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Evidence for an Intermediate in the Enzymatic Formation of Uroporphyrinogen III

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Abstract—Evidence for an azafulvene intermediate in the enzymatic formation of Uroporphyrinogen III has been obtained. Using conditions to slow down the enzyme activity (high pH, low temperature), the transient species was trapped with ammonium ions as aminomethylbilane and with sodium borohydride as methylbilane, and observed by ^{13}C-NMR.

Introduction

Uroporphyrinogen III (Uro'gen III) synthase (cosynthetase) is the enzyme in the porphyrinoid pathway responsible for the ring closure of the linear tetrapyrrole hydroxymethylbilane (HMB, **1**) with inversion of the ring D to Uro'gen III (**2**), an important biological intermediate for hemes, coenzyme F 430, vitamin B_{12} and chlorophyll.[1] In the absence of the enzyme, HMB cyclizes chemically without rearrangement of the ring D to form Uroporphyrinogen I (Uro'gen I, **3**) (Scheme 1A).

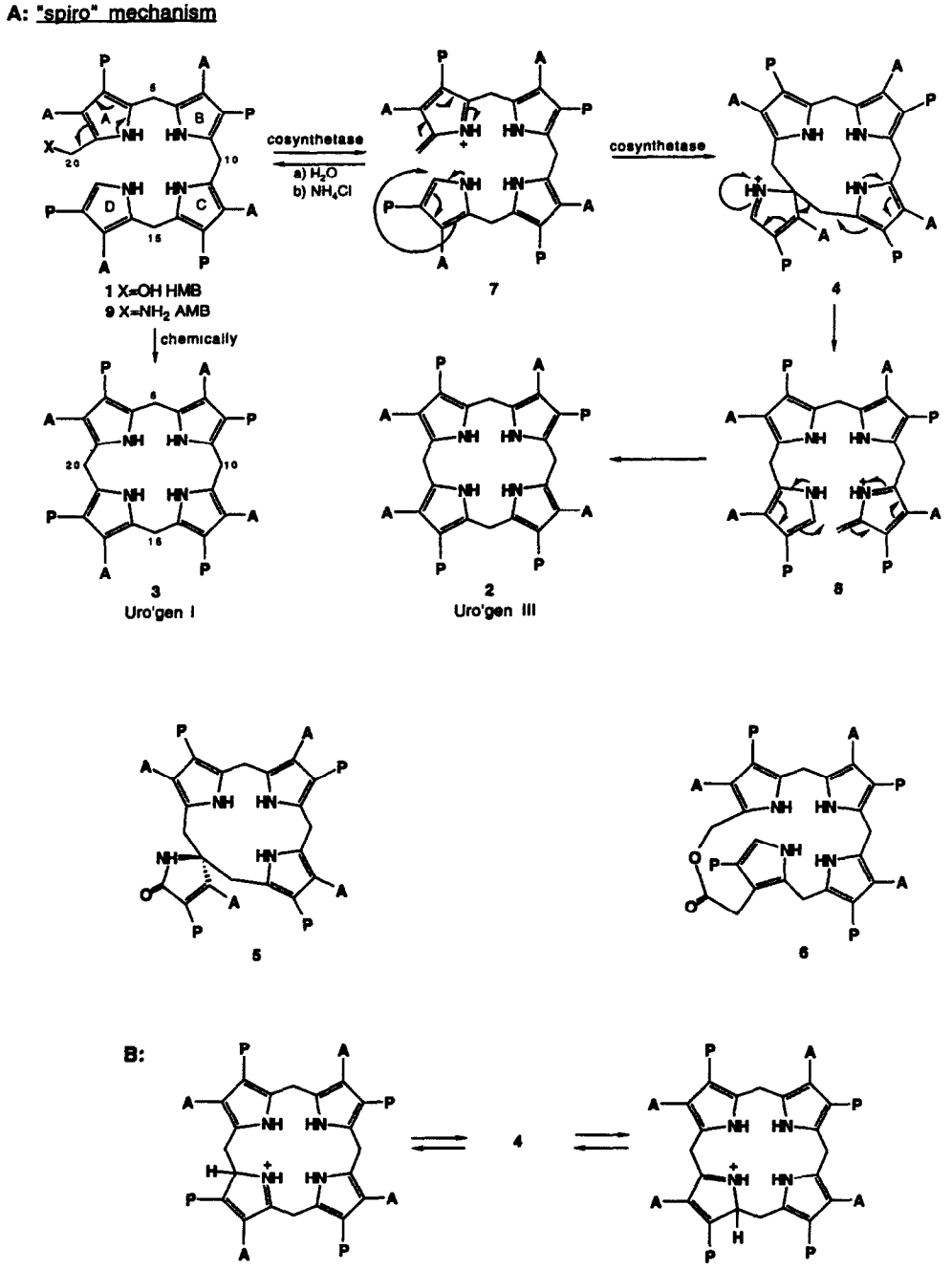
Over the years several mechanisms for this rearrangement have been proposed and investigated.[1c] Formation of a spiro intermediate (**4**)[2] followed by fragmentation–recombination[3] (Scheme 1A) is supported indirectly by the fact that one of the enantiomers of the synthetic spirolactam (**5**), analog of **4**, showed stronger enzyme inhibition (*ca* 20 times) than the other,[4] suggesting that **5** is a transition-state analog. However, X-ray crystallography has shown recently that what was thought to be the non-binding isomer of **5** has in fact a 28-membered ring dimer structure.[5] Alternatively, a lactonic intermediate (**6**) has been suggested[6] based on the failure of the synthetic bilane lacking the ring D acetate to undergo enzyme-catalyzed rearrangement.[7] More recently, theoretical calculations on the cyclization of tetrapyrroles to Uro'gens showed that direct formation of the spiro compound **4** was not energetically favored and led to the description of a new mechanism involving [1,5]-sigmatropic shifts, which took into account the experimental results (Scheme 1B).[8] Cryogenic NMR experiments using HMB ^{13}C-labelled at various positions have not so far permitted the observation of any intermediate (either through fragmentation or synchronous mechanisms),[6] probably due to the fact that the intermediate is enzyme-bound and therefore difficult to detect, and also to unfavorable kinetics which may not allow the accumulation of the transient species.

In all the fragmentation mechanisms two azafulvenes (**7** and **8**) are involved, one before the carbon–carbon bond formation to the intermediate and the second in the fragmentation–recombination process which follows, as shown in Scheme 1A. No observation of an azafulvene has ever been reported for cosynthetase, but it has been shown that porphobilinogen (PBG) deaminase, the enzyme responsible in the vitamin B_{12} pathway for condensing four units of PBG to generate the cosynthetase substrate HMB, produces the azafulvene **7**, which was trapped by ammonium ions as aminomethylbilane (AMB, **9**), by hydroxylamine as hydroxyaminobilane (**10**) or by methoxyamine as methoxyaminobilane (**11**) (Scheme 2).[9]

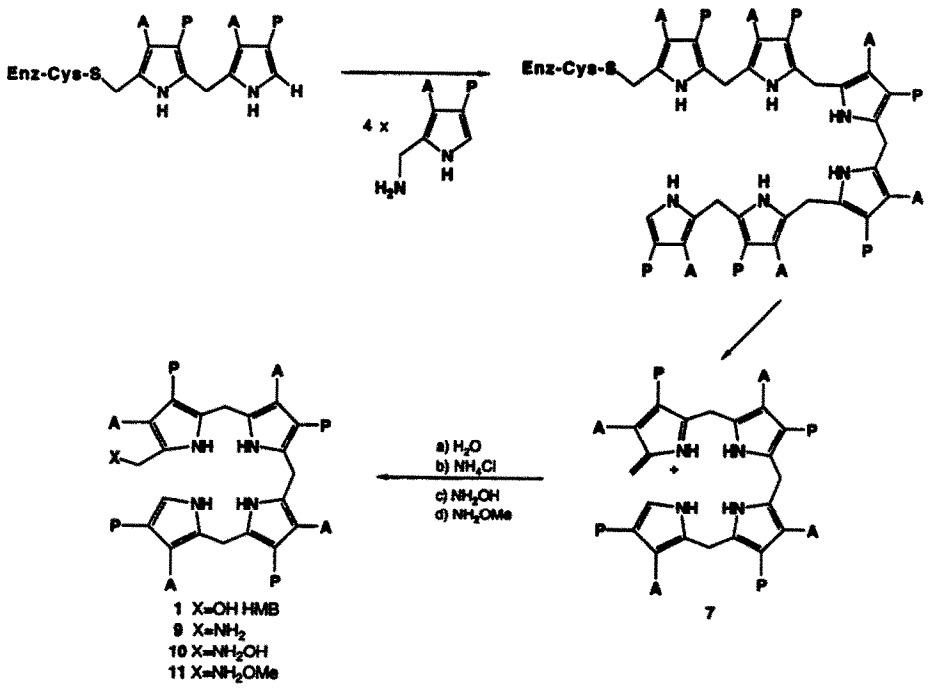
Availability of substantial quantities of pure cosynthetase (deaminase free), obtained by cloning and overexpressing the genes *hemC* and *D* together,[10] and of HMB ^{13}C-labelled specifically at the C-20 and C-15 positions has now allowed NMR investigations of the enzyme mechanism. The design of experiments to trap species such as **4** or the azafulvenes **7** and **8** relied on selecting specific reaction conditions which would slow down the action of the enzyme without causing inactivation. By using cryogenic solvents for low temperature studies, high pH levels, and by allowing the enzyme to turn over in the presence of high concentrations of an appropriate nucleophile, the chances of trapping a reactive species should be enhanced. This paper describes the successful trapping of an azafulvene generated by Uro'gen III synthase from its substrate HMB.

Syntheses of the ^{13}C-Labelled HMB's

In order to carry out these experiments, it was necessary to have in hand synthetic HMB ^{13}C-labelled specifically at the C-20 and C-15 positions, to ensure that the samples are deaminase-free and to provide a clear window for NMR detection, rather than using biosynthetic HMB generated from ^{13}C-labelled 5-aminolevulinic acid (ALA) by action of ALA dehydratase and PBG deaminase, which could contain traces of the latter enzyme, known to generate **7**.[9] In both cases, *N,N*-dimethylformamide-[^{13}C-carbonyl] (DMF) was used to introduce the labelled carbon by modifications of the HMB synthesis.[11]

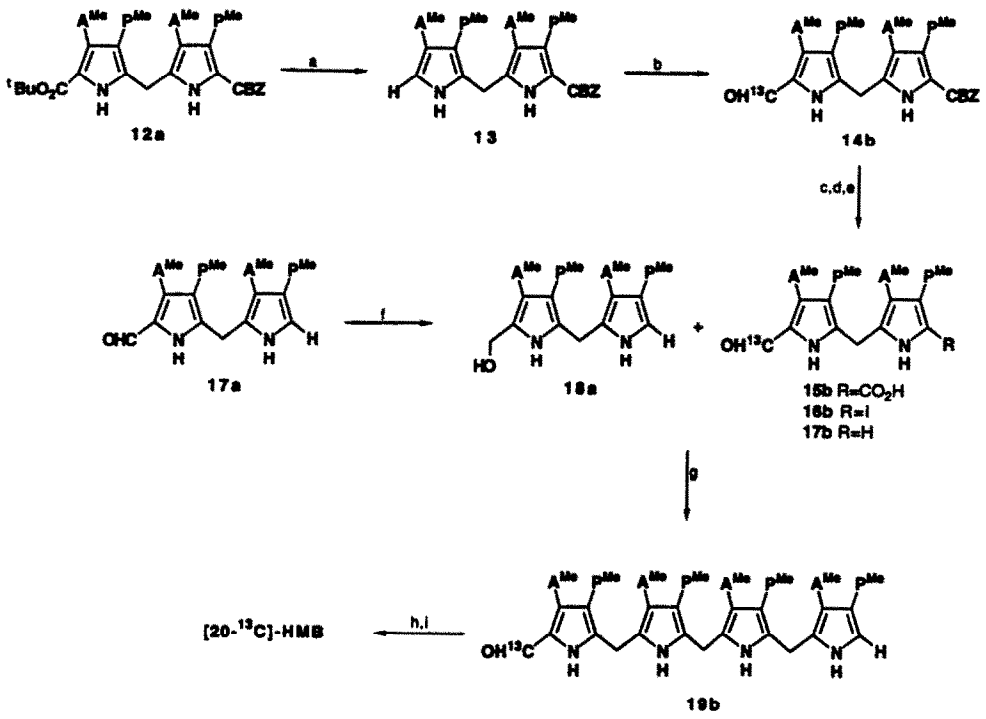


Scheme I. Proposed mechanisms for Uro'gen III synthase.



A=CH_2CO_2H; P=$CH_2CH_2CO_2H$

Scheme II. PBG deaminase mechanism.



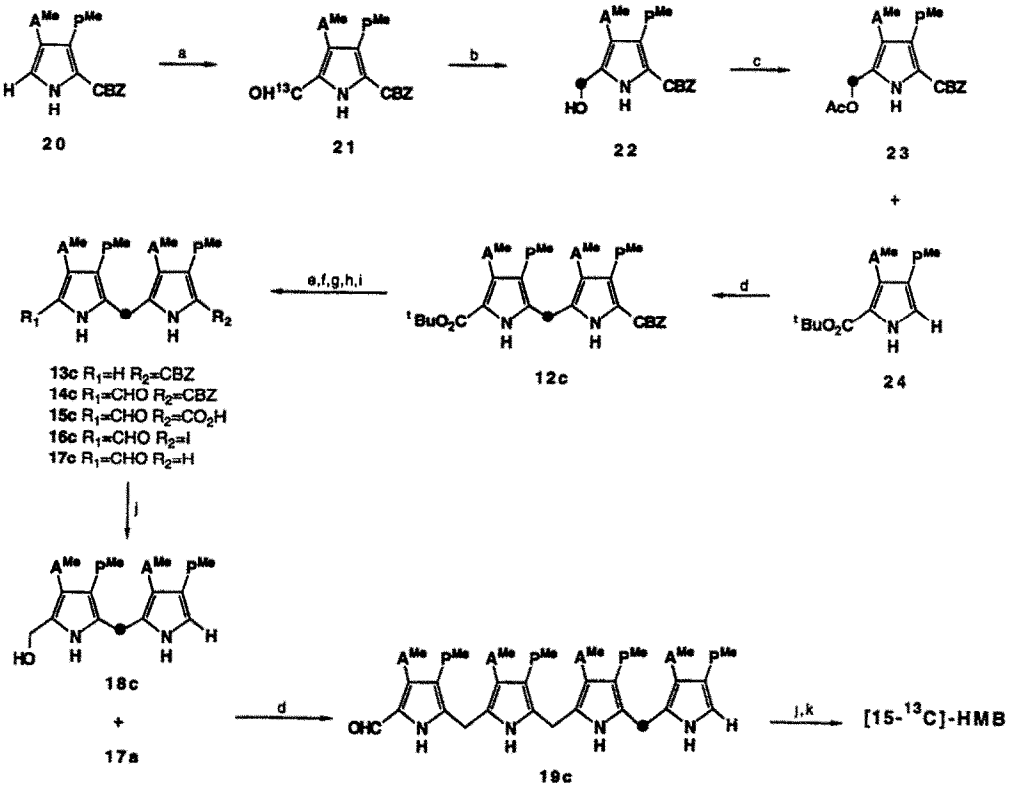
a) TFA; b) ^{13}C-DMF, $POCl_3$, CH_2Cl_2; c) H_2/Pd-C, Et_3N, THF; d) I_2, KI, $NaHCO_3$, H_2O-CH_2Cl_2; e) H_2/Pd-C, MeOH, NaOAc; f) $NaBH_4$, MeOH-CH_2Cl_2; g) Montmorillonite clay, CH_2Cl_2; h) $NaBH_4$, MeOH-CH_2Cl_2 + 1% Et_3N; i) 2N KOH.

A^{Me}=CH_2CO_2Me; P^{Me}=$CH_2CH_2CO_2Me$; CBZ=CO_2CH_2Bz

Scheme III. Synthesis of [20-^{13}C]-HMB.

The route to [20-^{13}C]-HMB is shown in Scheme 3. The dipyrromethane **12a** was prepared,[7a] the tertbutyl ester hydrolyzed and the resultant acid decarboxylated in trifluoroacetic acid to **13**. Vilsmeier formylation with ^{13}C-DMF afforded the ^{13}C-formyldipyrromethane **14b** in good yield. The next three steps followed the usual route,[11] i.e. debenzylation, oxidative decarboxylation and dehalogenation, providing the formyldipyrrole **17b**. The non-labelled equivalent **17a** (prepared as in Reference 11) was reduced with sodium borohydride to the hydroxymethyldipyrrole **18a** and coupled with an excess of **17b** using Montmorillonite clay as acid catalyst[12] to form the ^{13}C-formylbilane **19b**. After sodium borohydride reduction of the formyl group to hydroxymethyl, it was necessary to remove completely the boron impurities by washing the solid bilane with methanol, before saponification of the octamethyl ester to give [20-^{13}C]-HMB, ready for enzymatic experiments.

The second labelled bilane, [15-^{13}C]-HMB, was synthesized as shown in Scheme 4. The labelled carbon was also introduced by Vilsmeier formylation of compound **20**[7a] with ^{13}C-DMF to afford the ^{13}C-formylpyrrole **21**, which was reduced to the hydroxy-^{13}C-methylpyrrole **22** with a slight excess of sodium borohydride in methanol, buffered with ammonium chloride, at low temperature.[13] If the reaction is run with a large excess of the reducing reagent and in the absence of buffer, the reduction of the propionate methyl ester to alcohol is observed as side reaction. Acetylation of the hydroxy group with acetic anhydride/pyridine provided the pyrrole **23**, which was coupled to the α-free pyrrole **24**[7a] using again the Montmorillonite clay as a catalyst[12] to give the dipyrromethane **12c** in very good yield. The normal sequence of steps[11] (hydrolysis and decarboxylation of the tertbutyl ester, formylation, debenzylation, oxidative decarboxylation and finally hydrogenation) was then resumed to provide the ^{13}C-dipyrromethane **17c**. The formyl group was reduced by sodium borohydride to **18c** and coupled with **17a** (excess) with Montmorillonite clay to afford the ^{13}C-bilane **19c**. The [15-^{13}C]-HMB was then obtained after reduction of the aldehyde followed by alkaline hydrolysis of the methyl ester.



●^{13}C; A^{Me}=CH_2CO_2Me; P^{Me}=$CH_2CH_2CO_2Me$; CBZ=CH_2CO_2Bz

a) ^{13}C-DMF, $POCl_3$, CH_2Cl_2; b) $NaBH_4$, NH_4Cl, MeOH, 0°C; c) Ac_2O, Pyridine; d) Montmorillonite clay, CH_2Cl_2; e) TFA; f) $HC(OMe)_3$; g) H_2/Pd-C, THF, Et_3N; h) I_2, KI, $NaHCO_3$, H_2O-CH_2Cl_2; i) H_2/Pd-C, MeOH, NaOAc; j) $NaBH_4$, MeOH-CH_2Cl_2; k) 2N KOH.

Scheme IV. Synthesis of [15-^{13}C]-HMB.

Effects of Temperature, pH and Nucleophiles on Cosynthetase Activity

Effect of temperature

As lower temperatures slow down enzymatic reaction rates and should allow a better chance of trapping intermediates which could be observed by NMR,[14] a search for suitable cryo-solvents compatible with cosynthetase was performed. A study of the ability of cosynthetase to generate Uro'gen III in four potential solvents showed that cosynthetase remained reasonably active in up to 20% acetonitrile/water, 40% methanol/water, 20% ethanol/water and 20% DMF/water, where 55, 83, 100 and 100% respectively of the original enzyme activity was retained after one hour incubation (Figure 1). Having a choice of cryo-solvents, a temperature study was next performed, where no enzymatic turn over of HMB to Uro'gen III could be observed at temperatures below –5 °C in 30% methanol/water.

Effect of high pH

The effect of high pH on the enzyme was also studied as it increased the stability of the substrate HMB against chemical ring closure. Uro'gen III formation at pH 9 to 11 was monitored for two incubation times (10 minutes and 2 hours) (Figures 2A and 2B). The results showed that cosynthetase remains active at pH over 10.5, with inactivation occurring above 11. At pH 10.9 and 0 °C, both small (50 μg cosynthetase/20 μg HMB) and large (2 mg cosynthetase/1 mg HMB) scale incubations generated Uro'gen III over a period of 6 hours (Figure 2C), in contrast to less than 1 minute at the optimum conditions of 37 °C and pH 8 . The enzyme remains soluble at pH levels over 11, which is also the upper pH limit for activity.

Effect of various nucleophiles

1-Azafulvenes have been trapped with a variety of nucleophiles.[15] In earlier works on PBG deaminase/cosynthetase mixtures[9a,b] (later confirmed by Battersby with pure deaminase[9c]), the azafulvene **7** produced by the enzyme was trapped with ammonium ions, hydroxylamine or methoxyamine to give respectively aminomethylbilane (**9**), hydroxyaminomethylbilane (**10**) or methoxyaminomethylbilane (**11**) by running incubations in presence of high concentrations of these nucleophiles.

It was necessary to check the activity of cosynthetase in the presence of various nucleophilic salts. From these, only ammonium chloride appeared not to have a major effect on the enzyme (about 80% activity retained). With sodium borohydride, sodium cyanoborohydride, hydroxylamine and sodium hydrosulfite, considerable losses of activity were observed (less than 30% activity retained), while sodium azide and potassium cyanide partially inactivated the enzyme (30–50% activity retained).

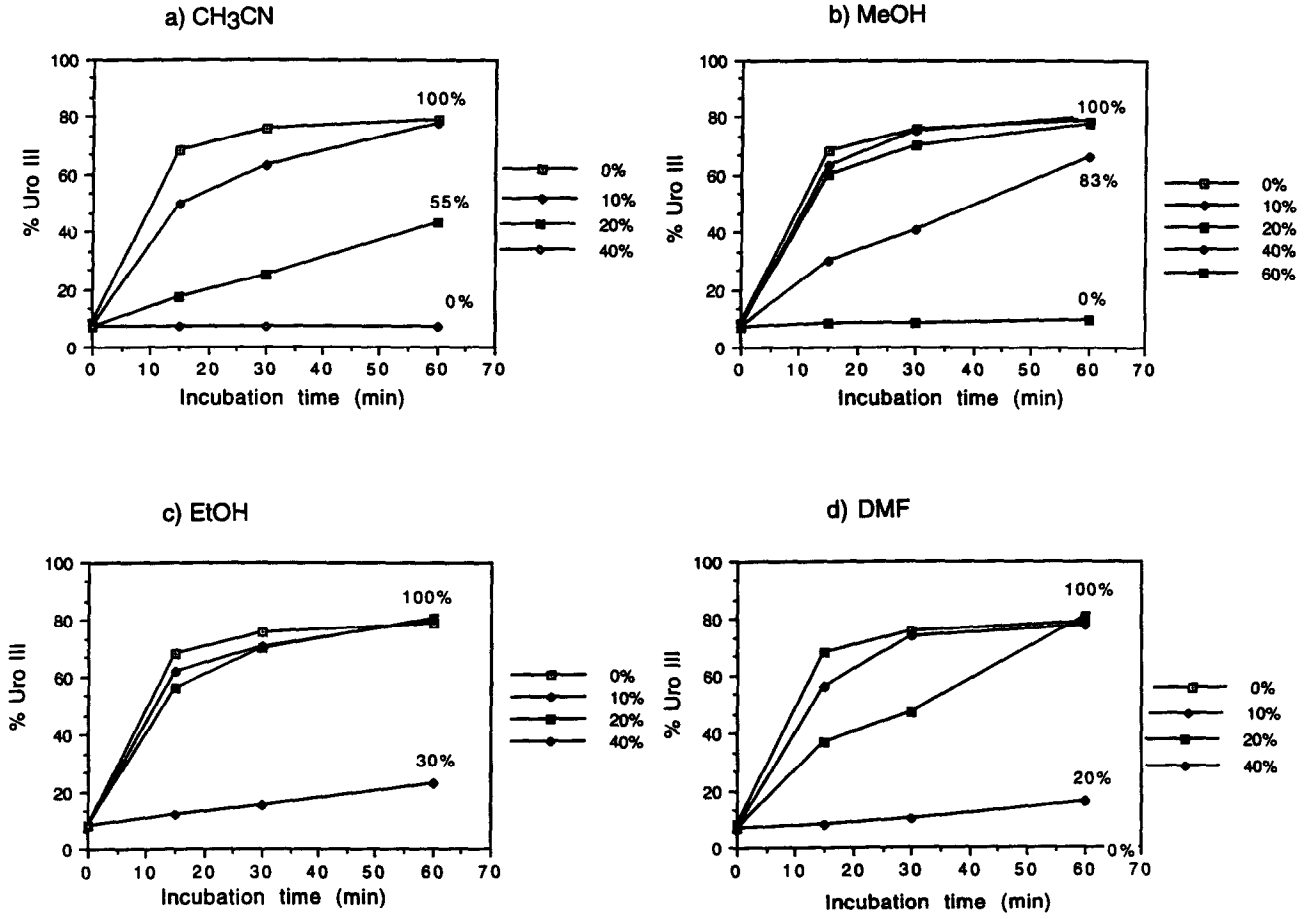
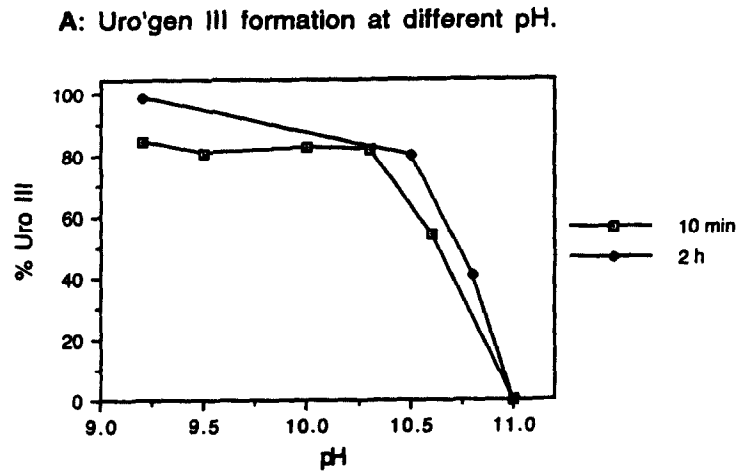
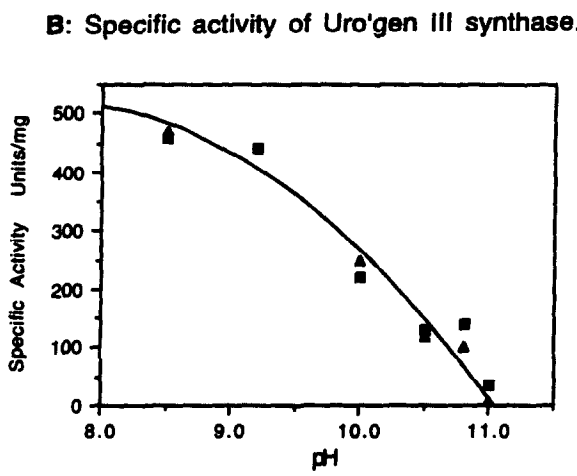


Figure 1. Effects of different solvents on Uro'gen III synthase.





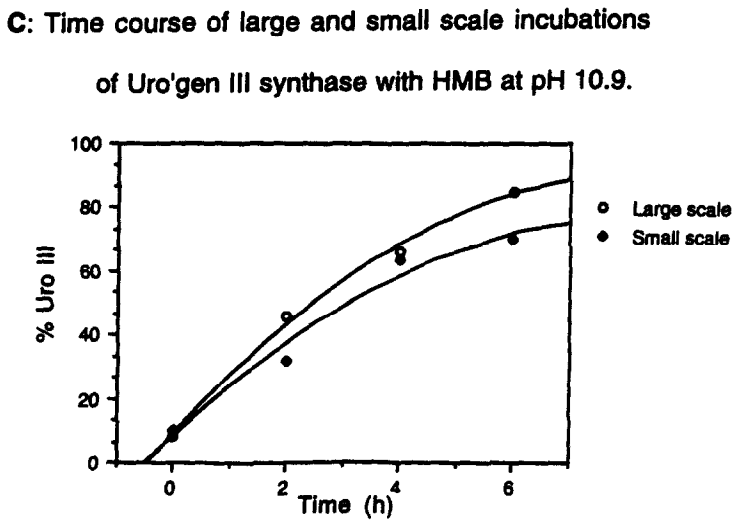


Figure 2. Effect of pH on Uro'gen III synthase.

NMR Studies

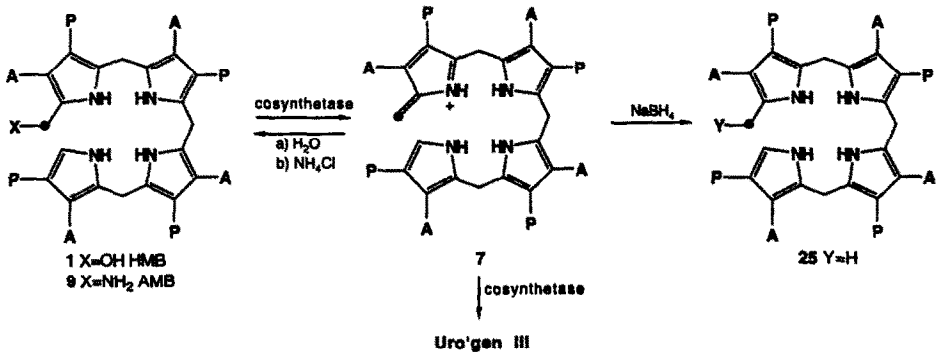
The main difficulty in carrying out azafulvene trapping experiments with cosynthetase (compared with deaminase) resides in the strong competition between the very fast enzymatic closure of the azafulvene to Uro'gen III, which takes place inside the active site, and the chemical trapping. If the azafulvene **7** was released, rapid exchange with the aqueous medium would form HMB. In slowing down the enzymatic rate, we may give more time for the nucleophile to attack **7** and thus form a stable species such as **9** (Scheme 5), which could be observed by ^{13}C-NMR. The rate was controlled by using high pH (10.8), low temperature (+5 °C) and low ratio of enzyme to substrate (1:40). To ensure that the cosynthetase solution was deaminase free, deaminase activity assays were performed prior to each incubation.

Incubations of cosynthetase with [20-^{13}C]-HMB in presence of 200 mM ammonium chloride were run in an NMR tube in 10% acetonitrile/buffer pH 10.8, the acetonitrile being preferred as cryo-solvent for its lack of interference in the NMR region of interest. Under these conditions, slow formation of Uro'gen III (δ = 22.0 ppm) from [20-^{13}C]-HMB (δ = 55.4 ppm) was observed. However, since the product of the trapping of azafulvene **7** by ammonium ions, aminomethylbilane (AMB, **9**), is itself a slow substrate of cosynthetase[6,7a] and obtained in small quantity (the normal product Uro'gen III being very strongly favored), ^{13}C-NMR observation of the corresponding signal at 35.7 ppm was only possible by increasing the pH to 11.5 (in order to inactivate the enzyme) and by overnight accumulation of data (Figure 3D). Verification of the assignment for AMB was confirmed by incubating [20-^{13}C]-HMB with PBG deaminase in presence of high concentration of ammonium chloride, which showed formation of some AMB (δ = 35.5 ppm). The amino group was then titrated by stepwise increase of pH and the aminomethyl peak shifted downfield by 0.8 ppm as the pH approached 12. A series of control experiments to probe the chemical reactivity of HMB with ammonium ions in the absence of enzyme and under the same conditions as for the enzymatic incubations (200 mM ammonium chloride, pH 10.8 increased later to 11.5) but without enzyme were run, in which *no* formation of AMB could be observed by ^{13}C-NMR (Figure 3B). This excludes a nucleophilic substitution of the hydroxy group by the ammonium ions and ensures that the generation of AMB via the azafulvene **7** is enzymatic.

Likewise, in the absence of ammonium chloride, cosynthetase incubations of HMB at pH 10.8 to 11.5 did not produce AMB (^{13}C-NMR, Figure 3C). To confirm that the amino group was in fact derived from the added ammonium ions, $^{15}NH_4Cl$ was used in the incubations. Because of the low ratio of AMB present and the small coupling constant expected,[9c] only special ^{13}C-NMR enhancement techniques enabled the observation of the signal corresponding to the $^{15}NH_2{}^{13}CH_2$-bilane as a doublet (δ = 35.4 ppm) with a coupling constant of 4 Hz (Figure 4).

Other nucleophiles, such as sodium azide and potassium cyanide, were also tested as trapping agents, however no NMR signal corresponding to a chemical reaction with the azafulvene **7** could be observed. This is possibly due to the loss of enzyme activity caused by these reagents, as mentioned earlier.

Sodium borohydride completely inactivated cosynthetase, although not immediately. By running the incubation in the presence of 400 mM sodium borohydride for 5 min at 37 °C, an appreciable turnover of HMB to Uro'gen III took place before complete enzyme inactivation. At this stage, a new peak at 11.0 ppm appeared in the ^{13}C-NMR spectrum (Figure 5C), which is assigned to the methylbilane **25**, resulting from the double bond reduction of the azafulvene **7** (Scheme 5). Again, this signal was not observed in any control experiments performed under identical conditions (400 mM sodium borohydride, 5 min, 37 °C, pH 10.8) without enzyme (Figure 5B). *Only* in acidic conditions, could the azafulvene be generated chemically and reduced to a methyl group by sodium cyanoborohydride or by hydrogenation over palladium-on-carbon. Thus, a synthetic sample of the [20-^{13}C]-methylbilane **25** was obtained from the chemical reduction of the [20-^{13}C]-HMB by sodium cyanoborohydride in the presence of Montmorillonite clay as acid catalyst. ^{13}C-NMR comparison of the synthetic bilane with the biosynthetic sample showed identical chemical shifts (Figure 6) and confirmed that the enzymatic product is the ^{13}C-methylbilane **25**.



• ^{13}C; A=CH_2CO_2H; P=$CH_2CH_2CO_2H$

Scheme V. Azafulvene trapping experiments with [20-^{13}C]-HMB.

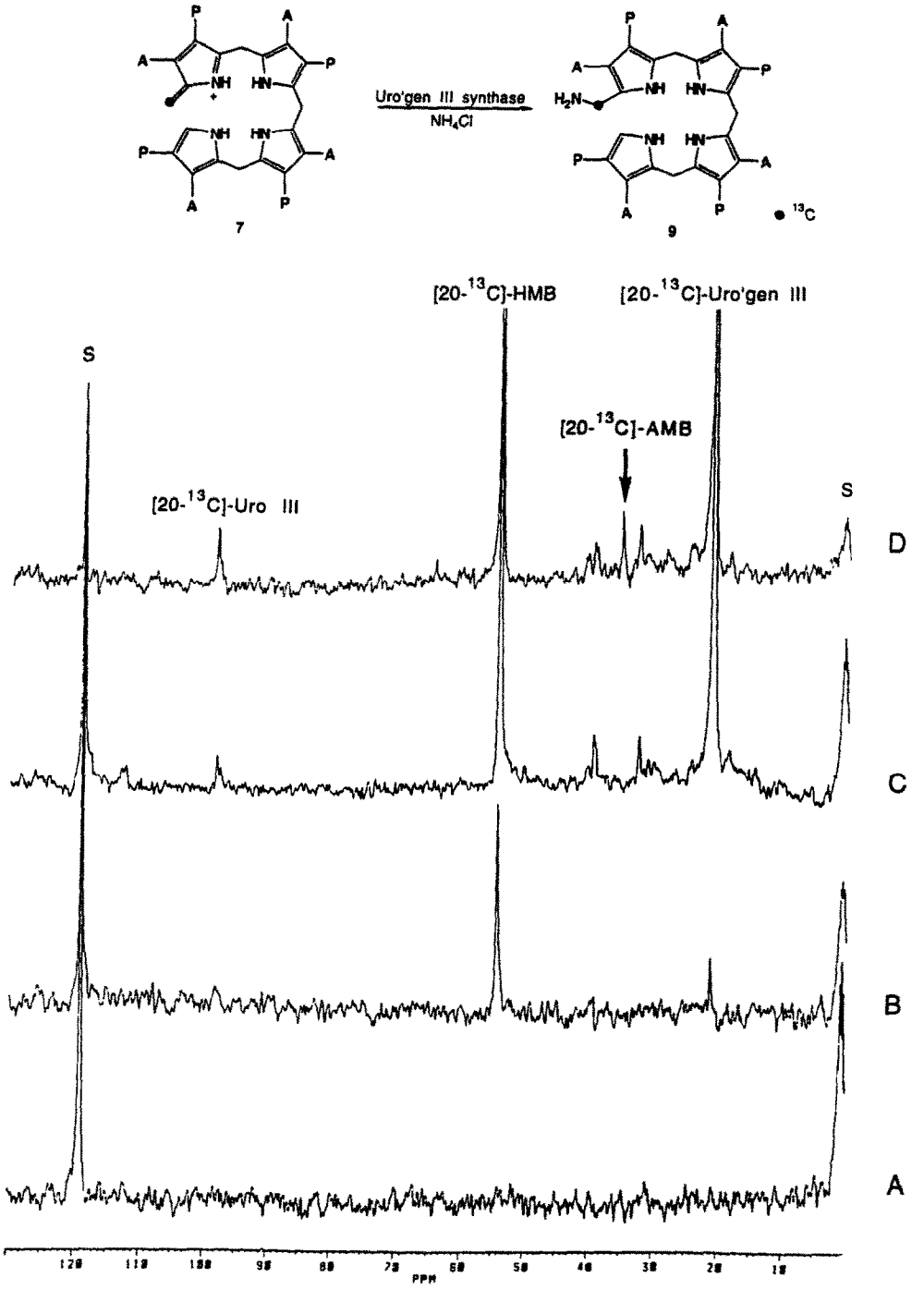


Figure 3. The NH_4Cl series of experiments. Proton decoupled 75.4 MHz ^{13}C-NMR spectra were recorded at 4°C and pH 11.5; A) Enzyme and NH_4Cl; B) [20-^{13}C]-HMB and NH_4Cl; C) [20-^{13}C]-HMB and enzyme; D) [20-^{13}C]-HMB, enzyme and NH_4Cl.

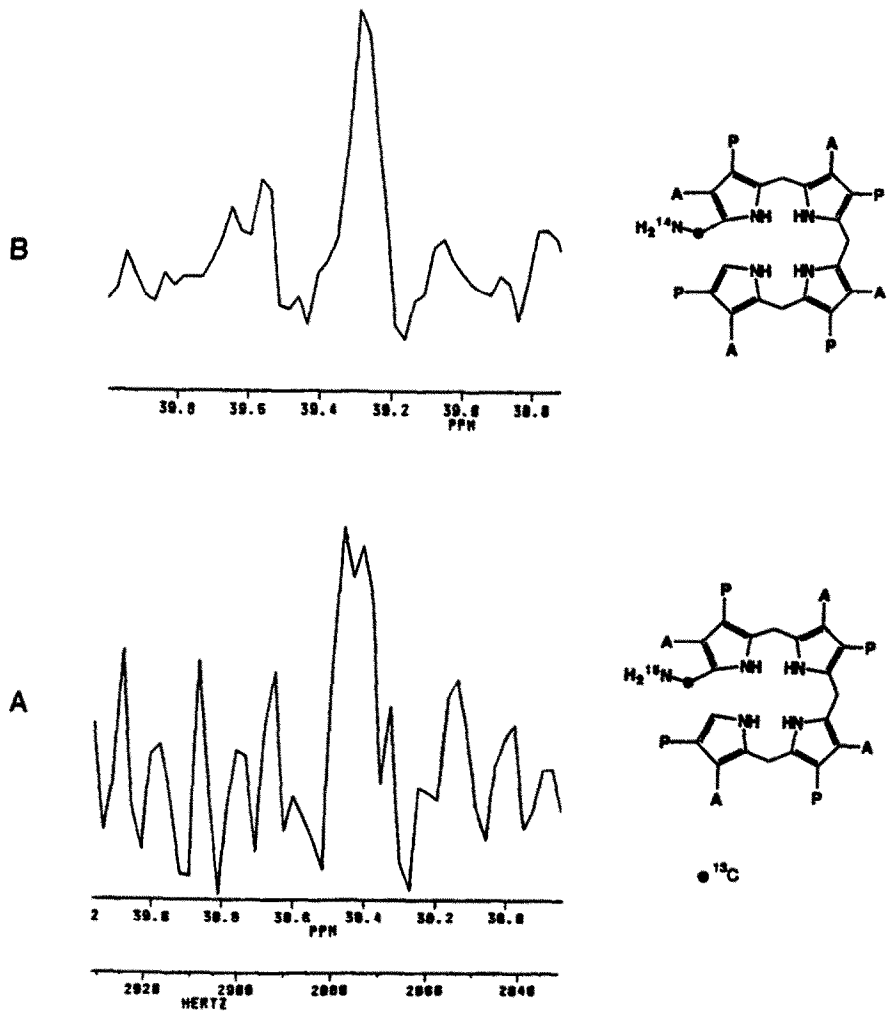


Figure 4. Comparison of $^{15}NH_4Cl$ versus $^{14}NH_4Cl$ in the trapping experiments. Selected region of 75.4 MHz ^{13}C-NMR spectra showing the aminomethyl signal resulting from the action of Uro'gen III synthase on [20-^{13}C]-HMB in the presence of A) $^{15}NH_4Cl$ at pH 12.6 and B) $^{14}NH_4Cl$ at pH 11.5. The shift in the ^{13}C-signals accounts for the different pH, as mentioned in the text.

A similar series of experiments was carried out using the [15-^{13}C]-HMB as substrate in order to attempt the trapping of the azafulvene **8** (Scheme 6). As before in the presence or absence of high concentration of ammonium chloride, after some turn over had occurred from HMB (δ = 22.5 ppm) to Uro'gen III (δ = 22.0 ppm), the pH was raised to 11–11.5 to allow NMR observation of new signals. These were expected to be around 55 ppm for the hydroxymethyl or 36 ppm for the aminomethyl groups (**26** and **27** respectively in Scheme 6). However none of these species was observed. Incubations were also run in the presence of sodium borohydride at 37 °C for 5 min, which did not show any peak at 11.0 ppm corresponding to the reduction of the azafulvene **8** to **28** (Scheme 6). Presumably, this second azafulvene could be short-lived or might be formed deep inside the active site pocket, rendering it unavailable to exchange with the medium to generate sufficient compound **26**, **27** or **28** to the level of NMR detection. On the other hand, a series of [1,5]-sigmatropic rearrangements instead of a fragmentation–recombination mechanism would not lead to the generation of the azafulvene **8** from the intermediate **4** and must still be considered as a possible biochemical mechanism.

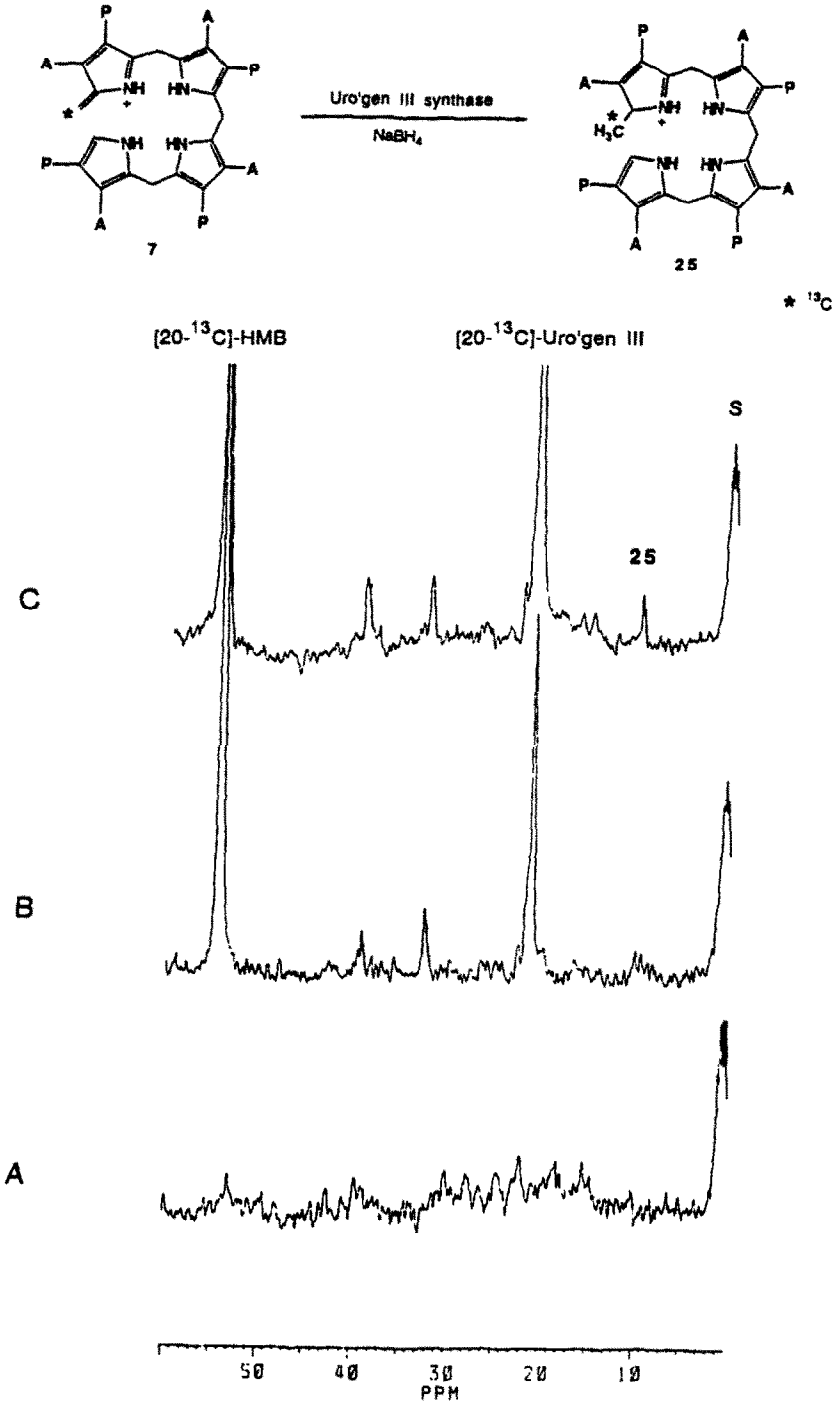


Figure 5. The $NaBH_4$ series of experiments. The proton decoupled 75.4 MHz ^{13}C-NMR spectra were recorded at 4°C and pH 11.5; A) enzyme and $NaBH_4$; B) [20-^{13}C]-HMB and $NaBH_4$; C) [20-^{13}C]-HMB, enzyme and $NaBH_4$.

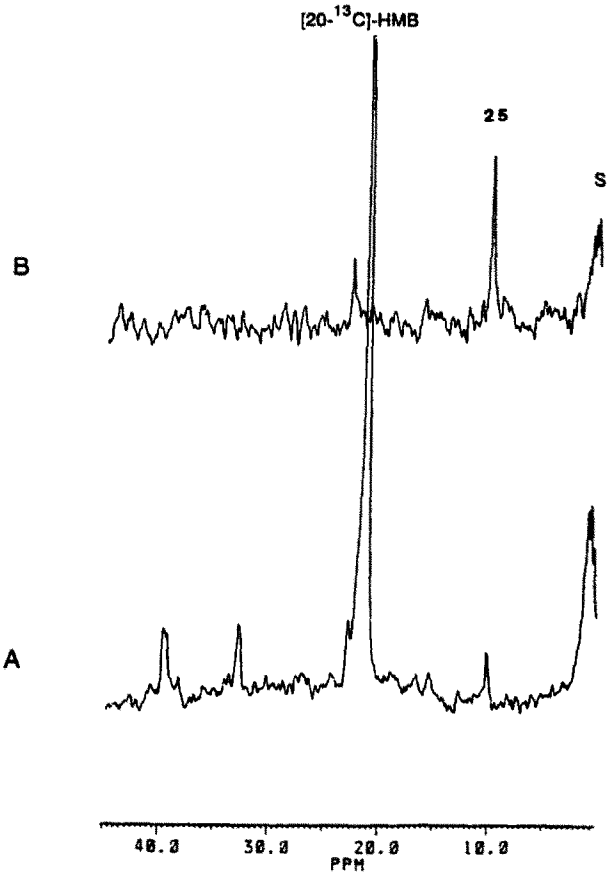
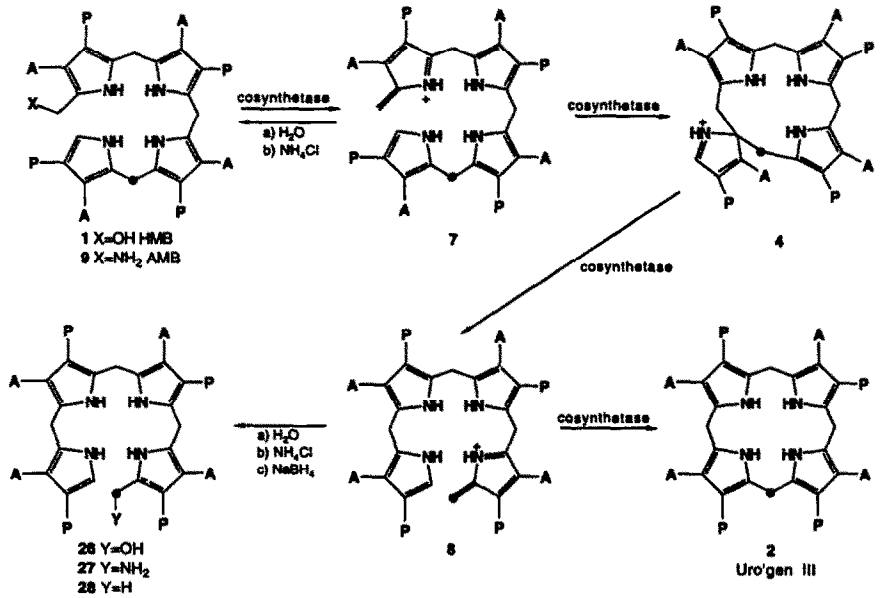


Figure 6. Comparison of the biosynthetic and synthetic samples of ^{13}C-methylbilane **25**. Selective region of 75.4 MHz ^{13}C-NMR spectra showing the methyl signal from the C-1 position of HMB derivative A) biosynthetically from Uro'gen III synthase in the presence of $NaBH_4$ and [20-^{13}C]-HMB and B) by chemical synthesis.



• ^{13}C; A=CH_2CO_2H; P=$CH_2CH_2CO_2H$

Scheme VI. Azafulvene trapping experiments with [15-^{13}C]-HMB.

Experimental

Chemistry

General procedures. All solvents and reagents were purified and dried when necessary by standard literature methods. Column chromatographies, TLC and preparative TLC (PLC) were carried out with silica gel. ^{1}H- and ^{13}C-NMR (200, 300 or 500 MHz) spectra were recorded in $CDCl_3$.

5-Benzyloxycarbonyl-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole (13). A solution of the tertbutoxycarbonyl dipyrromethane **12a**[7a] (5.21 g, 7.48 mmol) in TFA (30 mL) was stirred at R.T. for 1.5 h. The solution was diluted with AcOEt (130 mL) and washed with H_2O, 10% $NaHCO_3$ and saturated $NaHCO_3$ until the pH of the aqueous solution remained basic. After evaporation, the residue was chromatographed (AcOEt/Hexanes, 1/1). The product **13** was obtained as an oil (4.2 g, 7.05 mmol, 94%) ^{1}H-NMR δ 9.88, 9.34 (2s br, 2H, 2N*H*); 7.37–7.28 (m, 5H, Ph*H*); 6.55 (d, J = 2.5, 1H, *H*α); 5.23 (s, 2H, CH_2Ph); 3.87 (s, 2H, CH_2 *meso*); 3.73, 3.65, 3.60, 3.50 (4s, 12H, 4 CO_2CH_3); 3.55, 3.41 (2s, 4H, 2 CH_2CO_2Me); 2.99, 2.77 (2t, *J* = 7.8, 6.4, 4H, 2 $CH_2CH_2CO_2$Me); 2.57, 2.47 (2t, *J* = 6.4, 7.8, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 174.67; 174.31; 173.48; 172.78; 160.46; 136.03; 133.48; 129.89; 128.36; 128.25; 128.20; 128.08; 127.94; 125.77; 117.64; 116.29; 115.68; 113.87; 113.43; 65.55; 52.33; 51.71; 51.59; 51.28; 34.65; 34.31; 31.37; 29.18; 22.06; 20.51; 18.70.

Benzyl 5'-(^{13}C-formyl)-3',4-di-(2-methoxycarbonylethyl)-3,4'-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole-5-carboxylate (14b). A solution of ^{13}C-DMF (500 mg, 6.75 mmol) and phosphorus oxychloride (0.55 mL, 6.75 mmol) in CH_2Cl_2 (5 mL) was stirred at R.T. for 30 min. The solution was then added to a solution of the α-free dipyrromethane **13** (4.2 g, 7.05 mmol) in CH_2Cl_2 (5 mL). The reaction was stirred 5 h, aqueous $NaHCO_3$ was added until the pH remained basic. The product was extracted into CH_2Cl_2, the solvent evaporated, the residue redissolved in AcOEt and washed successively with 10% $NaHCO_3$, saturated $NaHCO_3$, H_2O and brine. The product **14b** was purified by chromatography (AcOEt/Hexanes, 1/1) (3.44 g, 5.50 mmol, 78%). ^{1}H-NMR δ 10.85, 10.46 (2s, 2H, 2N*H*); 9.46 (d, J_{CH} = 173.5, 1H, ^{13}C*H*O); 7.31–7.21 (m, 5H, Ph*H*); 4.00 (s, 2H, CH_2 *meso*); 3.69 (s, 2H, CH_2CO_2Me); 3.69, 3.65, 3.59, 3.55 (4s, 14H, 4 CO_2CH_3 + CH_2CO_2Me); 3.00, 2.77 (2t, *J* = 7.5, 6.7, 4H, 2 $CH_2CH_2CO_2$Me); 2.50 (m, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.23 (^{13}CHO); 173.47; 173.09; 172.69; 170.81; 160.26; 135.63; 135.26; 130.78; 129.97; 128.68 (d, *J* = 68.6, C_4·); 128.02; 127.88; 127.66; 120.80; 117.77; 114.37; 65.36; 51.93; 51.79; 51.31; 50.96; 34.25; 33.81; 29.41; 29.08; 22.24; 22.13; 20.21.

5'-(^{13}C-Formyl)-3',4-di-(2-methoxycarbonylethyl)-3,4'-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole-5-carboxylic acid (15b). The dipyrrole **14b** (2.89 g, 4.63 mmol) in THF (50 mL) containing Et_3N (2 mL) was hydrogenated over 10% Pd–C (300 mg) overnight. The solution was filtered through Celite and the catalyst washed with CH_2Cl_2. The filtrate was neutralized by washing with 0.1 N HCl. The compound **15b** (2.46 g, 4.60 mmol, 99%) was used without further purification in the next step. ^{1}H-NMR δ 11.43, 10.70 (m + s, 2H, 2N*H*); 9.31 (d, J_{CH} = 174.8, 1H, ^{13}C*H*O); 3.96 (s, 2H, CH_2 *meso*); 3.68, 3.49, (2s, 4H, 2 CH_2CO_2Me); 3.65, 3.61, 3.57 (3s, 12H, 4 CO_2CH_3); 2.97, 2.79 (2t, *J* = 7.7, 7.4, 4H, 2 $CH_2CH_2CO_2$Me); 2.53, 2.47 (2t, *J* = 7.7, 7.4, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.36 (^{13}CHO); 173.59; 173.30; 172.47; 170.92; 164.97; 136.29; 131.39; 131.19; 128.95 (d, *J* = 68.8, C_4·); 127.87; 121.57; 118.18; 114.73; 52.20; 52.11; 51.61; 51.29; 34.52; 34.45; 29.69; 29.45; 22.50; 20.40; 18.68.

5-(^{13}C-Formyl)-5'-iodo-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole (16b). A solution of I_2 (1.41 g, 5.56 mmol) and KI (1.38 g, 8.33 mmol) in H_2O (20 mL) was added to a mixture of **15b** (2.46 g, 4.60 mmol) and $NaHCO_3$ (1.56 g, 18.52 mmol) in H_2O/CH_2Cl_2 (20/40 mL). The reaction was stirred vigorously at R.T. for 15 min. Sodium bisulfite was added to destroy the excess of I_2 and the pyrrolic products extracted into CH_2Cl_2. After evaporation, the residue was purified by flash chromatography (AcOEt/Hexanes, 1/1) to give the iododipyrrole **16b** (2.53 g, 4.10 mmol, 89 %). ^{1}H-NMR δ 10.55, 9.58 (m + s, 2H, 2N*H*); 9.43 (d, J_{CH} = 173.1, 1H, ^{13}C*H*O); 3.85 (s, 2H, CH_2 *meso*); 3.67, 3.61, 3.59 (3s, 14H, 4CO_2CH_2 + CH_2CO_2Me); 3.47 (s, 2H, CH_2CO_2Me); 2.72, 2.59 (2t, *J* = 6.7, 7.9, 4H, 2 $CH_2CH_2CO_2$Me); 2.50, 2.36 (2t, *J* = 6.7, 7.9, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.32 (^{13}CHO); 174.08; 173.32; 173.15; 171.04; 136.26; 130.20; 128.84 (d, *J* = 68.5, C_4); 125.48; 120.40; 112.49; 63.79; 52.28; 52.05; 51.77; 51.33; 34.36; 33.99; 30.02; 29.75; 22.31; 21.84; 18.35.

5-(^{13}C-Formyl)-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole (17b). The iododipyrromethane **16b** (2.53 g, 4.10 mmol) was redissolved in MeOH (30 mL) and hydrogenated over 10% Pd–C (250 mg) in the presence of NaOAc (760 mg, 9.26 mmol) overnight. After filtration through Celite, the inorganic salts were removed by H_2O washings. After chromatography (AcOEt/Hexanes, 3/2), compound **17b** was obtained (1.64 g, 3.35 mmol, 82%). ^{1}H-NMR δ 10.34, 8.91 (m + s, 2H, 2 N*H*); 9.49 (d, J_{CH} = 172.8, 1H, ^{13}C*H*O); 6.42 (d, *J* = 2.1, 1H, H_α); 3.87 (s, 2H, CH_2 *meso*); 3.74, 3.65, 3.64, 3.61 (4s, 12H, 4 CO_2CH_3); 3.71, 3.48 (2s, 4H, 2 CH_2CO_2Me); 2.78, 2.70 (2t, *J* = 6.8, 7.7, 4H, 2 $CH_2CH_2CO_2$Me); 2.57, 2.51 (2t, *J* = 6.8, 7.7, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.37 (^{13}CHO); 174.16; 173.74; 171.30; 136.60; 128.93 (d, *J* = 68.6, C_4); 125.68; 121.12; 120.12; 114.55; 111.23; 52.53; 52.25; 51.79; 51.53; 34.63; 34.20; 29.95; 22.30; 20.55; 18.52.

1-(^{13}C-Formyl)-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetra-(methoxycarbonylmethyl)bilane (19b). A solution of the formyl dipyrromethane **17a**[11] (49 mg, 0.1

mmol) in CH_2Cl_2/MeOH (1/1, 1 mL) was reduced with $NaBH_4$ (50 mg) in the presence of NH_4Cl (60 mg) at R.T. for 15 min. The reaction was quenched with H_2O and the hydroxymethyldipyrrole **18a** was extracted into CH_2Cl_2. The residue of evaporation was added to a solution of the ^{13}C-formyldipyrrole **17b** (98 mg, 0.2 mmol) in CH_2Cl_2 (3 mL) and stirred over Montmorillonite clay (500 mg) at R.T. in the dark for 2 days. The solution was filtered and the catalyst was washed with CH_2Cl_2 + 5% MeOH. After evaporation of the solvent, the solid residue was suspended in MeOH and separated from the solvent by centrifugation. The bilane **19b** was further purified by PLC (2 x CH_2Cl_2/MeOH, 95/5)(44 mg, 0.045 mmol, 45%). ^{1}H-NMR δ 9.90 (m, 1H, N*H*); 9.46 (d, J_{CH} = 172.6, 1H, ^{13}C*H*O); 9.27, 9.11, 8.67 (3s, 3H, 3 N*H*); 6.37 (s br, 1 H, H_α); 3.78, 3.72 (2s, 6H, 3 CH_2 *meso*); 3.68, 3.66, 3.64, 3.63, 3.59, 3.55 (6s, 26H, 8 CO_2CH_3 + CH_2CO_2Me); 3.42, 3.37 (2s, 6H, 3 CH_2CO_2Me); 2.71 (m, 8H, 4 $CH_2CH_2CO_2$Me); 2.47 (m, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-HMB δ 177.05 (^{13}*C*HO); 174.89; 174.38; 174.19; 174.08; 173.83; 173.46; 171.38; 136.39; 128.53 (d, J = 66.9, C_1); 127.76; 126.18; 125.93; 125.10; 123.07; 121.01; 120.60; 116.09; 115.72; 113.68; 111.30; 110.24; 110.16; 52.29; 52.19; 51.56; 51.47; 35.25; 35.00; 34.73; 34.64; 30.21; 30.09; 29.77; 22.64; 22.22; 20.64; 19.42; 18.78.

*Reduction and hydrolysis of **19b** to [20-^{13}C]-HMB.* 2.3 mg of **19b** was dissolved in MeOH/CH_2Cl_2 + 1% Et_3N (1/1, 300 µL) and reduced with $NaBH_4$ (6 mg) at R.T. for 15 min. The reaction was quenched with H_2O and the hydroxymethylbilane extracted into CH_2Cl_2. The solid bilane was washed with MeOH and hydrolyzed with 2N KOH (150 µL) overnight. The pH was lowered to 10 with the IRC 50 Amberlite resin, the solution filtered and freeze-dried.

*Benzyl 5-(^{13}C-formyl)-3-(2-methoxycarbonylethyl)-4-(methoxycarbonylmethyl)pyrrole-2-carboxylate (**21**).* A solution of ^{13}C-DMF (250 mg, 3.37 mmol) and phosphorus oxychloride (273 µL, 3.37 mmol) in CH_2Cl_2 (2 mL) was stirred for 30 min, then added to a solution of the α-free pyrrole **20**[16] (1.21 g, 3.37 mmol) in CH_2Cl_2 (3 mL). After 6 h at R.T., the reaction was quenched with aqueous $NaHCO_3$ and extracted. The product **20** was purified by chromatography (AcOEt/Hexanes, 1/2) (906 mg, 2.33 mmol, 69%). ^{1}H-NMR δ 10.68 (s br, 1H, N*H*); 9.62 (d, J_{CH} = 178.0, ^{13}C*H*O); 7.32–7.23 (m, 5H, Ph*H*); 5.23 (s, 2H, CH_2Ph); 3.77 (s, 2H, CH_2CO_2Me); 3.59, 3.51 (2s, 6H, 2 CO_2CH_3); 2.93 (t, J = 7.6, 2H, $CH_2CH_2CO_2$Me); 2.47 (t, J = 7.6, 2H, C$H_2CH_2CO_2$Me). ^{13}C-NMR δ 179.98 (^{13}*C*HO); 172.90; 170.91; 159.79; 134.82; 130.55 (d, J = 65.6, C_5); 130.04; 128.16; 128.04; 124.40; 123.65; 66.51; 51.84; 51.10; 33.92; 28.71; 19.31.

*Benzyl 5-(hydroxy-^{13}C-methyl)-3-(2-methoxycarbonylethyl)-4-(methoxycarbonylmethyl)pyrrole-2-carboxylate (**22**).* A solution of the pyrrole **21** (628 mg, 1.62 mmol) in MeOH (5 mL) was cooled in an ice-bath and NH_4Cl (143 mg, 2.67 mmol) was added, followed by $NaBH_4$ (93 mg, 2.43 mmol). After 30 min, the reaction was quenched with H_2O and the product extracted into CH_2Cl_2. Compound **22** (557 mg, 1.43 mmol, 88%) was used in the next step without further purification. ^{1}H-NMR δ 9.97 (m, 1H, N*H*); 7.35–7.27 (m, 5H, Ph*H*); 5.24 (s, 2H, CH_2Ph); 4.51 (d, J_{CH} = 143.6, 2H, ^{13}CH_2OH); 3.65, 3.57 (2s, 6H, 2 CO_2CH_3); 3.48 (s, 2H, CH_2CO_2Me); 2.97 (t, J = 7.6, 2H, $CH_2CH_2CO_2$Me); 2.47 (t, J = 7.6, 2H, C$H_2CH_2CO_2$Me). ^{13}C-NMR δ 173.51; 173.26; 160.76; 135.80; 134.34 (d, J = 53.2, C_5); 130.02; 128.43; 128.20; 128.09; 117.82; 114.34; 65.95; 55.65 (^{13}CH_2OH); 52.26; 51.36; 34.68; 29.23; 20.20.

*Benzyl 5-(acetoxy-^{13}C-methyl)-3-(2-methoxycarbonylethyl)-4-(methoxycarbonylmethyl)pyrrole-2-carboxylate (**23**).* The hydroxy-^{13}C-methylpyrrole **22** (635 mg, 1.63 mmol) was dissolved in pyridine/Ac_2O (5/1 mL). The reaction was stirred at R.T. overnight, then diluted with AcOEt and washed with 0.1N HCl and $NaHCO_3$. The product **23** (688 mg, 1.59 mmol, 98%) was pure by TLC and used as such for the next step. ^{1}H-NMR δ 9.97 (s br, 1H, N*H*); 7.30–7.21 (m, 5H, Ph*H*); 5.19 (s, 2H, CH_2CO_2Me); 4.97 (d, J_{CH} = 149.0, 2H, ^{13}CH_2OH); 3.55, 3.49 (2s, 6H, 2 CO_2CH_3); 3.47 (s, 2H, CH_2CO_2Me); 2.91 (t, J = 7.8, 2H, $CH_2CH_2CO_2$Me); 2.43 (t, J = 7.8, 2H, C$H_2CH_2CO_2$Me); 1.91 (s, 3H, OCOC*H*3). ^{13}C-NMR δ 173.24; 171.68; 175.42 (d, J = 685, $^{13}CH_2OCOMe$); 160.53; 135.53; 129.08 (d, J = 89.9, C_5); 129.24; 128.23; 127.97; 118.68; 116.68; 65.87; 56.52 (^{13}CH_2OAc); 51.74; 51.14; 34.36; 29.02; 20.42; 20.16.

*t-Butyl 5'-benzyloxycarbonyl-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-(^{13}C-methylene)dipyrrole-5-carboxylate (**12c**).* A solution of the H_α-pyrrole **24**[7a] (620 mg, 1.91 mmol) and compound **23** (688 mg, 1.59 mmol) in CH_2Cl_2 (3 mL) was stirred over Montmorrillonite clay (2.5 g) at R.T. in the dark for 2 days. The solution was filtered and the catalyst washed with CH_2Cl_2 + 5% MeOH. After evaporation of the solvent, the oily residue was chromatographed (Et_2O/Hexanes, 7/3) and the dipyrromethane **12c** obtained (767 mg, 1.1 mmol, 69%). ^{1}H-NMR δ 10.03, 10.02 (2s, 2H, 2N*H*); 7.35–7.24 (m, 5H, Ph*H*); 5.25 (s, 2H, CH_2Ph); 3.89 (d, J_{CH} = 132.2, 2H, ^{13}CH_2 *meso*); 3.75 (s, 2H, CH_2CO_2Me); 3.73, 3.63, 3.58, 3.52 (4s, 12H, 4 CO_2CH_3); 3.50 (s, 2H, CH_2CO_2Me); 2.98, 2.72 (2t, J = 7.8, 6.5, 4H, 2 $CH_2CH_2CO_2$Me); 2.51, 2.45 (2t, J = 6.5, 7.8, 4H, 2 C$H_2CH_2CO_2$Me); 1.48 (s, 9H, C(CH_3)$_3$); ^{13}C-NMR δ 174.32; 173.56; 173.35; 171.94; 160.39; 160.14; 135.92, 132.04 (2d, J = 45.2, 46.1, C_2 + $C_{2'}$); 129.83; 128.26; 128.16; 128.10; 127.97; 127.86; 121.00; 120.55; 119.15; 117.82; 114.37; 80.31; 65.49; 52.35; 51.61; 51.56; 51.19; 34.54; 34.02; 30.64; 29.08; 28.13; 21.95 (^{13}CH_2 *meso*); 20.37; 18.40.

*Benzyl 5'-formyl-3',4-di-(2-methoxycarbonylethyl)-3,4'-di-(methoxycarbonylmethyl)-2,2'-(^{13}C-methylene)dipyrrole-5-carboxylate (**14c**).* The dipyrrole **12c** (767 mg, 1.1 mmol) was dissolved in TFA (4 mL) and stirred at R.T. for 3 h.

Then the solution was cooled in an ice-bath and trimethylorthoformate (1 mL) was added. After 30 min, the ice-bath was removed and 1 h later the reaction was worked up. The solution was diluted with AcOEt and washed with H_2O, 10% $NaHCO_3$ and saturated $NaHCO_3$ until the pH of the washings remained basic. The product **14c** was purified by chromatography (AcOEt/Hexanes, 1/1) (651 mg, 1.04 mmol, 95%). ^{1}H-NMR δ 10.60, 10.13 (2s br, 2H, 2 N*H*); 9.52 (s, 1H, C*H*O); 7.34–7.26 (m, 5H, Ph*H*); 5.23 (s, 2H, CH_2Ph); 3.98 (d, J_{CH} = 126.7, 2H, ^{13}CH_2 *meso*); 3.77, 3.67, 3.61, 3.54 (4s, 12H, 4 CO_2CH_3); 3.71, 3.57 (2s, 4H, 2 CH_2CO_2Me); 3.00, 2.79 (2t, J = 7.9, 6.4, 4H, 2 $CH_2CH_2CO_2$Me); 2.58, 2.49 (2t, J = 6.4, 7.9, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.51; 173.99; 173.41; 171.08; 160.47; 135.83; 135.32, 131.02 (2d, J = 44.8, 46.3, C_2 + $C_{2'}$); 130.03; 129.05; 128.31; 128.13; 127.97; 127.94; 120.68; 118.11; 114.56; 65.65; 52.43; 52.12; 51.71; 51.29; 34.52; 34.00; 29.79; 29.37; 22.25 (^{13}CH_2 *meso*); 20.43; 18.30.

*5'-Formyl-3',4-di-(2-methoxycarbonylethyl)-3,4'-di-(methoxycarbonylmethyl)-2,2'-(^{13}C-methylene)dipyrrole-5-carboxylic acid (**15c**).* The hydrogenation of **14c** was run as for **15b**. Product **15c** was obtained as a solid (550 mg, 1.03 mmol, 99%) and used directly in the next step. ^{1}H-NMR δ 11.49, 10.73 (m + s, 2H, 2 N*H*); 9.31 (s, 1H, C*H*O); 3.96 (d, J_{CH} = 129.4, 2H, ^{13}CH_2 *meso*); 3.69 (s, 2H, CH_2CO_2Me); 3.64, 3.62, 3.58 (3s, 12H, 4 CO_2CH_3); 3.49 (s, 2H, CH_2CO_2Me); 2.98, 2.80 (2t, J = 7.7, 7.1, 4H, 2 $CH_2CH_2CO_2$Me); 2.54, 2.48 (2t, J = 7.7, 7.1, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.42; 173.65; 173.33; 170.96; 164.99; 136.37,131.44 (2d, J = 46.3, 50.7, C_2 + $C_{2'}$); 129.01; 128.12; 121.61; 118.22; 114.75; 52.29; 52.18; 51.70; 51.37; 45.43; 34.55; 29.76; 29.51; 22.52 (^{13}CH_2 *meso*); 20.45; 18.73.

*5-Formyl-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-(^{13}C-methylene)dipyrrole (**17c**).* Oxidative decarboxylation followed by hydrogenolysis of **15c** were run and worked up as for **17b**. The dipyrromethane **17c** was obtained as a solid (491 mg, 1 mmol, 97%). ^{1}H-NMR δ 10.40 (m, 1H, N*H*); 9.42 (s, 1 H, C*H*O); 9.02 (s br, 1 H, N*H*); 6.39 (s, 1 H, H_α); 3.85 (d, J_{CH} = 128.6, 2H, ^{13}CH_2 *meso*); 3.69, 3.63, 3.61, 3.59 (4s, 14H, 4 CO_2CH_3 + CH_2CO_2Me); 3.45 (s, 2H, CH_2CO_2Me); 2.74, 2.67 (2t, 4H, J = 6.8, 7.6, 2 $CH_2CH_2CO_2$Me); 2.49 (m, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.27; 173.86; 173.62; 171.19; 136.66, 125.40 (2d, J = 44.7, 49.7, C_2+ $C_{2'}$); 128.70; 121.01; 120.27; 114.41; 111.04; 52.23; 52.07; 51.57; 51.34; 34.50; 34.11; 29.80; 29.72; 22.23 (^{13}CH_2 *meso*); 20.44; 18.44.

*5-Hydroxymethyl-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-(^{13}C-methylene)dipyrrole (**18c**).* The formyldipyrromethane **17c** was reduced as for **17a**. The hydroxymethyldipyrromethane **18c** was obtained and used directly in the coupling reaction. ^{1}H-NMR δ 9.29, 8.68 (2s, 2H, 2 N*H*); 6.32 (d, J = 1.7, 1H, H_α); 4.37 (s, 2H, CH_2OH); 3.76 (d, J_{CH} = 127.2, 2H, ^{13}CH_2 *meso*); 3.70, 3.65, 3.63, 3.59 (4s, 12H, 4 CO_2CH_3); 3.47, 3.42 (2s, 4H, 2 CH_2CO_2Me); 2.75, 2.69 (2t, J = 6.9, 8.3, 4H, 2 $CH_2CH_2CO_2$Me); 2.51 (m, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 174.38; 173.23; 128.48; 127.65, 125.94 (2d, J = 51.1, 51.0, C_2 + $C_{2'}$); 120.96; 116.01; 113.91; 111.45; 110.47; 55.76; 52.90; 51.55; 51.47; 35.02; 34.68; 30.06; 29.80; 22.01 (^{13}CH_2 *meso*); 20.60; 19.12.

*1-(^{13}C-Formyl)-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetra-(methoxycarbonylmethyl)-[15-^{13}C]-bilane (**19c**).* The hydroxymethyl-^{13}C dipyrromethane **18c** was coupled with the formyldipyrromethane **17a**, the reaction was treated as for **19b** to give the bilane **19c** (33 mg, 0.034 mmol, 34%). ^{1}H-NMR δ 9.95, 9.29, 9.12, 8.69 (m + 3s, 4H, 4 N*H*); 9.45 (s, 1H, C*H*O); 6.36 (s, 1H, H_α); 3.71 (d, J_{CH} = 127.2, 2H, ^{13}CH_2 *meso*); 3.78, 3.71 (2s, 4H, 2 CH_2 *meso*); 3.67, 3.65, 3.64, 3.63, 3.62, 3.59, 3.54 (7s, 26H, 8 CO_2CH_3 + CH_2CO_2Me); 3.41, 3.36 (2s, 6H, 3 CH_2CO_2Me); 2.70 (m, 12H, 4 $CH_2CH_2CO_2$Me); 2.46 (m, 12H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.04; 174.83; 174.32; 174.13; 174.00; 173.75; 173.10; 171.33; 136.33; 128.45; 127.69, 125.03 (2d, J = 51.0, 50.7, C_{14} + C_{16}), 126.12; 125.85; 123.02; 120.90; 120.54; 115.98; 115.60; 113.60; 111.20; 110.06; 52.23; 52.11; 51.49; 51.41; 35.18; 34.92; 34.64; 34.55; 30.12; 30.01; 29.68; 21.44 (^{13}CH_2 *meso* position 15); 20.55; 19.34; 18.69.

*3,8,13,18-Tetra-(2-methoxycarbonylethyl)-2,7,13,17-tetra-(methoxycarbonylmethyl)-1-(^{13}C-methyl)bilane (**25**).* The ^{13}C-formylbilane **19b** (3.4 mg) was dissolved in MeOH/CH_2Cl_2 + 1% Et_3N (1/1, 300 µL) and reduced with $NaBH_4$ (6 mg) at R.T. for 15 min. The reaction was quenched with H_2O and the product extracted into CH_2Cl_2. The volume was reduced to 500 µL, diluted with THF (500 µL) and stirred with Montmorillonite clay (25 mg) and $NaBH_3CN$ (25 mg) at R.T. in the dark for 2 days. The solution was filtered, the catalyst washed with CH_2Cl_2 + 5% MeOH and the filtrate washed with H_2O. The product **25** was purified by PLC (2 x CH_2Cl_2/MeOH, 95/5) (1.3 mg, 38%). ^{1}H-NMR δ 8.99, 8.91, 8.87, 8.64 (4s, 4H, 4 N*H*); 6.37 (s, 1H, H_α); 3.73 (s, 2H, CH_2 *meso*); 3.68, 3.64, 3.63, 3.59, 3.56 (5s, 28H, 8 CO_2CH_3 + 2 CH_2 *meso*); 3.43, 3.39, 3.34, 3.33 (4s, 8H, 4 CH_2CO_2Me); 2.69 (m, 8H, 4 $CH_2CH_2CO_2$Me); 2.50, 2.35 (2m, 8H, 4 $CH_2CH_2CO_2$Me); 2.08 (d, J_{CH} = 126.9, 3H, ^{13}CH_3). ^{13}C-NMR δ 11.09 (^{13}CH_3).

Enzyme assays

Enzyme preparation. The cosynthetase used in the NMR experiments was purified from recombinant *E. coli* strain CAR 211,[10] where a culture bearing a plasmid containing the *E. coli hemD* gene was innoculated into 28–30 L of *Luria Broth* (LB) medium containing ampicillin (50 mg/L) and grown at 37 °C overnight in an incubator–shaker at 300 rpm for good aeration. The cells were harvested by centrifugation at 7,000 *g* for 15 min. The pellet was resuspended in phosphate buffer (200 mL of 100 mM KH_2PO_4, pH = 8), lysed by sonication and centrifuged at 35,000 rpm for 30 min at 4 °C. The lysate was brought to 55% ammonium sulfate and again centrifuged (12,000 *g*

for 20 min). The pellet was suspended in 60 mL phosphate buffer, dialysed overnight against 100 mM phosphate buffer pH 8 and applied to a DEAE Sephacel resin (4 x 30 cm). The protein was eluted using 100 mM phosphate buffer and concentrated to 5 mL using an Amicon filtration system, then loaded onto a Sephadex G-50 gel filtration column (4 x 60 cm). After two column steps, 40–50 mg of cosynthetase was recovered at *ca.* 85% purity.

Determination of activity. The activity of cosynthetase in the cryo-solvents was determined by incubation of the enzyme (10 μL of 2mg/mL solution), HMB [generated from PBG (10 μg) and PBG deaminase (5 μL of a 2 mg/mL solution)] and the solvent mixture in 100 mM $NaHCO_3$ buffer at pH 10 and 0 °C in a total volume of 100 μL with 30 μL withdrawn at intervals of 15, 30 and 60 min. The reaction was stopped by addition of 70 μL of a 10% TCA mixture containing 0.5% I_2 and 1% KI. 3 min later, 10 μL of saturated sodium bisulfite was added to the solution to quench excess of I_2. Two controls were run: one to determine the activity of cosynthetase in the absence of cryo-solvent at pH 10 and 0 °C over the same period of time and the other to determine the background concentration of Uro'gen I formed by chemical closure over the time course. The amount of Uro'gen III generated was determined by UV-VIS and HPLC analysis following standard assay procedure.[18] The results are shown on Figure 1.

Effect of pH on activity. The effect of high pH levels on cosynthetase was determined by assaying the enzyme at low (50 μg/mL) and high (2 mg/mL) enzyme concentrations for different intervals of time at various pH levels using the standard assay procedure (Figure 2).[18]

Effect of different nucleophiles. The effect of various nucleophilic salts on Uro'gen III synthase was determined first at pH 9.2, 0 °C with 50 μL of 10 mM concentrations of NH_2OH, NaN_3, $NaBH_3CN$, $NaBH_4$, KCN and NaSH incubated in the presence of 50 μL of 1 mg/mL solution of enzyme for 1, 2 and 3 h. The enzyme was then assayed for activity. This was repeated at pH 10.8, 0 °C with 200 mM NH_4Cl, 10 mM NaN_3, 25 mM KCN and 25 mM NaSH (results not shown).

NMR experiments

Typically, Uro'gen III synthase (0.1 mM), labelled HMB (4 mM) and NH_4Cl (200 mM) in 10% CH_3CN/100 mM $NaHCO_3$ buffer pH 10.8 at 4–5 °C were incubated until 50 to 70% of Uro'gen III was formed, the reaction being monitored by NMR: δ = 55.4 ppm for [20-^{13}C]-HMB and 22.5 ppm for [15-^{13}C]-HMB, δ = 22.0 ppm for both [20-^{13}C]- and [15-^{13}C]-Uro'gen III. The pH of the solution was then adjusted to 11.5–12 and the NMR run overnight to collect up to 48,000 scans, where a 15 Hz exponential line broadening was applied to the 8K data point free induction decay before Fourier transformation. Bruker software on an Aspect 3000 computer was used to interpret the data. The experiments with $NaBH_4$ were done by incubating cosynthetase, labelled HMB (4 mM) and $NaBH_4$ (400 mM) in 10% CH_3CN/100 mM $NaHCO_3$ buffer pH 10.8, 37 °C for 5 min before starting NMR acquisition. All NMR spectra were acquired on a Bruker wide bore WM 300 spectrometer where the proton decoupled 75.4 MHz ^{13}C-NMR spectra were recorded at 4–5 °C under the following spectral conditions: WALTZ proton decoupling, SW = 16,667 Hz, AT = 0.247 s, RD = 0.5 s and NS = 48,000 for each experiment.

Conclusion

Using conditions to slow down the activity of Uro'gen III synthase, i.e. high pH and low temperature in 10% acetonitrile/buffer, we have been able to trap with ammonium ions and sodium borohydride, and observe by high field ^{13}C-NMR the azafulvene **7** generated at the C-1 position of HMB. This is the first evidence of an azafulvene intermediate in the enzymatic formation of Uro'gen III from HMB and shows that the formation of the intermediate in the Uro'gen III synthase mechanism probably does not involve a concerted nucleophilic attack.

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