under the same conditions as will be described for the removal of this group for compound **8a**. The same holds, apart from a slight modification, for the removal of the levulinoyl protecting group. Thus, after quenching with 2,4-pentanedione, the solution was diluted with chloroform (150 ml) and washed with water (75 ml) and TEAB (20 ml, 1 M, pH 7.5). The organic layer was then concentrated to an oil which was dissolved in chloroform (1 ml) and applied to a column of Kieselgel (5 g) suspended in chloroform. Elution of the column was effected with chloroform/methanol/TEAB (89/10/1 → 70/25/5, v/v). The appropriate fractions were concentrated to give a waxy compound. Yield 384 mg (88%); R_f 0.67 (chloroform/methanol/TEAB, 70/25/5, v/v); $[\alpha]_D^{25}$ +2.7 (*c* 0.7, chloroform/methanol/TEAB, 50/49.7/0.3). ^{1}H NMR ($CDCl_3/CD_3OD$): δ 0.89 (t, 6H, 2 × $C\underline{H}_3CH_2$, *J* 7.5 Hz); 1.2–1.5 (m, 50H, (2 × $(CH_2)_{12}$ and H2', H2''); 1.6 (m, 4H, 2 × $C\underline{H}_2CH_2C$=O); 1.94 (s, 3H, CH_3, T); 2.22–2.37 (m, 6H, 2 × CH_2C=O, H5', H5''); 3.98–4.12 (m, 4H, H3', H4', C1, glycerol); 4.33 (dd, 2H, C3, glycerol, $J_{H,H}$ 12 Hz, $^2J_{H-P}$ 7 Hz); 5.25 (m, 1H, C2, glycerol); 6.36 (dd, 1H, H1', $J_{1,2}$ 7 Hz); 7.72 (s, 1H, H6, T). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 9.0 (s, $C\underline{H}_3CH_2N$); 47.2 (s, $CH_3C\underline{H}_2N$); 12.5 (s, CH_3, T); 14.3, 23.3, 32.5, 29.7, 30.3, 25.5, 34.7 (m, $C\underline{H}_3C\underline{H}_2C\underline{H}_2$–$(C\underline{H}_2)_{10}$-$CH_2CH_2COO$); 63.0 (s, C2'); 64.1, 64.3 (d, CH_2OP, $^2J_{C-P}$ 5.3 Hz); 65.8, 65.6 (d, C5', $^2J_{C-P}$ 4.5 Hz); 71.0, 71.3 (d, HCOOCR, $^3J_{C-P}$ 9.1 Hz); 71.7 (s, C3'); 85.2 (s, C2'); 86.3, 86.6 (d, C4', $^3J_{C-P}$ 8.3 Hz); 111.4 (s, C5); 137.1 (s, C4); 151.6 (s, C3); 165.5 ppm (s, C2). ^{31}P{^{1}H} NMR ($CDCl_3/CD_3OD$): δ −0.7 (s, P–O$^-$).

*Thymidinyl-5' hexadecyl phosphate (***8b***; $R^1 = R^2 = H$)*

A solution of phosphorylating agent **1** (1.1 mmole) in dioxane (5.6 ml) was added, under anhydrous conditions, to nucleoside **2** (340 mg, 1 mmole). The mixture was stirred for 20 min at 20°C. TLC analysis (system B) of the reaction mixture showed that nucleoside **2** was completely converted into base-line material. To the intermediate **6** thus obtained were immediately added 1-hexadecanol (240 mg, 1.0 mmole) and *N*-methylimidazole (0.25 ml, 3.1 mmole). TLC analysis (system A), after 1 h at 20°C, showed the reaction to be complete. The reaction mixture was worked up as described earlier to give compound **8a** as a mixture of diastereomers. Yield 680 mg (90%); R_f 0.60 (system A). ^{1}H NMR ($CDCl_3$): δ 0.9 (t, 3H, CH_3–, *J* 6 Hz); 1.2 (m, 26H, $(CH_2)_{13}$); 1.6–1.7 (m, 2H, $C\underline{H}_2CH_2O$); 1.8 (s, 3H, CH_3, T); 2.1 (s, 3H, CH_3, lev.); 2.2–2.8 (m, 6H, CH_2CH_2, lev. and H2', H2''); 4.1–4.5 (m, 5H, H4', H5', H5'', $CH_2C\underline{H}_2O$); 5.2 (m, H3'); 6.4 (dd, 1H, H1'); 6.7–7.6 ppm (m, 5H, 4H, 2-ClC_6H_4 and H6, T). ^{31}P{^{1}H} NMR ($CDCl_3$): δ −6.73, −6.82 ppm (2 × s, P–O, 2 ClC_6H_4).

The 2-chlorophenyl group was removed by treating compound **8a** (680 mg, 0.9 mmole), after coevaporation with dioxane (2 × 20 ml), with *syn*-pyridine-2-carboxaldoxime (400 mg, 3.27 mmole) and N^1,N^1,N^3,N^3-tetramethylguanidine[9] (300 mg, 2.6 mmole) in dry acetonitrile. The reaction mixture was stirred for 12 h at 20°C, after which time Dowex 50W cation-exchange resin (100–200 mesh, ammonium form, 7 g per mmole oximate) was added. After 5 min, the resin was filtered off and washed with acetonitrile. After evaporation, the residual oil was dissolved in pyridine (10 ml) and a solution of hydrazine hydrate[10] (1.0 M) in pyridine/acetic acid (3/2, v/v, 10 ml) added. After 4 min at 20°C, the solution was cooled (ice-water bath) and 2,4-pentanedione (2 ml) added. The reaction mixture was then added to petroleum ether (40–60°C) with stirring to give the desired product as a precipitate. The precipitate was filtered off, dissolved in chloroform (2 ml) and applied to a column of Kieselgel (10 g) suspended in chloroform. Elution of the column was effected with chloroform/methanol/TEAB (89/10/1 → 70/25/5, v/v). The appropriate fractions were concentrated, dissolved in chloroform (50 ml) and washed with TEAB (10 ml, 1 M, pH 7.5). After evaporation of the solvent, the triethylammonium salt of compound **8b** was obtained as a white powder. Yield 465 mg (80%); R_f 0.54 (chloroform/methanol/TEAB, 70/25/5, v/v). $[\alpha]_D^{25}$ −0.21 (*c* 1, chloroform/methanol, 1/1, v/v). ^{1}H NMR ($CDCl_3/CD_3OD$): δ 1.9 (t, 3H, CH_3, alkyl, *J* 7.5 Hz); 1.25–1.4 (m, 28H, $(CH_2)_{14}$); 1.6 (m, 2H, H2', H2''); 1.97 (s, 3H, CH_3, T); 2.17–2.34 (m, 2H, H5', H5''); 3.87 (m, (H, CH_2–O–P, alkyl); 4–4.13 (m, 2H, H3', H4'); 6.33 (dd, 1H, H1', $J_{1,2}$ 7 Hz); 7.72 ppm (s, 1H, H6). ^{13}C{^{1}H} NMR ($CDCl_3$): δ 9.2 (s, $C\underline{H}_3CH_2N$); 46.6 (s, $CH_3C\underline{H}_2N$); 12.5 (s, CH_3, T); 14.3, 23.1, 31,4, 31.1, 29.8–30.1, 26.3, 32.4, 40.1, 39.7 (s, C1–C16); 65.5, 65.3 (d, CH_2OP, $^2J_{C-P}$ 4.5 Hz); 66.3, 66.1 (d, C5', $^2J_{C-P}$ 6.1 Hz); 67.4 (s, C2'); 71.5 (s, C3'); 85.33 (s, C1'); 86.3, 86.6 (d, C4', $^3J_{C-P}$ 8.3 Hz); 111.4 (s, C5); 137.1 (s, C4); 151.6, 165.5 ppm (s, 2 × C=O). ^{31}P{^{1}H} NMR ($CDCl_3/CD_3OD$): δ −0.5 ppm (s, P–O$^-$).

Acknowledgements

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