Biosynthesis of Porphyrins and Related Macrocycles. Part 17.[1,2] Chemical and Enzymic Transformation of Isomeric Aminomethylbilanes into Uroporphyrinogens: Proof that Unrearranged Bilane is the Preferred Enzymic Substrate and Detection of a Transient Intermediate

By **Alan R. Battersby,* Christopher J. R. Fookes, Kerstin E. Gustafson-Potter, Edward McDonald,** and **George W. J. Matcham,** University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW

Six isomeric aminomethylbilanes have been built by unambiguous synthesis. One bilane has the unrearranged structure corresponding to straightforward head-to-tail assembly of four units of porphobilinogen; the other five bilanes have one or more of the pyrrole rings reversed relative to the unrearranged bilane. The action of deaminase-cosynthetase on these bilanes has been studied and the results showed that (*a*) the enzyme system ring closes the unrearranged bilane far more efficiently than it closes any of the five isomeric bilanes, (*b*) under the best conditions the product formed enzymically from the unrearranged bilane is almost pure uroporphyrinogen-III, and (*c*) a transient intermediate is formed enzymically from the unrearranged bilane which can be detected kinetically. Further support is thereby given to the earlier conclusion that the intramolecular rearrangement which produces the natural type-III porphyrins occurs at the unrearranged tetrapyrrole level.

The enzymic results for the other bilanes are also described together with an improved method for analytical separation of the four isomeric esters of coproporphyrin.

THE specific incorporation of the unrearranged bilane (1) *intact* into uroporphyrinogen-III (7) [uro'gen-III] by the enzymes deaminase and cosynthetase from *Euglena gracilis* proved [1,3] that the single, intramolecular rearrangement established [4] for biosynthesis of the natural type-III porphyrins *occurs at the tetrapyrrole level.* Since this conclusion was quite different from that reached by others [5,6] who believed initial rearrangement to pyrromethane (10) occurred, it was important to learn whether the enzymes have a strong preference for bilane (1) and also how they would affect other isomeric bilanes. The set chosen comprised the five rearranged bilanes (2)—(6). These differ from the unrearranged bilane (1) by having one ring reversed [see (2)—(5)] or more than one for bilane (6). All five have acceptable structures in that schemes can be drawn for their formation from porphobilinogen (9), PBG, which satisfy the rigorous labelling requirements defined [4] by experiments with [2,11-$^{13}C_2$]PBG [*cf.* (9)].

Synthesis of the bilanes (2)—(6) followed the same strategy (Scheme 1) used previously [1] for construction of the unrearranged bilane (1). This involved condensation of the methyl ester of either PBG lactam (11) or of iso-PBG lactam [7] (12) with the formylpyrromethanes (13)—(16) to give the tripyrrenes (17)—(21). The best procedure was to treat a solution of the reactants with 2 equiv. of hydrogen chloride at room temperature and after evaporation of the solvents, the product crystallised in high yield. The five tripyrrenes (17)—(21) were then decarboxylated and condensed with the aldehydes (22) or (23) [8] and the resulting biladienes (24)—(28) were reduced with borohydride to yield the bilane lactam esters (29) [1]—(34) which were fully characterised (see *e.g.* Figure 1). These products were then ready for alkaline hydrolysis to produce the bilanes (2)—(6).

The pyrromethanes (14—(16) were synthesised by similar routes (see Scheme 2) which will be exemplified for compound (16). Treatment of the acid (35) [8] with isobutene and sulphuric acid gave the t-butyl ester (36) from which the acid (37) was derived by hydrogenolysis of the benzyl group. This was converted into the iodopyrrole (38) by the two-phase method.[9] Hydrogenation of compound (38) gave the α-free pyrrole (39) which reacted with the acetoxymethylpyrrole (40) under acid catalysis to furnish the pyrromethane (41) which has also been prepared independently.[10] The corresponding crystalline acid (42), obtained by hydrogenolysis, was decarboxylated, using toluene-*p*-sulphonic acid, and formylated with benzoyl chloride–dimethylformamide [11] to yield the aldehyde (16).

The routes to the other pyrromethanes are shown in Scheme 3. Several of the required building blocks were derived from the important pyrrole (43) which was made by the thallium rearrangement route of Kenner *et al.*[12] This method, with minor modifications (see Experimental section) allowed the pyrrole (43) to be made from diethyl 3-oxohexanoate with an overall yield close to 60%.

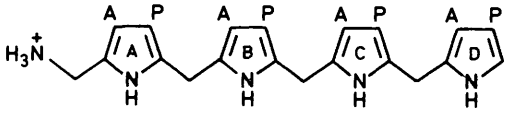
After the brief account of the work covered in this paper,[2] the synthesis of bilanes (1) and (6) has recently been reported [13] but no biological properties are described.

Samples of the unrearranged bilane lactam ester [1] (29) and of the five isomers (30)—(34) were hydrolysed with alkali and the resulting aminomethylbilanes (1)—(6) were incubated at 37 °C and pH 7.75 with deaminase-cosynthetase from *Euglena gracilis;* parallel chemical incubations (*i.e.* without enzyme) were also carried out. The methods used to obtain a partially purified preparation of deaminase-cosynthetase for these experiments are described in the Experimental section.†

Uro'gen formation in each of the foregoing solutions was monitored by treating aliquots first with iodine, then with acid and measuring absorption at the Soret band of

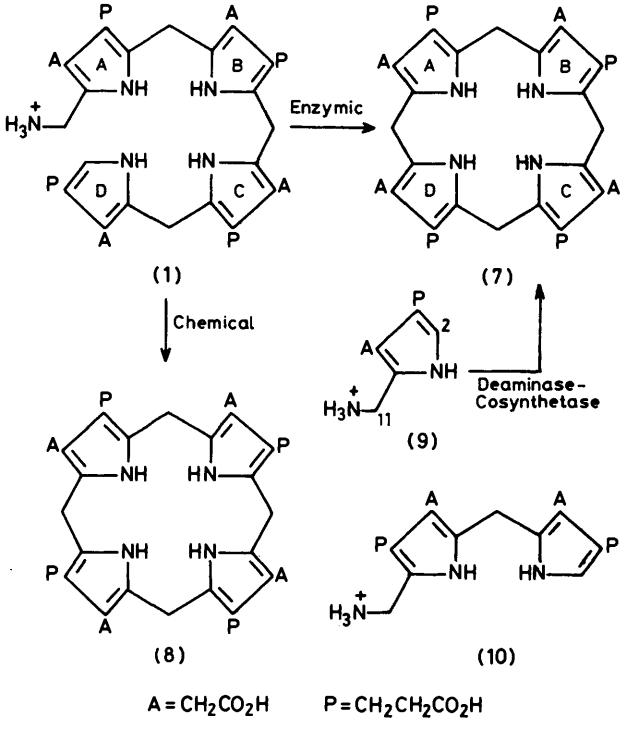
† Similar preparations of deaminase-cosynthetase were also used in Part 16 [1] and Part 15 [14] of this Series.

the uroporphyrin dication. Any remaining bilanes did not interfere with the assay since they were destroyed by the iodine treatment.

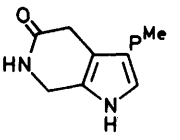


(1)

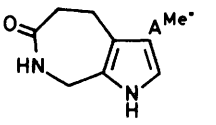
P A	A P	A P	A P	(2)
A P	P A	A P	A P	(3)
A P	A P	P A	A P	(4)
A P	A P	A P	P A	(5)
P A	P A	P A	A P	(6)



The results (see Figure 2, i—vi), showed that the ring-A reversed bilane (2) and the one with rings A, B, and C reversed (6) were not significant substrates for the enzyme system. In contrast it was found that the deaminase-cosynthetase enzyme system increased the rate of ring-closure of the unrearranged bilane (1) and far more effectively than for any of the remaining isomeric bilanes (3), (4), and (5). Indeed, for the ring-C reversed bilane (4), the enzymic acceleration of ring-closure was only just detectable above the non-enzymic rate of chemical cyclisation (Figure 2, iv). It is significant that the final yield of uro'gen from the enzymic cyclisation of the unrearranged bilane (1) was *ca.* double that for all the other incubations. This is because the rapid enzyme-promoted cyclisation competes very favourably with

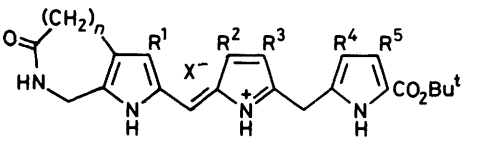


(11)

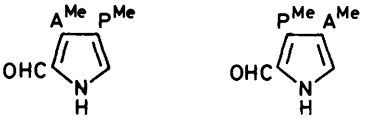


(12)

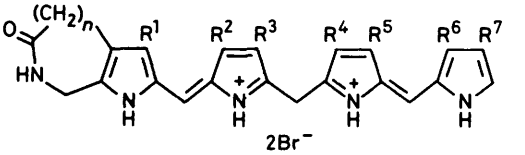
	R^1	R^2	R^3	R^4	
	A^{Me}	P^{Me}	A^{Me}	P^{Me}	(13)
	P^{Me}	A^{Me}	A^{Me}	P^{Me}	(14)
	A^{Me}	P^{Me}	P^{Me}	A^{Me}	(15)
	P^{Me}	A^{Me}	P^{Me}	A^{Me}	(16)



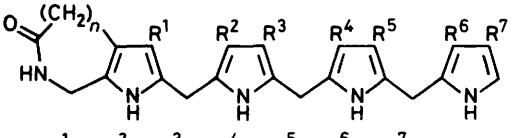
	n	R^1	R^2	R^3	R^4	R^5	X	
(12)+(13) →	2	A^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	Cl	(17)
(11)+(14) →	1	P^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	Cl	(18)
(11)+(15) →	1	P^{Me}	A^{Me}	P^{Me}	P^{Me}	A^{Me}	Cl	(19)
(12)+(16) →	2	A^{Me}	P^{Me}	A^{Me}	P^{Me}	A^{Me}	Cl	(20)
(11)+(13) →	1	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	Br	(21)



(22) (23)



	n	R^1	R^2	R^3	R^4	R^5	R^6	R^7	
(17)+(22) →	2	A^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	(24)
(18)+(22) →	1	P^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	(25)
(19)+(22) →	1	P^{Me}	A^{Me}	P^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	(26)
(21)+(23) →	1	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	P^{Me}	A^{Me}	(27)
(20)+(22) →	2	A^{Me}	P^{Me}	A^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	(28)



	n	R^1	R^2	R^3	R^4	R^5	R^6	R^7	
(29)	1	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	→ (1)
(24) → (30)	2	A^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	→ (2)
(25) → (31)	1	P^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	→ (3)
(26) → (32)	1	P^{Me}	A^{Me}	P^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	→ (4)
(27) → (33)	1	P^{Me}	A^{Me}	P^{Me}	A^{Me}	P^{Me}	P^{Me}	A^{Me}	→ (5)
(28) → (34)	2	A^{Me}	P^{Me}	A^{Me}	P^{Me}	A^{Me}	A^{Me}	P^{Me}	→ (6)

SCHEME 1 $A^{Me} = CH_2CO_2Me$ $P^{Me} = CH_2CH_2CO_2Me$

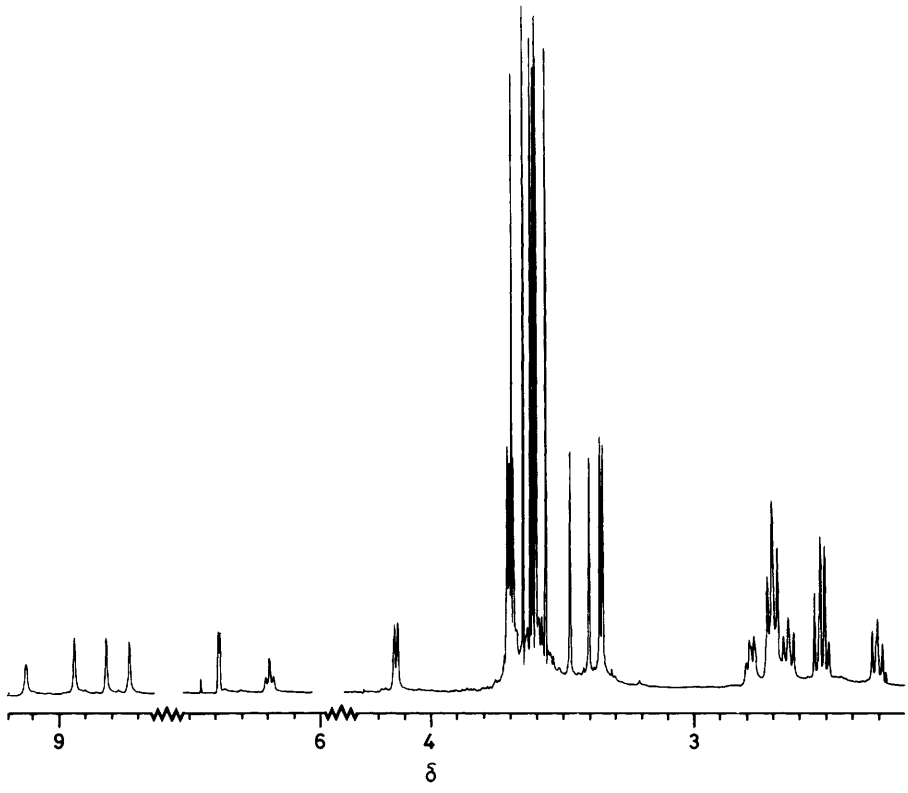
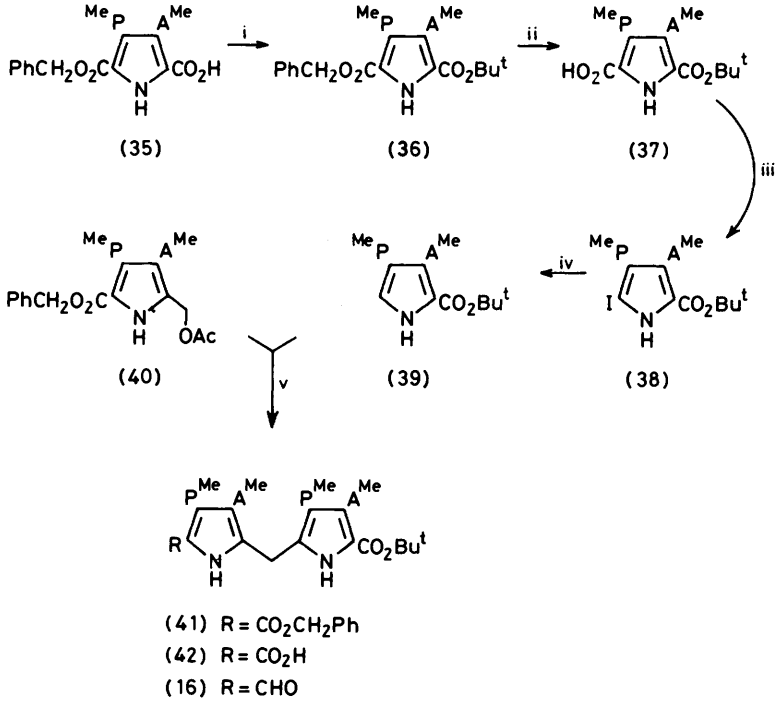


FIGURE 1 ^{1}H N.m.r. spectrum at 400 MHz of the bilane lactam ester (34) [rings-ABC reversed]; SW 4 000 Hz, 40° pulse, acquisition time 2.048 s, number of transients 1 240

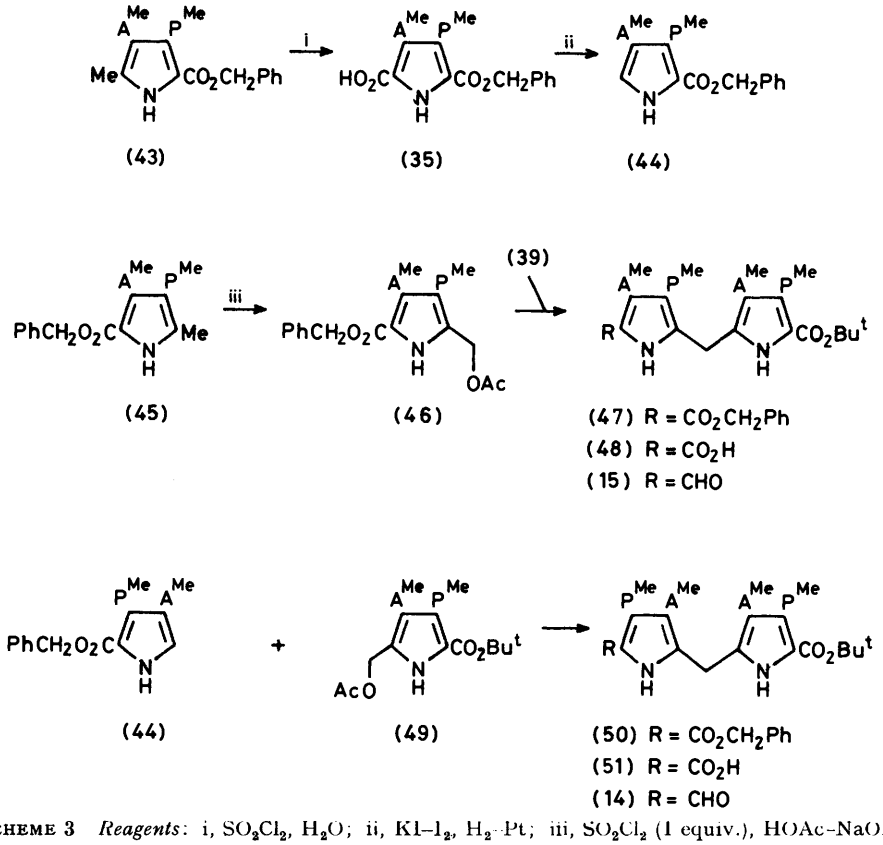


SCHEME 2 *Reagents*: i, H^+, isobutene; ii, H_2, Pd; iii, KI, I_2; iv, H_2, Pt; v, *p*-$MeC_6H_4SO_3H$, CH_2Cl_2

chemical breakdown; related chemical fragmentation had been observed previously.[1]

Sets of kinetic runs were carried out on the unrearranged bilane (1) and on the isomers (3) and (5) in the presence of deaminase and cosynthetase. These allowed determination of $V_{max.}$ and K_m in the standard way using double reciprocal plots (see Table 1).

The foregoing results not only show that the unrearranged bilane (1) is the best substrate in the set but they also provide further evidence against the claims [5,6] uroporphyrins were decarboxylated to yield coproporphyrins which were isolated as their tetramethyl esters. These can be analysed by high-pressure liquid chromatography [1] (h.p.l.c.) but an improved method was needed for the complete isomer analyses required in the present work. A significant development was the discovery that the zinc complexes of the four isomeric coproporphyrin esters could be cleanly separated by h.p.l.c. in a single pass by the appropriate choice of column and solvent (see Figure 3). The elution was in numerical order.



SCHEME 3 *Reagents*: i, SO_2Cl_2, H_2O; ii, KI–I_2, H_2–Pt; iii, SO_2Cl_2 (1 equiv.), HOAc–NaOAc

that rearrangement occurs at the beginning of the building process to form the head-to-head pyrromethane (10) as illustrated in Scheme 4. If this were true, bilanes (2) and (4) would be the products from straightforward attachment of two PBG units at one end or the other of pyrromethane (10). Also, a possible mechanism can be written [5] leading from (10) to bilane (6). Yet these three bilanes were not appreciable substrates for deaminase-cosynthetase.

Comment is needed here on the recent reported failure to observe enzymic conversion of the unrearranged bilane (1) into uro'gen.[10] This report is understandable since the authors used *ca.* 800 times less enzyme [10] (relative to bilane) than was used in the present work; the enzymic rate enhancement would under such conditions be lost in the experimental error.

The uro'gens produced in each of the foregoing incubations were aromatised with iodine and the resulting

The results obtained from isomer analysis by this new method are collected in Table 1. As expected,[1] there had been an essentially complete switch from chemical formation of uro'gen-I (8) to enzymic production of uro'gen-III (7) from the unrearranged bilane (1). For the ring-D reversed bilane (5), the terminal ring-D had also been enzymically inverted to a significant extent by deaminase-cosynthetase, to produce uro'gen-I (8). However, the ring-B reversed bilane (3), whose rate of ring-closure was appreciably increased by the enzyme system (Figure 2, iii) underwent little rearrangement.

A further study in this series involved treatment of the unrearranged bilane (1) with a much larger amount of enzyme than had been used above. The results in Figure 4 are self-explanatory and they emphasise the efficiency of the enzyme-catalysed ring-D-reversal to form uro'gen-III (7).

Lastly, it was demonstrated (*a*) that there is a linear

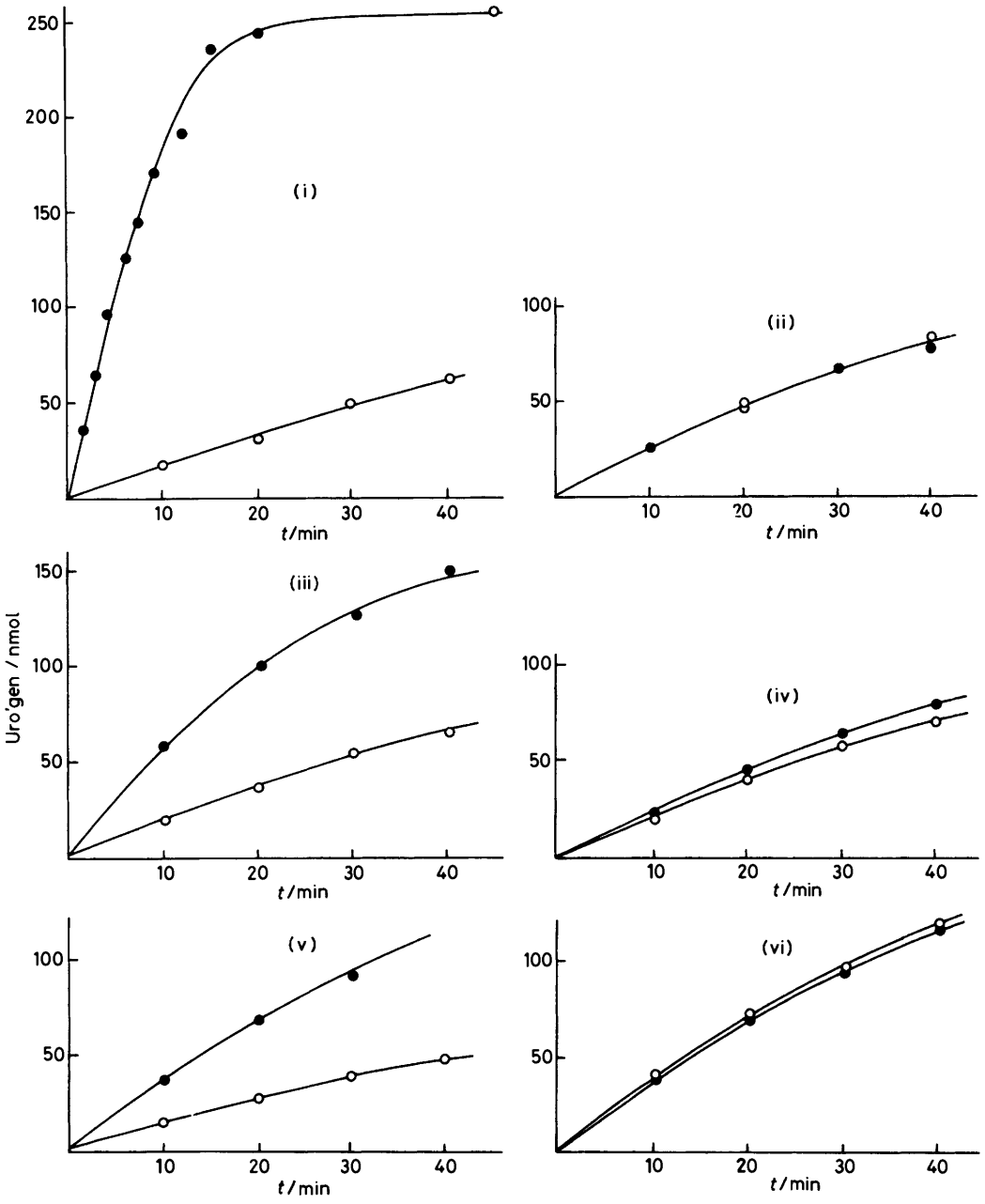


FIGURE 2 Experiments on rates of conversion of the isomeric aminomethylbilanes (1)—(6) [536 nmol in each run] into uro'gen macrocycles at pH 7.75, enzymically (—●—) or chemically (—○—). Unrearranged bilane (1); 27 500 units of deaminase-cosynthetase used. Ratio of initial rates, Enzymic : Chemical = 13.6. (ii) Ring-A reversed bilane (2); 22 200 units of deaminase-cosynthetase used. (iii) Ring-B reversed bilane (3); 59 500 units of deaminase-cosynthetase used. Ratio of initial rates, Enzymic : Chemical = 3.3. (iv) Ring-C reversed bilane (4); 22 200 units deaminase-cosynthetase used. Ratio of initial rates, Enzymic : Chemical = 1.1. (v) Ring-D reversed bilane (5); 22 200 units of deaminase-cosynthetase used. Ratio of initial rates, Enzymic : Chemical = 2.9. (vi) Rings-ABC reversed bilane (6); 22 200 units deaminase-cosynthetase used

relationship between the rate of uro'gen-III formation from bilane (1) and the amount of deaminase-cosynthetase enzyme system (Figure 5) and (*b*) that increasing the quantity of unrearranged bilane (1) used relative to enzyme produces for uro'gen-III (7) formation a typical Michaelis-Menten plot (Figure 6). As the bilane concentration was increased in Figure 6, correction for the chemically formed uro'gen-I (8) became increasingly important (see Experimental section).

At this stage, the following conclusions can be drawn: (*a*) the results from bilanes (4) and (5) show that inversion of the terminal ring of a bilane is carried out by the enzyme system even on an unnatural substrate; there were indications (Table 1) that slight enzymic reversal

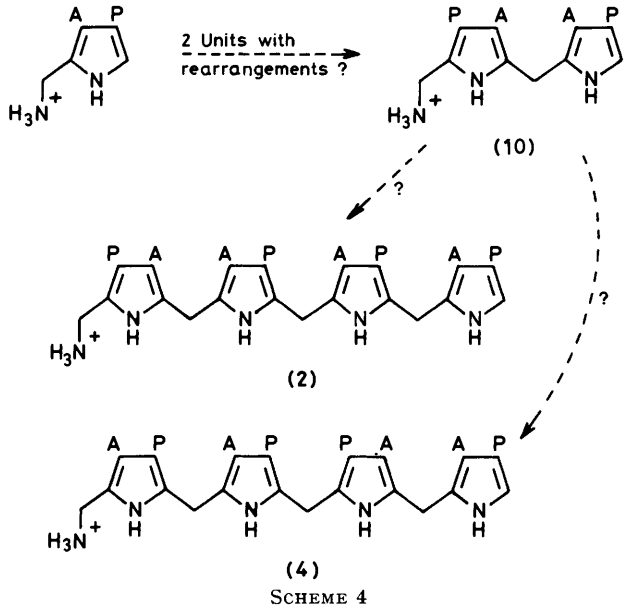
TABLE 1

Action of deaminase-cosynthetase from *Euglena gracilis* on isomeric bilanes

Substrate	$V_{max.}$[a]	K_m/μM	Uro'gen isomers produced (%)[b] Enzymic I	II	III	IV	Chemical I	II	III	IV
Unrearranged bilane (1)	64 ± 1	5.1 ± 0.2	8	0	90	2	95	0	4	1
Ring-A reversed (2)	—	—	3.5	0	91.5	5	3.5	0	94.5	2
Ring-B reversed (3)	25 ± 1	72 ± 3	0	8.5	90	1.5	0	5.5	93	1.5
Ring-C reversed (4)	—	—	0	2.5	90	7.5	0	2	94.5	3.5
Ring-D reversed (5)	7.7 ± 0.2	11 ± 1	22	0	76	2	9.5	0	88	2.5
Rings-ABC reversed (6)	—	—	2.5	0	94.5	3	2.5	0	95.5	2
PBG (9)	1 000	104 ± 7								

[a] $V_{max.}$ Is arbitrarily set at 1 000 for PBG and the rest are referred to this. [b] The experimental results have been rounded to nearest 0.5%.

of the α-free terminal ring may also occur for isomer (4); (*b*) the unrearranged bilane (1) is the best substrate for deaminase-cosynthetase in the tested set of isomeric bilanes (1)—(6). Not only did it bind best to the enzyme (lowest K_m) but it was also transformed into uro'gen-III (7) at the fastest rate. The earlier conclusion [1] that the biosynthesis of uro'gen-III (7) involves construction of an unrearranged bilane *followed by* intramolecular reversal of ring-D during cyclisation is thus strengthened; (*c*) conclusion (*b*) is rigorous but, nevertheless, the aminomethylbilane (1) is enzymically converted into uro'gen-III (7) less rapidly than is PBG (9). Therefore bilane (1) cannot be, in this exact form, a true intermediate on the biosynthetic pathway from PBG (9) to uro'gen-III (7).



SCHEME 4

All the foregoing results and conclusions, together with those derived from work on pyrromethanes,[14] are consistent with the view that the unrearranged aminomethylbilane (1) can be accepted by the enzyme and that it reacts to generate the intermediate (52) which is normally generated on the enzyme from four moles of PBG (9) (Scheme 5). Pointers as to what happens after formation of the intermediate (52) came from experiments with deaminase which had been considerably purified [15] and, in particular, was demonstrated to be free of cosynthetase.

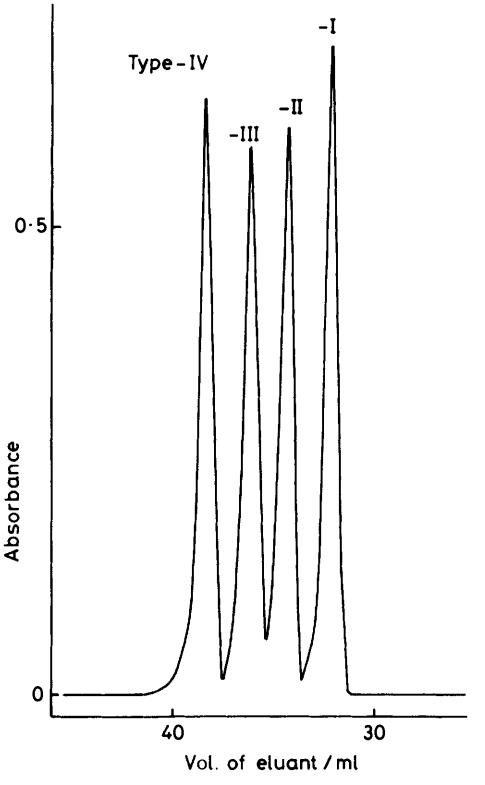


FIGURE 3 Separation of the Zn^{II} complexes of isomeric coproporphyrin tetramethyl esters on two 10μ Porasil columns in series. Eluant: dichloromethane, hexane, di-isopropylethylamine (80 : 19.7 : 0.3), flow rate 0.6 ml/min, u.v. detector at 400 nm

All the isomeric bilanes (1)—(6) were tested as substrates for this isolated deaminase. Roughly one quarter of the amount of enzyme was used for these experiments compared with the amounts used in the runs of Figures 2. Under these conditions, only the unrearranged bilane (1) and the ring-B reversed bilane (3) showed considerable enzymic acceleration of their ring-closures. The results for these two bilanes are collected in Table 2 and it should be noted that deaminase does not signifi-

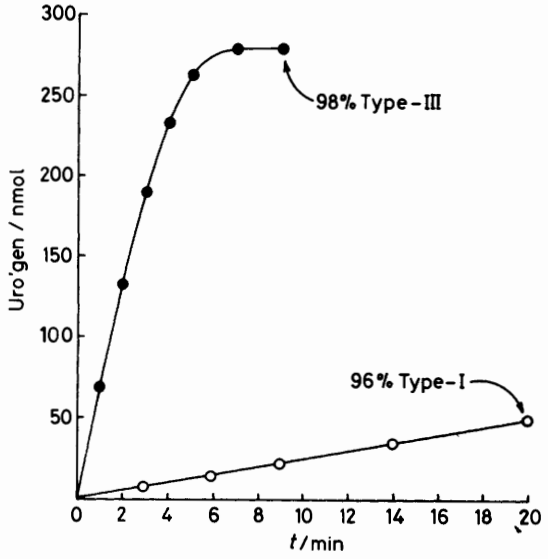


FIGURE 4 Enzymic (—●—) and chemical (—○—) conversion of the unrearranged bilane (1) (630 nmol) into uro'gen macrocycles at pH 7.75; 70 000 units of deaminase-cosynthetase used in total volume of 30 ml

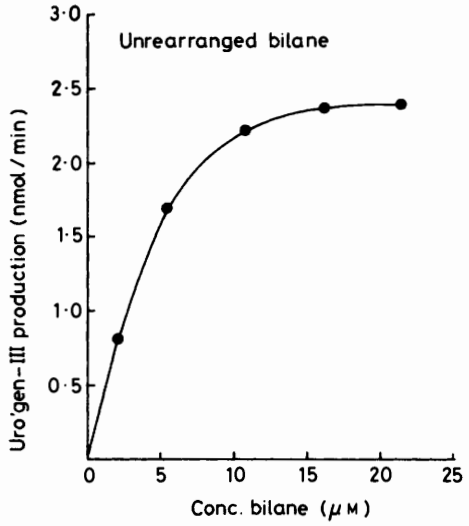


FIGURE 6 Relationship between initial rate of conversion by deaminase-cosynthetase of the unrearranged bilane (1) into uro'gen-III (7) and the concentration of bilane used

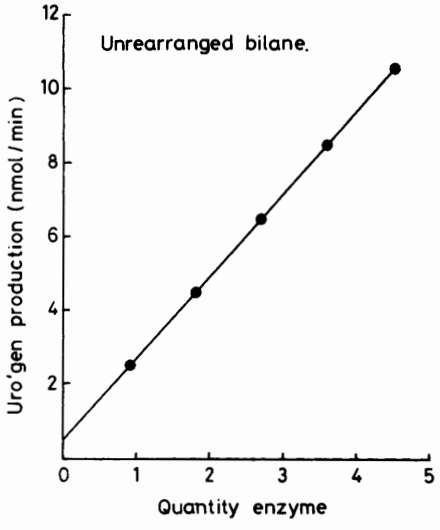
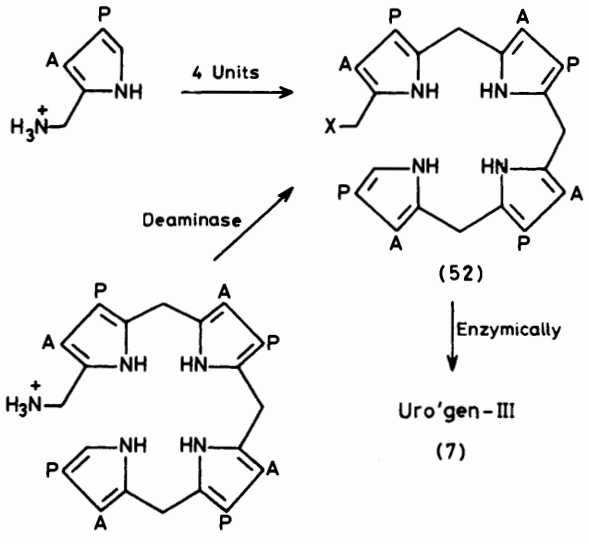


FIGURE 5 Linear relationship between initial rate of enzymic conversion of the unrearranged bilane (1) into uro'gen macrocycles and the amount of deaminase-cosynthetase used



SCHEME 5

TABLE 2

Action of deaminase from *Euglena gracilis* on isomeric bilanes

			Uro'gen isomers produced (%) [b]							
			Enzymic				Chemical			
Substrate	$V_{max.}$ [a]	K_m (μM)	I	II	III	IV	I	II	III	IV
Unrearranged bilane (1)	98 ± 19	14 ± 6	96	0	3	1	95	0	4	1
Ring-B reversed (3)	55 ± 16	24 ± 6	1	3	96		0	5.5	94.5	
PBG (9)	1 000	—	100	0	0	0	—	—	—	—

[a] $V_{max.}$ Is arbitrarily set at 1 000 for PBG and the rest are referred to this. [b] The experimental results have been rounded to the nearest 0.5%.

cantly change the isomeric composition of the uro'gens formed from that produced in the parallel chemical (non-enzymic) ring-closures. Cyclisation of the ring-D reversed bilane (5) was also somewhat accelerated by deaminase but with the smaller quantity of enzyme used, the rate increase was small.

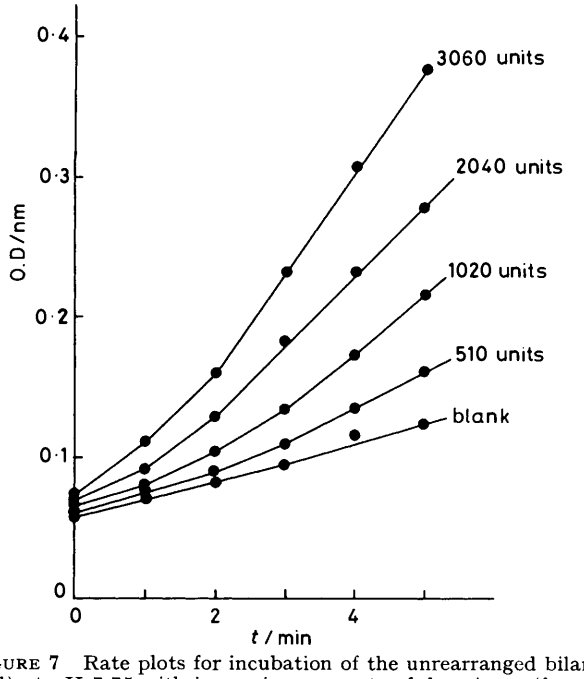


FIGURE 7 Rate plots for incubation of the unrearranged bilane (1) at pH 7.75 with increasing amounts of deaminase (free of cosynthetase); optical density at 406 nm is a measure of uro'gen-I (8) formed (see Experimental section)

There was a further important observation in the experiments with the unrearranged bilane (1). When deaminase-cosynthetase act on the unrearranged bilane (1), the enzymic acceleration of ring-closure to uro'gen-III (7) shows no lag phase (Figures 2i and 4). In contrast, when the same bilane (1) was incubated with deaminase alone, there was again enzymic acceleration of ring closure but there was a marked lag before formation of uro'gen-I (8) reached its maximum rate. The consistent clear lag for a set of runs with bilane (1) as substrate in which increasing quantities of deaminase alone were used can be seen in Figure 7.

One possible explanation for this observation was that slow displacement of ammonium ions (used in isolating the deaminase) might, perhaps, be occurring from the enzyme active site. However, the same lag was reproduced after extensive dialysis of the deaminase. It thus seemed likely that an intermediate was being released from the deaminase. A full study of the characterisation, structure, and synthesis of this intermediate is the subject of the following paper.

EXPERIMENTAL

General Directions.—Except where stated otherwise, the following procedures were adopted.

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Electronic spectra were measured with either a Pye-Unicam SP8-100 or SP800 spectrophotometer and refer to solutions in methanol. Infrared spectra are of Nujol mulls and were determined on Perkin-Elmer 257 or 297 instruments. ^{1}H N.m.r. spectra at 400 MHz were run on a Bruker WH-400 spectrometer. Solutions were normally in $CDCl_3$ and chemical shifts are quoted on the δ scale relative to tetramethylsilane (TMS) as $\delta = 0$. Mass spectra were obtained on an AEI-Kratos MS30 or MS50 machine.

Organic solutions which had been in contact with water were dried over anhydrous sodium sulphate prior to evaporation, which was normally carried out at *ca.* 30 Torr in a Büchi rotary film evaporator. Analytical thin layer chromatography (t.l.c.) was performed on Merck plates layered with Kieselgel GF_{254} (0.25 mm). Column chromatography on silica was carried out on short, fat columns (height ≒ diameter) of Merck Kieselgel H60 material. Alumina was Woelm grade III neutral. Diethylaminoethyl cellulose (DEAE-cellulose) was Whatman DE52. All solvents were redistilled; chloroform and dichloromethane were kept in the dark after distillation from anhydrous potassium carbonate. The light petroleum fraction b.p. 40—60 °C is referred to as petroleum. Reagents used in contact with porphyrins were of AnalaR quality and normal precautions to exclude light were taken.

Mixtures of isomeric coproporphyrin tetramethyl esters were analysed by high-pressure liquid chromatography (h.p.l.c.) on a Waters 6000 instrument fitted with a Cecil CE 272 variable-wavelength spectrophotometer operating at 400 nm. All four isomers were separated as their zinc complexes on two Waters μPorasil columns using dichloromethane, hexane, di-isopropylethylamine (80 : 19.7 : 0.3) as eluant, at 0.6 ml/min. Of the several commercial columns tested, only μPorasil columns were suitable. The isomers were eluted in the order I, II, III, and IV (Figure 3).

In the kinetic experiments, uroporphyrinogen was assayed by treating suitable aliquots (p μl) with water (750 − p μl) plus aqueous 0.5% iodine (0.4 ml) containing 1% potassium iodide. Two min later, excess of oxidant was destroyed with aqueous sodium hydrogen sulphite * (0.1 ml), and 7% trichloroacetic acid (1.75 ml) was added. The u.v. spectrum of the sample was scanned between 450 and 350 nm and an artificial baseline was drawn between the readings at 425 and 375 nm. Uro'gen was then calculated using $\sum_{MM} = 528$ for the uroporphyrin dication at 406 nm.[16]

Enzymes were assayed as earlier; [15] one unit of enzyme activity is defined as producing 1 mol of uro'gen per hour from PBG.

Synthesis of Pyrroles

Benzyl 4-Acetyl-3-(2-ethoxycarbonylethyl)-5-methylpyrrole-2-carboxylate.—A mixture of diethyl 3-oxohexanedioate [9] (216 g) and benzyl alcohol (270 g, 2.5 equiv.) was stirred and heated (air condenser) in an oil bath at 150—160 °C. A slow stream of nitrogen was passed over the liquid so that ethanol vapours were removed but less volatile components condensed and were returned to the mixture. After 3 h, excess of benzyl alcohol was distilled off at 0.5 Torr (bath temp. up to 160 °C) to leave 1-benzyl 6-ethyl 3-oxohexane-

* This solution was freshly prepared from sodium metabisulphite; 0.1 ml contained just sufficient reagent to decolourise 0.4 ml of the iodine solution.

dioate (271 g, 97.5%) as a colourless oil, used directly in the next stage; δ 1.20 (3 H, t, *J* 7 Hz, OCH_2CH_3), 2.40—3.00 (4 H, m, CH_2CH_2), 3.48 (2 H, s, $COCH_2CO_2$), 4.03 (2 H, q, *J* 7 Hz, OCH_2CH_3), 5.09 (2 H, s, CH_2Ph), and 7.28 (5 H, s, C_6H_5).

Sodium nitrite (70 g) in water (125 ml) was added during 30 min to a stirred solution of the foregoing diester (271 g) in acetic acid (400 ml) at 15—20 °C. Next day, this solution and a mixture of zinc dust (200 g) and ammonium acetate (200 g) were added simultaneously to a vigorously stirred solution of pentane-2,4-dione (115 g) in acetic acid (400 ml) at 90—100 °C. After 0.5 h at this temperature, the mixture was diluted with ice and water (to 3 l) and the product collected 2 h later. The solid was dissolved in dichloromethane (1 l), the filtered solution washed with water (1 l), evaporated, and without delay, the residual oil was dissolved in warm ether (400 ml). Next day, the title pyrrole (214.5 g, 60.1%) was collected as plates, m.p. 98—99 °C/104.5—105 °C (lit.,[13] m.p. 90—91 °C); δ 1.21 (3 H, t, *J* 7 Hz, OCH_2CH_3), 2.44 (3 H, s, $COCH_3$), 2.50 (3 H, s, pyrrole-CH_3), 2.51 (2 H, m, $CH_2CH_2CO_2$), 3.38 (2 H, m, $CH_2CH_2CO_2$), 4.08 (2 H, q, *J* 7 Hz, OCH_2CH_3), 5.31 (2 H, s, CH_2Ph), 7.36 (5 H, s, C_6H_5), and 9.05 (1 H, br, NH).

Benzyl 3-(Methoxycarbonylethyl)-4-methoxycarbonylmethyl-5-methylpyrrole-2-carboxylate (**43**).—Thallium(III) nitrate trihydrate (*ca.* 95% pure, 184 g) in methanol (500 ml) containing 17M-nitric acid (15 ml) was added to a solution of the foregoing acetylpyrrole (140 g) in methanol (1.5 l) and, 30 h later, trimethyl orthoformate (140 ml) was added. Next day, the thallium(I) nitrate (102.5 g, 98.3%) was filtered off and washed with methanol, and the filtrate partitioned between water (1.5 l) and dichloromethane (500 ml and 3 × 200 ml). The extracts were washed with water (500 ml), chromatographed on alumina (50 × 50 mm) and elution continued with ether (250 ml). Recrystallisation of the product from ether–hexane gave the title pyrrole (114.8 g) as colourless needles, m.p. 79—80 °C (lit.,[12] 78—79, and 80—81.5 °C). The yield was increased to 133.5 g (91.5%) by recrystallising the material in the mother-liquor from methanol–water.

5-Benzyloxycarbonyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylic Acid (**35**).—Freshly distilled sulphuryl chloride (42.5 g, 3.15 equiv.) was added during 1 min to a stirred solution of the foregoing pyrrole (**43**) (37.3 g) in dichloromethane (350 ml), and 2 h later, the mixture was added during 10 min to acetone–water (2 : 1; 1 500 ml), boiling gently in an open flask. The solution was cooled 15 min later, the acetone evaporated under reduced pressure, and the product extracted into dichloromethane (500 ml and 3 × 200 ml). The extracts were evaporated, the residue was dissolved in ether (750 ml), and the carboxylic acid extracted into aqueous sodium carbonate (10%; 4 × 250 ml). After the combined aqueous solution had been washed with ether (500 ml), it was acidified with 10M-hydrochloric acid and the product isolated by extraction with dichloromethane (500 ml and 3 × 250 ml). The residue left after evaporation of the washed (brine, 300 ml) extracts was recrystallised from methanol–water to give the title pyrrole (**35**) (32.3 g, 80.1%) as colourless prisms, m.p. 127.5—129 °C (lit.,[8] m.p. 124—125 °C).

5-t-Butoxycarbonyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylic Acid (**37**).—Liquid isobutene (100 ml) was added at 0 °C to a suspension of the foregoing pyrrolecarboxylic acid (**35**) (37.6 g) in dry, ethanol-free chloroform (240 ml). Sulphuric acid (18M; 1.5 ml) was then added dropwise and the mixture stirred at room temperature, in a sealed flask, for 40 h. The solution was cooled on ice and sufficient aqueous sodium hydrogen carbonate to make the mixture alkaline was added. Isobutene was allowed to evaporate at *ca.* 30 °C and the organic phase washed with water, and passed through an alumina column (50 × 25 mm). Elution was continued with ether (300 ml) and appropriate fractions evaporated to give *benzyl 5-t-butoxycarbonyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate* (**36**) (44.0 g) as a colourless oil, $\nu_{max.}$ (film) 3 475, 3 295br, 1 740, and 1 705 cm^{-1}; $\lambda_{max.}$ 281 nm; δ 1.53 [9 H, s, $C(CH_3)_3$], 2.52 (2 H, m, $CH_2CH_2CO_2$), 3.02 (2 H, m, $CH_2CH_2CO_2$), 3.58 and 3.65 (each 3 H, s, 2 × OCH_3), 3.83 (2 H, s, CH_2CO_2), 5.32 (2 H, s, CH_2Ph), 7.36 (5 H, s, C_6H_5), 9.93 (1 H, br, NH); *m/e* 459 (7%, M^+), 403 (23, $M^+ - C_4H_8$), 371 (37), 280 (100), and 252 (76).

All the foregoing benzyl ester (**36**) in methanol (200 ml) was hydrogenated at 35 °C over palladium-charcoal (10%; 1 g). The catalyst was removed by filtration through Celite and the residue left after evaporation of solvent was recrystallised from dichloromethane–ether–hexane to give the title *pyrrole* (**37**) (31.9 g, 92.7% over 2 steps) as prisms, m.p. 139—140.5 °C (Found: C, 55.2; H, 6.2; N, 4.0. $C_{17}H_{23}NO_8$ requires C, 55.3; H, 6.3; N, 3.8%); $\nu_{max.}$ 3 275br, 1 738, 1 709, and 1 668 cm^{-1}; $\lambda_{max.}$ 285sh and 279.5 nm; δ 1.58 [9 H, s, $C(CH_3)_3$], 2.60 (2 H, m, $CH_2CH_2CO_2$), 3.04 (2 H, m, $CH_2CH_2CO_2$), 3.67 and 3.70 (each 3 H, s, 2 × OCH_3), 3.85 (2 H, s, CH_2CO_2), and 9.92 (2 H, br, NH and CO_2H); *m/e* 369 (6%, M^+), 313 (34, $M^+ - C_4H_8$), 281 (98), and 253 (100).

t-Butyl 5-Iodo-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate (**38**).—Sodium hydrogen carbonate (21.6 g) was added to a *vigorously* stirred mixture of the foregoing carboxylic acid (**37**) (30.6 g), ethanol-free chloroform (300 ml) and water (250 ml) at *ca.* 50 °C. The mixture was then heated rapidly to reflux and a solution of iodine (24.4 g) and potassium iodide (25 g) in water (150 ml) was added during 5 min. Refluxing was continued, with vigorous stirring, for 25 min and then sufficient aqueous sodium hydrogen sulphite to destroy the excess of iodine was added. The organic phase was separated from the cooled mixture and passed over alumina (50 × 25 mm). Dichloromethane extracts (3 × 100 ml) of the aqueous phase were run through the column and elution was continued with ether (400 ml). The product was recrystallised from dichloromethane–ether–hexane to give the *iodopyrrole* (**38**) (34.3 g, 91.7%) as needles, m.p. 122—123 °C (Found: C, 42.8; H, 5.3; N, 2.9. $C_{16}H_{22}INO_6$ requires C, 42.6; H, 4.9; N, 3.1%); $\nu_{max.}$ 3 260br, 1 727, 1 716, and 1 694 cm^{-1}; $\lambda_{max.}$ 278 nm; δ 1.56 [9 H, s, $C(CH_3)_3$], 2.61 (4 H, m, CH_2CH_2), 3.71 (6 H, s, 2 × OCH_3), 3.86 (2 H, s, CH_2CO_2), and 9.27 (1 H, br, NH); *m/e* 451 (100%, M^+), 363 (57), and 336 (68).

t-Butyl 4-(2-Methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate (**39**).—A mixture of the foregoing iodopyrrole (**38**) (34.0 g), palladium-charcoal (10%; 3 g), sodium acetate (20 g), and methanol (250 ml) was stirred under hydrogen until uptake of gas ceased. The catalyst was removed by filtration through Celite and the residue left after evaporation of the solvent was partitioned between dichloromethane (200 ml) and aqueous sodium carbonate (5%; 200 ml). The organic phase was passed over alumina (50 × 25 mm) and elution continued with dichloromethane extracts (100 ml + 2 × 50 ml) of the

aqueous layer, and then with ether (300 ml). The product was recrystallised from dichloromethane–ether–hexane to give the α-free pyrrole (39) (22.4 g, 91.4%) as prisms, m.p. 87.5—88 °C (lit.,[10] m.p. 84—85 °C) (Found: C, 59.1; H, 6.9; N, 4.3. $C_{16}H_{23}NO_6$ requires C, 59.1; H, 7.1; N, 4.3%); $\nu_{max.}$ 3 378, 1 741, 1 726, and 1 688 cm^{-1}; $\lambda_{max.}$ 271 nm; δ 1.56 [9 H, s, $C(CH_3)_3$], 2.65 (4 H, m, CH_2CH_2), 3.66 and 3.69 (each 3 H, s, 2 × OCH_3), 3.81 (2 H, s, CH_2CO_2), 6.68 (1 H, d, *J* 3 Hz, pyrrole-H), and 9.57 (1 H, br, NH); *m/e* 325 (22%, M^+), 269 (40, $M^+ - C_4H_8$), 237 (100), 210 (35), and 209 (59).

Benzyl 5-Acetoxymethyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate (46).—Freshly distilled sulphuryl chloride (2.91 g, 1.03 equiv.) was added during *ca.* 1 min to a stirred solution of benzyl 4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl-5-methylpyrrole-2-carboxylate [9] (45) (7.80 g) in dichloromethane (50 ml). After 1.5 h, the solvent was evaporated and the residue stirred with sodium acetate (5 g) and acetic acid (150 ml) at 60 °C for 20 min. The solution was evaporated to dryness and the residue slurried with water (50 ml). The product was collected 1 h later, washed with water and purified by chromatography on silica (5 g), eluant dichloromethane and dichloromethane–ether (1 : 1), and by recrystallisation from dichloromethane–ether–hexane to give the acetoxymethylpyrrole (46) (8.04 g, 90.1%) as needles, m.p. 144—146 °C (lit.,[17] m.p. 145—146 °C), identical (mixed m.p., t.l.c., n.m.r.) with an authentic specimen.[9]

Pyrromethanes

5′-t-Butoxycarbonyl-4,3′-di-(2-methoxycarbonylethyl)-3,4′-bismethoxycarbonylmethyl-2,2′-methylenedipyrrole-5-carboxylic Acid (42).—Toluene-*p*-sulphonic acid monohydrate (1.0 g) was added to a solution of the α-free pyrrole (39) (20.0 g) and benzyl 5-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate [18] (40) (26.5 g) in dichloromethane (500 ml) and the mixture stirred at room temperature, under nitrogen, for 1.75 h. The red solution was passed through a silica column (20 g) and elution continued with dichloromethane–ether (1 : 1; 200 ml) and with ether (100 ml). Appropriate fractions were combined, washed with aqueous sodium carbonate, and passed over alumina (50 × 50 mm), elution being continued with dichloromethane extracts (3 × 50 ml) of the aqueous phase and then with ether (300 ml). Pyrromethane-containing fractions were evaporated to give an oil (37.0 g) which was stirred with methanol (100 ml) for 4 h at room temperature and then at −10 °C overnight. The solid was collected, washed with a little cold methanol, and recrystallised from chloroform–methanol to give a by-product, dibenzyl 4,4′-di-(2-methoxycarbonylethyl)-3,3′-bismethoxycarbonylmethyl-2,2′-methylenedipyrrole-5,5′-dicarboxylate (0.9 g), m.p. 145—147 °C (lit.,[18] m.p. 145—146 °C). Evaporation of the original methanolic mother liquor gave benzyl 5′-t-butoxycarbonyl-4,3′-di-(2-methoxycarbonylethyl)-3,4′-bismethoxycarbonylmethyl-2,2′-methylenedipyrrole-5-carboxylate (41) (36.1 g, 84.3%) as a foam which could not be induced to crystallise. A sample (1.5 g) was further purified by chromatography on silica (15 g) using benzene–petroleum (1 : 1) containing ether (0—60%) as eluant to give pure material, $\nu_{max.}$ (film) 3 330br, 1 735, and 1 692 cm^{-1}; $\lambda_{max.}$ 286 nm; δ 1.50 [9 H, s, $C(CH_3)_3$], 2.60 (6 H, m, $CH_2CH_2CO_2$, $CH_2CH_2CO_2$), 3.00 (2 H, m, $CH_2CH_2CO_2$ at C-4), 3.51, 3.57, 3.62 and 3.72 (each 3 H, s, 4 × OCH_3), 3.55 and 3.75 (each 2 H, s, 2 × CH_2CO_2), 3.89 (2 H, s, methane-CH_2), 5.21 (2 H, s, CH_2Ph), 7.28 (5 H, s, C_6H_5), and 9.86 (2 H, br, 2 × NH); *m/e* 696 (1%, M^+), 639 (16, $M^+ - C_4H_9$), 605 (10, $M^+ - C_7H_7$), 596 (10), 549 (69), and 505 (100).

A solution of the benzyl ester (41) (31.8 g) in methanol (250 ml) containing palladium-charcoal (1 g) was stirred under hydrogen until uptake of gas ceased. Catalyst was removed by filtration through Celite and the filtrate evaporated to dryness. The residue was chromatographed on silica (30 g) using dichloromethane–hexane (1 : 1), dichloromethane, and dichloromethane–ether (9 : 1 ⟶ 2 : 1). The residue from appropriate fractions was recrystallised from dichloromethane–ether–hexane and then twice from dichloromethane–ether to give the *pyrromethane acid* (42) (24.5 g, 88.6%) as needles, m.p. 158.5—159 °C (Found: C, 57.6; H, 6.3; N, 4.4. $C_{29}H_{38}N_2O_{12}$ requires C, 57.4; H, 6.3; N, 4.6%); $\nu_{max.}$ 3 354, 3 270br, 1 748, 1 725, 1 698, and 1 656 cm^{-1}; $\lambda_{max.}$ 277infl. and 286 nm; δ 1.51 [9 H, s, $C(CH_3)_3$], 2.45—3.16 (8 H, m, 2 × CH_2CH_2), 3.53 and 3.75 (each 2 H, s, 2 × CH_2CO_2), 3.64, 3.67, 3.68 and 3.71 (each 3 H, s, 4 × OCH_3), 3.95 (2 H, s, methane-CH_2), and 10.46 and 10.76 (each 1 H, br, 2 × NH); *m/e* 562 (33%, $M^+ - CO_2$), 506 (58), and 505 (100).

5′-t-Butoxycarbonyl-3,3′-di-(2-methoxycarbonylethyl)-4,4′-bismethoxycarbonylmethyl-2,2′-methylenedipyrrole-5-carboxylic Acid (48).—Benzyl 5-acetoxymethyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate (46) (2.16 g) was condensed with t-butyl 4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole-2-carboxylate (39) (1.63 g) as above to give *pyrromethane benzyl ester* (47) (3.20 g, 92.0%) as a colourless foam used directly in the next step, δ 1.49 [9 H, s, $C(CH_3)_3$], 2.2—2.95 (8 H, m, 2 × CH_2CH_2), 3.56 and 3.65 (each 6 H, s, 4 × OCH_3), 3.76 and 3.79 (each 2 H, s, 2 × CH_2CO_2), 3.94 (2 H, s, methane-CH_2), 5.21 (2 H, s, CH_2Ph), 7.31 (5 H, s, C_6H_5), and 9.36 and 9.69 (each 1 H, br, 2 × NH).

The benzyl ester (47) (3.20 g) was hydrogenated in methanol (40 ml) containing palladium-charcoal (0.1 g). Work-up and chromatography on silica (5 g), as before, followed by recrystallisation of the product from dichloromethane–ether–hexane gave the *pyrromethane acid* (48) (2.23 g, 79.8%) as needles, m.p. 147—148 °C (Found: C, 57.3; H, 6.4; N, 4.6%), $\nu_{max.}$ 3 335, 1 744, 1 729, 1 716, 1 693, and 1 646 cm^{-1}; $\lambda_{max.}$ 273infl. and 288 nm; δ 1.51 [9 H, s, $C(CH_3)_3$], 2.46 (4 H, m, 2 × $CH_2CH_2CO_2$), 2.77 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.67 (12 H, s, 4 × OCH_3), 3.72 and 3.83 (each 2 H, s, 2 × CH_2CO_2), 3.96 (2 H, s, methane-CH_2), and 10.86 and 11.36 (each 1 H, br, 2 × NH); *m/e* 562 (6%, $M^+ - CO_2$), 506 (50), and 505 (100).

5′-t-Butoxycarbonyl-4,4′-di-(2-methoxycarbonylethyl)-3,3′-bismethoxycarbonylmethyl-2-2′-methylenedipyrrole-5-carboxylic Acid (51).—Benzyl 3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate [8] (44) (1.80 g) and t-butyl 5-acetoxymethyl-3-(2-methoxycarbonylethyl)-4-methoxycarbonylmethylpyrrole-2-carboxylate [8] (49) (1.99 g) were condensed as above to give an oil which crystallised when triturated with methanol. Recrystallisation from ether–hexane gave the *pyrromethane benzyl ester* (50) (2.80 g, 80.5%) as needles, m.p. 104—106 °C (Found: C, 62.3; H, 6.2; N, 4.0. $C_{36}H_{44}N_2O_{12}$ requires C, 62.1; H, 6.4; N, 4.0%); $\nu_{max.}$ 3 300br, 1 741, 1 732, 1 719, and 1 698 cm^{-1}; $\lambda_{max.}$ 276infl. and 285.5 nm; δ 1.53 [9 H, s, $C(CH_3)_3$], 2.50 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.00 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.60 (12 H, s, 4 × OCH_3), 3.76 (4 H, s, 2 × CH_2CO_2), 3.79 (2 H, s, methane-CH_2), 5.23 (2 H, s, CH_2Ph), 7.33 (5 H, br, C_6H_5), and 10.12 and 10.31 (each 1 H, br, 2 × NH); *m/e* 696 (M^+).

Hydrogenolysis of the benzyl ester (50 (2.32 g) in methanol (40 ml) containing palladium-charcoal (0.2 g), followed by purification of the product by chromatography on silica (6 g), eluant ether (0 ⟶ 33%) in dichloromethane, and by recrystallisation from dichloromethane–ether–hexane gave the *pyrromethanecarboxylic acid* (51) (1.80 g, 89%) as prisms, m.p. 124—126 °C (Found: C, 57.2; H, 6.2; N, 4.5%), $\nu_{max.}$ 3 315br, 1 731, 1 697, and 1 657 cm^{-1}; $\lambda_{max.}$ 274infl. and 283.5 nm; δ 1.53 [9 H, s, $C(CH_3)_3$], 2.55 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.01 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.54 and 3.57 (each 2 H, s, 2 × CH_2CO_2), 3.64 and 3.74 (each 6 H, s, 4 × OCH_3), 3.87 (2 H, s, methane-CH_2), and 10.40 and 10.54 (each 1 H, br, 2 × NH); *m/e* 562 (40%, $M^+ - CO_2$), 506 (56), and 505 (100).

t-Butyl 5'-Formyl-3,4'-di-(2-methoxycarbonylethyl)-4,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate (16).—A solution of toluene-*p*-sulphonic acid monohydrate (0.5 g) in methanol (5 ml) was added to a solution of the pyrromethane acid (42) (1.00 g) in dichloromethane (25 ml) and the mixture kept under nitrogen, in the dark for 8 h at room temperature, and overnight at 5 °C. The red solution was washed with water (100 ml) and treated with sodium borohydride (0.1 g) in ethanol (5 ml). Excess of borohydride was destroyed by shaking with the original aqueous layer, after which the mixture was made basic with aqueous ammonia. The product from the organic solution was chromatographed on silica (5 g), using ether (0 ⟶ 50%) in dichloromethane as eluant, and Ehrlich-positive fractions were evaporated to give the corresponding α-*free pyrromethane* (950 mg) as a pale brown oil, pure by n.m.r. spectroscopy; δ 1.50 [9 H, s, $C(CH_3)_3$], 2.38—2.87 (8 H, m, 2 × CH_2CH_2), 3.49 (2 H, s, CH_2CO_2), 3.63 (3 H), 3.66 (6 H, 3.74 (3 H) (each s, 4 × OCH_3), 3.79 and 3.85 (each 2 H, s, CH_2CO_2 and methane -CH_2), 6.44 (1 H, d, *J* 2.5 Hz, pyrrole-H), and 8.65 and 9.69 (each 1 H, br, 2 × NH).

A solution of the foregoing α-free pyrromethane (950 mg) in dimethylformamide (15 ml) was added to a stirred mixture of dimethylformamide (10 ml), benzoyl chloride (7.5 ml), and calcium carbonate (2.5 g) and stirring continued at room temperature for 2 h. The mixture was then added slowly to a vigorously stirred solution of sodium acetate (7.5 g) in water (25 ml) and 1 h later, chloroform (250 ml) was added and the mixture filtered through Celite. The organic phase was washed with aqueous sodium carbonate, then water, and the isolated product purified by chromatography on silica (15 g) using benzene–light petroleum (1 : 1) containing ether (0—60%) as eluant. Appropriate fractions were combined and the residue, when kept under ether for several hours, crystallised. Recrystallisation from dichloromethane–ether–hexane gave the *formylpyrromethane* (16) (774 mg, 79.5%) as colourless needles, m.p. 138.5—139.5 °C (Found: C, 59.0; H, 6.6; N, 4.8. $C_{29}H_{38}N_2O_{11}$ requires C, 59.0; H, 6.5; N, 4.7%), $\nu_{max.}$ 3 350, 3 288, 1 742, 1 727, 1 684, and 1 648 cm^{-1}; $\lambda_{max.}$ 278 and 308.5 nm; δ 1.50 [9 H, s, $C(CH_3)_3$], 2.64 (6 H, m, $CH_2CH_2CO_2$ and $CH_2CH_2CO_2$), 3.03 (2 H, m, $CH_2CH_2CO_2$), 3.55 (2 H, s, CH_2CO_2), 3.65, 3.66, and 3.76 (each 3 H, s, 3 × OCH_3), 3.78 (5 H, s, CH_2CO_2 and OCH_3), 3.94 (2 H, s, methane-CH_2), 9.58 (1 H, s, CHO), and 9.85 and 10.09 (each 1 H, br, 2 × NH); *m/e* 590 (27%, M^+), 534 (100, $M^+ - C_4H_8$), 533 (43, $M^+ - C_4H_9$), 505 (55), 490 (25), and 461 (43).

t-Butyl 5'-Formyl-3,3'-di-(2-methoxycarbonylethyl)-4,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate (15).—The pyrromethane carboxylic acid (48) (1.00 g) was decarboxylated (5 h at room temperature) and formylated as for the previous example to give the *formylpyrromethane* (15) (672 mg, 69.1%) as colourless needles, m.p. 89—91 °C, from ether–hexane (Found: C, 59.0; H, 6.5; N, 4.7. $C_{29}H_{38}N_2O_{11}$ requires C, 59.0; H, 6.5; N, 4.7%); $\nu_{max.}$ 3 300, 3 235br, 1 738, 1 733, 1 664, and 1 628 cm^{-1}; $\lambda_{max.}$ 278 and 313.5 nm; δ 1.49 [9 H, s, $C(CH_3)_3$], 2.55 (4 H, m, 2 × $CH_2CH_2CO_2$), 2.76 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.67 and 3.69 (12 H, 4 × OCH_3), 3.74 and 3.76 (each 2 H, s, 2 × CH_2CO_2), 4.02 (2 H, s, methane-CH_2), 9.22 and 9.71 (each 1 H, br, 2 × NH), and 9.56 (1 H, s, CHO); *m/e* 590 (18%, M^+), 534 (100, $M^+ - C_4H_8$), 533 (51, $M^+ - C_4H_9$), 505 (40), 490 (58), and 461 (66).

t-Butyl 5'-Formyl-4,4'-di-(2-methoxycarbonylethyl)-3,3'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate (14).—The carboxylic acid (51) (1.00 g) was decarboxylated and formylated as above to yield the *formylpyrromethane* (14) (702 mg, 72.2%) as colourless needles, m.p. 99—100 °C, from ether–hexane (Found: C, 58.8; H, 6.5; N, 4.6. $C_{29}H_{38}N_2O_{11}$ requires C, 59.0; H, 6.5; N, 4.7%); $\nu_{max.}$ 3 295br, 3 246, 1 742, 1 728, 1 687, and 1 633 cm^{-1}; $\lambda_{max.}$ 277 and 308 nm; δ 1.52 [9 H, s, $C(CH_3)_3$], 2.54 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.02 (4 H, m, 2 × $CH_2CH_2CO_2$), 3.56 (4 H, s, 2 × CH_2CO_2), 3.64 (6 H), 3.79 (3 H), and 3.81 (3 H) (each s, 4 × OCH_3), 3.84 (2 H, s, methane-CH_2), 9.58 (1 H, s, CHO), and 9.92 and 10.32 (each 1 H, br, 2 × NH); *m/e* 590 (42%, M^+), 534 (100, $M^+ - C_4H_8$), 533 (42, $M^+ - C_4H_9$), 505 (41), 490 (24), and 461 (56).

Tripyrrenes *

t-Butyl 3,8-Di-(2-methoxycarbonylethyl)-2,7,12-trismethoxycarbonylmethyl-7'-oxo-1',2',6',7'-tetrahydro-5H-azepino[3',4'-m]tripyrrene-b-1-carboxylate Hydrochloride (20).—Hydrogen chloride in dichloromethane (0.2M; 2 ml) was added to a stirred solution of isoporphobilinogen lactam methyl ester [7] (12) (44.4 mg) and the formylpyrromethane t-butyl ester (16) (118 mg) in dichloromethane (13 ml) and methanol (1 ml). After 30 min dry benzene (20 ml) was added to the mixture and the solution evaporated to dryness below 40 °C. More benzene (20 ml) was added, and the evaporation repeated to leave an orange oil which crystallised when triturated with benzene–ether. Recrystallisation (without heating) from dichloromethane–benzene–ether gave the *tripyrrene hydrochloride* (20) (150.6 mg, 90.7%) as orange needles, m.p. 116—119 °C (Found: M^+ – HCl, 794.3366. $C_{40}H_{50}N_4O_{13}$ requires *M*, 794.3374); $\nu_{max.}$ 3 260br, 3 165br, 1 738, 1 666, and 1 622 cm^{-1}; $\lambda_{max.}$ (CH_3CO_2H) 493 nm; δ 1.52 [9 H, s, $C(CH_3)_3$], 2.10—3.10 (8 H, m, 2 × $CH_2CH_2CO_2$), 2.86 (4 H, s, CH_2CH_2CONH), 3.49, 3.72, and 3.75 (each 2 H, s, 3 × CH_2CO_2), and 3.61 (3 H), 3.64 (6 H), and 3.69 (6 H) (each s, 5 × OCH_3), 4.36 (2 H, s, methane-CH_2), 4.83 (2 H, d, *J* 6 Hz, CH_2NH), 6.06 (1 H, t, *J* 6 Hz, CH_2NH), 7.62 (1 H, s, methine-CH), 10.62 (1 H, br, pyrrole-NH), and 14.19 (2 H, br, 2 × pyrromethene-NH).

t-Butyl 2,7-Di-(2-methoxycarbonylethyl)-3,8,12-trismethoxycarbonylmethyl-7'-oxo-1',2',6',7'-tetrahydro-5H-azepino[3',4'-m]tripyrrene-b-1-carboxylate Hydrochloride (17).—Repetition of the previous procedure using isoporphobilinogen lactam methyl ester (12) (44.4 mg) and t-butyl 5'-formyl-4,3'-di-(2-methoxycarbonylethyl)-3,4'-bismethoxycarbonylmethyl-2,2'-methylenedipyrrole-5-carboxylate [8] (13) (118 mg) afforded the title *tripyrrene hydrochloride* (17) (148.7 mg,

* The non-systematic name ' tripyrrene ' has been retained in order to ensure consistency with earlier parts of the Series. Such compounds should be named according to the IUPAC rules as ' tripyrrins.'

89.6%) as orange needles, m.p. 125—127 °C (Found: M^+ − HCl, 794.3382. $C_{40}H_{50}N_4O_{13}$ requires M, 794.3374); $\nu_{max.}$ 3265br, 1 737, 1 688, 1 674, and 1 621 cm^{-1}; $\lambda_{max.}$ (CH_3CO_2H) 498 nm; δ 1.54 [9 H, s, $C(CH_3)_3$], 2.17—3.20 (8 H, m, 2 × $CH_2CH_2CO_2$), 2.86 (4 H, s, CH_2CH_2CONH), 3.55 (2 H, s, CH_2CO_2), 3.61, 3.62, 3.66, and 3.69 (19 H, 2 × CH_2CO_2 and 5 × OCH_3), 4.38 (2 H, s, methane-CH_2), 4.84 (2 H, d, *J* 6 Hz, CH_2NH), 6.07 (1 H, t, *J* 6 Hz, CH_2NH), 7.48 (1 H, s, methine-CH), 10.63 (1 H, br, pyrrole-NH), and 14.20 and 14.31 (each 1 H, br, 2 × pyrromethene-NH).

t-Butyl 2,8,12-*Tri*-(2-*methoxycarbonylethyl*)-3,7-*bismethoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*[3′,4′-m]*tripyrrene*-*b*-1-*carboxylate Hydrochloride* (18).—Porphobilinogen lactam methyl ester (11) (44.4 mg) was condensed with the aldehyde (14) (118 mg) as for the previous examples to give the *tripyrrene hydrochloride* (18) (149.8 mg, 90.2%) as orange prisms, m.p. 133—134.5 °C (Found: M^+ − HCl, 794.3390. $C_{40}H_{50}N_4O_{13}$ requires M, 794.3374); $\nu_{max.}$ 3 210-br, 1 738sh, 1 732, 1 662, and 1 609 cm^{-1}; $\lambda_{max.}$ (CH_3CO_2H) 489 nm; δ 1.53 [9 H, s, $C(CH_3)_3$], 2.63 (6 H, m, 3 × $CH_2CH_2CO_2$), 3.01 (6 H, m, 3 × $CH_2CH_2CO_2$), 3.47 (2 H) and 3.55 (4 H) (each s, 2 × CH_2CO_2 and CH_2CONH), 3.63 (12 H) and 3.70 (3 H) (each s, 5 × OCH_3), 4.36 (2 H, s, methane-CH_2), 4.87 (2 H, s, CH_2NH), 6.42 (1 H, s, CH_2NH), 7.78 (1 H, s, methine-CH), 10.53 (1 H, br, pyrrole-NH), and 14.07 and 14.23 (each 1 H, br, 2 × pyrromethene-NH).

t-Butyl 3,7,12-*Tri*-(2-*methoxycarbonylethyl*)-2,8-*bismethoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*[3′,4′-m]-*tripyrrene*-*b*-1-*carboxylate Hydrochloride* (19).—Porphobilinogen lactam methyl ester (11) (44.4 mg) and the pyrromethane aldehyde (15) (118 mg) gave the *tripyrrene hydrochloride* (19) (138.7 mg, 83.6%) as orange prisms, m.p. 125—126 °C (Found: M^+ − HCl, 794.3366. $C_{40}H_{50}N_4O_{13}$ requires M, 794.3374); $\nu_{max.}$ 3 180br, 1 737, 1 694, 1 668, 1 650sh, and 1 618 cm^{-1}; $\lambda_{max.}$ (CH_3CO_2H) 496 nm; δ 1.52 [9 H, s, $C(CH_3)_3$], 2.20—3.13 (12 H, m, 3 × $CH_2CH_2CO_2$), 3.53 (2 H, s, CH_2CONH), 3.65 (12 H) and 3.72 (3 H) (each s, 5 × OCH_3), 3.77 (4 H, s, 2 × CH_2CO_2), 4.40 (2 H, s, methane-CH_2), 4.83 (2 H, s, CH_2NH), 6.35 (1 H, s, CH_2NH), 7.62 (1 H, s, methine-CH), 10.89 (1 H, br, pyrrole-NH), and 14.19 (2 H, br, 2 × pyrromethene-NH).

Bilanes

7,12,18-*Tri*-(2-*methoxycarbonylethyl*)-3,8,13,17-*tetrakismethoxycarbonylmethyl*-7′-*oxo*-1′,2′,6′,7′-*tetrahydro*-5H-*azapino*[3′,4′-m]*bilane* (34).—The tripyrrene hydrochloride (20) (41.5 mg) and 2-formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethylpyrrole [9] (22) (12.7 mg) were dissolved in acetic acid (0.35 ml) and trifluoroacetic acid (0.5 ml) plus hydrogen bromide in acetic acid (48%, 4 drops) were added. The solution was kept in the dark, under nitrogen, for 2 h after which it was diluted with dry ether (5 ml). The precipitated solid was collected by centrifugation, washed several times with dry ether, and dried *in vacuo* to give the red biladiene dihydrobromide (28) (50.7 mg, 92.9%) which was reduced immediately.

Sodium borohydride (100 mg) was added during 5 min to a stirred solution of the above biladiene dihydrobromide (28) (35 mg) in methanol (3 ml), cooled on ice. Water (1 ml) was added to the pale yellow mixture which was then kept at 5 °C for 1 h. The product was collected by centrifugation, washed with methanol–water (2 : 1; 3 × 2 ml) and dried *in vacuo*; the title *bilane lactam* (34) (18.6 mg, 62.2%) was a pale yellow powder, m.p. 140—144 °C (Found: M^+, 933.4025. $C_{47}H_{59}N_5O_{15}$ requires C, 933.4007); δ (400 MHz) 2.30 (2 H, t, *J* 8 Hz), 2.52 (4 H, t, *J* 8 Hz), 2.64 (2 H, t, *J* 8 Hz), 2.70 (6 H, m,) and 2.78 (2 H, m) (4 × CH_2CH_2CO), 3.34, 3.36, 3.40, and 3.47 (each 2 H, s, 4 × CH_2CO_2), 3.57 (3 H), 3.59 (3 H), 3.60 (6 H), 3.62 (3 H), 3.65 (3 H), and 3.69 (3 H) (each s, 7 × OCH_3), 3.68, 3.70, and 3.71 (each 2 H, s, 3 × methane-CH_2), 4.13 (2 H, d, *J* 5.5 Hz, CH_2NH), 6.17 (1 H, t, *J* 5.5 Hz, CH_2NH), 6.39 (1 H, d, *J* 2.5 Hz, pyrrole-H), and 8.70, 8.84, 8.93, and 9.12 (each 1 H, br, 4 × NH).

8,13,18-*Tri*-(2-*methoxycarbonylethyl*)-3,7,12,17-*tetrakismethoxycarbonylmethyl*-7′-*oxo*-1′,2′,6′,7′-*tetrahydro*-5H-*azepino*[3′,4′-m]*bilane* (30).—Following the above procedure, the tripyrrene hydrochloride (17) (41.5 mg) and the α-free aldehyde (22) (12.7 mg) were converted into the biladiene dihydrobromide (24) (52.7 mg, 96.6%).

This material (35 mg) was reduced in the usual way to furnish the title *bilane lactam* (30) (23.5 mg, 78.6%) as a pale yellow powder, m.p. 156—160 °C (Found: M^+, 933.3992. $C_{47}H_{59}N_5O_{15}$ requires M^+, 933.4007); δ (400 MHz) 2.31, 2.48, and 2.51 (each 2 H, t, *J* 8 Hz) 2.70 (8 H, m) and 2.79 (2 H, m) (4 × CH_2CH_2CO), 3.29, 3.41, 3.43, and 3.44 (each 2 H, s, 4 × CH_2CO_2), 3.58, 3.59, 3.60, 3.61, 3.62, 3.65, and 3.71 (each 3 H, s, 7 × OCH_3), 3.67, 3.68, and 3.74 (each 2 H, s, 3 × methane-CH_2), 4.19 (2 H, d, *J* 6 Hz, CH_2NH), 6.11 (1 H, t, *J* 6 Hz, CH_2NH), 6.37 (1 H, d, *J* 2.5 Hz, pyrrole-H), and 8.63 (1 H), 8.95 (2 H), and 9.31 (1 H) (each br, 4 × NH).

3,7,13,18-*Tetra*-(2-*methoxycarbonylethyl*)-8,12,17-*trismethoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*-[3′,4′-m]*bilane* (31).—The tripyrrene hydrochloride (18) (41.5 mg) and the α-free aldehyde (22) (12.7 mg) gave the biladiene dihydrobromide (25) (47.9 mg, 87.7%) of which a portion (35 mg) was reduced to afford the title *bilane lactam* (31) (23.4 mg, 78.3%) as a pale yellow powder, m.p. 151—157 °C (Found: M^+, 933.4001. $C_{47}H_{59}N_5O_{15}$ requires M, 933.4007), δ (400 MHz) 2.42, 2.43, 2.44, and 2.52 (each 2 H, t, *J* 8 Hz), and 2.69 (8 H, m) (4 × $CH_2CH_2CO_2$), 3.35 (2 H, t, *J* 3.5 Hz, CH_2CONH), 3.36, 3.42, and 3.45 (each 2 H, s, 3 × CH_2CO_2), 3.59, 3.60, 3.61, 3.615, 3.62, 3.63, and 3.65, (each 3 H, s, 7 × OCH_3), 3.67, 3.74, and 3.75 (each 2 H, s, 3 × methane-CH_2), 4.33 (2 H, br, CH_2NH), 5.87 (1 H, br, CH_2NH), 6.38 (1 H, d, *J* 2.5 Hz, pyrrole-H), and 8.34, 8.53, 8.89, and 9.11 (each 1 H, br, 4 × NH).

3,8,12,18-*Tetra*-(2-*methoxycarbonylethyl*)-7,13,17-*trismethoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*-[3′,4′-m]*bilane* (32).—The tripyrrene hydrochloride (19) (41.5 mg) was condensed with the α-free aldehyde (22) (12.7 mg) as for the previous examples to give the biladiene dihydrobromide (26) (52.6 mg, 96.4%).

This material (40 mg) was reduced with sodium borohydride in the usual way to give the title *bilane* (32) (23.3 mg, 68.1%) as a pale yellow powder, m.p. 126—131 °C (Found: M^+, 933.4006. $C_{47}H_{59}N_5O_{15}$ requires M, 933.4007); δ (400 MHz) 2.20 (2 H), 2.22 (2 H), 2.51 (4 H), 2.59 (2 H), 2.63 (2 H), and 2.71 (4 H) (each t, *J* 8 Hz, 4 × $CH_2CH_2CO_2$), 3.35 (2 H, t, *J* 3.2 Hz, CH_2CONH), 3.41, 3.43, and 3.45 (each 2 H, s, 3 × CH_2CO_2), 3.48, 3.59, 3.60, 3.63, and 3.66 (each 3 H, s, 5 × OCH_3), 3.69 and 3.70 (each 5 H, s, 2 × OCH_3 and 2 × methane-CH_2), 3.72 (2 H, s, methane-CH_2), 4.38 (2 H, br, CH_2NH), 5.91 (1 H, br, CH_2NH), 6.39 (1 H, d, *J* 2.5 Hz, pyrrole-H), and 8.59, 8.93, 9.17, and 9.23 (each 1 H, br, 4 × NH).

3,8,13,17-*Tetra*-(2-*methoxycarbonylethyl*)-7,12,18-*trismethoxycarbonylmethyl*-6′-*oxo*-1′,2′,5′,6′-*tetrahydropyrido*-[3′,4′-m]*bilane* (33).—The tripyrrene hydrobromide (21) [1] (43.8 mg) and 2-formyl-3-(2-methoxycarbonylethyl)-4-

methoxycarbonylmethylpyrrole [8] (23) (12.7 mg) furnished the biladiene dihydrobromide (27) (49.0 mg, 89.8%).

Reduction of this material (35 mg) gave the title *bilane* (33) (19.8 mg, 66.2%) as a pale yellow powder, m.p. 154—159 °C (Found: M^+, 933.4006. $C_{47}H_{59}N_5O_{15}$ requires M, 933.4007); δ (400 MHz) 2.38, 2.40, 2.45, and 2.50 (each 2 H, t, J 8 Hz), 2.68 (8 H, m), (4 × $CH_2CH_2CO_2$), 3.35 (2 H, t, J 3.2 Hz, CH_2CONH), 3.36, 3.42, and 3.44 (each 2 H, s, 3 × CH_2CO_2), 3.52, 3.54, 3.57, 3.60, 3.64, 3.67, and 3.70 (each 3 H, s, 7 × OCH_3), 3.69, 3.71, and 3.75 (each 2 H, methane-CH_2), 4.38 (2 H, br, CH_2NH), 5.84 (1 H, br, br, CH_2NH), 6.48 (1 H, d, J 2.4 Hz, pyrrole-H), and 8.29, 8.46, 9.11, and 9.18 (each 1 H, br, 4 × NH).

Preparation of Deaminase-Cosynthetase from Euglena gracilis.—All operations were carried out at 2—4 °C. Four 250 ml pots of frozen *E. gracilis* cells in suspension [4] were thawed and centrifuged at 23 000 × g for 30 min. To the clear supernatant liquid (605 ml, 338 000 units of activity) were added 2-mercaptoethanol (0.6 ml), aqueous ammonia (to pH 7.5), and ammonium sulphate to 47% saturation (27.1 g/100 ml). The mixture was stirred for 30 min, centrifuged (10 min), and the supernatant liquid, after readjustment to pH 7.5, was treated as before with additional ammonium sulphate (10.6 g/100 ml) to 65% saturation. After centrifugation, the pellet was frozen to facilitate transferral to dialysis tubing (residual material was washed in with the minimum volume of distilled water) which was then dialysed against 10 mM phosphate buffer, pH 7.4, containing 10 mM 2-mercaptoethanol (2 × 2-l, 4-h each).

The resulting protein solution (28.5 ml, 239 000 units of activity) was applied to a column of Sephadex G-100 (4.5 × 97 cm) and at *ca.* 50 ml h^{-1}, with 10 mM phosphate buffer, pH 7.4 containing 10 mM 2-mercaptoethanol. Fractions (9 ml each) were collected and the peak of enzyme activity normally appeared around fraction 102; in the present case, 17 fractions were pooled to give 190 000 units of deaminase-cosynthetase.

Preparation of Deaminase.—A solution of deaminase (35 600 units, specific activity [16] 1 800) prepared from *E. gracilis* by heat treatment, ammonium sulphate fractionation, and chromatography on DEAE-cellulose as earlier,[15] was concentrated and further purified on G-100 exactly as for the mixed enzymes above. Deaminase of specific activity 9 000 (total 27 000 units) was obtained.

Kinetic Experiments with Isomeric Aminomethylbilanes: Formation of the Aminomethylbilanes (1)—(6).—Samples (1.0 mg) of each of the bilanes (29)—(34) were suspended in nitrogen-saturated aqueous 2M-potassium hydroxide (50 μl) in 2-ml screw-top vials sealed under nitrogen. The mixtures were kept in the dark, with occasional swirling, for 16 h by which time all solid had dissolved. The orange-brown solutions were then adjusted to pH 8 with aqueous 1M-potassium dihydrogen phosphate and diluted to 1.0 ml with water for immediate use below.

Incubation of the Aminomethylbilanes (1)—(6) *with Deaminase-Cosynthetase from* E. gracilis, *and Comparison with Chemical Cyclisations.*—Incubation conditions for the ring-A reversed (2), ring-C reversed (4), ring-D reversed (5), and rings-A,B,C reversed (6) bilanes were as follows.

Half of the solution of bilanes prepared as above (0.50 ml, 536 nmol bilane) was mixed with deaminase-cosynthetase (22 200 units) in 0.1M-phosphate buffer, pH 7.75 (18.2 ml) at 37 °C and the mixture was maintained at this temperature. The remaining half of the bilane solution was used in a parallel chemical cyclisation, *i.e.* without enzyme.

Aliquots (0.75 ml) of each solution were withdrawn at appropriate times and assayed for uro'gen (see *General Directions*); the results are presented in Figure 2, i—vi.

For the unrearranged bilane (1) and the ring-B reversed isomer (3), the enzymic reaction mixtures contained 27 500 units of deaminase-cosynthetase in total volume of 65.9 ml, and 59 500 units in 45.0 ml, respectively.

Analysis of Mixtures of Isomeric Uro'gens.—At the end of the kinetic experiments above, the uro'gens in each incubation were oxidised with iodine, the resulting uroporphyrins were isolated on DEAE-cellulose, and then decarboxylated in the usual way.[1] The coproporphyrins were esterified and the products, in chloroform (0.5 ml) were treated with saturated methanolic zinc acetate (4—5 drops) at 25 °C for 1 h. The solutions were evaporated with a stream of nitrogen and the residues chromatographed on silica columns, packed in Pasteur pipettes, using chloroform as eluant. Analysis of the resultant mixture of isomeric coproporphyrin esters (as their zinc complexes) was by h.p.l.c. (see *General Directions*). The results are collected in Table 1.

Incubation of Unrearranged Bilane (1) *with Various Amounts of Deaminase-Cosynthetase.*—Six samples of the aminomethylbilane (1) (each from 0.10 mg, 107 nmol of lactam heptamethyl ester) were incubated at 37 °C with deaminase-cosynthetase (2 690 units/ml; 0.0, 0.9, 1.8, 2.7, 3.6, or 4.5 ml) in 100 mM phosphate buffer, pH 7.75 (total 5 ml). Aliquots (0.75 ml) were taken at 1 min intervals for standard uro'gen assay, from which the initial rate of uro'gen formation in each incubate was determined. The results are presented in Figure 5.

Incubation of Deaminase-Cosynthetase with Various Concentrations of Unrearranged Bilane (1).—The deaminase-cosynthetase system (2 690 units per experiment) was incubated at 37 °C in 100 mM-phosphate buffer, pH 7.75 (5 ml) containing aminomethylbilane (1) (at concentrations of 2.14, 5.36, 10.72, 16.08, and 21.44 μM); a sixth incubate, containing 21.44 μM bilane but no enzyme, was run in parallel. The initial rate of uro'gen formation in each solution was determined as above. Appropriate fractions of the initial rate in the chemical run (non-enzymic) were subtracted from those of the enzyme-containing incubates to give the enzyme-catalysed velocities. The results are given in Figure 6.

Determination of K_m *and* $v_{max.}$ *for the Aminomethylbilanes* (1), (3), *and* (5) *and for Porphobilinogen* (9).—The experiment above was repeated with the ring-B reversed bilane (3) (5.35—107.0 μM), the ring-D reversed bilane (5) (2.5—50.0 μM) and with PBG (20—200 μM). Similar experiments, using deaminase alone (6 000 units) and the bilanes (1) and (3) (1.07—107 μM) were also carried out. Results from the six studies were converted into standard Lineweaver-Burk plots from which the kinetic constants K_m and $V_{max.}$ were obtained using regression analysis.[19]

Incubation of Unrearranged Aminomethylbilane (1) *with Varying Concentrations of Deaminase.*—The unrearranged aminomethylbilane (60 μl; 12.84 μM) in phosphate buffer (0.5 ml; 1M; pH 7.75) and water (440 μl) was incubated at 37 °C with deaminase (1 700 units/ml; 510, 1 020, 2 040, and 3 060 units) in phosphate buffer (0.01M; pH 7.4) to a total volume of 5 ml. Aliquots (0.75 ml) were taken every minute and were assayed for content of uro'gens as described earlier.

Grateful acknowledgement is made to the S.R.C., the Nuffield Foundation, and Roche Products Ltd. for financial

support and to Mr. Chris Sporikou and his staff for large-scale growth of *Euglena gracilis*.

[2/433 *Received*, 12*th March*, 1982]

REFERENCES

[1] Part 16, A. R. Battersby, C. J. R. Fookes, M. J. Meegan, E. McDonald, and H. K. W. Wurziger, *J. Chem. Soc., Perkin Trans. 1*, 1981, 2786.

[2] Preliminary account in part A. R. Battersby, C. J. R. Fookes, G. W. J. Matcham, and E. McDonald, *J. Chem. Soc., Chem. Commun.*, 1978, 1064.

[3] See also H.-O. Dauner, G. Gunzer, G. Heger, and G. Müller, *Z. Physiol. Chem.*, 1976, **357**, 147.

[4] A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald, and J. Saunders, *J. Chem. Soc., Perkin Trans. 1*, 1976, 273.

[5] R. B. Frydman A. Valasinas and B. Frydman, *Biochemistry*, 1973, **12**, 80; B. Frydman and R. B. Frydman, *Acc. Chem. Res.*, 1975, **98**, 1589.

[6] A. I. Scott, K. S. Ho, M. Kajiwara, and T. Takahashi, *J. Am. Chem. Soc.*, 1976, **98**, 1589.

[7] A. R. Battersby, J. F. Beck, and E. McDonald, *J. Chem. Soc., Perkin Trans. 1*, 1974, 160.

[8] A. R. Battersby, S. Kishimoto, E. McDonald, F. Satoh, and H. K. W. Wurziger, *J. Chem. Soc., Perkin Trans. 1*, 1979, 1927.

[9] A. R. Battersby, E. Hunt, E. McDonald, J. B. Paine III, and J. Saunders, *J. Chem. Soc., Perkin Trans. 1*, 1976, 1008.

[10] L. Diaz, R. B. Frydman, A. Valasinas, and B. Frydman, *J. Am. Chem. Soc.*, 1979, **101**, 2710.

[11] R. Chong, P. S. Clezy, A. J. Liepa, and A. W. Nichol, *Aust. J. Chem.*, 1969, **22**, 229.

[12] G. W. Kenner, K. M. Smith, and J. F. Unsworth, *J. Chem. Soc., Chem. Commun.*, 1973, 43; J.-H. Furhop and K. M. Smith, in 'Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, p. 758.

[13] L. Diaz, A. Valasinas, and B. Frydman, *J. Org. Chem.*, 1981, **46**, 864.

[14] A. R. Battersby, D. G. Buckley, D. W. Johnson, L. N. Mander, E. McDonald, and D. C. Williams, *J. Chem. Soc., Perkin Trans. 1*, 1981, 2779.

[15] D. C. Williams, G. S. Morgan, E. McDonald, and A. R. Battersby, *Biochem. J.*, 1981, **193**, 301.

[16] C. Rimington, *Biochem. J.*, 1960, **75**, 620.

[17] P. S. Clezy, T. T. Hai, and P. C. Gupta, *Aust. J. Chem.*, 1976, **29**, 393.

[18] A. R. Battersby, M. Ihara, E. McDonald, J. Saunders, and R. J. Wells, *J. Chem. Soc., Perkin Trans. 1*, 1976, 283.

[19] A. L. Lehninger, 'Biochemistry,' Worth, New York, 2nd edn., 1975, p. 183.