Mechanistic Studies on Thiaminase I. 3. Stereochemistry of the Thiaminase I and the Bisulfite-Catalyzed Degradation of Chiral Monodeuteriothiamin

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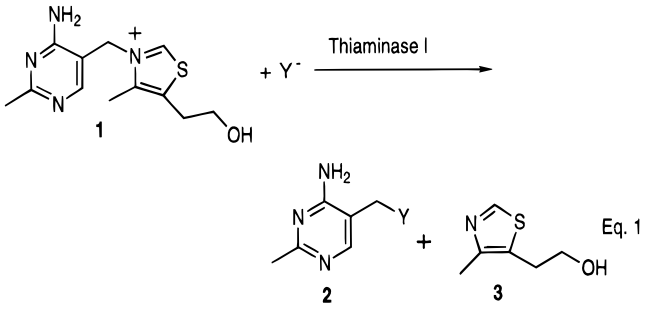
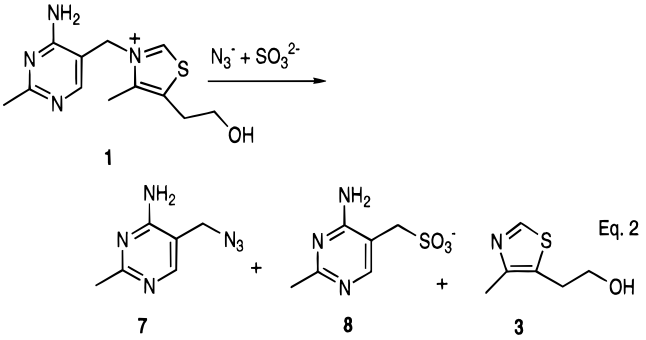
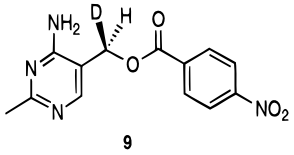
Thiaminase I catalyzes the displacement of the thiazole moiety of thiamin by a wide variety of nucleophiles (eq 1).[1] The presence of this thiamin-degrading enzyme in a variety of foods can result in thiamin deficiency in humans and other animals.[2]



This reaction is considered to proceed via the mechanism outlined in Scheme 1.[3] Nucleophilic attack at the planar methylene carbon of **5** may occur with overall retention, inversion, or racemization of stereochemistry. The bisulfite-catalyzed cleavage of thiamin proceeds by a similar mechanism (eq 2).[4]



We recently demonstrated that the thiaminase I-catalyzed substitution of the *p*-nitrobenzoate moiety of **9** by *p*-methoxybenzyl amine proceeds with retention of configuration.[5] Compound **9** did not react with bisulfite. We now report the first synthesis of chiral monodeuterated thiamin and its use in determining the stereochemistry of both the thiaminase I and the bisulfite-catalyzed thiamin cleavage reactions.



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The synthesis of (*S*)-monodeuteriothiamin (**14**) is outlined in Scheme 2. Condensation of the previously synthesized[5] amine **10** with triethyl orthoformate gave **11**, which when treated with thioketone **12**, followed by acid-catalyzed hydrolysis of the acetate, gave (*S*)-monodeuteriothiamin (**14**).[6] (*R*)-monodeuteriothiamin (**15**) was prepared in an identical manner starting from the (*R*)-monodeuterioamine **16**.

When (*S*)-monodeuteriothiamin (**14**) was treated with thiaminase I[7] in the presence of azide, the azidopyrimidine **17** was isolated in 82% yield (Scheme 3). Reduction with LAH followed by acylation with camphanic chloride gave camphanamide **19**, which gave an identical NMR spectrum to the camphanamide prepared from the (*S*)-monodeuterioamine **10** (Figure 1, parts A and B). A similar series of reactions with (*R*)-monodeuteriothiamin (**15**) gave a camphanamide with an identical NMR spectrum to the camphanamide prepared from the (*R*)-monodeuterioamine **16** (Figure 1, parts D and E). Thus, the thiaminase I-catalyzed degradation of thiamin proceeds with overall retention of configuration.

When (*S*)-monodeuteriothiamin (**14**) was treated with aqueous bisulfite and azide at 70 °C, the azidopyrimidine **17** was isolated in 34% yield.[8] Conversion to the camphanamide, by the same sequence of reactions used for the enzymatic product, demonstrated that the bisulfite-catalyzed reaction occurred with racemization of stereochemistry. This was confirmed by repeating the analysis using (*R*)-monodeuteriothiamin (**15**) (Figure 1, parts C and F). Thus, the bisulfite-catalyzed cleavage of thiamin proceeds with racemization.

Experimental Section

THF was distilled from sodium and benzophenone immediately before use. Anhydrous DMF was 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 EI mass spectra were recorded with a 70-VSE spectrometer.

Preparation of 3,4-Dihydro-5-deuterio-7-methylpyrimido-[4,5-*d*]pyrimidine (11). Amine **10** (114 mg, 0.82 mmol),[5] triethyl orthoformate (320 μL, 1.94 mmol), and dry *p*-toluenesulfonic acid (5 mg, 0.023 mmol) were added to a 10 mL round-bottom flask equipped with a small Vigreux column. The mixture was slowly heated to 110 °C until all of the solid had dissolved. The ethanol was removed *in vacuo*, the temperature

(1) a) Evans, W. C. *Vitam. Horm.* **1975**, *33*, 467−504. (b) Fujita, A. *Adv. Enzymol.* **1954**, *15*, 389−421.

(2) (a) Duffy, P.; Morris, H.; Neilson, G. *Am. J. Clin. Nutr.* **1981**, *34*, 1584−1592. (b) Earl, J. W.; McCleary, B. V. *Nature* **1994**, *368*, 683−684. (c) Fujita, A. *J. Vitaminol.* **1972**, *18*, 67−72. (d) Hayashi, R. *Nutr. Rev.* **1957**, *15*, 65−67.

(3) (a) Lienhard, G. *Biochemistry* **1970**, *9*, 3011−3020. (b) Puzach, S.; Gorbach, Z.; Ostrovskii, Y. *Biochemistry (USSR)* **1984,** *49*, 1010−1016. (c) Hutter, J. A.; Slama, J. T. *Biochemistry* **1987**, *26*, 1969−1973. (d) Kelleher, N. L.; Costello, C. A.; Begley, T. P.; McLafferty, F. W. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 981−984. (e) Costello, C. A.; Kelleher, N. L.; Abe, M.; McLafferty, F. W.; Begley, T. P. *J. Biol. Chem.* **1996**, *271*, 3445−3452.

(4) Uray, G.; Kriessmann, I.; Zoltewicz, J. A. *Bioorg. Chem.* **1993**, *21*, 294−308.

(5) Nicewonger, R.; Rammelsberg, A.; Costello, C. A.; Begley, T. P. *Bioorg. Chem.* **1995**, *23*, 512−518.

(6) Contant, P.; Forzy, L.; Hengartner, U.; Moine, G. *Helv. Chem. Acta* **1990**, *73*, 1300−1305.

(7) Thiaminase I was isolated from an *Escherichia coli* overexpression strain. Costello, C. A.; Kelleher, N. L.; Abe, M.; McLafferty, F. W.; Begley, T. P. *J. Biol. Chem.* **1996**, *271*, 3445−3452.

(8) Zoltewicz, J. A.; Kauffman, G. M. *J. Am. Chem. Soc.* **1977**, *99*, 3134−3142.

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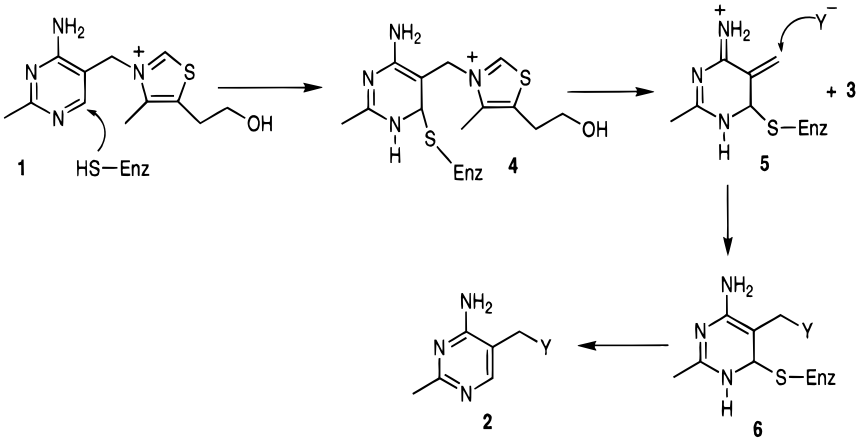
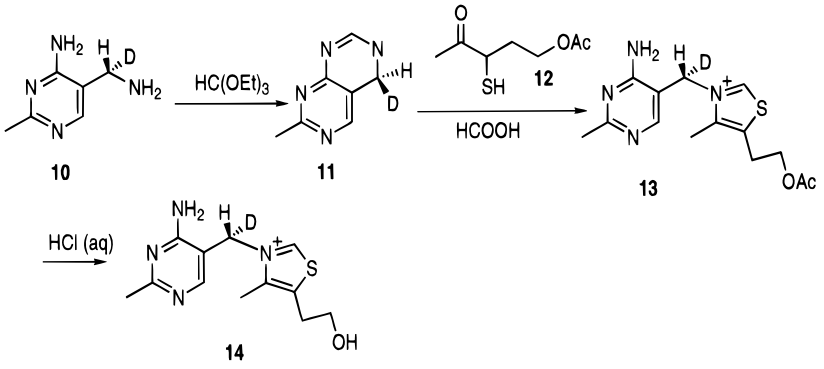
Scheme 1



Scheme 2



was maintained at 110 °C for another 30 min, 0.5 mL of toluene was added, and the mixture was further stirred for 1 h at 90 °C. The solvent was then removed *in vacuo*, and the resulting dark solid was sublimed under high vacuum (0.25 mmHg) at 150-160 °C to yield **11** as a white solid (79 mg, 65% yield). ^{1}H NMR (CD_3OD): 8.03 (s, 1H), 7.31 (s, 1H), 4.60 (s, 1H), 2.47 (s, 3H). MS (EI) 148 (M − 1), 121, 107, 95, 81.

Preparation of (*S*)-Deuteriothiamin (14). Freshly prepared thioketone **12** (92 mg, 0.53 mmol)[6] was added to a solution of **11** (79 mg, 0.53 mmol) in formic acid (88%, 1.1 mL). The solution was stirred for 30 min at room temperature, and a freshly prepared saturated solution of ethanolic HCl (0.25 mL) was added dropwise. The mixture was further stirred for 30 min at room temperature. All solvent was removed *in vacuo* at 50 °C. Absolute ethanol (1.1 mL) and aqueous HCl (25%, 0.30 mL) were added to the dark solid residue. The crude mixture was heated at 90 °C until all of the solid had dissolved and then cooled and kept overnight at 4 °C. The reaction mixture was filtered, and the resulting white solid was washed with ice-cold ethanol to give **14** as a white solid (80 mg, 45% yield). ^{1}H NMR (D_2O): 7.84 (s, 1H), 5.37 (s, 1H), 3.71 (t, 2H, J = 6 Hz), 3.01 (t, 2H, J = 6 Hz), 2.45 (s, 3H), 2.36 (s, 3H).

Enzymatic Reaction. Into a 1.5 mL Eppendorf tube was added monodeuteriothiamin chloride hydrochloride (30 mg, 0.089 mmol), water (300 μL), aqueous sodium azide (100 mg/mL, 320 μL, 0.49 mmol), and thiaminase I (9 mg/mL, 20 μL).[7] After incubating at room temperature for 2 h, the solution was extracted with ethyl acetate (5 × 2 mL). The combined organic extracts were washed with water (10 mL) and brine (10 mL). Evaporation of the solvent *in vacuo* followed by column chromatography (silica gel, 10% methanol in ethyl acetate) gave **17** as a white solid (12 mg, 82% yield). NMR ($CDCl_3$) 8.08 (s, 1H), 5.20 (br, 2H), 4.21 (s, 1H), 2.52 (s, 3H). MS (CI) 166 (M + 1), 138, 123, 97, 70.

Reduction of the Azidopyrimidine 17. To a solution of **17** (73 mg, 0.44 mmol) in 2 mL of 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

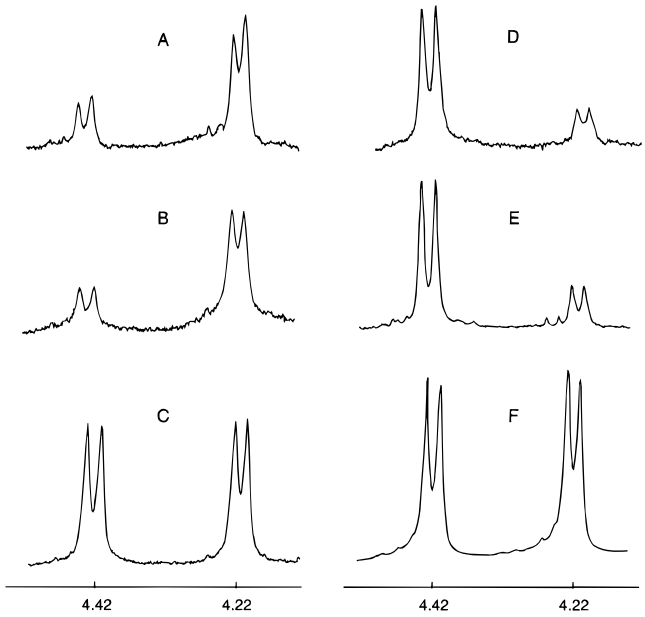
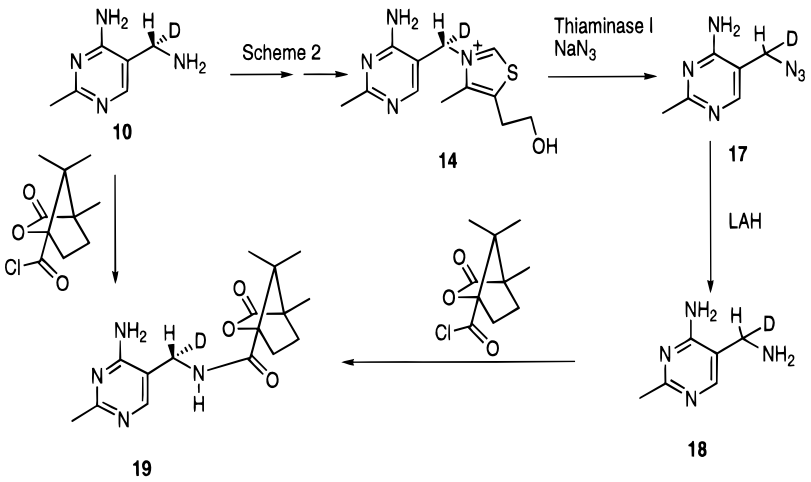


Figure 1. NMR analysis of the reaction product resulting from the cleavage of chiral monodeuteriothiamin with thiaminase I and with bisulfite. The partial NMR spectra show the signals for the pyrimidine C7-methylene protons of the pyrimidine camphanamide. (A) (*S*)-Monodeuteriopyrimidine camphanamide **19**. (B) Pyrimidine camphanamide derived from the enzymatic cleavage of (*S*)-monodeuteriothiamin (**14**). (C) Pyrimidine camphanamide derived from the bisulfite-catalyzed cleavage of (*S*)-monodeuteriothiamin (**14**). (D) (*R*)-Monodeuteriopyrimidine camphanamide. (E) Pyrimidine camphanamide derived from the enzymatic cleavage of (*R*)-monodeuteriothiamin (**15**). (F) Pyrimidine camphanamide derived from the bisulfite-catalyzed cleavage of (*R*)-monodeuteriothiamin (**15**).

Scheme 3



through a short plug of silica and washed with additional THF. All solvent was removed *in vacuo* to afford the crude amine as a slightly off-white solid (56 mg, 92% yield). ^{1}H NMR (CD_3OD) 7.95 (s, 1H), 3.65 (s, 1H), 2.38 (s, 3H). MS (EI) 139, 122, 110, 97, 81, 70, 55.

Preparation of Camphanamide 19. (1*S*)-(−)-camphanic chloride (20 mg, 0.093 mmol) was added to **10** (8 mg, 0.058 mmol) in THF (1 mL). After stirring for 2 h at ambient temperature, water (4 mL) was added and the mixture extracted with ethyl acetate (4 × 5mL). The combined extracts were washed with water (5 mL) and brine (5mL) and dried over sodium sulfate. Removal of solvent and column chromatography (silica gel, 5% methanol in ethyl acetate) afforded **19** as a white solid (8 mg, 44% yield). ^{1}H NMR ($CDCl_3$): 7.99 (s, 1H), 6.90 (br, 1H), 5.85 (br, 1H), 4.42 (d, 0.25H, J = 7 Hz), 4.22 (d, 0.75H, J = 7 Hz), 2.52 (m, 1H), 2.48 (s, 3H), 1.95 (m, 2H), 1.70 (m, 1H), 1.11 (s, 6H), 0.85 (s, 3H). MS (EI) 319, 138, 123, 83.

Bisulfite Cleavage. Into a stirring solution of monodeuteriothiamin chloride hydrochloride (91 mg, 0.27 mmol) in water (0.94 mL) at 70 °C was added aqueous sodium bisulfite (100 mg/mL, 75 μL) in five equal portions, one every 30 min. After heating for a total of 4 h, the mixture was extracted with ethyl acetate (5 × 2mL). The combined organic extracts were washed with water (10 mL) and brine (10 mL). Evaporation of the solvent *in vacuo* followed by column chromatography (silica gel, 2.5% methanol in ethyl acetate) gave **17** as a white solid (15 mg, 34% yield). NMR ($CDCl_3$) 8.08 (s, 1H), 5.20 (br, 2H), 4.21 (s, 1H), 2.52 (s, 3H). MS (CI) 166 (M + 1), 138, 123, 97, 70.

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