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Mechanistic Studies on Thiaminase I

1. The Stereochemical Course of the Reaction[1]

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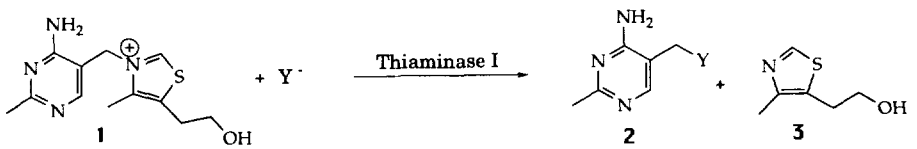
Thiaminase I catalyzes the displacement of the thiazole moiety of thiamin by a wide variety of nucleophiles. In this paper, we demonstrate that this reaction proceeds with overall retention of stereochemistry.

INTRODUCTION

Thiaminase I catalyzes the displacement of the thiazole moiety of thiamin by a wide variety of nucleophiles (Eq. [1]) (*1*).



EQUATION [1]

A mechanism for this substitution reaction is outlined in Scheme 1. Addition of an active-site nucleophile to the C6 position of the pyrimidine gives **4.** Loss of the thiazole followed by addition of the nucleophile Y gives **6.** Departure of the active site nucleophile completes the reaction. This mechanistic proposal is supported by the observation of ping-pong kinetics for the reaction (*2*) and by the mechanism-based inactivation of the enzyme with 6-chloro-2-methyl-4-aminopyrimidine (*3*). Nucleophillic attack at the planar methylene carbon of **5** may occur from the thiazole binding site, from a different site opposite to the thiazole binding site or without stereocontrol to give overall retention, inversion, or racemization of stereochemistry respectively. In this paper, we describe the stereochemical course of this reaction.

[1] This paper is dedicated to Professor Jeremy Knowles on the occasion of his 60th birthday.

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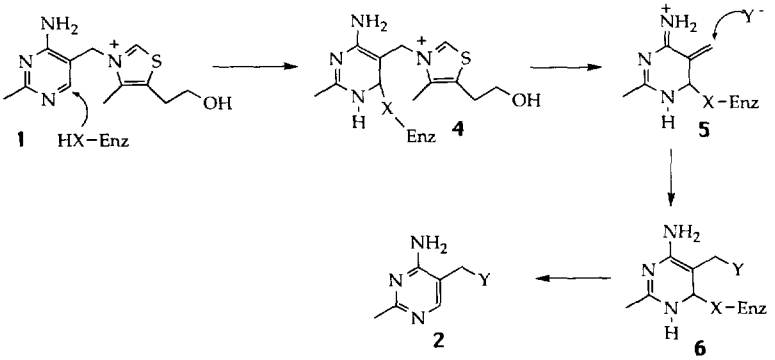
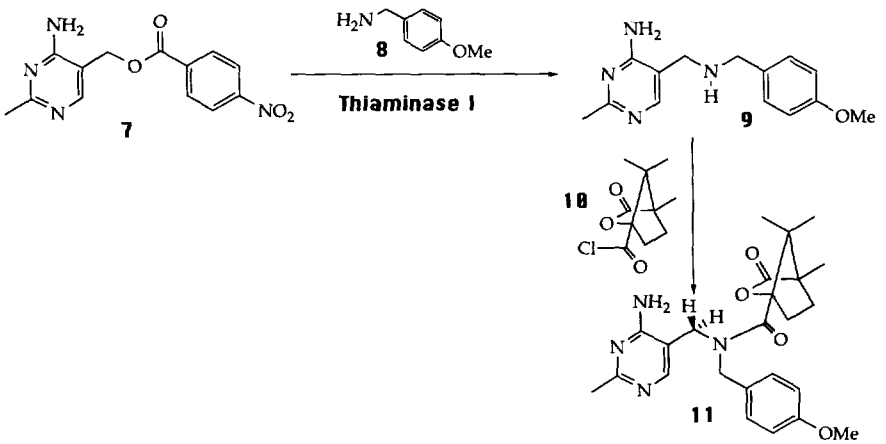


SCHEME 1

RESULTS AND DISCUSSION

Thiaminase I will catalyze the displacement of a variety of leaving groups from the pyrimidine (*4*). We have therefore been able to use the synthetically accessible *p*-nitrobenzoyl ester **7,** rather than thiamin, as our stereochemical probe (Scheme 2). This, when treated with the enzyme (*5*) in the presence of *p*-methoxybenzyl amine **8,** gives the substitution product **9** in 60% yield. This was converted to the camphanic amide **11** by treatment with (1S)-(-)-camphanic chloride. The diastereotopic C7 methylene protons of this compound can be readily differentiated by NMR analysis (Fig. 1).

The synthesis of the chiral monodeuterated ester (R)-**7** is shown in Scheme 3. Pyrimidine nitrile (*6*) **12** was reduced with $LiAlD_4$ to give deuterio-aldehyde **13.**



SCHEME 2

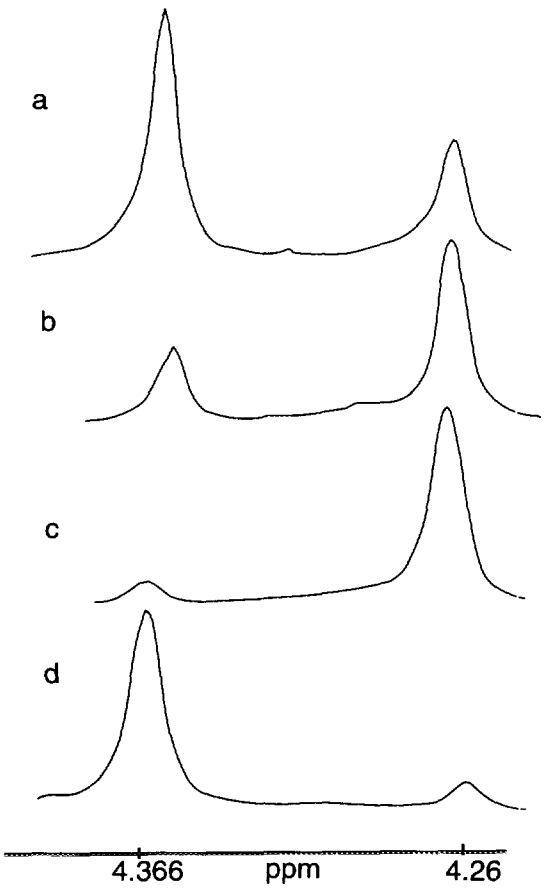
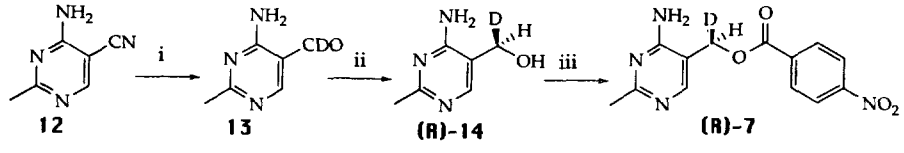
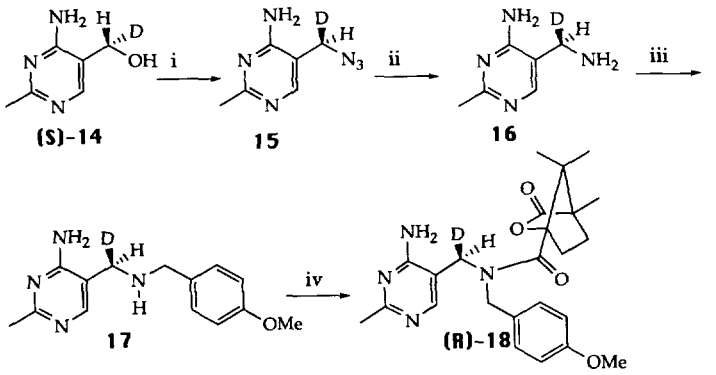


FIG. 1. Partial NMR spectra of the synthetic reference compounds and the enzymatic products: (a) reference compounds **18,** (b) reference compound **19,** (c) camphanic amide of the enzymatic product derived from (S)-monodeuterio-**7,** (d) camphanic amide of the enzymatic product derived from (R)-monodeuterio-**7.**



SCHEME 3. (i) $LiAlD_4$, THF_4; (ii) (S)-Alpine Borane; (iii) *p*-nitrobenzoyl chloride, TEA, THF.



SCHEME 4. (i) Diphenylphosphoryl azide, DBU, THF; (ii) $LiAlH_4$, THF_4; (iii) *p*-methoxybenzyl chloride, DMF; (iv) (1S)-(-)-camphanic chloride, THF.

Reduction of this with (S)-Alpine Borane (*7*) afforded (R)-**14** which was esterified by reaction with *p*-nitrobenzoyl chloride to give (R)-**7** (*8*). NMR analysis of the camphanic amide of the enzymatic product formed by treating (R)-**7** with **8** in the presence of thiaminase I demonstrated that the enzymatic reaction proceeded in a stereocontrolled manner (Fig. 1d). This was confirmed using the (S)-**7** which was prepared by reduction of aldehyde **13** with (R)-Alpine Borane followed by esterification (Fig. 1c).

To determine the absolute stereochemistry of the enzymatic reaction, it was necessary to correlate the enzymatic product with a reference compound of known absolute stereochemistry. The synthesis of this reference compound is shown in Scheme 4. Alcohol (S)-**14** was converted to azide **15,** with inversion of absolute stereochemistry, by treatment with diphenylphosphoryl azide (*9*). This reaction, under optimized conditions, gave a 4 : 1 mixture of R and S azides, presumably due to competing formation of the highly stabilized pyrimidine carbocation. Reduction with $LiAlH_4$ followed by alkylation with *p*-methoxybenzyl chloride and acylation with (1S)-(-)-camphanic chloride gave **18** (*10*). The diastereomer of **18** (compound **19**) was similarly prepared from the enantiomeric alcohol (R)-**14.**

Comparison of the partial NMR spectra of the enzymatic products (Figs. 1c and 1d) with the partial NMR spectra of the reference compounds **18** (Fig. 1a) and **19** (Fig. 1b) demonstrates that the enzymatic reaction proceeds with retention of configuration at the methylene position (*11*).

EXPERIMENTAL

THF was distilled from sodium and benzophenone immediately before use. Anhydrous DMF was used as purchased from Aldrich. All reactions in these solvents were conducted under a positive pressure of argon. Flash column chromatography was performed with EM Science silica gel 60 (230–400 mesh). NMR spectra were

recorded on a Varian 400 MHz spectrometer. Low-resolution CI mass spectra were recorded with a Finnigan-MAT CH5 spectrometer.

Preparation of 4-Amino-2-methylpyrimidine-5-carbaldehyde-d **(13)**

To a solution of **12** (500 mg, 3.7 mmol) in THF (50 ml) at −10°C was added solid lithium aluminum deuteride (147 mg, 3.7 mmol) in small portions. The mixture was stirred for 90 min before methanol (25 ml) was added. Solvent was removed *in vacuo*. Column chromatography (silica gel, 5% methanol in ethyl acetate) afforded **13** as a white solid (143 mg, 28%): ^{1}H NMR ($CDCl_3$) ∂ 8.57 (s, 1H), 8.14 (br, 1H), 5.97 (br, 1H), 2.57 (s, 3H); ^{13}C NMR ($CDCl_3$) ∂ 172.4, 163.7, 161.4, 110.5, 26.7. MS (CI) 138 (M).

Preparation of 4-Amino-5-(S)-hydroxydeuteriomethyl-2-methylpyrimidine **(14)** [(*S*)-**14**]

To a solution of **13** (540 mg, 3.9 mmol) in THF (43 ml) was added (R)-Alpine Borane (15 ml of a 0.5 M solution in THF, 7.5 mmol). The solution was stirred at ambient temperature overnight and quenched with methanol (10 ml) and water (10 ml). Solvent was removed *in vacuo*. Column chromatography (silica gel, 10% methanol in methylene chloride) gave (S)-**14** as a white solid in 81% yield (based on recovered starting material). ^{1}H NMR (CD_3OD) ∂ 7.95 (s, 1H), 4.46 (s, 1H), 2.40 (s, 3H); ^{13}C NMR (CD_3OD) ∂ 167.5, 163.9, 115.3, 59.6 (t, J = 22 Hz), 24.9.

Preparation of 4-Amino-5-(S)-hydroxydeuteriomethyl-2-methylpyrimidinyl-(4-nitrobenzoate) (*S*)-**7**

To a solution of (S)-**14** (118 mg, 0.84 mmol) in DMF (8 ml) was added *p*-nitrobenzoyl chloride (174 mg, 0.93 mmol). After 10 min triethylamine (150 μl, 0.93 mmol) was added and the reaction mixture was stirred for 3 h. Water (2 ml) was then added and the solution was extracted with ethyl acetate (3 × 20 ml). The combined organic phases were washed with water (20 ml) and brine (20 ml) and dried (Na_2SO_4). Column chromatography (silica gel, 5% methanol in methylene chloride) gave **7** as a yellow solid (104 mg, 43% yield): ^{1}H NMR (CD_3OD, 200 MHz) ∂ 8.33 (d, J = 9 Hz, 2H), 8.24 (d, J = 9 Hz), 8.15 (s, 1H), 5.27 (s, 1H), 2.43 (s, 3H).

Preparation of 4-Amino-5-(R)-azidodeuteriomethyl-2-methylpyrimidine **(15)**

To a stirring solution of (S)-**14** (153 mg, 1.10 mmol) in THF (10 ml) at ambient temperature were sequentially added diphenylphosphoryl azide (285 μl, 1.32 mmol) and DBU (215 μl, 1.44 mmol). After 10 min, water (5 ml) was added and the solution was extracted with ethyl acetate (4 × 15 ml). The combined organic phases were washed with water (15 ml) and brine (15 ml). Evaporation of the solvent *in vacuo* followed by column chromatography (silica gel, 10% methanol in ethyl acetate) gave (R)-**15** as a white solid (72 mg, 40%): ^{1}H NMR ($CDCl_3$) ∂ 8.08 (s, 1H),

5.20 (br, 2H), 4.21 (s, 1H), 2.52 (s, 3H); ^{13}C NMR ($CDCl_3$) ∂ 168.7, 161.8, 155.8, 108.3, 49.7 (t, J = 22 Hz), 25.9. MS (CI) 166 (M + 1).

Preparation of 4-Amino-2-methyl-5-(R)-(4-methoxybenzylamino) deuteriomethylpyrimidine **(17)**

To a solution of (R)-**15** (73 mg, 0.44 mmol) in 2 ml THF was added solid LAH (60 mg, 1.60 mmol) in small portions. After 10 min of stirring the reaction was quenched with water (60 μl), 1 N sodium hydroxide (60 μl), and water (60 μl). The mixture was filtered and washed with additional THF. All solvent was removed *in vacuo* to afford crude amine (R)-**16,** which was redissolved in 2 ml DMF. *p*-Methoxybenzyl chloride (75 μl, 0.55 mmol) was added and the solution was stirred for 1 h. Water (2 ml) was added and the mixture was extracted with ethyl acetate (3 × 5 ml). The combined organic phases were washed with water (5 ml) and brine (5 ml). Removal of the solvent *in vacuo* followed by column chromatography (silica gel, 20% methanol in ethyl acetate) afforded (R)-**17** as a colorless oil (8 mg, 8% yield): ^1H NMR ($CDCl_3$) ∂ 7.91 (s, 1H), 7.18 (d, J = 9 Hz, 2H), 6.87 (d, J = 9 Hz, 2H), 3.80 (s, 3H), 3.68 (s, 1H), 3.66 (s, 2H), 2.49 (s, 3H); ^{13}C NMR ($CDCl_3$) ∂ 162.7, 159.0, 154.7, 150.2, 127.3, 125.3, 109.8, 107.2, 51.2, 48.1, 43.9 (t, J = 20 Hz), 21.5.

Preparation of N,N-[(4-Amino-2-methyl-5-(R)-deuteriomethyl)-pyrimidinyl-(4-methoxybenzyl)]-1-(S)-camphanamide **(18)**

To a solution of (R)-**17** (8 mg, 0.031 mmol) in THF (1 ml) was added (1S)-(-)-camphanic chloride (18 mg, 0.083 mmol). After 1 h, water (2 ml) was added and the solution was extracted with ethyl acetate (3 × 3 ml). The combined organic phases were washed with water (3 ml) and brine (3 ml). Removal of the solvent *in vacuo* followed by column chromatography (20% methanol in ethyl acetate) afforded (R)-**18** as a white solid (5 mg, 37% yield): ^1H NMR ($CDCl_3$) ∂ 7.79 (s, 1H), 7.17 (d, J = 9 Hz, 2H), 6.91 (d, J = 9 Hz, 2H), 6.08 (br, 1H), 4.71 (d, J = 16 Hz, 1H), 4.58 (d, J = 16 Hz, 1H), 4.35 (s, 0.65H), 4.25 (s, 0.35H), 3.82 (s, 3H), 2.49 (s, 3H) 2.46 (m, 1H), 2.02 (m, 1H), 1.94 (m, 1H), 1.69 (m, 1H), 1.22 (s, 3H), 1.11 (s, 3H), 1.05 (s, 1H); ^{13}C NMR ($CDCl_3$) ∂ 177.9, 168.9, 168.0, 162.1, 159.6, 157.3, 157.2, 129.0, 127.3, 114.7, 108.1, 92.8, 55.7, 55.6, 54.0, 49.0, 42.1 (t, J = 19 Hz), 32.3, 29.9, 29.4, 25.7, 18.1, 17.1, 9.9.

Preparation of N,N-[(4-Amino-2-methyl-5-(S)-deuteriomethyl)-pyrimidinyl-(4-methoxybenzyl)]-1-(S)-camphanamide **(19)**

Compound **19** was prepared exactly as **18,** starting with (S)-**17:** ^1H NMR ($CDCl_3$) ∂ 7.79 (s, 1H), 7.17 (d, J = 9 Hz, 2H), 6.91 (d, J = 9 Hz, 2H), 6.08 (br, 1H), 4.71 (d, J = 16 Hz), 4.58 (d, J = 16 Hz, 1H), 4.35 (s, 0.28H), 4.25 (s, 0.72H), 3.82 (s, 3H), 2.49 (s, 3H), 2.46 (m, 1H), 2.02 (m, 1H), 1.94 (m, 1H), 1.69 (m, 1H), 1.22 (s, 3H), 1.11 (s, 3H), 1.05 (s, 1H).

Enzymatic Reaction

To a solution of (R)-monodeutero-**7** (200 μl of a 1 mg/20 μl solution in DMF) and *p*-methoxybenzyl amine (25 μl) in water (15 ml) was added thiaminase I (50 μl of a 6 mg/ml stock solution). The resulting mixture was incubated at ambient temperature for 3 h and the product was purified in the same manner as described for **17.**

ACKNOWLEDGMENT

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8. The enantiomeric excess was >90% by NMR analysis of the dicamphanate derivative.
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10. The enantiomeric excesses for **18** and **19** were 30 and 40%, respectively, by NMR analysis of the camphanate derivative.
11. With azide as the nucleophile, the substitution reaction also proceeds with retention.