Synthesis of Nucleoside 5'-O-(1,3-Dithiotriphosphates) and 5'-O-(1,1-Dithiotriphosphates)

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The synthesis of 3'-deoxy-3'-azidothymidine 5'-O-(1,3-dithiotriphosphate) (**5**) as the first example of a nucleoside 5'-O-(1,3-dithiotriphosphate) is described. 2-Chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one phosphitylates the 5'-hydroxy group of 3'-deoxy-3'-azidothymidine to form intermediate **1**. Reaction of **1** with thiopyrophosphate (**9**) and sulfur results in the formation of an unseparable mixture of 3'-deoxy-3'-azidothymidine 5'-O-(1,3-dithiotriphosphate) (**5**) and 3'-deoxy-3'-azidothymidine 5'-O-(1,2-dithiotriphosphate) (**3**). Selective hydrolysis of **5** allows isolation of **3**. However, reaction of **1** with P^1-O-(cyanoethyl)-P^1-thiopyrophosphate (**8**) and sulfur produced the diastereomers of **5** in good yield. Compound **8** is prepared by reaction of 3-hydroxyproprionitrile with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one, pyrophosphate, and sulfur to yield **10**, which is ring opened to **8** by reaction with ethylenediamine. Hydrolysis of this compound leads to **9**. An alternative route to nucleoside 5'-O-(1,3-dithiotriphosphates) consists of reacting a nucleoside 5'-O-(1-thiocyclotriphosphate) such as **13** with Li_2S. This reaction, when performed in pyridine/dioxane, leads to a mixture of the nucleoside 5'-O-(1,3-dithiotriphosphate) and the nucleoside 5'-O-(1,1-dithiotriphosphate). The latter is the only nucleotide product when the reaction is carried out in DMF.

Nucleoside phosphorothioate analogues of nucleotides have found wide application in biochemistry and molecular biology.[1,2] The S_p diastereomers of nucleoside 5'-O-(1-thiotriphosphates) are good substrates for RNA and DNA polymerases, thus allowing the incorporation of phosphorothioate groups into RNA and DNA. These triphosphate analogues are very slowly hydrolyzed by enzymes which attack the α-phosphorus such as snake venom phosphodiesterase.[3] Nucleoside 5'-O-(3-thiotriphosphates) are resistant to many enzymes which degrade nucleoside 5'-O-triphosphates by attack at the γ-position.[1] For example guanosine 5'-O-(3-thiotriphosphate) is resistant to many GTPases and has been found useful in establishing the role of various G-proteins (for an example see ref 4). These 3-thiotriphosphates are also substrates for polymerases.[5] We reasoned that nucleotide analogues containing phosphorothioate groups in the 1- as well as the 3-position of a nucleoside triphosphate could be expected to be stable against both types of hydrolytic enzymes and thus of interest for biochemical investigations. We describe here the first synthesis of such a dithiotriphosphate derivative of 3'-azido-3'-deoxythymidine. In addition the preparation of nucleoside 5'-O-(1,1-dithiotriphosphates) is presented.

Results and Discussion

Synthesis of Nucleoside 5'-O-(1,3-Dithiotriphosphates). Methods described so far for the chemical synthesis of nucleoside 5'-O-(3-thiotriphosphates) are multistep procedures which require activation of either S- or O-protected phosphorothioates with diphenyl phosphorochloridate and subsequent reaction with nucleoside 5'-diphosphates.[6,7] When such a method was applied to the synthesis 3'-deoxy-3'-azidothymidine 5'-O-(1,3-dithiotriphosphate) by reacting 3'-deoxy-3'-azidothymidine 5'-O-(1-thiodiphosphate) with a 2-fold excess of P^1,P^1-diphenyl-P^2-S-(cyanoethyl) thiopyrophosphate, the desired compound could only be isolated in 38% yield. Taking into account the low yield in the synthesis of the 3'-deoxy-3'-azidothymidine 5'-O-(1-thiodiphosphate) precursor, this approach is not very efficient.

We have previously shown that there is an alternative to this mixed anhydride method which employs acyl phosphites as educts for an efficient synthesis of thiophosphate–phosphate anhydrides.[8] Thus, a salicyl protected nucleoside monophosphite such as **1** can be converted to P^2,P^3-dioxo-P^1-nucleosidyl cyclotriphosphite by double displacement of the salicyl group by pyrophosphate. Oxidation of this phosphite with sulfur yields the nucleoside 5'-(1-thiocyclotriphosphate), which can be hydrolyzed to the diastereomeric mixture of nucleoside 5'-O-(1-thiotriphosphates). We attempted to adapt this procedure to the synthesis of nucleoside 5'-O-(1,3-dithiotriphosphates) by replacing pyrophosphate by thiopyrophosphate.

When **1** was reacted with thiopyrophosphate (**9**) and sulfur (Scheme I, A), 3'-deoxy-3'-azidothymidine 5'-(1,2-dithiocyclotriphosphate) (**2**) was formed. This compound was characterized by ^{31}P NMR spectroscopy (Figure 1A). In contrast to the spectrum of the nucleoside 5'-(1-thiocyclotriphosphate), **2** has a first-order spectrum because the chemical shifts of the $P\beta$-phosphoryl and -thiophosphoryl phosphorus atoms are separated by 50 ppm. However, the coupling pattern is complex due to the two asymetric centers at $P\alpha$ and $P\beta(S)$. Four diastereomeres can be distinguished by their slightly different chemical shifts and coupling constants. The J values for one diastereomer were determined to be $J_{\alpha,\beta(S)}$ = 46 Hz; $J_{\alpha,\beta(O)}$ = 35 Hz; $J_{\beta(O),\beta(S)}$ = 40 Hz. Hydrolytic ring opening of intermediate **2** leads to a mixture of 3'-deoxy-3'-azidothymidine 5'-O-(1,2-dithiotriphosphate) (**3**) and 3'-deoxy-3'-azidothymidine 5'-O-(1,3-dithiotriphosphate) (**5**), which could not be separated by DEAE-Sephadex ion exchange chromatography. However, the ^{31}P NMR spectrum of the mixture permitted the identification of **3** and **5**, which were present in a 6:1 ratio. The spectrum showed doublets at 43.47 and 34.06 ppm and a quartet at −23.54 ppm, which

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(7) Richard, J. P.; Frey, P. A. *J. Am. Chem. Soc.* **1982**, *104*, 3476-3481.
(8) Ludwig, J.; Eckstein, F. *J. Org. Chem.* **1989**, *54*, 631-635.

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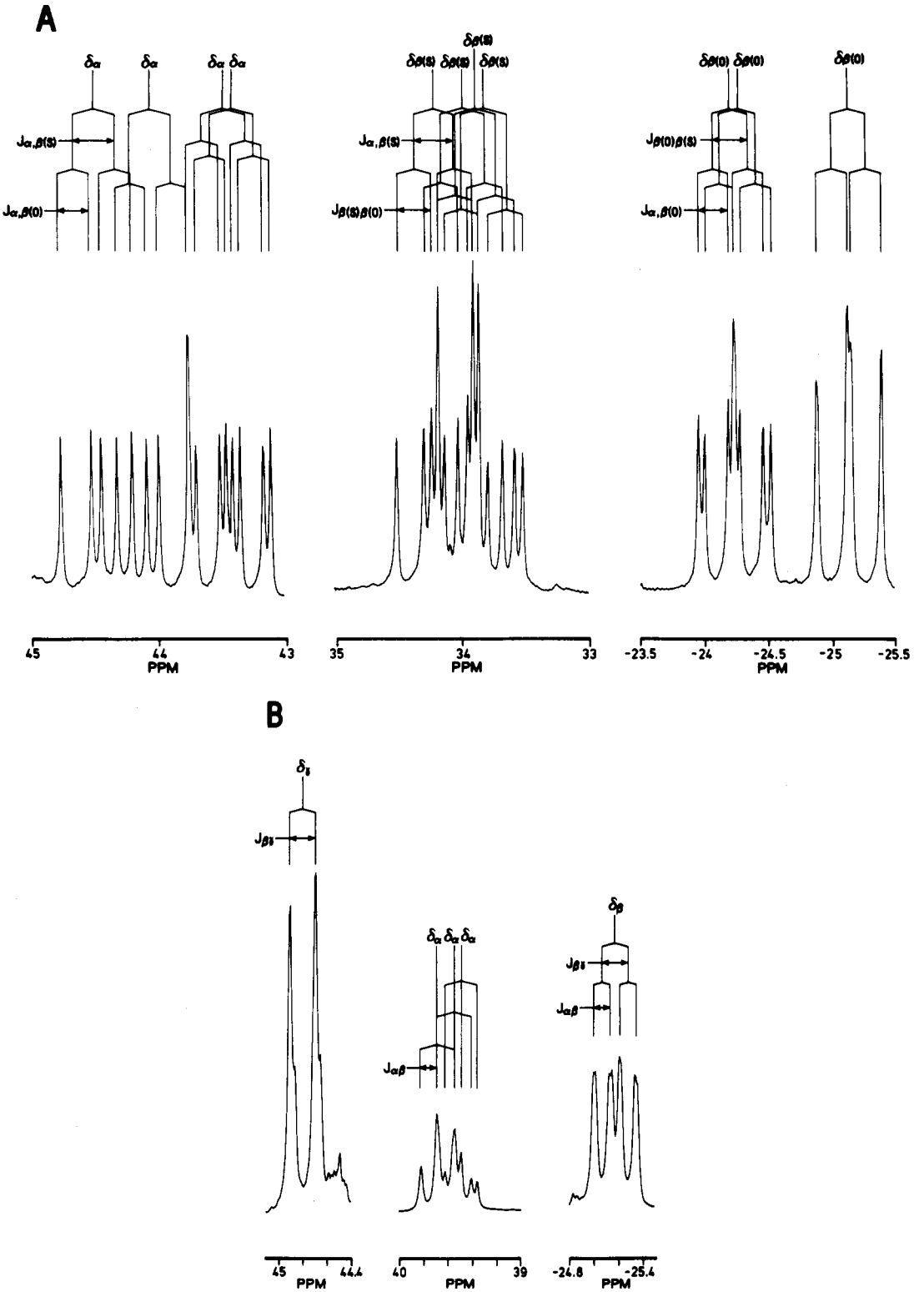

Figure 1. Selected regions of the ^{31}P NMR spectra of compounds **2** (spectrum A) and **4** (spectrum B). Parameters were as follows: offset, 500 Hz; sweep width, 2000 Hz; pulse width 4.5 μs; 32 K transients; acquisition time 0.819 s; line broadening, 3.0 Hz; number of transients, 545 for A and 547 for B.

were assigned to P^1, P^3, and P^2, respectively, of the S_p diastereomer of **5**. Doublets at 43.11, 34.06, and −23.58 ppm were assigned to the corresponding phosphoruses of the R_p diastereomer. Four doublets at 42 ppm, two quartets at 28 ppm, and one doublet at −7 ppm were assigned to P^1, P^2, and P^3, respectively, of the four diastereomers of **3**. Compound **3** is formed by attack of water on the phosphate Pβ(O) and **5** by attack on the phosphorothioate Pβ(S). The distribution of the two compounds in the reaction mixture reflects the different reactivities of the phosphoryl and thiophosphoryl centers in **2**.

The facile formation of thiometaphosphate from terminal phosphorothioate groups[9] was exploited to remove **5** from the product mixture. Thus, upon incubation of the

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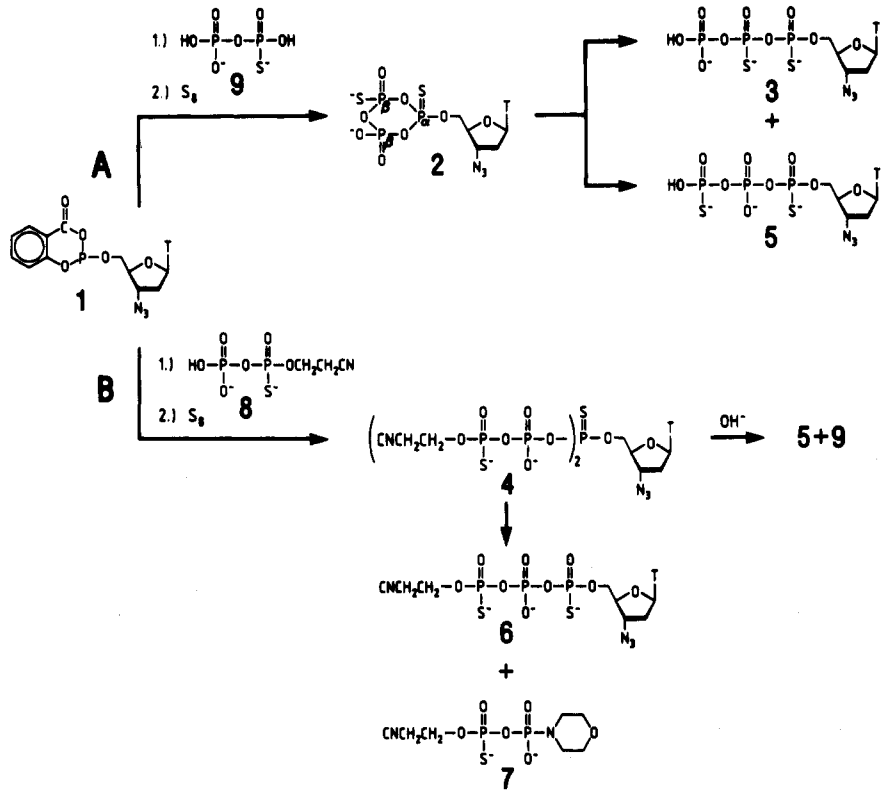
Scheme I



mixture at pH 4 at room temperature, 5 was converted to 3′-deoxy-3′-azidothymidine 5′-O-(1-thiodiphosphate) whereas 3 remained unchanged. This mixture was readily separable by DEAE-Sephadex chromatography.

Dialkyl cyclotriphosphates are known to hydrolyze by attack of water on the phosphorus of the uncharged phosphate with the second triester acting as the leaving group yielding P^1,P^3-dialkyl triphosphates.[10,11] Thus one would expect that hydrolysis of the $P\beta$(S)-2-cyanoethyl derivative of 2 would predominantly form 6. In an attempt to synthesize this derivative of 2, compound 1 was reacted with a 3-fold excess of P^1-O-(cyanoethyl)-P^1-thiopyrophosphate (8) (Scheme I, B). Analysis of the reaction mixture by ^{31}P NMR showed that instead the branched pentaphosphate 4 had been formed. Employing lower than a 2-fold excess of 8 resulted in incomplete conversion of 1. This indicates that the bifunctional derivate 1 does not react with pyrophosphate esters in an intramolecular double replacement reaction to form a stable cyclic product similar to 2, but that a second molecule of 8 reacts with the first reaction intermediate to form a branched compound which is subsequently oxidized with sulfur to 4.

The presence of 4 was verified by ^{31}P NMR spectroscopy of the reaction mixture. The spectrum recorded after the addition of a 3-fold excess of 8 to 1 followed by reaction with sulfur showed the expected 2:2:1 ratio of a doublet at 45 ppm due to Pγ(S) ($J_{\beta\gamma}$ = 30.4 Hz), a quartet at −25 ppm due to Pβ, and four partially overlapping triplets at 40 ppm due to Pα(S) ($J_{\alpha\beta}$ = 19 Hz) (Figure 1B). This spectrum is consistent with the structure of 4. The multiplicity of the Pα signal is a result of the two chiral centres at Pγ. The chemical properties of 4 are also in agreement with the proposed structure. Thus, when 4 was reacted with morpholine, the ^{31}P NMR spectrum of the reaction solution clearly showed the presence of P^1-O-(cyanoethyl)-P^2-morpholino-P^1-thiopyrophosphate (7) and 3′-deoxy-3′-azidothymidine 5′-[3-O-(β-cyanoethyl) 1,3-dithiotriphosphate] (6) in a 1:1 ratio as the sole reaction products. Compounds 6 and 7 were formed as the result of nucleophilic displacement at Pβ(O). This result is consistent with the known properties of trisubstituted pyrophosphates, where the anion of the stronger acid is displaced by the nucleophile. If a cyclic triphosphate derivative had been formed from 1 and 8, the amine would have been incorporated into the nucleotide. Compound 7 showed the expected doublets at 42.92 ppm for thiophosphorylphosphorus and at −3.2 ppm for the phosphoramidate phosphorus. In the absence of ^{1}H decoupling the phosphoramidate signal was split into a symmetrical quintet with J = 5.37 Hz. The spectrum of 6 is rather complex with eight doublets centered between 43 and 43.6 ppm for Pα and Pγ corresponding to the four diastereomers derived from the two asymmetric centers. Two triplets at −24.64 ppm are due to Pβ. Compound 6 was isolated by DEAE-Sephadex chromatography. It could quantitatively be converted into 5 by alkaline hydrolysis.

When 4 was reacted with NaOH under conditions necessary for the removal of the 2-cyanoethyl protecting group, the desired compound 5 was formed together with thiopyrophosphate (9). Although 5 and 9 have an equal number of negative charges, they separate well on DEAE-Sephadex. Compound 5 was formed as a 1:1 mixture of two diastereomers in this synthesis. In analogy to the behavior of NTPαS diastereomers the material with the shorter retention time on reverse-phase HPLC and with the more downfield shift for the α-phosphorus was assigned as the diastereomer with the S_p configuration.[1] The validity of this assignment is supported by the fact that only the S_p diastereomer of 5 was found to inhibit HIV reverse transcriptase (data not shown).

P^1-O-(Cyanoethyl)-P^1-thiopyrophosphate (8) is the key reagent in these syntheses. It was conveniently prepared

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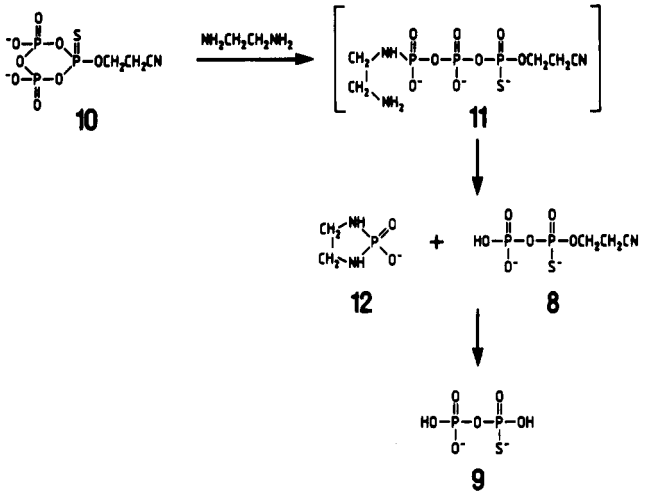
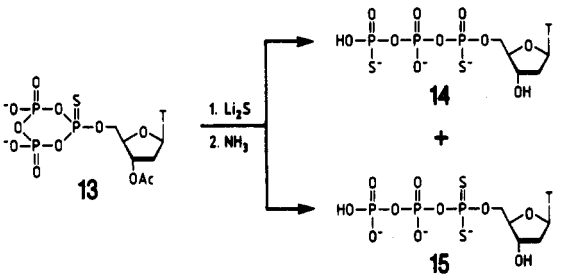
Scheme II



Scheme III



by reaction of 2-cyanoethanol with salicylphosphorochloridite as described for ethyl-(1-thiotriphosphate)[8] (Scheme II). The intermediate (2-cyanoethyl)-(1-thiocyclotriphosphate) (**10**) could be hydrolyzed to P^1-*O*-(2-cyanoethyl)-P^1-thiotriphosphate as followed by ^{31}P NMR spectroscopy (not shown). Reaction with excess ethylenediamine, however, resulted in dephosphorylation to P^1-*O*-(2-cyanoethyl)-P^1-thiopyrophosphate (**8**) in good yield. Its ^{31}P NMR spectrum showed the expected doublets at 42.79 ppm for P^1 and at −10.03 ppm for P^2. It was easily separated by DEAE-Sephadex chromatography from the phosphoramidate byproduct (**12**). The formation of theset two products has to be explained by ring opening of **10** first to **11** followed by a second, intramolecular nucleophilic displacement reaction.

Compound **8** could be quantitatively converted into thiopyrophosphate (**9**) by treatment with 0.5 M NaOH, identified by its ^{31}P NMR spectrum with two doublets at 30.77 and −5.85 ppm (J = 31.3 Hz). After neutralization with pyridinium ion exchanger this material could be used without further purification for the synthesis of the mixture of **3** and **5** (Scheme II). This synthesis presents an alternative to a previously described method where thiophosphate is heated to form **9**.[12]

Preliminary data (not shown) indicate that nucleoside 5′-*O*-(1,3-dithiotriphosphates) indeed combine the characteristics of nucleoside 5′-*O*-(1-thiotriphosphates) and nucleoside 5′-*O*-(3-thiotriphosphates) in their reactivity toward hydrolytic enzymes. Thus, the diastereomers of guanosine 5′-*O*-(1,3-dithiotriphosphates) are degraded by snake venom phosphodiesterase at similar rates to those of guanosine 5′-*O*-(1-thiotriphosphate) and by alkaline phosphatase with similar rates as guanosine 5′-*O*-(3-thiotriphosphate) (data not shown).

Synthesis of Nucleoside 5′-*O*-(1,1-Dithiotriphosphates). As an alternative to the synthesis of nucleoside 5′-*O*-(1,3-dithiotriphosphates) ring opening of thymidine 5′-(1-thiocyclotriphosphate) (**13**) with Li_2S was attempted (Scheme III). When this reaction was carried out in the presence of pyridine/dioxane as solvent a mixture of thymidine 5′-*O*-(1,3-dithiotriphosphate) (**14**) and thymidine 5′-*O*-(1,1-dithiotriphosphate) (**15**) was obtained in low yield. This mixture was only separable by HPLC. Interestingly, when DMF was employed as solvent, only the 5′-*O*-(1,1-dithiotriphosphate) **15** was formed. This solvent dependence of the product distribution was also observed in the reaction of the guanosine thiocyclotriphosphate.

The ^{31}P NMR spectrum of thymidine 5′-*O*-(1,1-dithiotriphosphate) shows a doublet at 99.7 ppm for P^1 with no indication of diastereomers. This chemical shift is consistent with the presence of two sulfur atoms at P^1 as it is similar to values reported for dinucleoside phosphorodithioates.[13] The chemical shifts of P^2 and P^3 correspond to those of a normal nucleoside 5′-triphosphate.

Thymidine 5′-*O*-(1,1-dithiotriphosphate) is the first example of a triphosphate analogue with two sulfur substituents on P^1 and may be regarded as a potential substrate for the enzymatic synthesis of the achiral phosphorodithioate analogues of oligonucleotides. The compound was therefore tested as substrate for the Klenow DNA polymerase in the presence of Mg^{2+}, with single-stranded circular M13 DNA as template and calf thymus derived oligonucleotides as primers.[14] The reaction was followed by agarose gel electrophoresis in the presence of ethidium bromide. There was no indication of elongation of the primers when TTP was replaced by thymidine 5′-*O*-(1,1-dithiotriphosphate) in the polymerization reaction (data not shown). Thus, this analogue is not a substrate for this polymerase. This result is consistent with the observation that only the S_p diastereomers of nucleoside 5′-*O*-(1-thiotriphosphates) are substrates for DNA and RNA polymerases.[1] This result, however, does not preclude the usefulness of nucleoside 5′-*O*-(1,1-dithiotriphosphates) in other enzyme-catalyzed reactions.

All compounds were characterized by ^{31}P NMR spectroscopy. The newly synthesized phosphorothioate derivatives **3**, **5**, and **15** were in addition characterized by their ^{13}C NMR spectra (Table I). Peak assignment is based on literature values.[15,16] The resonances of the pyrimidine moiety were essentially identical with those of the parent compound, 3′-deoxy-3′-azidothymidine 5′-triphosphate. Compounds with a chiral α-phosphorus such as 3′-deoxy-3′-azidothymidine 5′-*O*-(1-thiotriphosphate), which was also included for comparison, and compounds **3** and **5** showed a different set of resonances for each individual diastereomer at all sugar carbons. All four diastereomers of compound **3** were resolved only at the 3′-carbon. The configurational assignment of the resonances of the diastereomers of 3′-deoxy-3′-azidothymidine 5′-*O*-(1-thiotriphosphate) was made by recording the spectrum of a 2:1 mixture of the S_p/R_p diastereomers. The resonances of the S_p diastereomer appear at higher field than those of the R_p isomer. It is assumed that the same configurational assignment also applies to compound **5**.

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(16) Kalinowski, H. O., Berger, S., Braun, S., Eds. *^{13}C NMR Spektroskopie*; Georg Thieme Verlag: Stuttgart, 1984; p 400.

Table I. ^{13}C NMR Spectral Data

	chemical shifts, ppm (multiplicity, coupling constants, Hz)									
	C-4	C-2	C-6	C-5	C-1′	C-4′ ($J_{P\alpha C4'}$)	C-5′ ($J_{P\alpha C5'}$)	C-3′	C-2′	5 CH_3
N_3TTP[a]	171.12	156.29	141.90	116.43	89.47	87.63 (d, 9.3)	70.39 (d, 5.6)	65.67	40.90	16.26
N_3TTP αS[b]	171.08	156.25	141.93	116.43	89.39	87.59 (d, 9.6) (R_p) 87.48 (d, 7.4) (S_p)	70.79 (d, 5.7) (R_p) 70.33 (d, 6.1) (S_p)	65.85 (R_p) 65.57 (S_p)	40.90 (R_p) 40.79 (S_p)	16.31
N_3TTP $\alpha S\gamma S$[b,c] (5)	171.18	156.32	141.93	116.48	89.47 89.44	87.58 (d, 9.7) 87.47 (d, 9.1)	70.89 (d, 5.3) 70.44 (d, 6.5)	65.92 65.62	40.91 40.76	16.33
N_3TTP $\alpha S\beta S$[b] (3)	171.13	156.27	141.96	116.44	89.42 89.39	87.60 (d, 10.1) 87.50 (d, 9.8)	70.82 (d, 6.8) 70.39 (d, 5.9)	65.86 65.83 65.64 65.63	40.93 40.83	16.37
TTP $\alpha S\alpha S$[b] (15)	171.23	156.38	141.96	116.50	89.47	89.94 (d, 10.2)	70.29 (d, 8.3)	75.63	43.10	16.37

[a] Triethylammonium salt. [b] Na salt. [c] Sample was adjusted to pD 9 with NaOD in order to avoid hydrolysis to $N_3TDP\alpha S$, base carbon δ values are taken from a spectrum recorded at pH 7.

Compounds **3** and **5** were also analyzed by plasma desorption mass spectroscopy. They gave the expected molecular mass for the negative ion.

The methods described here for the synthesis of 3′-azido-3′-deoxythymidine 5′-*O*-(1,3-dithiotriphosphate) and thymidine 5′-*O*-(1,1-dithiotriphosphate) should be applicable to other nucleosides and thus make these analogues easily accessible for biochemical studies.

Experimental Section

Materials and Methods. The phosphitylating agent 2-chloro-4*H*-1,2,3-benzodioxaphosphorin-4-one and $LiEt_3BH$ (1 M solution in THF) were purchased from Aldrich. Sublimed sulfur was from Merck Darmstadt and dried over P_2O_5 in a desiccator before use. Dry dioxane and pyridine (containing less than 0.01% water) were also from Merck Darmstadt and were used as supplied. DMF and tri-*n*-butylamine were obtained from Fluka. All solvents were stored over 4-Å molecular sieves. 3′-Deoxy-3′-azidothymidine was purchased from Pharma Waldhof and 3′-acetylthymidine from Sigma.

^{31}P NMR spectra were recorded on a Bruker AM 360 spectrometer operating at 145.78 MHz with broad-band decoupling. Samples were recorded as 10 mM triethylammonium salts in aqueous solution containing 20% D_2O and approximately 10 mM EDTA, pH 8. For the detection fo intermediates **2** and **4**, spectra were recorded by adding DMF-d_7 to the reaction solution under argon. Spectrum accumulation was started 10 min after sulfur addition. Chemical shifts are given in ppm and are positive when downfield from the external standard of 80% aqueous phosphoric acid. ^{13}C NMR spectra were recorded at 90.55 MHz with ^1H broad-band decoupling of 60–100 mM solutions of the nucleotides in D_2O using the sodium salt of 3-(trimethylsilyl)propionic acid-d_4 as internal standard (δ 1.70 ppm).

Plasma desorption mass spectra were recorded with a Bio Ion 20 mass spectrometer from Applied Biosystems.[17] Acceleration voltage was 18 kV for positive and 15 kV for negative ions. A total of 2 × 106 spectra were recorded in an analysis time of 20 min. Samples (10 μL, approximately 100 nmol/μL in water) were adsorbed on nitrocellulose-coated aluminized mylar foils for 15 min and then dried by spinning. No signals could be detected in the positive ion mode. The negative ion spectra showed a significant deprotonated molecular ion with an accuracy of better than 0.1%.

Reverse-phase HPLC analyses were performed with a Waters 680 gradient controller and a Model 440 UV detector operating at 254 nm. Columns were packed with ODS Hypersil (5 μm, from Shandon Southern, Runcon, UK) and were eluted with 100 mM triethylammonium bicarbonate (TEAB), pH 7.5, containing a linear gradient of acetonitrile from 0% to 15% in 15 min. TLC was performed on Kieselgel 60 Platten (Merck, Darmstadt) eluted with 1-propanol/NH_4OH/H_2O (11/7/2, v/v). Chromatography on DEAE-Sephadex was carried out at 4 °C. Fractions containing product were combined and evaporated to dryness on a rotary evaporator, and the residue was coevaporated with methanol to remove traces of buffer.

Bis(tri-*n*-butylammonium) pyrophosphate (0.5 M solution in DMF) was prepared as described.[8] The Li_2S (0.5 M in THF) was prepared as described[18] by addition of 1 mL of $LiEt_3BH$ (1M in THF) with a dry syringe to sulfur (500 μmol, 16 mg) under argon. The yellow solution was stirred for 15 min before addition to the solution containing the nucleoside 5′-(1-thiocyclotriphosphate).

***P*^{1}-*O*-(Cyanoethyl)-*P*^{1}-thiopyrophosphate (8).** 3-Hydroxypropionitrile (710.8 mg, 10 mmol) was dissolved in dioxane/pyridine (3/1, v/v, 16 mL) under argon in a septum-sealed flask. A freshly prepared solution of salicylphosphochloridite (2.04 g, 10 mmol) in dioxane (5 mL) was then injected through the septum. Precipitation occurred immediately after addition of the phosphitylating agent. After stirring for 10 min at room temperature bis(tri-*n*-butylammonium) pyrophosphate (10 mmol) and tri-*n*-butylamine (4.76 ml, 20 mmol) in anhydrous DMF (20 mL) was pipetted into the reaction mixture. The resulting solution was stirred for 10 min before powdered sulfur (480 mg, 15 mmol) was added. After reaction for 10 min at room temperature the yellow suspension was pipetted into a solution of ethylenediamine (3.35 mL, 50 mmol) in DMF (10 mL).

The resulting heterogeneous mixture was stirred for 5 min, and water (20 mL) was added. After 30 min the reaction mixture was evaporated, the residue (approximately 20 mL) was diluted to 50 mL with water, and the solution was extracted with ether (2 × 50 mL). The pH of the aqueous phase was adjusted to 7 and the aqueous layer was applied onto a DEAE-Sephadex column (4 × 45 cm). The column was eluted with a linear gradient of 1500 mL each of 0.1 M TEAB and 0.8 M TEAB. Product-containing fractions were identified on the basis of R_f values by TLC. Spots were visualised with I_2 vapor (R_f of 8, 0.28; R_f of *P*^{1}-*O*-(2-cyanoethyl)-*P*^{1}-thiotriphosphate obtained by hydrolysis of **10**, 0.11). *P*^{1}-*O*-(cyanoethyl)-*P*^{1}-thiopyrophosphate (**8**) was eluted between 0.63 and 0.71 M buffer. The appropriate fractions were pooled, evaporated to dryness and repeatedly (4×) reevaporated with methanol to remove traces of buffer. The ratio of triethylammonium to cyanoethyl protons determined from the ^1H NMR spectrum corresponded to a 2:1 molar ratio. Yield of the bis(triethylammonium) salt of **8** was 2.21 g (49%). ^{31}P NMR (D_2O): δ 42.79 (d, P^1), −10.33 ppm (d, P^2); J = 28.32 Hz. In the ^1H coupled spectrum the P^1 signal was split into two triplets with J_{PH} = 8.78 Hz. ^1H NMR (DMSO-d_6): δ 3.9 (2 t, 2 H, CH_2), 2.82 (t, 2 H, CH_2), 3.03 (br s, 12 H, ethyl CH_2), 1.17 (br s, 18 H, ethyl CH_3). The compound can be stored as an aqueous solution for several weeks at −20 °C.

The bis(tri-*n*-butylammonium) salt was prepared as follows: *P*^{1}-*O*-(cyanoethyl)-*P*^{1}-thiopyrophosphate bis(triethylammonium) salt (300 μmol) was dissolved in methanol/DMF (1/1, v/v, 2 mL), and tri-*n*-butylamine (142.8 μL, 600 μmol) was added. The solution was evaporated to dryness, and the residue was redissolved in DMF (2 mL) and again evaporated to dryness. The resulting

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residue was then stored under dry argon. Immediately before use the material was dissolved in DMF (600 μL), and tri-*n*-butylamine (142.8 μL, 600 μmol) was added.

Bis(tri-*n*-butylammonium) Thiopyrophosphate (9). Bis(triethylammonium) P^1-*O*-(cyanoethyl)-P^1-thiopyrophosphate (200 μmol) was dissolved in H_2O (6 mL), and 1 N NaOH (6 mL) was added. After 30 min at room temperature the solution was passed through a column of DOWEX 50×8 (30 mL, pyridinium form). The column was washed with water (60 mL), the effluent and washings were evaporated, and the residue was dissolved in methanol (2 mL). DMF (2 mL) and tri-*n*-butylamine (95.2 μL, 400 μmol) was added, and the solution was evaporated. The residue was dried by coevaporation with DMF. It was finally dissolved in DMF (400 μL), and tri-*n*-butylamine (95.2 μL, 400 μL) was added before use. ^{31}P NMR (MeOH-d_4): δ 41.0 (d), −9.2 (d); J = 23.8 Hz.

Mixture of 3′-Deoxy-3′-azidothymidine 5′-*O*-(1,2-Dithiotriphosphate) (3) and 3′-Deoxy-3′-azidothymidine 5′-*O*-(1,3-Dithiotriphosphate) (5). 3′-Deoxy-3′-azidothymidine (26.7 mg, 100 μmol) was dissolved in pyridine (2 mL). The solution was evaporated to dryness, and the residue was dried overnight in vacuo over P_2O_5. It was then dissolved in a mixture of pyridine (100 μL) and dioxane (300 μL) under argon. All subsequent manipulations were performed under a small positive pressure of argon. Salicylphosphorochloridite (110 μL of a freshly prepared 1 M solution in dioxane, 110 μmol) was injected through a septum. After reaction for 10 min thiopyrophosphate bis(tri-*n*-butylammonium) salt (200 μmol) in DMF (400 μL) was added together with tri-*n*-butylamine (95.2 μL, 400 μmol). After 10 min of stirring a suspension of sulfur (6.4 mg, 150 μmol) was added, followed by water (2 mL) after an additional 10 min. The ^{31}P NMR spectrum presented in Figure 1A was recorded before the addition of water. After stirring for a further 30 min, the reaction mixture was evaporated to dryness and the residue was dissolved in water (10 mL). This solution was applied onto a DEAE-Sephadex column (2 × 40 cm) which was eluted with a linear gradient of 1000 mL each of 0.1 and 1.0 M TEAB. 3′-Deoxy-3′-azidothymidine 5′-*O*-(1,2-dithiotriphosphate) and 3′-deoxy-3′-azidothymidine 5′-*O*-(1,3-dithiotriphosphate) were eluted together between 0.69 and 0.78 M buffer. Product-containing fractions were pooled and evaporated to dryness, and the residue was coevaporated with methanol (4×) to remove traces of buffer. Yield 856 A_{267}-units (86 μmol, 86%).

3′-Deoxy-3′-azidothymidine 5′-*O*-(1,2-Dithiotriphosphate) (3). In order to obtain **3** from the above mixture, 220 μL of 30% aqueous acetic acid was added to 2.2 mL of an aqueous solution containing 300 A_{267} units of **3** and **5**. The solution was kept at room temperature for 20 h. After evaporation the residue was dissolved in water and chromatographed in the same system as above. 3′-Deoxy-3′-azidothymidine 5′-*O*-(1-thiodiphosphate) was eluted at 0.42–0.45 M buffer. Yield 39 A_{267}-units ^{31}P NMR (D_2O) S_p isomer: δ 41.84 (d, P^1), −6.15 (d, P^2). R_p isomer: δ 41.43 (d, P^1), −6.15 (d, P^2); $J_{P^1P^2}$ = 30.42 Hz. HPLC retention times (min): 10.05 (S_p), 10.25 (R_p).

Compound **3** was eluted 0.69–0.78 M buffer. Yield 256 A_{267}-units (86%). ^{31}P NMR (D_2O): δ 42.74 (d), 42.58 (d), 42.35 (d), 42.27 (d, P^1), 28.30 (q), 28.18 (q, P^2), −7.44 (d, P^3), $J_{P^1P^2}$ = 35.4 Hz; $J_{P^2P^3}$ = 28.4 Hz. ^{13}C NMR: Table I. Mass spectrum: M_r calculated 539.3, found 539.4. HPLC retention times (min): 11.54, 11.98, 12.39 (in a 2:1:1 intensity ratio).

3′-Deoxy-3′-azidothymidine 5′-*O*-(1,3-Dithiotriphosphate) (5). 3′-Deoxy-3′-azidothymidine (26.7 mg, 100 μmol) was reacted with salicylphosphorochloridite as described above for the synthesis of **3** and **5**. After stirring for 10 min, P^1-*O*-(cyanoethyl)-P^1-thiopyrophosphate bis(tributylammonium) salt (300 μmol) in DMF (600 μL) was added together with tri-*n*-butylamine (142.8 μL, 600 μmol). After the mixture was stirred for 10 min at room temperature a suspension of sulfur (6.4 mg, 150 μmol) in DMF (200 μL) was added, followed by water (2 mL) after an additional 10 min of stirring. The ^{31}P NMR spectrum presented in Figure 1B was recorded before the addition of water. After reaction for 30 min the reaction mixture was evaporated to dryness. The complete removal of pyridine is important at this step. The residue was dissolved in water (20 mL) and 1 N NaOH (20 mL) was added. After 90 min the solution was neutralized by passing it through a Merck cation exchange column (pyridinium form, 2 × 20 cm). The column was washed with two volumes of water, and the effluent was evaporated to dryness. The residue was dissolved in water (20 mL) and a few drops of ammonia were added to adjust the pH to approximately 9. This solution was applied onto a DEAE-Sephadex column (2 × 40 cm) which was eluted with a linear gradient of 1000 mL each of 0.1 and 1.0 M TEAB. 3′-Deoxy-3′-azidothymidine 5′-*O*-(1,3-dithiotriphosphate) was eluted between 0.68 and 0.79 M buffer. Product-containing fractions were pooled, evaporated to dryness, and coevaporated with methanol (4×) to remove traces of buffer. Yield 805 A_{267} units (81 μmol, 81%). ^{31}P NMR (D_2O) of S_p isomer: δ 43.43 (d, P^1), −23.56 (q, P^2), 34.05 (d, P^3); $J_{P^1P^2}$ = 27.06 Hz; $J_{P^2P^3}$ = 30.51 Hz. R_p isomer: δ 43.05 (d, P^1), −23.60 (q, P^2), 34.02 (d,P^3); $J_{P^1P^2}$ = 28.05 Hz; $J_{P^2P^3}$ = 30.48 Hz. ^{13}C NMR: Table I. Mass spectrum: M_r calculated 539.3, found 539.4. HPLC retention times (min): 10.77 (S_p), 11.19 (R_p).

3′-Deoxy-3′-azidothymidine 5′-[3′-*O*-(2-Cyanoethyl) 1,3-dithiotriphosphate] (6). 3′-Deoxy-3′-azidothymidine (26.7 mg, 100 μmol) was reacted with salicylphosphochloridite (110 μmol), P^1-*O*-(cyanoethyl)-P^1-thiopyrophosphate bis(*n*-tributylammonium) salt (300 μmol), and sulfur as described above for the synthesis of **5**. After reaction with sulfur for 10 min, morpholine (87 μL, 1 mmol) was pipetted into the reaction mixture. The resulting yellow solution was stirred for 30 min and then evaporated to dryness. The residual oil was dissolved in water (10 mL), and the solution was applied onto a DEAE-Sephadex column (2 × 40 cm) which was eluted with a linear gradient of 1000 mL each of 0.1 and 1.0 M TEAB. Compound **6** was eluted between 0.59 and 0.67 M buffer. Yield 657 A_{267}-units (68%). ^{31}P NMR (D_2O): 8 d in the 43.6–43 ppm region (2 P, Pα and Pγ); 2 t at −24.5 ppm (1 P, Pβ); $J_{\alpha\beta} = J_{\beta\gamma}$ = 27 Hz.

Thymidine 5′-*O*-(1,3-Dithiotriphosphate) (14) and Thymidine 5′-*O*-(1,1-Dithiotriphosphate) (15). Method A. 3′-*O*-Acetylthymidine (28.4 mg, 100 μmol) was reacted with salicylphosphorochloridite as described above for the synthesis of **5**. After reaction for 10 min a solution of 150 μmol of bis(tri-*n*-butylammonium) pyrophosphate in dioxane/pyridine (3/1, v/v) was added. This solution had been prepared by adding 2 mL of dioxane to a 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in anhydrous DMF (300 μL). It was evaporated to dryness in order to remove last traces of DMF. This procedure was repeated three times. The residual oil was finally dissolved in pyridine (200 μL), dioxane (600 μL), and tri-*n*-butylamine (200 μL). This solution was prepared immediately before use.

After reaction for 10 min powdered sulfur (4.8 mg, 150 μmol) was added to the reaction mixture, and stirring was continued for 30 min. Li_2S (500 μmol, 1 mL of a 0.5 M solution in THF) was injected into the reaction mixture, and the resulting yellowish suspension was stirred for 10 min. The reaction mixture was pipetted into water (3 mL), and concentrated ammonia (20 mL) was added. After 1 h the solution was evaporated, the residue was dissolved in water, and the solution was applied to a DEAE-Sephadex A-25 column (2 × 30 cm). Chromatography was performed with a linear gradient of 1250 mL each of 0.05 M and 1.0 M TEAB. Fractions of 17 mL were collected. Products were eluted between 0.55 and 0.65 M buffer. The residue was re-evaporated with methanol to remove traces of buffer. ^{31}P NMR spectral analysis indicated the presence of thymidine 5′-*O*-(1,1-dithiotriphosphate) and that of the diastereomers of thymidine 5′-*O*-(1,3-dithiotriphosphate). Total yield 220 A_{267}-units (22%). The products were separated by reverse-phase HPLC. S_p isomer of thymidine 5′-*O*-(1,3-dithiotriphosphate), retention time 8.23 min, (30%); R_p isomer, retention time 8.80 min (36%); thymidine 5′-*O*-(1,1-dithiotriphosphate), retention time 9.09 min (34%).

^{31}P NMR (D_2O) thymidine 5′-*O*-(1,1-dithiotriphosphate): δ 99.7 (d, P^1), −22.96 (q, P^2), −7.09 (d, P^3); $J_{P^1P^2}$ = 34.25 Hz; $J_{P^2P^3}$ = 20.25 Hz; $^3J_{P^1H^{5'}}$ = 7.8 Hz. Thymidine 5′-*O*-(1,3-dithiotriphosphate), S_p isomer: 43.50 (d, P^1), −23.30 (q, P^2), 34.40 (d, P^2); $J_{P^1P^2}$ = 27.60 Hz; $J_{P^2P^3}$ = 30.92 Hz; $^3J_{P^1H^{5'}}$ = 6.9 Hz. R_p isomer: 43.00 (d, P^1), −23.35 (q, P^2), 34.40 (d, P^3); $J_{P^1P^2}$ = 28.07 Hz; $J_{P^2P^3}$ = 30.22 Hz; $^3J_{P^1H^{5'}}$ = 6.7 Hz; $^4J_{P^1H^{4'}}$ = 1.7 Hz. ^{13}C NMR: Table I.

Thymidine 5′-*O*-(1,1-Dithiotriphosphate) (15). Method B. 3′-*O*-Acetylthymidine (100 μmol, 28.4 mg) was reacted with salicylphosphochloridite as described under method A. After reaction for 10 min, 250 μL of a 0.5 M solution of bis(tri-*n*-butylammonium) pyrophosphate in DMF and tri-*n*-butylamine (100

μL) was injected through a rubber septum into the reaction solution. The subsequent reactions with sulfur and Li_2S were performed exactly as described above for method A. Addition of the latter caused the appearance of a dark green suspension. Workup and DEAE-Sephadex A-25 chromtography was also carried out as above. Thymidine 5′-*O*-(1,1-dithiotriphosphate) eluted between 0.61 and 0.65 M buffer. Yield 125 A_{267} units (13%). This material was identical in all respects with that synthesized by method A.

Guanosine 5′-*O*-(1,1-Dithiotriphosphate). 2′,3′-*O*-Diacetylguanosine (100 μmol, 36.7 mg) was dissolved in pyridine (200 μL) and DMF (800 μL), and the solution was evaporated on a dry evaporator. The residue was dried over P_2O_5 for 1 h, dissolved in a mixture of pyridine (200 μL) and DMF (800 μL), and reacted with salicylphosphochloridite, pyrophosphate, sulfur, and Li_2S as described for compound **15** in method B. Purification on DEAE-Sephadex yielded 290 A_{254}-units (22%) of guanosine 5′-*O*-(1,1-dithiotriphosphate). Its ^{31}P NMR spectrum is virtually identical with that of thymidine 5′-*O*-(1,1-dithiotriphosphate). HPLC retention time, 7.53 min. For comparison that of R_p guanosine 5′-*O*-(1-thiotriphosphate) is 7.22 min.

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Enantioselective Synthesis of Both Enantiomers of Phosphinothricin via Asymmetric Hydrogenation of α-Acylamido Acrylates

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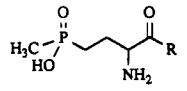
Both enantiomers of phosphinothricin (**1**), a naturally occuring amino acid that contains the unique methylphosphinate moiety, were prepared by asymmetric hydrogenation of α-acylamido acrylate precursors **7**. L-**1** and peptides containing L-**1** are inhibitors of the enzyme glutamine synthetase (GS). Inhibition of GS is responsible for the antibiotical and herbicidal properties of these compounds. Synthesis of substrates **7** and parameters influencing the enantioselectivity are discussed. Substrate concentration and solvent polarity appear to have the most marked effects on enantiomeric excesses for a given catalyst system. Enantiomeric excesses reach 91% for hydrogenations with (*R,R*)-NORPHOS- and (*S,S*)-CHIRAPHOS-derived catalysts.

Introduction

L-Phosphinothricin (L-**1**), which constitutes the N-terminal amino acid of the antibiotic tripeptides **2**[1] and **3**[2] produced by several streptomycete and actinomycete strains, exhibits strong herbicidal activity.[3]



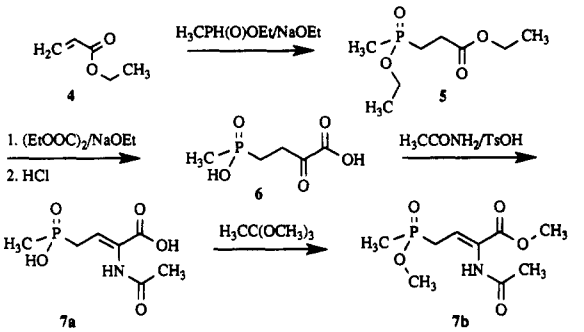
1 R= OH
2 R= Ala-Ala-OH
3 R= Ala-Leu-OH

The biological activity of these compounds is based on the inhibition of glutamine synthetase (EC 6.3.1.2), an enzyme that plays a pivotal role in the ammonia metabolism of plants[4] and bacteria.[5]

Several syntheses of racemic **1** have been reported[6] however L-**1**[7] is claimed to possess twice the biological activity of D,L-**1**.[6b,8]

L-**1** has been obtained with an enantiomeric excess (ee) of 79–94% by enantioselective alkylation of chiral glycine synthons[9] or by enzymatic resolution of racemic precursors.[10]

Scheme I



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