Thiamine Biosynthesis in Yeast—Evaluation of 4-Hydroxy-5-hydroxymethyl-2-methylpyrimidine as a Precursor

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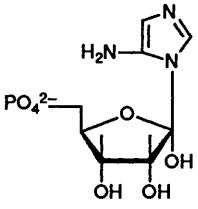
The putative role of 4-hydroxy-5-hydroxymethyl-2-methylpyrimidine **2** as a late precursor in the biosynthesis of thiamine **1** by *Saccharomyces cerevisiae* has been examined using the deuteriated derivative **2a**. To enable measurement of deuterium incorporation into the pyrimidine ring fragment of **1**, a procedure for the degradation of **1** to the aniline derivative **6** using thiaminase I from *Bacillus thiaminolyticus* has been developed. While 4-amino-2-methyl-5-hydroxy[$^2H_1$]methylpyrimidine **3a** was incorporated into thiamine, deuterium from hydroxypyrimidine **2a** was not incorporated to any significant extent, indicating that amination of compound **2** is not a late step in the biosynthesis of the pyrimidine ring.

The biosynthesis of both the pyrimidine and the thiazole rings of thiamine (vitamin B_1, **1**) occurs by different routes in prokaryotes and eukaryotes.[1] In the former, the elaboration of the pyrimidine of thiamine is linked to purine nucleoside biosynthesis by the role of 5′-aminoimidazole ribonucleotide (AIR) **4** which is an obligatory intermediate in both pathways.[2,3] A bacterial pathway from AIR to the pyrimidine



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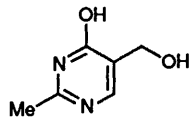
nucleus of thiamine which rationalises the incorporation of primary precursors has been proposed.[4] Much less is known of the precursors involved in eukaryotic thiamine biosythesis.[1] Earlier studies with yeast have shown that 4-amino-5-hydroxymethyl-2-methylpyrimidine **3** is incorporated, that the C-4 carbon of this unit is derived from formate,[5–7] and that the ring N-3 and the nitrogen of the 4-amino group are derived from the amido nitrogen of glutamine.[8] Recently, Tazuya *et al.*[9] have reported the enrichment of the N-3 and 4-amino nitrogens of the pyrimidine nucleus by ^{15}N from DL-[1,3-^{15}N]histidine. While this suggests that the introduction of both nitrogens may occur as an integral step in the elaboration of the ring system, introduction of the 4-amino nitrogen as a final step could also take place by transamination of the corresponding 4-hydroxypyrimidine **2** in a process analogous to that occurring in purine amination.[10] We have investigated the latter possibility by examining the incorporation of 4-hydroxy-5-hydroxy[$^2H_1$]-methyl-2-methylpyrimidine **2a** into thiamine in *Saccharomyces cerevisiae* and show here that while deuterium from 4-amino-5-hydroxy[$^2H_1$]methyl-2-methylpyrimidine **3a** was incorporated no significant deuterium incorporation from alcohol **2a** was observed.

Discussion

In view of the small amounts of thiamine (*ca.* 50 μg l^{-1}) produced by yeast cells and the relatively high detection limit of the underivatised metabolite (*ca.* 10 μg) by mass spectrometry,



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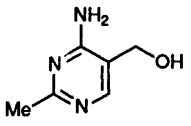

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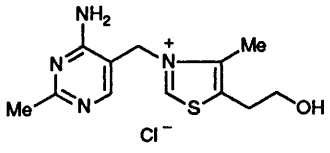

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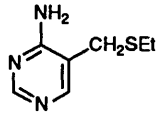
the analysis of the regiospecific incorporation of non-radioactive nuclides from enriched precursors into thiamine imposes severe experimental constraints. Most earlier studies on stable isotope incorporations into the pyrimidine ring have utilised the method developed by White and Rudolph[11] which involves reaction of partially purified thiamine with ethanethiol at elevated temperature in a sealed tube to afford the sulphide **5**



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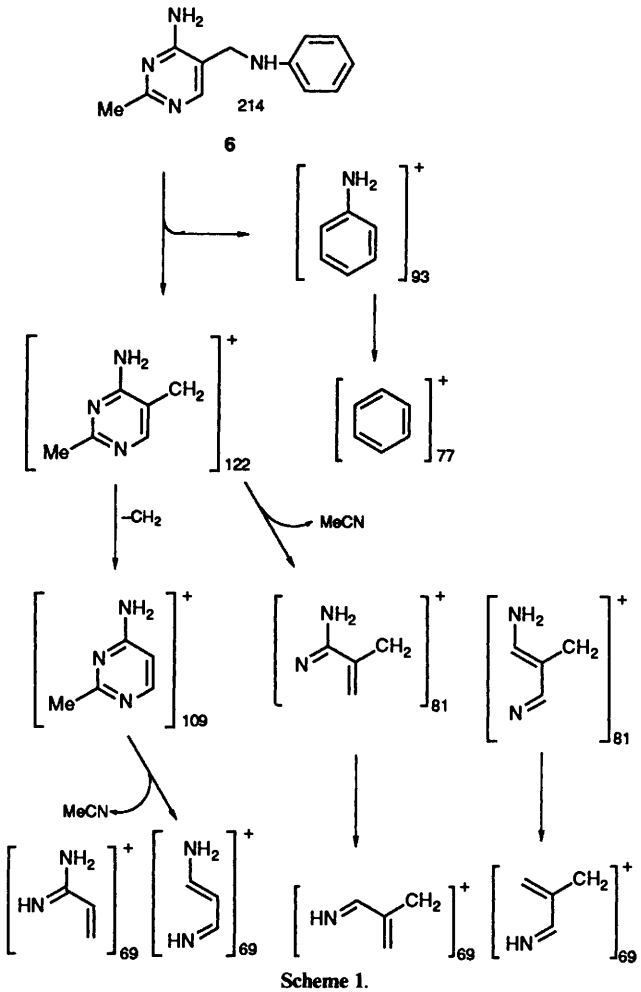
followed by isotope analysis by GCMS. In our hands, however, this approach gave variable results and we have consequently developed an alternative procedure, based on the enzyme-catalysed formation of the aniline derivative **6** which is also suitable for direct GCMS analysis.

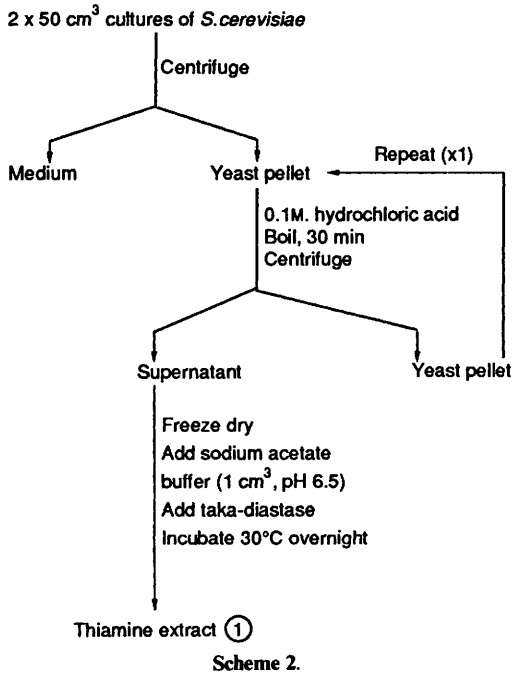
The mass spectral fragmentation of the aniline derivative **6**, prepared from thiamine by treatment with aniline and sodium metabisulphide,[12] is shown in Scheme 1. The fragmentation is based upon accurate mass measurements of each of the fragmentations. However application of this reaction to impure thiamine in extracts from yeast cultures gave only trace amounts of the derivative. In contrast, treatment of thiamine extracts with partially purified thiaminase I from *Bacillus thiaminolyticus*[13] in the presence of aniline afforded quantitative



Scheme 1.

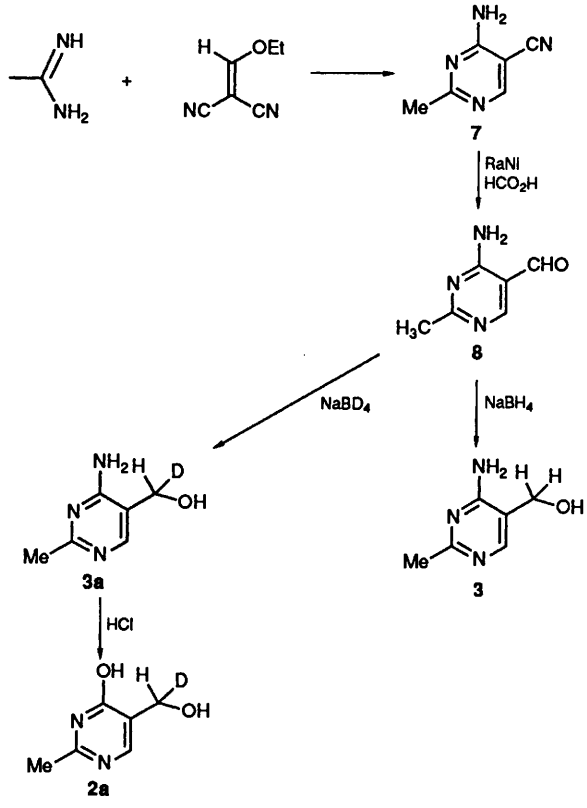
yields of **6**. The isolation and derivatisation protocol is shown in Scheme 2.

The known precursor 4-amino-5-hydroxymethyl-2-methyl-



Scheme 2.

pyrimidine **3** and its deuteriated analogue **3a** were synthesised by $NaBH_4$ and NaB^2H_4 reduction of the corresponding aldehyde **8**, which was prepared from acetamidine and ethoxymethylenemalonitrile by classical methods (Scheme 3).



Scheme 3.

4-Hydroxy-5-hydroxy[$^2H_1$]methyl-2-methylpyrimidine **2a** was prepared by acid hydrolysis of amine **3a**. Deuterium incorporations into compounds **3a** (95 atom %) and **2a** (85 atom %) were determined by MS and ^1H NMR. In trial feeding experiments, compound **2** proved to have an inhibitory effect on the growth of yeast at concentrations above 3mM. No inhibition of thiamine production by the organism was observed below this concentration.

Compounds **3**, **3a**, **2** and **2a** were each fed to *S. cerevisiae* at concentrations below the growth inhibitory level for compound **2**. After 20 h, the cells were extracted with acid, the extracts were treated with Taka-diastase, a commercial α-amylase preparation which also contains phosphatase activity,[9] to hydrolyse the thiamine pyrophosphate, and incubated with freshly prepared, partially purified, thiaminase I from *B. thiaminolyticus* and aniline to afford the aniline derivative **6** (Scheme 2). The incorporation of the deuteriated pyrimidine was determined by mass spectrometry of compound **6**. The product **6** derived from feeding unlabelled amino alcohol **3** had an $(M + 1)$ peak at 215 with 16% of the intensity of the molecular ion (214). The $(M + 1)$ peak of the de-anilino fragment (123) had an intensity of 17% of the fragment ion (122). In the product derived from feeding the deuteriated metabolite **3a**, the peak at 215 was enhanced to 28% compared with the molecular ion and the ion at 123 had an intensity of 25% of the fragment ion at 122. This enhancement corresponds to the contribution of the deuteriated anilinopyrimidine **6a** showing significant incorporation (*ca.* 8%) of **3a** into thiamine. The spectra of the product **6** derived from feeding the deuteriated 4-hydroxypyrimidine **2a** showed intensities at

215 and 123 ions indistinguishable from those of the unlabelled **6**.

Although it could be argued that diol **2** is not taken up by the cells as efficiently as amino alcohol **3**, the toxicity of compound **2** at higher concentrations suggests that this is not the case. Thus the lack of detectable incorporation of deuterium from compound **2a** under conditions where compound **3a** is effectively incorporated indicates that the 4-hydroxypyrimidine is not involved in the biosynthesis of the pyrimidine moiety in yeast. This result supports the hypothesis[9] that introduction of the 4-amino nitrogen occurs at an earlier stage of biosynthesis prior to formation of the pyrimidine ring system.

Experimental

4-Amino-2-methylpyrimidine-5-carbonitrile **7**.—Acetamidine hydrochloride (0.8 g, 8.5 mmol) was added to a 2.27M solution of NaOEt, prepared by adding sodium (0.21 g) to dry EtOH (4 cm^3). The solution was filtered through Celite and the filtrate mixed with ethoxymethylenemalonitrile (0.5 g, 4.1 mmol). The dense precipitate which formed immediately was filtered off and crystallised from EtOH to afford the nitrile **7** (0.23 g, 42%); m.p. 246–248 °C; δ(D_2O + DCl) 8.75 (1 H, s, 6-H), 2.85 (3 H, s, CH_3); *m/z* (EI), 134 (M^+) and 94 (M^{+1} – CH_3CN) (Found C, 53.4; H, 4.6; N, 42.0. $C_6H_6N_4$ requires C, 53.7; H, 4.5; N, 41.8%).

4-Amino-2-methylpyrimidine-5-carbaldehyde **8**.—Raney nickel (0.25 g) was activated by stirring with aqueous NaOH (2M, 6 cm^3) for 30 min. The supernatant liquid was decanted, the active catalyst was washed with water (2 × 10 cm^3) and added to a solution of the nitrile **7** (0.5 g, 1.1 mmol) in formic acid (98–100%; 2.5 cm^3). The reaction mixture was stirred at 80–100 °C for 45 min, then filtered through Celite, and the Celite was washed with EtOH–water (3:2; (2 × 5 cm^3). The combined filtrate and washings were evaporated under reduced pressure to remove the EtOH and the aqueous residue was neutralised with aq. $NaHCO_3$ and extracted with EtOAc (4 × 10 cm^3). The combined organic fractions were evaporated to dryness under reduced pressure to afford an off-white solid which crystallised from EtOH to give **8** (0.075 g, 20%); m.p., 192–194 °C [lit.[14] 195–196 °C]; δ$_H$([^{2}H]$_6$-DMSO); 9.8 (1 H, s, CHO), 8.6 (1 H, s, 6-H), and 2.4 (3 H, s, CH_3); *m/z* (EI) 137 (M^+) and 109 (M^+ – CO).

4-Amino-5-hydroxymethyl-2-methylpyrimidine **3**.—A solution of the aldehyde **8** (0.5 g, 3.7 mmol) in MeOH (25 cm^3) was treated with $NaBH_4$ (0.2 g, 5.3 mmol) and the reaction mixture stirred at room temperature for 30 min. After addition of further $NaBH_4$ (0.1 g, 2.6 mmol) the reaction mixture was stirred (30 min), quenched with aq. HCl (2M; 3.2 cm^3) and evaporated to dryness under reduced pressure. The residue was extracted with hot EtOH (3 × 10 cm^3) and the ethanolic extracts were combined and evaporated to dryness to afford a solid which crystallised from EtOH to give amino alcohol **3** (0.48 g, 93%); m.p. 198–200 °C [lit.,[14] 193–194 °C]; δ$_H$([^{2}H]$_6$-DMSO) 8.50 (2 H, br s, NH_2) 8.05 (1 H, s, 6-H), 4.35 (2 H, s, CH_2OH), 2.50 (3 H, s, CH_3); *m/z* (EI) 139 (M^+), 122 (M^+ – OH) and 110 (M^+ – CHO).

4-Amino-5-hydroxy[$^2H_1$]*methyl-2-methylpyrimidine* **3a**.—A solution of the aldehyde **8** (0.1 g, 0.7 mmol) in MeOH (8 cm^3) was treated with sodium borodeuteride in two portions as described above (0.04 g, 0.95 mmol; 0.02 g, 0.48 mmol). The reaction mixture was treated as described above and the product isolated and crystallised from EtOH to afford labelled compound **3a** (0.076 g, 76%); m.p. 196–198 °C; δ$_H$([^{2}H]$_6$-DMSO) 8.5 (2 H, br s, NH_2), 8.05 (1 H, s, 6-H), 4.35 (1 H, s, C*H*DOH), 2.50 (3 H, s, 2-CH_3); *m/z* (EI) 140 (M^+) and 110 (M^+ – CHDOH).

4-Hydroxy-5-hydroxy[$^2H_1$]*methyl-2-methylpyrimidine* **2a**.—A sample of the deuteriated amine **3a** (0.3 g, 2.16 mmol) was added to hydrochloric acid (6M; 5 cm^3) and heated at 100 °C for 30 min. The reaction mixture was then evaporated to dryness under reduced pressure and the residue crystallised from EtOH–EtOAc to afford compound **4a** (0.26 g, 77%); m.p. 198–200 °C, δ$_H$([^{2}H]$_6$-DMSO) 7.8 (1 H, s, 6-H), 4.20 (1 H, s, C*H*DOH) and 2.50 (3 H, s, 2-CH_3); *m/z* (EI) 141 (M^+) and 124 (M^+ – OH).

Ethyl 4-Hydroxy-2-methylpyrimidine-5-carboxylate **9**.—Acetamidine hydrochloride (1.24 g, 13 mmol) was added to a solution of NaOEt prepared by adding sodium (1.3 g) to dry EtOH (10 cm^3). The solution was filtered through Celite and the filtrate added to diethyl ethoxymethylenemalonate (2.84 g, 13 mmol) and then stirred at 0 °C for 3 h. The reaction mixture was treated with further NaOEt prepared by adding sodium (1.3 g) to dry EtOH (10 cm^3) and stirred at room temperature overnight. The reaction mixture was evaporated to dryness under reduced pressure and the yellow residue was suspended in water (20 cm^3) and washed with ether (2 × 10 cm^3). The aqueous fraction was acidified to pH 5–6 by addition of glacial HOAc then extracted with EtOAc (3 × 20 cm^3). The EtOAc extracts were combined, dried, and evaporated to dryness under reduced pressure to afford the crude product which was crystallised from acetone to give the ester **9** (1.18 g, 50%); m.p. 190–192 °C [lit.,[15] 191 °C]; δ$_H$($CDCl_3$) 8.70 (0.5 H, s, 6-H), 7.25 (0.5 H, s, 6-H), 4.50 (2 H, q, *J* 7 Hz, CH_2CH_3), 2.60 (3 H, s), and 1.35 (t, *J* 7 Hz, CH_2CH_3); *m/z* (EI) 182 (M^+).

5-Hydroxy-5-hydroxymethyl-2-methylpyrimidine **2**.—A solution of the ester **9** (0.46 g, 2.5 mmol) in dry THF (50 cm^3) was added to a suspension of lithium aluminium hydride (0.45 g, 12 mmol) in dry THF (20 cm^3). The mixture was stirred at room temperature for 1 h and then refluxed for 45 min until a faint yellow colour was observed. The reaction was cooled in ice and treated with water (0.5 cm^3). The precipitate was collected by filtration, added to phosphoric acid (*d* 1.71; 1.05 cm^3) in water (50 cm^3) then heated at 100 °C for 30 min. The hot solution was filtered, neutralised with aq. NaOH (0.1M), and evaporated to dryness under reduced pressure to afford a colourless residue. Extraction with boiling EtOH (3 × 60 cm^3; 20 min) and evaporation of the extracts to dryness gave a colourless solid which was recrystallised from dioxane to afford the diol **2** (0.02 g, 6%); m.p. 213–215 °C [lit.,[16] 212–213 °C]; δ$_H$([^{2}H]$_6$-DMSO), 7.80 (1 H, s, 6-H), 4.30 (2 H, s, CH_2OH) and 2.54 (3 H, s) *m/z* (EI) 140 (M^+) and 123 (M^+ – OH).

5-Anilinomethyl-4-amino-2-methylpyrimidine **6**.—Thiamine (0.5 g, 1.48 mmol) in EtOH–water (3:2; 12 cm^3) was treated with aniline (0.14 g, 1.5 mmol) and $Na_2S_2O_3$ (0.03 g, 0.15 mmol) and the mixture was refluxed for 1 h. The cooled reaction mixture was acidified with aq. HCl (6M), washed with diethyl ether, neutralised with aq. NaOH (4M), and extracted with EtOAc (3 × 20 cm^3). The EtOAc fractions were combined, dried and evaporated to dryness under reduced pressure to afford an oil which was purified by TLC on silica plates using $CHCl_3$–MeOH (9:1) as mobile phase. The purified compound was crystallised from EtOH to afford the aniline derivative **6** (0.08 g, 25%); m.p. 168–170 °C [lit.,[12] 167–169 °C]; *m/z* (EI) 214 (M^+) and 122 (M^+ – aniline).

Growth of the Organism.—S. cerevisiae was grown aerobically in a synthetic thiamine-free medium consisting of glucose (9 g), $(NH_4)_2HPO_4$ (6 g), KH_2PO_4 (1 g), sodium citrate (1 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), biotin (20 μg), calcium pantothenate (0.5 mg), inositol (10 mg), pyridoxine (1 mg), $ZnSO_4$

(0.4 mg), $CuSO_4 \cdot 5H_2O$ (25 μg) and $FeSO_4$ $(NH_4)_2 \cdot 6H_2O$ (150 μg) in 1 l water. The pH was adjusted to 5.0 with HCl.[15] Stock cultures were maintained on slopes of the same medium containing 2% agarose. Shake flask cultures (50 cm^3) were inoculated directly from slopes and growth of the cultures was monitored using a haemocytometer; a lag phase of 2 h was followed by rapid growth for 7 h; after 20 h the cell density was *ca.* 3×10^7 cm^{-3}.

Analysis of Thiamine.—At predetermined intervals cells were removed by centrifugation, boiled in 0.1M HCl (5 cm^3, 0.5 h) and the residue removed by centrifugation. The acid extraction was repeated and the pH of the combined supernatants was adjusted to 6.5 The thiamine extract was diluted with 0.1M acetate buffer to 20 cm^3 and treated with Taka-diastase at 30 °C for 18 h. Cultures were assayed for thiamine by conversion into the thiochrome derivative. Aliquots (5 cm^3) of the Taka-diastase treated extract were mixed with potassium ferricyanide solution (0.3M, 3 cm^3) in aq. NaOH (3.75M), the mixture was shaken for 2 min and set aside for a further 10 min, after which it was filtered and analysed by HPLC on a μBondapak column (250 × 4.6 mm i.d.) using 70% aq. MeOH as eluant (2 cm^3 min^{-1}) and fluorometric detection (excitation 365 nm; emission 435 nm). The thiamine concentration was found to maximise after 20 h growth at *ca.* 50 μg l^{-1}.

Production and Assay of Thiaminase I.—Cultures of *Bacillus thiaminolyticus* were maintained on nutrient agar slopes at 20 °C. Seed cultures were prepared by growth on nutrient broth (35 °C, 20 h) and an aliquot (5 cm^3) used to inoculate 100 cm^3 of defined medium.[13] Cultures were grown at 37 °C for 20 h. The culture was centrifuged (15 000 g, 15 min) and $(NH_4)_2SO_4$ added to the supernatant to 75% saturation (516 g l^{-1}) over 2 h at 4 °C. The precipitated protein was removed by centrifugation (15 000 g, 20 min), redissolved in water (10 cm^3) and desalted using an Amicon filter. Thiaminase I activity was assayed essentially as described.[13] An aliquot of the crude protein mixture (0.2 cm^3) was added to a solution of thiamine (1.7 mg) in sodium phosphate buffer (0.1M, pH 5.8) containing 25 mM aniline and the solution was incubated at 37 °C. Formation of the aniline derivative was followed spectrophotometrically at 248 nm.

Analysis of the Aniline Derivative **6** *from Yeast Cultures.*—Cellular extracts were prepared as above. Following treatment with Taka-diastase, the solution was freeze dried, the residue dissolved in phosphate buffer (2 cm^3, 0.1M, pH 5.8) containing 25 mM aniline, and treated with crude *B. thiaminolyticus* thiaminase at 37 °C for 18 h. The mixture was acidified to pH 2 with 6M HCl, washed with diethyl ether (2 cm^3), neutralised with 6M aq. NaOH and extracted with EtOAc (3 × 2 cm^3). The combined extract was evaporated under a stream of N_2 and the residue redissolved in 20 μl EtOAc. Samples (5 μl) were analysed by GCMS on a BP1 fused capillary column (12.5 m × 0.3 mm i.d.) linked to a Kratos MS80 Spectrometer. The inlet temperature of the GC was 260 °C and a temperature programme starting at 70 °C and increasing at 30 °C min^{-1} to 220 °C and 5° min^{-1} to 280 °C was employed. The aniline derivative **6** had a retention time of 8.0 min under these conditions.

Precursor Feeding Experiments.—Compounds **3**, **3a**, **2** and **2a** (20 mg) were each pulse fed in three equal aliquots at 5, 10 and 15 h after the initiation of growth to cultures of *S. cerevisiae* (50 ml) grown on a synthetic thiamine-free medium. After 20 h, the cells were extracted with acid, the extracts were treated with Taka-diastase and the thiamine derivatised as above and subjected to analysis using GCMS.

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