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19-Bromo-1-Hydroxymethylbilane, a Novel Inhibitor of Uro'gen III Synthase

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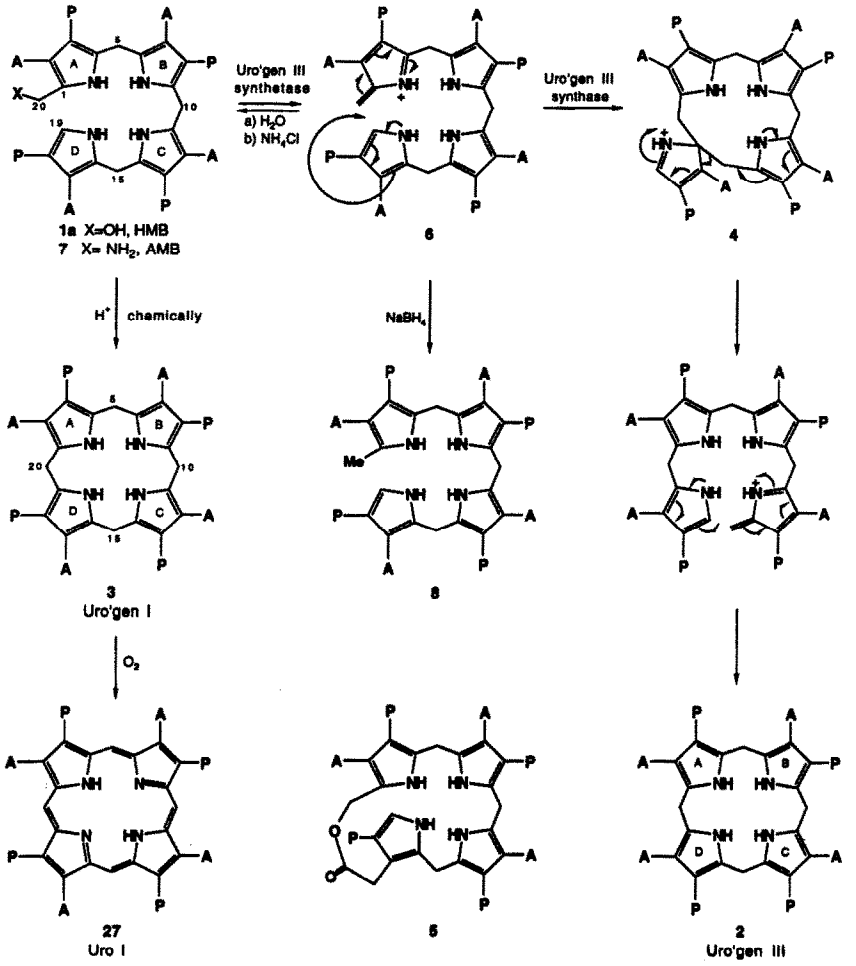
Abstract—A novel hydroxymethylbilane analog, 19-Br-HMB (**11**), has been synthesized. Its activity with the enzyme Uro'gen III synthase shows competitive inhibition.

Introduction

Uroporphyrinogen III (Uro'gen III) synthase (EC 4.2.1.75) cyclizes the linear tetrapyrrole hydroxymethylbilane (HMB, **1a**) to Uro'gen III (**2**) with intramolecular ring D rearrangement. In the absence of the enzyme, HMB ring-closes chemically to form Uroporphyrinogen I (Uro'gen 1, **3**) without ring inversion (Scheme I). Uro'gen III, from which haem, coenzyme F 430, chlorophyll and vitamin B_{12} are derived, is an important precursor in living systems[1,2] and consequently, extensive studies have been made concerning the enzyme catalyzing its formation.



A=CH_2CO_2H; P=$CH_2CH_2CO_2H$

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

Many mechanistic hypotheses have been proposed[2] for this unique ring D rearrangement, including the formation of a spiro intermediate **4**[3] followed by fragmentation–recombination[4] or via the lactone **5**[5] (Scheme I). An azafulvene **6** has been suggested as the first step towards the generation of any of these putative species. Although we have recently provided indirect evidence for the existence of **6** in trapping experiments with either ammonium chloride or sodium borohydride to form respectively the aminomethylbilane (**7**) and the methylbilane (**8**), observed by ^{13}C-NMR,[6] cryogenic NMR experiments using HMB ^{13}C-labelled at various positions have not so far permitted the direct observation of intermediates such as **4**, **5** or **6**.[1,5]

In addition to utilizing Uro'gen III synthase's true substrate HMB, it was anticipated that slow substrates or inhibitors could be designed to further probe the mechanism and specificity of the enzyme. Our intention was first directed towards bilanes bearing a substituent at the C-19 position, where the α-free position of the ring D was blocked, thus interfering with the cyclization process. The effect of two blocked bilanes, the 19-methylbilane **9** and the 19-cyanobilane **10** (Figure 1), on the enzymatic as well as chemical ring-closure have been described.[7] Both bilanes act as competitive inhibitors of Uro'gen III synthase, while under acidic conditions, cyclization at the C-19 position (type I form) was strongly favored. In this paper, we describe the syntheses of two specifically ^{13}C-enriched bilanes, the 19-bromo-1-hydroxymethylbilane (**11**, 19-Br-HMB) and the 1-hydroxymethyl-19-methylbilane (**9**, 19-Me-HMB) (Figure 1), and report on their interaction with Uro'gen III synthase.

Synthesis

The 19-bromobilane (11)

Scheme II describes the synthesis of the 19-Br-HMB (**11**). The preparation of the formyldipyrromethane precursor **12a** has been reported.[8] **12a** was brominated with copper bromide at low temperature (–20 °C) to afford the 5'-bromo-5-formyldipyrrole **13a**. Due to its great instability on purification, it was used as such in the next step. The aldehyde was reduced with sodium borohydride to give the 5'-bromo-5-hydroxymethyldipyrrole **14a**, which, again, was used without further purification to form the bilane. The classical procedure of coupling the hydroxymethyldipyrrole **14a** to an excess of α-free formyldipyrromethane **12a** catalyzed by acetic acid,[8] did not produce the expected 19-bromo-1-formylbilane **15a**. However, the use of Montmorillonite clay as a mild acid catalyst[9] provided the desired compound **15a** in moderate yield (25-30%). The acidity of the clay can be altered by washing with buffer solutions, but the best results were obtained with clay simply washed with distilled water.

To proceed to the enzymatic studies, the 19-bromo-1-formylbilane (**15a**) requires reduction of the aldehyde and hydrolysis of the methyl esters. While the reduction with sodium borohydride did not present any difficulty, alkaline hydrolysis of the ester functions in 2 N NaOH led to decomposition. However, 2 M piperidine gave the desired 19-bromo-HMB (**11a**) as its piperidinium salt, confirmed by ^{1}H- and ^{13}C-NMR. Yet, since assays (HPLC, NMR) performed with the piperidinium salt of HMB itself showed only a partial turnover to Uro'gen III, the methyl esters were hydrolyzed with 0.2 N KOH over a longer period of time to provide **11a** without any sign of decomposition, as checked by ^{1}H- and ^{13}C-NMR.

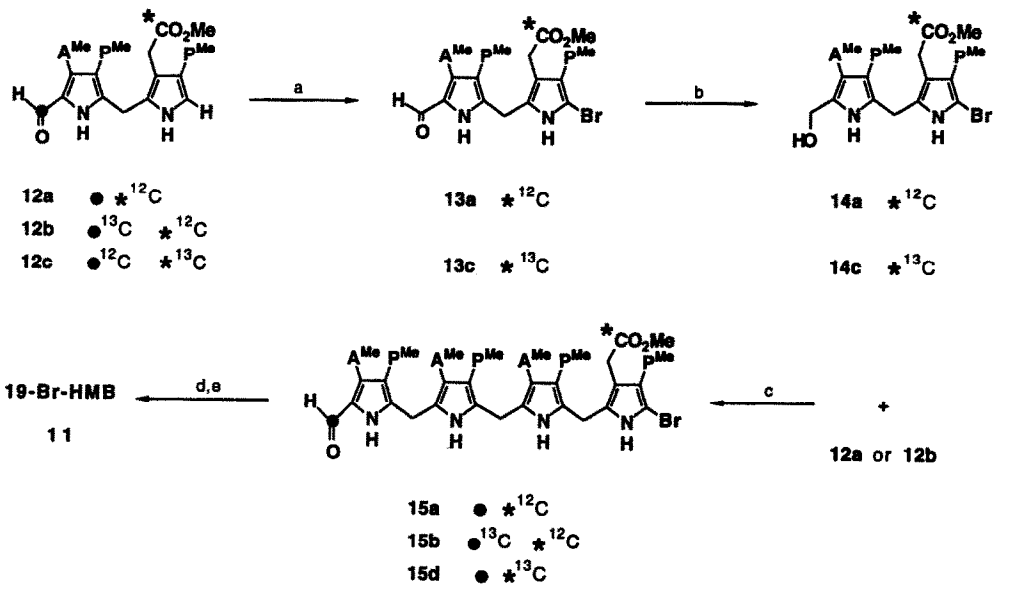
Doubly-labelled bilane 1d

In order to carry out ^{13}C-NMR investigations of the effect of 19-substituted HMB on Uro'gen III synthase, it was necessary to introduce ^{13}C-labels at specific positions in the HMB. A compromise between the synthetic difficulty of introducing the labels at specific positions and the amount of structural information to be derived from the experiments led us to choose C-20 and the carbonyl of the ring D acetate as the sites of ^{13}C-labelling.

The preparation of the [20-^{13}C]-HMB has recently been reported.[6] An appropriate modification of the published method for preparing HMB[8] provided the doubly ^{13}C-labelled HMB (**24d**) as shown in Scheme III. The precursor **16** was obtained as described elsewhere[10] and acetylated with [1-^{13}C]-acetyl chloride to give the 4-[1-^{13}C-acetyl]-pyrrole **17**. Acid catalysis during the rearrangement of the acetyl to the methyl acetate group with thallium nitrate in methanol transesterified the propionate group to produce the pyrrole **18**.[11] The usual sequence was then resumed. The pyrrole **18** was acetylated to **19**, then coupled to the α-free pyrrole **20**[12] using Montmorillonite clay as catalyst[9] to provide the dipyrromethane **21**. Hydrolysis and simultaneous decarboxylation of the tertbutoxycarbonyl in TFA followed by formylation gave the dipyrrole **22**. Debenzylation to **23**, oxidative decarboxylation and dehalogenation afforded the α-free dipyrromethane **12c**. After reduction of the aldehyde with sodium borohydride, the product was coupled to the ^{13}C-formyldipyrromethane **12b**, using Montmorrillonite clay as catalyst, giving the doubly ^{13}C-labelled bilane **24d** in a reasonable yield (30 %).

The 1-^{13}C-formyl-19-methylbilane (26)

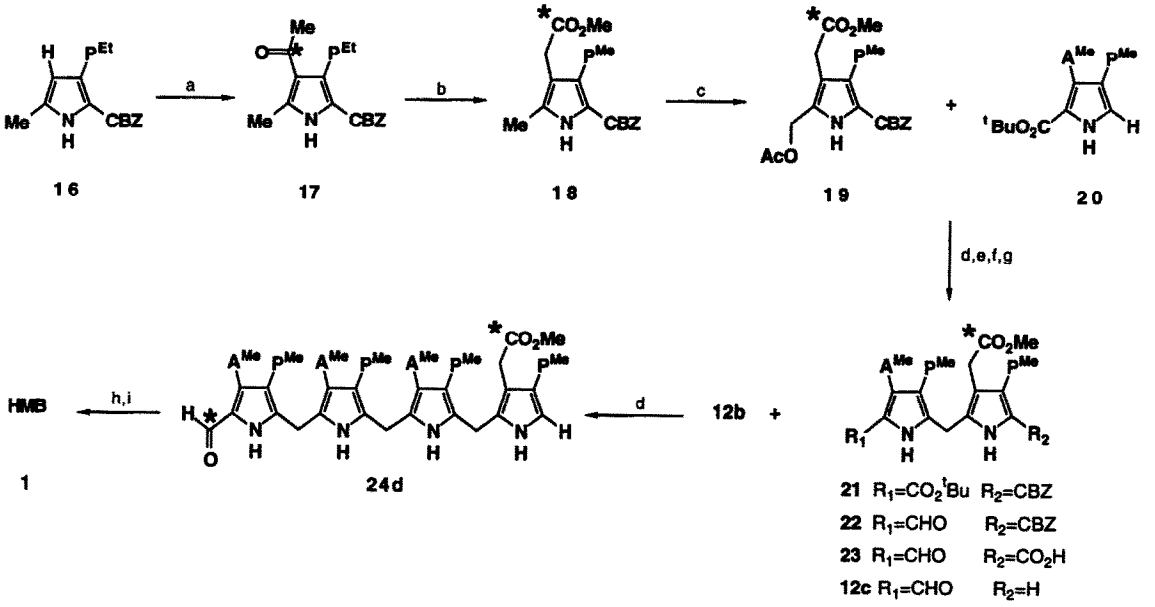
Battersby *et al.* have described the syntheses as well as the chemical and biological properties of the 19-methylbilane (**9**) and 19-cyanobilane (**10**).[7] The 19-methylbilane **9** showed an interesting inhibitory effect on Uro'gen III synthase. The availability of the ^{13}C-formyldipyrrole **12b** made it possible to prepare the [20-^{13}C]-19-methylbilane (**9b**) in a few steps and study its effect on the enzyme by ^{13}C-NMR. In a manner similar to the synthesis of doubly-labelled HMB (**24d**), the 5-formyl-5'-methyldipyrromethane **25**[7] was reduced with sodium borohydride and coupled to **12b** to give the ^{13}C-formyl-19-methylbilane **26**, which was then reduced and hydrolyzed to **9b** for enzymatic studies.



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

a) $CuBr_2$, CH_3CN, -20°C; b) $NaBH_4$, MeOH-CH_2Cl_2; c) Montmorillonite clay, CH_2Cl_2; d) $NaBH_4$, MeOH-CH_2Cl_2 + 1% Et_3N; e) 0.2N KOH.

Scheme II. Synthesis of the 19-bromobilane.



CBZ=CO_2CH_2Ph; A^{Me}=CH_2CO_2Me; P^{Et}=$CH_2CH_2CO_2Et$; P^{Me}=$CH_2CH_2CO_2Me$; ✱ ^{13}C

a) $CH_3{}^{13}COCl$, $SnCl_4$, CH_2Cl_2, 0°C; b) $Tl(NO_3)_3.3\ H_20$, MeOH, HNO_3; c) $Pb(OAc)_4$, AcOH-Ac_2O, 80°C;d) CH_2Cl_2, clay; e) 1.TFA; 2. $CH(OMe)_3$; f) THF, Et_3N, H_2/Pd-C; g) 1. KI_3, CH_2Cl_2-$NaHCO_3$; 2. MeOH, NaOAc, H_2/Pd-C; h) $NaBH_4$, MeOH-CH_2Cl_2 + 1% Et_3N; i) 2N KOH.

Scheme III. Synthesis of the doubly ^{13}C-labelled HMB.

Inhibition Studies

19-Br-HMB (**11a**) was found to inhibit Uro'gen III synthase. Preincubation of the enzyme with excess (10 to 20 fold) Br-HMB before addition of the substrate HMB resulted in inactivation of the enzyme (no Uro'gen III formation was observed). However, coincubations with Br-HMB and HMB showed a decrease in Uro'gen III formation without complete inactivation, suggesting competitive inhibition. To determine the type of inhibition, a dialysis experiment was performed, in which Uro'gen III synthase was incubated with excess of and without Br-HMB, dialyzed and assayed for Uro'gen III formation in a time course experiment. As the Br-HMB was removed by dialysis, the enzyme steadily regained activity (Figure 2), in agreement with reversible inhibition. The dissociation constant K_i could not be measured and this will be explained later in view of the following results.

NMR Studies

19-Br-HMB

The effect of the doubly ^{13}C-labelled Br-HMB (**11d**) on Uro'gen III synthase was investigated by ^{13}C-NMR to see if the formation and accumulation of an intermediate might occur and be detected, since 19-substituted bilanes are unable to proceed through cyclization and inversion of ring D to a Uro'gen III type product. When incubations of Uro'gen III synthase with Br-HMB (**11d**, δ = 181.0 and 55.4 ppm) were run and followed by ^{13}C-NMR, two new peaks at 180.5 and 98.0 ppm appeared (Figures 3A and 3B). HPLC analysis of this sample indicated the presence of Uroporphyrin I (Uro I, **27**). The signals were thus assigned respectively to the ^{13}C-carboxy group and [20-^{13}C]-*meso* carbon of Uro I (**27d**), which was confirmed by addition of chemically prepared doubly ^{13}C-labelled Uro I (**27d**) to the incubation mixture (Figure 3C). No intermediate in the reaction was detected.

This last result allowed us to understand the difficulties in obtaining kinetic data in the inhibition studies of Br-HMB with Uro'gen III synthase. Indeed, Uro I from chemical closure of HMB could not be distinguished in the UV-analysis from the Uro I product of the enzymatic reaction on Br-HMB.

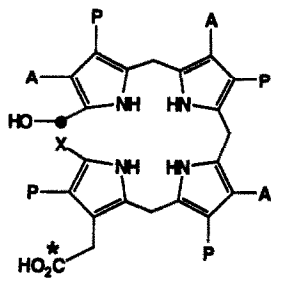
To explain the formation of Uro I from Br-HMB, the mechanism shown in Scheme IV is proposed: the first step involves the generation of the azafulvene **28**, which cyclizes at the C-19 position to form **29**, which rapidly eliminates HBr to generate **30** and after complete oxidation produces Uro I (**27**). Both compounds **27** and **30** are expected to show the same ^{13}C-NMR chemical shift of the [20-^{13}C]-*meso* carbon from the original sp^3-carbon of **11d**. The azafulvene may form an intermediate of the type **4** or **5**, in which, since rearrangement of ring D is precluded, an alternative pathway, i.e. cyclization at the C-19 position to form Uro I, is favored. On the other hand, it is possible that the putative spiro species might not be formed, due to a modification of the pyrrolic reactivity (deactivation of the C-16 position) related to the change in the π-electron density brought about by the presence of an electron-withdrawing substituent (Br) at the C-19 position and/or a different orientation of ring D in the catalytic site of the enzyme.

Indirect evidence of an azafulvene species has been previously observed by ^{13}C-NMR in trapping experiments with nucleophiles in the case of porphobilinogen deaminase, the enzyme in the vitamin B_{12} biosynthesis pathway which generates HMB via the azafulvene **6**,[13] and more recently, in the case of Uro'gen III synthase, where we have been able, under certain conditions, to partially divert **6** from its normal pathway to react with either ammonium chloride or sodium borohydride to form the aminomethylbilane (**7**) or the methylbilane (**8**) (Scheme I).[6] When enzyme incubations with Br-HMB (**11d**) in presence of 0.2 M ammonium chloride were performed under conditions similar to those described in the previous paper,[6] the azafulvene **28** was trapped to form the 1-aminomethyl-19-bromobilane (19-Br-AMB, **31**), which was observed by ^{13}C-NMR giving a signal for C-20 at 36.4 ppm (Figure 4). This experiment confirmed the involvement of the azafulvene **28** as the first step in the proposed mechanism.

The formation of Uro I from Br-HMB (**11b**) in buffer could also be detected by ^{13}C-NMR, but occurred at a more acidic pH (7) and apparently at a slow rate. Adding a large excess of sodium borohydride to the solution showed the very slow formation of the 19-bromo-1-^{13}C-methylbilane (δ = 12.6 ppm, **32**) resulting from the chemical reduction of the azafulvene double bond. Such reduction has been shown to occur with HMB, although at a basic pH (12).[8] The chemical cyclization of Br-HMB to Uro I should follow the same mechanism described for the enzyme reaction (Scheme IV). Thus the azafulvene **28** can be generated either chemically at acidic pH or enzymatically by Uro'gen III synthase.

19-Me-HMB

Similar studies were performed with the 19-Me-HMB. It was found to be a competitive inhibitor (K_i = 8 μM).[7] ^{13}C-NMR spectra of Uro'gen III synthase incubations with [20-^{13}C]-19-Me-HMB (**9b**, δ = 54.8 ppm) did not show any intermediate or product formation. However, in incubations run in presence of ammonium chloride, the appearance of a signal at 34.5 ppm, assigned to 19-Me-AMB, indicates that although the azafulvene can be generated by the enzyme, no lactone formation or cyclization (at C-16 or C-19) is observed.



1	HMB X=H	a ●★ ^{12}C	b ★ ^{12}C ● ^{13}C	d ●★ ^{13}C
9	Me-HMB X=Me	a ●★ ^{12}C	b ★ ^{12}C ● ^{13}C	
10	CN-HMB X=CN	a ●★ ^{12}C		
11	Br-HMB X=Br	a ●★ ^{12}C	b ★ ^{12}C ● ^{13}C	d ●★ ^{13}C

Figure 1. 19-Substituted HMB.

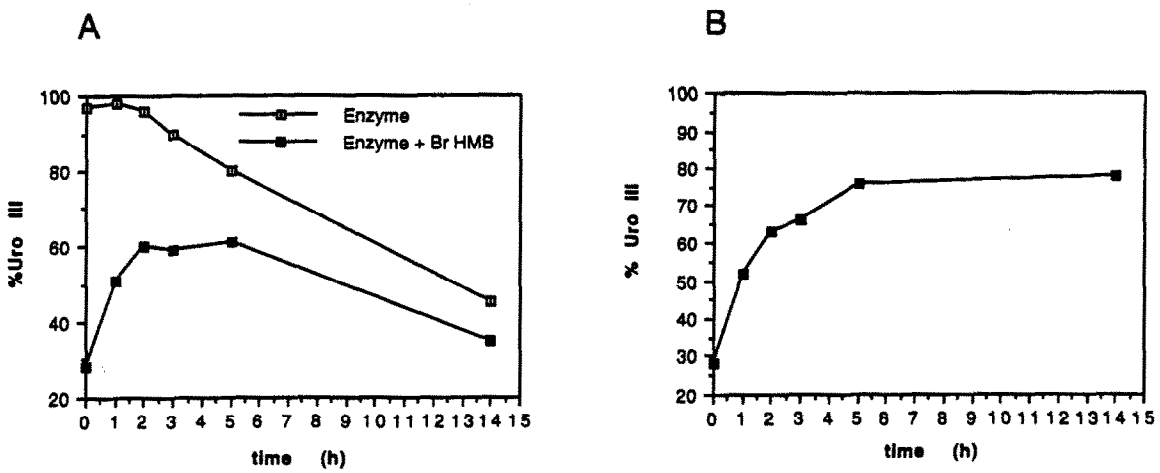


Figure 2. The dialysis experiment. A) comparison of the amount of Uro III formed in the presence and absence of Br-HMB after dialysis against $NaHCO_3$ buffer pH = 9.5. B) Uro III formation corrected for loss of enzyme activity over the time.

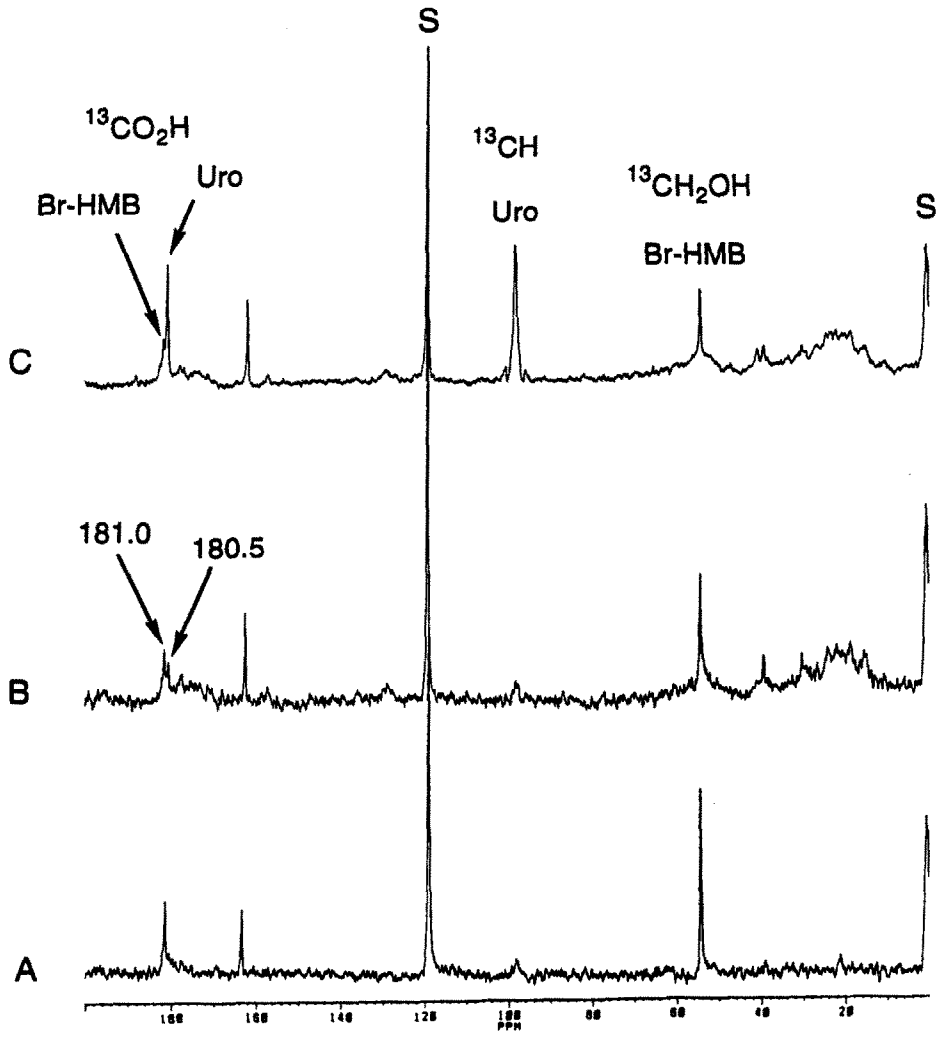
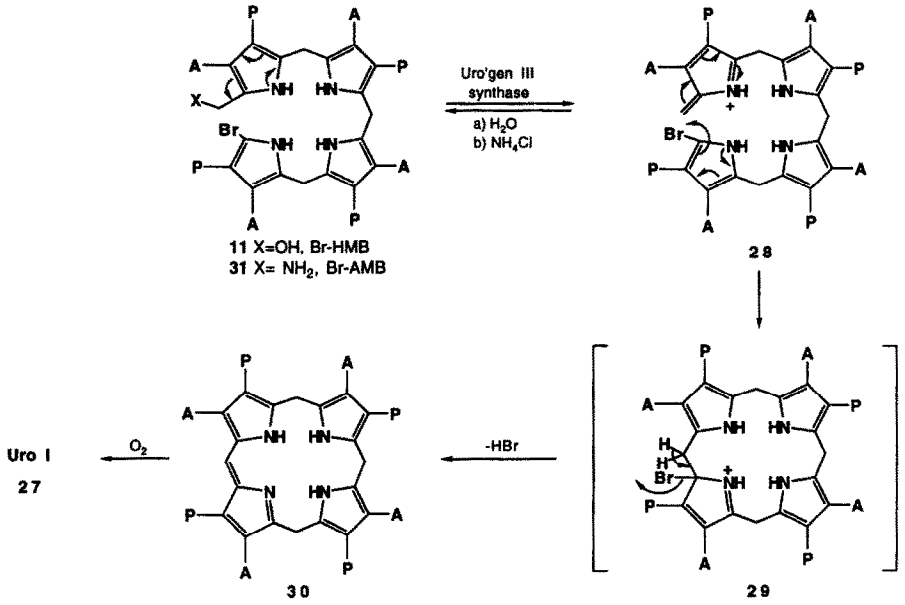


Figure 3. NMR spectra of A) Br-HMB (**11d**) in buffer pH = 10.0. B) Br-HMB (**11d**) and Uro'gen III synthase. C) Br-HMB (**11d**) and Uro'gen III synthase after addition of Uro I (**27d**).



Scheme IV. Proposed mechanism for the formation of Uro'gen I by action of Uro'gen III synthase on 19-Br-HMB.

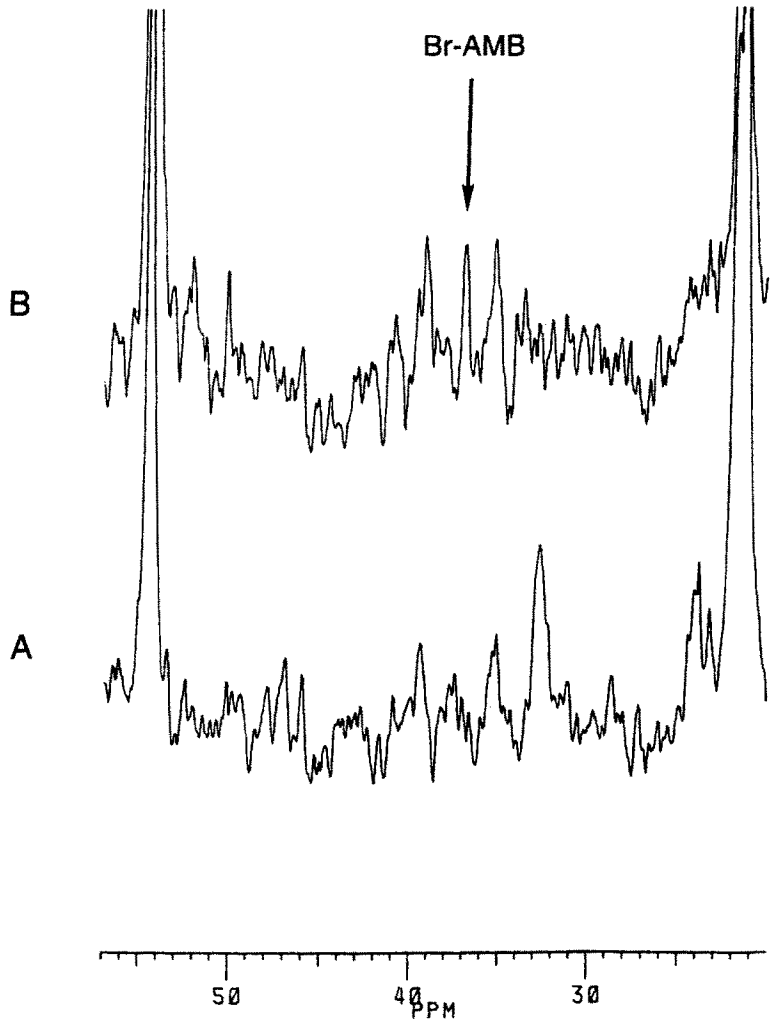


Figure 4. Selected part of NMR spectra for the azafulvene trapping experiment: A) [20-^{13}C]-19-Br-HMB (**11b**) with Uro'gen III synthase. B) [20-^{13}C]-19-Br-HMB (**11b**) with Uro'gen III synthase in the presence of NH_4Cl.

Conclusion

A novel reversible inhibitor of Uro'gen III synthase, 19-Br-HMB, has been prepared by a method which demonstrates that Montmorillonite clay is a mild acid catalyst useful for the formation of bilanes bearing acid labile substituents. ^{13}C-NMR investigations of its enzymatic interaction have not detected any intermediate, but instead, the formation of Uro I was observed. A mechanism for the latter process has been proposed involving the generation of an azafulvene catalyzed by the enzyme as the first step towards cyclization at the C-19 position. After loss of HBr and complete oxidation, Uro I is produced.

Experimental

Chemistry

General procedures. All solvents and reagents were purified when necessary by standard literature methods. Montmorillonite clay KSF was washed with H_2O and dried at 150 °C overnight before use. Column chromatography, TLC and preparative TLC (PLC) were carried out on silica gel. ^{1}H- and ^{13}C-NMR (300 and 500 MHz) spectra were recorded in $CDCl_3$. Mass spectra were obtained on a VG analytical 70s high-resolution double-focussing magnetic sector mass spectrometer.

*5'-Bromo-5-formyl-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole (**13a**).* A solution of $CuBr_2$ (24 mg, 0.108 mmol) in CH_3CN (2 mL) was added to a solution of the α-free dipyrromethane **12a**[4b] (49 mg, 0.1 mmol) in CH_3CN (2 mL) cooled at –20 °C. The reaction was stirred under N_2 for 30 min at –20 °C, then partitioned between H_2O (10 mL) and ether (20 mL). The aqueous phase was further extracted with ether. The etheral solutions were gathered and washed with brine. Due to its instability, the product **13a** was used without purification to the next step. ^{1}H-NMR δ 10.48 (m, 1H, N*H*); 9.54 (s, 1H, C*H*O); 9.38 (s br, 1H, N*H*); 3.86 (s, 2H, CH_2 *meso*); 3.80 (s, 3H, CO_2CH_3); 3.72 (s, 2H, CH_2CO_2Me); 3.67, 3.66, 3.65 (3s, 9H, 3 CO_2CH_3); 3.52 (s, 2H, CH_2CO_2Me); 2.80 (t, *J* = 6.1, 2H, $CH_2CH_2CO_2$Me); 2.67 (m, 4H, $CH_2CH_2CO_2$Me + $CH_2CH_2CO_2$Me); 2.44 (t, *J* = 7.7, 2H, $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.40; 174.85; 173.43; 171.28; 135.98; 129.35; 127.02; 119.99; 119.43; 112.50; 97.09; 52.78; 52.28; 52.06; 51.58; 34.32; 34.11; 30.22; 30.12; 22.25; 20.29; 18.43. MS(FAB) m/e 571, 569 (M + 3, M + 1); 570, 568 (M + 3-H, M + 1-H); 489 (M + 1-Br). HRMS(FAB) found: M + 3, M + 1, 571.1050, 569.1113; $C_{24}H_{29}BrN_2O_9$ requires: 571.1035, 569.1056.

*5'-Bromo-5-hydroxymethyl-3,4'-di-(2-methoxycarbonylethyl)-3',4-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole (**14a**).* The 5'-bromodipyrrole (**13a**) was dissolved in CH_2Cl_2/MeOH (1/1, 1 mL) and reduced with $NaBH_4$ (65 mg). After 15 min, the reaction was quenched with H_2O, the 5'-bromo-5-hydroxymethyldipyrrole **14a** was extracted into CH_2Cl_2 and used directly to the next step. ^{1}H-NMR δ 9.60, 9.35 (2m, 2H, 2 N*H*); 4.41 (s, 2H, CH_2OH); 3.74 (s, 2H, CH_2 *meso*); 3.71, 3.65, 3.64, 3.62 (4s, 12H, 4 CO_2CH_3); 3.49, 3.41 (2s, 4H, 2 CH_2CO_2Me); 2.73, 2.65 (2m, 4H, 2 $CH_2CH_2CO_2$Me); 2.56 (m, 2H, $CH_2CH_2CO_2$Me); 2.53 (m, 1H, O*H*); 2.42 (m, 2H, $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 175.01; 174.25; 173.87; 173.46; 128.88; 128.82; 125.90; 119.09; 115.85; 111.52; 111.36; 96.29; 52.39; 52.21; 51.79; 51.49; 34.70; 34.34; 30.19; 30.05; 21.94; 20.28; 18.89.

*5'-Bromo-5-hydroxymethyl-3,4'-di-(2-methoxycarbonylethyl)-4-(methoxycarbonylmethyl)-3'-(methoxy-^{13}C-carbonylmethyl)-2,2'-methylenedipyrrole (**14c**).* The compound **14c** was prepared as for **14a** and used without purification to obtain the corresponding bilane **15d**. ^{1}H-NMR δ 9.57, 9.31 (2s br, 2H, 2 N*H*); 4.41 (s, 2H, CH_2OH); 3.74 (s, 2H, CH_2 *meso*); 3.72, 3.65, 3.64, 3.62 (4s, 12H, 4 CO_2CH_3); 3.49 (d, J_{CH} = 7.0, 2H, $CH_2{}^{13}CO_2$Me); 3.41 (s, 2H, CH_2CO_2Me); 2.74, 2.65 (2m, 4H, 2 $CH_2CH_2CO_2$Me); 2.57, 2.42 (2m, 4H, 2 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 174.26, $^{13}CO_2$Me; 128.89; 128.84 ; 125.88 ; 119.12 ; 115.88; 111.55 ; 111.37 ; 96.29 ; 55.90; 52.39; 52.18; 51.76; 51.47; 34.64; 34.34; 30.25; 30.20 (d, *J* = 64.3, $CH_2{}^{13}CO_2$Me); 21.95; 20.28; 18.89.

*19-Bromo-1-formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetra-(methoxycarbonylmethyl)bilane (**15a**).* A solution of the 5'-bromo-5-hydroxymethyldipyrrole **14a** prepared above and the formyldipyrrole **12a** (49 mg, 0.1 mmol) in CH_2Cl_2 (2 mL) was stirred over Montmorillonite clay (500 mg) for 2 days in the dark. The solution was filtered, the catalyst washed with CH_2Cl_2 + 5% MeOH. After evaporation of the solvent, the bilane was precipitated in MeOH and separated from the solution by centrifugation. The solid obtained was further purified by PLC (2 x CH_2Cl_2/MeOH, 95/5) to give the pure 19-bromo-1-formylbilane (**15a**) (30 mg, 0.029 mmol, 26 % over 3 steps). ^{1}H-NMR δ 9.85 (m, 1 H, N*H*); 9.47 (s, 1 H, C*H*O); 9.28, 9.26, 9.20 (3s, 3H, 3 N*H*); 3.78, 3.70 (2s, 4H, 2 CH_2 *meso*); 3.69, 3.66, 3.64, 3.63, 3.59, 3.58 (6s, 28H, 8 CO_2CH_3 + CH_2CO_2Me + CH_2 *meso*); 3.45, 3.67 (2s, 6H, 2 CH_2CO_2Me); 2.78, 2.72, 2.68, 2.64 (4t, *J* = 7.8, 7.75, 6.6, 7.8, 8H, 4 $CH_2CH_2CO_2$Me); 2.52, 2.45, 2.41 (3t, *J* = 6.6, 7.75, 7.8, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 176.97; 174.90; 174.84; 174.16; 174.00; 173.46; 171.35; 136.34; 128.99; 128.56; 126.48; 125.89; 125.09; 123.05; 120.57; 119.21; 116.18; 115.54; 111.54; 111.37; 110.07; 96.07; 52.30; 52.17; 51.79; 51.55; 51.51; 51.44; 35.34; 34.66; 34.37; 30.23; 30.18; 30.02; 29.71; 22.65; 22.29; 22.09; 20.30; 19.45; 19.23; 18.79. MS(FAB) m/e 1045, 1043 (M + 3, M + 1); 964 (M + 1-Br); 741 (M + 1-$C_{11}H_{13}NO_4Br$). HRMS(FAB) found: M + 3, M + 1, 1045.3008, 1043.3119; $C_{48}H_{59}BrN_4O_{17}$ requires: 1045.3037, 1043.3057.

*19-Bromo-1-^{13}C-formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetra-(methoxycarbonylmethyl)bilane (**15b**).* The bromobilane **15b** was obtained (23 mg, 0.022 mmol, 22 %) following the same

preparation and purification as for the bromobilane **15a**. ^{1}H-NMR δ 9.85 (m, 1H, N*H*); 9.47 (d, J_{CH} = 172.5, 1H, ^{13}C*H*O); 9.25, 9.24, 9.18 (3s, 3H, 3 N*H*); 3.79, 3.71, 3.69 (3s, 6H, 3 CH_2 *meso*); 3.68, 3.66, 3.64, 3.63, 3.59, 3.57 (6s, 24H, 8 CO_2CH_3); 3.62, 3.45, 3.37 (3s, 8H, 4 CH_2CO_2Me); 2.78, 2.72, 2.68, 2.64 (4t, J = 8.0, 7.85, 6.85, 8.0, 8H, 4 $CH_2CH_2CO_2$Me); 2.52, 2.45, 2.41 (3t, J = 6.85, 7.85, 8.0, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.07, ^{13}*C*HO; 174.91; 174.85; 174.17; 174.02; 173.48; 171.36; 136.40; 129.00; 128.83; 128.29; 126.50; 125.89; 125.10; 123.06; 120.60; 119.23; 116.20; 115.56; 111.55; 111.38; 110.09; 96.09; 52.32; 52.19; 51.80; 51.57; 51.52; 51.46; 35.36; 34.67; 34.39; 30.24; 30.19; 30.04; 29.72; 22.67; 22.26; 22.12; 20.32; 19.46; 19.24; 18.80.

*19-Bromo-1-^{13}C-formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12-tri-(methoxycarbonylmethyl)-17-(methoxy-^{13}C-carbonylmethyl)bilane (**15d**).* The bromobilane **15d** was obtained (29 mg, 0.028 mmol, 28 %) following the same preparation and purification as for the bromobilane **15a**. ^{1}H-NMR δ 9.85 (m, 1H, N*H*); 9.46 (d, J_{CH} = 172.5, 1H, ^{13}C*H*O); 9.23, 9.21, 9.18 (3s, 3H, 3 N*H*); 3.78, 3.70, 3.69 (3s, 6H, 3 CH_2 *meso*); 3.68, 3.66, 3.64, 3.63, 3.58, 3.57 (6s, 24H, 8 CO_2CH_3); 3.63, 3.44, 3.36 (s + d + s, J_{CH} = 7.6, 8H, 3 CH_2CO_2Me + $CH_2{}^{13}CO_2$Me); 2.77, 2.72, 2.68, 2.64 (4t, J = 7.7, 7.8, 6.7, 8.0, 8H, 4 $CH_2CH_2CO_2$Me); 2.51, 2.45, 2.41 (2t + m, J = 6.7, 7.8, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.06, ^{13}*C*HO; 174.12, ^{13}*C*O_2Me; 138.19; 128.97; 128.56; 126.47; 125.86; 125.05; 123.06; 120.58; 119.24; 116.22; 115.60; 111.53; 111.38; 110.09; 96.06; 52.29; 52.17; 51.77; 51.55; 51.50; 51.43; 35.34; 34.70; 34.65; 34.37; 30.22; 30.15; 29.77; 29.71; 22.67; 22.29; 22.11; 20.31; 19.46; 19.24; 18.94.

19-Bromo-1-hydroxymethyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetra-(methoxycarbonylmethyl)-bilane. A solution of the 19-bromo-1-formylbilane **15a** (5 mg) in MeOH/CH_2Cl_2 + 1% Et_3N (1/1, 600 µL) was reduced with $NaBH_4$ (8 mg). After 15 min, the reaction was quenched with H_2O. The product was extracted into CH_2Cl_2. After evaporation of the solvent, the solid bilane was resuspended in MeOH and separated from the solution by centrifugation. ^{1}H-NMR δ 9.26, 9.21, 9.13, 8.89 (4s, 4H, 4 N*H*); 4.43 (s, 2H, CH_2OH); 3.70 (s, 4H, 2 CH_2 *meso*); 3.68, 3.66, 3.65, 3.64, 3.63, 3.61, 3.58, 3.54 (8s, 26H, 8 CO_2CH_3 + CH_2 meso); 3.45, 3.42, 3.38, 3.33 (4s, 8H, 4 CH_2CO_2Me); 2.76–2.63 (m, 8H, 4 $CH_2CH_2CO_2$Me); 2.51, 2.42, 2.35 (t + m + t, J = 6.5, 7.75, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 174.88; 174.37; 174.12; 174.02; 173.98; 173.89; 173.50; 128.98; 128.20; 126.44; 125.85; 125.34; 125.00; 124.84; 119.24; 116.16; 115.84; 111.78; 111.56; 110.63; 110.08; 96.11; 52.31; 52.21; 52.16; 51.79; 51.53; 51.46; 35.48; 35.40; 34.78; 34.41; 30.11; 30.07; 29.67; 22.52; 22.22; 22.14; 20.34; 19.59; 19.35; 19.25.

*19-Bromo-HMB (**11**).* A suspension of the bilane ester (2–3 mg) in 0.2 N KOH (1 mL) was stirred in the dark and under N_2 for 2 days. The pH was reduced to 10–10.5 by adding some resin Amberlite IRC 50. The solution was filtered and freeze-dried. ^{1}H-NMR (D_2O) δ 4.19 (s, 2H, CH_2OH); 3.52 (m, 6H, 3 CH_2 *meso*); 3.13, 3.08 (2s, 8H, 4 CH_2CO_2H); 2.45–2.37 (m, 8H, 4 $CH_2CH_2CO_2$H); 2.05–1.93 (m, 8H, 4 $CH_2CH_2CO_2$H). ^{13}C-NMR (D_2O) δ 184.73; 184.04; 183.71; 183.58; 183.38; 183.02; 130.32; 127.92; 127.80; 127.29; 127.13; 125.88; 121.97; 119.80; 119.14; 118.93; 117.08; 115.53; 114.97; 114.56; 99.80; 55.99; 42.51; 40.84; 39.82; 36.60; 34.25; 24.78; 23.40; 22.61.

*Benzyl 4-(1-^{13}C-acetyl)-3-(2-ethoxycarbonylethyl)-5-methylpyrrole-2-carboxylate (**17**).* A solution of $SnCl_4$ (1.27 mL, 11 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a mixture of the [1-^{13}C]-acetylchloride (1 g, 12.58 mmol), β-free pyrrole **16**[10] (3.15 g, 10 mmol) and anhydrous $CaCO_3$ (2 g, 20 mmol) in CH_2Cl_2 (25 mL) cooled in an ice-bath. At the end of the addition (10 min), the reaction was stirred 1 h more at 0 °C, then quenched with H_2O. The solution was filtered through Celite and the organic products extracted into CH_2Cl_2. The acetylpyrrole **17** was isolated by chromatography (AcOEt/-Hexanes/CH_2Cl_2, 1/1/1) (2.98 g, 8.32 mmol, 83 %). ^{1}H NMR δ 10.57 (s br, 1H, N*H*); 7.29–7.18 (m, 5H, Ph*H*); 5.21 (s, 2H, CH_2Ph); 3.96 (q, J = 7.1, 2H, $CO_2CH_2CH_3$); 3.32 (t, J = 8.05, 2H, CH_2CO_2Et); 2.49 (t, J = 8.05, 2H, $CH_2CH_2CO_2$Et); 2.43 (s, 3H, CH_3); 2.33 (d, J_{CH} = 5.6, 3H, $^{13}COCH_3$); 1.11 (t, J = 7.1, 3H, $CO_2CH_2CH_3$). ^{13}C-NMR δ 194.81, ^{13}*C*OMe; 172.83; 160.87; 138.51; 135.37; 132.99; 128.14; 127.82; 127.68; 122.39 (d, J = 63.1, C_4); 177.59; 65.86; 59.83; 34.65; 30.67 (d, J = 41.8, $^{13}COCH_3$); 21.07; 14.78; 13.80.

*Benzyl 3-(2-methoxycarbonylethyl)-4-(methoxy-^{13}C-carbonylmethyl)-5-methylpyrrole-2-carboxylate (**18**).* A solution of $Tl(NO_3)_3{\cdot}3H_2O$ (3.70 g, 8.32 mmol) in MeOH (20 mL) containing HNO_3 (0.3 mL) was added dropwise to a solution of the acetylpyrrole **17** (2.98 g, 8.32 mmol) in MeOH (20 mL). The reaction was stirred for 2 days, the thallium precipitate was filtered. The filtrate was diluted with CH_2Cl_2 and neutralized with aqueous $NaHCO_3$. After evaporation, the product **18** was purified by chromatography (AcOEt/Hexanes, 3/7)(2.54 g, 6.79 mmol, 82 %). ^{1}H-NMR δ 9.86 (s br, 1H, N*H*); 7.32–7.23 (m, 5H, Ph*H*); 5.22 (s, 2H, CH_2Ph); 3.58 (d, J_{CH} = 3.7, 3H, $^{13}CO_2CH_3$); 3.53 (s, 3H, CO_2CH_3); 3.37 (d, J_{CH} = 7.6, 2H, $CH_2{}^{13}CO_2$Me); 2.96 (t, J = 7.8, 2H, $CH_2CH_2CO_2$Me); 2.48 (t, J = 7.8, 2H, $CH_2CH_2CO_2$Me); 2.13 (s, 3H, CH_3). ^{13}C-NMR δ 173.37; 172.01, ^{13}*C*O_2Me; 160.84; 135.86; 131.99; 130.31; 128.20; 127.83; 116.27; 113.85; 65.49; 51.59; 51.10 (d, J = 4.4, $^{13}CO_2CH_3$); 34.55; 29.31 (d, J = 59.7, $CH_2{}^{13}CO_2$Me); 20.44; 11.05.

*Benzyl 5-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-(methoxy-^{13}C-carbonylmethyl)pyrrole-2-carboxylate (**19**).* A mixture of the 5-methylpyrrole **18** (1.74 g, 4.66 mmol) and $Pb(OAc)_4$ (2.27 g, 5.13 mmol) in AcOH/Ac_2O (4/1, 14/3.5 mL) was heated at 80 °C for 1.5 h, then stirred at rt overnight. Ethylene glycol (2 mL) was added to destroy the

excess of reagent. The reaction mixture was partitioned between H_2O and CH_2Cl_2. The organic phase was washed with saturated $NaHCO_3$. The 5-acetoxymethylpyrrole **19** was obtained after recrystallisation (CH_2Cl_2/Hexanes, 1.84 g, 4.26 mmol, 91 %). 1H-NMR δ 10.08 (s, 1H, N*H*); 7.33–7.23 (m, 5H, Ph*H*); 5.23 (s, 2H, CH_2Ph); 5.01 (s, 2H, CH_2OAc); 3.59 (d, J_{CH} = 3.8, 3H, $^{13}CO_2CH_3$); 3.53 (s, 3H, CO_2CH_3); 3.51 (d, J_{CH} = 7.85, 2H, CH_2 $^{13}CO_2$Me); 2.95 (t, J = 7.9, 2H, $CH_2CH_2CO_2$Me); 2.48 (t, J = 7.9, 2H, C$H_2CH_2CO_2$Me); 1.94 (s, 3H, $OCOCH_3$).^{13}C-NMR δ 173.23; 171.68, $^{13}CO_2$Me; 170.90; 160.54; 135.50; 129.65; 128.87; 128.20; 127.94; 127.89; 118.64; 116.64; 65.84; 56.49; 51.72; 51.12 (d, J = 3.9, $^{13}CO_2CH_3$); 34.33; 28.97 (d, J = 59.2, $CH_2^{13}CO_2$Me); 20.38; 20.14.

*Benzyl 5'-tertbutoxycarbonyl-4,3'-di-(2-methoxycarbonylethyl)-4'-(methoxycarbonylmethyl)-3-(methoxy-^{13}C-carbonylmethyl)-2,2'-methylenedipyrrole-5-carboxylate (**21**).* A solution of the 5-acetoxymethylpyrrole **19** (1.84 g, 4.26 mmol) and the α-free pyrrole **20**[12] (1.53 g, 4.7 mmol) in CH_2Cl_2 (10 mL) was stirred over Montmorillonite clay (10 g) in the dark for 2 days. The solution was filtered, the catalyst washed with CH_2Cl_2 + 5 % MeOH. The product **21** was isolated by chromatography (Et_2O/Hexanes, 7/3) (2.49 g, 3.58 mmol, 84 %). ^{1}H-NMR δ 10.03 (s br, 2H, 2 N*H*); 7.29–7.21 (m, 5H, Ph*H*); 5.20 (s, 2H, CH_2Ph); 3.86 (s, 2H, CH_2 *meso*); 3.73 (s, 2H, CH_2CO_2Me); 3.69 (d, J_{CH} = 3.6, 3H, $^{13}CO_2CH_3$); 3.60, 3.55 (2s, 6H, 2 CO_2CH_3); 3.50 (d, J_{CH} = 7.65, 2H, $CH_2^{13}CO_2$Me); 3.47 (s, 3H, CO_2CH_3); 2.95, 2.67 (2t, J = 7.55, 6.05, 4H, 2 $CH_2CH_2CO_2$Me); 2.49, 2.44 (2t, J = 6.05, 7.55, 4H, 2 $CH_2CH_2CO_2$Me); 1.44 (s, 9H, $CO_2C(CH_3)_3$). ^{13}C-NMR δ 174.46; 174.34; 173.50; 172.16, $^{13}CO_2$Me ; 160.60 ; 160.37 ; 136.09 ; 132.27; 130.05 ; 128.43 ; 128.34 ; 128.28; 128.14; 128.04; 121.22; 120.66; 119.41; 117.96; 114.54; 80.51; 65.68; 52.51; 51.77; 51.38; 34.70; 34.24; 30.80; 29.13 (d, J = 59.2, $CH_2^{13}CO_2$Me); 28.28; 22.13; 20.55; 18.60.

*Benzyl 5'-formyl-4,3'-di-(2-methoxycarbonylethyl)-4'-(methoxycarbonylmethyl-3-(methoxy-^{13}C-carbonylmethyl)-2,2'-methylenedipyrrole-5-carboxylate (**22**).* A solution of the dipyrrole **21** (1.75 g, 2.51 mmol) in TFA (8 mL) was stirred for 3 h. The solution was cooled in an ice-bath and trimethylorthoformate (2.25 mL) was added. After 30 min at 0 °C, the reaction was stirred at 0 °C for 1 h, then diluted with AcOEt and washed with H_2O, 10 % $NaHCO_3$, saturated $NaHCO_3$ and brine. The formyldipyrromethane **22** was isolated (1.33 g, 2.12 mmol, 84 %) by chromatography (AcOEt/hexanes, 1/1). ^{1}H-NMR δ 10.72, 10.30 (2s br, 2H, 2 N*H*); 9.46 (s, 1H, C*H*O); 7.31–7.22 (m, 5H, Ph*H*); 5.20 (s, 2H, CH_2Ph); 3.97 (s, 2H, CH_2 *meso*); 3.71 (d, J_{CH} = 3.75, 3H, $^{13}CO_2CH_3$); 3.69 (s, 2H, CH_2CO_2Me); 3.65, 3.58 (2s, 6H, 2 CO_2CH_3); 3.54 (d, J_{CH} = 8.5, 2H, $CH_2^{13}CO_2$Me); 3.53 (s, 3H, CO_2CH_3); 2.97, 2.76 (2t, J = 7.7, 6.8, 4H, 2 $CH_2CH_2CO_2$Me); 2.52, 2.48, (2t, J = 6.8, 7.7, 4H, 2 C$H_2CH_2CO_2$Me). ^{13}C-NMR δ 177.44; 173.17, $^{13}CO_2$Me; 171.02; 160.39; 135.74; 135.35; 130.94; 129.99; 128.90; 128.22; 127.87; 120.71; 117.96; 114.47; 65.54; 52.34; 52.09; 51.63; 51.25; 34.40; 33.93; 29.64; 29.24 (d, J = 59.6, $CH_2^{13}CO_2$Me); 22.19; 20.34; 18.24.

*5'-Formyl-4,3'-di-(2-methoxycarbonylethyl)-4'-(methoxycarbonylmethyl)-3-(methoxy-^{13}C-carbonylmethyl)-2,2'-methylenedipyrrole-5-carboxylic acid (**23**).* A solution of the dipyrromethane **22** (1.33 g, 2.12 mmol) in THF (20 mL) containing Et_3N (2 mL) was hydrogenated over 10 % Pd–C (140 mg) overnight. The solution was filtered, the catalyst washed with CH_2Cl_2, the filtrate further diluted with CH_2Cl_2 and neutralized by washing with 0.1 N HCl. The dipyrrolecarboxylic acid **23** (1.13 g, 2.12 mmol, 100 %) was used as such in the next step. ^{1}H-NMR δ 11.36, 10.64 (m + s br, 2H, 2 N*H*); 9.27 (s, 1H, C*H*O); 3.92 (s, 2H, CH_2 *meso*); 3.65 (s, 2H, CH_2CO_2Me); 3.60, 3.57, 3.54 (3s, 12H, 4 CO_2CH_3); 3.45 (d, J_{CH} = 7.25, 2H, C$H_2^{13}CO_2$Me); 2.93, 2.75 (2t, J = 7.1, 7.55, 4H, 2 $CH_2CH_2CO_2$Me); 2.50, 2.42 (2t, J = 7.55, 7.1, 4H, 2 C$H_2CH_2CO_2$Me). ^{13}C-NMR δ 177.27; 173.46; 173.26; 172.40, $^{13}CO_2$Me; 170.87; 164.60; 136.17; 131.60; 131.33; 128.78; 127.97; 121.46; 117.61; 114.70; 51.52; 51.47; 51.22; 51.17; 34.33; 34.22; 29.51; 29.25 (d, J = 62.9, $CH_2^{13}CO_2$Me); 22.36; 20.25; 18.49.

*5-Formyl-4',3-di-(2-methoxycarbonylethyl)-4-(methoxycarbonylmethyl)-3'-(methoxy-^{13}C-carbonylmethyl)-2,2'-methylenedipyrrole (**12c**).* a) A solution of I_2 (675 mg, 2.65 mmol) and KI (635 mg, 3.82 mmol) in H_2O (15 mL) was added to the mixture of the dipyrromethanecarboxylic acid **23** (1.13 g, 2.12 mmol) and $NaHCO_3$ (712 mg, 8.48 mmol) in CH_2Cl_2/H_2O (30/15 mL). The reaction was stirred vigorously for 15 min. Sodium bisulfite was added to destroy the excess of I_2 and the decarboxylated product extracted into CH_2Cl_2 and purified by flash chromatography (AcOEt/Hexanes, 1/1). b) The decarboxylated dipyrrole was redissolved in MeOH (40 mL). NaOAc (350 mg, 4.24 mmol) and 10 % Pd–C (100 mg) were added. The solution was hydrogenated overnight, filtered and washed with H_2O. The α-free dipyrrole **12c** (747 mg, 1.52 mmol, 72%) was obtained after chromatography (AcOEt/Hexanes, 1/1). ^{1}H-NMR δ 10.45 (m, 1H, N*H*); 9.39 (s, 1H, C*H*O); 9.03 (s, 1H, N*H*); 6.36 (d, J = 2.1, 1H, H_α); 3.84 (s, 2H, CH_2 *meso*); 3.66 (s, 2H, CH_2CO_2Me); 3.65 (d, J_{CH} = 3.8, 3H, $^{13}CO_2CH3$); 3.61, 3.59, 3.57 (3s, 9H, 3 CO_2CH_3); 3.43 (d, J_{CH} = 7.5, 2H, C$H_2^{13}CO_2$Me); 2.72, 2.65 (2t, J = 7.1, 7.7, 4H, 2 $CH_2CH_2CO_2$Me); 2.47 (m, 4H, 2 C$H_2CH_2CO_2$Me). ^{13}C-NMR δ 177.13, $^{13}CO_2$Me; 173.49; 171.14; 136.68; 128.57; 125.24; 120.96; 120.34; 114.36; 114.30; 110.99; 52.00; 51.46; 51.24; 34.43; 34.05; 29.70 (d, J = 58.9, $CH_2^{13}CO_2$Me); 29.59; 22.21; 20.37; 18.40.

*1-^{13}C-Formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12-tri-(methoxycarbonylmethyl)-17-(methoxy-^{13}C-carbonylmethyl)bilane (**24d**).* a) A solution of the formyldipyrromethane **12c** (49 mg, 0.1 mmol) in MeOH/CH_2Cl_2 (1/1, 1 mL) was reduced with $NaBH_4$ (60 mg) for 15 min. The reaction was quenched with H_2O and the hydroxydipyrromethane was extracted into CH_2Cl_2. b)

The crude hydroxymethyldipyrromethane and the formyldipyrromethane **12b** (98 mg, 0.2 mmol) were dissolved in CH_2Cl_2 (3 mL) and the solution stirred over Montmorillonite clay (500 mg) in the dark for 2 days. The solution was filtered, the catalyst washed with CH_2Cl_2 + 5 % MeOH. The bilane **24d** was precipitated in MeOH and purified by PLC (2 x CH_2Cl_2/MeOH, 95/5)(28 mg, 0.029 mmol, 29 %). ^{1}H-NMR δ 9.80 (m, 1H, N*H*); 9.48 (d, J_{CH} = 172.4, 1H, ^{13}C*H*O); 9.29, 9.11, 8.63 (3s, 3H, 3 N*H*); 6.37 (d, J = 2.25, 1H, H_α); 3.78, 3.72, 3.71 (2s + s br, 6H, 3 CH_2 *meso*); 3.69, 3.66 (2s, 6H, 2 CO_2CH_3); 3.65 (s, 2H, CH_2CO_2Me); 3.64 (2s, 6H, 2 CO_2CH_3); 3.63 (d, J_{CH} = 3.7, 3H, $^{13}CO_2CH_3$); 3.60, 3.59, 3.55 (3s, 9H, 3 CO_2CH_3); 3.43 (d, J_{CH} = 7.6, 2H, $CH_2{}^{13}CO_2$Me); 3.37 (s, 4H, 2 CH_2CO_2Me); 2.78, 2.70 (t + m, J = 7.7, 8H, 4 $CH_2CH_2CO_2$Me); 2.51, 2.46, 2.41 (t + m + t, J = 7.8, 7.5, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 177.05, ^{13}CHO; 174.96; 174.19, $^{13}CO_2$Me; 174.02; 173.87; 173.42; 172.89; 171.37; 136.50; 128.55; 127.82; 126.24; 125.97; 125.17; 123.11; 121.00; 120.62; 116.05; 115.65; 113.72; 111.30; 110.25; 52.37; 51.62; 35.28; 34.99; 34.74; 30.25; 30.14; 29.72; 22.64; 22.24; 20.66; 19.43; 18.79.

General procedure to prepare HMB for enzymatic studies. In a typical experiment, the 1-formylbilane (3 mg) was dissolved in MeOH/CH_2Cl_2 + 1 % Et_3N (300 µL) and reduced with $NaBH_4$ (6 mg) for 15 min. The reaction was quenched with H_2O and the 1-hydroxymethylbilane extracted into CH_2Cl_2. After evaporation of the solvent, the solid was washed with MeOH (300 µL) and hydrolyzed in 2 N KOH (150 µL) overnight. The pH was readjusted to 10–10.3 with the resin Amberlite IRC 50, the solution filtered and lyophylized.

Doubly ^{13}C-labelled Uro I (27d). The doubly ^{13}C-labelled formylbilane **24d** (4 mg) in solution in MeOH/CH_2Cl_2 + 1 % Et_3N (1/1, 400 µL) was reduced with $NaBH_4$ (6 mg). After 15 min, the reaction was quenched with H_2O and the hydroxymethylbilane extracted into CH_2Cl_2. The volume of the solution was reduced to 2 mL, Montmorillonite clay (50 mg) was added and the reaction stirred in the dark for 2 days. The solution was filtered and the catalyst washed with CH_2Cl_2 + 5 % MeOH. I_2 was added to the solution to oxidize Uro'gen I to Uro I (**27d**), which was then purified by PLC (2 x CH_2Cl_2/MeOH, 97/3)(1.4 mg, 35 %). ^{1}H-NMR δ 10.18 (s + d, J_{CH} = 156.1, 4H, 4 C*H* *meso*); 5.13 (s, 8H, 4 CH_2CO_2Me); 4.44 (t, J = 7.7, 8H, 4 $CH_2CH_2CO_2$Me); 3.77, 3.67 (2s, 24H, 8 CO_2CH_3); 3.36 (t, J = 7.7, 8H, 4 $CH_2CH_2CO_2$Me). ^{13}C-NMR δ 171.88, $^{13}CO_2$Me; 98.04, ^{13}CH *meso*.

1-^{13}C-Formyl-3,8,13,18-tetra-(2-methoxycarbonylethyl)-2,7,12,17-tetra-(methoxycarbonylmethyl)- 19-methylbilane (26). The 5-formyl-5'-methyl-4',3-di-(2-methoxycarbonylethyl)-4,3'-di-(methoxycarbonylmethyl)-2,2'-methylenedipyrrole **25**[7] (38 mg, 0.075 mmol) in MeOH/CH_2Cl_2 (1/1, 1 mL) was reduced with $NaBH_4$ (40 mg). After 15 min, the reaction was quenched with H_2O and the hydroxymethyldipyrrole extracted into CH_2Cl_2. The volume of solvent was reduced to 2 mL, the 5-^{13}C-formyldipyrrole **12b** (37 mg, 0.075 mmol) was added, followed by Montmorillonite clay (350 mg). The reaction was stirred in the dark for 2 days, then filtered and the catalyst washed with CH_2Cl_2 + 5 % MeOH. The 1-^{13}C-formyl-19-methylbilane (**26**) was isolated by PLC (2 x CH_2Cl_2/MeOH, 95/5)(28 mg, 0.028 mmol, 38 %).^{1}H-NMR δ 9.90 (m, 1H, N*H*); 9.46 (d, J_{CH} = 168.5, 1H, ^{13}C*H*O); 9.29, 9.19, 8.50 (3s, 3H, 3 N*H*); 3.78, 3.70, 3.66 (3s, 6H, 3 CH_2 *meso*); 3.69, 3.65, 3.64, 3.63, 3.62, 3.60, 3.59, 3.58 (8s, 24H, 8 CO_2CH_3); 3.61, 3.40, 3.36 (3s, 8H, 4 CH_2CO_2Me); 2.77, 2.70, 2.63 (t + m + t, J = 7.6, 8.0, 8H, 4 $CH_2CH_2CO_2$Me); 2.46, 2.40, 2.35 (m + 2t, J = 7.7, 7.9, 8H, 4 $CH_2CH_2CO_2$Me); 2.06 (s, 3H, CH_3). ^{13}C-NMR δ 177.00, ^{13}CHO; 174.93; 174.58; 174.48; 174.00; 173.78; 173.46; 171.34; 136.34; 128.52; 126.07; 125.95; 125.64; 125.18; 123.01; 122.83; 120.61; 116.06; 115.90; 115.36; 111.30; 110.28; 110.06; 52.28; 52.16; 51.54; 51.42; 35.46; 35.30; 34.92; 34.63; 30.20; 30.14; 29.97; 29.69; 22.62; 22.25; 21.98; 19.80; 19.41; 18.76; 10.97.

Enzymatic studies

Enzyme purification. The overexpression and purification of Uro'gen III synthase were carried out as described in Reference 6.

Inhibition studies. In the preincubation study, the Br-HMB (**11a**) was incubated with Uro'gen III synthase (5 µM) for 5 min at 0 °C in 100 mM $NaHCO_3$ buffer pH = 9.2 at concentrations ranging from 50–500 µM. The sample was then assayed for enzyme activity using UV and HPLC analysis by adding HMB (40 µM) generated from porphobilinogen (PBG) by PBG deaminase following the standard procedure.[14] Next, the Br-HMB concentration that appeared to cause inactivation (10–20 fold excess) was held constant and the HMB concentration varied (40–800 µM). Coincubation experiments were performed by incubating Br-HMB and HMB together before enzyme addition and assay. The dialysis experiments were run by incubating Uro'gen III synthase (80 µM, 500 units) both with and without Br-HMB (2 µM) and dialyzing the enzymatic mixture against 1 L of 100 mM $NaHCO_3$ pH = 9.7 at 0 °C. Aliquots were withdrawn after 1, 2, 3, 5 and 14 h and analyzed for enzyme activity.

NMR experiments. These were run on a Bruker wide bore WM 300 spectrometer. The proton decoupled 75.4 MHz ^{13}C-NMR spectra were recorded at temperatures from –5 °C to 5 °C under the following spectral conditions: WALTZ proton decoupling; SW = 16,667 Hz; AT = 0.247 s and RD = 0.8 s for each experiment. A 15 Hz exponential line broadening was applied to the 8K data point free induction decay before Fourier transformation. Bruker software on an Aspect 2000 computer was used to process the data.

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