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Direct Selective Pyrophosphorylation of the Primary Hydroxyl Group in (Hydroxyethyl)thiamin by Modified Phosphoric Acid–Cresol Solutions and Evaluation of Extension of the Method to Nucleosides

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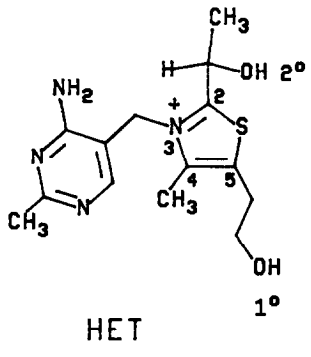
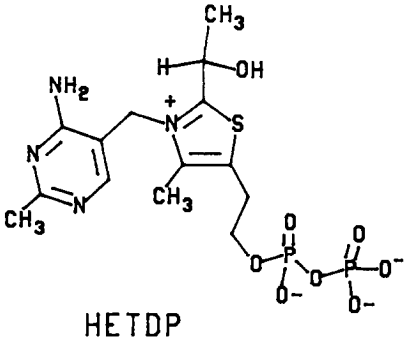
Thiamin is known to be converted to the coenzyme thiamin diphosphate by the action of partially dehydrated phosphoric acid. These conditions cause the unselective reaction of both the secondary and primary hydroxyl groups of 2-(1-hydroxyethyl)thiamin. The addition of *m*-cresol to the reaction solution leads to selective reaction at the primary hydroxyl. The use of the same combination of reagents permits the production of pyrimidine nucleoside 5′-diphosphates from unprotected pyrimidine nucleosides in low yield.

In earlier papers we reported studies which required the conversion of the enantiomers of 2-(1-hydroxyethyl)thiamin (HET)[1] to 2-(1-hydroxyethyl)thiamin diphosphate (HETDP) (*1–3*). The pyrophosphorylation of HET under the conditions used for thiamin (*4*) generated mixtures of products which we now have identified as having pyrophosphate and/or monophosphate groups in place of both



HET



HETDP

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[1] Abbreviations used: HET, 2-(1-hydroxyethyl)thiamin; HETDP, 2-(1-hydroxyethyl)thiamin diphosphate; PHETDP, a derivative of HETDP that is phosphorylated or pyrophosphorylated at the secondary hydroxyl position; PK, pyruvate kinase; LDH, lactate dehydrogenase; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; PEP, phosphoenolpyruvic acid; TMS, tetramethylsilane.

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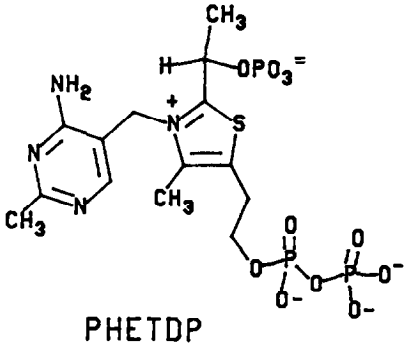

PHETDP

the primary and secondary hydroxyl groups (PHETDP). These materials were separated from the desired product, which was present in small quantities, with considerable difficulty. Since we intended to do further work we sought a method to pyrophosphorylate selectively the primary hydroxyl group.

Poulter and co-workers have shown that pyrophosphates can be made from alcohols by a two-step procedure. First the alcohol is converted to the tosylate (or the chloride if the alcohol is allylic). In the second step, the tosylate or chloride derivative is treated with tetrabutylammonium pyrophosphate (*5*). We were unable to extend this method to thiamin derivatives because the hydroxyl function of thiamin is resistant to such transformations under conditions where the rest of the thiamin molecule is stable. We therefore sought a one-step, regioselective method that would function under acidic conditions where thiamin derivatives are most stable.

It was reported by Gruber and Lynen that pyrophosphoryl tetrachloride in the presence of triethylamine converts 2′,3′-protected adenosine to the corresponding 5′-diphosphate but the reaction gives a mixture of materials in low yield and the product was not isolated (*6*). In addition, HET is not stable under those conditions. Gutowski (*7*) noted that the addition of *m*-cresol to Karrer's (*4*) partially dehydrated phosphoric acid improved the yield of thiamin thiazolone diphosphate from its precursor. Imai reported that the same cosolvent promoted selective production of 5′-monophosphates in reactions of pyrophosphoryl tetrachloride with nucleosides (*8*). We therefore expected that the combination reported by Gutowski might also be regioselective. In this paper we report that partially dehydrated phosphoric acid combined with *m*-cresol produces selective pyrophosphorylation of the primary hydroxyl group in HET. Since such a method might be useful where it is necessary to produce nucleoside 5′-diphosphates from nucleosides in a single step, we did a survey of the reactions of unprotected nucleosides. We find that acid-stable nucleosides give the desired products but in low yield while acid-sensitive nucleosides undergo hydrolysis.

EXPERIMENTAL

Materials. Thiamin chloride hydrochloride (USP) was obtained from Novopharm Limited (Scarborough, Ontario). Nucleosides, nucleotides, *m*-cresol,

Dowex-1-X8, Amberlite CG-50, and enzymes were obtained from Sigma Chemical Co. Phosphoric acid was obtained from Fisher Scientific. Bio-Gel P-2 was from Bio-Rad. Cellulose-coated thin-layer plates were Eastman No. 13254.

Spectra. Phosphorus and carbon NMR spectra were obtained with a Varian XL-200 spectrometer. Proton spectra were obtained on this instrument or a Varian T-60.

Thin-layer chromatography. Two alternative solvent systems were employed to develop the chromatograms: 1% ammonium sulfate : *i*-propanol (1 : 2, v/v) (System A) (*11*); absolute ethanol : *n*-butanol : 0.15 M, pH 4.0, sodium citrate buffer (10 : 1 : 6, v/v) (System B) (*7*).

Preparation of HET. The material was prepared from thiamin and acetaldehyde as reported previously (*1*). Authentic samples of HETDP were prepared by the addition of thiamin diphosphate to acetaldehyde according to Krampitz's procedure (*9*).

Identification of sites of modification of HET after reactions in the absence of m-cresol. This method produced a derivative of HETDP which is phosphorylated or pyrophosphorylated at the secondary hydroxyl position (PHETDP). HET was added to partially dehydrated phosphoric acid as described for the conversion of thiamin to thiamin diphosphate (*2, 4*). The ^{13}C NMR spectrum of material isolated from the reaction was compared to those of HET and HETDP (Table 1).

The spectrum of the reaction product (PHETDP) differs from that of HETDP by the chemical shift and splitting into a doublet of the signal of the methyl carbon attached to the secondary hydroxyl group of the 2(1-hydroxyethyl)side chain attached to C-2. In the spectrum of HETDP, the signal of that carbon is a singlet. In PHETDP we see splitting of peaks due to the presence of ^{31}P. In addition, the

TABLE 1

^{13}C Chemical Shifts in ppm from TMS of HET, HETDP, and PHETDP as Defined in Text

Carbon	TDP	HET	HETDP	PHETDP
C2′	163.7	162.8	162.2	162.3
C4′	163.5	162.2	161.1	160.9
C2	155.6	179.0	177.6	173.5 J = 7.2
C6′	144.8	139.4	139.7	140.0
C4	143.9	144.2	143.3	143.1
C5	136.2	135.0	134.0	133.9
C5′	106.9	109.3	109.1	108.3
CH_2—OR	65.5 J = 4.8	60.8	64.5 J = 3.0	64.3 J = 2.0
N3—CH_2	50.4	47.1	46.1	46.0
C5—CH_2	28.2 J = 5.8	29.6	28.7 J = 6.0	27.8 J = 6.0
C2′—CH_3	21.5	21.2	21.5	21.3
C4—CH_3	11.7	11.5	10.8	11.6
RO—CH—CH_3		64.8	63.9	68.0 J = 4.0
ROCH—CH_3		22.4	22.7	21.6 J = 6.8

Note. Carbon–phosphorus coupling, J (Hz), is listed for peaks which are doublets. Carbon numbers indicated with (′) are in the pyrimidine ring. "R" is H or a phosphate. The spectrum of TDP is included for comparison.

expected splitting of the single carbon attached to the pyrophosphate group of HETDP is also seen in PHETDP. The thiazolium C-2 signal is shifted upfield 5.5 ppm relative to HET and split into a doublet (J = 7.2 Hz). That splitting is typical of a group β to a phosphorus nucleus and this nucleus is too far from the primary hydroxyl group of the C-5 side chain to be affected by a pyrophosphate at that position.

The signal of the 1-carbon atom of the 2-(1-hydroxyethyl) group is a doublet (J = 4.0 Hz) and is shifted downfield 3.2 ppm relative to the same carbon in HET. The methyl group of the 2-(1-hydroxyethyl) substituent on C-2 is also β to the same phosphorus atom. Its signal is shifted upfield 0.8 ppm and is split into a doublet (J = 6.8 Hz). The spectrum of the carbons of the C-5 side chain (1-(2-hydroxyethyl) group) is the same as that in HETDP, showing that the primary hydroxyl group is also phosphorylated. (Phosphorus NMR indicated that the group on the C-2 side chain is a monophosphate and the group on the C-5 side chain is a diphosphate.) Addition of this material to the apoenzyme of pyruvate decarboxylase from wheat germ did not activate the enzyme and inhibited activation by thiamin diphosphate.

A genuine sample of the bis-monophosphorylated material, 2-(1-ethyl-1-phosphate)thiamin monophosphate was prepared through the reaction of pyrophosphoryl tetrachloride with 2-(1-hydroxyethyl)thiamin, following the procedure of Simoncsits and Tomasz (*10*). The ^{13}C NMR spectrum of this material was essentially identical to that of the bis-phosphorylated material PHETDP.

Conversion of HET to HETDP in the presence of m-cresol. HET (0.5 g) was dissolved in 2 ml of *m*-cresol by heating at 70°C with stirring. Phosphoric acid (85%, 1 ml) was heated in a flask with a flame until the solution became cloudy and then was allowed to cool to room temperature. The solution of HET was added and stirred with a glass rod for 30 min at 80°C. After cooling to room temperature, *m*-cresol was decanted. The residue was washed with two 5-ml portions of 95% ethanol. Water (5 ml) was added, and after 5 min stirring, the solution was transferred to a separatory funnel. The reaction vessel was washed with 2.5 ml of water which was added to the funnel. The solution was extracted with two 10-ml portions of ether. The aqueous layer was collected and centrifuged. The supernatant was transferred to a 250-ml flask and 50 ml of a solution of equal parts 95% ethanol and ether was added with stirring. This produced an oil and a cloudy supernatant. The oil was dissolved in 5 ml of water and triturated two times with the ethanol–ether solution. The residue was dissolved in a minimal volume of water and the acidity of the solution adjusted to pH 2 by the addition of sodium hydroxide. The solution was concentrated to about 3 ml and applied to an Amberlite CG-50 column. The mixture on the column was eluted with water and 7-ml fractions were collected. The fractions were analyzed by thin-layer chromatography according to the procedure of Gutowski (*7*) (solvent system B). The thin-layer chromatographic pattern for HETDP was determined using a genuine sample. Fractions 15 through 23 were pure as judged by this method. These were pooled and lyophilized to give 0.15 g of product. Enzymatic and proton NMR analyses of this material were identical to those obtained for HETDP prepared from TDP and acetaldehyde.

Pyrophosphorylation of nucleosides. The phosphoric acid solution with *m*-cresol was prepared as described above. Nucleoside (1.5 mmol) was added as a powder to the solution maintained at 75°C. The reaction mixture was stirred for 30 min and then cooled to room temperature and 5 ml of cold water added. The solution was worked up as for HETDP except with CCl_4 in place of ether. The white precipitate was collected by centrifugation, dissolved in water, and lyophilized. The residual white powder was purified by ion-exchange chromatography using Dowex 1-X8. Fractions were analyzed by thin-layer chromatography and by enzyme assays. Reactions of adenosine, guanosine, thymidine, and deoxyuridine under these conditions led to hydrolysis of the base-sugar linkage (see Results).

Uridine diphosphate. Uridine (360 mg) was subjected to the general reaction sequence described in the preceding section. This yielded between 250 and 300 mg of a mixture of esters of the primary alcohol consisting of oligomeric phosphate anhydrides. The uridine mono-, di-, tri-, and higher oligophosphates in the product mixture were separated by ion exchange chromatography. The preparation (400 mg) was dissolved in 0.5 ml of water and neutralized with dilute sodium hydroxide. The solution was placed on a Dowex-1-X8 column (2.5 × 16.0 cm, chloride form at 4°C) which had been equilibrated with distilled water. The column was washed with water, 0.01 M HCl, 0.015 M NaCl; 0.01 M HCl, 0.10 M NaCl; 0.01 M HCl, 0.25 M NaCl; and 1.0 M HCl. These washes eluted the uridine, uridine monophosphates, uridine diphosphates, uridine triphosphates, and higher order phosphates, respectively. The flow rate was 4 ml/min. The fractions were collected and lyophilized. The fraction containing uridine diphosphate was desalted by slurrying with water (4 ml), filtering through a sintered glass funnel, and passing through a Bio-Gel P-2 column. It was necessary to pass the resulting fractions through the column several times. The residual salt from the initial slurry was reextracted several times with water and the desalted fractions were then combined and lyophilized to yield uridine diphosphate. The yield (determined by enzymatic assay) was 2.2 mg of pure uridine diphosphate.

Cytidine diphosphate. Cytidine (360 mg) was reacted as described for UDP. This yielded 460 mg of a mixture of cytidine oligophosphates. Cytidine diphosphate was isolated by ion-exchange chromatography on Dowex-1-X8 at 4°C (*11*). The cytidine polyphosphate mixture was neutralized with 1 M NaOH and applied to the column. The column was then washed with water and the eluate was monitored at 252 nm. A linear gradient of water and 4 M formic acid was applied to the column. The flow rate was 3 ml/min. All fractions absorbing at 252 nm were collected and lyophilized. The fraction containing cytidine diphosphate was identified by thin-layer chromatography and enzymatic assay. This yielded 58 mg of pure cytidine diphosphate. When toluene was used as a cosolvent in place of *m*-cresol, the product was not obtained.

Enzymatic analysis. The nucleoside diphosphates isolated by these procedures were determined by a coupled assay utilizing the enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH) with NADH as a cofactor. The decrease in NADH concentration was measured by the change in optical density of 340 nm and is proportional to the amount of nucleoside diphosphate (NDP) converted to the corresponding triphosphate (NTP) as shown in Scheme I (*12*):

$$\text{NDP} + \text{PEP} \overset{\text{PK}}{\rightleftharpoons} \text{NTP} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{L-Lactate} + \text{NAD}^+$$

The assay contained imidazole buffer (0.05 M, pH 7.6), phosphoenolpyruvic acid (PEP, 5 mg/ml), NADH, 5 mg/ml, LDH, 0.4 units per test; PK (4.4 units/ml in 0.01 M KCl); and NDP standards ranging from 5 to 75 mg/ml. Buffer (2.6 ml), NDP (0.1 ml), PEP (0.1 ml), NADH (0.1 ml), and LDH (0.01 ml) were added to 1-cm quartz uv cells and incubated at 25°C. The reaction was initiated by addition of 0.1 ml of pyruvate kinase. The observed reaction velocities were then plotted against concentrations of NDP. An assay was then performed on a solution of NDP from the pyrophosphorylation procedure and the actual amount of NDP yielded was calculated from the standard curve.

Nucleotide fractions isolated from the ion-exchange columns were analyzed by thin-layer chromatography on cellulose plates with fluorescent indicator as described in the beginning of this section. The identity of the spots was determined by comparison with the authentic material.

RESULTS AND DISCUSSION

The reaction of HET with partially dehydrated phosphoric acid in the presence of *m*-cresol provides a regioselective introduction of the pyrophosphate function at a primary hydroxyl group. In the absence of *m*-cresol, reaction at both secondary and primary hydroxyl groups is demonstrated by analysis of the NMR spectrum and the failure of the material to be enzymatically active. This procedure also converts uridine and cytidine to the corresponding 5′-diphosphates but in poor yield. Attempts to pyrophosphorylate adenosine, guanosine, thymidine, and deoxyuridine were unsuccessful. Cytidine and uridine are not hydrolyzed under the strongly acidic conditions of the pyrophosphorylation reaction but the nucleosides for which the procedures fails undergo cleavage of the *N*-glycosidic linkage with subsequent transformation of the sugar moiety into furfural derivatives (*13*). Although the yields are low even for the stable nucleosides, in the absence of an alternative direct reaction, this method provides a necessary connection between the complex alcohols and the corresponding enzymatically active pyrophosphates. This should be useful when small amounts are needed for enzymatic analysis.

The origins of the selectivity induced by *m*-cresol are not known. It is likely that the hydroxyl group of *m*-cresol is an intermediate carrier of the phosphate or pyrophosphate function since we find that toluene does not promote the reaction. These phosphorylated cresols should be more sterically sensitive than the phosphoric anhydride and thus provide the observed selectivity.

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