SIMULTANEOUS FORMATION OF PEPTIDES AND NUCLEOTIDES FROM N-PHOSPHOTHREONINE

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Abstract. An intramolecular mutual activation between a phosphoryl group and carboxyl group results in the simultaneous formation of nucleotides and peptides by the reaction of nucleosides with N-(O,O-diisopropyl)phosphothreonine in anhydrous pyridine. These results suggest pathways for the simultaneous prebiotic synthesis of peptides and oligonucleotides.

1. Introduction

How proteins and nucleic acids originated under prebiotic conditions is still under investigation. Initially it was felt that peptides formed under prebiotic conditions (Miller and Orgel, 1974; Fox *et al.*, 1977; Kovacs and Nagy, 1961; Hushof and Ponamperuma, 1976); some polypeptides formed on heating amino acids in the absence of water; microspheres formed when an excess of one amino acid, such as glutamic acid, was fused with much smaller accounts of the other amino acids (Rolfing and Fox, 1969). From these data, it was concluded that life might have begun with polypeptides. Early in the 1960's, it was proposed that life on earth was initiated by the formation of RNA (Rich, 1962; Crick, 1968; Orgel, 1968). This proposal was not considered seriously until Cech and his coworkers found that a *Tetrahymena* RNA was capable of cleaving and ligating itself *in vitro* (Cech *et al.*, 1981). This discovery was followed by the observation of the broad range of RNA catalytic activity such as cleavage of DNA, catalysis of amide bond formation and cleavage of amide bond (Herschalg and Cech, 1990; Morl *et al.*, 1992; Noller *et al.*, 1992; Dai *et al.*, 1995). These findings challenged the concept that proteins alone catalyzed reactions in living systems. Consequently, the hypothesis that life might have begun with the 'RNA world' became more appealing.

In our previous research, it was found that N-(O,O-dialkyl) phosphoamino acids could be synthesized in aqueous-organic solutions from the reaction of dialkylphosphite and amino acids (Ji *et al.*, 1988). The N-(O,O-dialkyl) phosphoamino acids (DAP-aas) self-condensed to form phosphoryl peptides (Zhao *et al.*, 1995). In addition, the phosphorus of DAP-aa could exchange its ester groups with alcohol (Tan and Zhao, 1995). If there was a hydroxyl group in the DAP-aa, an intramolecular phosphoryl group migration from nitrogen to oxygen occurred (Yin *et al.*, 1994).

Since dialkylphosphite can be obtained by the hydrolysis reaction of $P(OR)_3$, which in turn might result from the reaction of PCl_3 with alcohol at slight basic

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pH and low temperature, N-(O,O-dilalkyl)phosphoamino acids might have been formed on the primitive earth. It is of importance to investigate the reaction between N-phosphoamino acids and some biomolecules. In this paper, the reaction between N-(O,O-diisopropyl) phosphothreonine (DIPPThr) and four nucleosides (adenosine, uridine, cytidine and guanosine) at room temperature in anhydrous pyridine is reported and the structures of the reaction products were determined by FAB-MS, HPLC, UV, CE-MS techniques. The results show that the peptides and nucleotides were formed simultaneously, a finding which suggests that peptides and oligonucleotides may have been formed simultaneously on the primitive earth.

2. Results and Discussion

2.1. HOMOPEPTIDE FORMATION

In general, amino acids themselves and their cBz-, Boc-protected or N-sulfonated derivatives are chemically stable. However, when amino acids are N-phosphorylated, the phosphoryl group activates the carboxyl group, and homo- and hetero-peptides are formed by the reaction of the DAP-aa in aqueous and organic media (Li *et al.*, 1992a; Ju *et al.*, 1995; Zhao *et al.*, 1995).

2.1.1. *Peptide Yields*

Since neither pure DIPPThr nor the nucleosides (A/U/C/G) themselves give a positive biuret reaction, the total yield of peptides produced in the reaction of DIPPThr with nucleosides can be determined by the biuret reaction without interference from the starting compounds. The reaction mixture formed on mixing DIPPThr with the nucleosides A, U, C or G gave a positive biuret test. The total peptide yields in the reactions were obtained by measuring the absorbance at 540 nm. The relative yields were calculated in reference to the absorbance at 540 nm for a standard glycyl glycine using the biuret procedure (Table I). The yield of N-(O,O-diisopropyl)phosphorylated oligopeptides of threonine was slightly higher in the presence of uridine and cytidine, as compared to the yield of oligopeptides when no nucleoside was added (Table I). Adenosine had no effect on the yield of oligopeptides while guanosine depressed the yield.

2.1.2. *Structure Analysis by FAB-MS*

The N-(O,O-dialkyl) phosphorylated oligopeptides showed intense molecular ions or quasi-molecular ions together with the successive alkene loss fragment ions in FAB-MS spectra. The masses of the ions afforded multiple checks of the molecular masses of the compounds (Yang *et al.*, 1992; Yin *et al.*, 1994). The masses of the peptides produced in the reaction of DIPPThr with nucleosides were also measured by positive and negative FAB-MS in order to establish their structures. Based on molecular ion peaks or quasi-molecular ion peaks and/or their corresponding

Table I

Relative yields of peptides and N-(O,O-diisopropyl)-phosphothreonine peptides after 50 h incubation in anhydrous pyridine at 40 °C

Peptides derived from	Relative yield (%)[a]
DIPPThr	6.52
DIPPThr + adenosine	6.52
DIPPThr + uridine	7.66
DIPPThr + cytidine	6.91
DIPPThr + guanosine	4.45

[a] Relative yields were calculated in reference to the absorbance at 540 nm by biuret test with the standard glycylglycine.

isopropene loss fragment ions (M–42) and dehydration fragment ions (M–18), the structures of the homopeptides derived from DIPPThr were established (Table II).

2.2. NUCLEOTIDE FORMATION

2.2.1. *Identification of 5′-nucleotides by HPLC*

In addition to the masses assigned the peptides by the FAB-MS spectra, there were other ion peaks which were assigned to the phosphorylated nucleosides formed by the ester exchange between DIPPThr and the hydroxyl groups of corresponding nucleosides (Table III). These compounds were formed by the replacement of one isopropyl group by the 5′-hydroxyl of the nucleoside to produce the 5′-phosphorylated nucleoside. The structures of the 5′-nucleotides were determined by their retention times on reverse phase HPLC. The presence of the 5′-mononucleotide was established by coinjection of the product mixture with authentic samples of the 5′-nucleotides. There were at least six peaks possibly related to the nucleotides in the products from the reaction of DIPPThr with adenosine as determined from the peaks when the HPLC eluate was monitored at 254 nm (Figure 1).

2.2.2. *Separation and Characterization of Products by Capillary Electrophoresis and UV Spectra*

Further effort was made to determine the structures of other possible nucleotide products detected by FAB-MS and HPLC. The products from the reaction of DIPPThr with uridine was separated by capillary electrophoresis (CE) (Figure 2); peak 1 and peak 13 were confirmed to be uridine and 5′-UMP respectively by coinjection with authentic standards. Other peaks were characterized by their UV absorbance at 190 nm ~ 350 nm (Figure 3). By comparison with the UV spectra of reactants DIPPThr and uridine (Figure 3A), it was established that all products had UV spectra that were similar to the reactant uridine (Figure 3B, C, D). Because

Table II
Positive and negative FAB-MS data for the main peptides formed (The relative intensities are given in parenthesis.)

Products	Reactions			
	DIPPThr + A	DIPPThr + U	DIPPThr + C	DIPPThr + G
$(Thr)_2$	220 (M+1, 2.0%)	219 (M–1, 20%)	219 (M–1, 6.0%)	219 (M–1, 16%)
	203 (M–18+1, 1.5%)	201 (M–18–1, 20%)	201 (M–18–1, 10%)	201 (M–18–1, 20%)
$(Thr)_3$	322 (M+1, 5.0%)		321 (M, 10%)	321 (M, 20%)
$(Thr)_4$	423 (M+1, 2.5%)		421 (M–1, 4.0%)	
	405 (M–18+1, 4.0%)			
DIPP $(Thr)_2$	385 (M+1, 3.5%)	384 (M, 6.0%)	383 (M–1, 11%)	383 (M–1, 10%)
	343 (M–42+1, 23%)	342 (M–42, 4.0%)	341 (M–42–1, 8.0%)	341 (M–42–1, 10%)
DIPP $(Thr)_3$	486 (M+1, 3.5%)		484 (M–1, 3.0%)	484 (M–1, 10%)
	402 (M–42*2+1, 5.0%)			442 (M–42–1, 3.0%)
DIPP $(Thr)_4$	587 (M+1, 2.5%)	585 (M–1, 4.0%)		585 (M–1, 3.0%)
				543 (M–42–1, 3.0%)

Table III

Negative FAB-MS data for the main nucleotides formed (The relative intensities are given in parenthesis.)

Products	Reactions			
	DIPPThr + A	DIPPThr + U	DIPPThr + C	DIPPThr + G
XMP	346 (M–1, 2.0%)	323 (M–1, 2.0%)	322 (M–1, 2.0%)	363 (M, 40%)
IsoPrO-XMP	388 (M–1, 3.0%)	365 (M–1, 5.0%)	364 (M–1, 15%)	404 (M–1, 5%)
	346 (M–42–1, 1.0%)			
XMP-Thr		424 (M–1, 1.0%)	423 (M–1, 20%)	465 (M, 9.0%)
				447 (M–18, 8.0%)
XpX	595 (M–1, 1.0%)	549 (M–1, 1.0%)	548 (M–1, 7.0%)	
$(Xp)_2$		629 (M–1, 1.0%)	628 (M–1, 3.0%)	
$(Xp)_2$-Thr		731 (M–1, 1.0%)		

Note: X represent A, U, C, G respectively.

the nucleoside, nucleotides and homooligonucleotides with the same base usually gave the similar UV spectra (Dunn and Hall, 1975), the products separated by CE were determined to be nucleotide species containing uracil.

2.2.3. *Structures Determined by Capillary Electrophoresis Electrospray Ionization-Mass Spectrometry (CE-ESI-MS)*

In order to get more structure information on the products, the CE-ESI-MS technique was used to analyze products of the reaction of DIPPThr with uridine. First the product mixture was separated by CE, then each separated product was flowed into the coupled Mass Spectrometry (MS) via a sheathflow Electrospray Ionization (ESI) interface. In this way, each product could be directly checked by the MS to obtain its m/z value (Figure 4). Based on the mass chromatography of each separated product (Figure 4, from top to the next to last box), the existence of peptides, nucleotides and other compounds were established (Table IV). These dates showed that in addition to the 5′-UMP, there were also the dimers UpU and $(Up)_2$, the complex $(Up)_2$ -Thr and diisopropylpyrophosphate.

2.3. COUPLING OF PEPTIDE AND NUCLEOTIDE SYNTHESIS

The reaction pathway for N-phosphoamino acids was suggested to proceed via a penta-coordinate transient transition state (Li *et al.*, 1992a, b) on the basis of studies of transition state analogues (Wang *et al.*, 1995). The carboxyl group forms an activated mixed anhydride with the phosphate and then the reaction proceeds via a pentacoordinate transition state. In the reaction of DIPPThr with uridine, the peptides were formed via this transition state and elongated according to the '1 + n' mechanism (Zhao *et al.*, 1995). In the presence of the uridine, DIPPThr reacts with the 5′ primary hydroxyl group of uridine to yield the monoester exchange product **1**.

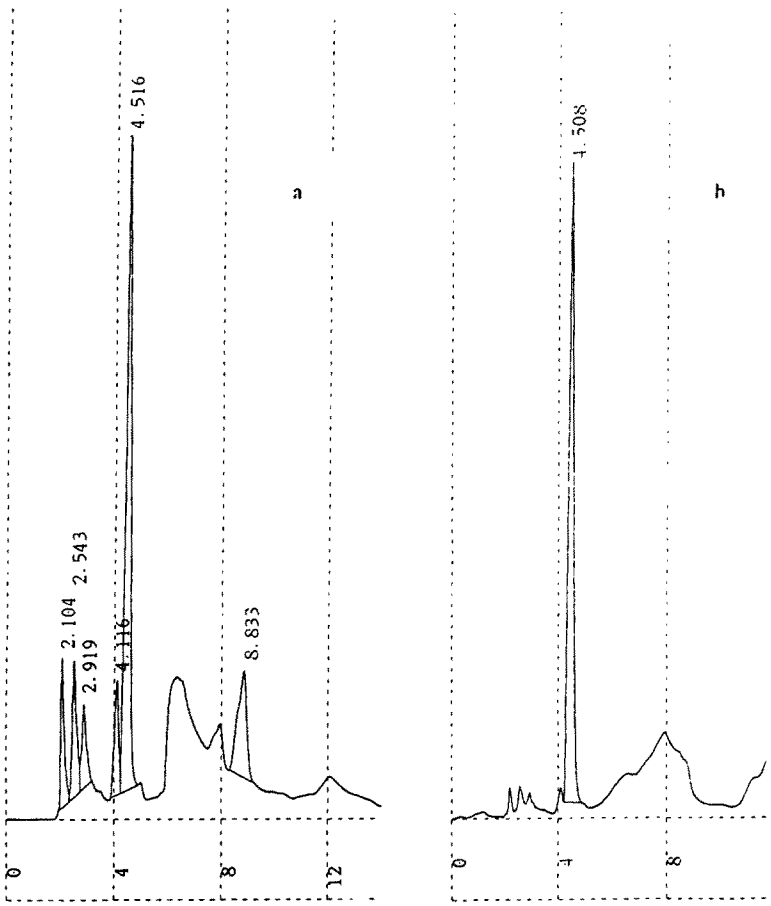


Figure 1. HPLC for (a): the products of the reaction of DIPPThr with adenosine; (b): the authentic 5′-AMP added to the reaction products. The retention time 4.516 min indicated the presence of 5′-AMP as a reaction product.

Hydrolysis of compound **1** results in the loss of threonine to give **2**, and isopropanol to give 5′-UMP (**3**) (Scheme 1). The DIPPThr can also exchange two ester groups with 2′- and 3′-hydroxyl groups of ribose to produce N-cyclophosphothreonine (**4**). Products **5** and **6** were obtained through the nucleophilic attack by the 5′-primary hydroxyl group of uridine on **4**. This results in the synthesis of the oligonucleotides.

In these processes, the N-phosphothreonine could not only react to form phosphopeptides but also exchange its ester groups with the 5′ hydroxyl group and 2′,3′-hydroxyl groups of nucleosides to form the nucleotides (Scheme 2). This shows that both peptide and nucleotide formation are driven by N-phosphothreonine.

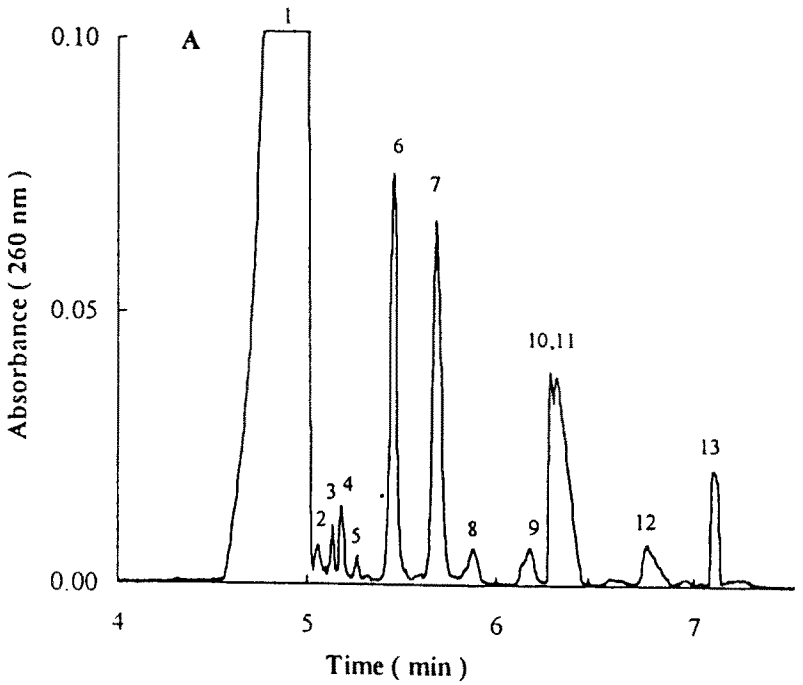


Figure 2. Capillary electrophoresis (CE) of the reaction products from the reaction of DIPPThr with uridine. Peak 13 is 5′-UMP and Peak 1 is uridine.

2.3.1. *The evidence for peptide and nucleotide formation in aqueous media*
When the N-phosphoalanine, uridine mixture was incubated in water/pyridine (7:1) solution at 40 °C for 72 h, the peptides and nucleotides were also detected by FAB-MS in the reaction system. Further investigation of the reaction in aqueous media will be continued.

3. Conclusion

N-phosphothreonine, due to the presence of the phosphoryl group and carboxyl group, can react to form peptides and nucleotides. These results might give insight into a possible interrelationship between the synthesis of peptides and oligopeptides on the prebiotic earth.

4. Experimental

4.1. General procedures

Capillary Electrophoresis: P/ACE™ system 5510 (Beckman, Palo Alto, CA, USA) automated CE instrument with a diode array detector (190~600 nm). P/ACE™

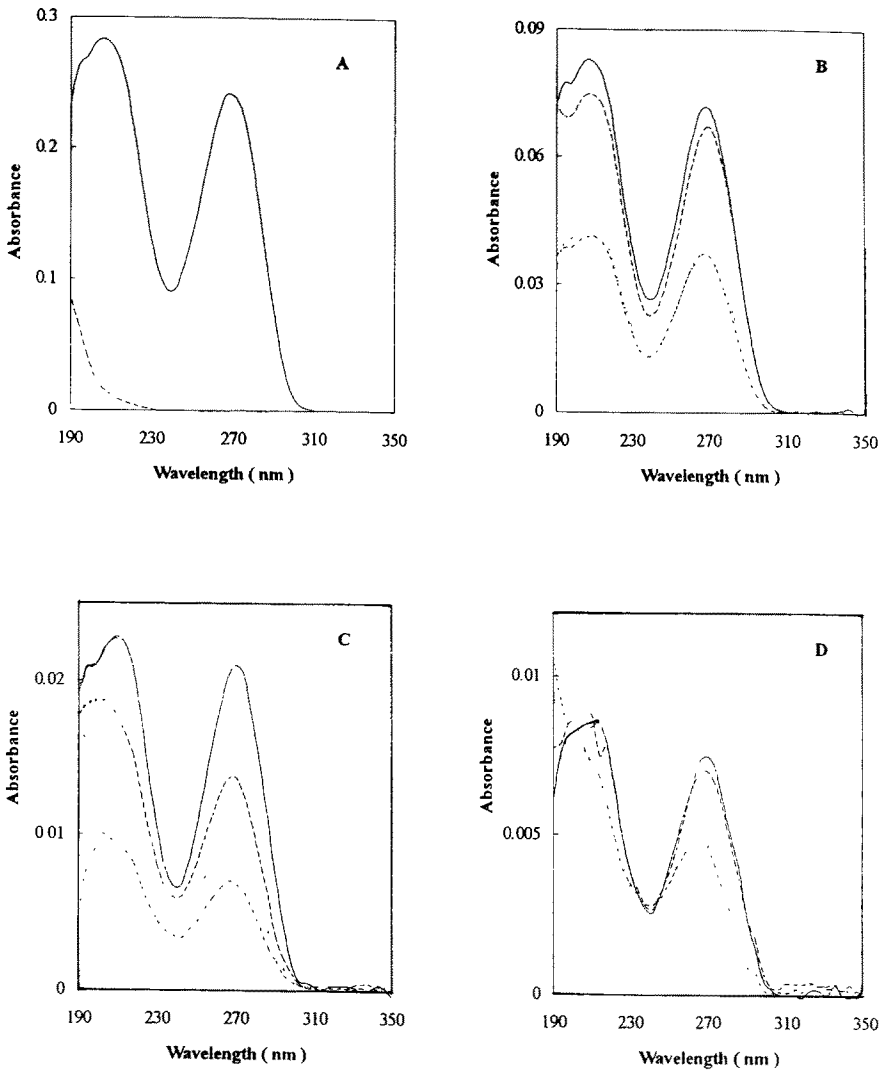


Figure 3. (A): the UV spectra of reactants. (—) uridine and (- -) DIPPThr. (B, C, D): the UV spectra of products separated by CE in Figure 2. (B): (—) peak 6, (- - -) peak 7, (· · ·) peak 11, (-· -·) peak 10; (C): (—) peak 13, (- - -) peak 8, (· · ·) peak 9, (-· -·) peak 12; (D): (—) peak 4, (- - -) peak 3, (· · ·) peak 2, (-· -·) peak 5.

system 5000 automated CE instrument coupled to a SSQ–710 system single quadruple mass spectrometer (Finnigan MAT, USA) with a sheathflow Electrospray Ionization interface. MS: Finnigan MAT 90 MS double focusing magnetic mass spectrometer. HPLC: Schimadzu L–2C, NMR: Bruker AC200p NMR spectrometry.

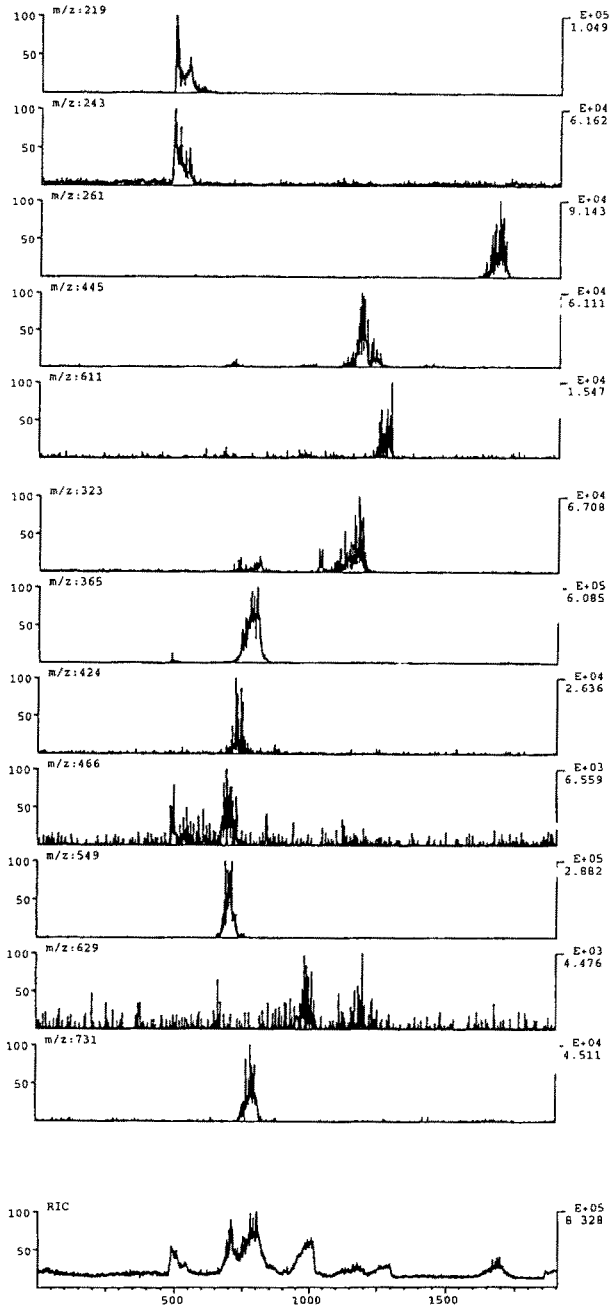
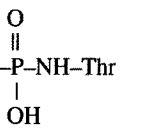
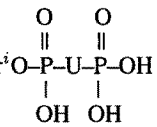


Figure 4. Rebuilt ion chromatogram (bottom graph) and mass chromatography of the separated products (top 12 graphs) from the reaction of DIPPThr with uridine. The horizontal scale is the scan numbers and vertical scale is the intensity of the molecular ions. The possible structure of each molecular ion peak was suggested in Table IV.

Table IV

The structure for each molecular ion peak in the mass chromatography given in Figure 4

m/z	Intensity	Structure
219	1.049 (E+05)	Thr-thr
243	6.162 (E+04)	Uridine
261	9.143 (E+04)	Diisopropylpyrophosphate
323	6.708 (E+04)	**3** 5′-UMP
365	6.085 (E+04)	**2**
424	2.636 (E+04)	
445	6.111 (E+04)	
466	6.559 (E+03)	**1**
549	2.882 (E+05)	**5** 3′,5′- and/or 2′,5′-UpU
611	1.547 (E+04)	Unknown
629	4.476 (E+03)	3′,5′- and/or 2′,5′-UpUp
731	4.511 (E+04)	$(Up)_2$-Thr

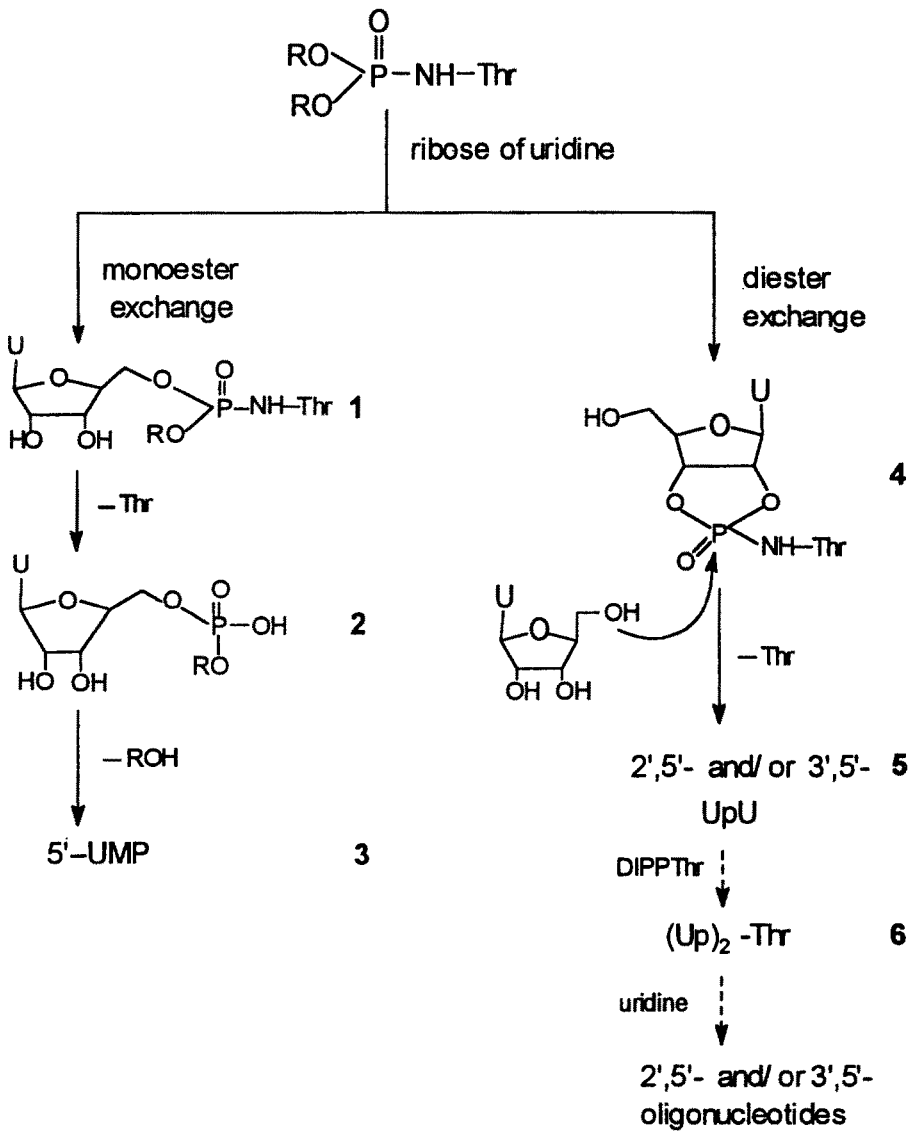
4.2. Preparation of N-(O,O-diisopropyl) phosphothreonine

N-phosphothreonine was synthesized according to the literature (Ji *et al.*, 1988). The solution of 10 mmol threonine in Et_3N (5 ml)/H_2O (10 ml)/EtOH (5 ml) was cooled to 0 °C. A mixture of diisopropyl phosphite (10 mmol) and CCl_4 (10 ml) was added dropwise and the mixture was kept stirring at 20 °C for 4 h. The reaction was quenched by acidifying the mixture to pH 3~4 with dilute HCl. The mixture was then extracted with EtOAc: t-Butyl alcohol (2:3) (5 × 20 ml), the extract was dried with $MgSO_4$, the solvent evaporated, and the oily residue purified by crystallization from EtOAc/petroleum ether.

All physical constants and spectroscopic data of the products were consistent with the literature values (Xue *et al.*, 1988).

4.3. Reaction of N-phosphothreonine with nucleosides

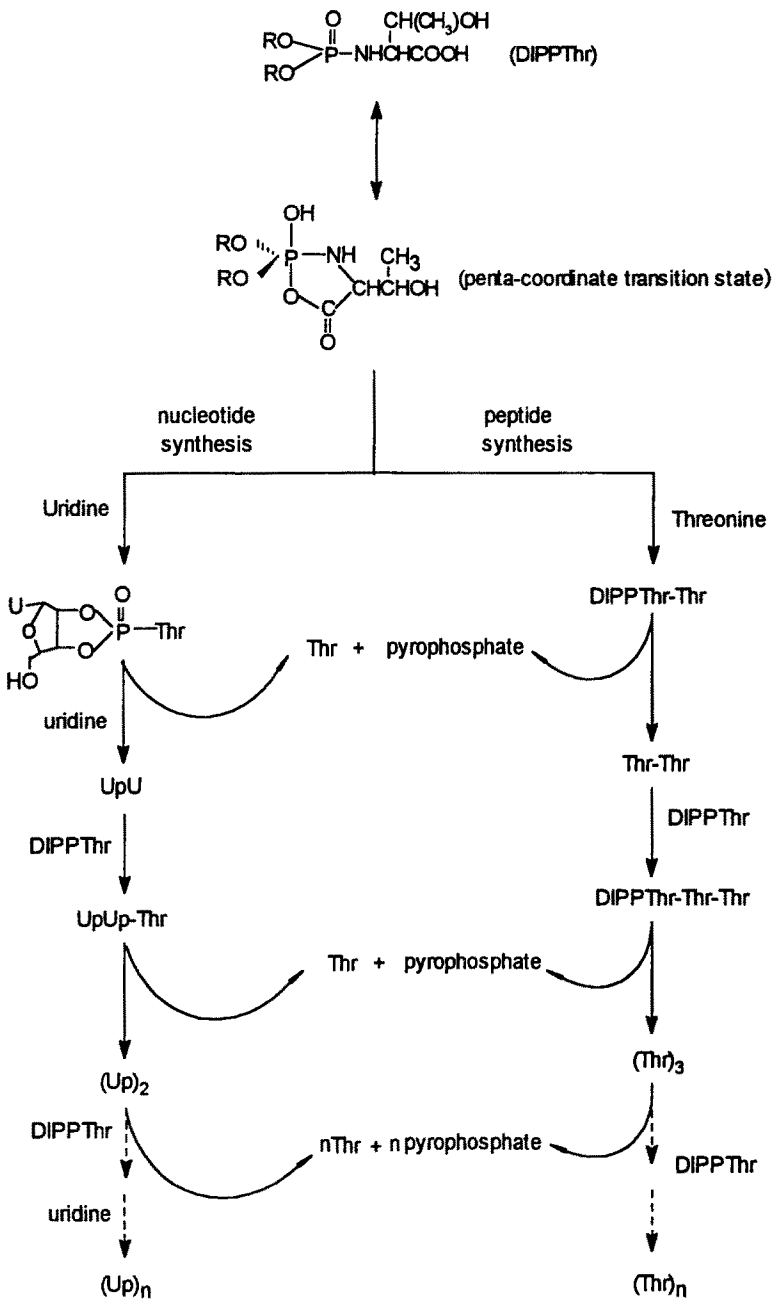
1 g of nucleoside (A/U/C/G) and 1 g of DIPPThr were stirred in anhydrous pyridine at 30 °C for a week. The pyridine was removed from product mixture under vacuum. The product mixture then were sent for FAB-MS, HPLC, CE, CE-MS.



Scheme 1 Proposed pathway for nucleotide formation in the reaction of N-phosphothreonine with uridine.

4.4. PREPARATION OF THE BIURET REAGENT AND PEPTIDE ANALYSIS

1.5 g $CuSO_4 \cdot 5H_2O$ and 6.0 g $KNaC_4H_4O_6 \cdot 4H_2O$ were dissolved into 500 ml double-distilled water. To it 300 ml 10% NaOH solution was added, followed by dilution to 1000 ml with double-distilled water, and 1 g KI was added to prevent Cu^{2+} from being reduced into Cu_2O. To a tube containing 4 ml biuret reagent



Scheme 2 Formation of nucleotides and oligopeptides. Pyrophosphate is diisopropylpyrophosphate.

added 0.1 ml of the reaction product mixture and the absorbance at 540 nm was recorded on UV 751G (made in Shanghai, China).

4.5. HPLC CONDITIONS

The reaction products were separated on a Zorbax ODS C_{18} column, 4.6 × 250 mm, UV detector set at 254 nm, using a mobile phase of 4% MeOH + 0.1 M NaH_2PO_4 pH 6, absorbance 0.08, flow of 0.8 ml/min at 25 °C.

4.6. CE CONDITIONS

The capillary was 27 cm in total length and 20 cm from the inlet to the detection window. Running buffer was 50 mM boric acid solution adjusted to pH 8.5 with 0.2 M NaOH. Injection was achieved by applying 3.0 KPa pressure at the inlet for 1 second, and then separation was performed at 10 Kv (370 v/cm) at 25 °C. The detector was set at 260 nm.

4.7. CE-ESI-MS CONDITIONS

An 80 cm capillary was used. Injection was achieved by applying 3.0 KPa pressure at the inlet for 5 sec. Running solution was 0.1 M pH 7.5 Tris buffer. Separation was performed at 20 Kv (250 v/cm) at 25 °C. ESI-MS was conducted in negative ion mode with a sheath liquid of isopropanol: water:acetic acid (60:40:1, v/v/v) delivered at 3 μl/min. The scan range was 100–750 amu at 100 amu/s.

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References

Cech, T. R., Zaug, A. T. and Grabowski, P. J.: 1981, *Cell* **27**, 487–496.
Crik, F. H. C.: 1968, *J. Mol. Biol.* **38**, 367.
Dai, X., Mesmaeker, A. D. and Joyce, G. F.: 1995, *Science*, 237–239.
Dunn, D. B. and Hall, R. H.: 1975, *CRC Handbook of Biochemistry and Molecular Biology*, CRC press, Cleveland, p. 65.
Fox, S. W. and Dose, K.: 1977, *Molecular Evolution and the Origins of Life*, 2nd, Marcel Dekker, New York.
Herschlag, D. and Cech, T. R.: 1990, *Nature* **344**, 405–409.
Hulshof, J. and Ponamperuma: 1976, *Orig. Life. Evol. Biosphere* **7**, 197.
Ji, G. J., Xue, C. B., Zeng, J. N., Li, L. P., Chai, W. G. and Zhao, Y. F.: 1988, *Synthesis* **6**, 444.
Ju, Y., Zhao, Y. F., Sha, Y. W. and Tan, B.: 1995, *Phosphorus Sulfur Silicon* **101**, 117.
Kovacs, J. and Nagy, H.: 1961, *Nature* **19**, 531.
Li, Y. M., Yin, Y. W. and Zhao, Y. F.: 1992a, *Int. J. Peptide Protein Res.* **39**, 375.
Li, Y. M., Zhang, D. Q., Zhang, H. W., Ji, G. J. and Zhao, Y. F.: 1992b, *Bioorganic Chemistry* **20**, 285–295.

Miller, S. L. and Orgel, L. E.: 1974, *The Origins of Life on the Earth*, Prentice Hall Inc., Englewood Cliffs, New Jersey, U.S.A.
Morl, M., Niemer, I. and Schmeltzer, C.: 1992, *Cell* **70**, 803–810.
Noller, H. F., Hoffarth, V. and Zimniak, L.: 1992, *Science* **256**, 1416–1419.
Orgel, L. E.: 1968, *J. Mol. Biol.* **38**, 381.
Rich, A.: 1962, M. Kasha and B. Pullman (eds.), *Horizons in Biochemistry*, Academic Press, p. 103.
Rolfing, D. L. and Fox, S. W.: 1969, *Advances in Catalysis* **20**, 373.
Tan, B. and Zhao, Y. F.: 1995, *Chinese Youji Huaxue* **15**, 30–34.
Wang, Q., Zhao, Y. F., An, F. L., Wang, Q. Z. and Mao, Y. Q.: 1995, *Science in China (Series B)* **25**(7), 683–689.
Xue, C. B., Yin, Y. W. and Zhao, Y. F.: 1988, *Tetrahedron Lett.* **29**, 1145–1148.
Yang, H. J., He, M. Y., Ye, Y. H. and Zhao, Y. F.: 1992, *Org. Mass Spectrometry* **27**, 746–749.
Yin, Y. W., Ma, Y., Zhao, Y. F., Li, Y. F., Feng, Y. P. and Mao, Y. Q.: 1995, *Science in China (Series B)* **25**(5), 472–479.
Yin, Y. W., Zhang, B. Z., Chen, Y. and Zhao, Y. F.: 1994, *Chinese Kexue Tongbao* **39**, 333.
Yin, Y. W., Zhao, Y. F., Chen, Y., Ma, Y., In, B. and Wang, G. H.: 1994, *Science in China (Series B)* **24**(9), 904–913cccc.
Zhao, Y. F., Ju, Y., Li, Y. M., Wang, Q., Yin, Y. W. and Tan, B.: 1995, *Int. J. Peptide Protein Res.* **45**, 514–518.