N.M.R. Spectroscopy as a Probe for the Study of Enzyme-catalysed Reactions. Further Observations of Preuroporphyrinogen, a Substrate for Uroporphyrinogen III Cosynthetase

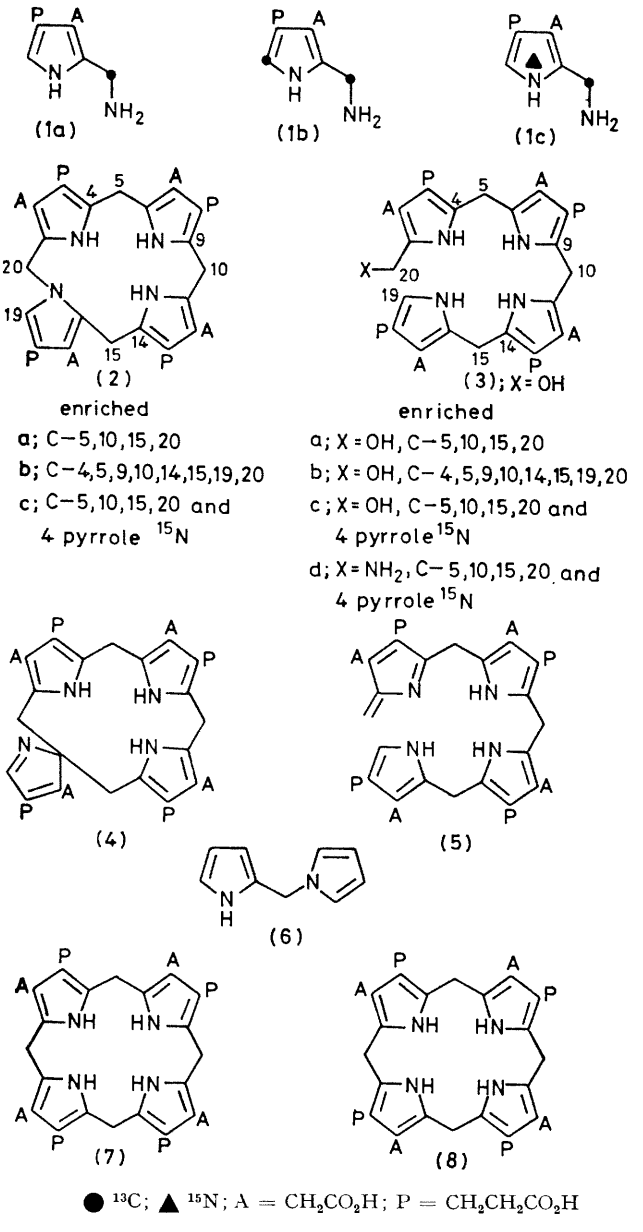
By A. IAN SCOTT,* GERARDO BURTON, PETER M. JORDAN, HIROO MATSUMOTO, PAUL E. FAGERNESS, and LANA M. PRYDE

(*Department of Chemistry, Texas A&M University, College Station, Texas* 77843)

Summary On the basis of n.m.r. and biochemical evidence, the hydroxymethylbilane structure (**3**) has been deduced for preuroporphyrinogen, the natural substrate for uro'gen III cosynthetase at pH 7·8—8·5 and 37 °C; at 0 °C formation of another species, *viz* the *N*-alkylpyrrole (**2**), is indicated, thus reconciling previously conflicting interpretation of these data.

IN earlier communications from this laboratory,[1,2] it was shown for the first time that application of ^{13}C and ^{15}N n.m.r. spectroscopy to the study of the reaction catalysed by porphobilinogen (PBG) deaminase (EC 4.3.1.8) from *Rhodopseudomonas spheroides* uncovered a novel, enzyme-free reactive species which we named preuro'gen. This substance ($t_{1/2}$ 3·5 min at 37 °C, pH 8·2) accumulated transiently in solution and was shown to be the substrate for cosynthetase, the second enzyme in the biosynthesis of uro'gen III (**7**). In the absence of cosynthetase, preuro'gen is chemically converted into uro'gen I (**8**).[3] These key experiments cast a new light on porphyrin biosynthesis, since until recently the view had been expressed[4] that deaminase and cosynthetase do not work as independent enzymes. The unique position occupied by a substrate for cosynthetase merited a detailed examination of its structure and reactivity, since this n.m.r. experiment showed that deaminase does not form uro'gen I directly but in fact synthesises preuro'gen which rearranges subsequently in a *chemical* reaction to uro'gen I. Thus although deaminase and cosynthetase have evolved to provide a coupled enzymatic system *under normal physiological conditions*, the process is sequential rather than synchronous *in vitro*.

Using [11-^{13}C]porphobilinogen (PBG) (**1a**) as substrate, the carbon-13 chemical shifts of preuro'gen [δ 22·00 (3C) and 54·78 (1C) p.p.m.] were consistent with *two* structures, the *N*-alkylpyrrole (**2a**) and the hydroxymethylbilane (**3a**)[1] observed at 0 °C. Evidence in favour of both structures (**2b**) and (**3b**) came from a double-labelled experiment in which [2,11-$^{13}C_2$]-PBG (**1b**) served as substrate. In this first experiment (Figure 1) the absence of coupling in the signals of the product at δ 54·78 and 22·12 p.p.m., together with the resonance at δ 113·69 p.p.m., are in accord with, but do not distinguish between, structures (**2b**) and (**3b**) derived from (**1b**) although structures such as (**3d**), (**4**), and (**5**) are eliminated. However, the doubly labelled ^{15}N–^{13}C substrate (**1c**), when incubated with deaminase at pH 8, gave a spectrum revealing clear evidence of one-bond ^{15}N–^{13}C coupling at δ 54·78 p.p.m. (J 6 Hz),[2] providing a strong case for the *N*-alkyl-tetrapyrrole labelled as in (**2c**), as the structure of preuro'gen at 0 °C, pH 7·8—8·5. The somewhat anomalous chemical shift[5] (*i.e.* 10—12 p.p.m. to lower field than expected) was ascribed to the unusual environment of $-CH_2-$ in the macrocycle (**2**). Moreover,



the 6 Hz splitting has been repeatedly observed (at 0 °C) in incubations of [1-^{15}N; 11-^{13}C]-PBG with deaminase at pH 7—8·5 with both 7·0 T and 4·7 T spectrometers. This result is to be contrasted with the two-bond coupling present in the substrate (**1c**) [^2J (^{13}C–^{15}N) 1·8 Hz] and several related pyrrolic substances synthesised in this

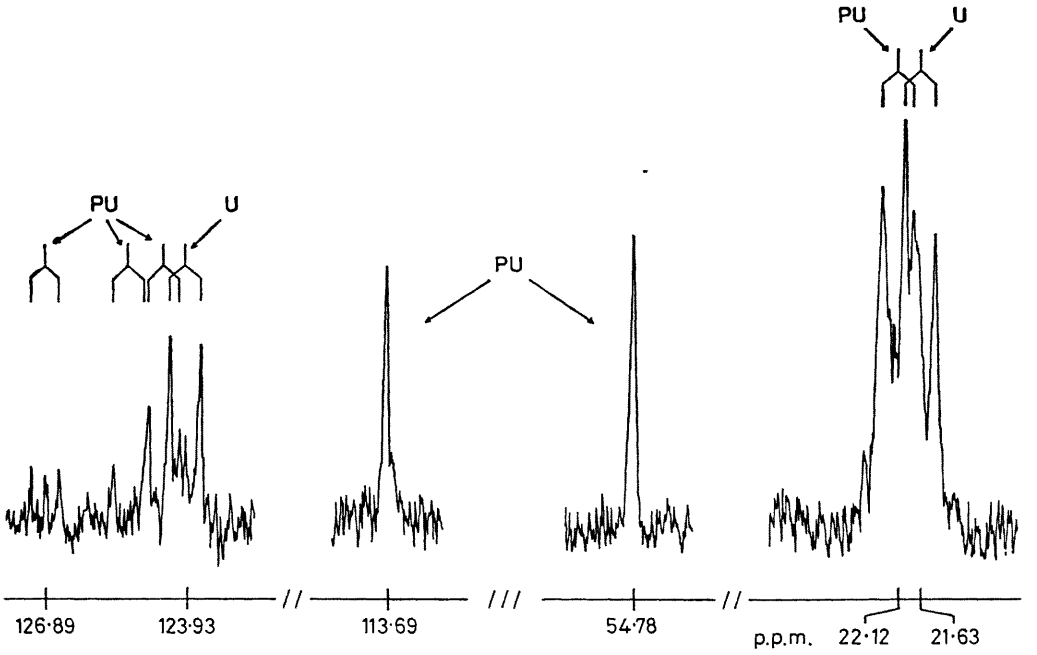


FIGURE 1. ^1H Decoupled ^{13}C n.m.r. spectrum at 75·5 MHz and 0 °C of a 3 min incubation of [2,11-$^{13}C_2$]-PBG with PBG deaminase (U = uro'gen I; PU = preuro'gen). 8000 pulses were accumulated over a spectral width of 10,000 Hz using a repetition rate of 0·6 s and 10% D_2O for internal lock. Chemical shifts referred to Me_4Si.

laboratory. These experiments were subsequently repeated at Cambridge, first using singly labelled [11-^{13}C]-PBG,[4] although in this case the pH was raised above physiological limits (pH 12) to provide a stable species with chemical shifts similar to those obtained originally in our laboratory. The hydroxymethylbilane structure (**3a**) also was suggested on the basis of good chemical shift analogy.[4] The close similarity of these spectra together with the intrinsic importance of the structure for the substrate of cosynthetase at pH 8 is now rationalised in the light of further experimentation.

We have shown that the reactive species formed from PBG by deaminase (after removal of deaminase) can tolerate reversible pH changes over the range pH 7·8—12 with little effect on its ability to act as a substrate for cosynthetase (at pH 7·8—8·5). Although the lifetime of the intermediate at pH 12 is increased ($t_{1/2}$ 40 min at 37 °C), its effectiveness to act as a substrate for cosynthetase remains unchanged. Thus we may assume that either the species at pH 7·8—8·5 discovered in our laboratory[1] and studied at Cambridge[4] are identical or that they are interconverted by the change in pH of the medium. To investigate the latter possibility, preuro'gen was enzymically generated by deaminase (pH 7·8), the pH adjusted to 12, and after 30 min at 37 °C the solution was cooled to 0 °C and rapidly brought to pH 7·8. On addition of cosynthetase there was immediate formation of uro'gen III (**7**), the amount of this isomer remaining constant for samples taken from the solution (at 0 °C) during 30 min, suggesting that the species observed at pH 7·8 and at pH 12 are either identical or in very rapid equilibrium. Furthermore, no difference in the rate of uro'gen III formation was observed using purified cosynthetase with a deaminase-free solution[3] of preuro'gen.

Information regarding the effect of pH on preuro'gen was obtained by incubation of [1-^{15}N; 11-^{13}C]-PBG (**1c**) with deaminase and observation of the ^{13}C and ^{15}N n.m.r. spectra between pH 7·8 and 8·5, and at pH 12. The 6 Hz doublet previously described (Figure 2a) at δ 54·78 p.p.m. in the ^{13}C spectrum[2] was not observed at pH 12, 0 °C (Figure 2b), indicating that either the structure of the intermediate was different at pH 7·8—8·5 and 12, or the ^{15}N–^{13}C coupling constant was reduced by a conformational change due to loss of H-bonding.[6] The latter hypothesis is supported by the ^{15}N n.m.r. spectrum where the four non-equivalent ^{15}N resonances in (**2c**) or (**3c**) observed at pH 8·2[2] coalesce to a single line at δ 154·5 p.p.m. when the pH is raised to 12 (reflecting the loss of H-bonds between the carboxylates and the pyrrole nitrogens) (Figure 3a,b).

The following data are also pertinent. (a) At pH 12, when treated with $^{15}NH_4OH$ or $^{15}NH_2OH$, the hydroxymethylbilane (**3a**) with CH_2 at position 20 labelled with ^{13}C did not exchange OH for ^{15}NHR (R = H, OH, *etc.*) as judged by the absence of ^{13}C–^{15}N coupling.[4] (b) At pH 7·8—8·5 preuro'gen (as **3c**) from [1-^{15}N; 11-^{13}C]-PBG reacted with NH_4^+ at 37 °C to give the amino-bilane (**3d**) as indicated by the upfield shift of the doublet at δ 54·78 to a singlet at δ 34·95 p.p.m. (*CH_2–NH_2) in the ^{13}C n.m.r. spectrum (Figure 2c), and the shifts and loss of ^{13}C–^{15}N coupling in the ^{15}N spectrum (Figure 3c). As this reaction [probably involving (**5**)[4]] is catalysed by deaminase, it would definitely fail to occur at high pH where the enzyme is not active.

In a recent publication,[7] the Cambridge group has confirmed our original finding[3] in that the species released from deaminase at pH 7—8·5 is the substrate for cosynthetase and has $t_{1/2}$ 4 min at 37 °C. Their substance has the same ^{13}C n.m.r. spectrum at pH 8·25 and 12, corresponding to a synthetic sample of the hydroxymethylbilane (**3**) whose

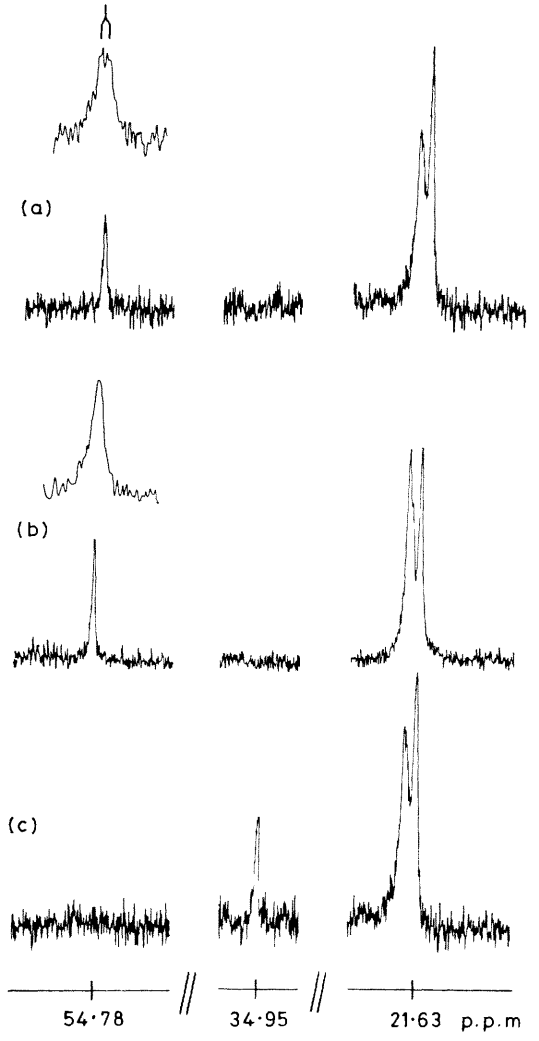


FIGURE 2. ^{1}H Decoupled ^{13}C n.m.r. spectra at 50·5 MHz and 0 °C of 3 min incubations of [1-^{15}N; 11-^{13}C]-PBG with PBG deaminase: (a) pH 8·2, (b) adjusted to pH 12 with NaOH, and (c) treated with NH_4Cl (final concentration 0·1 M) for 15 s at 37 °C. Each spectrum is the result of 14,000 pulses accumulated over a spectrum width of 9600 Hz using a repetition rate of 0·82 s and 6% CD_3OD for internal lock. Chemical shift referred to Me_4Si.

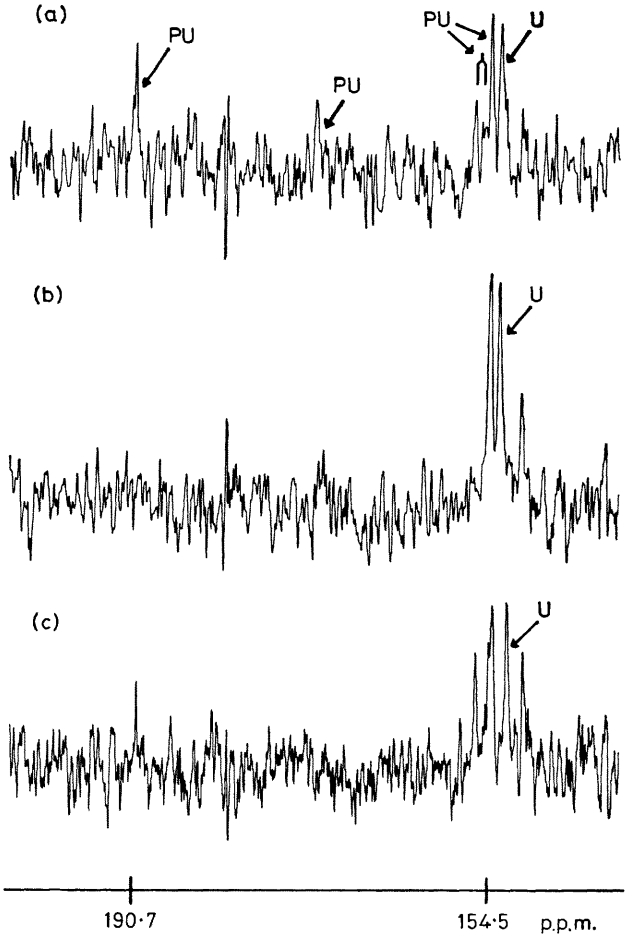


FIGURE 3. ^{1}H Decoupled ^{15}N n.m.r. spectra at 8·1 MHz and 0 °C of 3 min incubations of [1-^{15}N, 11-^{13}C] PBG with PBG deaminase: (a) pH 8·2, (b) adjusted to pH 12 with NaOH, (c) treated with NH_4Cl (final concentration 0·1 M) for 15 s at 37 °C. Each spectrum is the result of 20,000 pulses accumulated over a spectral width of 5000 Hz using a repetition rate of 0·82 s and 10% D_2O for internal lock. Chemical shift referred to anhydrous NH_3.

spectral characteristics over the range pH 8—12 are also unchanged and which serves as a good substrate for cosynthetase (at pH 8·25). Battersby *et al.* suggest[7] that the 6 Hz doublet in the spectrum of preuro'gen observed in our laboratory is an artifact, since the $-CH_2OH$ resonance for hydroxymethylbilane (**3c**) has a chemical shift of 57·0—57·1 p.p.m. (54·78 on our scale) and a *two-bond* coupling $^{2}J(^{13}C-^{15}N)$ of 2·4 Hz, and further postulate that both groups are handling the same substance. At this stage of the investigation we can offer the following comments. (i) The Cambridge spectrum[7] for the enzymically formed species shows a 2·4 Hz doublet (δ 57·1 p.p.m.) at pH 8·25 or $>$ 12 (no temperature stated). Under special conditions where high concentrations of substrate and enzyme are used [2·8 mg PBG (**1c**)/300 units of pure deaminase] we have recorded a resonance (*ca.* 3 Hz wide) at 55 p.p.m. which rapidly disappears as uro'gen I is formed at 37 °C. The intensity of the above signal can be enhanced in a more concentrated incubation (5 mg substrate/ml). Under the optimised conditions the n.m.r. signal at 54·78 p.p.m. (37 °C, pH 8·2) exhibits a *two-bond* coupling [$^{2}J(^{13}C-^{15}N)$ 1·8 $\pm$ 1 Hz; Figure 4], *i.e.* both groups are handling (**3c**) at 37 °C. (ii) If the Cambridge spectrum (Figure in ref. 7) was recorded at 0 °C (the conditions of our experiment), the two groups are not dealing with the same species since the observed doublet has a separation 5—6 Hz. (iii) The ^{15}N n.m.r. spectra of the $^{13}C-^{15}N$ enriched hydroxymethylbilane (**3c**; Figure 3b) and of the amino bilane (**3d**; Figure 3c) are quite distinct. (iv) While we agree that the chemical shift data for $-CH_2-N<$ in model compounds fall in the range 42—44 p.p.m., the two-bond coupling in these species $^{2}J(^{13}C-^{15}N)$ is in the range 1·8—2·4 Hz, whereas a 6 Hz coupling is always present in preuro'gen at pH 7—8·5 (0 °C). We consider that simple models may be misleading in that the unusual structure suggested for preuro'gen at 0 °C is not only conformationally distinct from a pyrrole-*N*-methy-

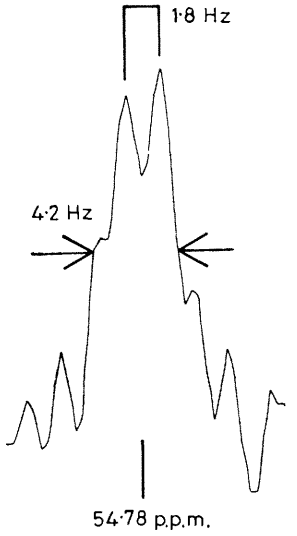
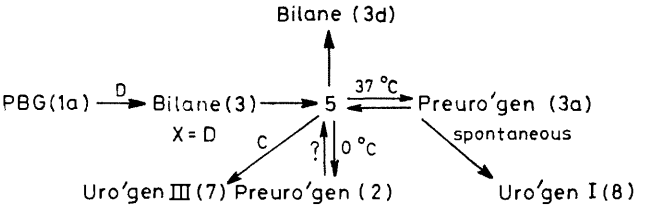


FIGURE 4. ^{1}H Decoupled ^{13}C n.m.r. spectrum at 50·3 MHz and 37 °C of an incubation of [1-^{15}N; 11-^{13}C]-PBG with PBG deaminase. The spectrum was obtained in 600 s at 37 °C and pH 8·2 with data acquisition beginning 120 s after reaction initiation. Shown is a 15·0 Hz portion of the 3000 Hz spectrum obtained with 750 90° pulses. 10% D_2O was added for internal lock and the free induction decay was zero-filled to 16 K points prior to Fourier transformation. No digital resolution or sensitivity enhancement was applied.

lene pyrrole [*e.g.*, (**6**)† and compound (**17**) in ref. 7] but may well be affected in the chemical shift for C-20 by the array of carboxylic acid groups in the most favoured conformation. Further experiments at 0 °C are now necessary in order to settle this point.

In our view, the accumulated evidence at present is still in accord with (**2**) as the structure for preuro'gen at 0 °C and pH 7—8·2, *i.e.* for the species observed under the conditions of our first experiments. The valuable synthetic work at Cambridge[7] has clearly defined structure (**3**) for the species present at pH 8—12 (37 °C) which is a good substrate for cosynthetase at pH 8·25 and on the basis of our own work at 37 °C reported herein we agree with this interpretation.

We believe that the above results now rationalise both current and previous experience, inasmuch as the coincidence of the chemical shift for C-20 (δ 54—57 p.p.m.) of *both* (**2**) and (**3**) has been revealed in the double labelling experiment with ^{15}N and ^{13}C by virtue of a 6 Hz doublet which has so far not been reported by the Cambridge group. The most likely species discharged into the medium by deaminase is thus (**5**) which can suffer (a) hydration to (**3**) and closure to uro'gen I (**8**) in the absence of cosynthetase, (b) addition of nucleophiles catalysed by deaminase (→**3d**), or (c) rearrangement to uro'gen III (**7**) catalysed by cosynthetase. Thus (**2**) and (**3**) correspond to internally and externally trapped forms of the reactive species (**5**). It now remains to devise additional diagnostic tests for interconversion of species such as (**2**), (**3**), and/or (**5**) during the course of the unperturbed enzyme-catalysed reaction at various temperatures and pH conditions using isotopic labelling and high sensitivity n.m.r. techniques, *i.e.* to probe the kinetics and equilibria of the working hypothesis shown in the Scheme.



SCHEME. D = deaminase; C = cosynthetase

In conclusion, it should be re-emphasised that contrary to the long-held opinion that deaminase and cosynthetase function *in tandem*, this viewpoint can no longer be held seriously, since the biochemical evidence[3] for sequential and independent operation of the two enzymes *in vitro* is now overwhelming. Thus while the Cambridge group has independently confirmed[7] our evidence[3] for sequential operation of deaminase and cosynthetase, our recent work leads to acceptance of structure (**3**) for preuro'gen at 37 °C. On the grounds of precedent [structure (**3**) for preuro'gen was first proposed in ref. 1], we suggest that the name for this species should be retained and further that the observation of a 6 Hz doublet at 0 °C cannot be simply explained as an artifact,[7] but rather represents the intervention of a structure with one-bond ^{13}C–^{15}N coupling. By elimination, this must correspond to (**2**). At the same time the above experiments, taken together with the Cambridge work, require that the conclusions regarding structure (**2**) for preuro'gen as stated in ref. 2 (but not in ref. 1) must be modified as far as observation at 37 °C is concerned.

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† Professor A. Gossauer (Berlin) has kindly informed us that the synthetic compound (**6**) has CH_2 at 46 p.p.m., $^{1}J(^{13}C–^{15}N)$ 12 Hz. Similar models have been reported to have CH_2 at 41—46 p.p.m., and $^{1}J(^{13}C–^{15}N)$ of 10—12 Hz. Full details of this work will be published separately.

‡ Received in revised form 18th February 1980.

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