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Studies on the formation of porphyrinogens from monopyrroles in presence of the enzymes PBG deaminase and/or Uro'gen III synthase

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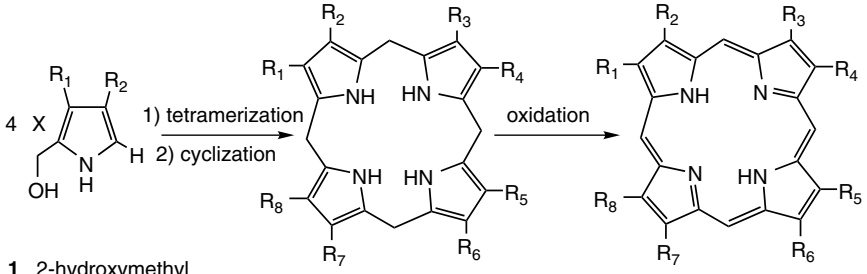
Abstract—The substrate-specificities of two enzymes in the biosynthetic pathway to vitamin B_{12}, PBG deaminase and Uro'gen III synthase, which are involved in the formation of Uro'gen III from the pyrrole PBG, are investigated for the preparation of Uroporphyrin analogs. Both enzymes display strong substrate-specificity. However, tetramerization of pyrroles with carboxylate β-substituents in mildly basic buffer represents the best and most rapid route to a family of Uro I analogs for enzymatic activity studies.

Numerous methods have been described for the synthesis of porphyrins.[1] The strategy used often depends upon the degree of symmetry of a particular type of porphyrin and its substituents, and ranges from monopyrrole tetramerization to routes involving dipyrrolic, tripyrrolic, or open-chain tetrapyrrolic intermediates. However, while the latter methods require numerous steps, the most direct preparation of porphyrins from monopyrroles with two different β-substituents generally leads to a mixture of the four possible isomers (Scheme 1), except for a handful of special cases.[1a,2]

Since our laboratory is especially interested in obtaining several analogs of Uroporphyrin (Uro) I with one or



Scheme 1. Preparation of porphyrins from 2-hydroxymethylpyrroles.

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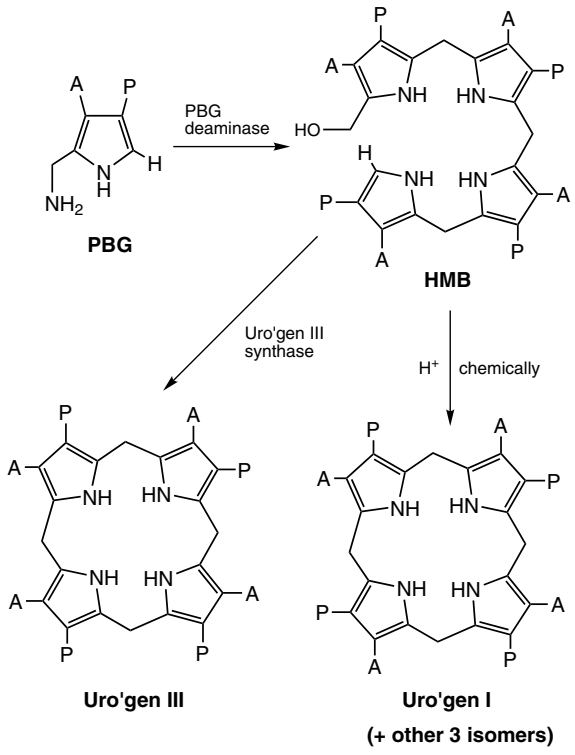

Scheme 2. Formation of Uro'gen I and III.

two carboxylate substituents (**3a**, Scheme 1) for subsequent use in enzymatic studies on the biosynthetic pathway to vitamin B_{12}, the most direct approach by polymerization of four pyrrole units followed by cyclization and oxidation to porphyrins seems the method of choice for us. Although Smith et al. reported formation of 100% type I coproporphyrin (**3f**),[2b] their reaction conditions do not work well with pyrroles bearing two electron-donating groups. Other conditions developed by Eschenmoser and co-workers[3] and in our group[4] give high ratios of type I (89%,[3] 72–80%[4]), but contamination with other isomers remains a concern. In nature however, porphobilinogen (PBG) deaminase, one of the early enzymes in the porphyrinoid pathway, performs the tetramerization of PBG to the tetrapyrrole 1-hydroxymethylbilane (HMB), which upon subsequent chemical cyclization and oxidation generates pure Uro I (Scheme 2).[5] Earlier studies in our laboratory on the enzyme have shown activity toward some substrate analogs[6] and we thought it would be of interest to investigate this approach further.

The next enzyme in the pathway, Uroporphyrinogen (Uro'gen) III synthase, uses the tetrapyrrole HMB as substrate and cyclizes it to Uro'gen III after rearrangement of the last pyrrole ring (Scheme 2).[5,7] The synthesis of type III porphyrins being rather lengthy,[1] it would certainly be interesting to explore the possibility of using this enzyme in combination with PBG deaminase to obtain some Uro III analogs.

The availability of such Uro I or III analogs would allow further probing and understanding of the enzymatic steps that follow in the biosynthesis of vitamin B_{12}, as well as the production of novel porphyrinoid compounds.

It has been demonstrated earlier that 2-hydroxymethyl PBG (**1a**) was a good substrate for the enzyme PBG deaminase.[8] We already had available from previous work a family of 2-formylpyrroles bearing one or two methoxycarbonate β-substituents (Scheme 1). For our experiments, the methyl esters were hydrolyzed and the formyl group reduced with sodium borohydride. The reaction completion and purity of the compounds were checked by ^1H and ^{13}C NMR in D_2O.

Overexpression and purification of PBG deaminase were conducted as described previously.[9] The enzyme was used pure or as a lysate after heat treatment (10 min, 50 °C)[10] to eliminate any trace of the next enzyme in the porphyrinoid pathway, Uro'gen III synthase.

The free-acid 2-hydroxymethylpyrroles were incubated with PBG deaminase under standard conditions.[11] Incubation conditions and work up were optimized for the 2-hydroxymethyl PBG (**1a**): it was found essential to oxidize the porphyrin products before esterification to avoid acid-catalyzed rearrangement of the porphyrinogens. It was also found important to remove all unreacted pyrroles before esterification to avoid additional chemical formation of porphyrinogens during the acid-catalyzed esterification process.

Results of the PBG deaminase incubation with 2-hydroxymethylpyrroles (Scheme 1) are shown in Table 1. Determination of type I ratios were based on ^1H NMR or HPLC analyses, depending on the products.[12] Comparison of the results (yields and type I ratios) of incubations with and without deaminase reveals a significant difference only in the case of pyrrole **1a**; with all other pyrroles, both yields and ratios do not appear significantly affected by the presence of enzyme. However, a comparison with other methods shows that porphyrin formation in buffer often gives better type I ratios. Thus, the conditions described in this paper constitute the best tetramerization method to obtain porphyrins with carboxylated β-substituents.

Although PBG deaminase seems to be too substrate-specific to be used in the synthesis of HMB analogs, in situ tetramerization of 2-hydroxymethylpyrroles bearing carboxylate as β-substituent in buffer (pH 7.8–8.0) proceeds reasonably well (Table 1) and allows the study of the effect of Uro'gen III synthase on the cyclization process (Scheme 2).

Overexpression of Uro'gen III synthase was conducted as described previously.[13] The enzyme was used as lysate, incubations were run in the same standard conditions as for PBG deaminase and worked up following the same protocol.[11] The results are shown in Table 2. With pyrrole **1a**, good yields were obtained with very high ratios of type III porphyrin. This was expected since the product formed in situ by the tetramerization of pyrrole **1a** is HMB, the natural substrate of Uro'gen III synthase (Scheme 2). With other pyrroles, yields of

Table 1. Tetramerization of pyrroles in buffer with or without PBG deaminase

Pyrrole	Conditions		PBG deaminase			In buffer		Other methods		
	pH	Time (h)	Purity	Yield	% I	Yield	% I	Conditions	Yield	% I
1a	7.8	3	Pure	86	100	26	81	0.01% TFA[4]	39	75
1a	8.0	6	Pure	77	100	24	82	CN, HCO_2H[3]	55	89
1a	8.0	18				38	87			
1b	7.8	3	Pure	32	89	28	87	0.01% TFA[4]	60	72
1b	7.8	6	Lysate	31	88	36	88			
1b	8.0	6	Lysate	30	90	34	86			
1c	7.8	3	Pure	31	92	28	89	0.01% TFA[4]	47	80
1c	8.0	6	Pure	19	88	25	86			
1d	8.0	6	Pure	18	92	13	93			
1e	7.8	6	Pure	26	86	27	79			
1f	7.8	3	Pure	22	91	18	86	Et_3N, K_3FCN_6[2b]	25	100

Table 2. Cyclization of HMB analogs formed in situ in presence of Uro'gen III synthase and in buffer

Pyrrole	Conditions		Uro'gen III synthase		In buffer	
	pH	Time (h)	Yield	% III[a]	Yield	% I
1a	7.5	6	53	93	26	84
1a	7.8	18	44	91	53	83
1a	8.0	6	29	91	24	82
1a	8.0	24	44	88	38	87
1b	7.5–8.0	6–24	29–49	30–33	26–55	79–93
1c	7.5	6–18	30–44	18–26	21–42	84–92
1d	7.5–8.0	6–18	33–56	32–51	17–36	81–96
1e	7.5–7.8	6–18	31–39	31–60	24–36	71–90
1f	7.5–8.0	6–24	40–67	27–44	18–32	76–89

[a] In all incubations, mainly type I and III isomers were formed and only the major isomer is reported. The other two isomers, type II and IV, were also present, but in small quantities.

porphyrins are higher than in absence of any enzyme, ratios of type III increase, but remain too low to be useful in the preparation of type III porphyrins.

In conclusion, from these studies both PBG deaminase and Uro'gen III synthase display strong substrate-specificity, preventing their use for preparation of Uro I and III analogs. However, tetramerization of pyrroles with carboxylate β-substituents in mildly basic buffer followed by cyclization and oxidation represents the best and most rapid method to prepare a family of Uro I analogs. These, as well as Uro III analogs, were used for activity studies with the first methyl transferases on the vitamin B_{12} biosynthetic pathway and the results will be published later elsewhere.

Acknowledgments

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11. Incubations were run in degassed 50 mM Tris-buffer, 20 mM $MgCl_2{\cdot}6H_2O$ and 50 mM KCl at 37 °C under argon. The 2-formylpyrrole methyl ester (5–10 mg) was dissolved in MeOH (200–500 μL) and hydrolyzed with KOH 2 N (200–500 μL) at rt overnight. The MeOH was

evaporated, the residual solution diluted with H_2O (200–500 μL) and the formyl group reduced by addition of $NaBH_4$ (5–10 mg) for 1 h at rt. The substrate concentrations for the enzymatic incubations were in the range of 0.5–1 mM, pH 7.5–8.1 and incubation times from 5 min to 24 h. Then, the incubation solution was saturated with O_2 and stirred under normal light at rt for 1 h to oxidize the porphyrinogens to porphyrins. The solution was absorbed on DEAE Sephadex A-25 resin (150 mg/5 mg substrate); the resin was washed with 0.15 M NaCl (10 mL) to remove all unreacted starting pyrrole, with acetone (20 mL), dried, and eluted with MeOH+5% H_2SO_4 (20–50 mL). The porphyrins were esterified at rt overnight, then after neutralization and extraction into CH_2Cl_2, the products were isolated by PLC (CH_2Cl_2+3% MeOH).

All products were characterized by ^1H and ^{13}C NMR spectroscopy and mass spectrometry. All porphyrins prepared, except **3d** and **3e**, are known compounds. Characterization data for **3d** (major isomer, I): ^1H NMR (500 MHz, $CDCl_3$) δ 10.13 (s, 4H, 4 *meso H*), 5.06 (s, 8H, $4CH_2CO_2Me$), 3.75 (s, 12H, $4CO_2CH_3$), 3.66 (s, 12H, $4CH_3$); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.56, 138.27, 136.51, 96.46, 51.74, 32.62, 11.70; MS (ESI) 655 $(M+H)^+$. Characterization data for **3e** (major isomer, I): ^1H NMR (500 MHz, $CDCl_3$) δ 10.10 (s, 4H, 4 *meso H*), 4.13 (t, 8H, $J = 7.1$ Hz, $4CH_2CH_2CH_2CO_2Me$), 3.70 (s, 12H, $4CO_2CH_3$), 3.64 (s, 12H, $4CH_3$), 2.67 (t, 8H, $J = 6.4$ Hz, $4CH_2CH_2CH_2CO_2Me$), 2.63 (m, 8H, $4CH_2CH_2CH_2CO_2Me$); ^{13}C NMR (500 MHz, $CDCl_3$) δ 173.58, 138.37, 136.67, 97.59, 51.78, 33.76, 28.16, 25.81, 11.75; MS (ESI) 767 $(M+H)^+$.

12. Integration of the *meso*-protons in the ^1H NMR spectra (500 MHz) provides a very good and rapid estimate of the type I ratio. This was confirmed in the case of Uro I samples by HPLC analysis (silica gel column, CH_2Cl_2/MeOH, 96/4).
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