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SYNTHESIS OF NUCLEOSIDE 3',5'-CYCLIC PHOSPHOROTHIOATES

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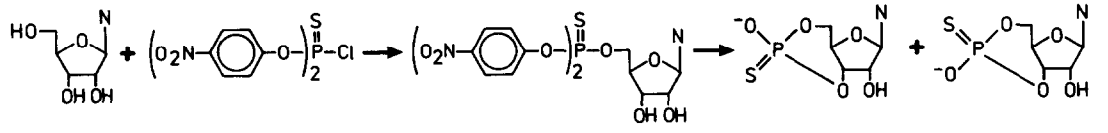
Abstract: A convenient and general method for the synthesis of nucleoside 3',5'-cyclic phosphorothioates using nucleosides as starting materials is described.

Phosphorothioate analogues of nucleosides have become powerful tools for the elucidation of stereochemical aspects of enzymatic phosphoryl- and nucleotidyl transfer reactions as well as for the inhibition of nucleases[1]. A particularly interesting class among these analogues are the nucleoside 3',5'-cyclic phosphorothioates since the corresponding phosphates, such as cAMP and cGMP, are important regulators of biochemical processes (for reviews see ref. 2 and 3). Indeed, the Rp- and Sp-diastereomers of cAMPS have been used successfully in a variety of biological systems[1] and those of cGMPS most recently as inhibitors of cyclic phosphodiesterase in rod outer segments[4]. Despite these applications and the increasing interest in these compounds, there is no convenient general synthesis available for these analogues. The Rp-diastereomers of cAMPS[5] and cGMPS[6] can be synthesized enzymatically from the corresponding nucleoside 5'-O-(1-thiotriphosphates) and the corresponding cyclases but this method does not provide the Sp-diastereomers and the enzymes are not easily accessible. A chemical synthesis of the diastereomers of cGMPS has been described in which guanosine 5'-phosphorothioate is activated by the reaction with diphenylphosphoryl chloride and subsequently cyclized but the yield of this method is extremely low[6]. Stec and coworkers have published a procedure for the synthesis of both diastereomers of cAMPS by converting N^6-dibenzoyl-2'-O-benzoyladenosine 3',5'-cyclic phosphate into a mixture of diastereomers of the anilidates which are separated by preparative TLC and then converted by reaction with CS_2 and potassium to the diastereomers of cAMPS[7]. They have also extended the method to the synthesis of the other nucleoside 3',5'-cyclic phosphorothioates[8,9]. The disadvantages of this method are that the starting materials are expensive, that it is a multistep procedure and the overall yields are low.

It seemed to us that the first reported synthesis of cAMPS[10] in which 2',3'-diacetyl adenosine was reacted with bis(p-nitrophenyl)phosphorochloridothioate and then cyclized to yield

the crystalline triethylammonium salt of cAMPS (the Sp-isomer as was later detected by comparison with material obtained from W.J. Stec) could be the basis for the development of a general and simple procedure.



N = nucleobase

Rp Sp

Preliminary experiments showed that it was not necessary to protect the cis-diol function of the nucleosides to obtain reaction specifically at the 5'-position with bis(p-nitrophenyl) phosphorochloridothioate[10] so that only the NH_2-functions of the nucleobases were protected[11] mainly to achieve solubility. N^6-Benzoyladenosine, N^2-isobutyrylguanosine or uridine (10 mmol) were dissolved in dry pyridine (20 ml) and bis(p-nitrophenyl)phosphorochloridothioate[10] (11 mmol) added. After reaction at room temperature for 3.5 h the solutions were evaporated to dryness. Because N^4-benzoylcytidine[11] was insoluble in pyridine at room temperature it was suspended in 60 ml pyridine, heated to 70°C in an oil bath and the bis(p-nitrophenyl)phosphorochloridothioate added. After stirring for 15 min the solution became clear. Stirring at room temperature was continued for another 3 h and the solution evaporated to dryness. The residues of the four reactions were flash-chromatographed on Kieselgel 60 (230 - 400 mesh; Merck, Darmstadt, FRG; column 4 x 30 cm) by elution first with 1.5 l $CHCl_3$, followed by 1 l of $CHCl_3$/MeOH (95:5, v/v) and then by 1 l of $CHCl_3$/MeOH (90:10, v/v). Fractions of 20 ml were collected. They were analyzed by TLC on Kieselgel 60F plates (Merck, Darmstadt, FRG) using $CHCl_3$/MeOH (90:10, v/v) as eluant. The products were eluted in fractions 80 - 120. The yields and characterizations of the products are given in Table I.

Table I Characterization of the nucleoside 5'-bis(p-nitrophenyl)phosphorothioates[a]

5'-Bis(p-nitrophenyl)phosphorothioate Derivative of	Yield (%)	^{31}P NMR[b] (ppm)	R_F-Values[c] starting nucleoside	product
N^6-Benzoyladenosine	56	57.5	0.39	0.58
Uridine	80	57.7	0.10	0.50
N^4-Benzolcytidine	44	57.4	0.36	0.58
N^2-Isobutyrylguanosine	69	57.7	0.10	0.40

[a] all compounds gave satisfactory elemental analyses; [b] recorded in d_6-acetone using 80 % H_3PO_4 as external standard on a Bruker WP 200 SY spectrometer operating at 81.01 Hz with ^1H broad band decoupling; [c] TCL on Kieselgel 60F plates with $CHCl_3$/MeOH (9:1, v/v).

The nucleoside 5'-bis(p-nitrophenyl)phosphorothioates were cyclized by dissolving 2 mmol in dry DMF (350 ml) and reaction with 35 ml of 1 M potassium tert.-butoxide for 20 min. After neutralization with acetic acid and evaporation to dryness, the residues were deblocked by dissolving in 100 ml conc. NH_4OH and the solutions kept at 50°C overnight in a securely stoppered flask. The solutions were then evaporated to dryness, the residues dissolved in H_2O and the solutions passed over ion exchange columns (Merck I, H^+-form, 3 x 30 cm) and the columns washed with water until no further uv-absorbing material was eluted. The NH_3-treatment was omitted for the synthesis of cUMPS and the reaction mixture was passed over the ion-exchange column directly. The eluates were evaporated to dryness, the residues dissolved in H_2O and converted to the triethylammonium salts by addition of sufficient 1 M triethylammonium bicarbonate solution to obtain a pH of 7.5. The solutions were evaporated to dryness, the residues dissolved in acetone/toluene/H_2O (8:2:0.75, v/v) (10 ml) and flash-chromatographed on Kieselgel 60 (column 3 x 30 cm) with the same solvent mixture until all the p-nitrophenol had been eluted. The solvent was then changed to acetone/MeOH/H_2O (13:2:2, v/v). Fractions of 20 ml were collected and analyzed by TLC on DC-Micro-Cards S/F (Riedel-de Haen, Seelze, FRG) using the same mixture. Products were eluted in fractions 3 - 12. They were evaporated to dryness, the residues dissolved in H_2O and the products purified by chromatography on DEAE-Sephadex columns (2 x 30 cm) by elution with a linear gradient of 1 l each of H_2O and 0.35 M triethylammonium bicarbonate. The product-containing fractions were evaporated and triethylamine removed by two evaporations with MeOH. Whereas the diastereomers of cAMPS and cGMPS were separated by this chromatography (concentration of buffer for elution of Rp-cAMPS 0.18 M, Sp-cAMPS 0.25 M, Rp-cGMPS 0.28 M and Sp-cGMPS 0.35 M) those of cUMPS and cCMPS (eluted at 0.20 and 0.12 M, respectively) were not. Sp-cAMPS was crystallized from EtOH [88 mg; mp 197 - 200° (ref. 9, 195 - 205°)]. The isomers of cUMPS and cCMPS could be separated by HPLC using a Waters system with a reversed-phase column (1 x 25 cm) and a linear gradient of 100 mM triethylammonium bicarbonate with increasing acetonitrile content from 0 to 9 % in 15 min. After HPLC separation Rp-cUMPS crystallized from EtOH (15 mg; mp 200 - 204°) as did the Sp-isomer (44 mg; mp 176-180°). The rentention times, yields and characterization of the cyclic phosphorothioates are summarized in Table II.

Table II Characterization of nucleoside 3',5'-cyclic phosphorothioates

Compound	Yield[a] (%)	Ratio of Sp/Rp	R_F-Values[b]		Retention Time[c] (min)		^{31}P NMR[d] (ppm)
cAMPS	17	4.7	cAMP	0.41	Sp	15.55	54.97
			cAMPS	0.83	Rp	14.41	56.52
cUMPS	18	4.1	cUMP	0.74	Sp	10.04	54.46
			cUMPS	0.87	Rp	8.97	56.04
cCMPS	17	4.4	cCMP	0.38	Sp	8.95	54.87
			cCMPS	0.81	Rp	8.30	56.50
cGMPS	18	3.2	cGMP	0.51	Sp	11.56	54.99
			cGMPS	0.80	Rp	10.25	56.69

[a] after DEAE-Sephadex chromatography; [b] TLC on DC-Micro-Cards S/F as in text; [c] on analytical column using the same gradient as for the preparative isolation; [d] in H_2O/D_2O, otherwise as described in Table I.

The configurational assignment for the diastereomers of cAMPS rests on the X-ray structural analysis of 2'-deoxyadenosine cyclic 3',5'-(Rp)-phosphoranilidate which is converted with retention of configuration to (Rp)-2'-deoxyadenosine 3',5'-cyclic phosphorothioate, and on the P-^{15}N spin-spin coupling constants in the ^{31}P NMR spectra of the two isomers of the ^{15}N-labelled deoxy-compound[12] and the ribo-compound[7]. These studies showed that the isomer of cAMPS with the low-field ^{31}P NMR resonance has the Rp- and the other the Sp-configuration. As the resonances for the other cyclic phosphorothioates have almost identical chemical shifts it is safe to assume that the configurations are the same as determined for those of cAMPS. The preference for the formation of the Sp-isomer seems to be thermodynamically controlled as reactions carried out additionally at 0° and 45° as well as up to one hour essentially produced the same ratio.

This paper is dedicated to Professor Morio Ikehara on the occasion of his retirement from Osaka University in March 1986.

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