Modified Substrates for Tetrapyrrole Biosynthesis: Analogues of Porphobilinogen Showing Unusual Inhibition of Porphobilinogen Deaminase

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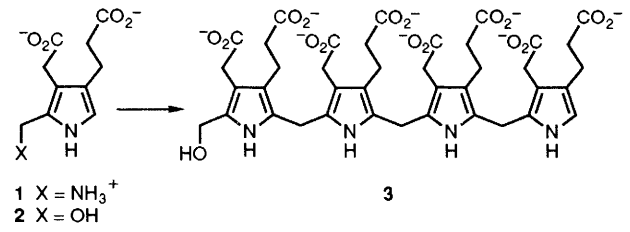
Syntheses are described of two analogues of porphobilinogen (PBG), a fluoro derivative **10** and a phosphonate **13**, which are the first known unnatural substrates of PBG deaminase; when they act as inhibitors of the reaction of PBG, these compounds produce unusual sigmoidal kinetics, explained by their involvement as poor substrates in the particular mechanism of this enzyme.

Porphobilinogen deaminase (hydroxymethylbilane synthase, EC 4.3.1.8) is the key enzyme of porphyrin biosynthesis, which assembles the linear tetrapyrrole, hydroxymethylbilane **3**, from four molecules of the monopyrrolic precursor, porphobilinogen **1** (PBG), Scheme 1.[1] In this process each of the pyrrolic units in turn becomes covalently attached to the enzyme by displacement of ammonia from the aminomethyl group. A remarkable feature of the mechanism, discovered recently, is that the growing pyrrolic chain is not attached directly to an amino acid residue but instead to a cofactor, which is itself formed from two molecules of PBG.[2]

Apart from PBG **1**, the corresponding alcohol **2** can also act as a substrate.[3] A number of other analogues of PBG have been reported[4–6] but although many have been competitive inhibitors, none have been observed to be substrates for the enzyme. In this paper, we describe the first two analogues of PBG that can act as substrates to produce modified bilanes. In addition these compounds show unusual kinetics in their inhibition when they compete with PBG as substrates.

The two analogues we aimed to synthesize were compounds **10** and **13**. The fluorinated analogue **10** was chosen because the substitution of hydrogen by fluorine involves a minimal increase in size and so it was thought that this analogue had the greatest likelihood of acting as a substrate. In addition it was possible that the α-fluorocarboxylic acid might act as an alkylating agent for some nucleophilic residue in the active site and thus allow the identification of some active site residue(s). We have previously reported the synthesis of the analogous fluorinated analogue of the immediate precursor, 5-amino-levulinic acid.[7] The phosphonate **13** was chosen because it has the same charge as a carboxylate and so might bind in the same way to the enzyme.

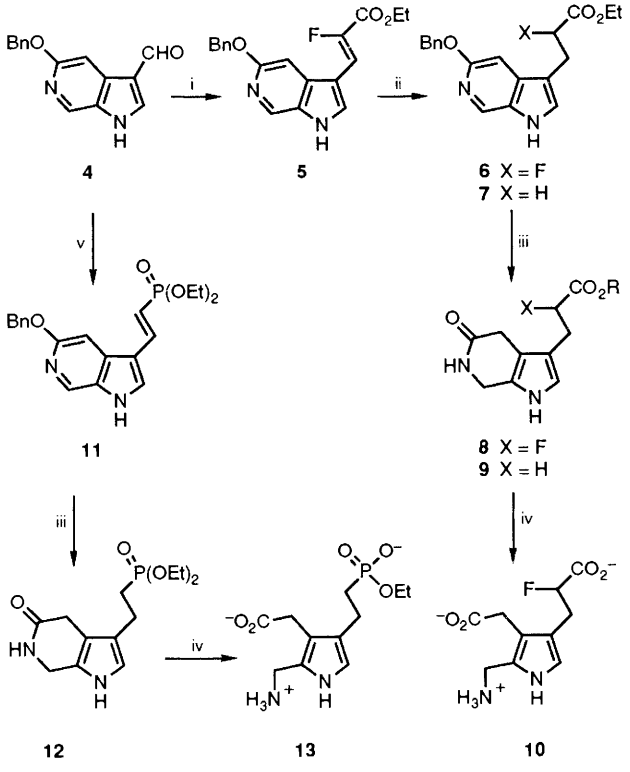
The two syntheses (Scheme 2) followed a previous synthesis of PBG[8] up to the stage of the formyl azaindole **4**. For the



1 X = NH_3^+
2 X = OH

3

Scheme 1



Scheme 2 *Reagents*: i, $HO_2CCHFCO_2Et$–pyridine–piperidine; ii, Mg–MeOH; iii, Pd/C/H_2; iv, KOH–H_2O; v, $CHLi[PO(OEt)_2]_2$

fluoro analogue, this aldehyde was condensed with monoethyl fluoromalonate to give the fluoroacrylate **5**. Unfortunately, catalytic hydrogenation to give the lactam **8** (R = Et) also resulted in extensive defluorination, giving the unsubstituted PBG lactam ethyl ester **9** as the major product. Loss of fluorine, however, was minimized by reducing the acrylate double bond first using magnesium in methanol. The fluorinated product **6** was now the major one and was completely separated by HPLC from the minor defluorinated product **7** (ratio 3:1). Subsequent catalytic hydrogenation caused no further loss of fluorine. Similar loss of fluorine during the hydrogenation of the unsaturated 2-fluoro-fumaric and -maleic acids but not from the saturated 2-fluorosuccinic acid has been described by Hudlicky.[9] Final hydrolysis of the lactam ester **9** (R = Me) was performed in KOD–D_2O so that the reaction could be followed by NMR. Clean conversion into the desired 9-fluoroPBG (FPBG) **10** was observed, accompanied by exchange of the hydrogen atoms of the acetate side-chain for deuterium.

For the phosphonate analogue, Wadsworth–Emmons reaction of aldehyde **5** with the anion from tetraethyl methylenebisphosphonate[10] gave the vinyl phosphonate ester **11**. Hydrogenation in this case proceeded normally to the lactam **12** and alkaline hydrolysis gave the phosphonate monoester analogue of PBG **13** (PPBG). Removal of both of the ethyl groups from the phosphonate diester **12** was also attempted using iodotrimethylsilane but although the ethyl groups were easily removed, some unidentified reaction of the pyrrole also occurred, evidenced by the loss of the signal for the α-hydrogen in the ^1H NMR spectrum.

Initially the two analogues, **10** and **13**, were tested as substrates for the enzyme PBG deaminase from *Escherichia coli*. Both analogues did show activity although FPBG **10** was *ca.* 50 times slower than PBG as a substrate and PPBG **13** *ca.* 500 times slower. The products from both analogues had absorption spectra virtually identical to that of uroporphyrin I. In the case of FPBG sufficient product was accumulated that it could be extracted and esterified (MeOH–H_2SO_4) to give a compound that had an identical R_F value to a uroporphyrin octamethyl ester but a molecular weight corresponding to a tetrafluoro derivative.† It was also possible to determine the K_m for FPBG as *ca.* 83 μmol dm^{-3} (*cf.* K_m for PBG,[11] 12 μmol dm^{-3}).

In order to show that the observed product formation from the two analogues was due to the normal catalytic process of the enzyme, tritium-labelled PPBG **13** (made from [*formyl*-^3H]azaindole **4**[12]) was incubated with deaminase. The amount of radioactivity that coeluted with the protein peak on gel filtration corresponded to 1.38 mol of PPBG per mol of enzyme.

For FPBG **10** a different approach was taken to demonstrate covalent attachment to the enzyme. When FPBG was incubated with deaminase and the mixture separated by FPLC, four peaks were observed, corresponding to Enz, Enz(FPBG), Enz(FPBG)$_2$ and Enz(FPBG)$_3$ (as previously reported for PBG[2]). These complexes were separated on a preparative scale and assayed with normal PBG as substrate. Each one showed a distinct lag before a rate of production of hydroxymethylbilane equal to that of the native enzyme was attained. The lag-times were *ca.* 30 s for Enz(FPBG)$_2$, 2 min for Enz(FPBG) and 3 min for Enz(FPBG)$_3$. This experiment shows that the FPBG does indeed form complexes with deaminase, which behave like normal enzyme–PBG complexes except that they are considerably slower to turnover.

As these substrate analogues form complexes with deaminase, which are slow to turnover, it would be expected that they would act as inhibitors when the enzyme is incubated with PBG. This was indeed found to be the case, *ca.* 50% inhibition being achieved with 7 μmol dm^{-3} FPBG or 50 μmol dm^{-3} PPBG. There was no evidence, however, for any time-dependent inactivation by either analogue.‡ When the enzymic rate was measured at various concentrations of PBG in the presence of the inhibitors, it was found that the rate no longer showed the usual hyperbolic dependence on substrate concentration but was sigmoidal at low [PBG]. The reason for this seems to be that once one of the substrate analogues becomes bound to the enzyme, that enzyme molecule remains affected until attachment of further PBG molecules has occurred to release a modified hydroxymethylbilane. Therefore, at lower concentrations of PBG the enzyme is reactivated more slowly and hence the inhibition appears greater. Indeed, a very similar sigmoidal curve was obtained by calculation of the kinetics of a simplified version of the mechanism of deaminase.

† The actual molecular weight, 1022 (by field desorption mass spectrometry), was eight higher than expected for a tetrafluorouroporphyrin owing to the deuterium atoms incorporated during the hydrolysis of **9**.

‡ Gradual loss of activity of deaminase is observed when deaminase is incubated for long periods with PBG but not in its absence. This is thought to be because some of the enzyme–PBG complexes are susceptible to oxidation by air whereas the native enzyme is not. Approximately the same loss of activity was observed with both of the analogues but there was no evidence for any more rapid inactivation process.

As a result of the sigmoidal kinetics, K_i values for the inhibitors could not be obtained. However, the concentration of FPBG that gives 50% inhibition (7 μmol dm^{-3}) is very much lower than the K_m value of FPBG as a substrate (83 μmol dm^{-3}). This could be explained if binding of the first FPBG molecule occurred with an intrinsic K_m value close to that of PBG (12 μmol dm^{-3}) but this then had the effect of raising the intrinsic K_m value for binding of the subsequent pyrrole(s). When FPBG acts as the sole substrate, it has to bind four successive times and so its overall K_m value would be considerably higher than for PBG alone.

In conclusion, the two PBG analogues **10** and **13** can act as substrates of PBG deaminase and this has been shown to be a way to synthesize novel porphyrins. However, the requirement for four analogue molecules to become attached to the enzyme makes turnover extremely slow and it was not possible to accumulate substantial quantities of these products. Inhibition of the reaction of deaminase with PBG, on the other hand, requires attachment of only one analogue molecule and is, therefore much more effective. With an excess of PBG present the inhibited enzyme turns over at a more reasonable rate, releasing a tetrapyrrole that is modified on one ring only. If this occurs *in vivo*, the modified tetrapyrrole may then inhibit the next enzyme or pass on to inhibit enzymes still later in the biosynthetic pathway. The accumulation of porphyrins resulting from inhibition of later enzymes in the pathway is known to be damaging due to photooxidative processes. Therefore, this type of substrate analogue may have interesting cytotoxic properties.

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