JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of 2-Methyl-4-amino-5-(2-methyl-3-furylthiomethyl)pyrimidine from Thermal Degradation of Thiamin

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Thiamin hydrochloride was thermally degraded in phosphate buffer (pH 6.5) at 110 °C for 2 h. A major decomposition product was isolated by column chromatography and structurally identified by spectrometric techniques (^{1}H NMR, ^{13}C NMR, 2D NMR, and MS) as 2-methyl-4-amino-5-(2-methyl-3-furylthiomethyl)pyrimidine (MAMP). The possible formation pathway of MAMP was studied using two model systems. It is proposed that MAMP is formed by nucleophilic attack of 2-methyl-3-furanthiol on the thiamin.

KEYWORDS: Thiamin; thermal degradation; 2-methyl-4-amino-5-(2-methyl-3-furylthiomethyl)pyrimidine

INTRODUCTION

Thiamin is one of the important precursors for odor-active sulfur-containing compounds. Flavor generation by thermal degradation of thiamin has been extensively studied (*1*−*3*). Upon heating, thiamin first forms reactive intermediates such as 5-hydroxy-3-mercapto-2-pentanone, which further reacts and leads to many sulfur-containing compounds that have a cooked and roasted meaty aroma. These reactive intermediates produce aroma compounds not only through transformation and interaction but also by reaction with other components of food, such as Maillard reaction products (*1*−*3*). 4-Methyl-5-(2-hydroxyethyl)thiazole (**6**), 4-amino-5-(aminomethyl)-2-methylpyrimidine, and 3-mercaptopropanol, along with hydrogen sulfide, ammonia, acetic acid, formic acid, formaldehyde, and acetaldehyde, have been reported as major compounds from thermally degraded thiamin (*1*, *2*). Additionally, many furans and thiophenes, including the character-impact meaty aroma compound 2-methyl-3-furanthiol (**7**), were identified from thermally degraded thiamin (**7**) (*3*). Although numerous volatile thermal degradation compounds have been identified from thiamin, limited information is available on nonvolatile or semivolatile compounds from thermally degraded thiamin.

Thiamin consists of two subunits, a pyrimidine unit, 4-amino-5-hydroxymethyl-2-methylpyrimidine, and a thiazole unit, 4-methyl-5-(2-hydroxyethyl)thiazole (**6**). Early studies reported that treatment of thiamin with sulfite led to the cleavage of thiamin and afforded 2-methyl-4-amino-5-pyrimidylmethanesulfonic acid and **6** (*4*, *5*). The substitution of thiamin by sulfite ion, which gave **6** as the leaving group, was proposed as a multistep process rather than an S_N2 mechanism (*5*). Zoltewicz et al. demonstrated that in methanol solution, several amine nucleophiles could replace the **6** in thiamin through a multistep mechanism (*6*). During the thermal degradation of thiamin, nucleophilic substitution to a bridged methylene carbon is rarely reported. The current study will discuss the isolation and characterization of a nucleophilic substituted novel compound, 2-methyl-4-amino-5-(2-methyl-3-furylthiomethyl)pyrimidine (MAMP; **1**), from thermally degraded thiamin in an aqueous solution and its possible formation pathway.

MATERIALS AND METHODS

Materials and General Procedures. Thiamin hydrochloride was a gift from Takeda Pharmaceuticals North America, Inc. (Lincolnshire, IL). 2-Methyl-3-furanthiol, all chemicals used for synthesis experiments, silica gel (130−270 mesh) for column chromatography, and TLC plates (250 μm thickness, 2−25 μm particle size) were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI). All solvents used for chromatographic isolation were of analytical grade and purchased from Fisher Scientific (Springfield, NJ). Thin-layer chromatography was performed on silica gel TLC plates with compounds visualized by spraying with 5% (v/v) H_2SO_4 in an ethanol solution.

Thermal Reaction of Thiamin. Thiamin hydrochloride (30 g) was dissolved in 0.1 M potassium phosphate buffer (300 mL, pH 6.5). The solution was heated, in a 1000 mL flask fitted with a condenser, at 110 °C in an oil bath for 2 h. After thermal reaction, the reaction mixture was cooled to room temperature and extracted with methylene chloride (300 mL) three times. The solvent was removed from the extract under reduced pressure.

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10.1021/jf011591v CCC: $22.00
Published on Web 05/31/2002

Table 1. ^{13}C and ^{1}H NMR Chemical Shift of MAMP and Thiamin[a]

	δ_C		δ_H	
C	MAMP	thiamin	MAMP	thiamin
2	167.1 s	164.1 s		
4	163.1 s	164.0 s		
5	112.0 s	106.9 s		
6	154.8 d	145.8 d	7.23 s	8.21 s
7	24.9 q	22.0 q	2.32 s	2.75 s
8	34.3 t	51.0 t	3.59 s	5.62 s
2′	158.3 s	155.5 d		9.86 s
3′	110.1 s			
4′	116.6 d	143.8 s	6.24 br s	
5′	142.3 d	137.4 s	7.29 br s	
6′	11.4 q	30.2 t	1.90 s	3.32 t
7′		61.2 t		4.03 t
8′		12.2 q		2.85 s

[a] MAMP was measured in CD_3OD. Assignments were based on HMQC, HMBC, and COSY experiments. Thiamin was measured in D_2O.

Purification of Thermally Degraded Compound. The methylene chloride extract (700 mg) was subjected to silica gel column chromatography with an ethyl acetate/methanol (10:0, 10:0.2, 10:0.4, 10:1, each 300 mL) solvent system, which afforded four fractions. Fraction 1 was further purified with RP-18 (MeOH/water, 3:2) to afford compound **1** (20 mg) as an amorphous powder: positive-ion HRFAB-MS, *m*/*z* 236.0865 $[M + H]^+$, calcd for $C_{11}H_{14}ON_3S$, 236.0858; EI-MS spectral data, *m*/*z* (relative intensity) 235 ($M^{\bullet+}$, 11), 122 (100), 113 (5), 81 (28), 80 (9), 59 (2), 54 (15), 42 (27), 28 (8); ^1H NMR (600 MHz, in methanol-d_4) see **Table 1** ; ^{13}C NMR (150 MHz, in methanol-d_4) see **Table 1**.

NMR, APCI-MS, and GC/GC-MS Analysis. ^1H NMR and ^{13}C NMR and all 2D NMR spectra were obtained on a Varian 600 instrument (Varian Inc., Palo Alto, CA). The compound was analyzed in CD_3OD, with TMS as the internal standard. HRFAB-MS was run on a JEOL HX-110 double-focusing mass spectrometer. Gas chromatography (GC) was performed on an HP 5790A gas chromatograph equipped with a flame ionization detector (FID) and a nonpolar fused silica capillary column (30 m, 0.32 mm i.d., 0.25 μm film thickness, DB-1, J&W Scientific Inc.). The column temperature was programmed from 40 to 260 °C at a rate of 2 °C/min. The injector temperature and FID temperature were set at 270 and 300 °C, respectively. The flow rate of the helium carrier gas was 1 mL/min. GC-MS was performed on an HP 5790A GC, which was coupled to an HP 5970A mass spectrometer. The ionization of the mass spectrometer was set at 70 eV.

Synthesis of 2-Methyl-4-amino-5-(2-methyl-3-furylthiomethyl)-pyrimidine (MAMP; 1). Thiamin hydrochloride (6 g) was dissolved in methanol (50 mL). Triethylamine (4 g) was slowly added into the solution with gentle shaking until the reaction mixture became clear. It was then concentrated under reduced pressure to approximately its half volume and left to stand overnight at room temperature. Thiamin monochloride was obtained by filtration from the reaction mixture. Subsequently, thiamin monochloride (1 g) and **7** (1 g) were dissolved in methanol (15 mL) and reacted in an oil bath at 80 °C for 2 h. After cooling, the solution was concentrated to dryness under reduced pressure. The residue was extracted with ethyl ether. The extract was then purified by column chromatographic methods to obtain compound **1** as described in the purification of compound **1** from thermal reaction of thiamin. This synthetic method was modified from the method of Shimahara et al. (*7*).

MAMP Formation Pathway Studies. Two model reactions were carried out to understand the formation pathway of **1**. The composition of model systems is described below. Model system I was prepared with thiamin monochloride (1 g) in methanol (15 mL). For model system II, the mixture of thiamin monochloride (1 g) and **7** (1 g) was dissolved in methanol (15 mL). Each reaction model system was heated at 80 °C for 2 h in an oil bath. The reaction mixtures were then extracted with methylene chloride and analyzed by GC-MS.

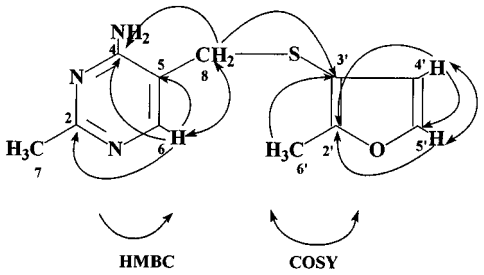


Figure 1. Significant HMBC (H–C) and ^1H–^1H COSY correlations of MAMP.

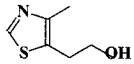
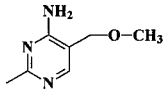
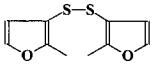
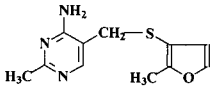
Reaction with Thiamin and Cysteine. Two model systems were prepared. For model system III, thiamin monochloride (1 g) was dissolved in water (15 mL). Model system IV was prepared with thiamin monochloride (1 g) and cysteine (1 g) in water (15 mL). Each reaction mixture was refluxed in an oil bath at 80 °C for 70 min. After cooling, 1 mL of 1000 ppm of tridecane (in methylene chloride) was added to each reaction mixture as internal standard. The solutions were then extracted with methylene chloride (15 mL). After drying with anhydrous $MgSO_4$, the organic fraction was concentrated by a stream of nitrogen and analyzed by GC-MS.

RESULT AND DISCUSSION

After thermal degradation of thiamin hydrochloride in aqueous solution, a major degradation compound was isolated with chromatographic methods. The structure was elucidated by interpretation of ^1H NMR, ^{13}C NMR, 2D NMR, and MS spectra. Compound **1** was isolated as an amorphous solid having a molecular formula of $C_{11}H_{13}ON_3S$, which was determined by positive-ion HRFAB-MS ($[M + H]^+$ at *m*/*z* 236.0865), as well as by its ^{13}C NMR data and EI-MS spectral data. The ^{13}C NMR spectrum showed 11 carbon signals. After careful comparison with the ^{13}C NMR spectrum of authentic thiamin and published data (*8*), one methyl carbon signal (δ 24.9) and four olefinic carbon signals (δ 112.0, 154,8, 163.1, and 167.1) were in agreement with the spectral characteristics of a pyrimidine moiety in thiamin. In addition, one methyl proton signal at δ 2.32 (3H, s, CH_3) and one olefinic proton signal at δ 7.23 (1H, s, CH) also agreed with a pyrimidine moiety when compared with the ^1H NMR spectrum of authentic thiamin and published data (*9*). Interestingly, bridged methylene carbon (C-8) was shifted upfield by ~16.7 ppm compared with the ^{13}C NMR spectrum of thiamin. This suggests that C-8, which is connected to the nitrogen atom of **6** in the thiamin, would be displaced with others.

The remaining five carbon peaks showed one methyl carbon (δ 11.4) and four olefinic carbons (δ 110.1, 116.6, 142.3, and 158.3) (**Table 1**). The ^1H NMR spectrum also gave one methyl proton signal at δ 1.90 (3H, s, CH_3) and two olefinic protons at δ 6.24 (1H, s, CH) and δ 7.29 (1H, s, CH) (**Table 1**). This spectral information suggests that the remaining moiety has a furan ring structure having one methyl group. According to the $^1H-^1H$ COSY spectrum, two olefinic proton signals have correlated with each other. Along with the NMR interpretation, the *m*/*z* 113 and 81 peaks in the mass spectrum suggest the presence of a 2-methyl-3-furanthiyl moiety. As we previously mentioned, the C-8 in the pyrimidine moiety shifted upfield. The evidence strongly supports the assumption that the pyrimidine unit and 2-methyl-3-furanthiol (**7**) connected with each other through the methylene bridge. The HMBC spectrum decisively showed a cross-peak between H-8 and C-3′ (**Figure 1**). Consequently, the compound was determined to be 2-methyl-4-amino-5-(2-methyl-3-furylthiomethyl)pyrimidine (MAMP; **1**)

Table 2. Thermal Degradation Products from Different Model Systems for MAMP Formation[a]

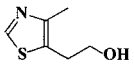
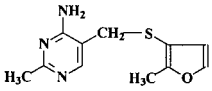
Compounds	Concentration (mg/mol of thiamin)	
	Model system I [1)]	Model system II [2)]
		211
	1147	163
	307	
		85
	32	211

[a] (1) thiamin monochloride; (2) thiamin monochloride and 2-methyl-3-furanthol.

(**Figure 1**). The structure suggested for compound **1** was ultimately confirmed by synthesis, which was performed according to modified methods of Shimahara et al. (*7*). The spectral characteristics of compound **1** were identical with those of the compound isolated from synthesis.

Formation of MAMP from Thiamin. It is of interest to understand the formation pathway of the novel compound **1**. Torward this end, we propose two possible formation pathways. One is formation of **1** through thiamin rearrangement during thermal degradation. The other possible pathway is substitution of **6** by **7**. Two model systems were, therefore, carried out to differentiate these two pathways. Model systems I and II consist of thiamin monochloride in methanol and thiamin monochloride and **7** in methanol, respectively. After the thermal reaction of each model system, thermal degradation products were determined by GC-MS analysis. As shown in **Table 2**, the formation of **1** was dramatically increased when thiamin monochloride was thermally degraded in the presence of **7**. These results favor the pathway that compound **1** results from the substitution of **6** by **7** rather than by the rearrangement of thiamin itself. Nucleophilic substitution of thiamin by sulfite ion has been kinetically proven to be a multistep process (*4*, *5*). Zoltewicz et al. also reported that amine nucleophiles such as pyridine, 4-aminopyridine, aniline, and diazabicyclo[2.2.2]octane could substitute the thiazole portion of the thiamin molecule in methanol with a multistep mechanism (*6*). Zoltewicz et al. (*5*, *6*) proposed that after the protonation of a pyrimidine ring, followed by a nucleophile attack on the pyrimidine ring by a hydroxy ion, the resulting group (**6**) is dissociated (**Figure 2**). The pyrimidine ring then forms a stabilized cation intermediate. Subsequently, the cation intermediate reacts with nucleophiles and gives nucleophilic substituted compounds (*5*, *6*). We presumed the formation pathway of **1** would be similar to that proposed by Zoltewicz et al. (*5*, *6*) as a multistep mechanism. Here, compound **7**, which is generated from thermal degradation

Table 3. Thermal Degradation Products from Reaction with Thiamin Monochloride and Cysteine[a]

Compounds	Concentration (mg/mol of thiamin)	
	Model system III [1)]	Model system IV [2)]
		11
		134
	1474	12894
	29	12

[a] (1) thiamin monochloride; (2) thiamin monochloride and cysteine.

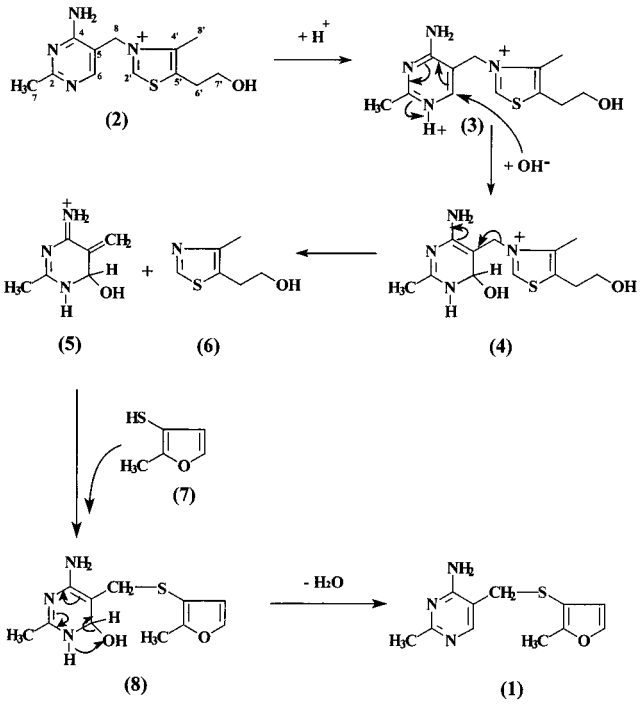


Figure 2. Proposed formation pathway of MAMP.

of thiamin as a nucleophile, reacts with the stabilized cation intermediate (**5**), which leads to the formation of compound **1** (**Figure 2**).

2-Methyl-3-furanthiol (**7**) is one of the important meaty aroma compounds from the thermal degradation of thiamin (*10*). According to the present study, formation of **7** would be decreased during the thermal degradation of thiamin because of its participation in the formation of **1**. However, **1** had only a very weak sulfury odor; however, its formation may affect the meatlike flavor profile during the thermal degradation of thiamin. Because thiamin is considered to be one of the most important ingredients for savory process flavors, the formation of **1** at the expense of **7** would be unfavorable. To develop an improved method for the production of meaty

aroma from the thermal reaction of thiamin, it is possible that the addition of another compound, having a nucleophilic character similar to that of **7**, into the reaction mixture may result in increased formation of **7**. Therefore, we carried out the analysis of two model systems. Model systems III and IV consist of thiamin monochloride in methanol and thiamin monochloride and cysteine (which was added as a nucleophilic competitor of **7**), respectively. After thermal reaction of each model system, degradation products were analyzed using GC-MS. As expected, when thiamin was thermally degraded in the presence of cysteine, the formation of **7** was increased, with a decreased amount of **1** (**Table 3**). The results strongly support the assumption that cysteine has the same nucleophilic character and can compete with **7** for reaction with thiamin.

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Received for review December 4, 2001. Revised manuscript received April 26, 2002. Accepted April 29, 2002.

JF011591V