Biosynthesis of Vitamin B_{12}: Analysis of the ^1H and ^{13}C N.m.r. Spectra of Heptamethyl Dicyanocobyrinate (Cobester)

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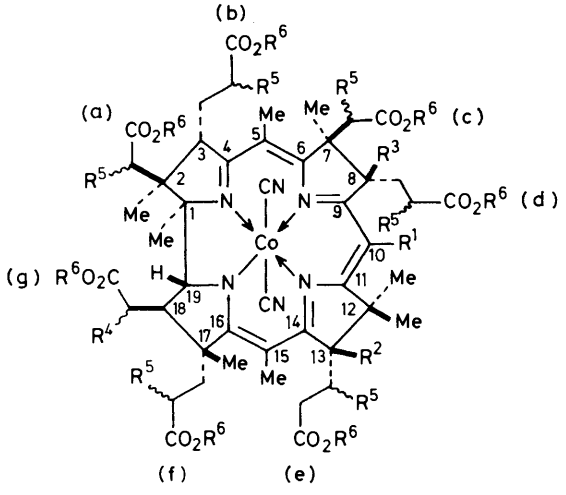
Heptamethyl dicyanocobyrinate (1), a key substance for biosynthetic researches on vitamin B_{12}, has been studied by ^1H and ^{13}C n.m.r. All the resonances in the ^1H spectrum (apart from those for the OMe groups) have been rigorously assigned using partially relaxed spectra and n.O.e. difference and decoupling difference techniques. The assigned ^1H resonances have been correlated with the ^{13}C spectrum to allow signal assignments for all the proton bearing carbons. The remaining signals, apart from the carbonyl groups, were assigned either by long-range proton decoupling or by specific labelling of cobester with ^2H to cause isotopic shifts of the ^{13}C signals across two to five bonds. The full set of assignments for ^1H and ^{13}C n.m.r. spectra form the basis for future biosynthetic studies on vitamin B_{12}.

HEPTAMETHYL DICYANOCOBYRINATE (1), commonly called cobester, is readily prepared from vitamin B_{12} by methanolysis [1] or by direct esterification of cobyrinic acid (2); the latter is a late biosynthetic precursor of vitamin B_{12}.[2] The important advantages of cobester (1) as the standard corrin for biosynthetic studies on vitamin B_{12} were soon recognised [3] and it is now the substance used for assay of radioactivity or for spectroscopic study in most investigations.[2] It was for these reasons that we chose to make a detailed study of the ^1H and ^{13}C n.m.r. spectra of cobester (1). Many of the signals assigned in the sequel are expected to be of value for future biosynthetic researches on vitamin B_{12}.

The ^1H *N.m.r. Spectrum.*—The advent of high-field n.m.r. spectrometers with powerful systems for data handling has opened the way to a full analysis of the ^1H n.m.r. spectrum of cobester (1). The spectrum at 100 MHz was not sufficiently resolved to allow this challenging problem to be solved. However at 400 MHz, the spectrum run in hexadeuteriobenzene was greatly clarified (Figure 1a); three of the five methine protons were clearly visible, in addition to the obvious signal from 10-H. Nevertheless, the bulk of the resonances appeared in the range δ 1.7—3.0 where more than twenty protons gave rise to severely overlapping multiplets though with an encouraging degree of visible detail (Figure 1b). It was clear that a combination of different approaches would be necessary to sort out individual signals.



	R^1	R^2	R^3	R^4	R^5	R^6
(1)	H	H	H	H	H	CH_3
(2)	H	H	H	H	H	H
(3)	D	H	H	H	H	CH_3
(4)	D	D	H	H	H	CH_3
(5)	H	D	H	H	H	CH_3
(6)	D	H	D	H	H	CD_3
(7)	H	H	H	H,D	H	CD_3
(8)	H	H	D	D_2	H,D	CD_3

Partially relaxed spectra helped to group the signals from methine, methylene, and methyl groups based on their differing relaxation times [4,5] and allowed some distinction within each group. But by far the most powerful method in this work has been that of Hall and Sanders [6] based on the detection, by difference spectroscopy, of small nuclear Overhauser effects (n.O.e.s). Most of the n.O.e.s gave <5% enhancement of the signal intensity. Similarly, difference spectroscopy greatly helped the decoupling experiments.

To simplify the discussion, Table 1 collects the signal assignments which have been made together with the the relaxation times, T_1, for the various hydrogen nuclei. These assignments were derived in the following way.

The starting point was the one-proton singlet at δ 5.74 which unambiguously corresponds to 10-H (see Figure 1a). A set of n.O.e. difference spectra was then determined in which 10-H and the other methine protons together with the 3-proton singlets from the eight *C*-methyl groups were each separately irradiated. The observed n.O.e. enhancements are collected in Table 2, and examples of the difference spectra appear in Figure 2; the unperturbed highfield portion of the ^1H n.m.r. spectrum is shown as 2(a). The notation {10-H} or {2-Me} means, respectively, that 10-H or the *C*-methyl group at position 2 have been irradiated in the experiment under discussion.

Irradiation at 10-H (Figure 2b) readily allowed

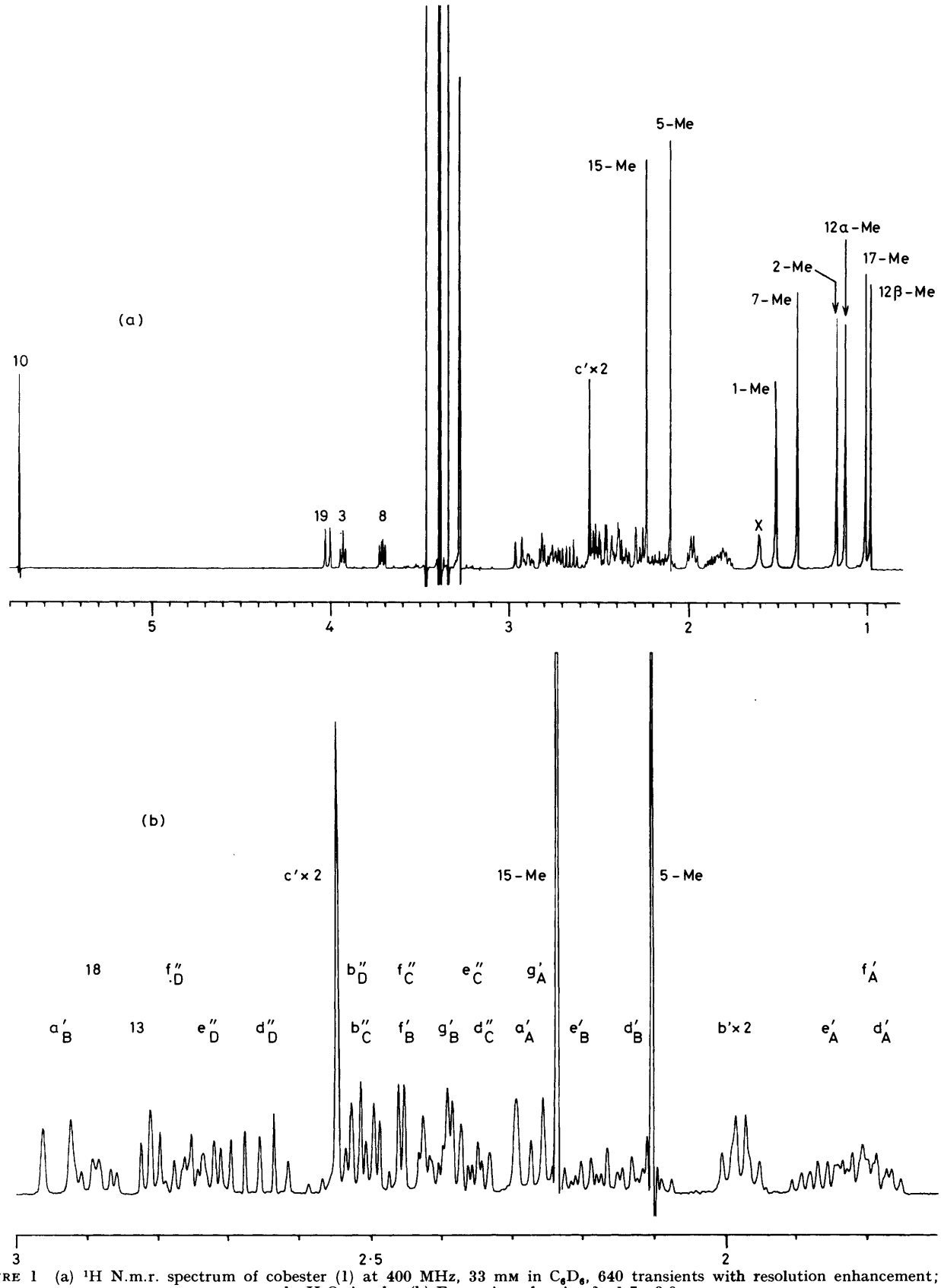


FIGURE 1 (a) ^1H N.m.r. spectrum of cobester (1) at 400 MHz, 33 mM in C_6D_6, 640 transients with resolution enhancement; X marks H_2O signal. (b) Expansion of region δ_H 1.7—3.0

assignment of the double doublet at δ 3.71 to 8-H and, in addition, selected the 3H singlets at δ 1.11 and 0.98 as those corresponding to the two methyl groups at C-12. Working from the latter two signals similarly allowed location of 13-H at δ 2.81. The *gem*-methyl groups at C-12 were distinguished by irradiation of 13-H which caused a far larger enhancement of the signal at δ 0.98 than of that at δ 1.11 (*ca.* 5 times larger). This result is consistent with the view that the signal at δ 0.98 arises from the 12β-methyl group and the one at δ 1.11 from its geminal partner (12α). Finally, in the experiment above where 10-H was irradiated, the signal now assigned to the 12α-methyl group showed an enhancement *ca.* 3.7 times larger than was observed for the signal from the 12β-methyl residue. This last result interlocks with information from recent *X*-ray analyses [8a,b] of different crystal forms of cobester (1). The average distance can be measured [8b] for the solid state between 10-H and the hydrogens of the 12α- and 12β-methyl groups which, as for related corrinoids,[8c] are respectively pseudo-equatorial and pseudo-axial. These distances are 3.56 Å for 12β and 2.89 Å for 12α. Using the r^{-6} dependence of n.O.e. on distance between the nuclei, these values lead to a calculated ratio of the n.O.e. from 10-H to the 12α-methyl group relative to the n.O.e. to the 12β-methyl group of *ca.* 3.5. The ratio found above was *ca.* 3.7.

TABLE 1

^{1}H Chemical shifts, coupling constants, and relaxation times for cobester (1) *

Proton(s)	Chemical shift(s) †	Coupling constant(s) ‡ (J/Hz)	T_1 §
12β-CH_3	0.98	---	0.23
17-CH_3	1.00	—	0.27
12α-CH_3	1.11	—	0.20
2-CH_3	1.17	—	0.25
7-CH_3	1.39	—	0.22
1-CH_3	1.51	—	0.14
d′$_A$	1.81	$J_{A,8}$ = 8.1, J_{AB} = 14.1, J_{AC} = 5.2, J_{AD} = 8.3	0.22
f′$_A$	1.82	J_{AB} = 15.4 ‖	(0.22)
e′$_A$	1.86	$J_{A,13}$ = 4.9, J_{AB} = 15.0, J_{AC} = 5.8, J_{AD} = 9.6	0.22
b′ (×2)	1.98	— ¶	0.22
5-CH_3	2.10	—	0.42
d′$_B$	2.12	$J_{B,8}$ = 5.0, J_{BA} = 14.1, J_{BC} = 8.1, J_{BD} = 8.3	0.22
e′$_B$	2.19	$J_{B,13}$ = 6.1, J_{BA} = 15.0, J_{BC} = 9.6, J_{BD} = 6.1	0.22
15-CH_3	2.24	—	0.39
g′$_A$	2.27	$J_{A,18}$ = 9.8, J_{AB} = 15.6	(0.26)
a′$_A$	2.28	J_{AB} = 15.6	0.23
d″$_C$	2.37	J_{CA} = 5.2, J_{CB} = 8.1, J_{CD} = 17.0	(0.29)
e″$_C$	2.38	J_{CA} = 5.8, J_{CB} = 9.6, J_{CD} = 16.8	(0.29)
g′$_B$	2.41	$J_{B,18}$ = 3.4, J_{BA} = 15.6	(0.25)
f′$_B$	2.46	J_{BA} = 15.4 ‖	(0.25)
f″$_C$	2.46	— ¶	(0.25)
b″ (×2)	2.52	— ¶	(0.40)
c′ (×2)	2.55	—	(0.29)
d″$_D$	2.65	J_{DA} = 8.3, J_{DB} = 8.3, J_{DC} = 17.0	0.35
e″$_D$	2.74	J_{DA} = 9.6, J_{DB} = 6.1, J_{DC} = 16.8	(0.36)
f″$_D$	2.77	— ¶	(0.40)
13	2.81	$J_{13,A}$ = 4.9, $J_{13,B}$ = 6.1	0.43
18	2.89	$J_{18,19}$ = 10.8, $J_{18,A}$ = 9.8, $J_{18,B}$ = 3.4	0.29
a′$_B$	2.94	J_{BA} = 15.6	0.25
OCH_3 (×7)	3.28—3.46	---	0.72—1.0
8	3.71	$J_{8,A}$ = 8.1, $J_{8,B}$ = 5.0	0.58
3	3.93	$J_{3,A}$ = 5.4, $J_{3,B}$ = 6.1	0.48
19	4.02	$J_{18,19}$ = 10.8	0.35
10	5.74	—	0.55

* 33 mM in C_6D_6 at 291 K. † ±0.01 p.p.m.; the residual solvent peak was used as an internal reference (δ_H 7.20). ‡ J ± 0.3 Hz; only geminal and vicinal coupling constants are recorded. § Relaxation times, T_1, ±0.03 s; values in brackets are probably less accurate because the signal had others overlapping. ¶ Not measured. ‖ Determined from 400 MHz ^{1}H spectrum of deuteriated cobester (8).

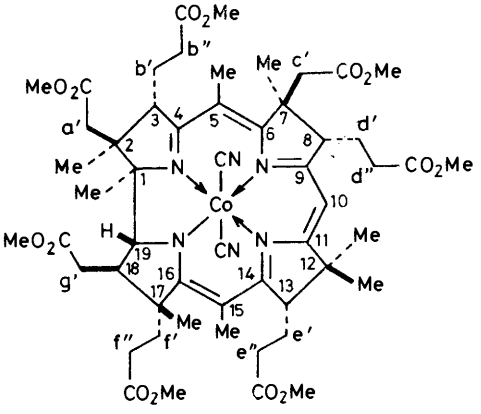
TABLE 2

Nuclear Overhauser enhancements for cobester (1) observed by difference spectroscopy

Signal irradiated	Signal(s) enhanced
1-CH_3	b′, 2-CH_3, 18
2-CH_3	a′$_A$, b′, b″, 3, g′$_B$, 1-CH_3
3	a′$_B$, b′, b″, 2-CH_3, 5-CH_3
5-CH_3	3, c′, 7-CH_3, (d′$_A$, d″$_C$) *
7-CH_3	c′, 8, 5-CH_3
8	c′, 7-CH_3, 10
10	8, d″$_D$, 12α-CH_3, 12β-CH_3
12α-CH_3	10, 12β-CH_3, 13, e′$_A$, e′$_B$, e″$_C$
12β-CH_3	10, 12α-CH_3, 13
13	12α-CH_3, 12β-CH_3, e′$_A$, e′$_B$, 15-CH_3
15-CH_3†	13, e′$_A$, 17-CH_3, (g′$_B$) †
17-CH_3	15-CH_3, g′$_A$, f′$_B$, f″$_C$, 19
18	1-CH_3

* Irradiation affected d′$_B$ also. † Irradiation affected g′$_A$ also.

Knowing the signal from 13-H, the {13-H} difference spectrum allowed assignment of the singlet at δ 2.24 to the 15-methyl group. Irradiation of this signal, in turn, produced among other changes, enhancement of the 3 H singlet at δ 1.00, which was tentatively assigned to the 17-methyl group. It was impossible here to achieve selective irradiation of the 15-methyl group because ther resonances coincide with it. However, confirmation of the signal assignment for the 17-methyl group came from irradiating the signal at δ 1.00 which could be achieved selectively; this produced a clear enhancement of the signal from the 15-methyl group. In addition, {17-CH_3} also caused enhancement of the doublet at δ 4.02, which was therefore assigned to 19-H by virtue of its proximity to the 17-methyl residue.



(1)

Assignments of the signals in rings A and B were established as follows. Irradiation of the singlet at δ 1.39 caused enhancement of the singlet at δ 2.10 and of the 8-H signal; therefore the δ 1.39 singlet arises from the 7-methyl residue and δ 2.10 singlet must be from the methyl at C-5. Since {5-Me} enhanced the triplet * at δ 3.93, this signal was assigned to 3-H. Again, selective irradiation of the 5-methyl group was complicated by overlapping signals but the foregoing tentative assignment was confirmed by irradiating 3-H, and observing enhancement of the signal from the 5-methyl group (see Figure 2c).

The remaining spectra in this set were those obtained by irradiation of the singlets at δ 1.51 and 1.17 which, by exclusion, must arise from the C-methyl groups at C-1 and C-2. It was expected that irradiation of each of these signals would enhance the other and this occurred. However, a CPK model of cobester (1) shows that only the 2-methyl group is near 3-H whereas that at C-1 is near 18-H. Thus, the signal at δ 3.93 already assigned to 3-H should appear only in the {2Me} difference spectrum and that at δ 2.89, corresponding to 18-H, should be present in the {1Me} difference spectrum. The experimental traces (Figure 2, d and e) show a clear distinction and therefore the 2-methyl group is assigned to the singlet at δ 1.17 and the 1-methyl group to that at δ 1.51. The assignment of 18-H to the multiplet at δ 2.89 is further supported below.

These assignments allow discussion of the relative relaxation times, T_1, of the methine groups of cobester (1). The nuclei 3-H and 13-H relax at a similar rate which is understandable from their very similar environments. Relaxation of 8-H is somewhat slower possibly because there is no methyl group at C-10. The T_1 values for 18-H and 19-H are the shortest, which is in accord with their being in the sterically most congested part of the molecule, the A,D-ring junction. The T_1 value for the 1-methyl group also reflects the close proximity of other groups.

These T_1 values have already been helpful in making assignments of ^{1}H n.m.r. signals from cobester (1) recorded in solvents different from the hexadeuteriobenzene used above. Finally, it should be noted that the *C*-methyl groups relax significantly faster than the ester *O*-methyl groups in agreement with observations on other macrocycles.[4]

Attention can now be focussed on the methylene protons of the acetate and propionate side-chains of cobester (1). The signals from these protons appeared in the foregoing set of n.O.e. difference spectra, but unequivocal assignments could only be made in conjunction with data from spectra obtained making use of decoupling difference and partial relaxation; some of these spectra are shown in Figures 3 and 4. The method of decoupling difference was particularly helpful in that most of the geminal and vicinal coupling constants could be determined. The clarity of the results can be seen in Figure 3b which shows the outcome of the {19-H} experiment.

* With greater resolution enhancement, this signal appeared as a doublet of doublets (see Table 1).

Assignment of the signals from the methylenes of the acetate side-chains was as follows, but first an explanation of the notation to be used is needed. The ester side-chains of cobester (1) are lettered a,b, . . . g as usual for substances related to vitamin B_{12}. The methylene carbon of a propionate side-chain which is attached directly to the corrin nucleus is given one prime *e.g.* C–b′ and the other methylene carbon, two primes, *e.g.* C–b″ [see (1)]. The hydrogen atoms of these methylene groups are similarly distinguished with the refinement that for a ' single prime ' methylene group, the hydrogen giving the higher field signal is marked A and that corresponding to the lower field signal is marked B, *e.g.* H-d′$_A$ and H–d′$_B$. For a ' double prime ' methylene group, C and D are similarly used.

The doublet at δ 2.94 was assigned to one of the C-a′ methylene protons H-a′$_B$ on the basis of the large coupling constant, J 15.6 Hz, and the fact that the doublet was enhanced when H-3 was irradiated. Its partner, H-a′$_A$, whose signal was enhanced when the 2-methyl group was irradiated, gave a doublet located upfield (δ 2.28); this assignment was confirmed by decoupling H-a′$_B$ (δ 2.94).

The methylene protons of C-g′, H-g′$_B$ and H-g′$_A$, were assigned to the two double doublets at δ 2.41 and δ 2.27, which appear in the {2-Me} and {17-Me} n.O.e. difference spectra, respectively. They showed the expected geminal coupling (15.6 Hz), as well as further coupling to 18-H; the geminal coupling was shown most clearly in the decoupling difference spectrum obtained from {18-H} (Figure 3e). The methylene protons on C-c′ gave rise to what appears to be a singlet at δ 2.55, which was clearly visible in the normal ^{1}H spectrum (Figure 1), and was enhanced when 8-H, and the 7-methyl group were irradiated. This ' singlet ' was unexpected in that the C-c′ methylene group is adjacent to a chiral centre; probably the signal arises from a strongly coupled AB system, where $\Delta\nu/J \leqslant 1$ and the outer lines are too small to be visible. Certainly, for solutions of higher concentration, coupling was observed between these two protons (*ca.* 15 Hz).

The signals from the methylene protons on the propionate side-chains were less easy to assign because each proton is coupled to at least three others. Here, decoupling difference spectra (Figure 3) proved to be the most effective method for making connections along each side-chain.

The protons on the b side-chain were tentatively assigned to the multiplets at δ 1.98 and δ 2.52, which were enhanced when the methyl groups on ring A were irradiated (Figure 2d and e). Decoupling 3-H perturbs only the multiplet at higher field, so it was assigned to the pair on C-b′ (Figure 3c). This trace also reveals a long-range coupling (<1 Hz) affecting one of the protons on C-b″. The other multiplet, δ 2.52 was then assigned to the protons on C-b″; the connection between these resonances was confirmed by decoupling at δ 1.98.

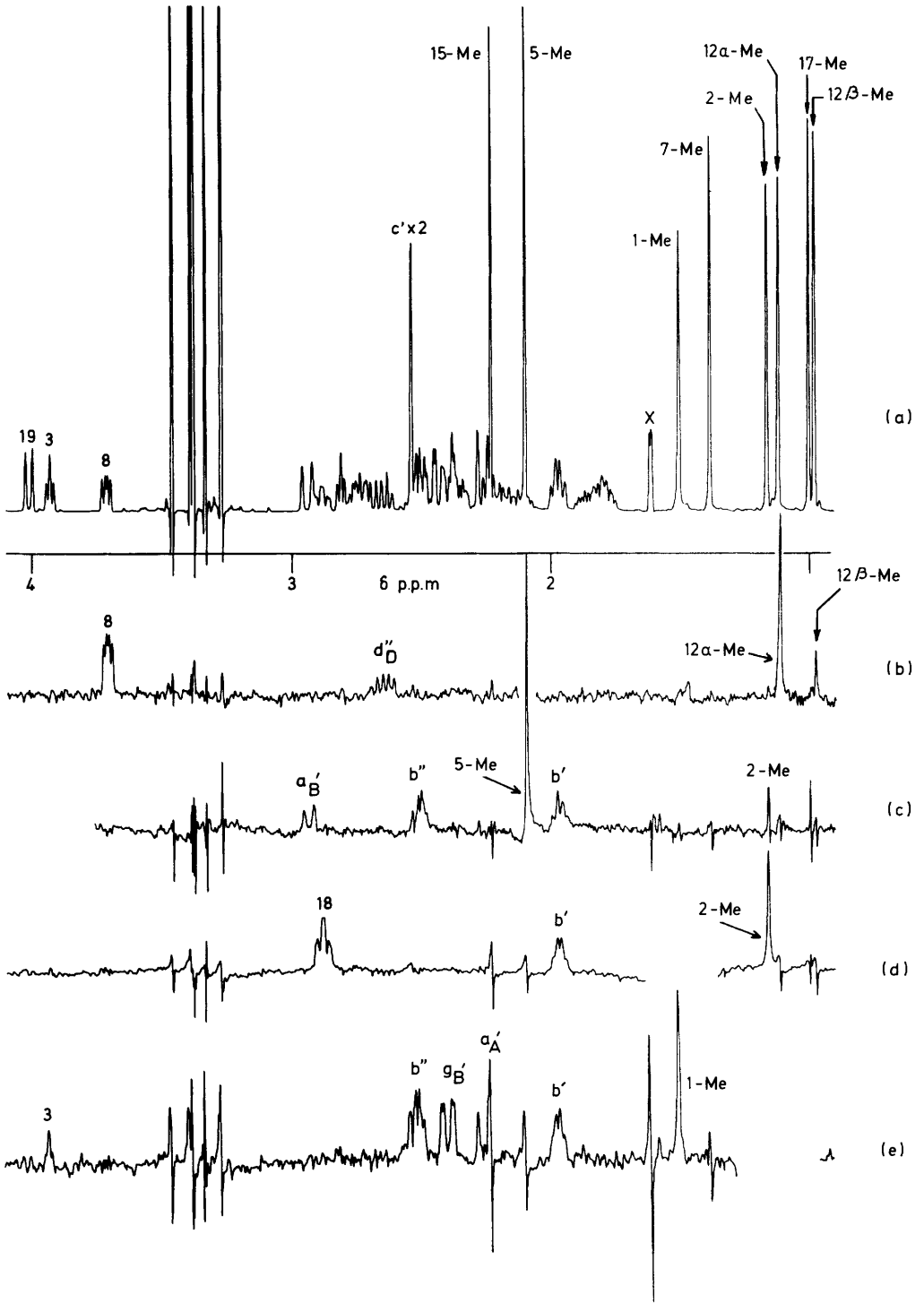


FIGURE 2 (a) Region δ_H 0.9—4.1 p.p.m. in 400 MHz ^1H n.m.r. spectrum of cobester (1) in C_6D_6 after resolution enhancement; X marks H_2O signal. (b)—(e) N.O.e. difference spectra for the same region obtained by irradiation at 10-H, 3-H, 1-Me, and 2-Me, respectively

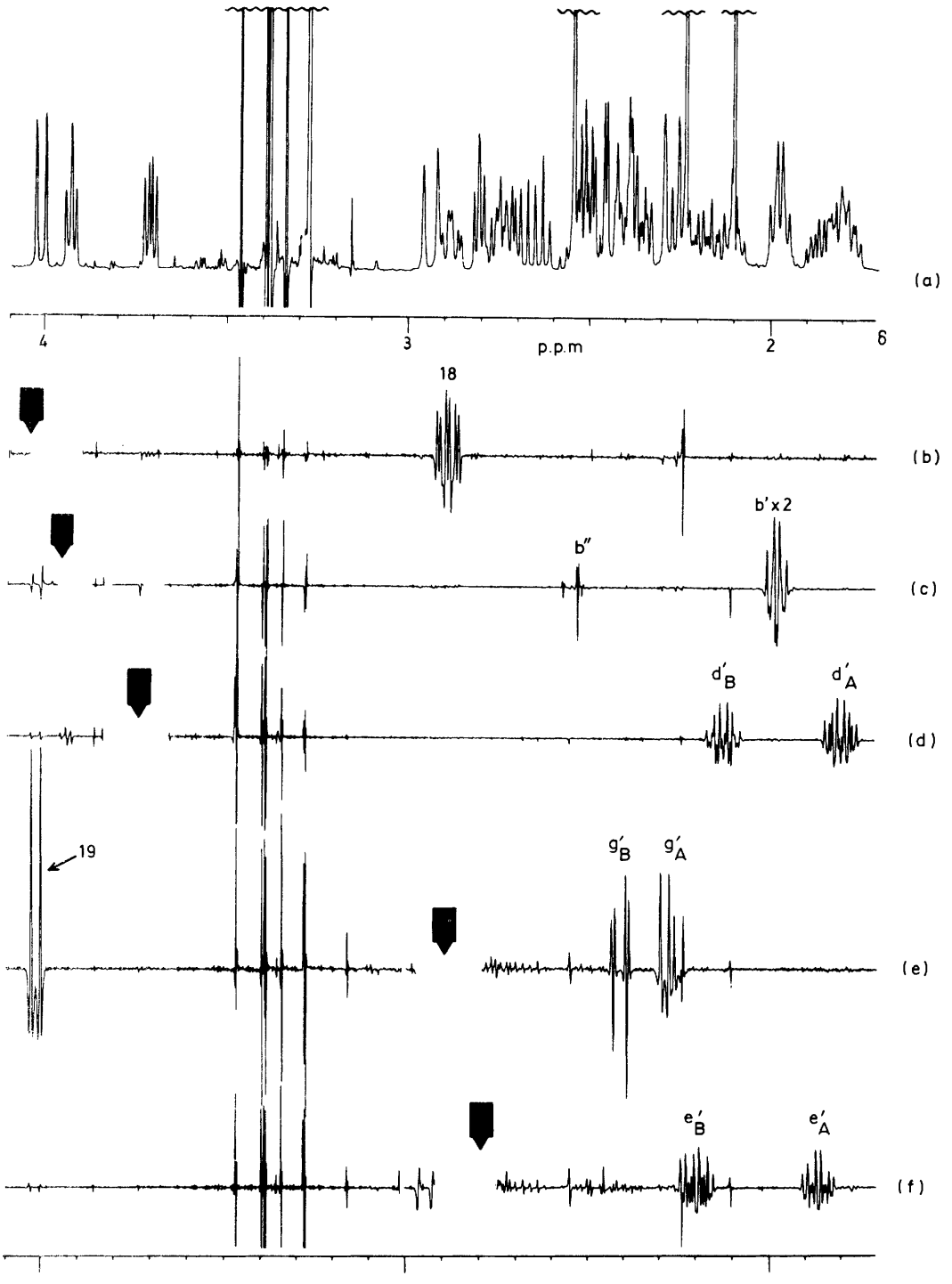


FIGURE 3 (a) ^{1}H N.m.r. spectrum, δ_H 1.7—4.1 p.p.m., of cobester (1) at 400 MHz in C_6D_6 after resolution enhancement. (b)—(f) Decoupling difference spectra for the same region obtained by irradiation of 19-H, 3-H, 8-H, 18-H, and 13-H, respectively

In contrast to the preceding case, the signals from the methylene protons on both the d and e side-chains approached first order ($\Delta\nu/J \gg 1$). The resonances from the pair on C-d', H-d'$_A$ and H-d'$_B$, were located at δ 1.81 and 2.12, respectively, by decoupling 8-H (Figure 3d). Of the geminal partners on C-d'', one H-d''$_D$, was assigned to the multiplet (dt) at δ 2.65, because this resonance showed a small but reproducible n.O.e. when 10-H was irradiated (Figure 2b). The signal at δ 2.37, was then assigned to H-d''$_C$ by decoupling either H-d''$_D$, or H-d'$_A$. While the result from the former experiment was clearer because selective irradiation was possible, the latter run

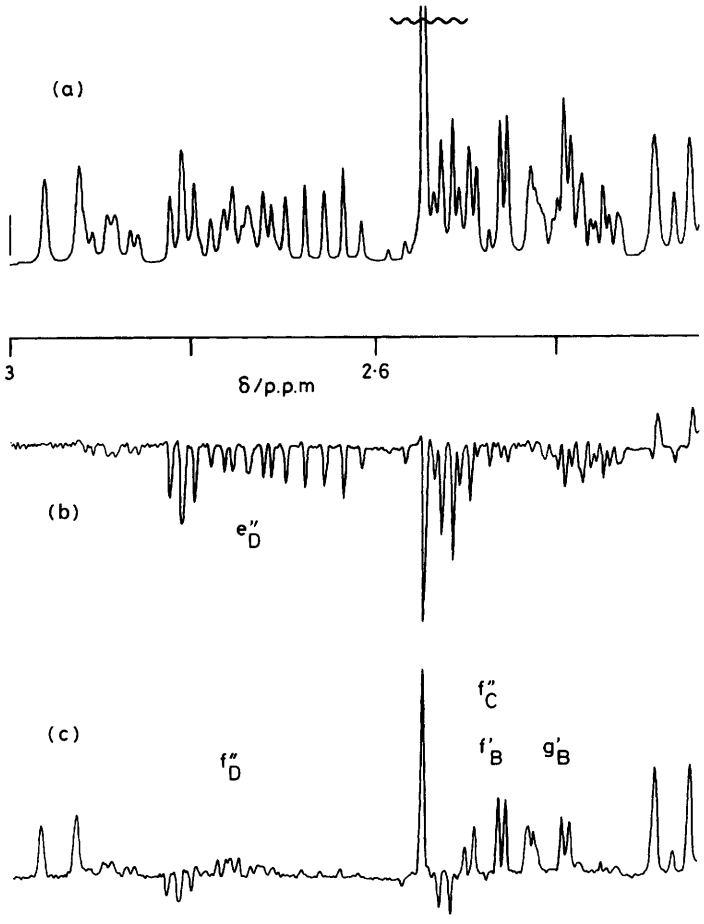


FIGURE 4 (a) Region δ_H 2.25—3.0 in 400 MHz ^{1}H n.m.r. spectrum of cobester (1) in C_6D_6 after resolution enhancement. (b) and (c) Partially relaxed spectra for same region using non-selective 180° pulse and then monitoring (b) after 0.17 s and (c) after 0.25 s

did help uncover H-f'$_A$ at δ 1.82 (see below). A similar approach was also used to assign the protons on the e side-chain. Decoupling 13-H picked out the protons on C-e' as corresponding to δ 1.86 and 2.19 (Figure 3 f). These protons also showed a significant n.O.e. enhancement when the 12α-methyl group was irradiated as did one of the vicinal partners, H-e''$_C$ (δ 2.38). This last assignment was confirmed by selectively decoupling H-e'$_A$ (δ 1.86), and this experiment established the additional connection to H-e''$_D$ (δ 2.74). The multiplet (ddd) from H-e''$_D$ was strikingly exposed in the partially relaxed spectrum shown in Figure 4 b.

The assignment of the methylene groups on the f side-chain posed the most difficult problem because of the virtual coincidence of the signals from the *vicinal* partners H-f'$_B$ and H-f''$_C$ at δ 2.46. These latter assignments and those of the signals from H-f'$_A$ and H-f''$_D$ were based on the following experiments. Irradiation of the 17-methyl group produced, together with others already mentioned, enhancements of signals at δ 2.46. These signals were most apparent in a partially relaxed spectrum (Figure 4c), in which most of the signals from protons having T_1 *ca.* 0.36 s were passing through their null points. This spectrum also showed a multiplet at δ 2.77, which was assigned to H-f''$_D$. The connection between these groups was then made by decoupling H-f'$_A$ at δ 1.82 and, apart from the expected effects (see earlier) on the resonances associated with the d side-chain, this experiment showed decoupling only at δ 2.77 and 2.46. A clearer result was obtained by performing the same decoupling (difference) experiment after the addition of some Eu([$^2H_9$]-fod)$_3$, which exposed H-f'$_A$ (δ 1.83) completely; irradiation at H-f'$_A$ then caused perturbation only of the resonances associated with the f side-chain.

Table 1 collects the complete assignments gained from the interlocking sets of experiments above.

The ^{13}C *N.m.r. Spectrum.*—The ^{13}C n.m.r. spectrum of cobester (1) run in hexadeuteriobenzene at 100.6 MHz gives an excellent dispersion of the signals (Figure 5). If one neglects the CO_2Me groups of the side-chains, the remaining 38 carbons all give fully resolved signals. A few of these signals had been uniquely assigned and others grouped into ' families ' on the basis of chemical-shift arguments, off-resonance decoupling, and biosynthetic ^{13}C-labelling experiments.[3] It was essential for future biosynthetic studies to make as complete an assignment as possible of this ^{13}C spectrum. Having the

foregoing thorough analysis of the ^1H n.m.r. spectrum, the way was open to make connections between the individual protons and the carbons to which they are attached. Several methods were used to achieve this.

The first studies involved selective proton decoupling at low power with the aim of identifying the carbon directly attached to the irradiated proton [9] and, in addition, to gain information about the carbon atoms separated by two or three bonds from the irradiated hydrogen.[9] This was partially successful. The assignments [3] of δ_C 91.5 to C-10 and δ_C 22.4 to the 1-methyl group were confirmed and δ_C 104.2 and δ_C 102.2 were assigned to C-5 and C-15, respectively. The signals from the latter two carbons appeared as quartets $^2J \sim 7$ Hz, in the fully coupled spectrum, which changed to sharp singlets when the appropriate methyl group, δ_H 2.10 (5-Me) or δ_H 2.23 (15-Me), was irradiated. The signal at δ_C 175.6 was found to arise from C-4 when irradiation of the protons on C-b' (δ_H 2.00) eliminated 3-bond coupling (H-b' to C-4) and left the 2-bond coupling (^2J 5 Hz) from H-3 to C-4.

A graphical method [10] for evaluation of spectra obtained by off-resonance decoupling was then applied. Briefly, this involves stepping the decoupler (at constant power level) through the proton spectrum at regular intervals. Observation of the series of ^{13}C spectra so obtained allows measurement of the residual 1-bond ^1H–^{13}C couplings which are plotted against the position of the decoupler on the frequency scale. The graphs then allow each carbon resonance to be connected with individual protons. Table 3 shows the δ_C values and the corresponding δ_H values derived from the graphs. This can be compared with the δ_H determined under exactly the same conditions (especially concentration of cobester) by direct ^1H n.m.r. The agreement between the two sets of values is excellent for all the methine and methyl groups. The values for the methylene groups were also satisfactory. At the higher concentration of cobester (1) used for these studies, all the protons of the methylene groups were found to be non-equivalent.

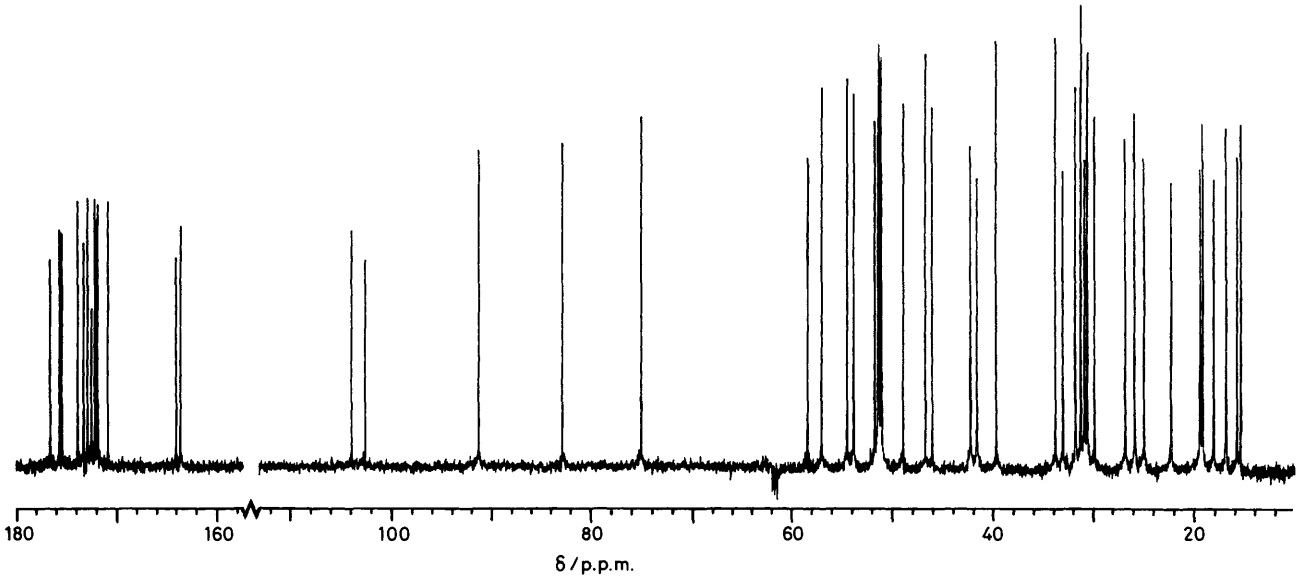


FIGURE 5 ^{13}C N.m.r. spectrum of cobester (1) at 100.6 MHz with proton-noise decoupling, 0.178M in C_6D_6, 5 990 transients, sweep width 20 000 Hz, 16 K data points, acquisition time 0.4 s

Where the chemical-shift difference between the signals from the two protons of CH_2 was large (*e.g.* for those at C-a' or C-f') the corresponding signal for the carbon appeared as a double doublet when the decoupler was off-set. This allowed the δ_H values for both protons to be determined.[11] However, where this chemical-shift difference was small (<0.1 p.p.m.), the carbon resonance appeared as a triplet (*e.g.* C-c' or C-g') and only an average δ_H value could be obtained for the two methylenic protons attached to that carbon (see Table 3). As a result, further study of C-g', C-c', and C-b'', was necessary and we will return to these carbons later.

Most of the remaining ^{13}C resonances were assigned by studying the ^{13}C-spectra of samples of cobester which had been selectively deuteriated;[12] the samples were (3)—(8). As expected, carbons bonded directly to the deuterium atom(s) gave little or no ^{13}C n.m.r. signal. But carbons two bonds away, and also up to five bonds away, gave signals which were shifted, generally to higher field,[13,14] relative to the corresponding signals from protio-cobester (1). These isotopic shifts were often less than 0.1 p.p.m. so they were best observed by adding a known amount of unlabelled compound (1) to the deuteriated sample before the spectrum was determined under conditions which maximised digital resolution. The observed shifts and the subsequent signal assignments are collected in Table 4.

The ^{13}C spectrum of a sample of cobester carrying some deuterium at C-10 (1) + (3) showed shifted signals alongside five of the resonances present in undeuteriated

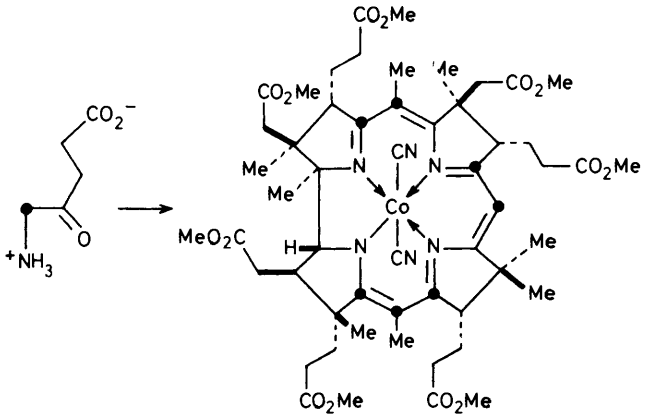
TABLE 3

Assignments of ^{13}C n.m.r. signals of cobester (1) using graphical analysis of partially decoupled spectra

^{13}C Resonance * δ_C in p.p.m. (multiplicity)	δ_H Determined graphically	δ_H From ^1H n.m.r. spectrum †	Assignment
75.3 (d)	3.99	3.98	C-19
57.2 (d)	3.89	3.90	C-3
54.6 (d)	3.68	3.68	C-8
54.0 (d)	2.84	2.83	C-13
42.4 (t)	2.53	2.53, 2.51	C-c′ ‡
41.8 (t)	2.88, 2.29	2.89, 2.29	C-a′
39.9 (d)	2.86	2.87	C-18
33.95 (t)	2.50	2.52	C-b″ ‡
33.2 (t)	2.43, 1.82	2.45, 1.80	C-f′
31.9 (t)	2.40	2.43, 2.33	C-g′ §
31.4 (t)	2.60, 2.36	2.61, 2.37	C-d″
31.0 (q)	0.97	0.99	12β-CH_3
30.7 (t)	2.65, 2.36	2.70, 2.35	C-e″
29.9 (t)	2.71, 2.43	2.74, 2.45	C-f″
26.9 (t)	2.08, 1.79	2.11, 1.79	C-d′
26.0 (t)	2.18, 1.86	2.17, 1.86	C-e′
25.1 (t)	1.99	2.00	C-b′
22.4 (q)	1.49	1.50	1-CH_3
19.6 (q)	1.12	1.13	12α-CH_3
19.3 (q)	1.38	1.40	7-CH_3
18.2 (q)	1.00	1.03	17-CH_3
16.9 (q)	1.17	1.20	2-CH_3
15.9 (q)	2.06	2.10	5-CH_3
15.6 (q)	2.25	2.23	15-CH_3

* δ_C For cobester (1) at 0.178M in C_6D_6 are reported relative to the centre line of the solvent (δ_C 128.0 p.p.m.). † δ_H For cobester (1) at 0.178M in C_6D_6 relative to δ_H 7.20 for C_6HD_5. ‡ These resonances could not be distinguished (see text). § Assignment confirmed by absence of this signal in the ^{13}C n.m.r. spectrum of deuteriated cobester (7).

cobester (1). Thus these five signals arise from carbon atoms around C-10. Of the three signals at low field (160—180 p.p.m.), one at δ 176.8 was assigned to C-11 because this signal was not enhanced in cobester produced biosynthetically from δ-amino[5-^{13}C]laevulinic



(9)

acid.[3] The labelling pattern for this ^{13}C-labelled cobester is shown in structure (9). The other two low-field signals, δ 172.2* and δ 163.8* were enhanced in the spectrum of the ester (9) and were assigned to C-9 and

* The original sample [3] of [^{13}C]cobester (9) was re-examined under the present conditions to give δ values differing slightly from those reported earlier [3] due to concentration differences. Also the assignment here of the signal for C-14 corrects the previous one [3] which had been to a signal 0.4 p.p.m. to lower field.

TABLE 4

Isotopic shifts induced by deuterium on the ^{13}C resonances of cobester and resultant signal assignments

[^2H]Cobester used in admixture with [^1H]cobester (1)	Unshifted resonance *	Isotopic shift(s) † (p.p.m.)	Assignment
[^2H]Ester (3)	176.8	−0.040	C-11
	172.2	−0.062	C-9
	163.8	+0.009	C-14
	54.6	−0.061	C-8
	46.9	−0.024	C-12
[^2H]Ester (5)	176.8	−0.012	C-11
	174.1	0.009 ‡	e-C=O
	163.8	−0.021	C-14
	46.9	−0.083	C-12
	31.0	−0.074	12β-CH_3
	30.7	−0.025	C-e″
	26.0	−0.101	C-e′
	15.6	−0.019	15-CH_3
[^2H]Ester (6)	49.1	−0.082	C-7
	42.4	−0.061	C-c′
	26.9	−0.109	C-d′
[^2H]Ester (7)	174.1	+0.017	C=O
	173.5	+0.021	C=O
	173.1	+0.021	C=O
	172.7	+0.024	C=O
	172.4	+0.017	C=O
	171.1	+0.020	C=O
	39.9	−0.060, −0.109	C-18,
	33.2	(signal broadened)	C-f′
[^2H]Ester (8)	174.1	+0.024	C=O
	173.5	+0.030	C=O
	173.1	+0.027	C=O
	172.7	+0.027	C=O
	172.4	+0.024	C=O
	171.1	+0.024	C=O
	58.6	−0.027	C-17
	57.2	−0.049, −0.088, −0.115	C-3
	54.6	−0.060	C-8
	49.1	−0.082, −0.165, −0.241	C-7
	46.2	−0.097, −0.153	C-2
	39.9	−0.109	C-18
	33.2	−0.033, −0.102	C-f′
	26.9	−0.164, −0.240	C-d′
	26.0	−0.061, −0.125	C-e′
	25.1	−0.077, −0.137	C-b′
	19.3	−0.065	7-CH_3
	16.9	−0.022, −0.033	2-CH_3
	15.9	0.017 ‡	5-CH_3

* δ_C Values are reported relative to centre line of the solvent (C_6D_6, δ_C 128.0 p.p.m.). † Isotopic shifts were measured relative to signals of [^1H]cobester (1); a negative sign indicates a shift to higher field. Accuracy of shift values is ±0.003 p.p.m. for δ 160—180 p.p.m. and ±0.006 p.p.m. for δ 10—60 p.p.m. ‡ Sign of shift not reliably determined.

C-14, respectively, the unique assignment requiring the results obtained with cobester (5) below. In the 'aliphatic' region, the signals at δ 54.6 and 46.9 were deshielded by deuterium at C-10. The former has been identified above as arising from C-8 so δ 46.9 was assigned to C-12.

In the ^{13}C spectrum of cobester partially deuteriated at C-13, (1) + (5) (Figure 6), the signal from C-12 was again isotopically shifted together with that from the 12β-methyl group (interestingly the signal from the 12α-methyl group was not significantly affected). The other resonances shifted by ^2H at C-13 were C-e′, C-e″, and the 15-methyl group; these effects supported earlier conclusions. In the region of the spectrum

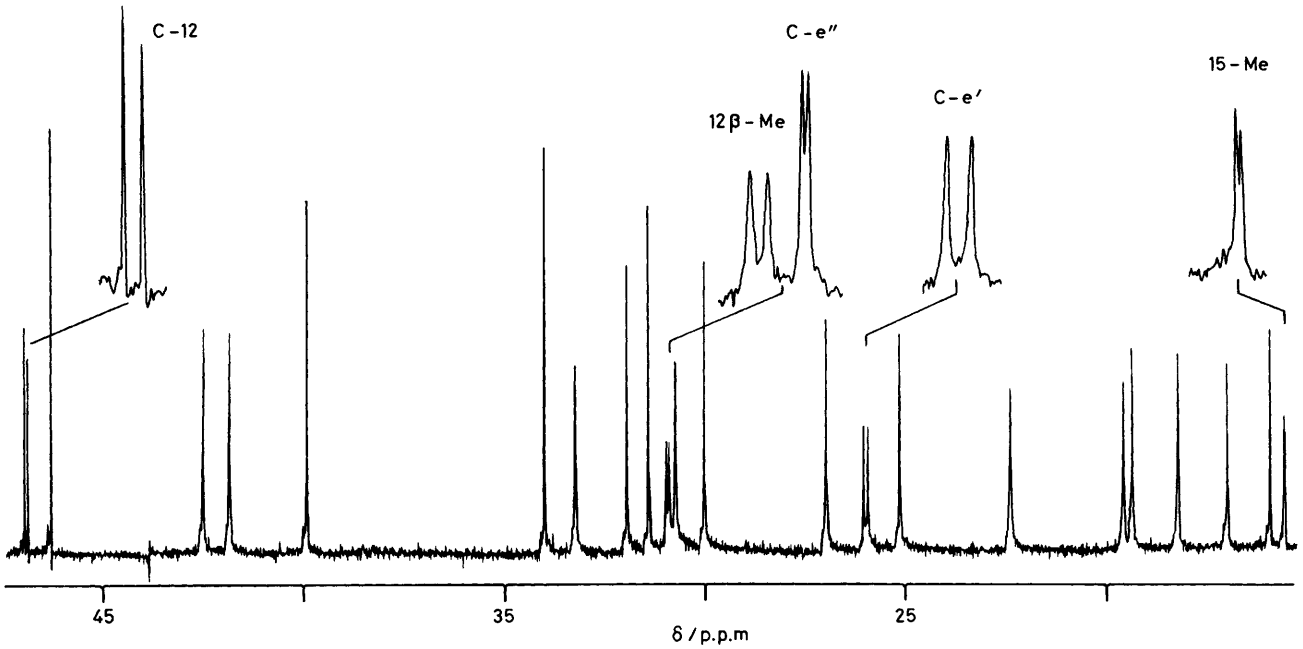


FIGURE 6 100.6 MHz ^{13}C N.m.r. spectrum after resolution enhancement from cobesters (1) + (5) in C_6D_6 over region δ_C 15—48 p.p.m.; 7 608 transients, sweep width 4 500 Hz, 16 K data points, acquisition time 1.82 s and digital resolution of 0.55 Hz per point

corresponding to C=O and C=N residues, three signals were shifted. One, δ 176.8, has already been assigned to C-11, another δ 163.8 was assigned to C-14 (see above) and the last, δ 174.1, must arise from the carbonyl group of the e side-chain since this signal was not enhanced in the ^{13}C spectrum of cobester (9). At this stage, the signals have been identified as corresponding to three of the four C=N systems of the corrin nucleus biosynthetically enriched [3] in cobester (9). Accordingly, the fourth signal, δ 175.9, was then assigned to C-16.

For cobester mixture (1) + (6) carrying some deuterium at C-8 and C-10, shifts due to both deuterium atoms were observed. However, since the effects arising from ^{2}H at C-10 have been identified above, clear deductions could be made. Of the ^{13}C signals from the four saturated quaternary centres (C-2, -7, -12, and -17), one at δ 49.1 was shielded by the ^{2}H at C-8 and so was assigned to C-7. Two others, δ 58.6 and 46.2 were unaffected whilst the signal from C-12 (δ 46.9, see above) was broadened. The signals from two CH_2 carbons were also shifted by ^{2}H at C-8; one, δ 26.9, was shown above to be from C-d' and the other, δ 42.4, was assigned to C-c'. This removes one earlier ambiguity and the other, concerning C-g', was cleared up when the signal at δ 31.9 shown by cobester (1) was greatly reduced in the spectrum of undiluted [^{2}H]cobester (7). In addition, the ^{13}C spectrum of the mixture (1) + (7) showed (i) clear isotopic shifts of the resonance from C-18, and (ii) six shifted signals between 171.1 and 174.1 p.p.m. (one of which has been assigned above to the carbonyl of the e side-chain) which can only be explained as being due to the signals of the carbonyl groups being shifted *downfield* by the trideuteriomethyl groups.

TABLE 5

Assignments of ^{13}C n.m.r. signals of cobester (1) based on selective ^{1}H-decoupling or ^{2}H-isotopic shifts

^{13}C-Resonance * δ_C (p.p.m.)	Assignment
176.8	C-11
175.9	C-16
175.6	C-4
172.2	C-9
164.1	C-6
163.8	C-14
104.2	C-5
102.2	C-15
91.5	C-10
82.8	C-1 †
58.6	C-17
49.1	C-7
46.9	C-12
46.2	C-2
42.4	C-c'
33.95	C-b''
31.9	C-g'

* δ_C Values are reported relative to the centre line of the solvent (C_6D_6, δ_C 128.0). † Assignment made earlier.[3]

We can now consider C-2 and C-17. For these two carbon atoms, the spectrum of cobester (1) + (8) extensively deuteriated α to the carbonyl groups and at C-8 was informative.

Previous work [13] and the results from the present study show, in general, that resonances of saturated carbon atoms are shifted 0.06—0.1 p.p.m. by ^{2}H at the β-position but only 0.0—0.07 p.p.m. by ^{2}H at the γ-position. In the spectrum of cobester mixture (1) + (8), the signal at δ 58.6 was shielded by 0.027 p.p.m. whereas that at δ 46.2 underwent two isotopic shifts of 0.097 and 0.153 p.p.m. These findings are consistent with δ 58.6 arising from C-17 (γ-shift by one or both deuterons at

C-f'') and δ 46.2 being from C-2 (β-shifts by $^2H_1$ and $^2H_2$ at C-a').

Finally, the two remaining signals at δ 164.1 and 172.1 must be due to C-6 and the seventh carbonyl group, almost certainly respectively, since C-14 (δ 163.8) and C-6 are expected to have similar chemical shifts. The fact that δ 172.1 is well within the region occupied by the six identified carbonyl resonances strengthens this assignment.

Conclusions.—The combined use of improved methods [6] has allowed assignment of all the skeletal proton resonances from cobester (1) and also of the 38 important ^{13}C resonances of this relatively complex system (1) (see Tables 1, 3, and 5). One can confidently make similar spectroscopic assignments for other tetrapyrrolic pigments and many such structures have already been determined in this laboratory on sub-milligram amounts using these techniques.[15] The ^{13}C assignments form the foundation for future biosynthetic researches on vitamin B_{12}.

After completion of the foregoing work on cobester (1), Ernst [16] reported a full assignment of the important signals in the ^{13}C spectrum of (1). Largely different methods were used but, independently of us, ^2H isotopic shifts were carefully explored. The conclusions of Ernst [16] and ours are in complete agreement and all the assignments thus gain complementary strength. We also agree with Ernst [16] that studies to render the shifts caused by ^2H on ^{13}C resonances more predictable [14] would be valuable.

EXPERIMENTAL

The ^1H n.m.r. spectra of heptamethyl dicyanocobyrinate, cobester, (1) and of its deuteriated derivatives (3)—(8) were obtained at 291 K on a Bruker WH400 spectrometer operating at 400 MHz under ASPECT 2000 computer control. The data presented in Tables 1 and 2 refer to 33 mM solutions of (1) (20 mg) in [$^2H_6$]benzene (0.55 ml) which were not degassed. For solutions at low concentration (33—0.8 mM), the chemical shifts and coupling constants were reproducible (±0.01 p.p.m.) whereas for higher concentrations (*e.g.* 178 mM) some variations in these values were observed. In all cases, the residual solvent peak was used as internal reference (δ 7.20 p.p.m.). Spectra were acquired with quadrature detection and phase cycling over 3 000 Hz; with 16 K data points, this gave an acquisition time of 2.73 s and a digital resolution of 0.37 Hz per point. The 90° pulse width was between 6—10 μs. Resolution enhancement was achieved by Gaussian multiplication of the original free induction decay (FID) prior to Fourier transformation.

Partially relaxed spectra of cobester (1) were obtained with an inversion-recovery sequence, (180°-t-90°); the delay time between each complete sequence was 4 s. The relaxation times, T_1, were then determined by the null point method from a series of these spectra.

Nuclear Overhauser and decoupling experiments on (1) and (3)—(8) were carried out in the difference mode using microprograms kindly made available by Dr J. K. M. Sanders.[6] The procedures are fully described in ref. 6, so only additional details are given: n.O.e.s were generated by continuous-wave irradiation at a chosen resonance frequency for 1.5—2 s before gating the decoupler off and acquiring the signal. One control irradiation satisfactorily served 10 or more on-resonance irradiations, the FID's of which were then acquired simultaneously. The decoupler was used at subsaturating power levels to ensure a selectivity of greater than 90%. For the decoupling experiments, the outcome of one control irradiation was accumulated for each on-resonance irradiation, the two irradiation frequencies being set as close together as possible.[6] The decoupler power was again chosen for selectivity so that couplings with $J > 4$ Hz were not completely removed. No advantage was found for the present work in subtracting spectra [6] so the n.O.e. and decoupling data were processed by subtracting the FID's of the on-resonance irradiation and the control to give a difference FID. For the n.O.e. experiments, the difference FID was line broadened (0.5—1.5 Hz), whereas for the decoupling experiments it was processed by resolution enhancement. Fourier transformation and phase adjustment then gave the required difference spectrum.

For the experiments involving use of shift reagent, Eu([$^2H_9$]-fod)$_3$ (2 mg) was added to a solution of cobester (1) (20 mg) in C_6D_6 (0.55 ml) and the spectrum was then recorded.

All ^{13}C n.m.r. spectra were obtained on a Bruker WH400 spectrometer operating at 100.6 MHz under ASPECT 2000 computer control using 0.178M-solutions of (1) (330 mg) in [$^2H_6$]benzene (1.5 ml) in a 10-mm broad band probe tuned to ^{13}C or with a 5 mm dedicated ^{13}C probe [110 mg of (1) in 0.5 ml]. Standard proton noise-decoupled, single frequency off-resonance decoupled, and fully coupled spectra were obtained using 16 K data points over 20 000 Hz, and an acquisition time of 0.4 s; quadrature detection and phase-cycling were employed in all cases. The original FID's were processed using 0.2—1.0 Hz line broadening. The spectra with off-resonance decoupling used for the graphical method of assignment (Table 3) were acquired with the above parameters and a decoupler power of *ca.* 900 Hz; 4 800 transients were collected for each decoupler frequency, and the decoupler was stepped under computer control through the ^1H spectrum from δ_H 0.5—4.5 p.p.m. at 0.25 p.p.m. intervals.

The deuteriated cobester samples (3)—(8) were examined by ^1H n.m.r. and then by ^{13}C n.m.r. with proton noise-decoupling to determine the site(s) of deuteriation. After dilution with unlabelled cobester (1), the ^{13}C spectra were acquired using 32 K data points over 20 000 Hz, giving an acquisition time of 0.8 s and a digital resolution of 1.2 Hz per point. These parameters were effective for observing ^2H isotopic shifts of >0.02 p.p.m.

For smaller ^2H isotopic shifts, resolution was improved by observing only the region of interest. Problems of 'fold-ins' were eliminated by judicious choice of spectral window and by setting the filter width equal to the sweep width: for observing the carbonyl-imine region (160—180 p.p.m.), a sweep width of 2 800 Hz over 16 K data points was used, acquisition time 2.91 s, digital resolution 0.34 Hz per point; for 74—108 p.p.m. sweep width 3 400 Hz, 16 K data points, acquisition time 2.41 s, digital resolution of 0.42 Hz per point; for 10—60 p.p.m., sweep width 4 500 Hz, 16 K data points, acquisition time 1.82 s, digital resolution of 0.55 Hz per point. The FID's accumulated using these three sets of parameters were processed by (i) resolution enhancement, then (ii) zero filling to 32 K, and (iii) Fourier transformation.

Preparation of Deuteriated Samples of Cobester.—(a) *General.* All the reactions described below were run under argon with exclusion of light. Cobester was prepared by methanolysis [1] of vitamin B_{12}; it crystallised from methyl acetate–hexane to give material with *ca.* 5% of a more polar corrin. This was removed by p.l.c. on silica (0.25 mm, 20 × 20 cm) in methanol–benzene (1 : 9) which had been saturated with KCN. The pure cobester band was eluted with methanol–methyl acetate (1 : 9) containing KCN; the eluate was evaporated and the residue dissolved in carbon tetrachloride. The filtered solution was evaporated and the residue recrystallised from methyl acetate–hexane to give cobester, m.p. 185—190 °C (decomp.).

A standard procedure was used to isolate the deuteriated samples of cobester below. The residue (or solution) from the labelling experiment was partitioned between water (20 ml) and carbon tetrachloride (20 ml) and the aqueous phase was made slightly basic when necessary with a saturated solution of sodium hydrogen carbonate, before addition of potassium cyanide (*ca.* 5 mg). The layers were equilibrated, separated, and the aqueous phase was extracted with carbon tetrachloride (3 × 20 ml). The combined extracts were washed with water (1 × 20 ml), dried over anhydrous sodium sulphate, and evaporated. The crude product was then purified as above. All the deuteriated samples of cobester (3)—(8) had properties (t.l.c., u.v.–vis., m.p.) identical with authentic cobester (1).

(b) [10-^{2}H]*Cobester* (3). [With Dr. R. Hollenstein] Cobester (200 mg) was heated under reflux for 18 h with a solution of 5% sulphuric acid in MeOD (2 ml) and the product was isolated directly from the resultant solution by the standard method to afford [10-^{2}H]cobester (3) (180 mg). This product showed 400 MHz ^{1}H n.m.r. and 100.6 MHz ^{13}C spectra identical with those of authentic cobester (1), except for the following: the signal at δ_H 5.74 was *ca.* 10% of its normal size, and in the ^{13}C spectrum, the signal assigned to C-10 (δ_C 91.5) was almost absent. This sample (3) (157 mg) was diluted with unlabelled cobester (1) (100 mg) for ^{13}C spectroscopy.

(c) [10,13-$^2H_2$]*Cobester* (4). A solution of cobester (1) (260 mg) in freshly distilled CF_3CO_2D was stirred at 20 °C for 19 h and then evaporated at <40 °C. The product was isolated by the standard method but was purified by column chromatography on silica (Kieselgel H; 5 g) eluting with benzene containing methanol (0—5%) and potassium cyanide. The resultant [10,13-^{2}H]cobester (4) (147 mg) showed δ_H 5.74 (10-H) and δ_H 2.81 (13-H) reduced to *ca.* half intensity and ^{13}C n.m.r. showed δ_C 91.5 (C-10) and δ_C 54.0 (C-13) similarly reduced.

(d) [13-^{2}H]*Cobester* (5). Cobester (4) (200 mg) was heated under reflux for 29 h in a solution of methanol (45 ml), trimethyl orthoformate (2.5 ml) and concentrated sulphuric acid (2.5 ml). The solution was then evaporated to *ca.* 25 ml under reduced pressure, and then worked up by the standard procedure to give [13-^{2}H]cobester (5) (167 mg). ^{1}H N.m.r. showed only the signal due to 13-H (δ 2.81) to be reduced in intensity. This sample was used without dilution for ^{13}C studies.

(e) *Hepta*[$^2H_3$]*methyl* [8,10-^{2}H]*cobyrinate* (6). A solution of cobyrinic acid (103 mg) in CD_3OD (8 ml) was treated with concentrated sulphuric acid (0.1 ml) and then heated under reflux for 48 h. The total solution was used directly for the standard work-up to give ester (6) (31 mg). The signals at δ_H 5.74 and 3.71 were absent from the ^{1}H spectrum and those at δ_C 91.5 and 54.6 were not present in the ^{13}C spectrum. Also, the two sets of OMe signals at δ_H 3.28—3.46 and δ_C 51—52 p.p.m. were absent. This total sample was mixed wtih unlabelled cobester (32 mg) for ^{13}C spectroscopy.

This preparation has been found to be capricious with respect to isotopic exchange at C-8; there is some factor, at present unknown, which causes it not to be reproducible.

(f) [g'-^{2}H]*Cobester* (7). Cobester (200 mg) and potassium cyanide (25 mg) in CD_3OD (1.5 ml) were stirred for 23 h at 20 °C before direct isolation of the product as usual to afford [g'-^{2}H]cobester (7) (165 mg). Its ^{1}H n.m.r. spectrum was identical with that of cobester (1) except that <5% of CH_3O signals were present and the signal from 18-H appeared as a double doublet, δ_H 2.88 (J 3.4 and 10.8 Hz) and a doublet δ_H 2.87 (J 10.8 Hz). In the ^{13}C spectrum, δ_C 31.9 and 39.9 p.p.m. were greatly reduced; the latter was replaced by two isotopically shifted peaks (Table 4).

This sample (143 mg) was diluted with unlabelled cobester (72 mg) for ^{13}C n.m.r.

(g) *Multi-deuteriated cobester* (8). Cobester (504 mg) and potassium cyanide (50 mg) in CD_3OD (5 ml) were heated under reflux for 14 h. Standard work-up of the final solution afforded multi-deuteriated cobester (8) (360 mg).

The ^{1}H n.m.r. spectrum of this product showed signals of considerably reduced intensity at δ_H 3.71 (8-H), δ_H 2.24—3.0 ($CH_2CO_2CD_3$), and δ_H 3.28—3.46 (OMe). The signal for 18-H, δ_H 2.89 appeared as a doublet (J 10.8 Hz), as did H-f'$_A$, δ_H 1.81, and H-f'$_B$, δ_H 2.44 ($J_{A,B}$ 15.4 Hz).

The ^{13}C n.m.r. spectrum showed loss of the following signals (relative to the spectrum from unlabelled cobester), the OMe signals and δ_C 33.95, 31.9, 31.4, and 29.9 p.p.m.; the following were greatly diminished in size, δ_C 54.6, 42.4, 41.8, and 30.7 p.p.m.; finally, several signals had partners caused by ^{2}H isotopic shift(s) (see Table 4).

Part of the foregoing sample (200 mg) was mixed with unlabelled cobester (1) (140 mg) before further purification by p.l.c. and recrystallisation to yield material (291 mg) for ^{13}C n.m.r. studies.

Grateful acknowledgement is made to Dr. J. K. M. Sanders for many helpful discussions, to Dr. R. Hollenstein for the indicated preparation, to Professor H.-H. Inhoffen and Dr. G. H. Gibbon (Glaxo) for valuable gifts, respectively, of cobester and vitamin B_{12}, and to the S.E.R.C. for financial support.

[2/161 *Received*, 27*th January*, 1982]

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