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# Chiral Intermediates in Thiamin Catalysis: Resolution and Pyrophosphorylation of Hydroxyethylthiamin[1]

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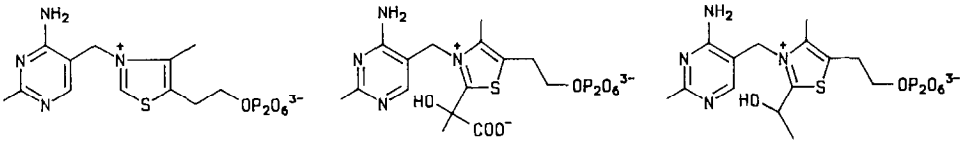
The improved preparation, resolution, and pyrophosphorylation of hydroxyethylthiamin [HET; 2-(1-hydroxyethyl)thiamin] is reported. HET is the chiral precursor to acetaldehyde, formed in the thiamin-catalyzed decarboxylation of pyruvate. The pyrophosphate, HETDP, is the precursor in the corresponding enzymatic process. Resolution of racemic HET was accomplished by formation of the dibenzoyltartrate salt, repeated crystallization from ethanol, and liberation of resolved HET from the resolving agent with 3 M hydrochloric acid. The optical rotation of the isolated material is comparable to that of the diphosphate derivative that has been isolated from an enzymatic reaction. Conversion of HET to the diphosphate provided material that was active in enzymic reactions. © 1985 Academic Press, Inc.

## INTRODUCTION

The conversion of pyruvate to acetaldehyde by pyruvate decarboxylase involves generation of chiral coenzyme derivatives from the achiral coenzyme and substrate (*1, 2*). The C-2 carbanion derived from thiamin diphosphate (TDP)[2] adds to the carbonyl group of pyruvate to form the chiral adduct, lactyl-thiamin diphosphate (LTDP). Loss of carbon dioxide followed by protonation generates the chiral adduct of TDP and acetaldehyde, hydroxyethylthiamin diphosphate (HETDP). Intermediates lacking the diphosphate moiety (LT and HET) have been implicated in the nonenzymatic reaction in which thiamin promotes the decarboxylation of acetaldehyde (*3*).




TDP LTDP HETDP

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[2] Abbreviations used: TDP, thiamin diphosphate; LTDP, lactyl-thiamin diphosphate; HET, hydroxyethylthiamin; HETDP, hydroxyethylthiamin diphosphate; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate.



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In order to utilize stereochemical studies as probes of mechanism in these systems, it is necessary to develop procedures for the resolution of the chiral intermediates. Ullrich and Mannschreck demonstrated that optically active HETDP can be isolated from the enzymatic reaction (*4*). Therefore, it is important to find means of generating the material nonenzymatically to determine the specificity of related processes. Our survey of the literature revealed one report of the use of optically active HET in nutrition studies but the report does not reveal the resolving procedure nor are the specific properties of the resolved material mentioned (*5*). The paper notes that the resolution will be reported later but we have not found that report.

For these reasons, we have undertaken the development of procedures for the convenient synthesis and resolution of chiral intermediates. We now report an improved synthesis of HET, a procedure for resolution of HET, and a method for converting HET to the enzymatically active material, HETDP.

## EXPERIMENTAL PROCEDURES

### *Materials*

Thiamin chloride hydrochloride, ethyl pyruvate, and (+) and (−)-2,3-dibenzoyltartaric acid monohydrate were purchased from the Sigma Chemical Company and were used without further purification. All other chemicals were reagents purchased from Sigma or Fisher Scientific. Amberlite CG-50 ion-exchange resin, hydrogen form (100–200 mesh), was obtained from Sigma. Dowex 2X8 (100–200 mesh) ion-exchange resin was purchased from Bio-Rad Laboratories.

### *Methods*

Proton NMR spectra were recorded on Varian T-60 (60 MHz) and XL-200 (200 MHz) spectrometers. Sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) and tetramethylsilane (TMS) were used as internal references. Phosphorus NMR spectra (32 MHz) were recorded on a Bruker WP-80 instrument with chemical shifts reported relative to trimethyl phosphate. UV–vis spectra and kinetic determinations were recorded on a Unicam SP1800 instrument. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. Circular dichroism spectra were obtained with a Jasco spectrometer and were recorded by Mr. R. S. Hinks. Melting points are uncorrected.

### *2-(1-Hydroxyethyl)thiamin Chloride Hydrochloride (Racemic HET)*

Two procedures, which are modifications of published methods, were used.

*Method 1.* This is a modification of the procedure reported by Risinger *et al.* (*6*). To a three-neck flask equipped with a pressure-equalizing dropping funnel and gas inlet and outlet tubes with a condensor were added 750 ml absolute ethanol and 25 g (0.074 mol) thiamin chloride hydrochloride. The suspension was stirred with a magnetic stirrer and cooled to −5°C in a bath maintained by a Neslab RTE-8

circulating refrigeration system. Freshly distilled acetaldehyde (25 ml) was added followed by two equivalents of sodium ethoxide (3.41 g sodium in 100 ml absolute ethanol) under a nitrogen atmosphere over a period of 30 min. The mixture was stirred under nitrogen for 16 h and then acidified to pH 3 with hydrogen chloride gas (produced by the addition of concentrated hydrochloric acid to sulfuric acid in an external reactor). After stirring for 10 min, the mixture was filtered with suction. The filtrate was stored at −5°C for 48 h during which time HET precipitated. The product was collected and washed with cold absolute ethanol and then with ether. Further crops of product were obtained by concentration of the original filtrate and cooling. Yield, 20 g; mp 228–230°C. The proton NMR spectrum agreed with that reported by Risinger *et al.*

*Method 2.* The adduct of ethyl pyruvate and thiamin (ethyl lactyl-thiamin) was prepared as reported (*7*). This material (4 g) was dissolved in 12 M hydrochloric acid (30 ml) and kept at room temperature for 24 h to convert the ester to the parent acid, lactyl-thiamin. The solution was evaporated and the resulting solid was dissolved in 30 ml of water and left for 4 days. The water was removed by lyophilization. HET was obtained in over 95% yield. The resulting material was recrystallized from acidic ethanol. The properties and spectra agree with the material prepared by the alternative procedure. The absence of a peak at 8.1 ppm in the proton NMR spectrum indicates that less than 2% unreacted thiamin is present.

*Resolution of HET*

Two different adducts of HET and dibenzoyltartaric acid were used to complete the resolution.

*Method 1.* Via a 1 : 1 adduct: 3.9 g (+)-2,3-dibenzoyl-D-tartaric acid (0.01 mol) was dissolved in 50 ml ethanol. To this was added 25 ml water and 2 ml 10 M sodium hydroxide. HET (3.8 g) was added to the magnetically stirred solution and then the solution was taken to dryness on a rotary evaporator under vacuum. To remove the last traces of ethanol, 50 ml of water was added to the pale yellow oil and the solution was again rotary-evaporated. After initial precipitation of crystals the residue takes on the appearance of a slurry. The slurry was diluted with 50 ml of water and warmed to cause complete solution. The material was then kept at room temperature for 2 days, during which time white crystals formed. The crystals were collected by filtration and washed with small volumes of chilled water. The yield of the first crystallization was 40% based on starting material, or 80% based on a single enantiomer. The material thus obtained was analyzed by proton NMR and shown to be the 1 : 1 adduct of HET and dibenzoyltartaric acid. The resolving agent was then removed. The crystals were suspended in ethanol and the solution was made acidic with concentrated HCl dissolved in ethanol (the solution indicated as pH 3 on pH paper). The solvent was removed by rotary evaporation. Absolute ethanol was added to dissolve the residue and was removed by evaporation. The residual material was dissolved in a minimal amount of absolute ethanol, followed by the addition of a small amount of ether to turn the solution cloudy. Storage at −5°C led to the precipitation of white crystals. The

crystallization procedure was repeated once more. The resulting material (3.8 g) had a proton NMR spectrum that indicated the absence of the resolving agent. The specific rotation agreed with material prepared by the alternative method.

*Method 2.* Via the tris-dibenzoyltartaric acid adduct of HET. To a solution of 2 g (0.0052 mol) HET in 7 ml water was added 15 ml 2 M sodium hydroxide followed by a solution of 6 g (0.013 mol) of (+)-2,3-D-dibenzoyltartaric acid monohydrate in 12 ml methanol. The resulting precipitate was collected by filtration. Further material was obtained by addition of the resolving acid to the filtrate. The crops were combined and dried in a vacuum dessicator. The integrated NMR spectrum of the product indicated that it was the tris-dibenzoyltartaric acid adduct with HET. The material is insoluble in water but is soluble in methanol and dimethyl sulfoxide. The salt was recrystallized three times from 95% ethanol, giving 2.7 g (37%) of the pure diastereomeric salt, tris-(2,3-D-dibenzoyltartartrate) HET. After each recrystallization, HET was liberated from a small sample by ion exchange (described below), and then converted to the chloride hydrochloride salt by the addition of ethanolic HCl. The specific rotation was then determined. After three recrystallizations, optical rotation remained constant (See Results). The salt was converted to HET by dissolving it in 50% aqueous ethanol and then passing it through a column (2.0 × 45.0 cm) of Dowex AG-2X8 anion-exchange resin, chloride form (100–200 mesh) suspended in the same solvent. Fractions containing HET (detected by uv absorbance) were pooled. Ethanol was removed by rotary evaporation and water was removed by freeze-drying. The residual solid was recrystallized from acidic ethanol and dried *in vacuo* to give (−)-HET in 35% yield from HET; mp, 234°; specific rotation, −12.5° ($c$ = 0.7, water). CD spectrum (0.1 mM in water): $\theta$ (300) 0; $\theta$ (272.5) −1480; $\theta$ (260) 0; $\theta$ (243) 3360.

The other diastereomeric salt of HET and dibenzoyltartaric acid was isolated by concentrating the mother liquor from the first recrystallization. The crude salt (4.1 g) was converted to HET as above. The specific rotation was 6.80° ($c$ 0.7, water). This partially resolved material was then reacted with (−)-2,3-dibenzoyltartaric acid monohydrate. The solid was collected by filtration and recrystallized twice from 95% ethanol (mp, 128°C). The (+) enantiomer of HET was freed of the resolving agent as described for the (−) species and recrystallized [mp, 234°C; specific rotation, +12.0° ($c$ 1.0, water)].

### *Conversion of HET to HETDP*

The procedure developed for the pyrophosphorylation of thiamin thiazolone was used (*8*). Orthophosphoric acid (85%, 2 ml) was heated over an open flame in a round-bottom flask until the solution became cloudy. The liquid was cooled to room temperature and 0.5 g (−)-HET was added. The mixture was heated in an oil bath at 105°C for 15 min and stirred with a glass rod. The solution was then cooled to room temperature, dissolved in 6 ml water, and centrifuged at low speed in a test tube. The supernatant was applied to a 2.6 × 45-cm column of Amberlite CG-50 cation-exchange resin (H form, 100–200 mesh, suspended in water). The column was eluted with water and the effluent was collected in fractions and analyzed by thin-layer chromatography, as described for the thiazolone (*8*). Frac-

tions containing HETDP were pooled and the pH was adjusted to 5.5 with 12 M sodium hydroxide. The solution was lyophilized. The proton NMR spectrum (deuterium oxide relative to internal DSS) was identical to that of HET except for the change in splitting patterns due to proton–phosphorus coupling: δ 3.35 (2H, t, $-CH_2CH_2OP-$) and δ 4.20 (2H, t, $-CH_2CH_2OP-$). The proton-decoupled phosphorus NMR spectrum showed three peaks (chemical shifts relative to trimethyl phosphate) δ −4.10 (monophosphate), δ −13.0 (diphosphate), and δ −24.7 (triphosphate). From the integrated intensities it was estimated that the diphosphate accounted for 70% of the total phosphate. Attempts to further purify the material using reverse-phase high-performance liquid chromatography led to the fragmentation of HETDP. A comparison sample of racemic HETDP was prepared by condensation of acetaldehyde with TDP (*9*).

*Enzyme Assay*

The HETDP that we prepared was tested for its ability to be converted enzymatically to TDP. Since pyruvate dehydrogenase and pyruvate decarboxylase are specific for the diphosphate (*10*), successful formation of the diphosphate is indicated by the ability of the material to activate an apoenzyme. Thus, the product was tested for activity with TDP-free *Escherichia coli* pyruvate dehydrogenase (a gift from Professor P. A. Frey of the University of Wisconsin) (*11*). The material was also tested with the apoenzyme of wheat germ pyruvate decarboxylase, prepared and analyzed as described previously (*12*).

## RESULTS AND DISCUSSION

The improved methods for preparation of HET and the procedures for resolution of the racemate are described in detail under Experimental Procedures. In addition to the methods reported there, we attempted to prepare other diastereomeric salts for the purpose of the resolution. These included salts of L-malic acid, D-10-camphorsulfonic acid, and D-tartaric acid. None of the salts were crystalline. Our preparations of the 2,3-dibenzoyltartrate salts are based on analogy to the procedures reported by Murakami *et al.* for the preparation of salts of thiamin (*13*). The specific rotation of HET that had been cocrystallized with the resolving agent and then freed of the dibenzoyltartrate is summarized in Table 1. Solvents that were less satisfactory than our final choice are also indicated.

The yields reported in Table 1 are overall results from unresolved HET. The final specific rotation of the (−) enantiomer is −12.5°. This compares with the specific rotation of −10 ± 2° reported by Ullrich and Mannschreck for the HETDP they isolated from pyruvate decarboxylase (*4*). The material used by Shiobara *et al.* in nutritional experiments had specific rotations of −13.5° and +12.5° (*5*).

The wavelength dependence of the specific rotation of (−)-HET is presented in Table 2 and the results are compared with those of Ullrich and Mannschreck for HETDP isolated from the enzymatic reaction. (Professor Ullrich has informed us

TABLE 1

FRACTIONAL CRYSTALLIZATION OF HET SALTS

| Solvent | No. of rxt. | Percentage yield | $[\alpha]_D^{22}$ |
|---|---|---|---|
| DMSO-ethanol | 1 | 45 | −7.3 |
| Methanol-water | 1 | 48 | −7.0 |
| | 2 | 42 | −7.8 |
| 95% Ethanol | 1 | 45 | −8.1 |
| | 2 | 41 | −11.2 |
| | 3 | 37 | −12.5 |
| | 4 | 33 | −12.5 |

*Note.* Specific rotations are for solutions in water (0.7%, 22°C).

that the his values have since been revised to higher rotations after adjusting for instrumental problems.)

The results in Table 2 clearly indicate that the pyrophosphate group does not significantly affect the optical rotation of the rest of the molecule. Therefore, determination of the absolute structure of the enzymatically active material can be accomplished by analysis of the structure of the thiamin derivative. We are developing degradative procedures for conversion of chiral thiamin derivatives to compounds of known absolute configuration.

Circular dichroism spectroscopy permits measurement of optical activity in the region in which the molecule absorbs light. The circular dichroism spectrum of (−)-HET is presented in Fig. 1. The two peaks coincide with the aborbances due to the thiazolium (low wavelength) and pyrimidine (high wavelength) moieties of HET. This confirms that the activity is not due to residual resolving agent.

The resolution of HET will permit the stereospecificity of enzymatic processes to be determined if the material can be converted to the diphosphate. Although thiamin is routinely converted to the diphosphate (*14*), we could find no procedure

TABLE 2

SPECIFIC ROTATION OF (−)HET AND HETDP AS A FUNCTION OF WAVELENGTH

| Wavelength (nm) | $[\alpha]_D^{22}$ Degrees[a] | |
|---|---|---|
| | HET | HETDP[b] |
| 589 | −12.5 | −10.0 |
| 578 | −12.8 | −11.0 |
| 546 | −14.7 | −11.5 |
| 436 | −25.2 | −17.5 |
| 365 | −43.6 | −25.0 |

[a] Determined at 22°.

[b] From Ref. (*4*).

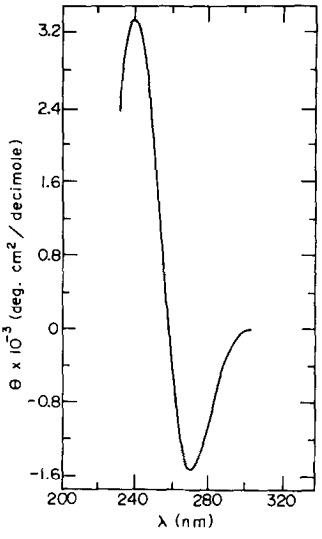


FIG. 1. The circular dichroism spectrum of (−) 2-(1-hydroxyethyl)thiamin.

for the conversion of HET to HETDP. The original synthetic preparation of HETDP by Krampitz and co-workers involved the addition of acetaldehyde to TDP (*9*). This procedure produces racemic material. These workers report that they did not attempt the conversion of HET to HETDP because they felt that the hydroxethyl group might not survive the pyrophosphorylation reaction. We find that the modified pyrophosphorylation conditions do not lead to decomposition of HET and do not cause reaction of the secondary hydroxyl group, as evidenced by the NMR spectrum of the product. The HETDP produced by our method is accompanied by small amounts of monophosphate and triphosphate derivatives, as seen in the phosphorus NMR spectrum. We use ion-exchange chromatography to separate the materials.

Thus, (−)-HET was converted to (−)-HETDP. This was tested for its ability to activate the apoenzyme of *E. coli* pyruvate dehydrogenase and wheat germ pyruvate decarboxylase. The material successfully activated both enzymes, indicating that the synthetic material is enzymatically competent. Our further studies will determine the stereochemical requirements of the enzymes and the relationship to the HETDP produced by the enzyme to its chiral precursor.

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