Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 9, pp. 1105-1110, 1994

0960-894X/94 $6.00+0.00

0960-894X(94)E0109-R

STUDIES ON URO'GEN III SYNTHASE WITH MODIFIED BILANES

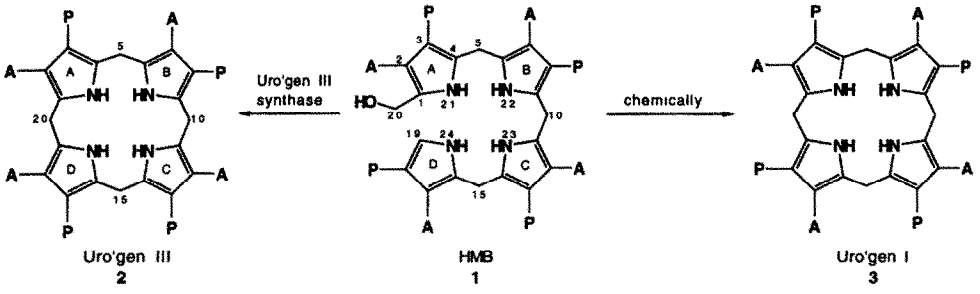
Clotilde Pichon*, Barbara P. Atshaves, Tianhan Xue, Neal J. Stolowich and A. Ian Scott*.

Center for Biological NMR, Department of Chemistry, Texas A&M University, College Station, Texas 77843-3255, USA.

Abstract. Four new hydroxymethylbilane analogs have been prepared and their activities as substrates/inhibitors of Uroporphyrinogen III synthase investigated.

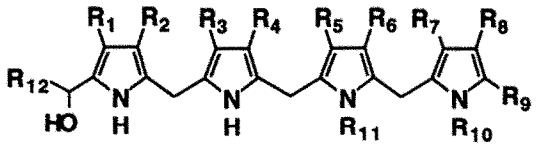
Uroporphyrinogen III (Uro'gen III) synthase (EC 4.2.1.75) is the enzyme in the porphyrinoid pathway which cyclizes the linear tetrapyrrole hydroxymethylbilane (HMB, **1**) to Uro'gen III (**2**) with intramolecular rearrangement of the ring D. In the absence of the enzyme, HMB ring-closes chemically without ring inversion to form Uro'gen I (**3**, scheme 1). Since Uro'gen III is a key precursor of many vital pigments present in living system (heme, chlorophyll, coenzyme F 430, vitamin B_{12}), numerous studies on the substrate specificity and the mechanism of Uro'gen III synthase have been performed.[1]

As a part of our continuing effort to unravel the mechanism of this fascinating enzyme, several modified bilanes (**4** to **7**)[three substituted by a methyl group at the C-20 (HEB, **4**), N-24 (N_D-Me-HMB, **5**) and N-23 (N_C-Me-HMB, **6**) positions and the fourth by elongation of the ring D propionate side-chain to a butyrate (butyrate-HMB, **7**)(table 1)] have been synthesized.[2] Their ability to function as substrates or inhibitors have then been determined with the goal of gaining indirect information about the active site and eventually of leading to the preparation of novel Uro'gen III analogs, otherwise difficult to obtain.



A=CH_2CO_2H; P=$CH_2CH_2CO_2H$

Scheme 1



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}	R_{12}	I	S	ref
1	A	P	A	P	A	P	A	P	H	H	H	H		++	8
4	A	P	A	P	A	P	A	P	H	H	H	*Me*	+	-	
5	A	P	A	P	A	P	A	P	H	*Me*	H	H	+	-	
6	A	P	A	P	A	P	A	P	H	H	*Me*	H	+	-	
7	A	P	A	P	A	P	A	*B*	H	H	H	H		+	
8[9]	*P*	*A*	A	P	A	P	A	P	H	H	H	H		-	3
9	A	P	*P*	*A*	A	P	A	P	H	H	H	H		-	4
10	A	P	A	P	*P*	*A*	A	P	H	H	H	H		+	4
11	A	P	A	P	A	P	*P*	*A*	H	H	H	H		+	4
12	*P*	*A*	*P*	*A*	*P*	*A*	A	P	H	H	H	H		-	4
13	A	P	A	P	A	P	*Me*	P	H	H	H	H		(+)	5
14	A	P	A	P	A	P	A	*Et*	H	H	H	H		+	5
15	A	P	A	P	A	P	A	P	*Me*	H	H	H	+	-	6
16	A	P	A	P	A	P	A	P	*CN*	H	H	H	+	-	6
17	A	P	A	P	A	P	A	P	*Br*	H	H	H	+	-	7

A=CH_2CO_2H; P=$CH_2CH_2CO_2H$; B=$CH_2CH_2CH_2CO_2H$
I=inhibitor; S=substrate; +=yes; -=no; (+)=poor substrate

Table 1

Similar studies have been conducted elsewhere with other HMB analogs, such as bilanes with different acetate and propionate side-chain arrangements (**8**,[3] **9**-**12**[4]), 17-methylbilane (**13**),[5] 18-ethylbilane (**14**)[5] and 19-substituted HMB [19-methyl-HMB (**15**) and 19-cyano-HMB (**16**)][6]. Recently, our group synthesized the 19-bromo-HMB (**17**) and studied its reactivity with Uro'gen III synthase.[7] Table 1 summerizes these results together with those obtained with our new synthetic analogs.

The Uroporphyrin (Uro) content of incubations of bilanes **4** to **7** with Uro'gen III synthase were analyzed by HPLC-UV to determine the ratio of type I/type III[10] following the usual assay procedure.[11] Uro III analog production was observed only with the butyrate-HMB **7**. Kinetic data using a Lineweaver-Burk plot gave a K_m of 20 μM for this substrate versus 3 μM for HMB. Analog **7** appeared to be turned over at about half the rate of the natural substrate, HMB.

Inhibition studies were carried out with the three other new bilanes (**4**, **5** and **6**), all of which exhibited a decrease of enzyme activity consistent with a reversible inhibition with respect to HMB. This effect was overcomed by increasing the substrate concentration, showing the inhibition to be competitive. The kinetic parameters (K_i) were not formally determined, since no extra information concerning the active site or the enzymatic mechanism would be gained. However, HPLC analysis showed clearly that N_D-Me-HMB (**5**) is a better inhibitor than N_C-Me-HMB (**6**) or HEB (**4**) (less Uro'gen III formation observed).

The activity of Uro'gen III synthase with the butyrate-HMB (**7**) and with N_D-Me-HMB (**5**) was also investigated by ^{13}C-NMR. Both bilanes were synthesized with a ^{13}C-label at the C-20 position.[12] The incubations were run in an NMR tube using conditions described earlier[13] (millimolar concentration, 5°C, in 20% methanol or 10% acetonitrile/sodium bicarbonate buffer, pH 10 to 10.8). As expected, the butyrate bilane (**7**, δ=55.5 ppm for C-20) was observed to turn over slowly at pH 10 generating the corresponding Uro'gen type product (δ=22 ppm), confirmed as type III by HPLC analysis of the sample.[10] No new peak consistent with an intermediate was seen. With N_D-Me-HMB (**5**, δ=55.4 ppm for C-20), no

change in the NMR spectrum [no Uro'gen (δ=22.0 ppm) or Uro product (δ=98.0 ppm)] was perceived during overnight incubation.

Recently, we have been able to provide evidence of the generation of an azafulvene at C-1 as the first step of the Uro'gen III synthase mechanism by trapping this species with ammonium chloride to form the 1-aminomethylbilane.[13] An experiment with analog **5** under similar conditions (200 mM ammonium chloride) showed no signal at ca. 36 ppm expected for the aminomethylbilane, indicating that the N_D-Me-HMB is unable to form an azafulvene at C-1. It can be speculated that the presence of the N-methyl group does not allow the correct conformation of the bilane inside the active site pocket.

A survey of all the results to date of substrate/inhibition studies with HMB analogs (table 1) indicates that only bilanes bearing modified side-chains (**7**, **10**, **11**, **13** and **14**) can be alternate substrates, while other substituted HMB's (**4** to **6** and **15** to **17**) act as inhibitors, showing that Uro'gen III synthase is not an absolutely specific enzyme. Nevertheless, HMB remains the best substrate, as these side-chain modifications produced a substantial adverse effect on the reaction rate and/or efficiency of the inversion process (ratio of ring D inversion: 80% for **7**, 95% for **10**, 45% for **11** and 65% for **14**).

In contrast to other side-chain permutations, inversion of the propionate and acetate groups on ring A (**8** and **12**) and on ring B (**9**) bring about more drastic changes, as the bilanes **8**, **9** and **12** are not substrates of the enzyme. Any change in the side-chains creates some electronic effect in the pyrrolic ring and particularly concerning ring A, a variation of the pK of the hydroxy group could be expected. In the light of recent evidence involving the generation of an azafulvene at C-1, *i.e.* at ring A, as the first step of the enzymatic mechanism,[13] it is likely that the inversion of the side-chains on ring A would affect the formation of the azafulvene. The result for ring B is more difficult to interpret.

As for the ring D, substitution of the acetate group by a methyl (**13**) yields a very poor substrate, indicating that the ring D acetate carboxyl

group must have an important function, whereas the ring D propionate carboxyl group apparently plays a less critical role (**7** and **14**).

Uro'gen III synthase appears not to tolerate any other changes than side-chain modifications. Indeed, inhibition is brought about by substitution at the C-19 position (**15** to **17**), methylation of the ring C and D pyrrolic nitrogen (**5** and **6**) and by substitution at the hydroxymethyl side-chain (**4**). The first effect is easily understood, as the substitution will directly influence the cyclization process. A distortion in the conformation of the enzyme-bound bilane engendered by the presence of the methyl group on the pyrrolic nitrogen might explain the second case. The inhibition by **4** could equally be explained on the basis of steric hindrance, which may not allow the enzymatic formation of the azafulvene at C-1.

In summary, the activities as substrates/inhibitors of Uro'gen III synthase of four new bilanes have been studied. Of these, one (butyrate-HMB) is acting as a slow substrate to give a Uro'gen type III product while the three others (HEB, N_C-Me-HMB and N_D-Me-HMB) show competitive inhibition. A comparison of substrate specifity/inhibition effect of diverse bilanes with Uro'gen III synthase in view of all the structure-activity results to date suggests that the synthase can tolerate side-chain modifications to some degree, however other changes usually result in inhibition of the enzyme. No conclusive information about the enzyme mechanism could be obtained, warranting further studies to probe this unique mechanism.

Acknowledgment. We thank the National Institute of Health (N.I.D.D.K.) for financial support of this work.

References and notes

(1) a) Scott, A. I. *Angew. Chem. Int. Ed. Engl.* **1993**, *32* 1223. b) Battersby, A. R; Leeper, F. J. *Chem. Rev.* **1990**, *90*, 1261.

(2) The four bilanes (**4** to **7**) were prepared following the general procedure described in reference 5. Details of their syntheses and spectroscopic data will be reported elsewhere.

(3) a) Battersby, A. R.; Fookes, C. J. R; Gustafson-Potter, K. E.; McDonald, E.; Matcham, G. W. J. *J. Chem. Soc., Perkin Trans. I* **1982**, 2413. b) Sburlati, A.; Frydman, R. B.; Vlasinas, A.; Rosé, S.; Priestap, H. A.; Frydman, B. *Biochemistry* **1983**, *22*, 4006.

(4) Battersby, A. R.; Fookes, C. J. R.; Matcham G. W. J.; Pandey, P. S. *Angew. Chem. Int. Ed. Engl.* **1981**, *20*, 293.

(5) Battersby, A. R.; Fookes, C. J. R.; Pandey, P. S. *Tetrahedron* **1983**, *39*, 1919.

(6) Anderson, P.A.; Battersby, A. R.; Broadbent, H. A.; Fookes, C. J. R.; Hart, G. J. *Tetrahedron* **1986**, *42*, 3123.

(7) Pichon, C.; Atshaves, B. P.; Danso-Danquah, R.; Stolowich, N. J.; Scott, A. I. *Bio. & Med. Chem.*, submitted.

(8) Battersby, A. R.; Fookes, C. J. R.; Gustafson-Potter, K. E.; McDonald, E.; Matcham, G. W. J. *J. Chem. Soc., Perkin Trans. I* **1982**, 2427.

(9) The activity study was run with the aminomethyl derivative of **8**. However, it was shown that the aminomethyl analog of HMB, AMB, was also a slow substrate of Uro'gen III synthase.[3a] Xue T., Ph. D. Thesis, Texas A&M University, 1992.

(10) Uro I and III standards corresponding to each bilane were prepared chemically following known procedures to measure their HPLC retention times in acetonitrile/ammonium acetate buffer with UV-Vis detection at λ_{max}=405 nm for **4** and **7** and λ_{max}=415 nm for the N-methylbilanes **5** and **6**.

(11) Jordan, P. M. *Enzyme* **1982**, *28*, 158.

(12) The preparation of [20-^{13}C]-butyrate-HMB (**7**) and [20-^{13}C]-N_D-Me-HMB (**5**) followed the same procedure used for the synthesis of [20-^{13}C]-HMB, as described in reference 13.

(13) Pichon, C.; Atshaves, B. P.; Stolowich, N. J.; Scott, A. I. *Bio. & Med. Chem.*, in press.

(*Received in USA* 8 *February* 1994; *accepted* 18 *March* 1994)