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A new synthesis of porphobilinogen analogues, inhibitors of hydroxymethylbilane synthase †

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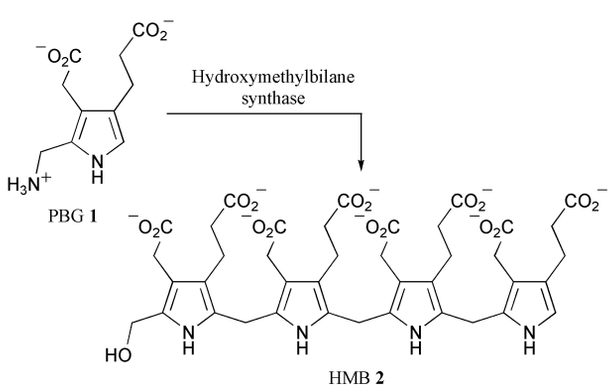
Two analogues of porphobilinogen, the 6-methyl and 6,11-ethano derivatives, have been made by a new synthetic route and the 6-methyl analogue has proved to be the most potent inhibitor of hydroxymethylbilane synthase yet reported (K_i = 3 μM).

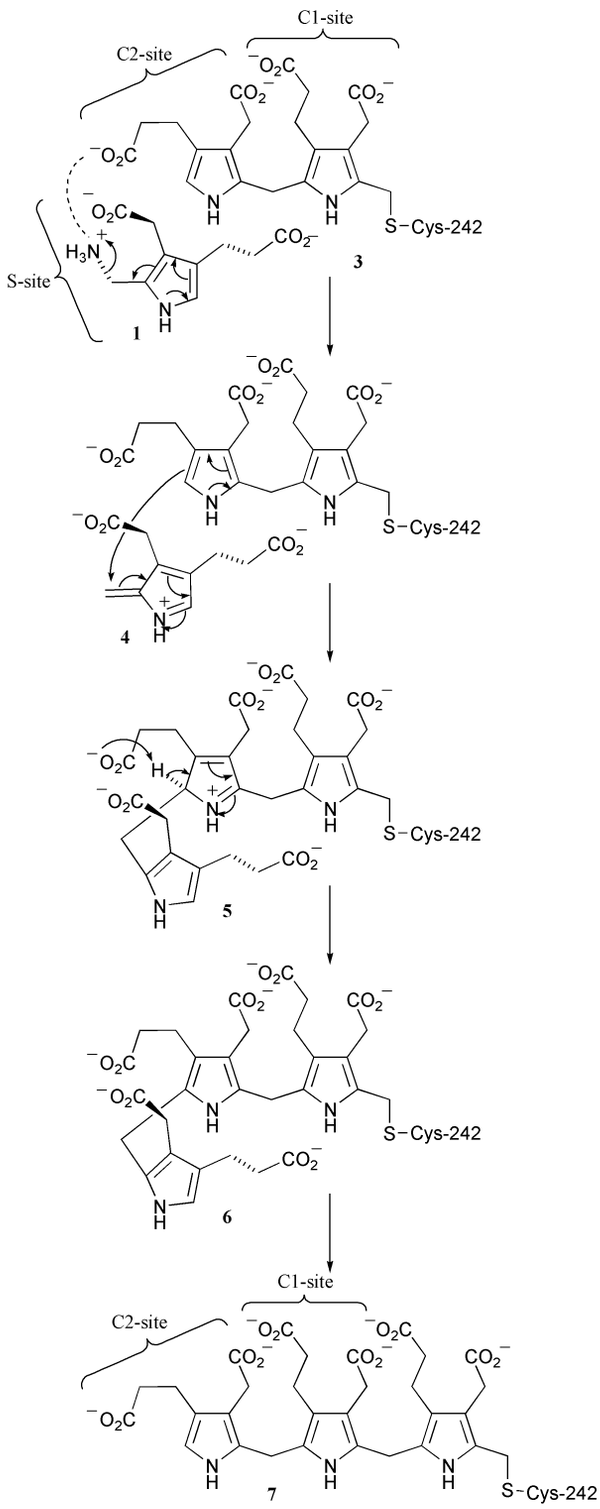
In the biosynthesis of tetrapyrroles a key step is the one in which four molecules of porphobilinogen (PBG) **1** are combined to give the first tetrapyrrole, the linear hydroxymethylbilane (HMB) **2** (Scheme 1).[1,2] The enzyme that catalyses this



Scheme 1

step is hydroxymethylbilane synthase (HMBS), also known as PBG deaminase. HMBS has a unique dipyrromethane cofactor **3**, made from two molecules of PBG (Scheme 2), which is covalently attached to the enzyme *via* a thioether linkage to cysteine-242. Each molecule of PBG becomes attached to the end of this cofactor, with elimination of NH_3. The mechanism for the attachment of the first PBG molecule is thought to be as shown in Scheme 2.[2] After four PBG molecules have been attached in turn, giving a hexapyrrole, the tetrapyrrolic product **2** is then detached, leaving the dipyrromethane cofactor still attached to the enzyme, ready for the start of the next turnover.

The crystal structure of HMBS has been solved both with the active dipyrromethane cofactor[3] and with an inactive oxidised form of it.[4] In the latter structure the terminal pyrrolic ring of the cofactor appears to occupy a binding site which is assumed to be where the substrate molecules normally bind (the S-site shown in Scheme 2). Modelling studies, using molecular mechanics, have suggested[5] a probable orientation for the non-covalently bound PBG which allows it to undergo the required covalent attachment to the end of the cofactor. In this orientation there is a cavity in the active site of the enzyme, which is to the side of the PBG molecule, close to the acetate and aminomethyl side-chains. We thought it possible, therefore, that analogues of PBG with additional groups on either of these side-chains might fit in well to the active site and thus



Scheme 2 Proposed mechanism for hydroxymethylbilane synthase.

† Electronic supplementary information (ESI) available: Dixon plot. See http://www.rsc.org/suppdata/ob/b2/b209613g/

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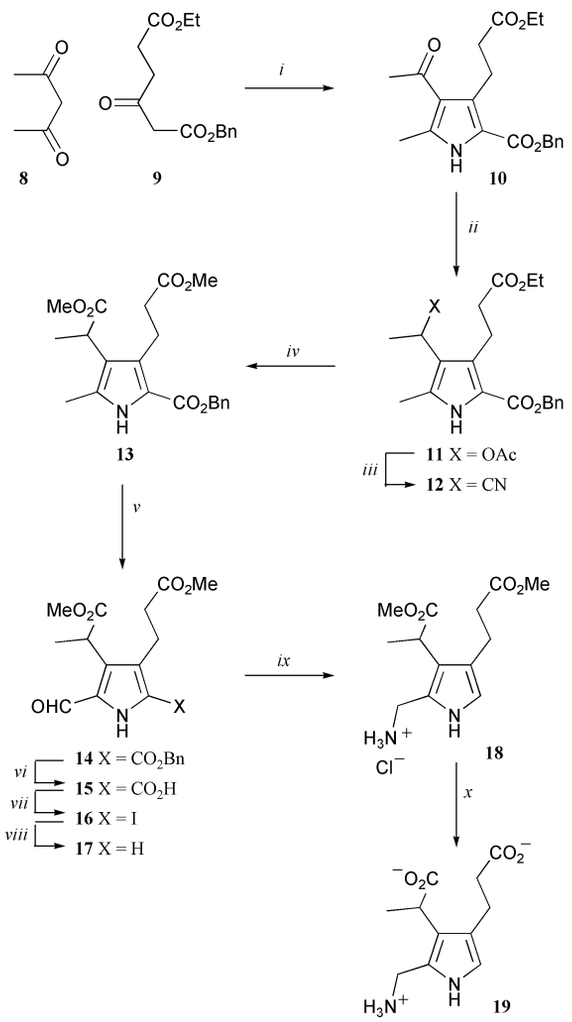
inhibit the enzyme. Here we describe the synthesis of two such analogues, 6-methyl-PBG **19** and 6,11-ethano-PBG **22**.

A review of published syntheses of PBG[6] suggested that the easiest route to 6-methyl-PBG **19** would be a modification of the Knorr pyrrole synthesis route first reported by Kenner *et al.*[7] Thus treatment of the β-ketoadipate diester **9** with $NaNO_2$ in acetic acid–water, followed by reduction of the resulting oxime with zinc in acetic acid and concomitant condensation with pentane-2,4-dione **8** results in the pyrrole **10** (Scheme 3).[7]

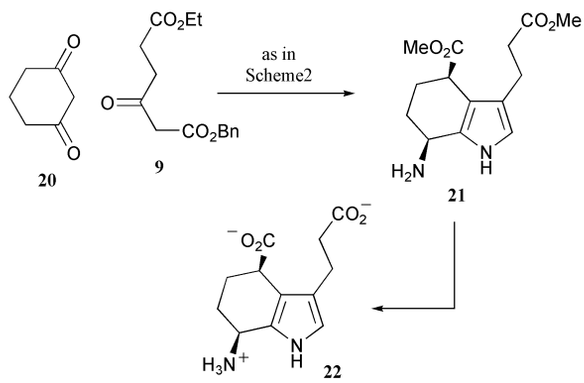


Scheme 3 *Reagents: i*, $NaNO_2$ then Zn, AcOH; *ii*, $NaBH_4$ then Ac_2O, pyridine; *iii*, Me_3SiCN, $TiCl_4$; *iv*, MeOH, HCl; *v*, SO_2Cl_2; *vi*, H_2SO_4, TFA; *vii*, I_2, KI, $NaHCO_3$; *viii*, H_2, Pt_2O; *ix*, NH_2OH then Pd, HCl; *x*, NaOH then Dowex 50 (NH_4^+).

In Kenner's synthesis of PBG the acetyl group of **10** was rearranged to a methoxycarbonylmethyl group using thallium trinitrate. Here we want to keep the methyl group of the acetyl side-chain and introduce a methoxycarbonyl group in place of the carbonyl. This was achieved by initial reduction of the ketone and acetylation to give the acetate **11** (58%). The key step then was treatment of **11** with trimethylsilyl cyanide and titanium tetrachloride, which results in replacement of the acetoxy group by cyanide in 92% yield, presumably *via* an S_N1 mechanism. The cyano group of **12** was then methanolysed using hydrogen chloride in methanol to give the methyl ester **13** (73%), with transesterification of the ethyl ester also occurring.

The remainder of the route to 6-methyl-PBG **19** followed published procedures for the synthesis of PBG and related compounds.[7–9] Thus the α-methyl group of **13** was oxidised to a formyl group **14** in 74% yield by chlorination with sulfuryl chloride followed by hydrolysis of the dichloride. The benzyl ester was removed in three steps and 71% overall yield by acid-catalysed cleavage of the benzyl group, iodinative decarboxylation of acid **15** and then hydrogenolysis of the iodide **16**. The formyl group of **17** was converted to the amine hydrochloride **18** by hydrogenation of the corresponding oxime (69% over two steps). Finally hydrolysis of the esters with NaOH gave 6-methyl-PBG **19**, isolated as its mono-ammonium salt in 91% yield after treatment with Dowex 50 (NH_4^+ form).

The 6,11-ethano derivative **22** of PBG (Scheme 4) was synthesised in the same way from β-ketoadipate diester **9** and cyclohexane-1,3-dione **20**, with similar yields all through the synthesis. The only significant difference was that the oxidation of the α-methylene group to a ketone was best performed using ceric ammonium nitrate[10] (84% yield) instead of sulfuryl chloride. In the reduction of the oxime that generates the amine **21**, two diastereoisomers could be formed. In fact only one product was observed (84% isolated yield), which was proved using a NOESY spectrum to have the *cis* orientation of the amino and ester groups. Presumably the hydrogenation occurs from the opposite side to the methoxycarbonyl group because of steric hindrance from this group. The NOESY spectrum showed that the methoxycarbonyl group is predominantly in an axial position, where it avoids an allylic 1,3-interaction with the adjacent propionate side-chain, and for this reason it would be very hindering of the top face of the carbocyclic ring.



Scheme 4

The two PBG analogues were tested[11] as inhibitors of HMBS from *Escherichia coli* (kindly supplied by Dr N. P. J. Stamford). Whereas the 6,11-ethano-PBG **22** showed no inhibition of the enzyme at concentrations up to 900 μM, 6-methyl-PBG **19** showed significant inhibition in the 2 to 10 μM range. Accordingly assays were performed with a range of concentrations of both substrate (PBG) and inhibitor (6-methyl-PBG). The Michaelis–Menten plots of this data show that the inhibitor both decreases the V_{max} value and increases the K_m value of the enzymic reaction. This indicates mixed inhibition, which is not surprising given the complexity of the reaction mechanism. A Dixon plot of the data † gave convergent lines with a reasonably good intersection point corresponding to an apparent K_i value of 3 μM (*cf.* K_m for PBG = 20–40 μM). Before this the best reported inhibitor of HMBS was 9-fluoro-PBG with an apparent K_i value of 6 μM.[11] It would be interesting to determine whether this inhibition is primarily due to only one of the two enantiomers of 6-methyl-PBG **19**, as one might expect.

The inhibition caused by **19** might be simply due to non-covalent binding, but equally well it might be due to covalent attachment to the dipyrromethane cofactor, in the same way as PBG normally binds, if this causes subsequent steps to be slower. To investigate this HMBS was incubated with three molar equivalents of 6-methyl-PBG **19** for 20 min and the sample then injected onto a MonoQ FPLC column and eluted under conditions known[11] to separate the various enzyme–substrate complexes. The elution profile, measuring A_{280}, showed clearly the formation of three complexes in addition to some remaining native holoenzyme (Fig. 1). Enzymic assay

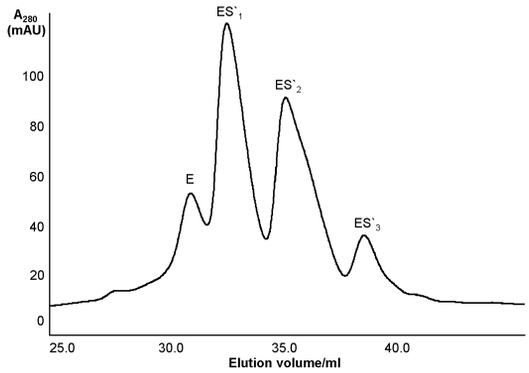


Fig. 1 FPLC elution profile of the complexes of HMBS with 6-methyl-PBG **19**.

showed activity in all four peaks and LC-electrospray MS analysis of the fractions showed holoenzyme at a mass of 34270 (calc. 34270), ES′$_1$ complex at 34493 (calc. 34493), and ES′$_2$ complex at 34715 (calc. 34716).‡ Thus it is clear that **19** does become covalently attached to the enzyme and the slowness of the further reactions of such complexes is probably the cause of the observed inhibition.

It is thought that after covalent attachment of the first PBG molecule all the pyrrole rings move along one place (as in **6** ⟶ **7** in Scheme 2) so as to leave the substrate binding site free for the second molecule of PBG. It is understandable therefore that the extra methyl group present in **19**, although well accommodated in the substrate binding site, does not fit well into the subsequent sites into which this pyrrole ring needs to fit (the C1 and C2 sites occupied by the two rings of the cofactor in the native enzyme) and hence subsequent steps in the mechanism are significantly retarded.

In view of the potent inhibition observed with **19** it seems at first surprising that no inhibition was observed with the 6,11-ethano analogue **22**. However **22** has the *cis* arrangement of amino and carboxylate groups on the carbocyclic ring, whereas molecular modelling[5] suggested that these two groups should be *trans* to each other when PBG binds in the active site (as shown in Scheme 2). Therefore the lack of inhibition by **22** is readily explained and, furthermore, supports the proposed *trans* conformation of the side-chains of bound PBG. It would be interesting to see if the *trans* isomer of **22** is a potent inhibitor but unfortunately this was not accessible with the synthetic strategy used here.

In summary, we have described a new synthetic route to PBG analogues that bear extra substituents on C-6 and/or C-11. 6-Methyl-PBG **19** is the most potent inhibitor of HMBS yet reported. As all organisms have a requirement for one or more of the tetrapyrroles (haem, chlorophyll, vitamin B_{12} *etc*.) HMBS activity is vital. Therefore potent inhibitors of HMBS could potentially be effective antibiotics and/or herbicides.

Notes and references

‡ ES′$_1$ implies enzyme with one molecule of substrate analogue **19** covalently bound, ES′$_2$ implies enzyme with two molecules of **19** bound *etc*. No molecular ion of the ES′$_3$ complex, assumed to be the fourth peak on FPLC, was observed, perhaps because it is unstable.

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