Synthesis of (11*S*)- and (11*R*)-[11-$^2H_1$]Porphobilinogen; Stereochemical Studies on Hydroxymethylbilane Synthase (PBG Deaminase)

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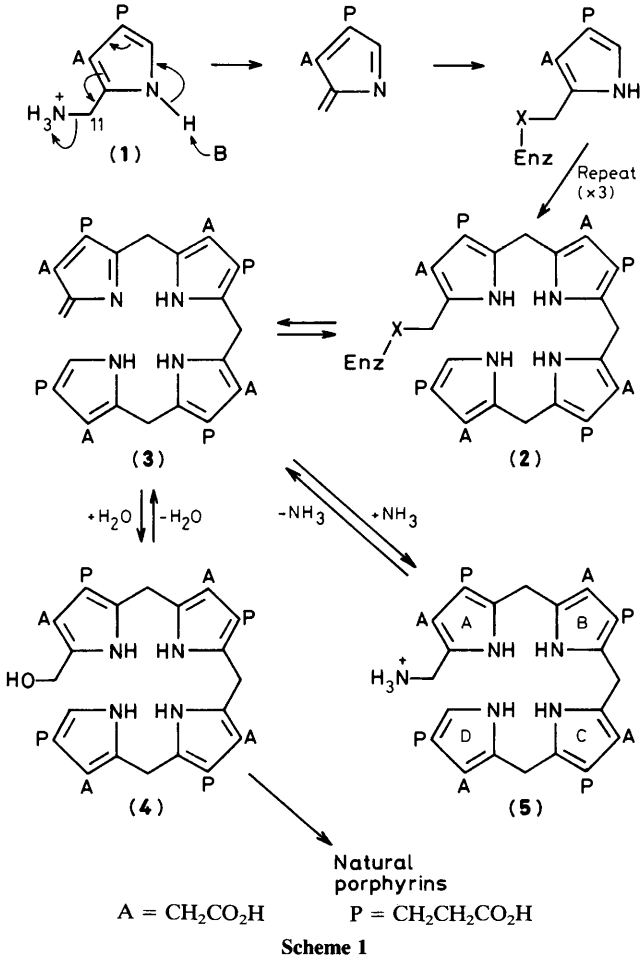
(11*S*)- and (11*R*)-[11-$^2H_1$]Porphobilinogen (PBG) are synthesized and their configurations are established by degradation to a derivative of $^2H_1$-glycine; they are used to prove that when hydroxymethylbilane synthase (PBG deaminase) acts on PBG in the presence of ammonia, aminomethylbilane is formed with overall *retention of configuration* at the NH_2CHD-group.

Hydroxymethylbilane synthase (EC 4.3.1.8), previously called porphobilinogen deaminase, acts in the early stages of biosynthesis of the natural porphyrins.[1] Its role is to assemble four molecules of porphobilinogen (**1**), PBG, to form the unrearranged hydroxymethylbilane[1,2] (**4**), Scheme 1. There is strong evidence[3] that the bilane (**4**) is formed *via* the azafulvene (**3**) and the trapping of this highly reactive intermediate by ammonia accounts for the formation[4] of aminomethylbilane (**5**) when PBG (**1**) is incubated with the enzyme in the presence of ammonia (introduced as NH_4^+ ions). It was demonstrated[3] that the amino group of this aminomethylbilane (**5**) is not that of the original PBG (**1**) but is derived from the added ammonium ions.

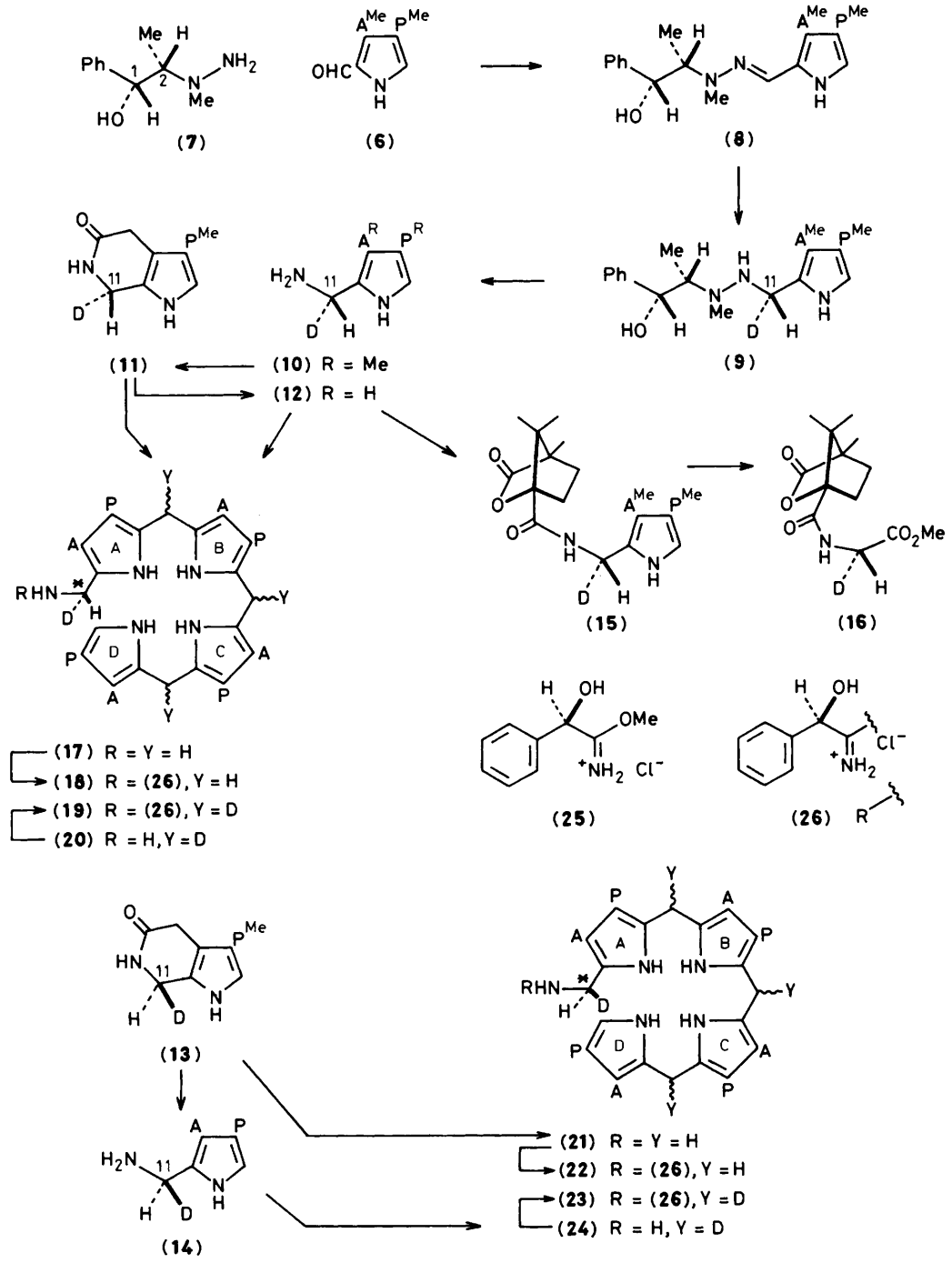
As part of our detailed study of the stereochemistry of action of hydroxymethylbilane synthase, we planned first to discover the overall stereochemical change as the aminomethyl group of PBG (**1**) is converted through several steps (see Scheme 1) into the aminomethyl group of the bilane (**5**). Accordingly, chiral samples of PBG, as (**1**), labelled with ^2H at the 11-methylene group were synthesized as follows; (11*S*)-[11-$^3H_1$]PBG has been previously made by enzymic methods.[5]

The aldehyde[6] (**6**) condensed with the hydrazine (**7**) derived[7] from (−)-(1*R*,2*S*)-ephedrine to yield the hydrazone (**8**), 90%. This was reduced under high dilution conditions in tetrahydrofuran with $NaBD_3CN$ in the presence of deuteriated toluene-*p*-sulphonic acid ($ArSO_3D$). ^1H N.m.r. spectroscopy showed that the deuteriated product (95% of total material) consisted of the pyrrole (**9**), 83%, proved later to have the illustrated (11*S*)-configuration. Thus, 17% of the deuteriated material had the (11*R*)-configuration [(**9**) epimeric at C-11] and only 5% of the total sample consisted of undeuteriated molecules.

This sample was cleaved by hydrogenation over platinum in 15% v/v acetic acid in methanol to yield the dimethyl ester of (11*S*)-[11-$^2H_1$]PBG (**10**) as its acetate salt, 83% of the



Scheme 1



deuteriated material. When this was heated in methanol containing sodium carbonate, it cyclized to give the crystalline (11*S*)-[11-$^2H_1$]PBG lactam ester (**11**), 65% yield over (**9**) → (**11**).

A strictly complementary synthesis based on (+)-(1*S*,2*R*)-aminoephedrine [enantiomer of (**7**)] afforded (11*R*)-[11-$^2H_1$]PBG lactam ester, (**13**). This represented 83% of the deuteriated material which again formed 95% of the total product.

The configuration (**11**) for the major (11*S*)-component (83% of $^2H_1$-species) in the PBG lactam ester was established by degradation, the first step being alkaline hydrolysis to yield (11*S*)-[11-$^2H_1$]PBG (**12**). The corresponding camphanamide, prepared using (−)-(1*S*,4*R*)-camphanic acid chloride in a two-phase acylation (toluene–aqueous KOH), was esterified with diazomethane and the amide (**15**) was spread on silica[8] and oxidized with ozone (−70 °C for 0.5 h, then −70 °C to 18 °C during 1 h). After treatment of the products with

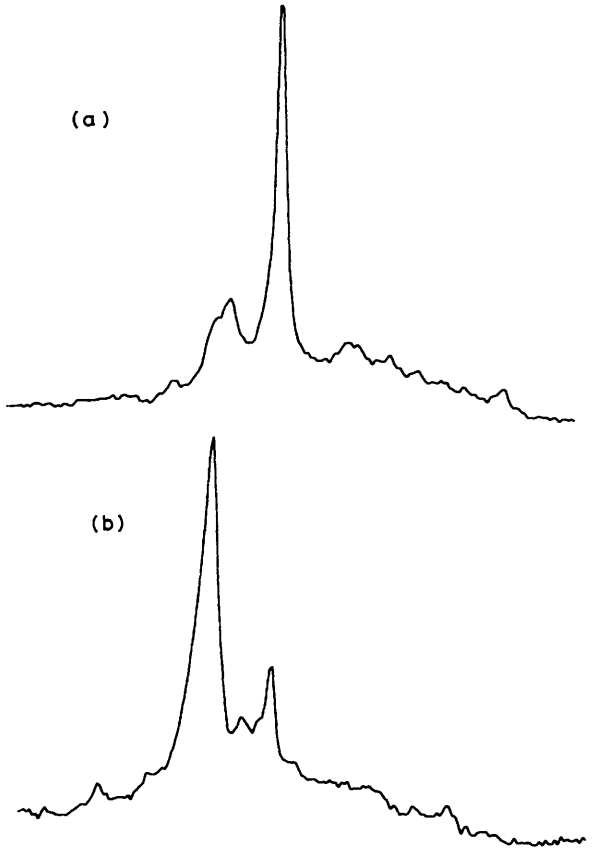


Figure 1. ^{1}H N.m.r. signals (400 MHz) from the asterisked centre of (a) (*R*)-amidine (**22**) and (b) (*S*)-amidine (**18**). Both spectra run in CD_3OD.

diazomethane, *N*-camphanoyl glycine methyl ester[9] was isolated (20% yield), shown by n.m.r.[9] to have the (2*S*)-configuration (**16**) for its major component. The same degradative sequence was carried out on the (11*R*)-lactam (**13**) to yield (2*R*)-camphanoyl-[2-$^2H_1$]glycine methyl ester as the major product as shown by n.m.r.[9]

Earlier syntheses[6,10] of the aminomethylbilane (**5**) used the unlabelled form of the lactam, as (**11**), to provide ring-A of (**5**). This same approach was followed but now using the labelled (*S*)-lactam (**11**) to afford the bilane having the (*S*)-configuration at the asterisked centre (**17**). The (*R*)-aminomethylbilane (**21**) was similarly synthesized from the (*R*)-lactam (**13**).

These samples of $^2H_1$-labelled (*S*)- and (*R*)-bilane (**17**) and (**21**), of known configuration by synthesis, were prepared as standards to allow a method to be developed for assay of the configuration at the asterisked centre in samples of unknown stereochemistry. Attachment of a chiral residue to the NH_2CHD-group of (**17**) and (**21**) should in principle afford an assay by n.m.r. spectroscopy. However, many obvious and less obvious methods for attaching such a chiral group all failed owing to the strongly hydrophilic nature of these bilanes and the ease with which they undergo non-enzymic ring-closure to uro'gen-I.[10] The chiral imino ether hydrochloride (**25**) prepared from (+)-mandelonitrile[11] provided the solution. This reacted smoothly in water at pH 9.7 with the (*S*)-aminomethylbilane (**17**) to yield the amidine (**18**) which was readily freed from excess of reagent; the solution of amidine was finally adjusted to pH 7.5. Figure 1(b) shows the ^{1}H n.m.r. signal from the asterisked centre in (**18**), Figure 1(a)

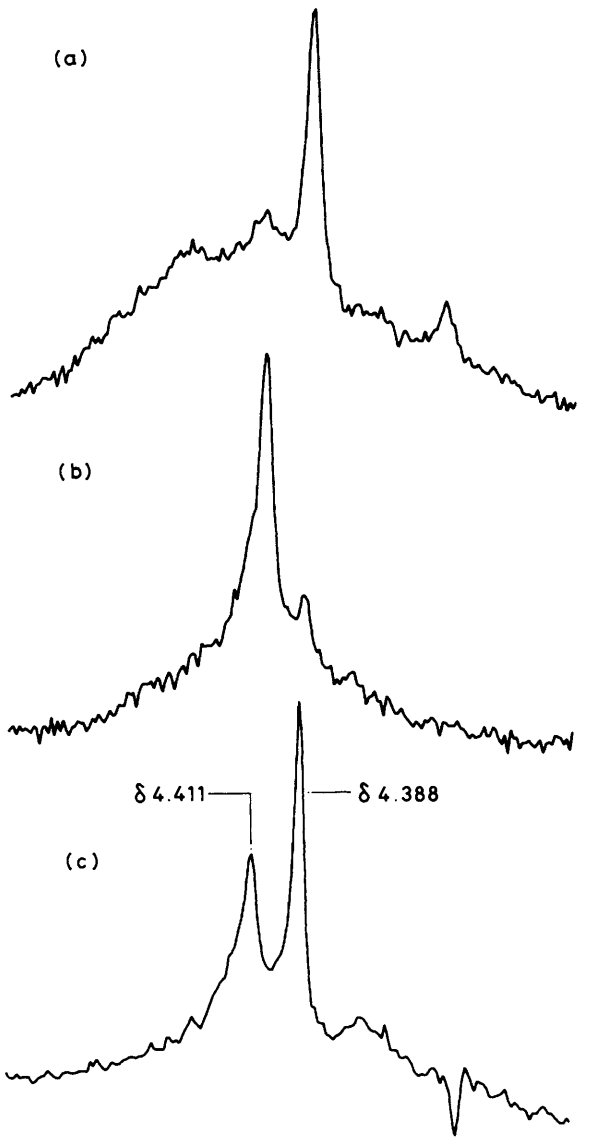


Figure 2. ^{1}H N.m.r. signals (400 MHz) from the asterisked centre (a) of the (*R*)-amidine (**23**) from the enzymic experiments, (b) of the (*S*)-amidine (**19**) from the parallel enzymic run, and (c) of a mixture of the synthetic (*R*)-amidine (**22**) with a smaller quantity of the synthetic (*S*)-amidine (**18**).

gives the corresponding signal from the amidine (**22**) similarly derived from the (*R*)-bilane (**21**), and Figure 2(c) shows the pattern when the (*S*)-amidine (**18**) was mixed with a larger quantity of the (*R*)-amidine (**22**). The assay method was thus available.

(11*S*)-[11-$^2H_1$]PBG (**12**) was incubated at pH 8.05 in the presence of 0.2 M NH_4Cl with more than sufficient hydroxymethylbilane synthase to consume >95% of the PBG in 0.5 h. The solution was adjusted to pH >13 and then freeze dried to remove ammonia; the resultant bilane (**20**) was converted as above into the amidine (**19**). The ^{1}H-signal from the asterisked centre in this product is shown in Figure 2(b) which matches Figure 1(b). It follows that the amidine has the configuration (**19**) and thus, (11*S*)-PBG (**12**) has been converted enzymically into the (*S*)-bilane (**20**).

This result was confirmed by repeating the entire sequence above now starting with (11*R*)-PBG (**14**). The final amidine

(**23**) showed a strong high-field signal, Figure 2(a), and even though the expected weak low-field signal was somewhat obscured, the match with Figure 1(a) was good. Finally, to the solution of the enzymically formed (*S*)-amidine (**19**) was added an aliquot of the enzymic (*R*)-amidine (**23**). The small high-field signal [see Figure 2(b)] increased in size; addition of a second portion of (*R*)-amidine (**23**) caused a further increase in the size of this signal. This precaution eliminates any uncertainty about signal-assignments by comparing (*S*) and (*R*) samples under identical conditions.

These experiments prove that when hydroxymethylbilane synthase acts on PBG (**1**) and the product is trapped as the aminomethylbilane (**5**), the overall stereochemical outcome is *retention of configuration.*

Scheme 1 summarises present knowledge of hydroxymethylbilane synthase.[3] PBG (**1**) is first covalently bound to the enzyme through a nucleophilic group. The normal product, hydroxymethylbilane (**4**), is in equilibrium with the bound form (**2**) *via* a highly reactive intermediate, probably the azafulvene[3] (**3**), which can be trapped by ammonia yielding the bilane (**5**). Thus two new covalent bonds are formed (one to X and one to NH_3) during the conversion (**1**) → (**5**), which as shown above occurs overall with retention of configuration. The separate processes for making these two covalent bonds must therefore each occur with inversion or *both* bond-forming processes must take place with retention. The finding that the aminomethylbilanes (**20**) and (**24**) formed above are not racemised is also important. On the basis that the azafulvene (**3**) is indeed the highly reactive intermediate leading to the bilane (**5**), Scheme 1, then the stereochemical results show that only one of the enantiotopic faces of (**3**) is available to ammonia. Thus, the azafulvene (**3**) reacts in the active site of the enzyme and is not first released into the medium.

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