106.12, 93.36, 64.36, 54.69, 13.59, 10.88 ppm; mass spectrum, m/z (relative intensity) 183 (4), 182 (M^+, 34), 167 (4), 165 (8), 164 (15), 151 (3), 150 (7), 149 (6), 140 (5), 139 (34), 135 (28), 125 (18), 121 (15), 111 (90), 109 (15), 107 (16), 100 (9), 97 (10), 95 (36), 91 (9), 82 (28), 79 (23), 77 (16), 72 (25), 69 (16), 65 (12), 57 (13), 55 (43), 53 (23), 51 (16), 45 (19), 43 (100), 41 (28). Anal. Calcd for $C_{10}H_{14}O_3$: C, 65.91; H, 7.74. Found: C, 66.19; H, 7.88.

Acknowledgment. We are grateful to Hoechst-Roussel Pharmaceuticals Inc., Hoffmann-La Roche Inc., Lederle Laboratories, Rutgers University (Charles & Johanna Busch Memorial Fund), and the NIH (Grant MBRSG 1-S06-RR08223) for supporting this research. We also thank M. N. Agnew, A. Rizwaniuk, and T. Kaminski, Hoechst-Roussel, Somerville, NJ, for the ^{13}C and ^1H NMR spectra and MS data.

Registry No. 1, 13169-00-1; **2**, 100-52-7; **2a**, 99309-82-7; **2b**, 16183-45-2; **3**, 939-97-9; **3a**, 99327-11-4; **3b**, 99309-83-8; **4**, 123-11-5; **4a**, 99309-84-9; **4b**, 99309-85-0; **5**, 120-57-0; **5a**, 99309-86-1; **5b**, 99309-87-2; **6**, 14371-10-9; **6a**, 99309-88-3; **6b**, 99309-89-4; **7**, 122-78-1; **7a**, 99309-90-7; **7b**, 99309-91-8; **8**, 630-19-3; **8a**, 99309-92-9; **8b**, 88522-71-8; **9**, 78-84-2; **9a**, 99309-93-0; **9b**, 6986-73-8; **10**, 2043-61-0; **10a**, 99309-94-1; **10b**, 99309-95-2; **11**, 1192-58-1; **11a**, 99309-96-3; **12**, 5834-16-2; **12a**, 99309-97-4; **13**, 620-02-0; **13a**, 99309-98-5; methyl propargyl ether, 627-41-8; (*E*)-2-methoxy-1-(4-*tert*-butylphenyl)-2-butene, 99309-99-6; 1-(4-*tert*-butylphenyl)butane, 14011-00-8.

[^{18}O] Chiral Phosphate in d(CpA) and d(TpA): Synthesis via Phosphite Triesters and Assignment of Configuration

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The dinucleoside monophosphates d(TpA) (**11a**) and d(CpA) (**19a**)—both chirally labeled with ^{18}O at phosphorus and representing cleavage sites in a number of restriction endonuclease recognition sequences—have been synthesized via the fully protected phosphate triesters **8a/b** and **16a/b**. The latter were obtained from condensation of the pyrimidine phosphoramidites **3a/b** and **5a/b** with an appropriately protected adenosine derivative (**1**). Oxidation of these intermediates with [^{18}O]H_2O and iodine furnished diastereomeric mixtures of the fully protected triesters (**8a/b** and **16a/b**). Chromatographic separation of the diastereomeric triesters was achieved by using flash chromatography on the detritylated compounds **9a/b** and **17a/b**. Complete deprotection of the materials of the "fast" migrating zones furnished stereochemically pure [^{18}O]d(TpA) as well as [^{18}O]d(CpA) (**11a** and **19a**). The absolute configuration at phosphorus was deduced from hydrolysis experiments in which dimers were digested with nuclease P1 in [^{17}O]H_2O to yield [^{16}O,^{17}O,^{18}O]dAMP by inversion. The configuration was determined after cyclization to cyclic 3′,5′-phosphate followed by methylation and NMR analysis and was found to be S_P. Consequently [^{18}O]d(TpA) and [^{18}O]d(CpA) obtained from the "fast" migrating zones on triesters both have the R_P configuration. From this analysis it is apparent that the detritylated pairs of phosphate triesters with identical configurations show the same trends in their shieldings of ^1H, ^{13}C, and ^{31}P NMR signals. Although it has been shown that the ^{31}P NMR chemical shifts of methylated stereochemically pure ^{18}O dinucleoside monophosphates **26a/b** and **28a/b** do not follow simple principles,[8,9] the stereochemical assignment of [^{18}O]d(TpA) or [^{18}O]d(CpA) can now be established by comparing the ^{31}P NMR shifts of their methylation products with those of the methyl esters **25** and **27** described in this manuscript.

Oxygen chirally labeled nucleoside phosphates are useful tools for the elucidation of the stereochemistry of reactions catalyzed by enzymes.[1] With the help of such substrates the stereochemical course of mechanisms of several nucleases has been elucidated.[1] Moreover, a study by Reed[2] has shown that chiral [α-^{16}O,^{17}O]ADP can be employed to investigate the first coordination sphere of the Mn^{2+} ion in the active site of kreatine kinase.

Until recently the elucidation of the stereochemical course of enzymatic phosphodiester hydrolysis was achieved by the use of stereochemically pure phosphorothioates.[3] However, due to the altered van der Waals radii of sulfur compared to oxygen, phosphorothioates are sometimes processed less efficiently, which can obscure the interpretation of stereochemical data.

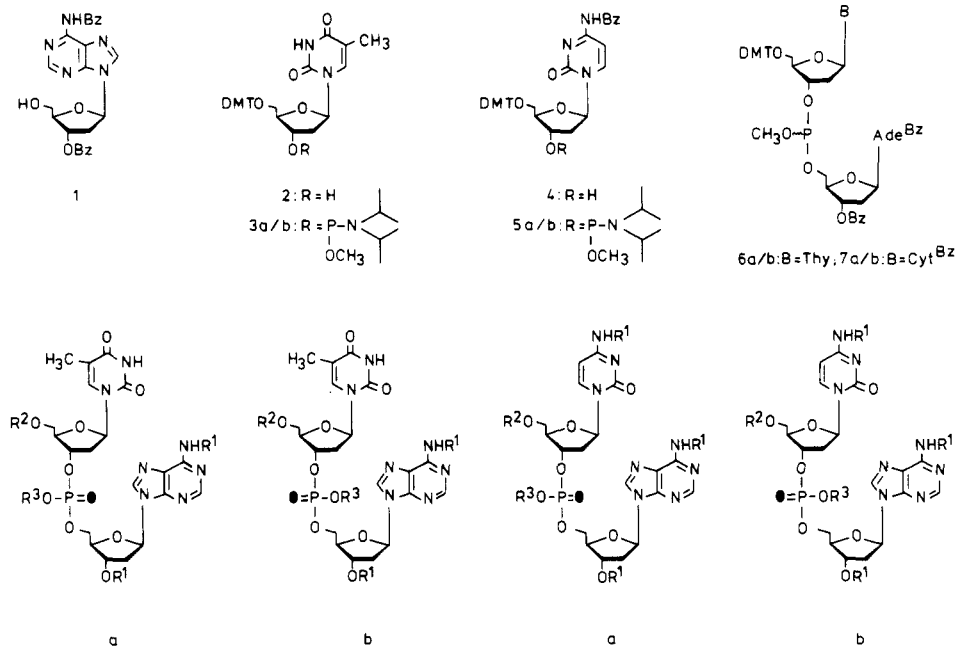
Lowe[4] and Knowles[5] were the first to report the synthesis of phosphate monoesters chiral by virtue of oxgen isotopes. Chiral [^{16}O,^{17}O,^{18}O] phosphate monoesters were subsequently employed in many studies of phosphoryl transfer reactions.[1,6] The synthesis of a chirally ^{18}O-labeled dinucleoside monophosphate has been reported by Eckstein et al.[7] These authors converted diastereomerically pure phosphorothioates by a sulfur exchange into a chirally ^{18}O-labeled phosphate. By employing this chirally labeled product, (R_P)-[^{18}O]d(TpT), they were successful in elucidating the stereochemical course of phosphodiester hydrolysis by nuclease P1.

A direct approach using ^{18}O as well as ^{17}O labeling has been developed by our laboratory using phosphite inter-

(1) Gerlt, J. A.; Coderre, J. A.; Mehdi, S. In "Advances in Enzymology"; Meister, A., Ed.; Interscience: New York, 1983; Vol. 55, pp 291–380.
(2) Leigh, T. S.; Sammons, R. D.; Frey, P. A.; Reed, G. H. *J. Biol. Chem.* **1982**, *257*, 15047–15053.
(3) Burgers, P. M. J.; Eckstein, F. *Annu. Rev. Biochem.* **1985**, *54*, 367–402.
(4) Cullis, P. M.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1978**, 512–514.
(5) Abbott, S. J.; Jones, S. R.; Weinmann, S. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1978**, *100*, 2558–2560.
(6) Jones, S. R.; Kindman, L. A.; Knowles, J. R. *Nature (London)* **1978**, *275*, 564–565.
(7) Potter, B. V. L.; Connolly, B. A.; Eckstein, F. *Biochemistry* **1983**, *22*, 1369–1377.

0022-3263/85/1950-5314$01.50/0

Scheme I



8 - 15

	R^1	R^2	R^3	P=O ●=[^{18}O]	O=[^{16}O]
8a/b	Bz	DMT	Me	●	
9a, 9b	Bz	H	Me	●	
10a, 10b	Bz	H	H	●	
11a, 11b	H	H	H	●	
12a/b	Bz	DMT	Me		O
13a, 13b	Bz	H	Me		O
14	Bz	H	H		O
15	H	H	H		O

16 - 23

	R^1	R^2	R^3	P=O ●=[^{18}O]	O=[^{16}O]
16a/b	Bz	DMT	Me	●	
17a, 17b	Bz	H	Me	●	
18a, 18b	Bz	H	H	●	
19a, 19b	H	H	H	●	
20a/b	Bz	DMT	Me		O
21a, 21b	Bz	H	Me		O
22	Bz	H	H		O
23	H	H	H		O

a: faster migrating zone; b: slower migrating zone; a/b: diastereomeric mixture.

mediates and employing either [^{17}O]H_2O or [^{18}O]H_2O/iodine in the oxidation reaction. Chromatographic separation of protected diastereomeric triesters allowed the separation of oxygen chirality at phosphorus. Deprotection led to the oxygen-labeled chiral ribonucleoside monophosphates. By this route (R_P)-[^{18}O]UpA[8] and (R_P)-[^{18}O]d(GpA)[9] have been prepared.

In this paper we report the synthesis of the chirally ^{18}O-labeled deoxyribodinucleoside monophosphates, namely, d(TpA) and d(CpA).[10] Both dinucleotides are the cleavage sites of a number of restriction endonucleases.[11] For example, the restriction enzyme HpaI from *Haemophilis parainfluenzae* cleaves the sequence GTTAAC between thymidine and 2′-deoxyadenosine and the enzyme BstNI from *Bacillus stearothermophilis* cuts between 2′-deoxycytidine and 2′-deoxyadenosine in the sequence CCAGG. The incorporation of chirally labeled dimers will allow the elucidation of the stereochemistry of phosphodiester hydrolysis occurring at the active site of these enzymes (Scheme I).

Results and Discussion

The synthesis of chirally ^{18}O-labeled deoxyribodinucleoside monophosphates such as [^{18}O]d(TpA) and [^{18}O]d(CpA) was achieved by direct phosphite triester oxidation.[8] Starting materials for the preparation of the dinucleoside monophosphates **11a** and **19a** were the phosphoramidites **3a/b**[12] and **5a/b**.[12] The latter compounds were prepared by phosphitylation of the 5′-protected nucleosides **2** and **4** in dichloromethane solution and were obtained pure after flash chromatography on silica gel. The ^{31}P NMR spectra of the phosphoramidites **3a/b** and **5a/b** demonstrate the existence of diastereomeric mixtures. The same conclusion was drawn from the proton-decoupled ^{13}C NMR spectra (Table I). At the field strength (62.9 MHz) used for these experiments the signals of the chromophore moieties of the diastereomers were not separated. However, almost all of the sugar signals were separated. One difficulty arose from the fact that the chemical shielding of the carbon signals was in the same range as the splitting caused by carbon–phosphorus coupling. It is worth mentioning that the phosphoramidite group of compounds **3a/b** and **5a/b** shifts the carbon-3′ signal by about 1–2 ppm downfield compared to the corresponding signals from the nucleosides **2** and **4**. This shielding is much smaller than that induced by phosphate residues (6–7 ppm) linked to this carbon. Except for the C-3′ signal all the other carbon signals were practically unaffected.

The condensation of the phosphoramidites **3a/b** and **5a/b** with compound **1** was carried out in anhydrous THF solution in the presence of tetrazole as catalyst. The

(8) Seela, F.; Ott, J.; Potter, B. V. L. *J. Am. Chem. Soc.* **1983**, *105*, 5879–5886.
(9) Potter, B. V. L.; Eckstein, F. *Nucleic Acids Res.* **1983**, *11*, 7087–7103.
(10) Seela, F.; Ott, J.; Potter, B. V. L.; Herdering, W. *Nucleosides Nucleotides* **1985**, *4*, 131–133.
(11) Kessler, C.; Neumaier, T. S.; Wolf, W. *Gene* **1985**, *33*, 1–102.
(12) McBride, L. J.; Caruthers, M. H. *Tetrahedron Lett.* **1983**, *24*, 245–248.

Table I. ^{13}C NMR Chemical Shifts of Proton-Decoupled NMR Spectra of Detritylated [^{18}O]d(TpA) and [^{18}O]d(CpA) Diastereomers and of the Parent Nucleoside Phosphoramidites in $CDCl_3$ Solution[a]

compd		C-2	C-4	C-5	C-6	C-8	C-1′	C-2′	C-3′	C-4′	C-5′	CH_3	OCH_3	CH	$(CH_3)_2$
1		152.11	150.92	124.44	150.30	142.29	87.18	38.12	76.47	87.18	63.04				
2		150.52	163.82	111.14	135.58		84.88	40.89	72.35	86.22	63.66	11.77			
3a/b		150.25	163.60	110.98	135.54		84.94	40.21 (d)[b]	73.42 (2d)[b]	85.70 (2d)[b]	63.30 (d)[b]	11.66	50.32 (2d)[b]	43.19 (2d)[b]	24.55 (d)[b]
4		154.89	162.24	96.65	144.50		87.24	41.99	70.94	86.56	62.93				
5a/b		154.48	162.14	96.55	144.39		87.08 (d)[b]	41.15 (2d)[b]	72.17 (2d)[b]	85.98 (2d)[b]	62.52 (d)[b]		50.27 (d)[b]	43.16 (d)[b]	24.50 (d)[b]
9a	T	150.49	163.78	111.09	136.32		85.84	38.49 (d, 4.9)	78.54 (d, 4.7)	83.62 (d, 7.3)	61.95	12.22	54.73 (d, 5.5)		
	A	152.62	152.07	124.01	150.02	141.70	84.68	37.39	74.79	85.84	67.40 (d, 5.5)				
9b	T	150.51	163.84	111.11	136.25		85.79	38.54 (d, 3.2)	78.24 (d, 4.7)	83.56 (d, 7.5)	61.82	12.25	54.80 (d, 6.2)		
	A	152.57	151.87	123.84	150.04	141.61	84.85	37.59	74.79	85.62 (d, 6.2)	67.17 (d, 4.9)				
17a	C	154.52	162.37	96.98	145.25		88.00	39.75 (d, 4.8)	78.19 (d, 4.8)	83.71 (d, 6.6)	61.70		54.71 (d, 7.4)		
	A	152.72	151.96	124.02	149.86	141.66	84.72	37.58	74.83	86.62 (d, 3.7)	67.48 (d, 4.8)				
17b	C	154.80	162.52	97.04	145.14		87.98	39.83 (d, 3.2)	78.02 (d, 5.1)	83.68 (d, 7.5)	61.65		54.88 (d, 5.5)		
	A	152.69	151.87	123.88	149.92	141.53	84.92	37.86	74.92	86.52 (d, 5.6)	67.30 (d, 5.0)				

[a] δ values are given in parts per million relative to Me_4Si as internal standard. Multiplicities and coupling constants (in hertz) are in parentheses. [b] Splitting pattern is due to phosphorus coupling and/or the differences of the chemical shifts of the diastereomers. The assignment of signals of almost identical shifts is tentative.

Table II. ^1H NMR Chemical Shifts of Detritylated [^{18}O](TpA)/[^{18}O]d(CpA) Diastereomers in $CDCl_3$ Solution[a]

compd		2-H	5-H	6-H	8-H	1′-H	2′-H_a	2′-H_b	3′-H	4′-H	5′-H	CH_3	OCH_3
9a	T			*b*		6.15 dd (6.2, 8.0)	2.24 m	2.39 m	4.96 m	4.39 m	3.82 m	1.78 s	3.77 d (11.3)
	A	8.47 s			8.80 s	6.66 dd (5.9, 8.0)	3.18 m	2.82 m	5.73 m	4.18 m	4.48 m		
9b	T			*b*		6.18 pt (6.9)	2.36 m	2.44 m	5.08 m	4.48 m	3.78 m	1.79 s	3.77 d (11.3)
	A	8.47 s			8.81 s	6.64 pt (6.4)	3.12 m	2.84 m	5.78 m	4.10 m	4.44 m		
17a	C		*b*	8.30 d (7.4)		6.23 pt (6.6)	2.32 m	2.70 m	5.03 m	4.42 m	3.87 m		3.77 d (11.2)
	A	8.49 s			8.74 s	6.68 pt (6.6)	3.18 m	2.82 m	5.78 m	4.28 m	4.46 m		
17b	C		*b*	8.28 d (7.4)		6.19 pt (5.8)	2.46 m	2.78 m	5.14 m	4.48 m	3.85 m		3.77 d (11.3)
	A	8.42 s			8.77 s	6.64 pt (6.6)	3.08 m	2.87 m	5.77 m	4.26 m	4.44 m		

[a] δ values are given in parts per million relative to Me_4Si as internal standard. Multiplicities are set behind. Coupling constants (in hertz) are in parentheses. Splitting pattern: s, singlet; d, doublet; pt, pseudo triplet. [b] Masked by benzoyl protons.

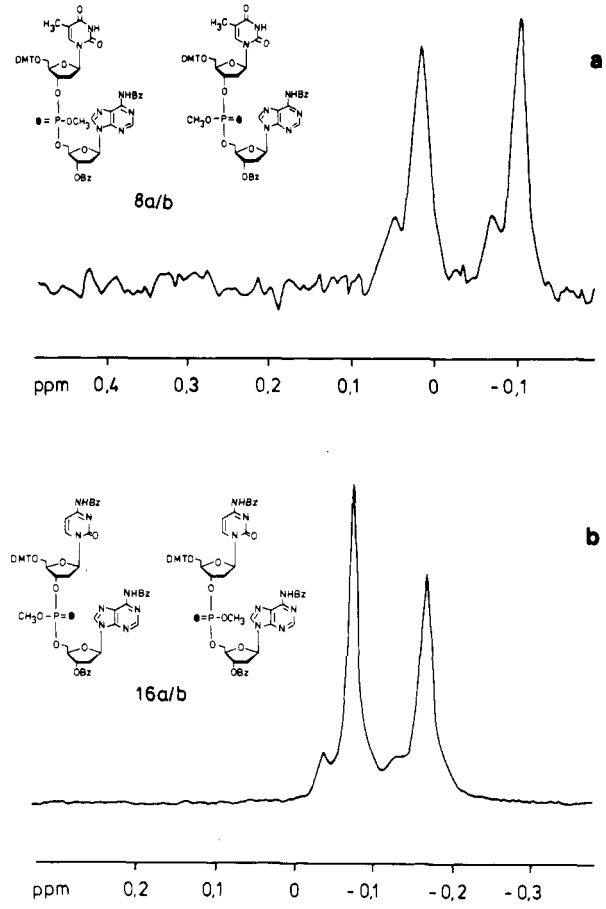


Figure 1. ^{31}P NMR spectra of the fully protected ^{18}O-labeled dimers **8a/b** and **16a/b** in $CDCl_3$. The small peaks relate to unlabeled materials, the large peaks to ^{18}O-labeled materials.

phosphoramidites were employed in excess in order to capture any last traces of unlabeled water. The condensation step itself led to the diastereomeric phosphite triesters **6a/b** and **7a/b**, respectviely. Without further "workup" of the reaction mixtures these intermediates were oxidized by iodine/lutidine/[^{18}O]water to the phosphotriesters **8a/b** and **16a/b**, which were obtained in a pure form with 80% yield. HPLC on an analytical scale separated the fully protected diastereomeric mixtures of **8a/b** and **16a/b**. However, separation on a preparative scale was extremely difficult. From the ^{31}P NMR data of **8a/b** and **16a/b** (Figure 1) it became apparent that the mixtures contained about equal amounts of diastereomers.

One might assume that the use of a stereochemically pure phosphoramidite such as **3a** or **5a** would lead to only one diastereomeric phosphite triester in a stereospecific reaction which would then give stereochemically pure phosphate triester by a stereospecific oxidation reaction. As Stec[13] demonstrated, however, diastereomeric mixtures of phosphite triesters are formed from stereochemically pure phosphoramidites indicating either that the condensation step and/or the oxidation reaction occurs in a nonstereospecific manner. As recently shown by Cullis,[14] the oxidation of phosphite triesters with iodine–water is stereospecific and occurs with retention of configuration thus confirming that the condensation step is the nonstereospecific part of the overall process.

It was of interest to know how much of the ^{18}O isotope was incorporated in the phosphate triesters when using

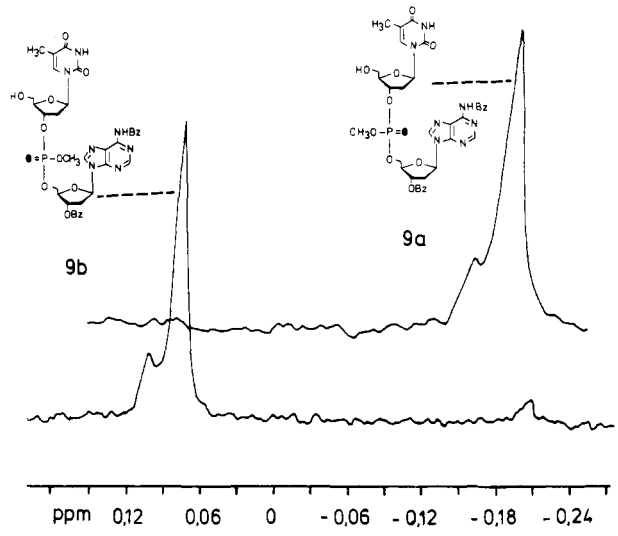


Figure 2. ^{31}P NMR spectra of the fast migrating diastereomer **9a** and the slow migrating **9b** in $CDCl_3$. The small peaks relate to unlabeled materials, the large peaks to ^{18}O-labeled materials.

90% [^{18}O]H_2O during oxidation. Since ^{18}O causes an upfield shift[15] of the ^{31}P NMR resonance (**8a/b**, 3.5 Hz; **16a/b**, 4.0 Hz) the extent of ^{18}O labeling can be calculated from the ratio of the [^{18}O]P=O to the [^{16}O]P=O signal. Integration of the signal (Figure 1) revealed an ^{18}O labeling of 85%. The apparent loss by about 5% of the ^{18}O label is most likely due to traces of [^{16}O]H_2O or [^{16}O]O_2 present in the reaction mixture.

Upon storing the diastereomeric mixtures (**8a/b**, **16a/b**), it became apparent that new compounds were formed which separated well on TLC plates. Examination of these products indicated that trityl residues had been lost. Consequently the diastereomeric mixtures of **8a/b** and **16a/b** were detritylated to yield **9a/b** and **17a/b**. These mixtures exhibited R_f values different enough to separate the diastereomers of **9a/b** and **17a/b**, respectively, on a preparative scale using flash chromatography on silica gel columns.

The signals in ^{31}P NMR spectra (Figure 2) of the diastereoisomers that were present in the fast migrating zone (**9a** and **17a**) are shifted upfield by about 0.2 ppm compared to those of the slow migrating zones (**9b** and **17b**). Typical differences of chemical shifts are also found in the ^1H (Table II) and ^{13}C NMR spectra (Table I), particularly for nuclei close to the phosphate group. A typical example is the H-3′ atom in the deoxythymidine and the deoxycytidine residues. The signals of these protons of the faster migrating compounds are shifted downfield by about 0.1 ppm compared to those of the slower migrating diastereomers. The great similarity amongst the fast migrating diastereomers and amongst the slow migrating compounds is further clearly shown in the ^1H and ^{13}C NMR spectra. We concluded that flash chromatography separates groups of diastereomers of the same configuration. The phosphate triesters of the fast and the slow migrating zones, which were pure according to TLC or ^{31}P NMR (Figure 2), were demethylated by thiophenol/dioxane/triethylamine to yield the phosphodiesters **10a,b** and **18a,b**, respectively.

After removal of the phosphate protecting group the benzoyl residues of **10a** and **18a** were split off using aqueous ammonia to yield [^{18}O]d(TpA) and [^{18}O]d(CpA),

(13) Stec, W. J.; Zon, G. *Tetrahedron Lett.* **1984**, *25*, 5279–5282.
(14) Cullis, P. M. *J. Chem. Soc., Chem. Commun.* **1984**, 1510–1512.
(15) (a) Cohn, M.; Hu, A. *J. Am. Chem. Soc.* **1980**, *102*, 913–916. (b) Jarvest, R. L.; Lowe, G. *J. Chem. Soc., Chem. Commun.* **1979**, 364–366. (c) Lowe, G.; Potter, B. V. L.; Sproat, B. S.; Hull, W. E. *J. Chem. Soc., Chem. Commun.* **1979**, 733–735.

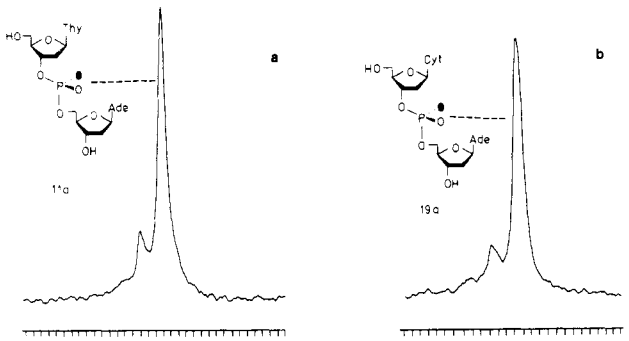
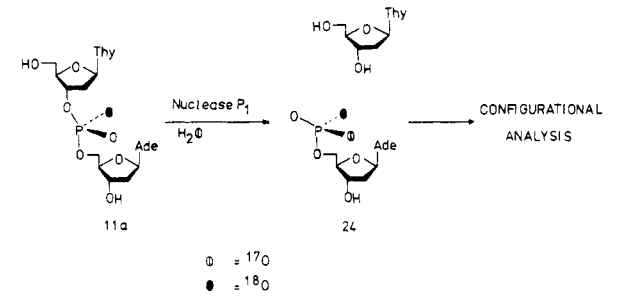


Figure 3. ^{31}P NMR spectra in D_2O/0.1 M sodium EDTA (R_P)-[^{18}O]d(T$_p$A) [a] (δ, 0.25) and (R_P)-[^{18}O]d(C$_p$A) [b] (δ, 0.20). Isotopic shifts between nonlabeled and labeled materials are 2.7 Hz; scale, 1 Hz = 1 division.

Scheme II



both of which were purified on DEAE-Sephadex A 25 ion exchange resin. From HPLC (LiChrosorb RP 18 column, 0.1 M triethylammonium acetate/acetonitrile, 91:9, v/v), it became apparent that the dinucleoside monophosphates **11a** and **19a** showed identical mobilities to the dimers **15** and **23**, thus confirming their structures. Further structural evidence came from hydrolysis experiments with snake venom phosphodiesterase. This furnished 5′-dAMP and thymidine from **11a** and 2′-deoxycytidine from **19a**. In order to quantify the cleavage products they were converted into the nucleosides with alkaline phosphatase demonstrating that the oxygen-labeled dinucleoside monophosphates are chemically pure (99%). Methylation of 2′-desoxythymidine residues as described by Gao[16] was not detected.

Figures 3a,b demonstrate that the extent of labeling of the fully deprotected dimers was 85%. This demonstrates that no loss of ^{18}O occurred during the deprotection procedure. With the successful synthesis of chiral [^{18}O]d(TpA) (**11a**) and [^{18}O]d(CpA) (**19a**) it now remained to determine their absolute configuration. It has recently been demonstrated that it is possible to determine the absolute isotopic configuration of an ^{18}O-labeled dinucleoside monophosphate by digestion of the dimer in ^{17}O-labeled water using an enzyme whose stereochemical course of reaction is known. The stereochemical course of the enzyme nuclease P1 has been determined by Potter et al.[7] This enzyme is active toward ribodinucleoside monophosphates[8] and also toward deoxyribonucleoside monophosphates.[9] Therefore, we decided to cleave the ^{18}O-labeled chiral dimer **11a** with this enzyme in ^{17}O-enriched water. Cleavage of this compound in ^{17}O,^{18}O-labeled water produced, in addition to achiral isotopomers, [^{16}O,^{17}O,^{18}O]dAMP (**24**) of one configuration (Scheme II). The configuration was analyzed by cyclization to the isotopomer of [^{16}O,^{17}O,^{18}O]dcAMP followed by methylation

Table III. Configurational Analysis of (R_P)-[^{18}O]d(TpA) (11a)[a]

	axial diastereomer calcd for [^{18}O]d(TpA)			equatorial diastereomer calcd for [^{18}O]d(TpA)		
	obsd	R_P	S_P	obsd	R_P	S_P
MeOP=O	0.81	0.34	0.34	0.73	0.34	0.34
Me●P=O	0.70	0.52	1.00	1.00	1.00	0.52
MeOP=●	1.00	1.00	0.52	0.76	0.52	1.00
Me●P=●	0.38	0.34	0.34	0.61	0.34	0.34

[a] The observed relative peak intensities of the ^{31}P NMR resonances (from Figure 4a/b) of the isotopomers of the diastereomeric triesters obtained via the cyclization and methylation of [^{16}O,^{17}O,^{18}O]dAMP derived from nuclease P1 catalyzed hydrolysis of [^{18}O]d(TpA) (**11a**) are compared with the calculated values for the hydrolysis of both isotopomers with inversion of configuration at phosphorus. The values were calculated on the basis of following assumptions: (R_P)-[^{18}O]d(TpA) starting material contained 15% ^{16}O, 85% ^{18}O, and the hydrolysis reaction with nuclease P1 was performed in water of the following isotopic composition: ^{16}O, 13.8%, ^{17}O, 50.7%, ^{18}O, 35.5%.

according by the procedure of Jarvest et al.[17] The ^{31}P NMR spectrum of the resulting isotopomers of N^1-methyl[^{16}O,^{17}O,^{18}O]dcAMP methyl ester are shown in Figure 4a,b and their relative signal intensities are in Table III.

These spectra are interpreted as follows.[15,18] When ^{18}O is bonded to phosphorus it causes an upfield shift in the ^{31}P NMR signal that is greater for double than for single bonds. Additionally, when ^{17}O is bonded to phosphorus, the quadrupolar moment of the ^{17}O nucleus causes a broadening of the ^{31}P NMR signal to such an extent that this resonance disappears. Random loss of any one of the three labels from an [^{16}O,^{17}O,^{18}O]dAMP molecule during cyclization gives rise to only one species that does not contain ^{17}O and which is therefore not quenched in the ^{31}P NMR spectrum. This species is that derived from the loss of ^{17}O during the cyclization process. Inspection of the ^{31}P NMR intensity patterns of the signals due to the ^{16}O,^{18}O isotopomers of the methyl esters of N^1-methyl-[^{16}O,^{17}O,^{18}O]dcAMP permits the localization of the residual ^{18}O atom to either a bridging or doubly bonded position in the species that has lost ^{17}O on cyclization. Only this resonance will have no quenched component. Since the cyclization reaction inverts the configuration[17] of [^{16}O,^{17}O,^{18}O]dAMP, its original configuration can be deduced. Thus the isotopic configuration of [^{18}O]d(TpA) (**11a**) can also be deduced since the cleavage by nuclease P1 also proceeds by inversion of configuration.[7]

Examination of the intensity pattern of the ^{31}P NMR signals in Figure 4a and Table III shows that the highest peak in the equatorial series of triesters is derived from the isotopomer possessing ^{18}O in a single bond and in the axial series (Figure 5b) from the isotopomer possessing ^{18}O in a double bond. The intensity patterns (Table III) correlate well with those predicted and provide an unambiguous stereochemical answer. Exact conformity of peak heights should not be expected since the isotopomer signals in the final spectra (Figures 4a,b) are not completely resolved. The heights of the peaks of the ^{16}O isotopomers methyl esters are also slightly larger than expected. This may represent a small further contamination by ^{16}O during the analytical procedure or an error incurred during calculation the isotopic content of [^{18}O]d(T$_p$A) (**11a**) from the ^{31}P NMR spectrum. These data permit the precursor [^{16}O,^{17}O,^{18}O]dAMP (**24**) to be designated to the S_P con-

(16) Gao, X.; Gaffney, B. L.; Senior, M.; Riddle, R. R.; Jones, R. A. *Nucleic Acids Res.* **1985**, *13*, 573–584.

(17) Jarvest, R. L.; Lowe, G.; Potter, B. V. L. *J. Chem. Soc., Perkin Trans 1* **1981**, 3186–3195.

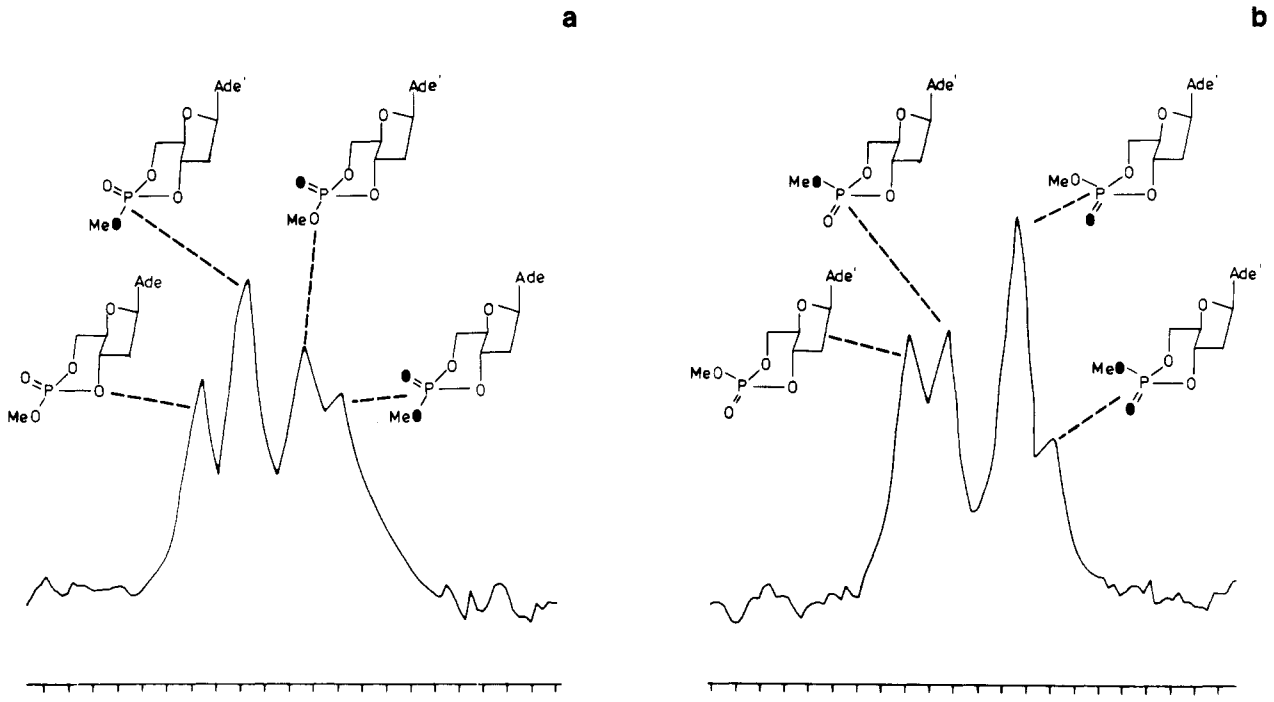


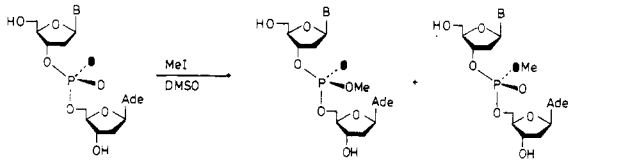
Figure 4. ^{31}P NMR spectrum of the isotopomers of N^1-methyl-dcAMP derived from [^{16}O,^{17}O,^{18}O]dAMP via nuclease P1 digestion of (R_P)-[^{18}O]d(T_pA). The spectrum on the left shows the isotopomers of the equatorial diastereomer (δ −2.88, −2.90, −2.93, −2.94) [a]; the spectrum on the right shows the isotopomers of the axial diastereomer (δ −4.35, −4.37, −4.39, −4.41) [b]. Spectra were obtained by using a 20 mM solution in Me_2SO-d_6 containing 8-hydroxyquinoline. NMR parameters were as follows: sweep width, 434 Hz; pulse width, 7 μs; aquisition time, 2.36 s; data collection in 2K, Fourier transform in 16K; number of transients, 2900; line broadening 0.5 Hz; scale, 1 Hz = 1 division. Ade′ = N^1-methyl-2′-deoxyadenosine.[9]

figurated[19] at phosphorus. Since this molecule was formed by an inversion of configuration at phosphorus, the original [^{18}O]d(TpA) (**11a**) must have the R_P configuration. Thus, an S_P configuration can be assigned to the fast migrating product of the detritylation reaction (**9a**) and an R_P configuration to the slow migrating zone (**9b**).[19] A configuration analysis of chiral [^{18}O]d(CpA) was also made, establishing an R_p configuration for the ^{18}O-labeled dimer **19a**. Correspondingly, the content of the slow migrating zone **17a** has the S_p configuration. So in fact the fast migrating zones **9a** and **17a** both possess the same configuration at phosphorus as was expected from their NMR (^1H, ^{13}C, ^{31}P) spectra (vide supra).

It has been discussed earlier[8,9] that simple methylation and NMR analysis of unlabeled and stereochemically pure oxygen labeled ribo- or deoxydinucleoside monophosphates can be used as a guide in the determination of the absolute configuration at phosphorus of such diesters. If this route is followed the more rigorous and difficult stereochemical analysis as presented above, requiring perhaps 10–20 times more material, became unneccessary.

In order to study the methylation method the dimers d(TpA) and (R_P)-[^{18}O]d(TpA) as well as d(CpA) and (R_p)-[^{18}O]d(CpA) were used. Treatment of the dimers with methyl iodide in the presence of the phase-transfer catalyst 18-crown-6 in Me_2SO according to a method of Potter et al.[7] yielded the corresponding methyl phosphates (Scheme III). Because methylation of the nucleobases could not be excluded[20] an excess of the methylation agent was used to get a homogenous product. Complete methylation

Scheme III



would not effect NMR analysis. Figures 5a,b show the results of the ^{31}P NMR tube experiments for d(TpA) and its ^{18}O derivative taken in Me_2SO-d_6. Figure 5a shows two singlets from the unlabeled diastereomeric d(TpA) methyl esters **25a/b** with a difference of 7.0 Hz in their chemical shifts. A mixture of the unlabeled methyl esters **25a/b** and their ^{18}O derivatives **26a/b**, at a ratio of 1:1, gives rise to the ^{31}P NMR spectrum in Figure 5b in which four resonances can be observed. The fact that an ^{18}O-phosphorus double bond generates a high field shift that is greater than that caused by an ^{18}O in a bridging position allows interpretation of the spectrum in Figure 5b. The first and the third signal were identified as resonances of the unlabeled diastereomers due to their differences in their chemical shifts of about 7.0 Hz. Consequently, the second and the fourth resonances are assigned to the ^{18}O-labeled diastereomers. Next we had to establish whether ^{18}O was in a bridging position or double bonded to the phosphorus. From the stronger shielding (4.1 Hz) indicated by the second signal we deduced that ^{18}O is located in a double bond. As a result of the small difference (1.3 Hz) between the third and the fourth signal, the latter has to be assigned to a [^{18}O]d(TpA) methyl ester with ^{18}O in a bridging position.

Depending on the configuration of the starting material, (R_p)-[^{18}O]d(TpA) (**11a**), and the conditions used in the

(18) Jarvest, R. L.; Lowe, G.; Potter, B. V. L. *J. Chem. Soc., Chem. Commun.* **1980**, 1142–1145.
(19) Cahn, R. S.; Ingold, S. C.; Prelog, V. *Angew. Chem.* **1966**, *78*, 413–447.
(20) Kochetkov, N. K.; Budovskii, E. J.; Sverdlov, E. D.; Simukova, N. A. "Organic Chemistry of Nucleic Acids", Part B; Kochetkov, N. K., Budovskii, E. J., Eds.; Plenum Press: London, New York, 1972.

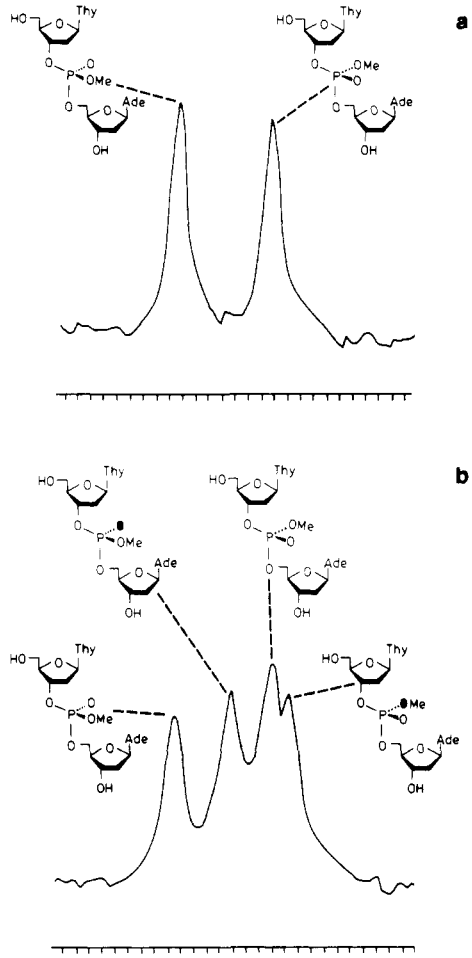


Figure 5. ^{31}P NMR spectrum of (R_P,S_P)d(T_pA) methyl ester (**25a/b**) and (R_P,S_P)-[^{18}O]d(T_pA) methyl ester (**26a/b**). The upper spectrum (a) is of d(T_pA) methyl esters (**25a/b**) [δ 0.08 (S_P) and 0.01 (R_P)]. Assignments were made after interpretation of spectrum 6 (b). The lower spectrum (b) represents the mixtures of **25a/b** and **26a/b**. Isotope shifts are as follows: for downfield resonances, 4.1 Hz; for upfield resonances, 1.3 Hz. Spectra were recorded using a 10 mM solution in Me_2SO-d_6 containing 8-hydroxyquinoline; NMR parameters are as follows: sweep width, 421 Hz; pulse width, 7 μs; aquisition time, 2.62 s; data collection in 2K; Fourier transform in 16K; line broadening 0.3 Hz; number of transients 310 (a) and 581 (b); scale, 1 Hz = 1 division. Thy = thymine; Ade = adenine.

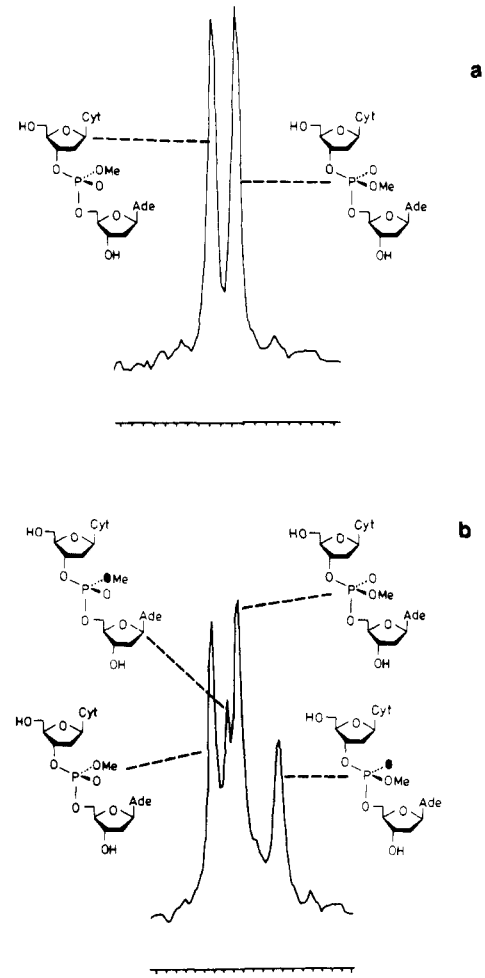


Figure 6. ^{31}P NMR spectrum of (R_P,S_P)d(C_pA) methyl esters (**27a/b**) and (R_P,S_P)-[^{18}O]d(C_pA) methyl esters (**28a/b**). The upper spectrum (a) is of d(C_pA) methyl esters (**27a/b**) [δ −0.06 (R_P) and −0.09 (S_P)]. The lower spectrum (b) represents the mixtures (2:1) of **27a/b** and **28a/b**. Assignments were made after interpretation of spectrum 7 (b). Isotope shifts are as follows: for downfield resonances, 1.6 Hz; for upfield resonances, 4.0 Hz. Spectra were recorded using a 10 mM solution in Me_2SO-d_6–MeOH (50:50, v/v) containing 8-hydroxyquinoline. NMR parameters were as follows: sweep width, 350 Hz; pulse width, 7 μs; aquisition time, 3.10 s; data collection in 2K; Fourier transform in 16K; line broadening, 0.2 Hz; scale, 1 Hz = 1 division. Cyt = cytosine; Ade = adenine.

methylation experiments leading to retention at phosphorus (see Experimental Section), the configuration of **11a** and its methylated derivative with ^{18}O in a bridging position have to be the same. Since the sequence rules of Cahn, Ingold, and Prelog[19] gives the substituents priority over the isotopes the methyl ester with the double-bonded ^{18}O at phosphorus has an S_P configuration.

The ^{31}P NMR spectrum of unlabeled d(CpA) methyl esters **27a/b** taken in Me_2SO-d_6 showed only a broad singlet. On the other hand in a mixture of Me_2SO-d_6/MeOH (1:1) the resonances were resolved (Figure 6a). This behavior has already been described for UpA methyl esters.[8] An approximately 2:1 mixture of the unlabeled and the labeled methyl esters of d(CpA) (**27a/b**, **28a/b**) gave two sets of resonances (Figure 6b), which were assigned to the diastereomeric methyl esters by the method mentioned above.

From previous results with methylated d(TpT),[7] UpA,[8] and d(GpA),[9] each of which has been independently assigned stereochemically, we assumed that the ^{31}P NMR signal from a methyl ester in the R_P configuration resonates upfield from the S_P diastereomer.[9] This trend is supported by the results obtained here on d(TpA) methyl esters (Figure 5b). However, that the assumption cannot be taken as a general rule is shown by the methylation experiments on d(CpA) (**23**) and (R_p)-[^{18}O]d(CpA) (**19a**) where a Me_2SO-d_6/methanol mixture instead of pure Me_2SO-d_6 had to be used for the resolution of the signals. From Figure 6b it clearly can be seen that the pair of ^{31}P NMR signals with the small isotopic shift, indicating ^{18}O in a bridging position, is now located downfield from the methyl esters with the R_p configuration and therefore opposite to the spectra mentioned above. This observation demonstrates that the earlier assumption that methyl esters of dinucleoside monophosphates in the R_P configuration resonate upfield from S_P diastereomers cannot be used as a method of assignment.

The failure of this method for d(CpA) methyl esters could be caused by the fact that the ^{31}P NMR spectra must be taken in a Me_2SO-d_6/MeOH mixture to obtain a resolution of signals. As demonstrated earlier for d(TpT) methyl esters,[7] it is now shown for d(T_pA) methyl esters that addition of methanol to a Me_2SO-d_6 solution of this

compounds reduces the difference between ^{31}P NMR shifts of the stereoisomers from 7 to 4 Hz. The relative location of the ^{31}P NMR signals did not change, which has been concluded from the ^{31}P NMR spectrum of a mixture (1:1) of **25a/b** and **26a/b**. The strong influence of the solvent used for these NMR experiments has also been shown on fully protected d(GpA) methyl esters. In this case a change from $CDCl_3$ to Me_2SO-d_6 leads to an interchange of ^{31}P NMR signals.[9] Nevertheless, methylation of deprotected ^{18}O chirally labeled dinucleoside monophosphates is a good measure of the stereochemical purity of ^{18}O-labeled compounds; nonstereospecifically labeled material would give rise to two additional signals.[7]

An important feature of the methylation procedure employed on the sterochemically assigned labeled and unlabeled compounds **11a/15** and **19a/23**, respectively, is the use of their ^{31}P NMR patterns in determining the configurations at phosphorus for assignment of chiral [^{18}O]-d(TpA) and [^{18}O]d(CpA) of unknown configuration. If such partially labeled materials are isolated from oligomers by enzymatic processing and then methylated at phosphate, comparison of their ^{31}P NMR patterns with those of Figures 5b and 6b allow a direct assignment of configuration at phosphorus. This is possible because the relative location of the pairs of ^{31}P NMR signals (^{16}O and ^{18}O) is different in the R_P and S_P series.

In summary, we have described the syntheses of deoxydinucleoside monophosphates d(TpA) (**11a**) and d(CpA) (**19a**), which are ^{18}O chirally labeled at phosphorus, and we have determined their absolute configuration. The incorporation of ^{18}O into the dimers can be achieved simply by oxidation of phosphite triesters in the presence of ^{18}O-labeled water. Stereochemically pure ^{18}O-labeled dimers were obtained from detritylated diastereomeric phosphate triesters by preparative column chromatography. The deprotected sterochemically pure ^{18}O-labeled dimers **11a** and **19a** will be useful in the elucidation of the stereochemical mechanism of phosphodiester cleavage. Incorporation of stereochemically pure **11a** and **19a** as appropriately protected O-3′ phosphoramidite dimers[21] into oligonucleotides is under investigation.

Experimental Section

Elemental analyses were performed by Mikroanalytisches Labor Beller (Göttingen, FRG). UV spectra were measured on an Uvikon 810 spectrophotometer (Kontron, Switzerland). NMR spectra were recorded on a Bruker WM 250 spectrometer. Chemical shifts are in ppm relative to Me_4Si for ^{1}H and for ^{13}C nuclei, relative to 85% phosphoric acid for the ^{31}P nucleus. Chemical shifts are positive when downfield to the appropriate standard. ^{13}C and ^{31}P NMR spectra were recorded with ^{1}H broad-band decoupling. ^{31}P NMR spectra of the fully protected and partially deprotected dinucleoside monophosphates were measured in $CDCl_3$. Samples of the fully deprotected nucleotides are recorded in 100 mM EDTA solution adjusted to pH 9.0 with triethylamine containing 20% D_2O. The methyl esters of cdAMP and of the methylated dinucleoside monophosphates are taken in Me_2SO-d_6 and Me_2SO-d_6–MeOH (1:1), respectively, containing 8-hydroxyquinoline (0.03 M).

TLC was performed on silica gel SIL G-25 UV_{254} plates (Macherey & Nagel, FRG) with solvents as indicated. Column and flash chromatography (0.5–1.0 bar) was carried out on silica gel 60 H (Merck, FRG); DEAE-Sephadex A-25 (Pharmacia, Sweden) was used as an anion exchanger. The columns were connected to a UV detector (254 nm) and a fraction collector. HPLC was performed on prepacked columns (Merck LiChrosorb RP-18, 4 × 250 (10 μm) using a LKB HPLC system with two pumps (Model 2150), a variable wavelength monitor (Model 2152), and a controller (Model 2151) connected to an integrator (Hewlett Packard 3390^A).

Pyridine, lutidine, and diisopropylamine were distilled from toluene-4-sulfonyl chloride, redistilled from CaH_2 and stored over 4-Å molecular sieves. Dichloromethane was distilled from P_2O_5 and tetrahydrofuran was distilled from KOH and then redistilled from Na–K alloy (5:1)/benzophenone. Dimethylformamide was dried with barium oxide and redistilled under reduced pressure.

N^6,3′-*O*-Dibenzoyl-2′-deoxyadenosine (1) was prepared as described.[22] The nucleoside 2′-deoxyadenosine, 2′-deoxythymidine, and 2′-deoxycytidine were purchased by Pharma Waldhof (Düsseldorf, FRG), and trimethylsilyl chloride and dimethoxytrityl chloride were purchased from Fluka AG (Switzerland). Chloro(diisopropylamino)methoxyphosphine was prepared as described[12] and was stored at −18 °C. Nuclease P1 (EC 3.1.30.1) from *Penicillium citrum* was obtained from Sigma (Munich) as lyophilized powder. d(TpA), d(CpA), snake venom phosphodiesterase (EC 3.1.16.1, Crotallus durissus), and alkaline phosphatase (EC 3.1.3.1, *E. coli*) were obtained from Boehringer (Mannheim, FRG). ^{18}O-enriched water (^{18}O, 90%) was purchased from Ventron Ltd. (Karlsruhe, FRG) and ^{17}O-enriched water (^{16}O, 13.8%; ^{17}O, 50.7%; ^{18}O, 35.7%) from the Monsanto Research Corp. (Miamisburg, OH, USA).

(R_P,S_P)-5′-*O*-(Dimethoxytrityl)-3′-*O*-[(diisopropylamino)methoxyphosphino]-2′-deoxythymidine (3a/b). 2′-Deoxythymidine was tritylated and then converted into the phosphoramidite **3a/b** with chloro(diisopropylamino)methoxyphosphine.[12] After flash chromatography[23] on a silica gel column (10 × 5 cm) with ethyl acetate/dichloromethane/triethylamine (45:45:10 v/v/v), compound **3a/b** was isolated as a colorless foam in 74% yield; ^{13}C NMR data see Table I.

(R_P,S_P)-N^4-Benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-[(diisopropylamino)methoxyphosphino]-2′-deoxycytidine (5a/b). 2′-Deoxycytidine was benzoylated and tritylated as described.[24] The resulting N^6-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidine (4) was converted into the phosphoramidite **5a/b** under conditions as described for compound **3a/b**. After flash chromatography on a silica gel column (10 × 5 cm) with ethyl acetate/dichloromethane/triethylamine (45:45:10 v/v/v) as eluent, compound **5a/b** was obtained in 81% yield as a colorless foam; ^{13}C NMR data see Table I.

(R_P,S_P)-5′-*O*-(Dimethoxytrityl)-2′-deoxythymidylyl(3′→5′)-N^6,3′-*O*-dibenzoyl-2′-deoxyadenosine Methyl Ester (12a/b). To a mixture of compound 1 (500 mg, 1.09 mmol) and **3a/b** (1.42 g, 2.01 mmol), both thoroughly dried over KOH (desiccator), was added freshly sublimed tetrazole (500 mg, 7.10 mmol). The solid materials were placed in a round-bottomed flask equipped with a septum under argon atmosphere. Dry THF (8 mL) was added by a syringe yielding a clear solution. This was stirred for 5 h at 20 °C. Oxidation of the resulting phosphite **6a/b** was accomplished by addition of iodine (522 mg, 2.01 mmol) in THF/2,6-lutidine/H_2O (4:3:1 v/v/v). The solution was stirred for another 30 min and then diluted with 100 mL of ethyl acetate. The organic layer was separated and washed with 1% aqueous sodium bisulfite (20 mL), dried with Na_2SO_4, and evaporated to dryness. The residue was dissolved in dichloromethane and applied to a silica gel column (7 × 5 cm). Flash chromatography with dichloromethane/acetone (55:45 v/v) yielded the diastereomers **12a/b** as colorless foam, 950 mg (81%): TLC (silica gel, CH_2Cl_2/acetone 55:45 v/v) R_f 0.62; UV (CH_2Cl_2) λ_{max} 274 (ε 28 800); ^{31}P NMR ($CDCl_3$) δ 0.04 (s), −0.08 (s) (1:1). Anal. Calcd for $C_{56}H_{54}N_7O_{14}P$: C, 62.27; H, 5.04; N, 9.08. Found: C, 62.05; H, 5.04; N, 9.14.

(R_P,S_P)-N^4-Benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxycytidylyl(3′→5′)-N^6,3′-*O*-dibenzoyl-2′-deoxyadenosine Methyl Ester (20a/b). The diastereomeric mixture of **20a/b** was synthesized as described for **12a/b**. Starting with compound 1 (500 mg, 1.09 mmol) and **5a/b** (1.60 g, 2.01 mmol) the pure material was isolated after flash chromatography as colorless foam, 1050 mg (82%): TLC (silica gel, CH_2Cl_2/acetone 55:45 v/v) R_f 0.54;

(21) Kumar, G.; Poonian, M. S. *J. Org. Chem.* **1984**, *49*, 4905–4912.

(22) Ogilvie, K. K. *Can. J. Chem.* **1973**, *51*, 3799–3807.

(23) Dörper, T.; Winnacker, E. L. *Nucleic Acids Res.* **1983**, *11*, 2575–2584.

(24) Ti, G. S.; Gaffney, B. L.; Jones, R. A. *J. Am. Chem. Soc.* **1982**, *104*, 1316–1319.

UV (CH_2Cl_2) λ_{max} 259 (ϵ 32 500); ^{31}P NMR ($CDCl_3$) δ −0.03 (s), −0.12 (s) (1.1:1). Anal. Calcd for $C_{62}H_{57}N_8O_{14}P$: C, 63.69; H, 4.91; N, 9.58. Found: C, 63.56; 5.18; N, 9.40.

Detritylation of 12a/b and Separation of the Diastereomers 13a and 13b. To the diastereomeric mixture of **12a/b** (900 mg, 0.833 mmol) was added a saturated solution of $ZnBr_2$ in nitromethane/methanol (95:5 v/v) (10 mL). After being stirred for 45 min at 20 °C, the reaction was stopped by addition of 5% aqueous ammonium acetate solution (50 mL). The detritylated diastereomers **13a/b** were extracted with chloroform (2 × 80 mL). The pooled organic layers were dried over Na_2SO_4 and filtrated and a reduced volume was then applied to a silica gel column (25 × 4 cm). Flash chromatography with dichloromethane/2-propanol (91:9 v/v) separated the reaction product into two zones.

(S_P)-2′-Deoxythymidylyl(3′→5′)-N^6,3′-O-dibenzoyl-2′-deoxyadenosine Methyl Ester (13a); "Fast" Migrating Zone. The fast migrating zone **13a** (260 mg, 40%) was obtained as a colorless solid: TLC (silica gel, CH_2Cl_2/2-propanol, 91:9 v:v) R_f 0.30; UV (MeOH) λ_{max} 274 (ϵ 26 500); ^1H NMR, see Table II; ^{13}C NMR, see Table I; ^{31}P NMR ($CDCl_3$) δ −0.15 (s). Anal. Calcd. for $C_{35}H_{36}N_7O_{12}P$: C, 54.06; H, 4.67; N, 12.61. Found: C, 54.22; H, 4.68; N, 12.51.

(R_P)-2′-Deoxythymidylyl(3′→5′)N^6,3′-O-dibenzoyl-2′-deoxyadenosine Methyl Ester (13b); "Slow" Migrating Zone. The slow migrating zone yielded **13b** (243 mg, 38 %) as a colorless amorphous material: TLC (silica gel, CH_2Cl_2/2-propanol, 91:9 v:v) R_f 0.24; UV (MeOH) λ_{max} 274 (ϵ 26 900); ^1H NMR, see Table II; ^{13}C NMR, see Table I; ^{31}P NMR ($CDCl_3$) δ 0.11 (s). Anal. Calcd for $C_{35}H_{36}N_7O_{12}P$: C, 54.06; H, 4.67; N, 12.61. Found: C, 54.16; H, 4.84; N, 12.56.

Detritylation of the Diastereomeric Mixture 20a/b and Separation of the Diastereomers 21a and 21b. The diastereomeric mixture **20a/b** (1.00 g, 0.855 mmol) was detritylated as described before. Flash chromatography on silica gel (column, 25 × 4 cm) with dichloromethane/2-propanol (92:8 v/v) separated the product into two zones.

(S_P)-N^4-Benzoyl-2′-deoxycytidylyl(3′→5′)-N^6,3′-O-dibenzoyl-2′-deoxyadenosine Methyl Ester (21a); "Fast" Migrating Zone. The fast migrating zone **21a** (320 mg, 43%) was obtained as a colorless solid: TLC (silica gel, CH_2Cl_2/2-propanol 92:8 v/v) R_f 0.30; UV (MeOH) λ_{max} 260 (ϵ 33 800); ^1H NMR, see Table II; ^{13}C NMR, see Table I; ^{31}P NMR ($CDCl_3$) δ −0.14 (s). Anal. Calcd for $C_{41}H_{39}N_8O_{12}P$: C, 56.81; H, 4.54; N, 12.93. Found: C, 56.90; H, 4.73; N, 12.80.

(R_P)-N^4-Benzoyl-2′-deoxycytidylyl(3′→5′)-N^6,3′-O-dibenzoyl-2′-deoxyadenosine Methyl Ester (21b); "Slow" Migrating Zone. The slow migrating zone yielded **21b** (280 mg, 38%) as a colorless solid: TLC (silica gel, CH_2Cl_2/2-propanol, 92:8 v/v) R_f 0.25; UV (MeOH) λ_{max} (ϵ 32 300); ^1H NMR, see Table II; ^{13}C NMR, see Table I; ^{31}P NMR ($CDCl_3$) δ 0.26 (s). Anal. Calcd for $C_{41}H_{39}N_8O_{12}P$: C, 56.81; H, 4.54; N, 12.93. Found: C, 56.81; 4.17; N, 12.78.

2′-Deoxythymidylyl(3′→5′)-2′-deoxyadenosine Triethylammonium Salt (15). (R_P,S_P)-2′-Deoxythymidylyl(3′→5′)-N^6,3′-O-dibenzoyl-2′-deoxyadenosine methyl ester (**13a/b**) (250 mg, 0.322 mmol) was suspended in a mixture (8 mL) of dioxane/triethylamine/thiophenol (2:1:1, v/v/v). The solution was stirred for 2 h at room temperature. Deprotection was monitored on TLC (silica gel, $CHCl_3$/MeOH, 9:1 v/v)). The reaction mixture was concentrated to a small volume and applied to a silica gel column (7 × 5 cm). Dichloromethane eluted the excess of thiophenol and CH_2Cl_2/MeOH/triethylamine (85:10:5 v/v/v), the demethylation product **14**. Compound **14** was isolated as colorless amorphous material (228 mg, 0.264 mmol) in 82% yield as the triethylammonium salt. To afford deblocking of the benzoyl groups, the product was dissolved in 20 mL of a mixture of 25% aqueous ammonia–dioxane (1:1) and stored for 2 days at 37 °C. The solution was evaporated to dryness and the residue was dissolved in H_2O (10 mL). The resultant was applied to a Sephadex A-25 column (15 × 3 cm) and chromatographed with a linear gradient of triethylammonium bicarbonate (20–200 mM). The slow migrating main zone, containing compound **15**, was pooled and evaporated to dryness. To remove excess of triethylammonium bicarbonate the residue was coevaporated 5 times with 50 mL of water to dryness. Compound **15** was isolated as glassy triethylammonium salt in 78% yield (135 mg, 0.206 mmol). This material was pure (99%) according to HPLC (retention time 5.6 min at a flow rate of 1.0 mL/min) and coeluted with an authentic sample of d(T_pA). Digestion by snake venom phosphodiesterase followed by alkaline phosphatase as described[25] yielded 2′-deoxythymidine and 2′-deoxyadenosine in an equimolar ratio: ^{31}P NMR (D_2O, EDTA, pH 9) δ 0.28 (s).

2′-Deoxycytidylyl(3′→5′)-2′-deoxyadenosine Triethylammonium Salt (23). As described for compound **15**, dinucleoside monophosphate **23** was prepared from (R_P,S_P)-N^4-benzoyl-2′-deoxycytidylyl(3′→5′)-N^6,3′-O-dibenzoyl-2′-deoxyadenosine methyl ester (**21a/b**) (300 mg, 0.273 mmol). The demethylation product **22** was obtained in 79% yield (260 mg, 0.273 mmol). Deblocking of the benzoyl groups with ammonia–dioxane and workup as described before yielded d(C_pA) as a glassy triethylammonium salt in 81% yield (142 mg, 0.221 mmol). This material was pure (98%) according to HPLC pattern of Figure 3 (retention time 4.6 min at a flow rate of 1.0 mL/min). Compound **23** coeluted with an authenic sample of d(C_pA). Digestion by snake venom phosphodiesterase followed by alkaline phosphatase[25] yielded 2′-deoxycytidine and 2′-deoxyadenosine in an equimolar ratio: ^{31}P NMR (D_2O, EDTA, pH 9) δ 0.23 (s).

(R_P)-[^{18}O]-2′-Deoxythymidylyl(3′→5′)-2′-deoxyadenosine Triethylammonium Salt (11a). (R_P)-[^{18}O]d(T_pA) (**11a**) was synthesized following the same sequence of reaction steps as used for the unlabeled compound **15** starting with the coupling reaction by the same amounts of compound **1** and **3a/b** but replacing unlabeled water by [^{18}O]H_2O (90%) in the oxidation step. The ^{31}P NMR spectrum of the diastereomeric mixture **8a/b** (Figure 1a) showed that ^{18}O labeling was 85%. Upfield shift of **8a/b** to **12a/b** was 3.5 Hz. After detritylation and separation of the detritylated diastereomers **9a/b**, compound **9a** (234 mg, 0.301 mmol; >95% pure according to TLC and ^{31}P NMR) was deprotected with thiophenol and ammonia as described before. The labeled compound **11a** (130 mg, 0.198 mmol) was pure (99%) according to the HPLC pattern. Digestion by snake venom phosphodiesterase followed by alkaline phosphatase yielded 2′-deoxythymidine and 2′-deoxyadenosine in an equimolar ratio. The ^{18}O labeling according to ^{31}P NMR was 85%. Upfield shift of **11a** to **15** = 2.7 Hz.

(R_P)-[^{18}O]-2′-Deoxycytidylyl(3′→5′)-2′-deoxyadenosine Triethylammonium Salt (19a). Compound **19a** was synthesized following the same sequence of reaction steps as used for the unlabeled compound **23** starting with the coupling reaction by using the same amounts of compounds **1** and **5a/b** but replacing unlabeled water by [^{18}O]H_2O (90%) in the oxidation step. The ^{31}P NMR spectrum of the diastereomeric mixture **16a/b** (Figure 1b) showed that ^{18}O labeling was 85%. Upfield shift of **16a/b** to **20a/b** = 3.9 Hz. After detritylation and separation of the detritylated diastereomers **17a/b**, the fast migrating zone **17a** (280 mg, 0.323 mmol, >95% pure according to TLC and ^{31}P NMR) was deprotected with thiophenol and then with ammonia as before described. The labeled compound **19a** (140 mg, 0.218 mmol) was pure (99%) according to the HPLC pattern. Digestion by snake venom phosphodiesterase followed by alkaline phosphatase yielded 2′-deoxycytidine and 2′-deoxyadenosine in an equimolar ratio. The ^{31}P NMR spectrum showed that ^{18}O labeling was 85%. Upfield shift of **19a** to **23** = 2.7 Hz.

Configurational Analysis of (R_P)-[^{18}O]-2′-Deoxythymidylyl(3′→5′)-2′-deoxyadenosine Triethylammonium Salt (11a). (a) Hydrolysis of (R_P)-[^{18}O]d(TpA) by Nuclease P1 in [^{17}O]H_2O. Hydrolysis of [^{18}O]d(T_pA) (**11a**) (60 μmol) by nuclease P1 in ^{17}O-enriched water (^{16}O, 13.8; ^{17}O, 50.7%, ^{18}O, 35.5%) and isolation of [^{16}O,^{17}O,^{18}O]dAMP was carried out as described by Potter et al.[7] The enzymatic digestion was carried out in 300 μL of ^{17}O-enriched water with 300 μg of enzyme and was complete after incubation at 37 °C for 5 h. After chromatography on DEAE Sephadex A-25, a colorless glass of [^{16}O,^{17}O,^{18}O]dAMP (**24**) was obtained (85%).

(b) Configurational Analysis of (S_P)-[^{16}O,^{17}O,^{18}O]dAMP (24). [^{16}O,^{17}O,^{18}O]dAMP triethylammonium salt (51.5 μmol) was converted to the tri-*n*-butylammonium salt via the pyridinium salt and cyclized with diphenylphosphorochloridate and *tert*-

(25) Seela, F.; Driller, H. *Nucleic Acids Res.* **1985**, *13*, 911–925.

butoxide to the isotopomers of [^{16}O,^{17}O,^{18}O]dcAMP by the method of Jarvest et al.[17] Purification by DEAE Sephadex A-25 chromatogrpahy as described gave [^{16}O,^{17}O,^{18}O]dcAMP (10.1 μmol, 20%). After evaporation of the solvent in vacuo and conversion to the potassium-18-crown-6 salt, the product was methylated by using methyl iodide in Me_2SO-d_6 as described. The ^{31}P NMR spectrum and the data thereof are presented in Figure 4.

(R_P,S_P)-2′-Deoxythymidylyl(3′→5′)-2′-deoxyadenosine Methyl Ester (25a/b). Compound **15** (10 μmol) was methylated as described[8] for U_pA to give the methyl esters **25a/b** as a solution in Me_2SO-d_6: ^{31}P NMR (Me_2SO-d_6) δ 0.08 (s) for the S_P diastereomer and 0.01 (s) for the R_P diastereomer; ^{31}P NMR (Me_2SO-d_6/MeOH, 1:1) δ −0.06 (s) for the S_P diastereomer and −0.10 (s) for the R_P diastereomer.

(R_P,S_P)-[^{18}O]-2′-Deoxythymidylyl(3′→5′)-2′-deoxyadenosine Methyl Ester (26a/b). (R_P)-[^{18}O]-2′-Deoxythymidylyl(3′→5′)-2′-deoxyadenosine (**11a**) was methylated as described for the unlabeled dimer **15** to give the methyl esters **26a/b**: ^{31}P NMR (Me_2SO-d_6) δ 0.04 (s) for the S_P diastereomer and 0.00 (s) for the R_P diastereomer. After mixing with an approximately equal amount of unlabeled material the following isotope shifts were recorded: P—[^{18}O]Me, 1.3 Hz; P=[^{18}O], 4.1 Hz; ^{31}P NMR (Me_2SO-d_6/MeOH, 1:1) δ −0.10 (s) for the S_P diastereomer and −0.11 (s) for the R_P diastereomer.

(R_P,S_P)-2′-Deoxycytidylyl(3′→5′)-2-deoxyadenosine Methyl Ester (27a/b). 2′-Deoxycytidylyl(3′→5′)-2′-deoxyadenosine (**23**) (10 μmol) was methylated as described[8] for U_pA to give the methyl esters **27a/b** as a solution in Me_2SO-d_6: ^{31}P NMR (Me_2SO-d_6/MeOH, 1:1) δ −0.06 (s) for the R_P diastereomer and −0.09 (s) for the S_P diastereomer.

(R_P,S_P)-[^{18}O]-2′-Deoxycytidylyl(3′→5′)-2′-deoxyadenosine Methyl Ester (28a/b). (R_P)-[^{18}O]-2′-Deoxycytidylyl(3′→5′)-2′-deoxyadenosine (**19a**) was methylated as described for the unlabeled compound **23** to give the methyl esters **28a/b** as a solution in Me_2SO-d_6: ^{31}P NMR (Me_2SO-d_6/MeOH, 1:1) δ −0.08 (s) for the R_P diastereomer and −0.13 for the S_P diastereomer. After mixing with an approximately twofold amount of unlabeled material, the following isotope shifts were recorded: P—[^{18}O]Me, 1.6 Hz; P=[^{18}O], 4.0 Hz.

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General Synthesis of (1→3)-β-D-Galacto Oligosaccharides and Their Methyl β-Glycosides by a Stepwise or a Blockwise Approach

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All intermediates for the chemical synthesis of (1→3)-β-D-galacto oligosaccharides or their methyl β-glycosides are prepared from one readily available substance, namely methyl 2,4,6-tri-*O*-benzoyl-3-*O*-benzyl-β-D-galactopyranoside (**1**). Debenzylation of **1** gives **2**, the initial nucleophile for the synthesis of methyl β-glycosides of (1→3)-β-D-galacto oligosaccharides. Reaction of **1** with 1,1-dichloromethyl methyl ether affords the key glycosyl donor **3** permitting the extension of the oligosaccharide chain through HO-3. 1,1-Dichloromethyl methyl ether is a suitable reagent also for the conversion of derivatives of higher oligosaccharides into the corresponding glycosyl chlorides, and these are sufficiently reactive under the conditions of silver triflate promoted glycosidation reactions. Reaction of the halide **3** with silver acetate, followed by reductive cleavage of the benzyl group from the formed 1-*O*-acetyl-2,4,6-tri-*O*-benzoyl-3-*O*-benzyl-β-D-galactopyranose (**4**), gives **5**, the initial nucleophile for the synthesis of free (1→3)-β-D-galacto oligosaccharides. The sequential or blockwise synthesis of higher title oligosaccharides using the above intermediates is demonstrated by the preparation of various (1→3)-β-D-galacto oligosaccharides and their methyl β-glycosides. The per-*O*-benzoate of the methyl β-glycoside of (1→3)-β-D-galactoheptaose (**26**) was obtained in 62% yield by a condensation of a trisaccharide nucleophile with a glycosyl chloride derived from (1→3)-β-D-galactotetraose. The structure of all mono- and disaccharide intermediates was confirmed by 2D NMR carbon–proton correlation experiments, and that of higher oligosaccharides was verified by comparison of their ^{13}C NMR spectra with those of the lower members of the respective series.

The *O*-β-D-galactopyranosyl-(1→3)-D-galactopyranosyl or 3-*O*-substituted β-D-galactopyranosyl sequence occurs widely in nature.[2-7] 3-*O*-β-D-Galactopyranosyl-D-galactose has been synthesized,[4,8,9] but a systematic synthesis of higher members of this series or of their methyl β-glycosides has not been carried out due to the difficulties involved in the preparation of suitable intermediates. We have reported[10] a synthesis of methyl 3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside, a compound previously obtained[11] only in admixture with its α-, 2-*O*-α-, and 2-*O*-β-isomers. Here we describe efficient syntheses of intermediates needed to prepare virtually *any* of the compounds of the above series by either a stepwise or a blockwise synthesis. The use of the present approach is demonstrated by the syntheses of a series of the title methyl β-glycosides up to and including the heptasaccharide.

(1) (a) NIADDK. (b) University of Missouri.
(2) Rodén, L. "Metabolic Conjugation and Metabolic Hydrolysis"; Fishman, W. H., Ed.; Academic Press: New York, 1970; Vol. 2, pp 345–442.
(3) Bailey, R. W. "Oligosaccharides"; Pergamon Press: Oxford, 1965; p 48.
(4) Benzing-Nguyen, L.; Roden, L. *Carbohydr. Res.* **1977**, *53*, 123–128 and references cited therein.
(5) Marcus, D. M. *Mol. Immunol.* **1984**, *21*, 1083–1091.
(6) Roy, N.; Glaudemans, C. P. J. *Carbohydr. Res.* **1978**, *63*, 318–322.
(7) Collins, J. G.; Bradbury, J. H.; Trifonoff, E.; Messer, C. R. *Carbohydr. Res.* **1981**, *92*, 136–140 and references cited therein.
(8) Ball, D. H.; Jones, J. K. N. *J. Chem. Soc.* **1958**, 905–907.
(9) Chacon-Fuertes, M. E.; Martin-Lomas, M. *Carbohydr. Res.* **1975**, *43*, 51–56.
(10) Kováč, P.; P.; Glaudemans, C. P. J.; Taylor, R. B. *Carbohydr. Res.* **1985**, *142*, 158–164.
(11) Gorin, P. *Carbohydr. Res.* **1982**, *101*, 13–20.