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BIOSYNTHESIS OF CLAVINE ALKALOIDS: PROTON MAGNETIC RESONANCE STUDIES*

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Abstract—A detailed proton magnetic resonance spectral analysis has been made for the clavine alkaloids setoclavine, penniclavine, agroclavine, and elymoclavine as a necessary preliminary to a biosynthesis study. The PMR spectra are used to establish the isotopic composition of elymoclavine biosynthesized in D_2O with selected hydrogen substrates. These results indicate a large, and previously unsuspected, degree of solvent participation in the biosynthesis of the alkaloid. The PMR data also establish the conformation of the normal alkaloids.

INTRODUCTION

THE ISOTOPIC composition of compounds biosynthesized by deuterated organisms using hydrogen substrates has been found to yield useful biogenetic information.[1-4] The compounds so obtained contain both hydrogen and deuterium, and the isotopic composition of the compound at many molecular positions can be inferred from proton magnetic resonance (PMR) spectroscopy. Such an approach to biogenetic problems appears particularly useful in providing an insight into mechanisms of precursor utilization. In such an experiment, carbon–hydrogen precursor compounds are supplied to an organism growing in D_2O, and proton magnetic resonance spectroscopy of the resulting isotopic hybrid compound[2] is used to ascertain the fate of the hydrogen. A necessary preliminary to the application of this procedure is the complete PMR spectral assignment for the compound whose biosynthesis is being studied. In conjunction with studies we are carrying out on the biosynthesis of clavine alkaloids[5] we present here a detailed description of the PMR spectra for the clavine alkaloids setoclavine, penniclavine, agroclavine and elymoclavine, and also report some results obtained on the biosynthesis of elymoclavine in D_2O-replacement culture.

Relatively little has been published on the nuclear magnetic resonance spectroscopy of clavine alkaloids.[6] Relevant to work described here are the studies of Stauffacher and Tscherter[7] who have analyzed the PMR spectra of various isomers of chanoclavine. The

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6 J. W. EMSLEY, J. FEENEY and L. H. SUTCLIFF, *High Resolution Nuclear Magnetic Resonance Spectroscopy*, Vol. II, p. 806, Pergamon Press, New York (1966).

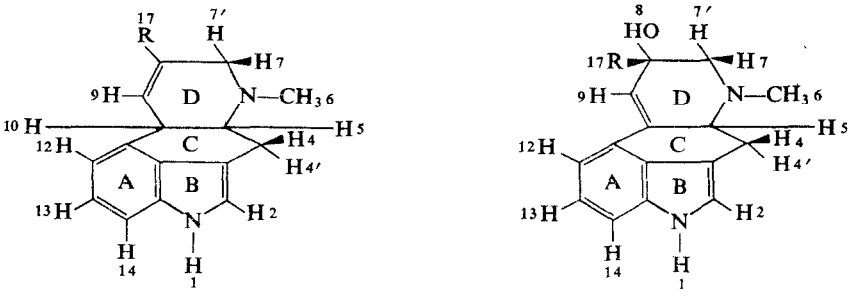
7 D. STAUFFACHER and H. TSCHERTER, *Helv. Chim. Acta.* **47**, 2186 (1964).

spectra these workers[7] present, however, are not sufficiently resolved at 60 Mc to measure the chemical shift of all of the protons. Chanoclavine also is unusual in that it is a naturally occurring clavine alkaloid that possesses an incomplete D ring, so that the PMR spectra of this alkaloid differ considerably from those observed for alkaloids with a complete D ring. We have assigned chemical shifts and coupling constants to all the protons in the alkaloids under study and have also applied these data to a conformational analysis of these compounds.

RESULTS AND DISCUSSION

Structures and Assignment of Chemical Shifts

Figure 1 shows the structure and proton numbering of the clavine alkaloids that were used in this study. The chemical shifts (δ, ppm from tetramethylsilane) for all the protons and the integral equivalents are presented in Table 1. The assignment of the protons of the methyl



R = CH_3, Agroclavine

R = CH_2OH, Elymoclavine

R = CH_3, Setoclavine

R = CH_2OH, Penniclavine

FIG. 2. STRUCTURAL FORMULAS AND PROTON NUMBERING OF CLAVINE ALKALOIDS.

TABLE 1. CHEMICAL SHIFTS AND MULTIPLICITIES* OF RESONANCES IN CLAVINE ALKALOIDS (δ, ppm FROM TMS)

	Setoclavine $C_{16}H_{18}ON_2$		Penniclavine $C_{16}H_{18}O_2N_2$		Agroclavine $C_{16}H_{18}N_2$		Elymoclavine $C_{16}H_{18}ON_2$	
Proton	δ	No. of protons	δ	No. of protons	δ	No. of protons	δ	No. of protons
6	2·43	3 (*s*)	2·46	3 (*s*)	2·38	3 (*s*)	2·41	3 (*s*)
7	2·45	1 (*d*)	2·79	1 (*d*)	2·73	1 (*d*)	3·05	1 (*d*)
4	2·67	1 (d^3)	2·73	1 (d^3)	2·90	1 (d^3)	2·92	1 (d^3)
7′	2·89	1 (d^2)	3·17	1 (d^2)	3·18	1 (*d*)	3·61	1 (*d*)
4′	3·10	1 (d^3)	3·20	1 (d^3)	2·51	1 (d^3)	2·59	1 (d^3)
5	3·53	1 (d^2)	3·53	1 (d^2)	3·36	1 (d^2)	3·35	1 (d^2)
10	—	0	—	0	3·89	1 (*m*)	3·94	1 (*m*)
17	1·42	3 (*s*)	4·00	2 (*s*)	1·68	3 (*s*)	4·36	2 (*s*)
17 (OH)	—	0	5·2–6·2	2	—	0	4·75	1
8 (OH)	5·2–6·2	1			—	0	—	0
9	6·65	1 (*m*)	6·85	1 (*m*)	6·30	1 (*m*)	6·71	1 (*m*)
Arom H	7·1–7·4	4 (*m*)	7·1–7·4	4 (*m*)	7·1–7·4	4 (*m*)	7·1–7·4	4 (*m*)
1	11·44	1 (*s*)	11·43	1 (*s*)	11·43	1 (*s*)	11·40	1 (*s*)

* The multiplicity is indicated by the letter in parenthesis. For a singlet, *s*; for a doublet, *d*; for coupling to two or three non-equivalent protons, d^2 or d^3; for a multiplet, *m*.

group at positions 6 and 17, the aromatic protons, the vinyl proton at 9 and the exchangeable protons is straightforward for all four alkaloids.

Setoclavine

Figure 2 shows the PMR spectrum of setoclavine (3·6 per cent w/v in deuterio-pyridine-d_5). The methine proton at position 5 appears as a quartet and is common to the spectra of all clavine alkaloids. In setoclavine and penniclavine, this quartet is centered at 3·53 ppm, while in agroclavine and elymoclavine a diamagnetic shift of 17–18 Hz is observed, presumably because the double bond in the D ring is shifted to the 8-9 position. Double resonance studies in which proton 5 is externally irradiated indicate that the multiplets centering at

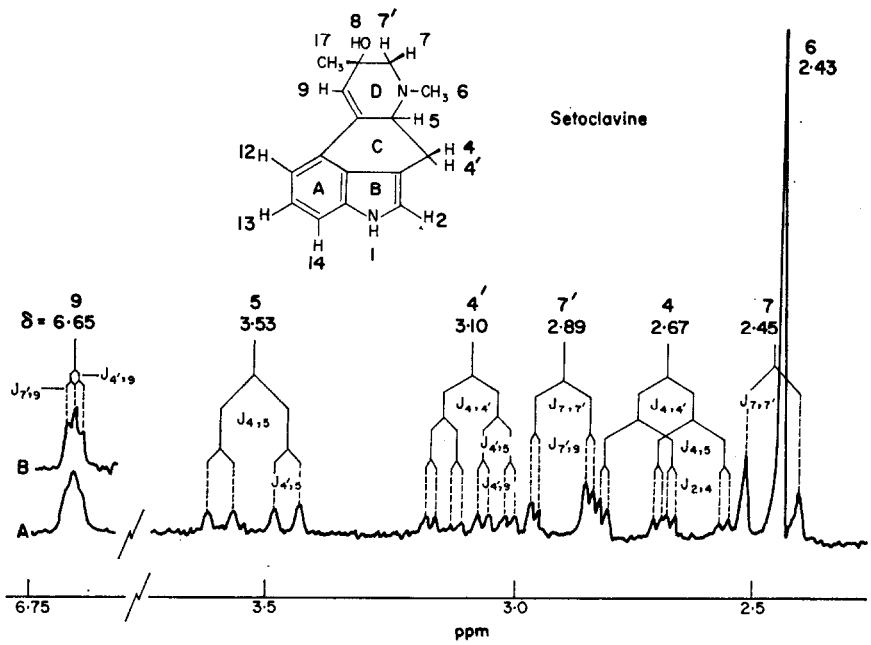


FIG. 2. A PORTION OF THE PMR SPECTRUM OF SETOCLAVINE.

The multiplicities and chemical shifts are indicated by the pitchfork diagrams. Spectrum A was recorded without decoupling; spectrum B (proton 9 only) was recorded with irradiation at proton 5.

2·67 ppm and 3·10 ppm are due to protons 4 and 4′. The assignments of protons 4 and 4′ were based on the postulates of Lemieux *et al.*[8] which state that for geminal protons, the chemical shift of the axial (4) should be at a higher field than the equatorial (4′) proton; consequently, proton 4 is assigned a chemical shift of 2·67 ppm and proton 4′ a value of 3·10 ppm. Lemieux[8] has also shown that the absolute value of diaxial–vicinal couplings may be expected to be larger than for the axial–equatorial or diequatorial–vicinal coupled systems in cyclohexane-type ring systems. Decoupling of protons 4 and 4′ from proton 5 produces vicinal coupling constants that follow this relationship. A summary of these and other spin–spin coupling constants for setoclavine is given in Table 2.

[8] R. U. LEMIEUX, R. K. KULLNIG, H. J. BERNSTEIN and W. G. SCHNEIDER, *J. Am. Chem. Soc.* **80**, 6098 (1958).

TABLE 2. SPIN–SPIN COUPLING CONSTANTS IN SETOCLAVINE

Coupling	J* (Hz)
$J_{4,5}$	13·7
$J_{7,7'}$	11·2
$J_{4,4'}$	10·9
$J_{4',5}$	5·4
$J_{4',9}$	1·9
$J_{2,4}$	1·8
$J_{7',9}$	1·6
$J_{5,9}$	<0·5

* ±0·2 Hz.

The center of a doublet of doublets, $|J| = 11{\cdot}2$ and 1·6 Hz, appears at 2·89 ppm. A doublet, $|J| = 11{\cdot}2$ Hz, is centered at approximately 2·47 ppm. Double resonance experiments indicate that the groups of signals are mutually coupled. The higher-field resonance ($\delta = 2{\cdot}47$ ppm) is assigned to the axial proton at 7, and the quasi-equatorial geminal proton at 7′ is assigned a chemical shift of 2·89 ppm. We then have $|J_{7,7'}| = 11{\cdot}2$ Hz and $|J_{7',9}| = 1{\cdot}6$ Hz.

Penniclavine, Agroclavine, and Elymoclavine

In penniclavine, the C-17 methyl group of setoclavine is replaced by a hydroxymethyl group. The C-17 hydroxymethyl substituent produces significant paramagnetic shifts in the protons which are nearest to it (see δ for 7,7′ and 9 in Table 1). This paramagnetic shift brings the chemical shift of proton 7 very close to that of proton 4 and that of proton 7′ close to proton 4′ (Table 1). Unequivocal decoupling experiments involving these protons could not be performed, but straightforward assignments could be made by analogy with the setoclavine spectrum.

Agroclavine, a Δ8-9 clavine alkaloid, possesses a C-17 methyl group instead of the hydroxymethyl present in penniclavine. As is to be expected, structural changes in the D ring do not affect the chemical shift of either the indole proton or the aromatic protons of the A and B rings. The C-9 proton of agroclavine, however, has experienced a diamagnetic shift of 35 Hz with respect to the C-9 proton of setoclavine. The increased shielding of this proton in agroclavine may be due to the change from a conjugated (9-10) double bond to an isolated (8-9) double bond, and the absence of the (deshielding) oxygen at position 8. In the high-field region of the agroclavine spectrum, the C-17 methyl is assigned a chemical shift of 1·68 ppm. In comparison with the C-17 methyl of setoclavine (1·42 ppm), the agroclavine methyl resonance is shifted to lower-field by 0·26 ppm because of deshielding by the double bond at the 8-9 position.

Six protons of agroclavine appear as multiplets in a nearly continuous absorption envelope extending from 2·4 to 3·6 ppm. This region contains the resonances of proton 7 which overlap with the signals produced by 4 and 4′, and the resonances of 7′, which overlap partially with the spin pattern of proton 5. A similar, but less complex set of overlapping resonances, is found in the spectrum of elymoclavine; here, however, only portions of the resonance envelopes of 4 and 4′ overlap. In the cases of agroclavine and elymoclavine, suitable double and triple resonance studies allow definition of the multiplicities and chemical shifts involved.

Long Range Coupling

Sternhell[9] and Barfield[10] have cited many examples of long range coupling in both aliphatic and cyclic systems. Several conditions which may result in long range spin–spin interactions can be found in the clavine alkaloids. Absolute values of the coupling constants are most easily measured in the spectrum of setoclavine because in this alkaloid the resonance signals do not overlap.

In setoclavine, spin–spin interactions through four bonds in an allylic type arrangement are observed between protons 2 and 4, $|J| = 1{\cdot}8$ Hz, and protons 5 and 9, $|J| < 0{\cdot}5$ Hz. The magnitudes of these coupling constants are consistent with those given by Barfield[10] for allylic coupling constants in cyclic hydrocarbons and with the structures of Fig. 1. The data indicate that protons 2 and 4 should approach an "in-plane" configuration and that proton 5 is considerably out of the plane defined by proton 9 and the intervening bonds. The relatively large coupling constants between proton 7′ and 9, $|J| = 1{\cdot}6$ Hz, suggests that these diequatorial protons are arranged in a W configuration and are coplanar with the intervening bonds.[10,11] A more unusual interaction is seen in the coupling of protons 4′ and 9, $|J| = 1{\cdot}9$ Hz, (but not of protons 4 and 9) which occurs through five bonds.

In setoclavine and penniclavine, proton 7′ is in a W configuration with proton 9, and the replacement of CH_3 with CH_2OH at position 17 gives a larger paramagnetic shift to proton 7 than to 7′. These data indicate that the D ring in these two alkaloids is in a quasi-chair conformation with proton 7′ in an equatorial position and 7 in an axial position. Similarly, the D ring in agroclavine and elymoclavine should be in a quasi-chair conformation, as 7′ receives a greater paramagnetic shift than proton 7. However, it is possible to satisfy the PMR data with structures in which proton 10 is axial or equatorial. We favor the structure shown in Fig. 1 because of a very small coupling constant, less than 0·5 Hz, between protons 10 and 5. This small coupling constant between protons 10 and 5 in agroclavine and elymoclavine indicates a *cis* fusion between rings C and D.

Biosynthesis in Replacement Culture

Elymoclavine has been isolated from replacement cultures in 98–99 per cent D_2O using succinic acid and phenylalanine as substrates.[12] The technique of replacement culture circumvents the inability of fully deuterated ergot to synthesize alkaloids. In the replacement culture technique, mycelium from ergot cultures grown in H_2O on protio-nutrients is transferred to D_2O and then supplied with substrates that will elicit alkaloid production. Alkaloids produced in this way contain both H and D, and are referred to as isotope hybrid compounds. Table 3 lists the proton chemical shifts observed on isotope hybrid elymoclavine which was isolated from replacement culture in D_2O, with deuterio-succinic acid and protio-phenylalanine as substrates. Some small differences in chemical shifts between the protio and the hybrid elymoclavine can be noted; these probably are due to deuterium isotope effects on the proton chemical shifts. The general agreement between chemical shift and the chromatographic behavior of the isotope hybrid compound[12] establishes the identity of the isolated substance as elymoclavine.

Table 4 shows the isotopic composition of two isotopic hybrid elymoclavine preparations as determined by integration of PMR spectra. These data show two features of interest:

[9] S. STERNHELL, *Rev. Pure Appl. Chem.* **14**, 15 (1964).
[10] M. BARFIELD, *J. Chem. Phys.* **41**, 3825 (1964).
[11] J. MEINWALD and A. LEWIS, *J. Am. Chem. Soc.* **83**, 2769 (1961).
[12] R. G. MRTEK, H. L. CRESPI, M. I. BLAKE and J. J. KATZ, *J. Pharm. Sci.* **56**, 1234 (1967).

(1) the overall similarity in isotopic composition of the two products, despite the utilization of protio-succinic acid in one experiment and deuterio-succinic acid in the other; (2) the variation in the isotopic composition at positions 7, 7′, 9, and 17. These data indicate that phenylalanine contributes to a considerable extent to the formation of the precursors of isopentenylpyrophosphate, thus minimizing the expected increase in deuterium incorporation because of the use of deuterio-succinate. Further, the data strongly suggest almost complete equilibration of the methylene group of acetoacetate-CoA with the medium. This equilibration leads to complete deuteration of position 2 in mevalonate. In mevalonate itself, these

TABLE 3. CHEMICAL SHIFTS OF ISOTOPIC HYBRID ELYMOCLAVINE

Proton	δ (ppm)
6	2·38
7	3·05
4	2·92
7′	3·64
4′	2·60
5	3·31
10	3·94
17	4·37
9	6·68
Arom. H	7·1–7·3
1	11·40

TABLE 4. ISOTOPE ANALYSIS OF ELYMOCLAVINE OBTAINED FROM REPLACEMENT CULTURES IN D_2O

		Deuterium incorporation*	
Proton	Substrates:	Protio-succinic acid Protio-phenylalanine	Deuterio-succinic acid Protio-phenylalanine
6		69	78
7		87	85
7′		79	70
9		86	90
10		98	99
17		98	100
4,4′		85	82
5		74	70

* Data presented as per cent deuterium at each position.

hydrogen atoms are non-exchangeable.[13] Consequently, any equilibration of the —CH_2— group with the medium must occur at an even earlier stage of biosynthesis. An alternative but less likely interpretation requires labilization of hydrogen at position 17 in elymoclavine during oxidation. The isotopic composition of our elymoclavine can only be explained in terms of a very large degree of equilibration of all molecular sites with the solvent water. Exchange reactions must occur at many stages in the biosynthesis of elymoclavine and solvent water contributes a surprisingly large proportion of the hydrogen in the final product.

[13] A. HOFFMAN, *Die Mutterkornalkaloide*, p. 132, Enke, Stuttgart (1964).

The usual picture in which precursor molecules are incorporated in large intact blocks clearly is inadequate to the chemistry of hydrogen in this biosynthesis situation, and is undoubtedly inadequate in other biosynthesis schemes as well.

EXPERIMENTAL

Spectra were measured on a Varian HA 100 NMR spectrometer in the HA mode. Samples of alkaloids* were dissolved in pyridine-d_5 (98 per cent minimum isotopic purity†) in sealed, evacuated tubes, which also contained approximately 10 per cent tetramethylsilane. The PMR spectra were collected at about 33°. Proton spin–spin decoupling data were obtained with the instrument operating in the frequency-sweep mode. Proton spectra of highly deuterated alkaloids were enhanced by repetitive scanning techniques. Integration of the deuterium-decoupled spectra was performed manually with an Ott planimeter.

* Authentic samples of alkaloids were obtained from Pierce Chemical Co., Rockford, Illinois.
† Volk Radiochemical Company, 803 North Lake Street, Burbank, California.