Pergamon

Tetrahedron Letters 43 (2002) 5397–5400

TETRAHEDRON LETTERS

Bis(hydroxymethyl)phosphinic acid analogues of acyclic nucleosides; synthesis and incorporation into short DNA oligomers

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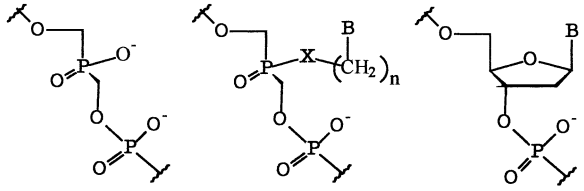
Received 30 April 2002; revised 27 May 2002; accepted 7 June 2002

Abstract—Novel analogues of acyclic nucleosides based on a bis(hydroxymethyl)phosphinic acid (BHPA) backbone were obtained by condensation of the bis(4,4′-dimethoxytrityl) derivative of BHPA with *N*-1- or *N*-3-(2-hydroxyethyl)thymine in the presence of MSNT, or by an Appel reaction with *N*-1- or *N*-3-(2-aminoethyl)thymine. After selective deprotection and phosphitylation they were used as monomers for automated solid support synthesis of short oligomers by the phosphoramidite approach.

Acyclic analogues of nucleosides are of interest due to their attractive chemical and pharmacological properties. Up to now, several purine and pyrimidine linked aliphatic derivatives have been found to be effective antiviral agents.[1–3] Acyclic derivatives of nucleosides are also considered as components of novel DNA analogues. The aim of synthesizing such analogues, including those derived from isosteric glyceronucleosides, is to identify potential antisense or antigene therapeutics with improved nucleolytic stability and binding affinity toward complementary DNA or RNA strands.[4–7]

In the presented analogues **1** and **2**, bis(hydroxymethyl)-phosphinic acid (BHPA) replaces the 3′-, 4′- and 5′-carbon atoms of the sugar–phosphate backbone of DNA (**3**) and provides a site for attachment of nucleobases via a one- or two-carbon linker. Esters or amides of BHPA (**2**, X = O or NH) possess an additional hydrogen bond acceptor center at the phosphinyl oxygen atom. The non-ionic nature of such a unit, as in **2**, may enhance cellular uptake, while attached nucleobases (B) may interact via stacking and/or hydrogen bonds with complementary DNA or RNA.

We report here a general method for the synthesis of bis(hydroxymethyl)phosphinic acid derivatives carrying *N*-1- or *N*-3-ethylthymine and the incorporation of such analogues into a short oligonucleotide chain.



1 **2** **3**

The synthesis of the monomers is outlined in Scheme 1. Both OH functions of bis(hydroxymethyl)phosphinic acid methyl ester[8] **4** were protected with acid-labile 4,4′-dimethoxytrityl (DMT) groups. Fully protected derivative **5** was demethylated with *tert*-butylamine at 100°C (sealed tube). BHPA esters **7a** and **7b** could be synthesized in good yields (74 and 75%, respectively) by condensation of **6** with *N*-1-[9] or *N*-3-(2-hydroxyethyl)thymine[10] in the presence of 1-(2-mesitylensulfonyl)-3-nitro-1,2,4-triazole (MSNT) as activator. To obtain **7c** and **7d**, phosphinate **6** was subjected to an Appel reaction[11] with *N*-1- or *N*-3-(2-aminoethyl)-thymine.[10] The reaction was carried out in pyridine in the presence of triphenylphosphine (3 equiv.) and carbon tetrachloride (5 equiv.). The yields of the desired products were rather low (25–30%), as was reported by Appel for such reactions.[11] Selected physicochemical characteristics of **7a–d** are presented in Table 1.

Keywords: phosphinic acid derivatives; nucleic acid analogues.

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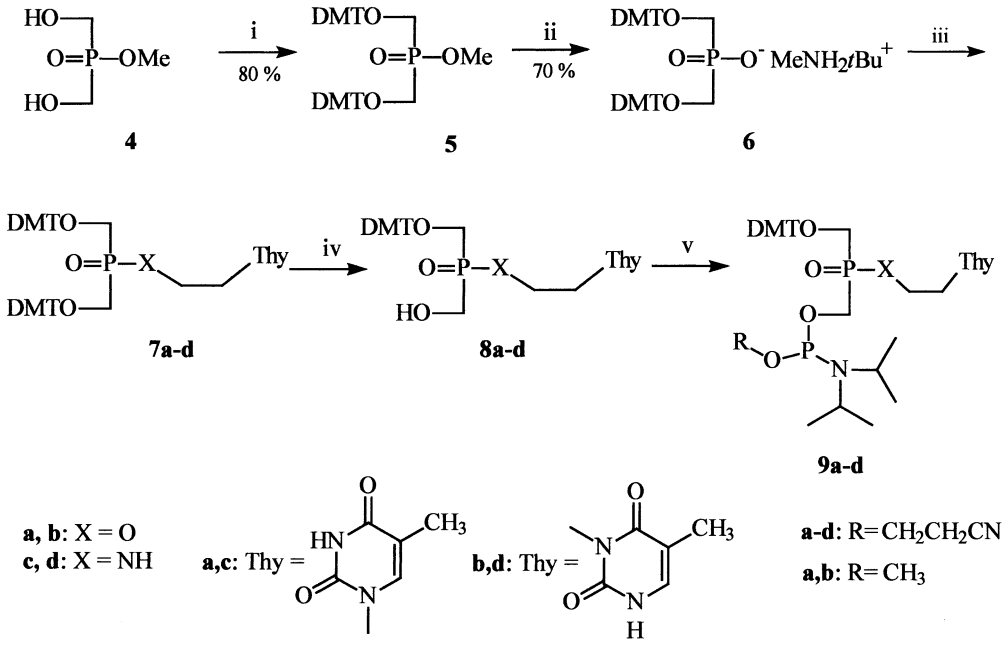
PII: S0040-4039(02)01067-5



Scheme 1. *Reagents and conditions*: (i) 4,4′-dimethoxytrityl (DMT) chloride (2.5 equiv.)/pyridine, 12 h, rt; (ii) *tert*-butylamine (6 equiv.), 100°C, 10 h; (iii) *N*-1 or *N*-3-(2-hydroxyethyl)thymine (1 equiv.), MSNT (1 equiv.) in pyridine, 24 h, rt or (2-aminoethyl)thymine (1 equiv.), PPh_3 (3 equiv.), CCl_4 (5 equiv.) in pyridine, rt, 24 h; (iv) toluene-4-sulfonic acid in methanol (0.02 M, 0.8 equiv.), rt, 5 min, then pyridine, (v) *O*-2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (1.2 equiv.) and 2-ethylthio-1*H*-tetrazole (2.4 equiv.) in acetonitrile, rt, 1 h.

Table 1. Spectral and chromatographic data for bis(hydroxymethyl)phosphinic acid derivatives **7**–**9**

Comp.	Yield (%)	R_f (TLC)[a]	FAB MS *m*/*z*	δ ^{31}P NMR (ppm)
7a	74	0.34	882.6 [*M*+H] 881.8 [*M*−H]	49.10
7b	75	0.22	881.8 [*M*−H]	48.43
7c	30	0.58	882.8 [*M*+H] 880.3 [*M*−H]	38.55
7d	25	0.42	880.6 [*M*−H]	37.35
8a	35	0.61	579.3 [*M*−H]	48.75
8b	33	0.50	579.1 [*M*−H]	50.65
8c	32	0.82	578.2 [*M*−H]	38.26
8d	30	0.73	578.3 [*M*−H]	37.51
9a[c]	70	0.32		153.37; 153.0; 46.96; 46.59[b]
9b[d]	60	0.42		152.69; 152.31; 46.32; 46.16[b]
9c[d]	75	0.48		152.10; 152.00; 151.96; 151.86; 36.59; 36.53; 36.44; 36.39[b]
9d[d]	50	0.50		151.92; 151.61; 36.37; 36.00[b]

[a] $CHCl_3$/MeOH, 9:1, v/v.
[b] The presence of multiple resonances (four or eight signals) may be due to the presence of *threo–erythro* diastereoisomerism and/or P–C–O–P spin couplings.
[c] Methyl-protection of phosphoramidite.
[d] 2-Cyanoethyl-protection of phosphoramidite.

The resulting derivatives **7a**–**d** were subjected to the selective removal of one DMT group by brief treatment with toluene-4-sulfonic acid in methanol (0.02 M, 0.8 equiv.). The reaction had to be stopped after 5–6 min while ca. 60% of substrate was still present in the reaction mixture. Longer reaction times led to significant amounts of fully deprotected BHPA ester or amide derivatives and to their further decomposition resulting from P–O and P–N bond cleavage. Thus, the reactions were terminated by addition of an excess of pyridine. The desired products were isolated by means of preparative TLC (yield ca. 30–35%), and about 60% of starting material was recovered.

Phosphitylation[12] of the free OH group in **8a**–**d** by *O*-2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (1.2 equiv.) was carried out in anhydrous acetonitrile in the presence of 2-ethylthio-1*H*-tetrazole (2.4 equiv.) providing the novel acyclic nucleoside amidite synthons **9a**–**d**.

Table 2. Characteristics of short DNA analogues possessing bis(hydroxymethyl)phosphonic acid units (Y)

Comp. no.	Compound	HPLC[c] R_t (min)	MALDI TOF	
			Calc.	Found
10c[a]	5′-dApYpdC-3′	16.15	878	879
10d	5′-dApYpdC-3′	17.10	878	878
11	5′-dApY⁻pdC-3′[b]	13.49	725	727
12a	TpTpYpYpTpT-3′	20.06	1828	1835
12b	TpTpYpYpTpT-3′	20.63	1828	1833

[a] **a**–**d** are according to Scheme 1.
[b] Y^- is ionic bis(hydroxymethyl)phosphinic acid residue as in **1**.
[c] Analytical HPLC was performed on a C18 (4.6×250 mm, ThermoQuest) column with a linear gradient of acetonitrile in 0.1 M triethylammonium bicarbonate pH 7.5 buffer (0–20 min/0–20% CH_3CN, 20–25 min/20–40% CH_3CN, flow 1 mL/min).

The phosphoramidite monomers **9** were used for the synthesis of modified trinucleotides 5′-dApYpdC-3′ (where Y is a modified BHPA unit) by automated solid phase methodology.[13] The synthesis of oligomers **10**–**12** was performed on an ABI 394 synthesizer (Applied Biosystem Inc., Foster City, CA) using succinyl-linked LCAA-CPG solid support. The only difference in the manufacturer recommended procedure was a prolonged coupling time (up to 600 s). The coupling efficiency of **9a**–**d**, determined by DMT-ion assay, was in the range of 95–97%. The 5′-terminal DMT group was removed before cleavage of the product from the solid support. A standard ammonium deprotection and one-step RP HPLC purification led to the desired trimers **10c** and **10d** for which HPLC showed no separation of diastereomeric trimers (P-epimers, Table 2). Unfortunately, this approach did not yield the desired trimers **10a** and **10b**, derived from esterified BHPA. As revealed by MALDI-TOF analysis, both isolated products were in fact the same compound **11**, possessing no alkylnucleobase moiety. Hydrolytic deprotection conditions (concentrated aqueous ammonia, 16 h, 55°C) resulted in cleavage of the ester bond of the phosphinic acid derivatives **10a** and **10b**, giving rise to anionic DNA analogues with an abasic site (as in the structure **1**). This instability of the BHPA ester bond of **10a** and **10b** was also observed under less harsh conditions, routinely used for cleavage of oligomers from the solid support (aq. NH_4OH, 1 h, 20°C). Despite the observed stability of the ester bond of **7a** and **7b** in concentrated aqueous ammonia for 2 h at rt (data not shown), it was expected that such compounds, as base-labile phosphotriester analogues, may not survive the deprotection conditions.

Thus we could not use the routine phosphoramidite methodology for the synthesis of the modified oligonucleotides **10a**,**b** with protected nucleobases, which demand the use of basic deprotection conditions. However, using oxalyl-LCAA CPG solid support[14] and methyl-phosphoramidite methodology[13] we obtained hexamers 5′-TpTpYpYpTpT-3′ (**12a** and **12b**). Removal of the phosphate protecting methyl groups was achieved by treatment of solid support-bound oligomers with thiophenol/dioxane/triethylamine mixture (2:1:2, v/v) for 5 min at rt followed by the cleavage of oligomers form the solid support with 1% aqueous triethylamine for 7–10 min at rt.

These oligomers were also obtained by using oxalyl-LCAA CPG solid support and protection of the phosphate function with a 2-cyanoethyl group. In this case, treatment of solid support-bound oligomers with 1% aqueous triethylamine for 10 min at room temperature resulted in simultaneous cleavage of the oligomer from the solid support and removal of the 2-cyanoethyl protecting groups. Hexamers **12a** and **12b** were purified by HPLC and their structure was confirmed by MALDI TOF analysis (Table 2).

Anionic analogues of DNA, such as **11**, were also obtained by selective protection of one OH function with the DMT group and subsequent phosphitylation of the second OH group of BHPA methyl ester **4**, followed by introduction of such monomer into the oligonucleotide by the solid phase methodology with two-step deprotection/purification.[15]

In summary, acyclic nucleoside analogues **7a**–**d** derived from bis(hydroxymethyl)phosphinic acid linked via an amide or ester bond to an alkylnucleobase moiety were synthesized and then introduced into an oligonucleotide chain using the phosphoramidite methodology. The routine synthetic strategy has proved to be successful for **10c** and **10d**, and oxalyl-LCAA CPG afforded the homo-thymidylate oligomers **12a** and **12b**. Studies on the application of the aforementioned methodology for the synthesis of longer oligonucleotide analogues, their hybridization properties as well as nuclease resistance are underway.

Experimental

Typical procedures for the synthesis of compounds 7

Preparation of 7a (MSNT procedure): MSNT (0.44 g, 1.5 mmol) was added to a solution of **6** (0.80 g, 1.0 mmol) and *N*-1-(2-hydroxyethyl)thymine (0.17 g, 1 mmol) in anhydrous pyridine (8 mL) and the mixture was stirred at rt for 24 h. The solvent was evaporated and the crude product was isolated by column chromatography on silica gel with a gradient of 0–5% MeOH in $CHCl_3$ to give 0.66 g (75%) of pure **7a** in the form of a white foam.

Preparation of 7c (Appel procedure): CCl_4 (0.54 mL, 5.6 mmol) was added with stirring under argon at rt to a solution of **6** (0.90 g, 1.1 mmol) and PPh_3 (0.88 g, 3.4 mmol) in anhydrous pyridine (8 mL). Stirring was continued for 30 min and a solution of *N*-1-(2-aminoethyl)thymine (0.19 g, 1.1 mmol) in anhydrous pyridine (4 mL) was added. The resulting solution was stirred for 24 h at rt and the solvent was removed under

reduced pressure. The crude product was purified by silica gel column chromatography with a gradient of 0–5% MeOH in $CHCl_3$ to give 0.27 g (30%) of pure **7c** as white crystals.

^{1}H NMR characterization of products 7a–d (200 MHz, $CDCl_3$)

7a: 7.38–6.71 (m, 26H), 7.32 (q, $J=1.1$ Hz, 1H), 4.04 (m, 4H), 3.78 (s, 6H), 3.77 (s, 6H), 3.69 (dd, $J=9.5$ Hz, $J=12.5$ Hz, 2H), 3.41 (dd, $J=9.7$ Hz, $J=12.5$ Hz, 2H), 1.64 (d, $J=1.1$ Hz, 3 H).

7b: 7.37–6.71 (m, 27H), 4.13 (m, 4H), 3.77 (s, 6H), 3.76 (s, 6H), 3.60 (dd, $J=9.0$ Hz, $J=12.5$ Hz, 2H), 3.42 (dd, $J=9.0$ Hz, $J=12.5$ Hz, 2H), 1.77 (d, $J=1.1$ Hz, 3H).

7c: 7.40–6.70 (m, 26H), 7.08 (q, $J=1.0$ Hz, 1H), 3.77 (s, 6H), 3.76 (s, 6H), 3.73–3.58 (m, 4H), 3.28 (dd, $J=9.6$ Hz, $J=11.5$ Hz, 2H), 3.14 (dd, $J=9.6$ Hz, $J=11.5$ Hz, 2H), 1.76 (d, $J=1.0$ Hz, 3H).

7d: 7.38–6.7 (m, 26H), 7.34 (q, $J=1.0$ Hz, 1H), 4.03 (t, $J_{PCH}=5.7$ Hz, 4H), 3.55 (dd, $J=9.3$ Hz, $J=12.0$ Hz, 2H), 3.77 (s, 6H), 3.76 (s, 6H), 3.32 (dd, $J=9.4$ Hz, $J=12.0$ Hz, 2H), 1.76 (d, $J=1.0$ Hz, 3H).

Typical procedures for the synthesis of compounds 8

Preparation of 8c: To a solution of compound **7c** (0.13 g, 0.22 mmol) in MeOH (4 mL), a methanolic solution of toluene-4-sulfonic acid (0.13 mL, 0.02 M) was added, with stirring. After 5 min the reaction was terminated by addition of pyridine (0.4 mL). The product and unreacted substrate were isolated by means of preparative TLC on silica gel with $CHCl_3$/MeOH (9:1, v/v). The recovered substrate was again reacted as above. After two-fold repetition of the procedure, pure product **8c** was obtained in 35% yield as a white solid.

^{1}H NMR characterization of products 8a–d (d_5-Py):

8a: 7.77–6.80 (m, 14H), 4.98 (br. s, OH), 4.69–4.52 (m, 4H), 4.18–4.01 (m, 2H), 3.84 (t, $J=3.3$ Hz, 2H), 3.65 (s, 3H), 3.64 (s, 3H), 1.85 (d, $J=1.0$ Hz, 3H).

8b: 7.78–6.74 (m, 14H), 4.90 (br. s, OH), 4.46–4.20 (m, 2H), 4.17–4.03 (m, 4H), 3.79 (s, 3H), 3.78 (s, 3H), 3.61–3.23 (m, 2H), 1.87 (d, $J=1.0$ Hz, 3H).

8c: 7.92–6.93 (m, 14H), 4.98 (br. s, OH), 4.55 (d, $J=3.0$ Hz, 2H), 4.45–4.25 (m, 2H), 3.97–3.67 (m, 4H), 3.65 (s, 6H), 1.88 (d, $J=1.0$ Hz, 3H).

8d: 7.81–6.86 (m, 14H), 5.19 (br. s, OH), 4.57 (m, 2H), 4.45 (t, $J=6.0$ Hz, 2H), 3.98–3.67 (m, 4H), 3.64 (s, 3H), 3.63 (s, 3H), 1.87 (d, $J=1.0$ Hz, 3H).

Acknowledgements

The results presented here were obtained within the project funded by the State Committee for Scientific Research (KBN Grant 3T09A 03917 for B.N.).

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