Biosynthesis of porphyrins and related macrocycles. Part 53.[1,2] Stereochemical studies on the enzymic formation of hydroxymethylbilane, the precursor of uroporphyrinogen III

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A new synthesis of porphobilinogen **1** (PBG) is described that allows the preparation of (11*R*)-[11-$^3H_1$]PBG **1a** and its (11*S*)-enantiomer **1b**. Their enantiomeric purities are determined by degradation of their immediate synthetic precursors by way of ^{3}H-labelled glycines to yield two samples of ^{3}H-labelled glycolic acid **16**. The enzyme glycolate oxidase, known to remove H_R stereospecifically from the methylene group of glycolic acid in forming glyoxylic acid **17**, is then used to assay the configurations of these two samples. Each ^{3}H-labelled PBG **1a** and **1b** is converted by hydroxymethylbilane synthase into hydroxymethylbilane **5a** and **5b**. Methods are devised for the isolation of this labile product from water and for its subsequent degradation to two further samples of glycolic acid. These are assayed enzymically to prove that there is overall retention of configuration as the aminomethyl carbon of PBG **1** enzymically affords the hydroxymethyl centre of the bilane **5**. Thus, the two covalent bonds that are formed in this whole process must both involve reactions with retention of configuration or both with inversion. The significance of these results is discussed.

The importance of uroporphyrinogen III **7** (shortened to uro'gen III) for the biosynthesis of the natural porphyrins, chlorins and corrins was brought out in the Introduction to the preceding paper.[1] Fuller details were also given there of the biosynthetic pathway,[3] shown in Scheme 1, by which uro'gen III **7** is built from four molecules of porphobilinogen, PBG, **1** by two enzymes acting in sequence. Hydroxymethylbilane synthase, HMBS (E.C. 4.3.1.8), builds hydroxymethylbilane **5** ready for conversion into uro'gen III **7** by uropoporphyrinogen III synthase. There is good evidence[4] that breakdown of the bound bilane **3** occurs by way of the azafulvene **4** which reacts with water to yield hydroxymethylbilane **5**. The present paper reports our work on the overall stereochemistry at the starred centres as PBG **1** is converted into the bilane **5**.

Labelling with deuterium was used in the preceding paper.[1] However, pilot experiments showed that this isotope would not serve for the present research because the yields likely to be realised for the production of the bilane **5** and for its stereochemical assay by degradation were far too low. It was clear that the higher sensitivity afforded by tritium labelling would be needed. As is often the case, this switch from labelling with ^{2}H to ^{3}H required a new synthesis of stereoselectively labelled samples of [11-$^3H_1$]PBG, **1a** and **1b**. The latter but not the former had earlier been prepared enzymically.[5]

Results and discussion

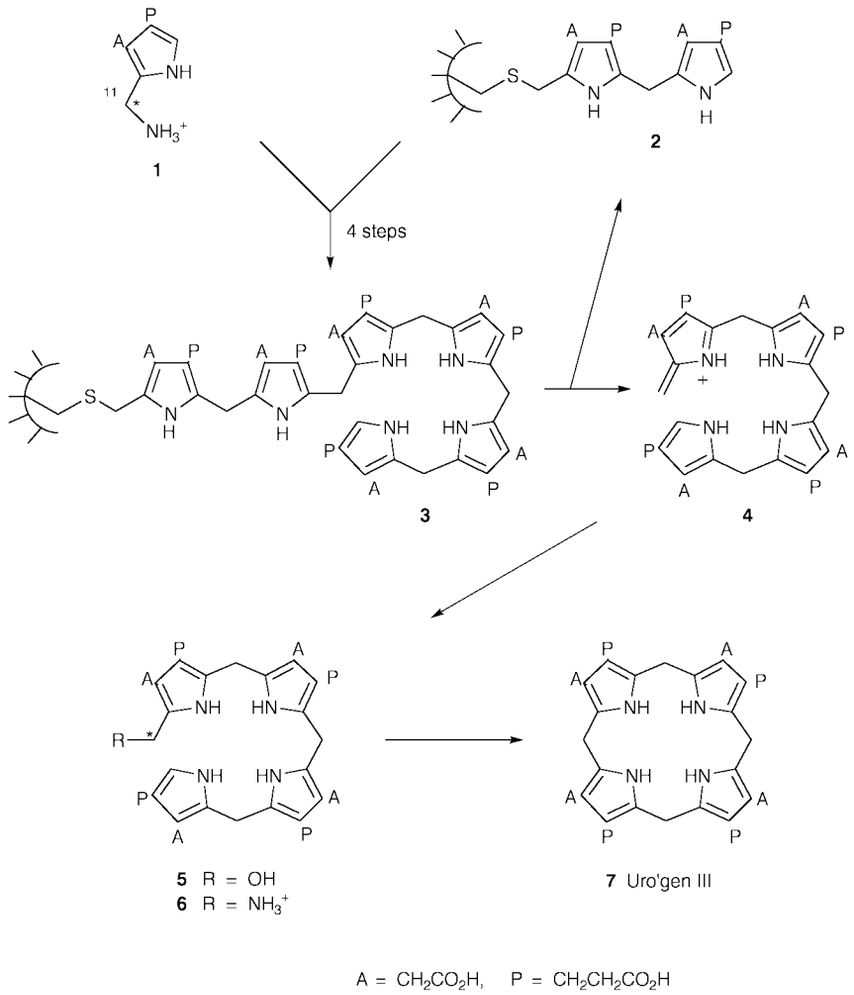
Syntheses of (11*R*)-[11-$^3H_1$]PBG 1a and of its (11*S*)-enantiomer 1b

Initially, an attempt was made to adapt the route described in the preceding paper[1] for synthesis of the ^{2}H-labelled analogues of **1a** and **1b** to the production of the corresponding ^{3}H-labelled species but several practical difficulties arose. These were mainly due to the fact that whereas ^{2}H-labelling can be carried out with stoicheiometric quantities of materials on a reasonable scale, ^{3}H-labelling generally involves minute quantities of ^{3}H-reagents in order to maintain the high specific radioactivities needed for the subsequent work. Accordingly, the following new route was devised, Scheme 2, which took account of the special requirements for tritium labelling.

The deactivated pyrrolic aldehyde **8** had been prepared earlier[6] and a separate study had shown[7] that reduction of the ^{2}H-labelled form **8a** with (*R*)-Alpine-borane prepared from (1*R*)-(+)-α-pinene,[8] gave material containing 95% of the (*S*)-alcohol† **9a** with 5% of the (*R*)-isomer **9b**. This product had a marginally higher configurational purity than that from the complementary preparation using (*S*)-Alpine-borane which contained a 9 : 1 mixture,† respectively, of the (*R*)-alcohol **9b** and the (*S*)-enantiomer **9a**. It had also been shown[7] that the *N*-triflyl group of **9a** and **9b** deactivated the pyrrole ring sufficiently to allow S_N2 displacement of an activated form of the hydroxy group by a carboxylate anion, using Mitsunobu conditions,[9] with *ca.* 90% inversion of configuration. Without the *N*-triflyl group, attempted substitution reactions (S_N2) would cause racemisation due to facile S_N1 reaction *via* the azafulvenium ion[10] (*cf.* **4**). Knowledge of this chemistry laid the foundation for synthesis of the labelled PBG molecules **1a** and **1b** as in Scheme 2.

Reduction of the aldehyde **8** with sodium borotritiide under special conditions to maximise the transfer of tritium (see Experimental section) yielded the randomly tritiated alcohol (**9c** + **9d**). This was oxidised with pyridine–chromium trioxide[11] to regenerate the [^{3}H]aldehyde **8b** which retained *ca.* 80% of the original ^{3}H-activity due to the kinetic isotope effect. Reduction of **8b** using (*S*)-Alpine-borane then afforded the alcohol **9**, which because of results reported in the foregoing paper[1] for the strictly parallel [^{2}H]series, consisted of a mixture of 9 parts of the (*R*)-alcohol **9d** with 1 part of the (*S*)-enantiomer **9c**. The complementary product, similarly expected to consist of mainly (96%) the (*S*)-alcohol **9c** with a small amount (4%) of the (*R*)-species **9d**, was obtained by reduction of **8b** with (*R*)-Alpine-borane. The work described

† The quoted proportions derive from direct measurements by NMR in the ^{2}H series.[7] All the enantiomeric purities for the ^{3}H-series are collected in Table 2, where our best estimate of the experimental errors involved in the NMR and enzymic assays are included.



Scheme 1

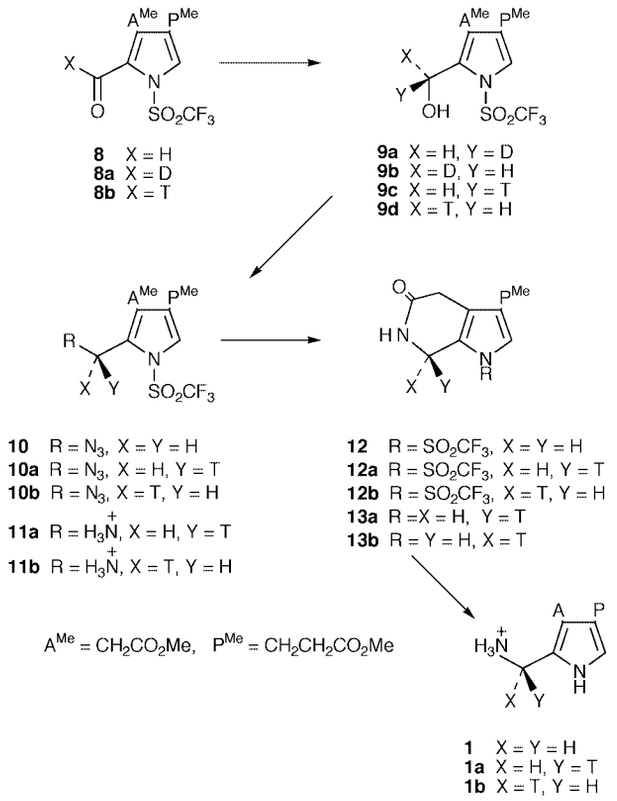
below confirmed that, as expected, this product had a somewhat higher enantiomeric purity than the mainly (*R*)-alcohol generated by (*S*)-Alpine-borane.

Treatment of the (*S*)-alcohol **9c** with triphenylphosphine and hydrazoic acid followed by diethyl azodicarboxylate (Mitsunobu reaction) led to the (*R*)-azide **10a** in 80% yield. Hydrogenation of the (*R*)-azide **10a** under acidic conditions using palladium black yielded the amine salt **11a** and this material, without purification, was treated with sodium carbonate to release the free base which readily cyclised to the lactam **12a**, 88%. Chromous chloride was also effective for reduction of the azide **10**, five runs giving yields >90% overall to the lactam **12**. The triflyl group was then removed from **12a** using sodium methoxide to afford a product (80%) containing mainly the (11*R*)-[11-$^3H_1$]PBG lactam ester **13a**, the rest being the (11*S*)-isomer **13b**. Repetition of this entire sequence starting from the *R*-alcohol **9d** going *via* **10b**, **11b** and **12b** provided a lactam containing mainly (11*S*)-[11-$^3H_1$]PBG lactam ester **13b** with a small amount of the (11*R*)-isomer **13a**, the yields throughout being similar to those recorded for the enantiomeric series. It was now necessary to show that the high proportion of the major enantiomers present in the alcohols **9c** and **9d** had largely been preserved through to the lactams **13a** and **13b**.

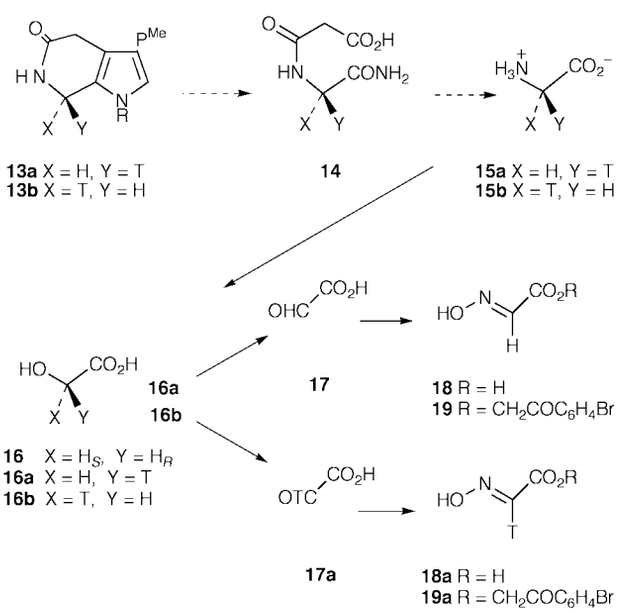
Assay of the enantiomeric purity of the lactams 13a and 13b

The (11*R*)-[11-$^3H_1$]PBG lactam ester **13a** was adsorbed onto finely divided silica gel and oxidatively degraded by treatment in the absence of solvent with a large excess of ozone[12] initially at low temperature, Scheme 3. It was expected that a mixture of glycine amides would be formed (*e.g.* **14** is a likely product) so the crude products were hydrolysed with hot 6 M hydrochloric acid. These conditions are used for the breakdown of peptides and proteins to amino acids without significant racemisation. Unlabelled glycine was then added to act as carrier for the small amount of ^{3}H-labelled glycine **15a** that had been formed. An aliquot of the isolated glycine was converted into its *N*-benzoyl derivative to allow crystallisation to constant specific activity. The value found showed the yield of glycine from this and several repeat degradations was surprisingly good at 20–35%. The rest of the glycine was converted into ^{3}H-labelled glycolic acid **16a** by treatment with nitrous acid, a reaction shown[13] to occur with retention of configuration. Again a portion of this glycolic acid, 37% yield, was converted after dilution with unlabelled material, into its crystalline *p*-bromophenacyl ester[13] for purification to constant specific activity. This provided an accurate value for the specific activity of the glycolic acid **16a** ready for the stereochemical assay. Exactly the same procedures were then used for the degradation of (11*S*)-[11-$^3H_1$]PBG lactam ester **13b** to a second sample of glycolic acid **16b**.

The plan for the stereochemical assay of the foregoing glycolic acids **16a** and **16b** was based on the work of Arigoni and Besmer[13] using glycolate oxidase,[14] a flavoenzyme (E.C. 1.1.3.1) which uses oxygen as the terminal oxidising agent. It converts glycolic acid **16** into glyoxylic acid **17** with the production of one equivalent of hydrogen peroxide. The enzyme stereospecifically removes H_R from the methylene group of glycolic acid[15] **16**. Accordingly, the acids **16a** and **16b**, together with the randomly tritiated sample (**16a** + **16b**), were separately oxidised using glycolate oxidase isolated from spinach.[14] The resultant three samples of glyoxylic acid, mainly expected to be respectively **17**, **17a** and (**17** + **17a**), were isolated and the



Scheme 2



Scheme 3

corresponding crystalline oximes, **18**, **18a** and (**18** + **18a**) were prepared from them. After purification, each was converted into its *p*-bromophenacyl ester **19**, **19a** and (**19** + **19a**) for further recrystallisation to constant specific activity. These precautions are important to ensure radiochemical purity. The results, collected in Table 1, were unexpectedly high and puzzling. In particular, the high value for apparent ^{3}H-retention found for the randomly tritiated glycolic acid (**17** + **17a**), rather than 50% retention, made it clear that something was fundamentally wrong. Repetition of these enzymic oxidations gave variable results but always the ^{3}H-retentions were significantly too high.

The reasons for these puzzling results became clear when a Warburg apparatus was used to measure the oxygen consumed

Table 1 Anomalous results from initial stereochemical assays using glycolate oxidase

Glycolic acid	Apparent ^{3}H-retention in glyoxylic acid derivative (%)	Expected values for ^{3}H-retention (%)
(2*R*)-[2-$^{3}H_1$]Isomer[a] **16a**	**19** 40	17[b]
Random ^{3}H (**16a** + **16b**)	(**19** + **19a**) 72	50
(2*S*)-[2-$^{3}H_1$]Isomer[a] **16b**	**19a** 110	83[b]

[a] This is the major component. [b] The [^{3}H]PBG samples that had been degraded to yield these two glycolic acids were synthesised (with difficulty) by the route developed for ^{2}H-labelling in the preceding paper.[1] [b] The quoted "expected values" are based on the proportions of (*R*)- and (*S*)-forms shown to be present in the equivalent synthesis with ^{2}H-labelling.[1]

in the enzymic oxidation. Catalase was included in the incubation mixture to decompose the hydrogen peroxide formed in the oxidative step with regeneration of 0.5 moles of oxygen. Thus the enzymic oxidation should use 0.5 moles of oxygen for each mole of glyoxylate formed. It was found that an excess of oxygen was always consumed, the extra amount varying over the range 16–30%; over-oxidation was occurring. Furthermore, these enzymic oxidations to give glyoxylic acid **17** were found to be fairly rapid and so were stopped after 20–30 min. The incubations yielding the results in Table 1 were run for 3.5 h so even greater over-oxidation almost certainly occurred.

The high apparent ^{3}H-retentions in Table 1 can now be understood. A survey of the literature on glycolate oxidase showed that it is capable of oxidising glyoxylic acid **17**, probably as its hydrate, to oxalic acid.[16] For the randomly tritiated sample (**16a** + **16b**), the glyoxylic acid formed initially will indeed have the expected 50% retention of tritium. However, in the over-oxidation step, the conversion of unlabelled molecules **17** (which form almost the entire sample) into oxalic acid will be faster than the oxidation of the minute amount of ^{3}H-labelled ones **17a** because of the tritium isotope effect. Thus, at the end of the incubation, the *remaining unoxidised glyoxylic acid* will have a higher proportion of labelled molecules than the glyoxylic acid initially formed. Hence it will have a higher specific activity and so apparently a high retention of tritium. This explanation obviously holds true also for the results in Table 1 from the stereoselectively labelled glycolic acids **16a** and **16b**.

The solution to the problem was to trap the glyoxylic acid as it was formed by including hydroxylamine in the mixture; hydroxylamine was tolerated by the enzyme. Under these conditions, randomly tritiated glycolic acid (**16** + **16a**) was converted into the oxime of glyoxylic acid (**18** + **18a**), purified finally as its *p*-bromophenacyl ester (**19** + **19a**) which, within experimental error, reproducibly retained 50% of the original tritium.

The foregoing experiments included a further refinement which involved adding a suitable quantity of [1-^{14}C]glycolic acid to the ^{3}H-labelled material to give a satisfactory ^{3}H : ^{14}C ratio for radio-assay *i.e.* generally in the range 1 to 10. By referring the ^{3}H-labelling to an internal ^{14}C-standard, dilution with unlabelled material necessary to provide enough substance for later transformations is readily done because the ^{3}H : ^{14}C ratio remains unchanged. In the absence of the ^{14}C-label, it would be impossible to allow for the inevitable fall in the ^{3}H-specific activity caused by dilution without introducing unacceptable errors.

The glycolic acids **16a** and **16b** derived from the lactams **13a** and **13b** were ^{14}C-labelled in this way and were assayed again under the new conditions. The results in Table 2 showed that though some configurational purity had been lost during the synthesis of the lactams **13a** and **13b** from the alcohols **9c** and **9d**, the enantiomeric excess was amply sufficient to solve the stereochemical problem.

Table 2 Configurational assays on precursors and products

	Alcohols **9c** and **9d**[a]	PBG Lactam esters **13a** and **13b**[b]	Hydroxymethylbilanes **5a** and **5b**[b]
Series involving (11*R*)-[11-$^3H_1$]PBG **1a**	**9c**, 96 ± 5% (*S*)	**13a**, 85 ± 5% (*R*) **13a**, 84 ± 5% (*R*)	**5a**, 74 ± 5% (*R*)
Series involving (11*S*)-[11-$^3H_1$]PBG **1b**	**9d**, 88 ± 5% (*R*)	**13b**, 74 ± 5% (*S*) **13b**, 74 ± 5% (*S*)	**5b**, 68 ± 5% (*S*)

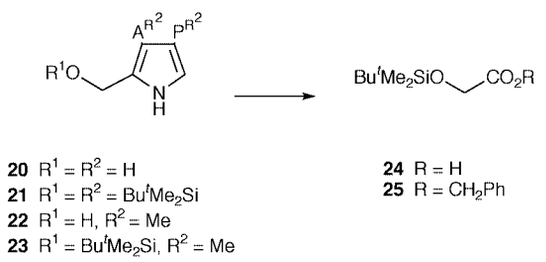
[a] Enantiomeric ratio is based on the strictly parallel ^{2}H-series described in the preceding paper.[1] [b] Configurational assays (in duplicate for **13**) by degradation to glycolic acid and assay with glycolate oxidase as described in the text.

Enzymic conversion of (11*R*)-[11-$^3H_1$]PBG 1a and the (11*S*)-enantiomer 1b into two samples of hydroxymethylbilane and determination of their absolute configurations

This stage of the research required solutions to three problems. They were (a) how to produce the labile hydroxymethylbilane **5** in a workable quantity; (b) how to isolate **5** from aqueous solution in a form suitable to allow (c) its degradation to a derivative of glycolic acid from which it could be regenerated for enzymic stereochemical assay.

Hydroxymethylbilane **5** is produced enzymically at *ca.* pH 8.0 where its half-life is around 4 min.[3] It is stable[10] above pH 12 but the enzyme is inactivated under these conditions. The solution was to prepare a column of Sepharose to which the enzyme had been covalently bound. By passing a solution of PBG **1** through the column directly into aqueous potassium hydroxide, the bilane **5** was generated and quickly stabilised.

Trial experiments involving pyrroles **20**–**23**, described briefly in the Experimental section, had already shown that the hydroxymethylpyrrole **20**, Scheme 4, could be fully silylated



20 $R^1 = R^2 = H$
21 $R^1 = R^2 = Bu^tMe_2Si$
22 $R^1 = H$, $R^2 = Me$
23 $R^1 = Bu^tMe_2Si$, $R^2 = Me$

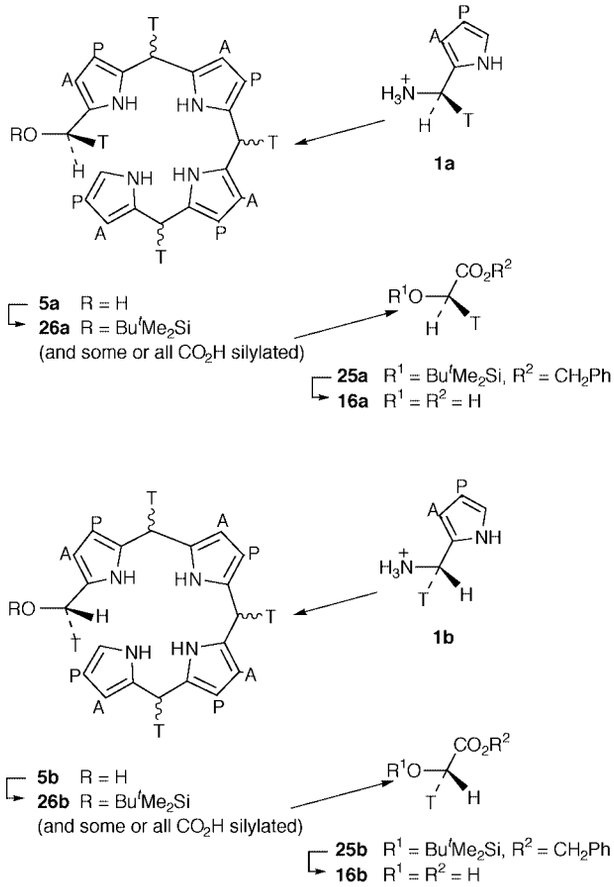
24 R = H
25 $R = CH_2Ph$

Scheme 4

using *tert*-butyldimethylsilyl chloride and the trisilyl derivative **21** was not washed out of organic solvent by water. Accordingly, synthetic[17] hydroxymethylbilane **5** in aqueous solution was silylated under the same basic conditions and though it was not known whether all eight carboxy groups had been fully silylated, the crucial point was established that the silylated bilane was extractable from water. Application of this silylation procedure to the bilane **5** produced enzymically was complicated by the presence of a relatively huge amount of Tris-buffer since Tris [tris(hydroxymethyl)methylamine] is also susceptible to silylation. The problem was overcome by chromatographic removal of the buffer on Sephadex G-10.

Attention then turned to degradation of the silylated bilane. Again, trial experiments established that the silyl protecting group of the silylated pyrrole **23** withstood dry ozonolysis well enough to allow isolation of the silyl ether of glycolic acid **24** as its benzyl ester **25**, 10% overall yield. The latter was prepared for comparison from authentic glycolic acid **16**. That these protecting groups were appropriate ones was confirmed by their mild removal involving treatment of **25** with fluoride ion then hydrogenolysis to yield glycolic acid ready for stereochemical assay.

These procedures were then applied to the conversion of (11*R*)-[11-$^3H_1$]PBG **1a** into the bilane **5a**, Scheme 5, which was isolated in the silylated form **26a**. This was degraded by



Scheme 5

ozonolysis and the isolated product **25a** was deprotected to afford glycolic acid **16a**. Stereochemical assay of this sample by the reliable enzymic procedure went *via* **17**, **18** and **19**, Scheme 3. The ester **19** had lost most of its tritium so proving that material assayed was mainly (2*R*)-[2-$^3H_1$]glycolic acid **16a**, see Table 2.

The entire sequence was then repeated starting from (11*S*)-[11-$^3H_1$]PBG **1b**, the silylated bilane **26b** being converted as before *via* **25b** into a second sample of glycolic acid. Glycolate oxidase converted this material into **17a**, trapped as **18a** and finally purified as **19a**. This largely retained the ^{3}H-label, Table 2, so proving that the original glycolic acid had mainly the (2*S*)-configuration **16b**.

It follows that (11*R*)-PBG **1a** had been converted by hydroxymethylbilane synthase into the bilane **5a** having the (*R*)-configuration at the hydroxymethyl group. The results from the (11*S*)-PBG **1b** gave the complementary result *i.e.* the bilane had the (*S*)-configuration **5b**. Thus, the overall process occurs with *retention of configuration* as had also been found[1] when the aminomethylbilane **6** had been formed by trapping with ammonia.

Looking back at the mechanism of action of hydroxymethylbilane synthase, Scheme 1, the first PBG unit becomes

attached to the dipyrrolic cofactor and when the tetrapyrrole **3** has been built, it has to be detached almost certainly *via* the azafulvene **4**. Two conclusions can be drawn from our stereochemical studies. (a) When the azafulvene **4** reacts with water to form the hydroxymethylbilane **5** or is trapped by ammonia to afford the bilane **6,** the stereochemical outcome in both cases shows that only one of the enantiotopic faces of the terminal double bond in **4** is available to water or ammonia. Thus, the azafulvene cannot be released into the medium (this would lead to racemisation) and it must react in the enzymic active site. (b) Building the hydroxymethylbilane **5** involves two covalent bonds being formed at the centre that was initially C-11 of PBG **1**; these are one to the cofactor and one to water and the same holds true, but with ammonia rather than water, for formation of the aminomethylbilane **6** . Overall retention of configuration shows that either both reactions must occur with retention of configuration or both must occur with inversion. This can be understood if the mechanism of release of the tetrapyrrole system is the exact reverse of that involved in attaching the first PBG unit. A more detailed scheme proposed for the enzymic mechanism uses two retention steps[18] and draws on the determinations of the structure of the inactive oxidised form of hydroxymethylbilane synthase[19] and of the biologically active form of the enzyme.[20,21]

Experimental

General

For general directions, see ref. 1. The abbreviation dpm means disintegrations per minute. NMR and IR spectra were determined in $CDCl_3$ and $CHCl_3$, respectively, unless otherwise stated.

[*formyl*-^{3}H]-2-Formyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl-1-trifluoromethylsulfonylpyrrole 8b

To a solution of the formylpyrrole[6,7] **8** (1.54 g) in dichloromethane (64 cm^3) and methanol (32 cm^3) under argon at 0 °C was added sodium borohydride (12 mg) and the mixture was stirred at 0 °C for 5 min. Sodium [^{3}H]borohydride (3.35 mg, 25 mCi) was then added and after stirring for a further 5 min, sodium borohydride (270 mg) was added in small portions over *ca*. 5 min. Aqueous oxalic acid (1 g in 10 cm^3) was added 5 min later and the resultant aqueous phase was extracted with dichloromethane (4 × 20 cm^3). The combined organic phases were washed with water and saturated brine, dried and evaporated to yield the [^{3}H]alcohol (**9c** + **9d**) as an oil (1.52 g). Total activity 4.7×10^{10} dpm, spec. act. 1.2×10^{10} dpm mmol^{-1}.

All this product in dry dichloromethane (75 cm^3) was stirred under argon with pyridine–chromium trioxide[11] (3 g). After 15 h, the mixture was filtered through a short column of silica which was washed with diethyl ether and the filtrate was evaporated. Purification of the residue by flash chromatography with pentane–diethyl ether (1 : 1) gave the ^{3}H-formylpyrrole **8b** (1.0 g), spec. act. 9.5×10^9 dpm mmol^{-1}.

The foregoing procedures were developed using unlabelled materials and the products **9** and **8** were identified by comparison with standard samples.[6,7]

(*S*)-[*methylene*-^{3}H$_1$]-2-Hydroxymethyl-4-(2-methoxycarbonylethyl)-3-methoxycarbonylmethyl-1-trifluoromethylsulfonylpyrrole 9c

A mixture of 0.5 M 9-borabicyclo[3.3.1]nonane in tetrahydrofuran (THF, 2.5 cm^3) and (1*R*)-(+)-α-pinene (175 mg) under argon was heated at reflux for 2 h then cooled to room temperature. A solution of the foregoing [^{3}H]aldehyde (0.44 g) in dry THF (5 cm^3) was added and stirred for 3 h before mixing with acetaldehyde (0.5 cm^3). The THF was evaporated and the less volatile materials were removed at 0.1 mmHg pressure and 40 °C. To a solution of the residue in diethyl ether (20 cm^3) was added ethanolamine (0.1 cm^3) at 0 °C. The white precipitate was filtered off and washed twice with diethyl ether, the total organic solution then being washed twice with water, dried and evaporated. The residue was purified by PLC on silica using diethyl ether to give the mainly (*S*)-alcohol **9c** (385 mg, 88%), identified by comparison with an authentic unlabelled sample.

Similarly, use of (1*S*)-(−)-α-pinene in the foregoing procedure afforded mainly the (*R*)-alcohol **9d**.

(11*R*)-[11-^{3}H$_1$]Porphobilinogen lactam methyl ester 13a and its (11*S*)-enantiomer 13b

A solution of hydrazoic acid in benzene was prepared by first making a paste of sodium azide (19.5 g) with warm water (19.5 g). Benzene (120 cm^3) was added and the stirred mixture at 0 °C was treated dropwise with concentrated sulfuric acid (total 8.3 cm^3) at such a rate that the cooled mixture was held below 10 °C. It was then cooled to 0 °C, the organic layer was decanted and dried. Titration of this solution showed it contained 1.11 mmol cm^{-3} of hydrazoic acid.

The foregoing solution (3.3 cm^3) was added to triphenylphosphine (393 mg) in dry benzene (20 cm^3) and to this mixture at 5 °C was added the (*S*)-alcohol **9c** (385 mg) in dry THF (4 cm^3) followed by diethyl azodicarboxylate (261 mg). This solution was warmed to room temperature, stirred for 18 h and then evaporated. The residue was purified by PLC on silica using dichloromethane–ethyl acetate (9 : 1) to give the azide **10a**, mp 63–64 °C from diethyl ether–hexane (333 mg, 80%), spec. act. 9.4×10^9 dpm mmol^{-1}. ν_{max}/cm^{-1} 2140 (N_3); δ_H 2.59 and 2.75 (each 2 H, t, *J* 7.2, CH_2CH_2), 3.50 (2 H, s, CH_2CO), 3.67 and 3.70 (each 3 H, s, OMe), 4.53 (2 H, s, CH_2N_3) and 6.94 (1 H, s, 2-H pyrrole); δ_C 20.1 (CH_2CH_2CO), 29.8 (CH_2CO), 32.8 (CH_2CH_2CO), 43.3 (CH_2N_3), 51.7 and 52.4 (2 × OMe), 121.0 (2 overlapped), 125.1, 128.2 (C=C), 119.0 (q, *J* 321, CF_3) and 169.9 and 172.6 (CO).

A solution of the azide **10a** in ethanol (26 cm^3) containing concentrated hydrochloric acid (0.16 cm^3) was stirred for 1 h at room temperature under hydrogen with palladium black (80 mg). The filtered solution was evaporated to leave the crystalline hydrochloride **11a** (351 mg). δ_H ([^{2}H$_6$]DMSO, 90 MHz) 2.35 and 3.16 (each 2 H, br, CH_2CH_2), 3.46 and 3.51 (each 3 H, s, 2 × OMe), 3.69 (2 H, s, CH_2CO), 4.00 (2 H, s, $CH_2\overset{+}{N}$), 7.20 (1 H, s, 2-H pyrrole) and 8.45 (3 H, br, $\overset{+}{N}H_3$).

The foregoing salt, without purification, was dissolved in dichloromethane–methanol (9 : 1, 7 cm^3) and stirred for 5 min at room temperature with saturated aqueous sodium carbonate (1 cm^3). The mixture was shaken with water (3 cm^3) and the separated aqueous layer was extracted five times with dichloromethane. After the combined organic solution had been washed with saturated brine, it was dried and evaporated. The residue by PLC on silica using chloroform–methanol (9 : 1) afforded the lactam **12a** (225 mg, 80%). δ_H 2.54–2.71 (4 H, m, CH_2CH_2), 3.37 (2 H, t, *J* 4, CH_2CO), 3.68 (3 H, s, OMe), 4.58 (2 H, br t, CH_2N) and 6.87 (1 H, s, 2-H pyrrole); δ_C 19.9 (CH_2CH_2CO), 28.2 (CH_2CO) 32.8 (CH_2CH_2CO), 40.7 (CH_2N), 51.7 (OMe), 119.9, 121.3, 123.3, 126.8 (C=C), 119.0 (q, *J* 321, CF_3) and 169.1 and 172.4 (CO). Reduction of the azide *e.g.* **10a** could also be carried out with chromous chloride, all solvents being deoxygenated with argon. To a stirred solution of the azide (51 mg) in acetone–water (2 : 1, 3 cm^3) at 0 °C was added dropwise chromous chloride (61 mg) in water (1 cm^3). The solvents were evaporated and the resultant crude salt **11a** was converted as above into the lactam **12a** (41 mg, 90%). This product was identical to the foregoing one.

The lactam **12a** (225 mg, 0.64 mmol) in dry methanol (16 cm^3) was stirred with sodium methoxide in methanol (2 cm^3, 3.2 mmol) for 3.5 h at 18 °C. Saturated aqueous ammonium chloride (2 cm^3) was added and the solution was extracted seven times with chloroform–methanol (9 : 1). The combined organic

solution was washed once with water, dried ($MgSO_4$) and evaporated. Purification of the residue by column chromatography on silica, eluant chloroform–methanol (95 : 5) and PLC on silica with same solvents (85 : 15) gave *(11R)-[11-$^3H_1$]porphobilinogen lactam methyl ester* **13a** (96 mg, 80%) as white crystals after crystallisation (precipitation) from chloroform–methanol. It was identified by spectroscopic and chromatographic comparison with authentic material;[22] specific activity 9×10^9 dpm $mmol^{-1}$.

The (*R*)-alcohol **9d** was carried through the equivalent steps **10b**→**11b**→**12b** finally to yield *(11S)-[11-$^3H_1$]porphobilinogen lactam methyl ester* **13b**, specific activity 8.9×10^9 dpm $mmol^{-1}$. The yields were at least as good as those reported for the (11*R*)-series.

Degradation of (11*R*)-[11-$^3H_1$]PBG lactam methyl ester 13a and of its (11*S*)-isomer 13b to ^3H-labelled glycolic acids

Silica (5 g, 60–120 mesh) was added to a solution of the (11*R*)-lactam **13a** (10 mg, total activity 4.05×10^8 dpm) in chloroform–methanol (7 : 3, 10 cm^3) and the solvents were evaporated. The solid was resuspended in the same mixed solvent which was evaporated again; this was repeated twice more and finally thrice using chloroform. The coated silica was cooled to −78 °C and ozonised oxygen (40 l of O_2 per h) from the generator at full power was passed into it for 2.5 h. After the silica had warmed to room temperature, it was extracted first with methanol–ethyl acetate (1 : 1, 50 cm^3) then with ethanol (30 cm^3) and the extracts were evaporated. A solution of the residue in 6 M hydrochloric acid (3 cm^3) was heated in a sealed tube for 10 h at 120 °C; the tube had been cleared of oxygen before sealing. The cooled contents of the tube were filtered, unlabelled glycine (50 mg) was added and the solution was adjusted to pH 7 using 5 M aqueous potassium hydroxide initially then 1 M KOH. It was then run onto an ion-exchange column (Amberlite IR 120, H^+ form; 1 × 14 cm) which was washed with water (70 cm^3) and eluted with 2 M aqueous ammonia (150 cm^3). The fractions containing glycine, detected by testing aliquots with ninhydrin, were pooled and evaporated. Pure glycine (28 mg) was isolated from the residue by sublimation at 200–210 °C (0.1 mmHg) and an aliquot (1.378 mg) was accurately diluted with unlabelled glycine (54.073 mg) and then converted into its *N*-benzoyl derivative by the standard method. After this derivative had been multiply recrystallised from aqueous methanol, it showed a constant specific activity of 1.07×10^7 dpm $mmol^{-1}$, corresponding to a value of 4.3×10^8 dpm $mmol^{-1}$ for the sublimed glycine. Thus the total activity in the [2-$^3H_1$]glycine **15a** produced by the ozonolysis–hydrolysis was 2.9×10^8 dpm (yield 71%); this high yield was out of line with the usual ones reported in the text (20–35%).

The (11*S*)-isomer **13b** was degraded in the same way to afford sublimed [2-$^3H_1$]glycine **15b** (38 mg) having specific activity 1.28×10^8 dpm $mmol^{-1}$, yield 21%.

The [2-$^3H_1$]glycine **15a** from the (*R*)-series (26 mg) was diluted with unlabelled glycine (49 mg) and then was dissolved in 1 M hydrochloric acid (1 cm^3). This was added dropwise under argon to a stirred solution of sodium nitrite (100 mg) and sodium chloride (105 mg) in water (1 cm^3) at room temperature.[13] After 15 h, the mixture was adjusted to *ca.* pH 1 with 1 M hydrochloric acid and continuously extracted with diethyl ether overnight to afford [2-$^3H_1$]glycolic acid **16a**. This was sublimed twice at 60 °C/0.1 mmHg and the pure glycolic acid (10 mg) was mixed with [1-^{14}C]glycolic acid (16 mg) and resublimed to give [2-$^3H_1$,1-^{14}C]glycolic acid. An aliquot (1.311 mg) was diluted with unlabelled glycolic acid (10.298 mg) before being converted as usual into its *p*-bromophenacyl ester[13] for crystallisation from dichloromethane–hexane to constant specific activities and ^3H : ^{14}C ratio of ^3H 1.91×10^6, ^{14}C 8.85×10^5 dpm $mmol^{-1}$, ^3H : ^{14}C 2.16. Thus the [2-$^3H_1$, 1-^{14}C]glycolic acid before the final dilution had specific activities ^3H 1.69×10^7, ^{14}C 7.84×10^6 dpm $mmol^{-1}$ and ^3H : ^{14}C ratio 2.16. Treatment of the [2-$^3H_1$]glycine **15b** from the (*S*)-series in the same way gave [2-$^3H_1$,1-^{14}C]glycolic acid (as **16b**) having specific activities ^3H 1.46×10^7, ^{14}C 1.38×10^7 dpm $mmol^{-1}$, ^3H : ^{14}C ratio 1.06.

Stereochemical assay of the labelled glycolic acids 16a and 16b by glycolate oxidase

The reliable conditions for the enzymic oxidation were developed using (2*RS*)-[2-$^3H_1$]glycolic acid (**16a** + **16b**), prepared below, which had been mixed with [1-^{14}C]glycolic acid to give a ^3H : ^{14}C ratio of 2.49, ^{14}C activity 5×10^4 dpm mg^{-1}.

The foregoing glycolic acid (1.55 mg), oxalic acid (5.92 mg), K_2HPO_4 : KH_2PO_4 mixture corresponding to a pH 8.3 buffer (37 mg) and hydroxylamine hydrochloride (3.45 mg) were dissolved in water (1.5 cm^3), the pH was adjusted to 8.3 by addition of 1 M sodium hydroxide and water was added to give a total volume of 2.8 cm^3. To this solution were added flavine adenine mononucleotide (0.18 mg) and catalase (0.6 mg), the stirred mixture was warmed to 30 °C and glycolate oxidase[14,15] (50 units in 0.7 cm^3 of water) was then added. The reaction was stopped after 26 min by adding aqueous 5% (w/v) trichloroacetic acid (0.5 cm^3) followed by the unlabelled oxime of glyoxylic acid (60 mg) in water (2 cm^3). After the precipitated protein had been removed by centrifugation, the solution was adjusted to pH 1 with 1 M sulfuric acid, saturated with ammonium sulfate and extracted continously overnight with diethyl ether. Evaporation of the dried ethereal solution left a residue which was sublimed at 110 °C/0.1 mmHg to give the oxime of glyoxylic acid[23] (52 mg), mp 139 °C. This was converted by the standard method into its *p*-bromophenacyl ester for recrystallisation twice from hexane–ethyl acetate, then twice from aqueous methanol and finally once from hexane–ethyl acetate. The ^3H : ^{14}C ratio remained constant after the first 2 recrystallisations at 1.25 corresponding to 50% retention of tritium.

The [2-$^3H_1$,1-^{14}C]glycolic acid (as **16a**) from degradation of the PBG lactam methyl ester **13a** in the (*R*)-series (1.55 mg), ^3H : ^{14}C ratio 2.16, was oxidised exactly as above save that less oxime of glyoxylic acid (50 mg) was added. The isolated oxime (27 mg) as its *p*-bromophenacyl ester was purified as before to a constant ^3H : ^{14}C ratio of 0.34 corresponding to 16% (and in a repeat run, 15%) retention of tritium. Thus this sample contained 84% (85%) (*R*)-[2-$^3H_1$]glycolic acid **16a** and 16% (15%) of the (*S*)-isomer **16b** (second column, Table 2).

Enzymic oxidation of the (*S*)-glycolic acid (1.55 mg), ^3H : ^{14}C ratio 1.06, in the same way gave the oxime of glyoxylic acid as its *p*-bromophenacyl ester showing a ^3H : ^{14}C ratio of 0.78 (repeat 0.78) corresponding to 74% (74%) retention of tritium. This showed that the original sample contained 26% (*R*)-[2-$^3H_1$]glycolic acid **16a** with 74% of the (*S*)-isomer **16b** (second column, Table 2).

Preparation of (2*RS*)-[2-$^3H_1$]glycolic acid 16a + 16b

Freshly distilled glyoxylic acid benzyl ester[24] (492 mg), showing δ_H 5.28 (2H, s, ArCH_2), 7.35 (5H, s, ArH), 9.35 (1H, s, CHO), in dry THF (15 cm^3) at 0 °C was stirred while unlabelled sodium borohydride (56 mg) in admixture with $NaBH_3T$ (*ca.* 2 mg, 350 mCi $mmol^{-1}$) was added. After 15 min, the mixture was partitioned between saturated brine and dichloromethane and the aqueous layer was further extracted with dichloromethane. The dried organic extracts by evaporation gave a mixture of benzyl alcohol (116 mg) and the benzyl ester of (2*RS*)-[2-$^3H_1$]glycolic acid (273 mg, 55%), specific activity 4.9×10^{10} dpm $mmol^{-1}$ which were separated by PLC on silica using hexane–ethyl acetate (8 : 2).

The foregoing benzyl ester (187 mg) in THF (10 cm^3) was shaken under hydrogen with 10% palladium on carbon (30 mg) for 45 min. Evaporation of the filtered solution and sublim-

ation of the residue as above afforded (2*RS*)-[2-$^3H_1$]glycolic acid (77 mg, 88%), identified by comparison with an authentic sample.

Brief description of trial experiments and preparation of simple derivatives of glycolic acid

(a) Silylations. The alcohol[6] **22** (97 mg) was dissolved in a mixture (5 cm^3) prepared from aqueous 0.6 M KOH (50 cm^3) and THF (25 cm^3) and stirred at room temperature for 3 h. After the THF had been evaporated, the solution was adjusted to pH 10.2 with aqueous 6% perchloric acid then filtered and freeze dried to yield a solid (162 mg). Part of the residue (44 mg) containing the sodium salt of **20** was dissolved in dimethylformamide, DMF, (0.5 cm^3) and treated first with Hünig's base (150 mg) then with *tert*-butyldimethylsilyl chloride (132 mg). The mixture was stirred under argon at room temperature for 10 min then the addition of base and silyl chloride was repeated and after 1 min more, ethyl acetate (10 cm^3) was added. After the solution had been washed with water (3 × 5 cm^3), it was dried and evaporated to yield the silyl derivative **21** as an oil (29 mg, 52%) which showed only minor impurities by NMR. δ_H (CD_2Cl_2, 100 MHz) 0.09 (6 H, s, $SiMe_2$), 0.22 and 0.24 (each 6 H, s, CO_2SiMe_2), 0.90 (9 H, s, Bu^t), 0.94 (18 H, s, CO_2SiBu^t), 2.62 (4 H, m, CH_2CH_2CO), 3.41 (2 H, s, CH_2CO), 4.66 (2 H, s, CH_2O), 6.50 (1 H, s, 2-H pyrrole), 8.0 (1 H, br s, NH): *m*/*z* 569, $C_{28}H_{55}NO_5Si$ requires 569.

The pyrrole **22** (50 mg) in dry DMF (0.5 cm^3) was silylated using Hünig's base (0.1 g) and *tert*-butyldimethylsilyl chloride (60 mg) as above and worked up in a similar way to give the silyl ether **23** as an oil (61 mg, 82%) which was essentially pure by NMR. δ_H (CD_2Cl_2, 100 MHz) 0.10 (6 H, s, $SiMe_2$), 0.93 (9 H, s, Bu^t), 2.65 (4 H, m, CH_2CH_2CO), 3.43 (2 H, s, CH_2CO), 3.66 (6 H, s, 2 × OMe), 4.67 (2 H, s, CH_2O), 6.51 (1 H, d, *J* 2, 2-H pyrrole), 8.1 (1 H, br s, NH).

(b) Ozonolysis. The silyl ether **23** (37 mg) was spread on silica (2 g) as earlier but using dry diethyl ether as solvent and the ozonolysis was carried out in the same way but for 30 min at −70 °C and 1 h at room temperature. The products were eluted from the silica with ethyl acetate and the residue from evaporation in methanol (3 cm^3) was treated with an excess of phenyldiazomethane. Purification of the product by PLC on silica using diethyl ether–pentane (1:6) gave the benzyl ester **25** (2.8 mg, 10%) identified by comparison with the standard sample prepared below.

(c) Protection and deprotection of glycolic acid 16. The benzyl ester of glycolic acid was prepared in the way above. Alternatively, glycolic acid (665 mg) in diethyl ether (40 cm^3) was stirred while a solution (40 cm^3) of phenyldiazomethane (prepared[25] in diethyl ether from 1.86 g azibenzil) was added dropwise over 30 min. The small excess of reagent was removed by addition of a little glycolic acid and the pale yellow solution was washed with saturated aqueous sodium hydrogen carbonate, dried and evaporated. Chromatography on silica (3 × 15 cm) eluting with ethyl acetate–hexane (1:9) removed yellow material and further elution with ethyl acetate–hexane (1:1) afforded benzyl glycolate. Distillation (Kugelrohr) at 170 °C/2 mmHg gave the pure ester as an oil (1.3 g, 67%). δ_H 2.38 (1 H, br s, OH), 4.19 (2 H, s, CH_2CO), 5.22 (2 H, s, CH_2Ar) and 7.36 (5 H, s, ArH).

A solution of this ester (1.0 g) in dry DMF (7 cm^3) was stirred for 12 h with imidazole (1.0 g) and *tert*-butyldimethylsilyl chloride (1.17 g) and then mixed with ethyl acetate (50 cm^3). The solution was washed thrice with brine, dried ($MgSO_4$) and evaporated. The residue by Kugelrohr distillation at 120 °C/0.3 mmHg afforded the silylated ester **25** as an oil (1.67 g, 92%). δ_H 0.08 (6 H, s, $SiMe_2$), 0.90 (9 H, s, Bu^t), 4.28 (2 H, s, CH_2CO), 5.17 (2 H, s, CH_2Ph) and 7.35 (5 H, s, Ar).

The silyl protecting group was removed from **25** by stirring it (100 mg) for 45 min at room temperature in a mixture (0.5 cm) prepared from acetonitrile (9.5 cm^3) and 40% aqueous hydrogen fluoride (0.5 cm^3). Saturated aqueous sodium hydrogen carbonate (2 cm^3) was added and the mixture was extracted thrice with dichloromethane to give benzyl glycolate (45 mg) identical to the material above. The benzyl group was then removed by hydrogenolysis as already described to yield glycolic acid which was sublimed at 90 °C/0.1 mmHg (19 mg, 88%).

Enzymic conversion of (11*R*)-[11-$^3H_1$]PBG 1a and the (11*S*)-isomer 1b into the bilanes 5a and 5b and determination of their configurations

(d) Immobilised hydroxymethylbilane synthase. Sepharose, activated by cyanogen bromide (Sigma, 2 g) was swollen in 1 M hydrochloric acid (50 cm^3) and washed on a sinter with 1 mM hydrochloric acid (400 cm^3) then with a solution (300 cm^3) containing sodium hydrogen carbonate (0.1 M) and sodium chloride (0.5 M). Purified hydroxymethylbilane synthase[4] was dialysed against the second of the washing solutions above and part of it (2 × 10^5 units) was diluted to 25 cm^3 with the dialysis solution and cooled to 5 °C. Unlabelled PBG was then added to *ca*. 2.2 mol PBG per mol of enzyme and after 20 min, the above washed Sepharose was added. The mixture was shaken at 5 °C for 23 h at such a rate that the gel remained suspended and it was then collected; assay of the filtrate showed the absence of enzyme. The gel was resuspended in aqueous 0.2 M glycine (30 cm^3, pH 8.2) and shaken at 5 °C as before for 23 h to block any remaining reactive groups on the Sepharose.

After the gel had been washed with the above dialysis solution (300 cm^3), it was equilibrated with 50 mM Tris-HCl buffer, pH 8.0 and packed into a jacketed column.

(e) Enzymic production of bilanes 5a and 5b. The foregoing column was held at 36–37 °C while a solution (300 cm^3) of the (11*R*)-PBG **1a** (0.20 mg cm^{-3}) in 50 mM Tris-HCl buffer, pH 8 was passed through at 1.1 cm^3 min^{-1} into stirred aqueous 2 M potassium hydroxide (15 cm^3) containing sodium dithionite (300 mg) under argon at 0 °C. The preparation of **1a** from the lactam **13a** was as usual.[1] Assay[26] showed that hydroxymethylbilane **5a** was present (6.4 mg). The alkaline solution was evaporated on a high vacuum rotatory evaporator (water bath < 30 °C) to *ca*. 10 cm^3 and run onto a Sephadex G-10 column (4 × 14 cm) eluting with 0.1 M aqueous KOH to separate the Tris. The Ehrlich-positive fractions (30 cm^3) were combined, freeze dried and the residue was assayed again which showed that sufficient hydroxymethylbilane **5a** (1.6 mg) had survived. A solution of this solid in water (10 cm^3) was *carefully* adjusted to pH 12 by dropwise addition of 0.6 M aqueous perchloric acid. The filtered solution was then freeze dried and the residue dissolved under argon in dry DMF (1.8 cm^3) containing Hünig's base (870 μl). The following additions were made at the indicated times: *tert*-butyldimethylsilyl chloride (525 mg, start), Hünig's base (870 μl, 10 min), *tert*-butyldimethylsilyl chloride (525 mg, 13 min), Hünig's base (670 μl) and the silyl chloride (525 mg) together at 20 min. After 30 min, ethyl acetate (40 cm^3) containing Hünig's base (0.5 cm^3) was added and the solution was extracted twice with half saturated aqueous sodium hydrogen carbonate then with brine, dried and evaporated to an oil (250 mg).

(f) Degradation of 5a and 5b to glycolic acids followed by stereochemical assay. A solution of the foregoing oil in dichloromethane (50 cm^3) was mixed with silica (20 g) as for earlier examples and the solvent was evaporated. The dissolution and evaporation were repeated twice from dichloromethane (2 × 30 cm^3) and this solid was dried at 0.1 mmHg. The ozonolysis was carried out as above but for 1 h at −78 °C and 15 min at room temperature. Then the silica was

extracted with methanol–ethyl acetate (1:1, 40 cm^3) and methanol (30 cm^3), the combined extracts were evaporated to 10 cm^3 and mixed with unlabelled glycolic acid silyl ether **24** (57 mg). This solution was treated with phenyldiazomethane in diethyl ether as under (c) above and evaporated to an oil (600 mg) which was purified by flash chromatography on silica eluting with hexane–ethyl acetate (95:5), then by PLC twice with the same solvent and finally by PLC with pentane–diethyl ether (9:1). The resultant oil was the chromatographically pure benzyl ester of silylated glycolic acid **25a** (60 mg, total ^{3}H-activity 1.5×10^5 dpm). Both protecting groups were removed as under (c) and the product was sublimed as earlier to give [2-$^3H_1$]glycolic acid **16a** (11.2 mg, total ^{3}H-activity 7.9×10^4 dpm). This was mixed with sufficient [2-^{14}C]-glycolic acid to give a ^{3}H:^{14}C ratio of *ca*. 2 and an aliquot (0.862 mg) was mixed with unlabelled glycolic acid (4.864 mg) and converted into its *p*-bromophenacyl ester as usual. Purification twice by PLC using hexane–ethyl acetate (3:7) followed by multiple recrystallisation from hexane–ethyl acetate gave material of constant specific activity for ^{3}H and ^{14}C with a ^{3}H:^{14}C ratio of 2.01.

The main sample of the foregoing glycolic acid **16a** (6.2 mg) was used as substrate for glycolate oxidase essentially as described earlier save that less unlabelled glyoxylic acid oxime (8 mg) was used as diluent. The final *p*-bromophenacyl ester of the labelled oxime **19** after purification to constant ^{3}H and ^{14}C activities showed a ^{3}H:^{14}C ratio of 0.53. Thus the original glycolic acid contained 74% (*R*)-species **16a** and 26% (*S*)-species **16b** and so the bilane from which it was derived was mainly (*R*)-bilane **5a** (Table 2).

The entire procedure described under (e) and (f) was then repeated exactly starting from (11*S*)-PBG **1b** (61 mg); just the quantities of key products and their activities are reported. Hydroxymethylbilane **5b** (3.5 mg) was preserved through to the silylation step and the product was degraded to the protected and diluted glycolic acid **26b** (41 mg, total ^{3}H-activity 1.75×10^5 dpm). This was deprotected to afford [2-$^3H_1$]glycolic and **16b** (7.3 mg, total ^{3}H-activity 1.3×10^4 dpm) which was mixed with [2-^{14}C]glycolic acid to give a ^{3}H:^{14}C ratio of 1.06 determined as before on the *p*-bromophenacyl ester. Stereochemical assay with glycolate oxidase afforded finally the oxime of glyoxylic acid as its *p*-bromophenacyl ester (mainly **19a**) showing a ^{3}H:^{14}C ratio of 0.72. It follows that the original glycolic acid contained 68% (*S*)-species **16b** and 32% (*R*)-species **16a**, so the bilane from which it was derived was mainly the (*S*)-bilane **5b** (Table 2).

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