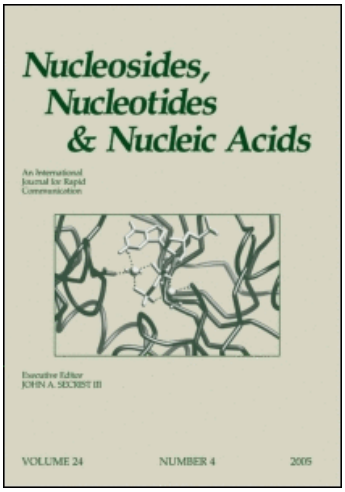

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

3'-3',3'-5' and 5'-5' TpT-Amides: Synthesis, Characterization and Alkaline Hydrolysis

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To cite this Article Tomasz, J.(1983) '3'-3',3'-5' and 5'-5' TpT-Amides: Synthesis, Characterization and Alkaline Hydrolysis', Nucleosides, Nucleotides and Nucleic Acids, 2: 1, 51 — 61
To link to this Article: DOI: 10.1080/07328318308078849
URL: http://dx.doi.org/10.1080/07328318308078849

NUCLEOSIDES & NUCLEOTIDES, 2(1), 51-61 (1983)

3'-3', 3'-5' AND 5'-5' TpT-AMIDES: SYNTHESIS, CHARACTERIZATION AND ALKALINE HYDROLYSIS

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Abstract. A simple procedure is described for the preparation of the title compounds 1, 8 and 9. 3'-3' or 3'-5' or 5'-5' TpT was reacted with a twofold molar excess of TPS in anhydrous DMF, at room temperature, for 5 min, followed by a 1 min *in situ* treatment of the reaction mixture with excess 7.0 N NH_4OH, at 0°C. The alkaline hydrolysis of 1, 8 and 9 proceeds without the assistance of 3'- and 5'-hydroxyl groups resulting in equimolar mixtures of thymidine (4) and thymidine 3'-phosphoramidate (6)(for the 3'-3' isomer) or thymidine 5'-phosphoramidate (7)(for the 5'-5' isomer) or 6 and 7 in equal quantities (for the 3'-5' isomer).

INTRODUCTION

We have recently reported a synthesis and the alkaline hydrolysis of thymidylyl-(3'-5')-thymidine (P-N) amide (1)[1]. We found that in alkali, 1 was readily hydrolyzed to a 1:1 mixture of thymidine (4) and isomeric thymidine phosphoramidates (6 and 7) /$t_{1/2}$~20 min, in 0.1 N NaOH, at 25°C/. The ratio of 6 to 7 was approximately 1:1. We formulated the alkaline hydrolysis as an $S_N1(P)$ process *via* the metaphosphorimidate intermediates 2 and 3 (SCHEME 1), as suggested by Westheimer for the alkaline hydrolysis of those phosphoramidates having at least one ionizable hydrogen atom attaching to the amide nitrogen atom[2]. However, the fact that 6 and 7 were formed in approximately equal quantities, did not definitely indicate that the breaking of the P-O(3') and P-O(5') bonds of 1 occurred with approxi-

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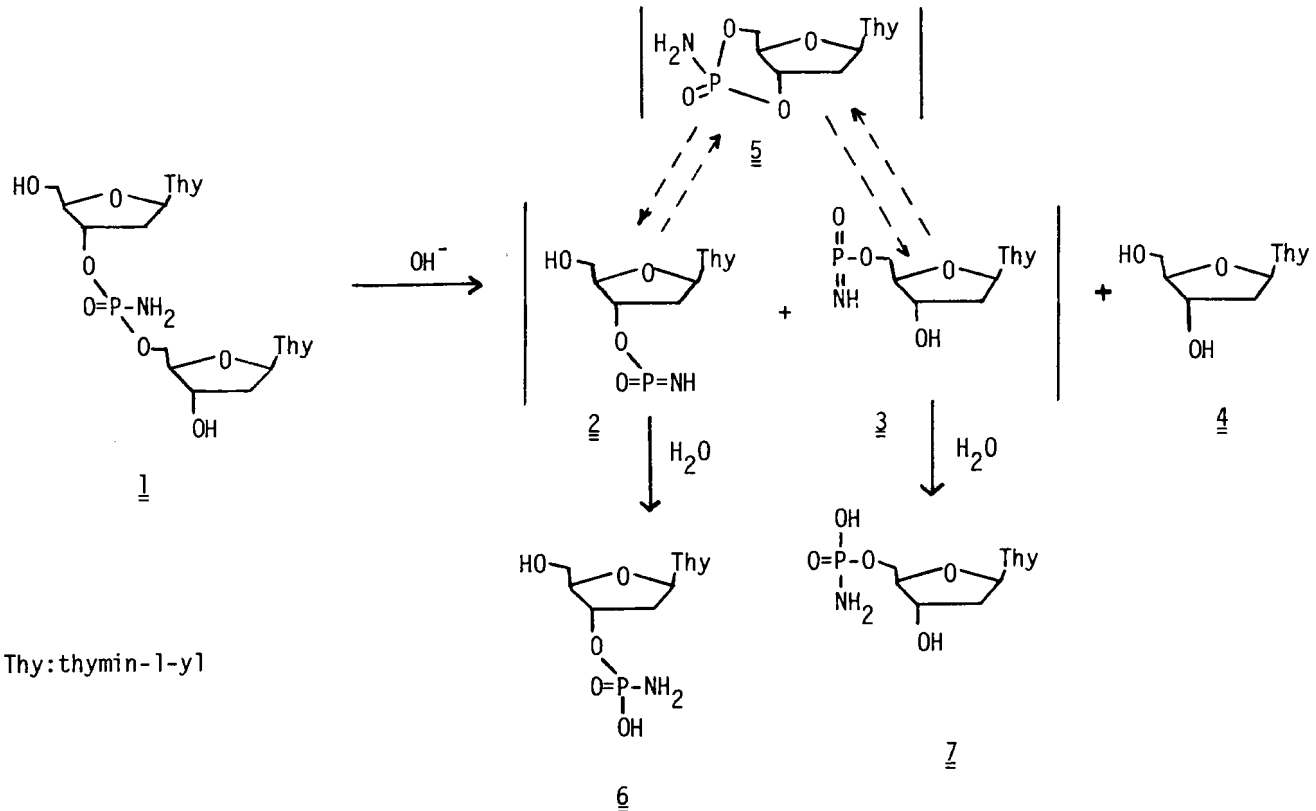
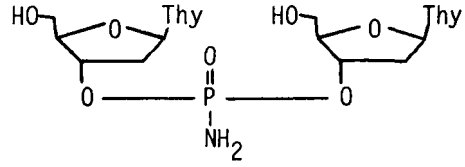


Thy:thymin-1-yl

SCHEME 1

mately equal rates, since a different speed interconversion of the intermediates 2 and 3 via thymidine 3',5'-cyclic phosphoramidate (5) could not be excluded (SCHEME 1). 5 may be formed by the intramolecular trapping action of the 3'-hydroxyl group of 3 or the 5'-hydroxyl group of 2 on their metaphosphorimidate residue[3]. Such a participation of the 3'- and/or 5'-hydroxyl groups might be demonstrated by determining the molar quantities of 6 and 7 produced in the alkaline hydrolysis of the isomeric thymidylyl-(3'-3')-thymidine (P-N) amide (8) and thymidylyl-(5'-5')-thymidine (P-N) amide (9). When the hydroxyl groups



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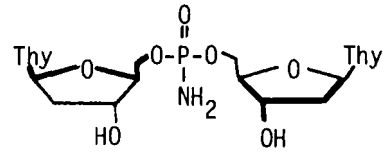


9

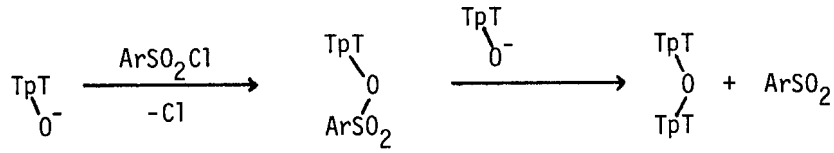
do not participate in the reaction, from 8 exclusively 6, while from 9 only 7 should be formed. These studies had been performed and the results are presented in this paper.

RESULTS AND DISCUSSION

Synthesis

The synthesis of TpT-amides 1, 8 and 9 was performed by reacting the respective TpT-s with a twofold molar excess of 2,4,6,-triisopropylbenzenesulfonyl chloride (TPS) in anhydrous N,N-dimethylformamide (DMF), in the presence of tri-n-butylamine, at room temperature for 5 min, followed by an in situ treatment of the reaction mixture with excess 7.0 N NH_4OH, at 0^oC, for 1 min.

In the first step, the TpT-sulfonic acid mixed anhydride (10) may be formed, which in turn is transformed into the symmetrical TpT-anhydride (11) as a result of a very rapid exchange reaction with the unreacted TpT (SCHEME 2)[4,5]. The ammonolysis of 11 should result in equimolar quantities of TpT-amide and TpT. Consequently, the theoretical yield is only 50% for the starting TpT, and it seems very probable



Ar:2,4,6-triisopropylphenyl

SCHEME 2

that 0.5 molar equivalent of TPS gives the same result as the twofold molar excess does[5]. The short reaction time may be responsible for the fact that the sulfonation of 5'-hydroxyl group[6] is negligible, if it occurs at all.

The TpT-amides were isolated by column chromatography, first on DEAE-cellulose (HCO_3^-) then on cellulose, in yields of 22.0-29.3% as TLC pure, white solids.

This procedure is a simplified version of our previous method[1] when we used diphenyl-phosphorochloridate instead of TPS, and a much more longer reaction time (2.5 h) for the preparation of 1.

Characterization

TpT-amides 1, 8 and 9 were characterized by UV, CD and ^{31}P NMR spectroscopies as well as acid hydrolysis.

The UV spectra at pHs 2.0, 7.0 and 11.0 were identical with those of the respective TpTs.

The CD spectrum between 220-300 nm, at pH 7.5, closely resembled that of the parent TpT (FIG. 1). This indicates conformational similarity between the phosphate and the phosphoramidate.

The ^{31}P NMR spectrum in DMSO-d_6 of the symmetrical TpT-amides 8 and 9 contained one single signal, while that of 1 showed two close signals of about equal intensity. As expected, 1 with a chiral phosphorus atom, was an approximately 1:1 mixture of two diastereoisomers. All signals were located around 10-12 ppm, i.e. about 11-13 ppm downfield from the signal of 3'-5' TpT, in D_2O[1,7]. A downfield chemical shift of such magnitude is characteristic of a nitrogen-for-oxygen displacement at tetracoordinate phosphorus atom[8]. On the other hand, the ^{31}P chemical shift values found for 1, 8 and 9 agreed well with those of 10.85 and

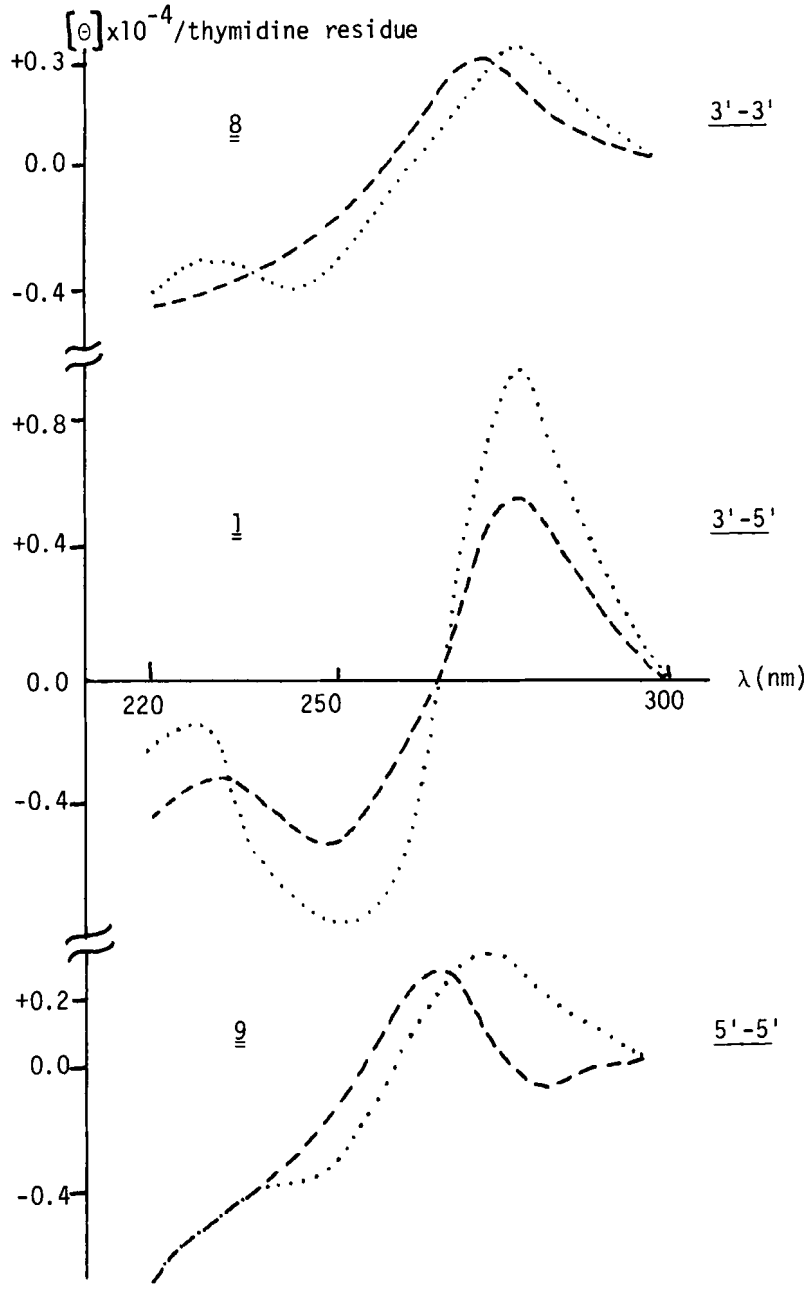


FIG.1. CD spectra of TpT-amides 8, 1 and 9 (---) and the respective TpTs (...) in 0.01 M NaH_2PO_4-Na_2HPO_4 + 0.1 M $NaClO_4$, pH 7.5, buffer, at room temperature.

11.00 ppm observed for 5'-O-trityl-thymidylyl-(3'-5')-3'-O-acetyl-thymidine (P-N) amide diastereoisomers, in pyridine[10]. Similarly to isomeric dinucleoside phosphates[7], the 5'-5' isomer 9 had the relatively largest and the 3'-3' isomer 8 had the relatively smallest downfield chemical shift. - In D_2O, only one single signal near 12.00 ppm can be observed for 1 (see also ref. 1). The rapid exchange of amide hydrogens with those of water[11], may contribute to the apparent coincidental equality of the ^{31}P chemical shifts of the diastereoisomers of 1.

Acid hydrolysis of TpT-amides 1, 8 and 9 resulted in the formation of TpTs, as expected on the basis of the sensitivity of P-N bonds towards acid[12]. The phosphodiester-amide bond of 1, 8 and 9, is, however, more resistant towards acid than the phosphomonoester-amide bond of thymidine phosphoramidates and the phosphomonoester-diamide bond of thymidine phosphorodiamidates. For example, the latter compounds were quantitatively hydrolyzed to thymidine phosphates in 50% aqueous acetic acid, at 50°C, within 5 h[13]. At the same time, more than 95% of 1, 8 and 9 remained unaltered under identical conditions, and more vigorous conditions (1.0 N HCl, 25°C, 22 h) were necessary for their complete conversion to TpTs. A similar phenomenon, the decreased reactivity of phosphodiester morpholidates compared to phosphomonoester morpholidates, has recently been reported[14].

Alkaline hydrolysis

The alkaline hydrolysis was studied in 0.1 N NaOH, at room temperature. Complete conversion of 1, 8 and 9 to a mixture of 4 and 6 and/or 7, was observed in 7 h. Hydrolysis mixtures were separated by ion-exchange (4 from 6+7) and subsequent partition column chromatography (6 from 7), and the molar quantities of hydrolysis products were determined by UV spectrophotometry.

As shown in TABLE 1, practically exclusive formation of 6 from 8 and that of 7 from 9 occurred, while an approximately 1:1 mixture of 6 and 7 was formed from 1. The ratio of 4 to 6+7 was 1.00 ± 0.09.

These results clearly show, that neither the 3'- nor the 5'-hydroxyl groups take part in the alkaline hydrolysis of 1, 8 and 9. If we accept that the alkaline hydrolysis of these compounds proceeds according to an $S_N1(P)$-mechanism, it means that the 3'- and the 5'-

TABLE 1

Molar percentages of thymidine (4), thymidine 3'-phosphoramidate (6) and thymidine 5'-phosphoramidate (7) formed during the alkaline hydrolysis of isomeric TpT-amides 8, 1 and 9.

TpT-amide	Hydrolysis products		
	4	6	7
8 (3'-3')	52	47.5	0.5
1 (3'-5')	52	23	25
9 (5'-5')	49	1	50

hydroxyl groups cannot act as intramolecular traps for metaphosphorimidate intermediates 3 and 2, i.e. the interconversion of 2 and 3 via 5, does not occur. At the same time, the participation of the 3'- and 5'-hydroxyl groups in the alkaline hydrolysis of the phenyl esters of isomeric TpTs, going on very probably according to an $S_N2(P)$-mechanism, is well known[15]. This observation indirectly supports our previous assumption, that the alkaline hydrolysis of 1, 8 and 9 is an $S_N1(P)$-type process.

EXPERIMENTAL

Materials. Isomeric TpTs were of generous gifts of Dr. A. Simoncsits (Institute of Genetics, Biological Research Centre). DMF was distilled from P_2O_5 and stored over 4Å molecular sieve. Chemicals were of reagent grade, H_2O was deionized.

Methods. Evaporations were carried out using a rotary evaporator (Rotavapor, Büchi), at 0.2 kPa with a bath temperature of 30°C. - Absorption spectra were recorded on a Cary 15 spectrophotometer at pHs 2.0 (0.01 N HCl), 7.0 (0.2 M sodium phosphate buffer) and 11.0 (0.003 N NaOH). Molar quantities of compounds were determined by UV spectrophotometry at 260 nm and pH 7.0 by using the following molar extinction coefficients: 16.800[16] (for 3'-3' and 3'-5' TpTs[17], 8 and 1), 16.200[15] (for 5'-5' TpT and 9) and 8700[16] (for 4, 6 and 7). - CD spectra were obtained on a JASCO J-40C spectropolarimeter in 0.01 M NaH_2PO_4-Na_2HPO_4 + 0.1 M $NaClO_4$, pH 7.5, buffer, at room temperature. - ^{31}P NMR spectra

were taken at 32.2 MHz on a Varian FT-80A NMR spectrometer, using external H_3PO_4, as reference. Positive chemical shift values are given for compounds that absorb at lower fields than H_3PO_4 does. - Column chromatography was performed in the following systems: D1, D2 and D3, column: DEAE-cellulose /DE-32, Whatman, HCO_3^- form, 1.6x53.0 cm (D1 and D2) or 1.4x30.0 cm (D3)/, eluent: linear gradient of 0.0-0.15 M (D1) or 0.0-0.30 M (D2 and D3) aqueous triethylammonium hydrogencarbonate, pH 7.5 /2000 mL (D1) or 1000 mL (D2 and D3), for D2 the column was washed with 130-150 mL H_2O before the gradient/, elution rate: 18.0 mL (D1) or 13.2 mL (D2) or 12.0 mL (D3)/20 min/fraction; C1 and C2[13], column: cellulose /CC 31, Whatman, 2.2x40.0 cm (C1) or 1.4x30.0 cm (C2)/, eluent: n-butanol - ethanol - 0.1 M aqueous triethylammonium hydrogencarbonate, pH 7.5 (16:2:5, v/v), elution rate: 4.5 mL (C1) or 2.5 mL (C2)/20 min/fraction. - TLC was performed on precoated cellulose chromatosheets (Cellulose F_{254}, Merck) in n-butanol - 5.0 M aqueous acetic acid (2:1, v/v)[15]. Spots were visualized by UV absorption.

General procedure for the preparation of isomeric thymidylyl-thymidine (P-N) amides 1, 8 and 9.

TpT (about 0.2 mmol) was purified by column chromatography, first in system D1 then in C1 to obtain a TLC homogeneous triethylammonium salt free of the other two isomers. R_f values were: 0.41 (3'-3' TpT), 0.31 (3'-5' TpT) and 0.24 (5'-5' TpT).

The TpT purified above (0.1 mmol) was dissolved in DMF (1.0 mL) containing tri-n-butylamine (47.6 µL, 0.2 mmol). This solution was dropped into a vigorously stirred solution of TPS (60.4 mg, 0.2 mmol) in DMF (1.0 mL), at room temperature, for 1 min. Stirring was continued with the exclusion of atmospheric moisture for an additional 5 min. Then the solution was quickly poured into ice-cold 7.0 N NH_4OH (10 mL) under vigorous stirring. The mixture was evaporated to dryness. The residue was dissolved in H_2O (50 mL), and the solution was extracted with ether (2x20 mL). The aqueous phase - after concentration to 5-10 mL - was chromatographed in system D2. Altogether about 1400-1500 $A_{260}^{pH=7}$ units of UV absorbing materials were present in the aqueous phase. Four peaks emerged from the column. Peak I (<1% of total A_{260} units separated) and peak II (39-49%) were eluted by H_2O in fractions

4-6 and 7-12, respectively. Peak III (47-58%) and peak IV (3-4%) appeared in the gradient fractions 18-27 and 28-36, respectively. The recovery was near quantitative. Peaks I and IV were not identified and discarded. Peak III was TpT, as proved by TLC comparison with the starting material. Peak II was evaporated to dryness. The residue was dissolved in ethanol - H_2O (1:1) mixture (200 μL). The solution was diluted with the eluent of system C1 (200 μL) and was chromatographed in the same system. Besides several small peaks, one large peak (85-90% of total A_{260} units separated) covering 6-10 fractions between the fractions 25-45, was eluted. The exact position of this peak was determined by the 8>1>9 relative elution order of isomers. The peak was pooled and evaporated to dryness. Triethylammonium hydrogencarbonate was removed by repeated evaporation with H_2O. The residue was dissolved in H_2O (1-2 mL) and freeze-dried to yield 12-16 mg (22.0-29.3%) of white, TLC homogeneous solid. R_f values were: 0.57 (8), 0.50 (1) and 0.44 (9).

^{31}P NMR (DMSO-d_6) δ(ppm): 10.37 (8), 10.98 and 11.11 (two signals of approximately equal intensity, 1) and 11.67 (9).

(D_2O) δ(ppm): 12.06 (1).

On treatment with 1.0 N HCl, at room temperature, the product was quantitatively converted to the starting TpT in 22 h. Decomposition was less than 5% in 50% aqueous acetic acid, at 50°C, during 5 h, as detected by TLC in both cases.

Alkaline hydrolysis of 1, 8 and 9.

0.02-0.03 M solutions of 1, 8 and 9 in 0.1 N NaOH were hydrolyzed at room temperature, for 3 h. 50-100 A_{260} units of compounds were used for each experiment. After a tenfold dilution with H_2O, the hydrolysates were chromatographed in system D3. Two peaks emerged from the column in fractions 4-6 (4) and 14-19 (6 and 7). The second peak was evaporated to dryness. The residue was dissolved in ethanol - H_2O (1:1) mixture (50-100 μL). The solution was diluted with the eluent of system C2 and chromatographed in this system. The compounds emerged from the column in the order of 6>7, between fractions 30-50). The structure of 6 and 7 was verified by TLC comparison with authentic samples[18]. Molar percentages of hydrolysis products are presented in TABLE 1.

ACKNOWLEDGMENTS

The author thanks Dr. A. Simoncsits (Institute of Genetics, Biological Research Centre, Hungarian Academy of Sciences) for the preparation of isomeric TpTs, Dr. K. Nelson (Department of Chemistry, University of Utah) for the ^{31}P NMR measurements and Ms. E. Rádi for the excellent technical assistance.

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Received October 22, 1982