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Synthesis of the 2-Fluoro-11-hydroxy Analog of Porphobilinogen, a New Suicide Inhibitor of the Enzyme Porphobilinogen Deaminase

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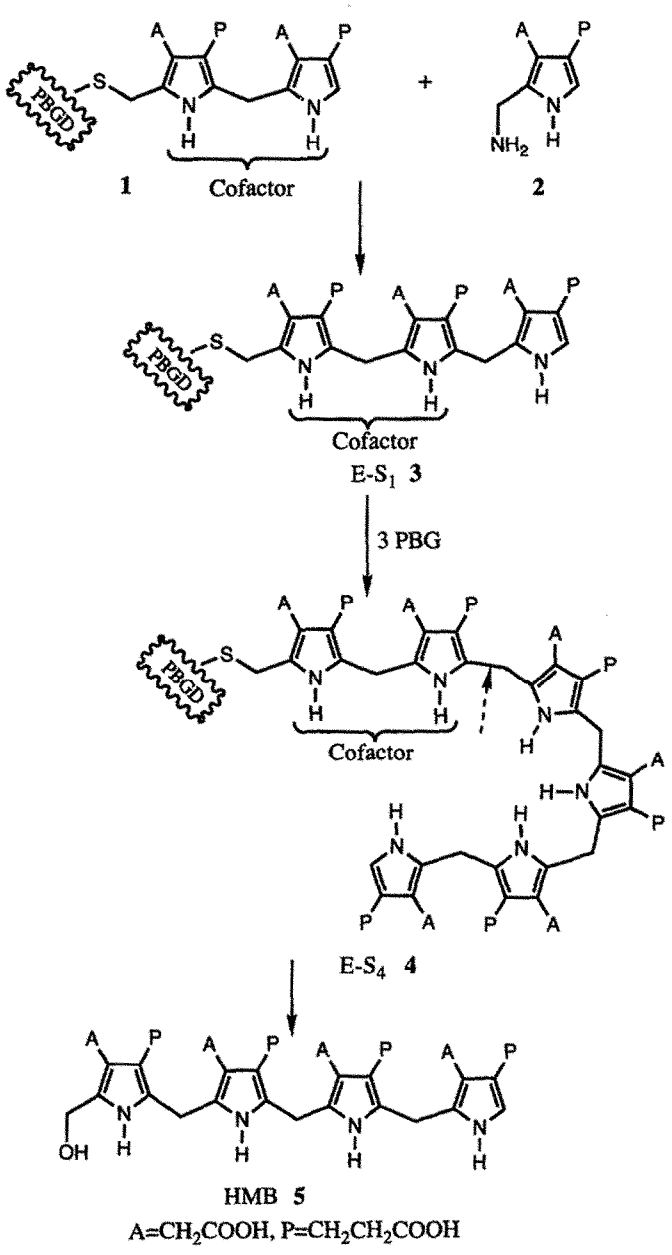
Key Words: Porphobilinogen Deaminase; Analog of Porphobilinogen; Fluoropyrrole; Inhibitor.

ABSTRACT: The 2-fluoro-11-hydroxy analog of porphobilinogen (PBG) **15** was designed and synthesized by the direct fluorination of the pyrrole ring with the fluorinating reagent, xenon difluoride, in the first successful preparation of fluoropyrroles without N-H protection. Incubation of porphobilinogen deaminase (PBGD) with the 2-fluoro derivative **15** showed that the latter is a suicide inhibitor of PBGD which reacts with the enzyme to form a stable covalent complex. This should facilitate further studies of the inhibitor-enzyme complex by X-ray analysis and ^{19}F-NMR.

INTRODUCTION

Porphobilinogen deaminase (PBGD, EC 4.3.1.8, also known as hydroxymethylbilane synthase or uroporphyrinogen I synthase) catalyzes the stepwise polymerization of four molecules of porphobilinogen **2** (PBG) into the linear tetrapyrrole, 1-hydroxymethylbilane (HMB **5**) (Scheme 1), a key intermediate in the biosynthesis of heme, chlorophylls, vitamin B12 and related macrocycles.[1,2] The catalytic mechanism of PBGD has been studied intensively for more than decade,[3,4] and it was established by NMR spectroscopy that the active site of the enzyme contained a unique dipyrromethane (DPM) cofactor.[5] More recently, this was



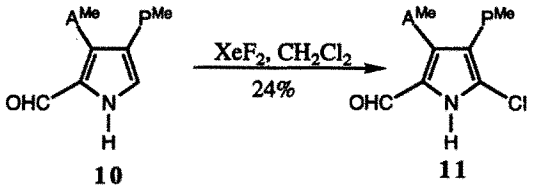
Scheme 1. Biosynthetic formation of hydroxymethylbilane (HMB) from PBG

confirmed by investigations of the crystal structure of this enzyme,[6,7] which showed that the DPM cofactor is covalently linked to Cys 242 as previously demonstrated by site directed mutagenesis. However, the precise mechanism of the polymerization process[8,9] by which the growing polypyrrole chain is assembled by PBGD is still an unsolved problem, although it is clear from NMR studies of the covalent enzyme-substrate (ES) complexes that the α-free position of each incoming PBG unit serves as the nucleophile for the reiterative covalent tetramerization. Based on the following reasons, we have designed and synthesized a new suicide inhibitor of PBGD, the 2-fluoro-11-hydroxy analog of PBG **15**. Firstly, the Van der Waal's radius of fluorine (1.35 Å) and the length of C-F bond (1.39 Å) closely resemble hydrogen (1.20 Å) and the C-H bond length (1.09 Å) respectively. Thus, the structure formed by this inhibitor and PBGD should be also close to that formed by PBG and PBGD, leading to a more accurate structural analysis for such complexes compared with other enzyme-inhibitor (E-I) complexes. Secondly, ^{19}F is a very sensitive NMR nucleus. Its receptivity (4730, relative to ^{13}C, 1) is close to hydrogen (5680). It will therefore be interesting to observe the formation of enzyme-inhibitor complexes by ^{19}F-NMR spectroscopy, as the fluorine atom's direct attachment to the pyrrole ring should be an ideal probe to detect the change of electronic properties on the pyrrole ring during the course of reaction. Finally, in earlier experiments[10] it was found that with the E-I complex of 2-bromo-PBG suitable crystals for X-ray diffraction could not be obtained, due to instability. The fluoro analog **15** therefore appeared to be the most attactive structure for our purposes.

RESULTS AND DISCUSSION

Synthesis

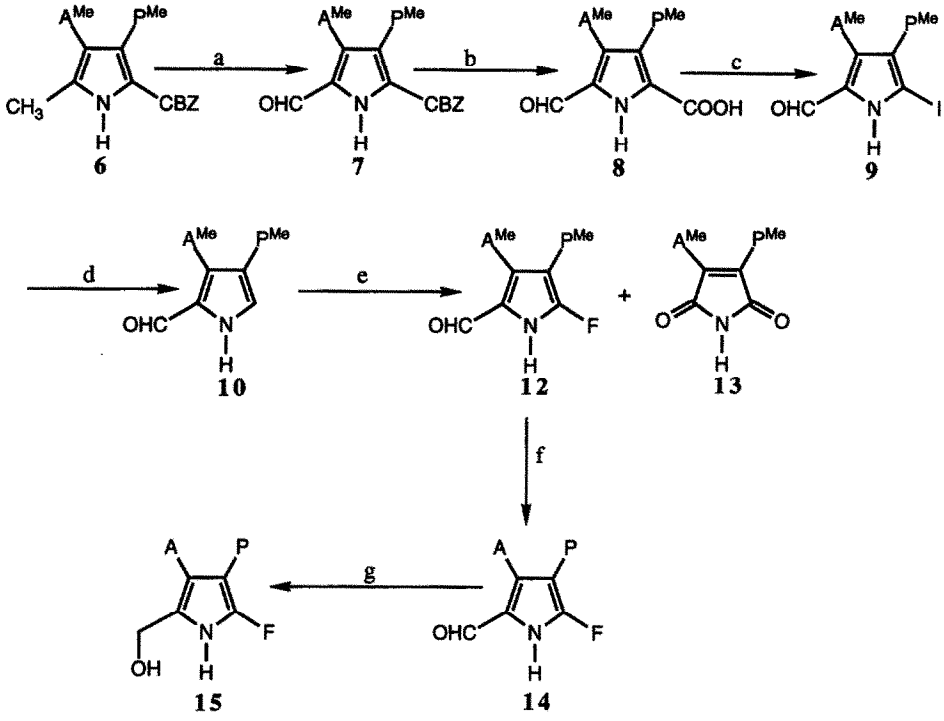
Very little is known about the synthesis of fluoropyrroles.[11] Either the high reactivity of the pyrrole ring towards electrophiles and oxidizing reagents or oxidization of most of the electrophilic fluorinating reagents makes the direct fluorination of the pyrrole ring problematic, nor is there a successful case of introducing fluorine into the pyrrole ring by the classic Balz-Schiemann reaction[12]. Clearly, one must use the right fluorinating reagents, solvents, catalysts and temperature which are important for the reaction to succeed. Our initial attempt to obtain the expected product **12** by direct fluorination of compound **10** (which was prepared



(equation 1)

$$XeF_2 + CH_2Cl_2 \longrightarrow ClF + CH_2ClF + Xe$$

(equation 2)



A^{Me}=CH_2COOCH_3, P^{Me}=$CH_2CH_2COOCH_3$, CBZ=$COOCH_2Ph$

(a) 2 equiv SO_2Cl_2; (b) 10% Pd-C/H_2, Et3N; (c) $NaHCO_3$, CH_2Cl_2/H_2O, reflux, I_2/KI; (d) 10%, Pd-C/H_2, NaOAc; (e) XeF_2, CH_3CN; (f) 1. KOH, 0.2N; 2. HCl, 2N; (g) $NaBH_4$, CH_3OH

Scheme 2. Synthesis of the 2-fluoro-11-hydroxy PBG analog **15**

following known procedures[13,14] from **6**; Scheme 2) using xenon difluoride in dichloromethane as solvent at room temperature gave instead of **12**, the chloro derivative **11** in 30% yield (equation 1). This result may be explained by initial reaction of xenon difluoride with the solvent, dichloromethane, yielding chlorofluoride, a strong electrophile (equation 2) which then reacts with compound **10** to give chloride **11**. However, the expected fluoride **12** could be obtained in modest yield only when acetonitrile was used as solvent. We have also used several other fluorinating reagents, for example, N-fluoropyridium triflate,[15] p-toluyl-N-fluoro-N-propylsulfonamide,[16] and found xenon difluoride to be the best reagent for fluorinating the pyrrole rings with electron-withdrawing substituents, such as, -CHO, -CN, -COR and -COOR.[17]

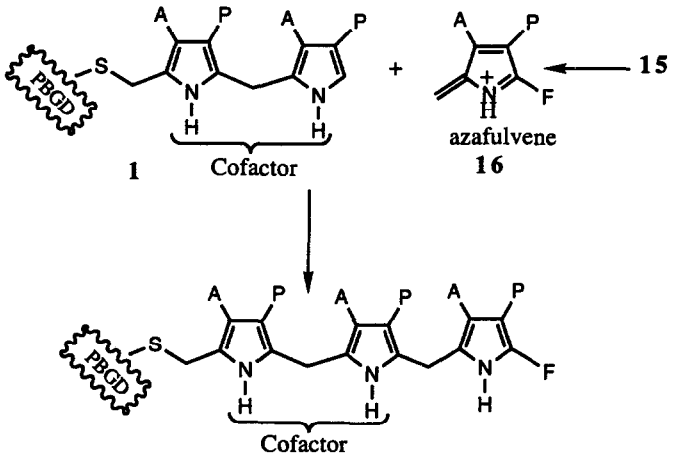
The synthesis of the 2-fluoro-11-hydroxy analog of PBG **15** is outlined in Scheme 2. The fluoride **12** was obtained in 32 % yield from **10** along with the oxidized product **13** (20 % yield). Addition of Lewis acid catalysts, for example, TfOAg,[18] BF_3-diethyl ether,[19,20] to the reaction mixture resulted in decreasing yields of **12** accompanied by an increase of by-product **13**. Compound **12** was hydrolyzed with potassium hydroxide (0.2 N) and after neutralization with hydrochloric acid (2 N), gave the 2-fluoro-5-formylpyrrole **14** in 62 % yield. All attempts to reduce the aldehyde **14** in aqueous solution with sodium borohydride failed. However, the successful reduction of the aldehyde could be carried out using methanol as solvent. The structure of the final product **15** was determined by ^{1}H-NMR and ^{19}F-NMR. The disappearance of the CHO peak (singlet; 8.71 ppm) and the appearance of new peak (singlet; 4.01 ppm) indicated that the aldehyde in compound **14** was reduced to alcohol **15**. A high-field shift of 14.6 ppm in the ^{19}F-NMR spectra on going from **14** to **15** also confirmed that the formyl group had been reduced to hydroxymethyl.

Enzymology

Incubation of PBGD with the 2-fluoro-11-hydroxy analog of PBG **15** was carried out at 37°C in PE buffer (100 mM KH_2PO_4, 2 mM EDTA adjusted to pH 8.0 with NaOH). Native polyacrylamide gel eletrophoresis (PAGE) analysis of this enzymatic reaction showed that the 2-fluoro derivative **15** inhibited PBGD by forming a covalent bond (Scheme 3). The resultant E-I complex **17** blocked the growing polypyrrole chain as the α-position was occupied by the fluorine atom, resulting in the inactivation of the enzyme (Fig. 1). Thus, the multiple bands associated with the enzyme and its ES complexes are clearly visible in Fig. 1A. Lanes 1-4

are accompanied by the corresponding fluorescence of the product uro'gen I (Fig. 1B). However, lanes 5-7 while showing the shift of the PBGD bands on complexation with **15** (Fig. 1A) reveal (Fig. 1B) that no porphyrin product is formed. Isolation and crystallization of this inhibitor-enzyme complex and the tripyrrolic derivative, which could be obtained by the incubation of ES_2[21] with 2-fluoro-11-hydroxy analog of PBG **15**, should permit the X-ray analysis of these complexes to cast light on the process of assembling the polypyrrole chain by PBGD.

Another interesting experimental result showed that the 2-fluoro-11-hydroxy analog of PBG **15** reacted with PBGD in about 1 h, whereas the hydroxymethyl analog of PBG reacts three times slower than PBG itself with PBGD (10 sec).[22] This indicates that the fluorine atom at the 2-position deactivates the pyrrole ring toward displacement of the 11-hydroxy group, i.e. suppresses azafulvene**16** formation (Scheme 3). This retardation of reaction rate may provide enough time to detect, by ^{19}F-NMR, intermediates involved in the formation of the inhibitor-enzyme complexes.



Scheme 3. The reaction of the 2-fluoro-11-hydroxy PBG analog **15** with PBGD

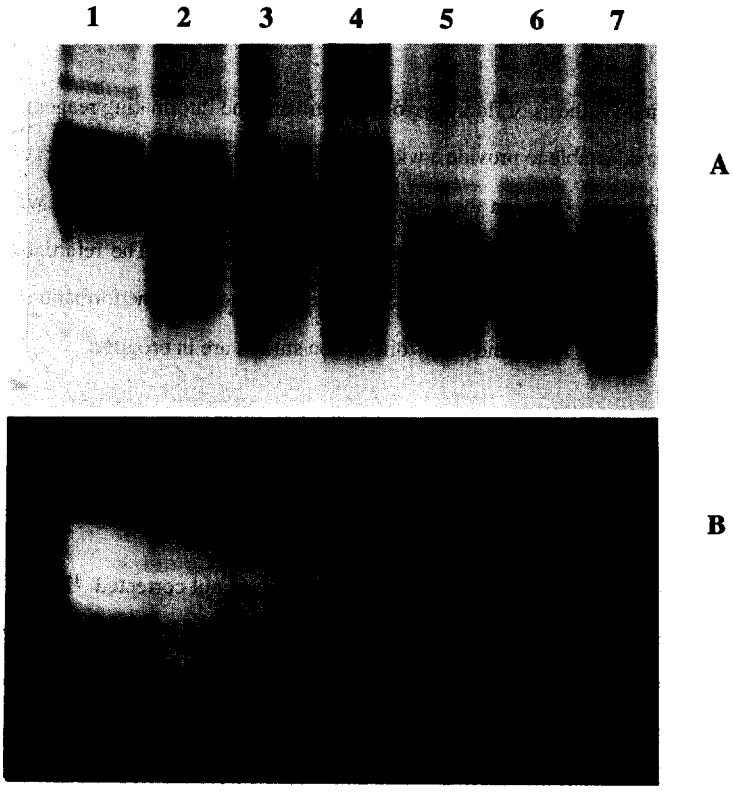


Figure 1. Native PAGE of PBGD incubated with PBG and the 2-fluoro-11-hydroxy PBG analog (**15**). The gel was stained with (A) Coomassie blue or (B) for activity as determined by fluorescence of the uroporphyrin produced.
lane 1: PBGD.
lane 2: PBGD + PBG (1:1).
lane 3: PBGD + PBG (1:2).
lane 4: PBGD + PBG (1:4).
lane 5: PBGD + **15** (1:1).
lane 6: PBGD + **15** (1:2).
lane 7: PBGD + **15** (1:4).

CONCLUSIONS

We report herein the first synthesis of fluoropyrrole **15**, utilising the fluorinating reagent, xenon difluoride, a process which should be adaptable to provide a wide range of novel fluoropyrrole compounds for mechanistic studies. The covalent bonding between the 2-fluoro-11-hydroxy analog of PBG **15** with PBGD will now allow the further X-ray analysis of analog inhibitor-enzyme complexes. The retardation of reaction rate between inhibitor and enzyme PBGD also provides the opportunity to study the formation of inhibitor-enzyme complexes by ^{19}F-NMR. These NMR and X-ray diffraction studies are in progress.

EXPERIMENTAL PART

Chemistry

Instrumentation and general procedures. Melting points (m. p.) are not corrected. ^{1}H and ^{13}C-NMR spectra were recorded with Bruker-WM300MHz spectrometer with tetramethylsilane as an internal standard. ^{19}F-NMR chemical shifts were reported in ppm upfield from trichlorofluoromethane as an internal standard.$CDCl_3$ was used as a solvent for ^{1}H, ^{13}C and ^{19}F-NMR, unless other noted. EI (High res.) and FAB-ms were obtained by VG 70S mass spectrometer. All solvents and reagents were purified and dried before use.

2-Fluoro-5-formyl-3-((methoxycarbonyl)ethyl)-4-((methoxycarbonyl)methyl)pyrrole (12). In a 10-ml two-necked round bottom flask, **10** (170 mg, 0.67 mmol) was dissolved in degassed acetonitrile (2.5 ml) under the nitrogen atmosphere. After the solution was cooled to 0°C, xenon difluoride (110 mg, 0.65 mmol) was added rapidly in one-portion to the above solution. The resulting mixture was stirred and warmed gradually to room temperature. The reaction was monitored for the disappearance of xenon difluoride using KI-starch paper (4-5 h). The reaction mixture was then partitioned between saturated sodium bicarbonate and dichloromethane. The organic phase was dried with anhydrous sodium sulfate and evaporated to remove the solvent. The resulting residue was purified by chromatography on silica gel. Two fractions eluted with ether/dichloromethane (1:1, v/v) were collected. Removal of the solvents gave the first fraction **13**

(34 mg, 20%), m.p. 143-145°C (ether) and second fraction **12** (58 mg, 32 %), m.p. 87-89°C (ether). Compound **12**: ^{1}H-NMR δ 9.47 (br., s, 2H, N-H and CHO), 3.77 (s, 2H, C*H*$_2$COOMe), 3.72, 3.67 (2 s, 2x3 H, 2 COO*Me*), 2.73 (t, ^{3}J = 6.8 Hz, 2H, CH$_2$C*H*$_2$COOMe), 2.57 (t, ^{3}J = 6.8 Hz, 2H, C*H*$_2$CH$_2$COOMe); ^{13}C-NMR δ 176.78 (CHO), 173.04 (COO), 170.69 (COO), 150.67 (d, $J_{C\text{-}F}$ = 274 Hz, C-2), 127.77, 120.93, 103.68 (C-3), 52.48 (OCH3), 51.72 (OCH3), 33.59, 29.64, 17.32. ^{19}F-NMR δ -130.60 (s). HRMS (EI) Calcd for $C_{12}H_{14}FNO_5$ (M^+) 271.0856, Found 271.0851. Compound **13**: ^{1}H-NMR δ 7.48 (br., s, 1H, N-H), 3.71, 3.64 (2 s, 2x3 H, 2 COO*Me*), 3.52 (s, 2H, C*H*$_2$COOMe), 2.70 (t, ^{3}J = 6.9 Hz, 2H, CH$_2$C*H*$_2$COOMe), 2.64 (t, ^{3}J = 6.9 Hz, 2H, C*H*$_2$CH$_2$COOMe); ^{13}C-NMR δ 172.52, 170.63, 170.27, 168.97, 142.90, 135.48, 52.62, 51.94, 31.42, 28.59, 19.53.

3-(2-Carboxyethyl)-4-(carboxymethyl)-2-fluoro-5-formylpyrrole (14). **12** (25 mg, 0.092 mmol) was treated with degassed potassium hydroxide (0.2 M, 1.5 ml). The suspension was stirred under argon in the dark at room temperature for 24 h. The resulting homogeneous alkaline solution was neutralized with hydrochloric acid (2 M) to pH 2. The precipitate was collected by filtration and washed with water (2x 0.5 ml). Removal of the water by evaporation gave the product **14** (14 mg, 63 %) as a white solid, m. p. 170°C dec. For n.m.r. multiple evaporations from D_2O were performed. ^{1}H-NMR (D_20) δ 8.71 (s, 1H, CHO), 3.15 (s, 2H, C*H*$_2$COOMe), 2.09 (t, ^{3}J = 7.4 Hz, 2H, CH$_2$C*H*$_2$COOH), 1.98 (t, ^{3}J = 6.8 Hz, 2H, C*H*$_2$CH$_2$COOH); ^{13}C-NMR (D_2O) δ 184.21 (CHO), 178.94 (COOH), 178.47 (COOH), 151.83 (d, $J_{C\text{-}F}$ = 274 Hz, C-2), 134.20, 127.09, 108.19, 36.68, 32.55, 18.28; ^{19}F-NMR (CD_3OD) δ -133.90 (s). FAB-MS m/e 244 ($(M+H)^+$, 57); 266 ($(M+Na)^+$, 78), 289 ($(M+2Na)^+$, 32), 226 (15); 192 (60), 176 (94), 154 (100), 136 (87), 120 (17), 107 (33).

3-(2-Carboxyethyl)-4-(carboxymethyl)-2-fluoro-5-hydroxy methylpyrrole (15). Sodium borohydride (6 mg, 0.16 mmol) was added portion-wise into a stirred solution of **14** (3 mg, 0.012mmol) in deuteriated methanol (0.5 ml) at 0°C. After the addition was completed, the solution was continued to stir for 0.5 h and then allowed to warm up to room temperature. The resulting solution was transferred to an n.m.r. tube and the structure of **15** was determined by ^{1}H-NMR and ^{19}F-NMR spectra. ^{1}H-

NMR (CD_3OD) δ 4.01 (s, 2H, CH_2OH), 3.17 (s, 2H, CH_2COOH), 2.18 (t, ^3J = 7.6 Hz, 2H, CH_2CH_2COOH), 1.96 (t, ^3J = 7.6 Hz, 2H, CH_2CH_2COOH); ^{19}F-NMR (CD_3OD) δ -148.49 (s).

2-Chloro-5-formyl-3-((methoxycarbonyl)ethyl)-4-((methoxycarbonyl)methyl) pyrrole (11). Using the procedure described in the synthesis of **12** except using dichloromethane instead of CH3CNas solvent, **11** was obtained in 24% yield as a white solid, m. p. 104-106°C (ether). ^{1}H-NMR δ 9.87 (br. s, 1H, N-H), 9.44 (s, 1H, CHO), 3.73 (s, 2H, CH_2COOMe), 3.67, 3.60 (2 s, 2x3 H, 2 COO*Me*), 2.71 (t, ^3J = 7.6 Hz, 2H, CH_2CH_2COOMe), 2.49 (t, ^3J = 7.6 Hz, 2H, CH_2CH_2COOMe); ^{13}C-NMR δ 176.84 (CHO), 173.14 (COO), 170.86 (COO), 128.34, 126.83, 123.84, 120.97, 52.53, 51.78, 33.47, 29.67, 19.55; GC-MS m/e 287 (M^+, 78), 255 (70), 228 (80), 192 (100), 168 (97), 156 (100), 140 (45), 104 (33).

Enzymology

PBGD Purification

The enzyme was purified from overproducing *E. coli* as previously described,[23] except for final desalting on a PD-10 column (G-25 M sephadex), followed by lyophylization from water and storage at -20°C.

Enzymatic Reactions

Reaction of PBG and the 2-fluoropyrrole **15** with PBGD were carried out in PE buffer at pH 8.0 with an enzyme concentration of 2 mg/ml. Incubations were carried out at 37°C for 1 h.

PBGD Assay

PBGD activity *in situ* in native polyacrylamide gels was determined by incubation the gel for 20 min. at 37°C in PE buffer, containing 0.2 mg/ml PBG followed by oxidation in 0.01% iodine in 1 N HCl and observation of the resulting fluorescent uroporphyrins on an ultraviolet transilluminator at 300 nm.

Analysis of Reactions

Analysis of complexes was carried out by non-denaturing PAGE. The stacking gel was 3% acrylamide, Tris hydrochloride, pH 6.8, with a separating gel of 7.5% acrylamide, Tris hydrochloride, pH 8.8; electrophoresis was at 180 V and 4°C. The gel were stained with a solution of 50% trichloroacetic acid containing 0.12% Coomassie brilliant blue R250 and destained with 7% acetic acid.

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