Biosynthesis of Ergot Alkaloids. Studies on the Mechanism of the Conversion of Chanoclavine-I into Tetracyclic Ergolines
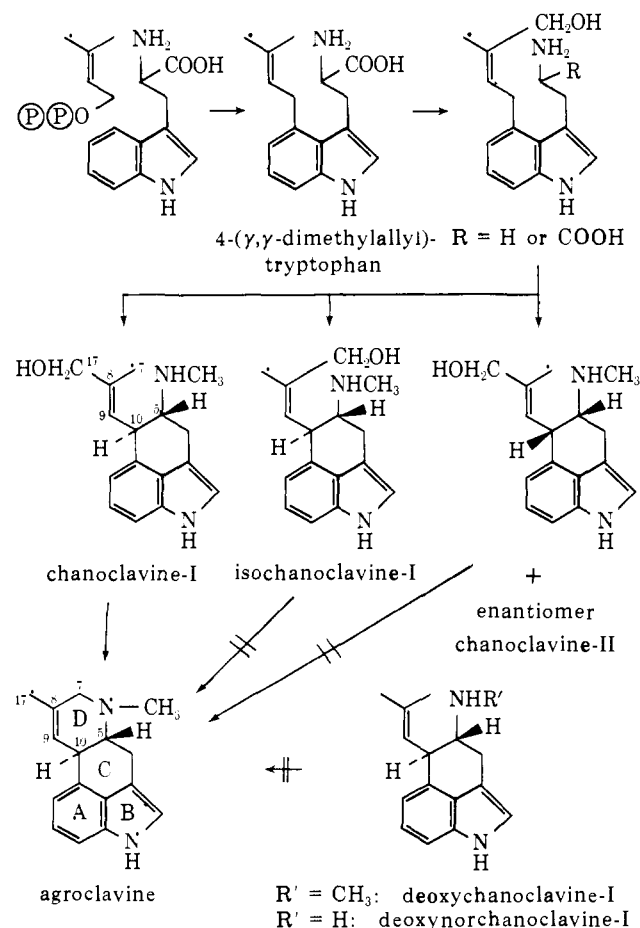
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Abstract: As indicated by double labeling experiments with stable isotopes, the cyclization of chanoclavine-I to tetracyclic ergolines involves an intermolecular transfer of the hydrogen at C-9 into the same position of a new molecule. Also in this process, one of the methylene hydrogens at C-17 of the precursor is exchanged, the newly introduced hydrogen occupying the *pro-R* position at C-7. This and the fact that chanoclavine-I-aldehyde is efficiently and specifically converted into elymoclavine by the ergot fungus suggest that the cyclization involves aldehyde intermediates. Experiments with $(3R,4R)$-mevalonic-2-^{14}C-4-t acid indicate that an isotope effect in the further metabolism of chanoclavine-I leads to an enrichment of tritium at C-9 of the unreacted chanoclavine-I. A mechanism involving double bond isomerization at the aldehyde stage is proposed which accounts for all the observations.

The biosynthesis of the tetracyclic ergoline ring system of ergot alkaloids from tryptophan, mevalonic acid, and the methyl group of methionine has been found to involve an unexpectedly complex sequence of reactions which is still not very well understood.[1] The pathway as we see it now (Scheme I) involves two cis–trans isomerizations at the allylic double bond, one occurring in the reaction sequence leading to chanoclavine-I and the other in the conversion of chanoclavine-I into the tetracyclic ergoline, agroclavine. It is attractive to speculate that these two isomerizations are somehow tied to the corresponding ring closure reactions leading to the formation of the 5,10 and the 6,7 single bonds, respectively. An attempt has been made to establish such a relationship for the first isomerization and the formation of ring C but without producing the expected results.[2,3] Various pieces of information are available, which relate to the mechanism of the second isomerization and the formation of ring D. It is known that the conversion of chanoclavine-I to agroclavine and elymoclavine proceeds with complete retention of the hydrogen at C-10.[4,5] The retention of the hydrogen at C-9, on the other hand, is not complete. Tritium retentions of 70% were observed for the incorporation of both $(3R,4R)$-mevalonic-2-^{14}C-4-t acid and chanoclavine-I-7-^{14}C-9-t into elymoclavine.[5] The chanoclavine-I reisolated from the latter experiment had the same $T/^{14}C$ ratio as the starting material. The same mevalonic acid was converted into chanoclavine-I with 100% tritium retention using a different ergot strain which produces chanoclavine-I as the terminal alkaloid.[5] In the present paper we would like to present results which give further insight into the mechanism of the transformation of chanoclavine-I into tetracyclic ergolines. Some of these results have been communicated in preliminary form.[6]

Results

In a previous publication[5] we discussed, as one explanation for the above mentioned tritium retention

Scheme I

4-(γ,γ-dimethylallyl)- R = H or COOH
tryptophan

chanoclavine-I   isochanoclavine-I   + enantiomer chanoclavine-II

agroclavine   R' = CH₃: deoxychanoclavine-I
R' = H: deoxynorchanoclavine-I

(1) For reviews see (a) R. Voigt, *Pharmazie*, **23**, 285, 353, 419 (1968); (b) E. Ramstad, *Lloydia*, **31**, 327 (1968); H. G. Floss, *Abh. Deut. Akad. Wiss. Berlin, Kl. Chem., Geol., Biol.*, 395 (1972).

(2) M. Seiler, W. Acklin, and D. Arigoni, *Chem. Commun.*, 1394 (1970); *Chimia*, **24**, 449 (1970).

(3) C. I. Abou-Chaar, H. F. Guenther, M. F. Manuel, J. E. Robbers, and H. G. Floss, *Lloydia*, **35**, 272 (1972).

(4) S. Agurell, *Acta Pharm. Suecica*, **3**, 71 (1966).

(5) H. G. Floss, U. Hornemann, N. Schilling, K. Kelley, D. Gröger, and D. Erge, *J. Amer. Chem. Soc.*, **90**, 6500 (1968).

(6) H. G. Floss, D. Gröger, and D. Erge, paper presented at the 5th International Symposium on the Chemistry of Natural Products, London, July 1968, Abstracts, p 72; B. Naidoo, J. M. Cassady, G. E. Blair, and H. G. Floss, *Chem. Commun.*, 471 (1970).

data, the possibility of a mechanism involving an intermolecular hydrogen transfer. Such a mechanism can, for example, be visualized as shown in Scheme II.

Scheme II

Scheme III
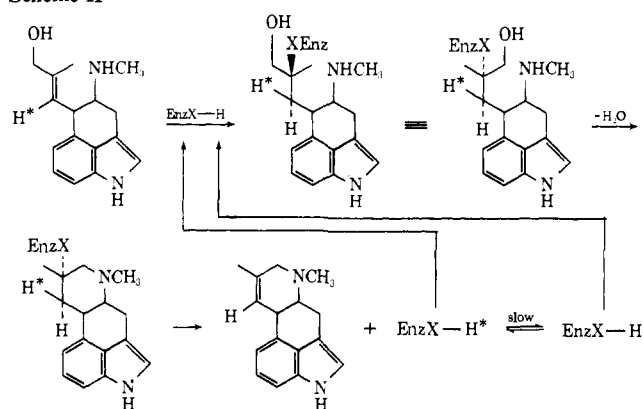
By addition of some XH group of the enzyme across the double bond of chanoclavine-I, a second hydrogen would be introduced at C-9 and, with the rotation around the 8,9 bond required to bring the hydroxymethyl group close to the nitrogen, the original hydrogen from this position would be transferred to the enzyme in the subsequent elimination step. If this hydrogen on the enzyme were to undergo only slow exchange with solvent protons, it could be introduced into a new molecule of bound substrate, where it would now occupy the enantiotopic position and would thus end up in the product molecule upon completion of the second turnover of the enzyme. To test for such an intermolecular transfer of the hydrogen at C-9 of chanoclavine-I we carried out experiments with mevalonic acid intramolecularly labeled with deuterium at C-4 (indicator label) and C-3' (reference label). This material was prepared as shown in Scheme III. Base-catalyzed equilibration of 1,1-dimethoxybutan-3-one with D_2O gave, after four exchanges, the pentadeuterated compound containing 100% deuterium in the methylene group and over 90% deuterium in the C-methyl group, as determined by pmr. This material was then used in a Reformatsky reaction to give the acetal of mevaldic acid ethyl ester, which was converted to mevalonic acid essentially as described by Eggerer and Lynen.[7] As shown by mass spectral analysis, the latter consisted of 80% pentadeuterated, 11% tetradeuterated, 7% trideuterated molecules, and less than 1% each of molecules containing 2, 1, and 0 atoms of deuterium. The rest of Scheme III indicates the presumed metabolic fate of these labeled hydrogens in the ergot fungus. One atom of deuterium from C-4 would be lost in the transformation of mevalonic acid to dimethylallyl pyrophosphate and another one from the methyl group in the further transformation of the latter into chanoclavine-I. This alkaloid, then, should contain the majority of its deuterium in molecules carrying 3 atoms of deuterium. In addition there would be varying amounts of unlabeled molecules, depending on the ratio of added to endogenously synthesized mevalonic acid, and, because of the incomplete deuteration of the precursor, a small percentage of di- and monodeuterated species. If the conversion

(7) H. Eggerer, F. Lynen, E. Rauenbusch, and J. Kessel, *Justus Liebigs Ann. Chem.*, 608, 71 (1957).
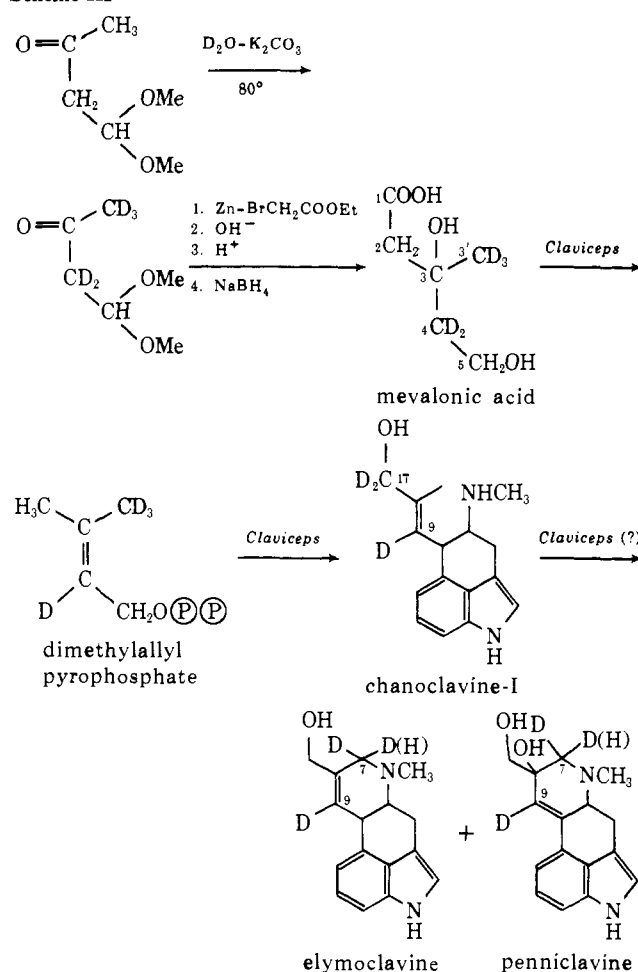
of chanoclavine-I into the tetracyclic alkaloids is straightforward, then elymoclavine and its immediate conversion product penniclavine should have essentially the same deuterium distribution, except for a slight decrease in deuterium content due to exchange or an isotope effect. If on the other hand this conversion involves an intermolecular hydrogen transfer as outlined above, then a deuterium atom removed from C-9 could be introduced either into a molecule already containing deuterium, in which case there would be no net change, or into an unlabeled molecule, in which case one D_3 molecule and one D_0 molecule would give rise to one D_2 and one D_1 molecule. The operation of an intermolecular hydrogen transfer mechanism would thus increase the amount of D_2 and D_1 species in the tetracyclic alkaloids at the expense of the D_3 and the D_0 species. This increase would depend on the ratio of labeled to unlabeled species in chanoclavine-I, and the expected deuterium distribution of the tetracyclic alkaloids can be estimated from the observed deuterium distribution of chanoclavine-I and the degree of tritium retention. To determine the latter individually in each experiment, the deuterated mevalonic acid was mixed with $(3R,4R)$-mevalonic-2-^{14}C-4-t[8] acid (final specific activity 1.44 × 10^{7} dpm ^{14}C/mmol, T/[14]C = 2.48). This precursor was then fed to cultures of *Claviceps* strain SD 58 under several different con-

(8) "$(3R,4R)$-mevalonic-2-^{14}C-4-t acid" always refers to the 1:1 mixture of the 3R,4R and 3S,4S isomers, of which only the 3R,4R isomer is biologically active.[5] The radioactivities given are always those of the mixture.

Table I. Conditions of Feeding Experiments with Intramolecularly Double Labeled Deuteriomevalonic Acid in *Claviceps* strain SD 58 (T/^{14}C of precursor, 2.48)

Expt no.	Feeding conditions	Alkaloid formed	Radioactivity of product (T/^{14}C = T retn)[a]
1	0.2 mmol mevalonate-2-^{14}C-4R-4-t-3',4-d_5 to one 100-ml NL 406 medium replacement culture	4.7 mg	Chanoclavine-I: 3.33 = 134% Elymoclavine: 1.35 = 54% Penniclavine: 1.28 = 52%
2	0.5 mmol mevalonate-2-^{14}C-4R-4-t-3',4-d_5 to two 25-ml NL 406 medium replacement cultures	7.6 mg	Chanoclavine-I: 3.37 = 136% Elymoclavine: 2.04 = 82% Penniclavine: 1.83 = 74%
3	0.05 mmol mevalonate-2-^{14}C-4R-4-t-3',4-d_5 + 0.02 mmol L-tryptophan to two 25-ml phosphate buffer replacement cultures	692 μg	Chanoclavine-I: 3.1 = 127% Elymoclavine: 1.0 = 40%
4	0.025 mmol mevalonate-2-^{14}C-4R-4-t-3',4-d_5 + 0.025 mmol mevalonate + 0.02 mmol L-tryptophan as in expt 3	719 μg	Chanoclavine-I: 3.5 = 141% Elymoclavine: 0.96 = 39%

[a] T retn (tritium retention) = {[T/^{14}C of product]/[T/^{14}C of precursor]}100%.

Table II. Deuterium Distribution in Clavine Alkaloids Biosynthesized from Mevalonic-3',4-d_5 Acid by *Claviceps* strain SD 58

%[a]	Found in chanoclavine-I	Exchange at C-9	Intermolecular transfer at C-9	Exchange at C-9 and loss of 1H from C-17	Intermolecular transfer at C-9 and loss of 1H from C-17	Found in Elymoclavine	Found in Penniclavine
				Experiment 1			
D$_3$	28.4	15	6	0	0	0.7	0.3
D$_2$	12.4	20	25	18	6–8	9.1	6.1
D$_1$	1.8	7	23	21	25–42	28.7	28.9
D$_0$	57.4	58	46	61	51–69	61.5	64.7
				Experiment 2			
D$_3$	46.0	36	24	0	0	2.5	6.9
D$_2$	19.0	25	32	43	24–29	36.3	28.8
D$_1$	2.9	6	27	22	32–50	26.5	30.8
D$_0$	32.1	33	17	35	21–44	34.7	33.5
				Experiment 3			
D$_3$	33.6	13	7	0	0	0.5	
D$_2$	16.5	27	30	17	7–9	11.4	
D$_1$	5.7	12	24	31	30–46	32.0	
D$_0$	44.2	48	39	52	45–63	56.1	
				Experiment 4			
D$_3$	27.9	11	5	0	0	0.4	
D$_2$	6.0	19	24	12	5–6	3.2	
D$_1$	14.7	9	17	26	24–38	20.7	
D$_0$	51.4	61	54	62	56–71	75.7	

[a] The figures given are the percentages of the molecules containing 3, 2, 1 and 0 atoms of deuterium, respectively.

Table III. Incorporation of ^{14}C- and Tritium-Labeled Precursors into Elymoclavine by *Claviceps* Strain SD 58

Expt no.	Precursor fed	Radioactivity fed	Alkaloid formed, mg	Radioactivity of elymoclavine	Incorporation, % T retn, %
5	18.6 mg chanoclavine-I-^{14}C-17-t	1.19 × 10^6 dpm ^{14}C, T/^{14}C = 5.15	141	2.10 × 10^5 dpm ^{14}C, T/^{14}C = 2.72	17.6 53
8	(3R,4R)-Mevalonolactone-2-^{14}C-4-t	4.4 × 10^7 dpm ^{14}C, T/^{14}C = 5.55	128	6.15 × 10^6 dpm ^{14}C, T/^{14}C = 3.16	14 57
14	0.25 mg chanoclavine-I-aldehyde-17-t	1.01 × 10^7 dpm	142	4.05 × 10^6 dpm	40.1
15	0.06 mg chanoclavine-I-17-t	4.88 × 10^6 dpm	141	4.83 × 10^5 dpm	9.9
16	0.12 mg chanoclavine-I-aldehyde-17-t	5.0 × 10^6 dpm	127	1.17 × 10^6 dpm	23.3
17	0.12 mg chanoclavine-I-17-t	1.0 × 10^7 dpm	127	5.74 × 10^5 dpm	5.7
18	5.2 mg chanoclavine-I-aldehyde-^{14}C-17-t	3.60 × 10^5 dpm ^{14}C, T/^{14}C = 1.78	71	1.24 × 10^5 dpm ^{14}C, T/^{14}C = 1.61	34.4 91

ditions and the alkaloids chanoclavine-I, elymoclavine, and, in some experiments, penniclavine were isolated, purified chromatographically, and analyzed for their T/^{14}C ratios. The data are summarized in Table I. Aliquots of these alkaloids were then subjected to mass spectral analysis to determine from the isotope com-position of the molecular ion the number of unlabeled, mono-, di-, and trideuterated molecules. In Table II the observed values are compared with the expected figures or the expected range of values estimated for various mechanisms. In accordance with expectations, the chanoclavine-I does indeed carry most of its

Table IV. Degradation of Biosynthetically Labeled Elymoclavines

Expt no.	Precursor	Elymoclavine	N-Methylsecoelymoclavine	Acetic acid
5	Chanoclavine-I-^{14}C-17-t	T/^{14}C = 2.72	2.71 × 10^5 dpm T/mmol, T/^{14}C = 2.69	2.61 × 10^5 dpm T/mmol = 93%
8	(3R,4R)-Mevalonolactone-2-^{14}C-4-t	2.22 × 10^6 dpm T/mmol, T/^{14}C = 3.16	2.18 Y 10^6 dpm T/mmol, T/^{14}C = 3.18	3.0 × 10^3 dpm T/mmol = 0.14%; 4.7 × 10^3 dpm T/mmol = 0.21%
14	Chanoclavine-I-aldehyde-17-t	2.08 × 10^5 dpm T/mmol	N.d.	2.05 × 10^5 dpm T/mmol = 98.5%
16	Chanoclavine-I-aldehyde-17-t	2.75 × 10^5 dpm T/mmol	N.d.	2.59 × 10^5 dpm T/mmol = 94%

deuterium in the form of trideuterated molecules. However, the distribution of deuterium in elymoclavine and penniclavine is quite different. Particularly striking is the virtual absence of trideuterated molecules. This is definitely not in agreement with the expected distribution for a simple exchange of some of the label from C-9 and it also does not fit the values expected for just an intermolecular transfer. However, a much better fit is obtained if the assumption is made that the reaction involves not only an intermolecular transfer of the hydrogen at C-9 but also loss of one of the deuterium reference labels at C-17. Assuming loss of one of the deuterium atoms from C-17 also improves the fit between observed and calculated data for the case of a mere exchange of some label from C-9, although the agreement is not as good as for the intermolecular transfer case.

In order to test the assumption that one of the hydrogens at C-17 is replaced during the transformation of chanoclavine-I into tetracyclic ergolines, we prepared chanoclavine-I-17-t by the following reaction sequence.[9,10]
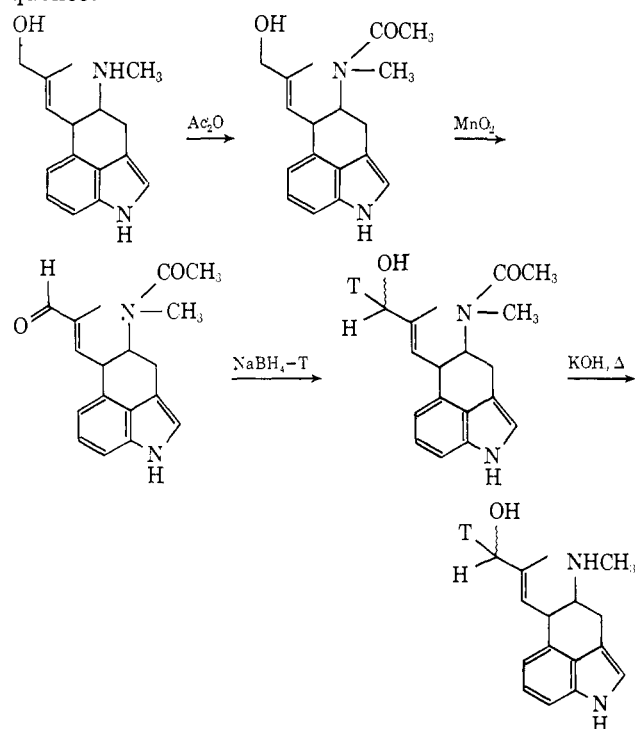

The last step, the alkaline hydrolysis of N-acetylchanoclavine-I, is very inefficient[10] and in later work we therefore used direct reduction of chanoclavine-I-aldehyde for the preparation of chanoclavine-I-17-t. The tritiated material was mixed with chanoclavine-I-^{14}C

obtained biosynthetically from tryptophan-$^{14}C^{11}$ to give a T/^{14}C = 5.15 and then fed to two cultures of *Claviceps* strain SD 58. The resulting elymoclavine (experiment 5, Table III) had a T/^{14}C ratio of 2.72 (tritium retention 53%) confirming the loss of one of the two labeled hydrogens from C-17 of chanoclavine-I. The elymoclavine from this experiment was diluted with carrier material and degraded by the following reaction sequence[5,12] to locate the tritium in the molecule.
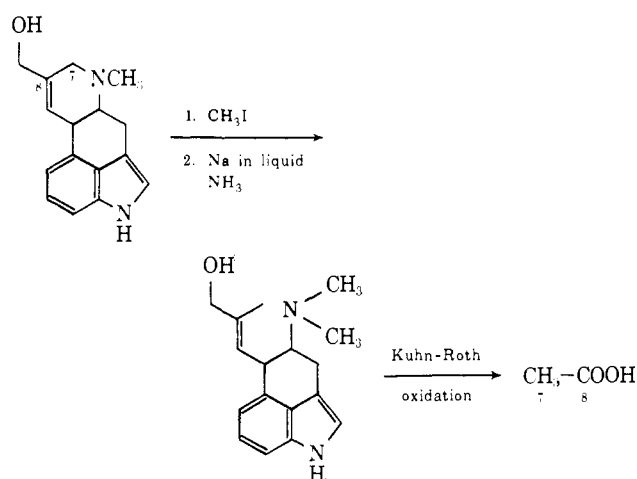
The result of this degradation (Table IV) shows that essentially all the tritium is confined to the 7 position as expected. Thus, the experiment with chanoclavine-I-^{14}C-17-t confirms the correctness of the assumption that one of the hydrogens at C-17 is replaced during the conversion into tetracyclic ergolines.

While the data from the experiments with mevalonate-3',4-d_5, in light of the above, favor a process involving an intermolecular hydrogen transfer, the predicted deuterium distributions for exchange at C-9 and loss of one deuterium from C-17 are not sufficiently different to exclude this possibility. Furthermore, the evidence for an intermolecular hydrogen transfer in this type of experiment is based on the separation of two isotopic labels which in the precursor are present in the same molecule. A much stronger case could be made on the basis of an experiment of the opposite type, in which two isotopic labels which are present in separate molecules in the precursor end up in the same molecule in the product. To carry out such an experiment we synthesized mevalonic-4-d_2 acid from acetic-d_4 acid via methyl bromoacetate and mevaldic acid essentially as described by Cornforth, et al.,[13] for the synthesis of

(9) T. Fehr, Ph.D. Dissertation, ETH Zürich, 1967.

(10) A. Hofmann, R. Brunner, H. Kobel, and A. Brack, *Helv. Chim. Acta*, 40, 1358 (1957).

(11) D. Gröger, D. Erge, and H. G. Floss, *Z. Naturforschg. B*, 21, 827 (1966).

(12) S. Bhattacharji, A. J. Birch, A. Brack, A. Hofmann, H. Kobel, D. C. C. Smith, H. Smith, and J. Winter, *J. Chem. Soc.*, 421 (1962).

(13) J. W. Cornforth, R. H. Cornforth, A. Pelter, M. E. Horning, and G. Popjak, *Tetrahedron*, 5, 311 (1959).

mevalonic-4-^{14}C acid. This material (99% D_2) was mixed in a ratio of 1:1 with mevalonic acid-2-^{13}C which had been synthesized from sodium acetate-2-^{13}C (86.8 atom % excess ^{13}C) via ethyl acetate and its condensation with 1,1-dimethoxybutan-3-one according to Pichat, et al.,[14] to give the ethyl ester of mevaldic acid dimethyl acetal, which was then converted to mevalonic acid by a modification of the method of Eggerer, et al.[7] This mixture was fed in each of two separate experiments to two 20-ml medium NL 406 replacement cultures of Claviceps strain SD 58. Chanoclavine-I and elymoclavine were isolated and purified as before and analyzed by mass spectrometry. Since the quantitative determination of the isotope composition of elymoclavine is somewhat complicated by the presence of a strong M-1 peak in the mass spectrum, a portion of each of the two elymoclavine samples was treated with horseradish peroxidase and hydrogen peroxide as described by Ramstad and coworkers[15] and the resulting penniclavine was purified and also subjected to mass spectral analysis. The absence of an M − 1 peak in $\Delta^{9,10}$-clavines like penniclavine considerably simplifies the calculation of the isotope composition. The percentage of tritium retention from (3R,4R)-mevalonic-2-^{14}C-4-t acid was in this case determined in a separate identical experiment and found to be 65%. The results of these two experiments are shown in Table V, which

Table V. Isotope Distribution in Clavine Alkaloids Biosynthesized from a Mixture of Mevalonic-2-^{13}C and -4-d_2 Acid (43 Atom % ^{13}C Excess and 50% D_2 in Mixture)[a]

		Percentage of		
	Unlabeled	Single labeled (D or ^{13}C)	Double labeled (D + ^{13}C)	Triple labeled
Experiment 6				
Chanoclavine-I	58	42	0	0
Elymoclavine	67.1	26.1	6.8	0
Penniclavine	67.0	25.4	7.1	0.5
Predicted for elymoclavine and penniclavine			2.9–14	0
Experiment 7				
Chanoclavine-I	50.2	49.8	0	0
Elymoclavine	61.7	30.0	8.2	0.1
Penniclavine	59.0	31.8	8.8	0.5
Predicted for elymoclavine and penniclavine			4.0–14	0

[a] Amount of precursor fed: experiment 6, 190 μmol; experiment 7, 200 μmol. Alkaloid formed 19.3 and 30.1 μmol.

lists the isotope distribution in excess of the natural abundance. As expected the chanoclavine-I from both experiments shows isotope excess only in the M + 1 peak; i.e., it only contains molecules labeled with deuterium or with ^{13}C in addition to unlabeled ones but none containing both ^{13}C and deuterium. In contrast, the elymoclavine from these two experiments as well as the penniclavine derived from it showed appreciable enrichment in the M + 2 peak indicating the presence of molecules containing both ^{13}C and deuterium. High resolution peak matching showed

(14) L. Pichat, B. Blagoev, and J. C. Hardouin, Bull. Soc. Chim. Fr., 4489 (1968).

(15) W.-n. Chan-Lin, E. Ramstad, and E. H. Taylor, Lloydia, 30, 202 (1967); W.-n. Chan-Lin, Ph.D. Thesis, Purdue University, 1967.

that the exact mass of the major peak at m/e 256 in the elymoclavine spectrum corresponded to the composition $C_{15}^{13}CH_{17}DN_2O$.[16] The percentage of doubly labeled molecules in both experiments is well within the calculated range. The lower limit of the predicted range is obtained by making the assumption that the ratio of labeled to unlabeled molecules in chanoclavine-I measured at the end of the experiment represents that throughout the culture period. The upper limit follows from the opposite extreme, assuming that all the labeled alkaloid was synthesized at one time and all the unlabeled at a different time. As would be expected the observed values lie between these extremes. These data thus strongly support the idea of an intermolecular transfer of the hydrogen at C-9 during the conversion of chanoclavine-I into tetracyclic ergolines.

If the hydrogen at C-9 undergoes an intermolecular transfer, the question arises whether this transfer occurs back into the same position or whether the label from C-9 of chanoclavine-I appears at a different position in the product. Extensive studies by various groups[1b,17] have established the origin of almost all the hydrogens in the tetracyclic ergolines to be the corresponding hydrogens of the respective precursor. The only two positions in the molecule for which such proof is missing, and to which a hydrogen from C-9 could therefore potentially have migrated, are position 2 at the indole ring and position 7. Transfer of hydrogen from C-9 to C-2 appears as an extremely unlikely prospect, but transfer to C-7 has to be seriously considered in view of the finding that one of the hydrogens from C-17 of chanoclavine-I is replaced in the process. To examine this possibility, we prepared elymoclavine from (3R,4R)-mevalonate-2-^{14}C-4-t (experiment 8, Table III) and degraded it to determine the amount of tritium present at C-7. The results (experiment 8, Table IV) clearly show that C-7 of elymoclavine carries no tritium, thereby excluding the possibility of a hydrogen transfer from C-9 to C-7. By way of exclusion this suggests that the hydrogen from C-9 of chanoclavine-I is transferred to the same position in the tetracyclic product. This tentative conclusion was confirmed by feeding mevalonate-4-d_2 (0.57 mmol of 99% D_2) to 40-ml replacement cultures of Claviceps strain SD 58 and analyzing the resulting elymoclavine by high resolution proton nmr. Mass spectrometry of the isolated elymoclavine indicated the presence of 51.5% monodeuterated molecules ($D_2 < 1$%), and the decrease in the intensity of the pmr signal for H-9 (6.80 ppm)[18] showed that essentially all the deuterium (47%) was located in this position.

Some further, although circumstantial, evidence for a partial intermolecular hydrogen transfer is obtained from an inspection of the data in Table I. The tritium retention values for the tetracyclic clavines vary from

(16) There is one ambiguity. The composition of this ion could also be $C_{15}^{13}CH_{19}N_2O$, because the mass of two H is almost identical with that of one D and these two possibilities cannot be distinguished with the normal present day high resolution mass spectrometers. However, unlabeled elymoclavine produces almost exactly the theoretical distribution of the isotope satellites of the molecular ion and there is thus no indication for a proton addition process in the mass spectrometer.

(17) B. J. Wilson, Ph.D. Dissertation, Purdue University, 1970.

(18) A complete analysis and reinterpretation of the pmr spectra of agroclavine and elymoclavine has been done (E. C. Kornfeld, N. J. Bach, H. E. Boaz, C.-j. Chang, H. G. Floss, E. W. Hagaman, and E. Wenkert J. Org. Chem., in press).

experiment to experiment in a way which seems to correlate with the amount of alkaloid formed. At these very low rates of alkaloid synthesis the tritium retention seems to decrease with decreasing rate of alkaloid formation. This could be very plausibly explained by the working hypothesis illustrated in Scheme II, because at low rates of alkaloid synthesis the enzyme would be idle for longer periods of time between turnovers and labeled hydrogens on the enzyme would have more time to undergo exchange with solvent protons. However, it is somewhat difficult to judge how valid this observation and its interpretation is, because the tritium retention values for the tetracyclic ergolines do show some general fluctuation throughout these experiments. More significant is the finding that chanoclavine-I in all four experiments has a higher $T/^{14}C$ ratio than the precursor. A systematic experiment with $(3R,4R)$-mevalonic-2-^{14}C-4-t acid (Table VI) indicates that

Table VI. Incorporation of $(3R,4R)$-Mevalonic-2-^{14}C-4-t Acid into Clavine Alkaloids by *Claviceps* Strain SD 58 (Experiment 9)[a]

Compd	$T/^{14}C$	T retn, %
Precursor	5.00	
Agroclavine	3.14	63
Elymoclavine	2.61	52
Chanoclavine-I	7.29	146
(−)-Chanoclavine-II	5.25	105
Isochanoclavine-I	4.97	99

[a] Total alkaloid formed 126.5 mg, ^{14}C incorporation 13.75%.

chanoclavine-I is the only clavine showing this behavior. The tetracyclic clavines show the usual decrease in the $T/^{14}C$ ratio and the other two chanoclavine isomers, which are not precursors for the tetracyclic alkaloids, have the same $T/^{14}C$ ratio as the starting mevalonate.[19] This result implies that an isotope effect operates in the further metabolism of chanoclavine-I which leads to enrichment of tritium in the unreacted chanoclavine-I. This requires the addition of another hydrogen at C-9, followed by competition between these two hydrogens in a subsequent elimination step and complete reversibility of the entire reaction sequence from chanoclavine-I to this elimination step. The following system would satisfy these requirements.
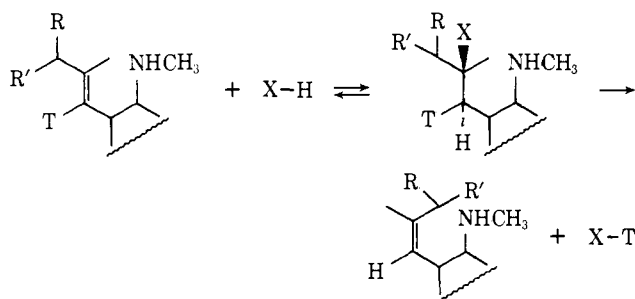
Elimination of X–H would be favored over elimination of X–T, increasing the relative rate of back reaction for those molecules of the addition product which were generated directly from tritiated precursor. If this is true, addition of X–D instead of X–H should decrease the enrichment of tritium in unreacted chanoclavine-I,

(19) The same experiment was carried out by Arigoni and coworkers with a different ergot strain and produced almost identical results. We thank Professor Arigoni for informing us of his results.

because the competition would now be between tritium and deuterium rather than between tritium and hydrogen. An indication that this might be the case can already be seen in experiments 3 and 4 (Table I). These two experiments are identical except for the fact that the mevalonate added in one case was all (except for the traces of radioactive material) mevalonate-3',4-d_5 whereas in the other it was a mixture of equal parts of pentadeuterated and nondeuterated material. In the latter case chanoclavine-I has a tritium retention of 141%, which in the presence of all deuterated mevalonate is decreased to 127%. Experiments 10–13 (Table VII) with mevalonate-4-d_2 show the same trend. The

Table VII. Tritium Retention in Clavines from $(3R,4R)$ Mevalonate-2-^{14}C-4-t in the Presence of Deuterated Carrier Mevalonate[a]

Expt no.	Additive	$T/^{14}C$ of mevalonate	Elymoclavine $T/^{14}C$	Elymoclavine T retn, %	Chanoclavine-I $T/^{13}C$	Chanoclavine-I T retn, %
10	186 μmol mevalonate-4-d_2	2.50	1.58	63	2.84	114
11	Same as 10	2.46	1.65	67	2.87	117
12	93 μmol mevalonate-4-d_2 + 93 μmol unlabeled mevalonate	2.56	1.50	59	3.19	125
13	Same as 12	2.36	1.55	66	3.08	130

[a] In each experiment 4.5×10^6 dpm ^{14}C precursor were fed.

tritium retention in chanoclavine-I of 125 and 130% in the presence of equimolar mixtures of deuterated and nondeuterated mevalonate is decreased to 114 and 117% upon adding only deuterated mevalonate. Somewhat surprisingly the tritium retention of elymoclavine shows little change. It will be noted, of course, that these four experiments also again support the idea of an intermolecular transfer of the hydrogen at C-9. Since the tritium and the deuterium as well as the ^{14}C are present strictly in separate molecules, the observed effect can only have come about if deuterium (or tritium) had been transferred into different molecules.

The loss of one of the labeled hydrogens from C-17 of chanoclavine-I during the conversion into tetracyclic ergolines can be explained if one assumes that this hydroxymethyl group goes through the oxidation stage of an aldehyde in the process. To examine this possibility, we prepared chanoclavine-I-aldehyde. Treatment of chanoclavine-I with MnO_2 in acetone at room temperature gave no reaction, but upon refluxing the mixture for 45 min the aldehyde was produced in 50–60% yield. It was purified by preparative layer chromatography and could be converted back into chanoclavine-I by reduction with $NaBH_4$. Its structure was proven[6] on the basis of the spectroscopic data given in the Experimental Section, which are in agreement with literature data of tricyclic and tetracyclic ergolines.[9,20,21] A sample of chanoclavine-I-aldehyde tritiated at C-17 was then prepared by reducing nonlabeled aldehyde

(20) M. Barger, J. A. Weisbach, B. Douglas, and G. O. Dudek, *Chem. Ind. (London)*, 1072 (1965).

(21) D. Stauffacher and H. Tscherter, *Helv. Chim. Acta*, 47, 2186 (1964).

with NaBH$_4$-t and reoxidizing the tritiated chano-clavine-17-t with MnO$_2$. This material was fed to two cultures of *Claviceps* strain SD 58 and as a control two parallel cultures received chanoclavine-I-17-t (experiments 14 and 15, Table III). The incorporation of tritium from the aldehyde into elymoclavine was about four times higher than that from chanoclavine-I-17-t. Even after correction for loss of half of the tritium from chanoclavine-I-17-t in the process, the aldehyde is still twice as efficient as chanoclavine-I as a precursor to elymoclavine. The high rate of incorporation leaves little doubt in a very direct precursor relationship of the aldehyde. Degradation of the elymoclavine from experiment 14 (Table IV) confirmed that the tritium was entirely located in the expected position and thus indicated that the incorporation of the aldehyde was specific. A complete repetition of these two experiments (experiments 16 and 17, Table III and IV) produced very similar results. In another experiment it was examined whether the hydrogen at C-17 of chano-clavine-I-aldehyde is completely retained during the conversion into elymoclavine. Within the limits of experimental error this seems to be the case, since chanoclavine-I-aldehyde-^{14}C-17-t, prepared by oxidation of chanoclavine-I-^{14}C-17-t (T/^{14}C = 3.50), is incorporated with little change of the T/^{14}C ratio (experiment 18, Table III).

To further confirm that the loss of one of the methylene hydrogens from the hydroxymethyl group of chanoclavine-I is not merely an unphysiological process which occurs when the alkaloid is added to the cultures, we fed mevalonate-$3'$-d_3 (>98% D$_3$) and determined the number of deuterium atoms in chanoclavine-I and elymoclavine. The precursor was synthesized by reaction of the THP ester of acetic acid-d_4 with allyl-magnesium bromide, followed by ozonolysis of the resulting methyldiallylcarbinol and reductive cleavage of the ozonide with NaBH$_4$.[22] The final oxidation of the 3-methylpentane-1,3,5-triol with silver carbonate–Celite[22] did not work in our hands, but oxidation with CrO$_3$ in acetic acid as described by Goeggel[23] did produce mevalonolactone, albeit in low yield. This material (570 μmol, as sodium salt) was fed to 40 ml replacement cultures and the resulting chanoclavine-I and elymoclavine were analyzed by mass spectrometry (Table VIII). It is evident that while chanoclavine-I

Table VIII. Deuterium Distribution (%) in Chanoclavine-I and Elymoclavine after Feeding Mevalonate-$3'$-d_3 to *Claviceps* Strain SD 58

| | Labeled species in | |
	Chanoclavine-I	Elymoclavine
D$_0$	21.7	28.2
D$_1$	11.3	67.9
D$_2$	63.0	3.9
D$_3$	4.0	

consists of predominantly dideuterated molecules, the deuterium in elymoclavine is present almost exclusively in monodeuterated molecules. The presence of about 4% D$_3$ species in chanoclavine-I and D$_2$ species in

(22) M. Fetizon, M. Golfier, and J.-M. Louis, *Chem. Commun.*, 1118 (1969).
(23) H. Goeggel, Ph.D. Dissertation No. 3923, ETH Zürich, 1966.

elymoclavine can be accounted for by the known[1] slight (5–10%) scrambling of the label between the carbon atoms derived from C-2 and C-3' of mevalonic acid. In support of this, the 220-MHz ^{1}H nmr spectrum of the elymoclavine integrates for about 12.5% D in the C-17 protons (4.45 ppm). The majority of the deuterium (37.8%) resides in the equatorial hydrogen at C-7 (3.65 ppm) with little or no deuterium (5.5%) in the axial hydrogen (3.08 ppm).[24] Thus the deuterium occupies the *pro-7S* position whereas the newly introduced hydrogen is the *pro-7R* hydrogen.

Discussion

The results of this study clearly indicate that chano-clavine-I-aldehyde can be efficiently and specifically converted into tetracyclic ergolines by the ergot fungus. The fact that it is a more efficient precursor than chano-clavine-I, the finding that the latter is incorporated with loss of half of the tritium from the hydroxymethyl group, and the observation that mevalonate-$3'$-d_3 gives rise to chanoclavine-I-d_2 but elymoclavine-d_1 all suggest that the aldehyde is a normal intermediate in the pathway. The conversion of chanoclavine-I into elymo-clavine could then be written as shown in Scheme IV.

Scheme IV
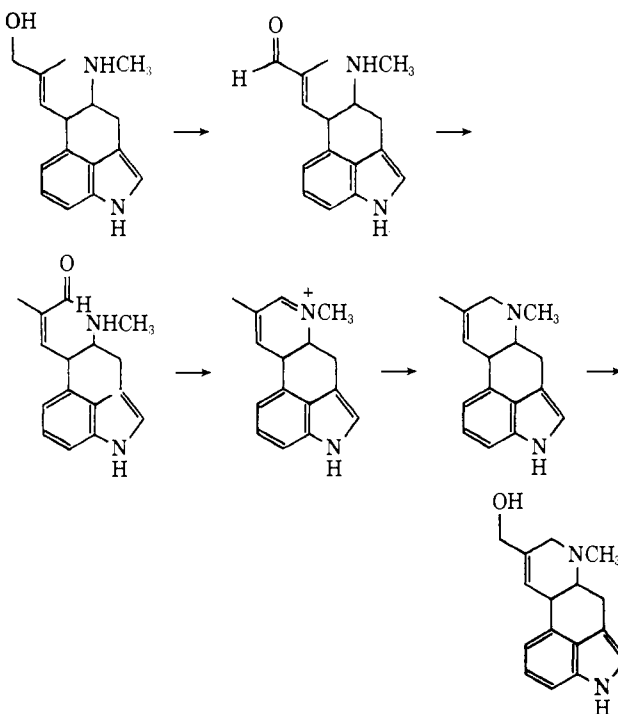
The double bond isomerization would take place at the aldehyde stage, leading to isochanoclavine-I-aldehyde which by Schiff base formation and reduction would give rise to agroclavine. Presumably, isochanoclavine-I cannot be dehydrogenated to the aldehyde and is therefore not converted into agroclavine and elymoclavine. Alternatively, as discussed below, the cyclization could lead directly to the carbinolamine. However, there is no definitive evidence that the reaction really proceeds through chanoclavine-I-aldehyde and this pathway therefore still has to be considered as hypothetical.

(24) The numerical discrepancy with the mass spectral analysis of elymoclavine could well be the result of an isotope effect. As the fragmentation of elymoclavine is most likely initiated by loss of a hydrogen from C-7, which in this sample carried both H and D, one might expect to find a too high percentage of D$_1$ species in the molecular ion.

For example, the aldehyde could be reduced to chano-clavine-I and then be converted to tetracyclic ergolines by a different route. A number of trapping experiments, in which labeled aldehyde was fed together with nonlabeled chanoclavine-I and *vice versa* and the unlabeled component reisolated to determine whether it had acquired radioactivity, did indicate some conversion of aldehyde to chanoclavine-I but were inconclusive regarding the formation of the aldehyde from chanoclavine-I, because we were unable to recover any aldehyde from the cultures. If the added aldehyde were indeed incorporated *via* chanoclavine-I, the loss of half of the tritium from C-17 of chanoclavine-I would require a different explanation. A very rapid redox reaction either at the stage of chanoclavine-I or at the tetracyclic stage could account for this finding, provided the tritium on the reduced form of the coenzyme could rapidly equilibrate with a large pool of nonlabeled hydrogens. Such a rapid exchange is unlikely at the chanoclavine-I stage, because in experiments 1-4 (Tables I and II) the chanoclavine-I isolated contains the majority of its deuterium in trideuterated molecules and because the material from mevalonate-3'-d_3 is largely dideuterated (Table VIII). There appears to be no experimental data which would allow an evaluation of the possibility of exchange at the agroclavine or elymoclavine stage. However, this alternative explanation of the experimental observations, although it cannot be excluded, seems to be considerably less likely.

The data on the mechanism of the double bond isomerization seem to clearly support the involvement of an intermolecular hydrogen transfer. While the experiments with mevalonate-3',4-d_5 are little more than indicative, the results of the feeding experiments with a mixture of mevalonate-2-^{13}C and -4-d_2 can hardly be explained in any other way, and the conclusion is further supported by the effect which mevalonate-4-d_2 has on the tritium enrichment at C-9 of unreacted chanoclavine-I. An intermolecular hydrogen transfer requires that the enzyme either binds two molecules of substrate and transfers a hydrogen directly between them or, more likely, that the hydrogen is transferred from one substrate molecule to some acceptor, where it undergoes only little equilibration with other hydrogens while the product dissociates from the enzyme, and then back to a newly bound molecule of substrate. If the hydrogen is transferred as a hydride, the assumption of only limited equilibration with other hydrogens presents no problem. However, even if the hydrogen is transferred as a proton, the assumption of only limited exchange with solvent protons during almost one entire cycle of the enzyme reaction is not unreasonable in view of the recent demonstrations of such very slow proton exchanges, for example, in the aconitase reaction[25] and in the fumarase reaction.[26] Scheme V, which is more or less a modification and extension of the original Scheme II, represents an attempt to accommodate all these various results and considerations in one chemical mechanism. Other schemes can certainly be drawn which also account for all the observations, and we do not mean to imply that the one outlined here is necessarily the best

or the only one to describe this process. For example, all the data can also be accommodated by a mechanism involving reversible 1,4-reduction of the α,β-unsaturated carbonyl system of the aldehyde. Modifying Scheme II to account for an aldehyde intermediate obviously presents the problem of having to carry out an addition of X–H across the double bond in an "anti-Michael" direction. However, this problem can be overcome by suitably masking the carbonyl group on the enzyme. In the proposed model the α,β-unsaturated carbonyl portion of the substrate would insert into the crevice of the enzyme in such a way that the groups —Y: and —B:H of the enzyme are situated on one side of the plane of the π system and the groups —X: and —A:H on the other. Following binding through —Y: and —X: and protonation of the oxygen and C-9, partial rotation around the C-8/C-9 axis on the side opposite —B: will bring N-6 in proximity to the aldehyde carbon and the group —A: on the enzyme. The nitrogen atom could thus transfer its proton to —A: and displace —Y: at the aldehyde carbon atom (pathway a). Subsequent removal of —X: and the asterisked hydrogen from C-9 would then release the carbinolamine from the enzyme. —B:H* would then introduce the original hydrogen (H*) from C-9 of this chanoclavine molecule into the next molecule of substrate which binds to the enzyme. As an alternative, the reaction sequence may lead to the release of isochanoclavine-I-aldehyde rather than the carbinolamine from the enzyme as shown in pathway b. One of the attractions of this scheme is that it explains readily the occurrence of an isotope effect leading to tritium enrichment in unreacted chanoclavine-I without any need of resorting to nonstereospecific reactions. All that is required is that every reaction in the scheme and also the conversion of chanoclavine-I to the aldehyde is reversible and that the elimination of a proton from C-9 (at least in the forward reaction) occurs in a rate-limiting step. Both these are entirely plausible assumptions.

Finally, let us consider some of the other mechanisms which have been proposed for the formation of ring D in light of the above data. Clearly, mechanisms which involve isomerization and ring closure merely *via* formation of a carbonium ion at C-17, as both Agurell[4] and we[27] have discussed, are insufficient to account for the new data. Ogunlana, *et al.*,[28] obtained a cell-free system from *Claviceps* which converted chanoclavine-I, but not agroclavine, into elymoclavine in the presence of oxygen, ATP, Mg^{2+}, and NADPH. On the basis of these findings they propose a mechanism which involves (1) phosphorylation of the hydroxyl group, (2) double bond shift from the 8,9 to the 7,8 position, (3) epoxidation of the 7,8 double bond, (4) elimination of phosphate and reaction of the resulting carbonium ion with N-6, (5) opening of the epoxide ring with loss of a proton from either C-9 or from the original C-17 followed by shift of the double bond into the 8,9 position. This mechanism is difficult to reconcile with the above data. To account for the intermolecular hydrogen transfer, steps 2-5 would all have to occur at the same active site of just one enzyme, which is not a very
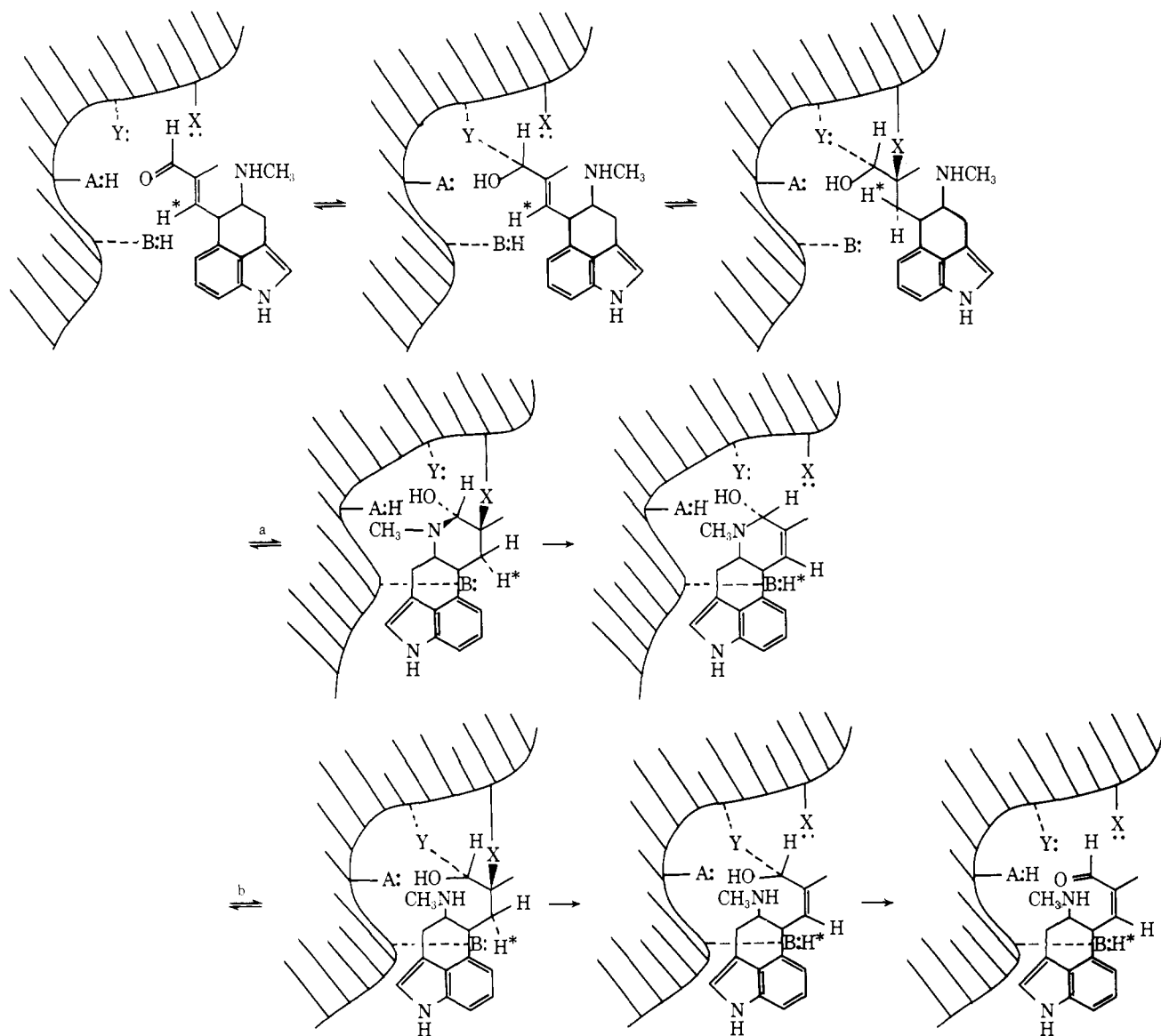
(25) I. A. Rose and E. L. O'Connell, *J. Biol. Chem.*, 242, 1870 (1967).
(26) J. N. Hansen, E. C. Dinovo, and P. D. Boyer, *J. Biol. Chem.*, 244, 6270 (1969).
(27) H. G. Floss, U. Hornemann, N. Schilling, D. Gröger, and D. Erge, *Chem. Commun.*, 105 (1967).
(28) E. O. Ogunlana, B. J. Wilson, V. E. Tyler, and E. Ramstad, *Chem. Commun.*, 775 (1970).

Scheme V

likely prospect. To account for the isotope enrichment in unreacted chanoclavine-I, all the steps would have to be completely reversible, which is even less likely.[29-32] Another mode of cyclization of the tricyclic to the tetracyclic ring system involves the transformation of dihydrochanoclavines into dihydroagroclavines in *Claviceps*, which has recently been demonstrated by Voigt and coworkers.[33] The idea of cyclization at the di-

(29) Gröger and coworkers[30] have since obtained a cell-free system from *Claviceps* which cyclizes chanoclavine-I under conditions similar to those of Ogunlana, *et al.*,[28] but produces only agroclavine rather than elymoclavine. Their system is not oxygen dependent and does convert chanoclavine-I-aldehyde to agroclavine. We[31] have also obtained a cell-free system from *Claviceps* strain SD 58 which converts chanoclavine-I into tetracyclic ergolines, but our system produces both agroclavine and elymoclavine and, unlike that of Ogunlana, *et al.*, also converts agroclavine into elymoclavine. The difference seems to be primarily in the relative amount of agroclavine hydroxylase[32] present in the system. We therefore see no need to invoke a mechanism for the direct formation of elymoclavine from chanoclavine-I without agroclavine as an intermediate.

(30) D. Gröger and P. Sajdl, *Pharmazie*, **27**, 188 (1972); D. Erge, W. Maier, and D. Gröger, *Biochem. Physiol. Pflanz.*, **164**, 234 (1973).

(31) P. F. Heinstein, R. Ledesma, and H. G. Floss, unpublished results.

(32) J. C. Hsu and J. A. Anderson, *Biochim. Biophys. Acta*, **230**, 518 (1971).

(33) R. Voigt and P. Zier, *Pharmazie*, **26**, 494 (1971); R. Voigt, P. Zier, and G. Rabitzsch, *ibid.*, **27**, 175 (1972).

hydro stage is not incompatible with most of the above results, if one assumes that reduction, ring closure, and dehydrogenation all take place reversibly on the same enzyme; *i.e.*, the dihydro alkaloids would probably not be free intermediates. However, Voigt's group has not observed formation of agroclavine in their experiments[33] and we are therefore inclined to consider the cyclization at the dihydro stage as not to be involved in the transformation of chanoclavine-I to agroclavine. This view is supported by recent experiments of Gröger, Voigt, and their coworkers.[34]

Experimental Section

Feeding Experiments. *Claviceps sp.*, strain SD 58,[35,36] was used throughout this study. The organism was grown in shake culture at 25° in 500-ml Erlenmeyer flasks containing 100 ml of medium NL 406[35,36] for 5 days before the addition of labeled precursors. The normal feeding experiments (experiments 5, 8, 9, 14–18) were carried out and the alkaloids were isolated from them exactly as described previously.[3,36] All alkaloid samples were purified to constant specific radioactivity or constant $T/^{14}C$ ratio either by

(34) S. Johne, D. Gröger, P. Zier, and R. Voigt, *Pharmazie*, **27**, 801 (1972).

(35) D. Gröger, *Arch. Pharm.*, **292**, 389 (1959).

(36) H. G. Floss and D. Gröger, *Z. Naturforsch. B*, **18**, 519 (1963).

recrystallization (if necessary with carrier) or by repeated chromatography in different systems. For replacement cultures, mycelia were grown in NL 406 to the age of 5–7 days, filtered and washed with water under sterile conditions, and resuspended at twice the ratio of cells to medium in either fresh medium NL 406 (experiments 1, 2, 6, 7, 10–13 and experiments with mevalonate-4-d_2 and -3′-d_3) or $^1/_{15}$ M phosphate buffer (Soerensen), pH 7.3 (experiments 3 + 4). Labeled precursors were then added immediately and the cultures were incubated with shaking for another 3 days (buffer replacement) or 7–8 days (medium NL 406 replacements). In all the replacement experiments the alkaloids were extracted with 2-propanol–chloroform from the alkalinized culture filtrate and partitioned between 2% succinic acid and methylene chloride. However, instead of the usual column chromatography they were then resolved by preparative tlc on silica gel H with ethanol–chloroform 1:3 as the developing solvent. Except in experiments 3 and 4, the alkaloids were then rechromatographed on silica gel H, chanoclavine-I with tert-butyl alcohol–CHCl₃ 1:3 in an atmosphere of NH₃, and elymoclavine and penniclavine with ethyl acetate–ethanol–dimethylformamide 85:10:5 as developing solvents. The alkaloids were eluted from the silica gel with 2% succinic acid and the eluate was extracted twice with ether (reagent grade), made alkaline with concentrated NH₄OH, and extracted five times with 4 ml of ether. These operations were carried out in test tubes using Pasteur pipets for the transfers in order to avoid contamination with silicone grease or plasticizer. The combined second ether extract was dried over sodium sulfate and evaporated to dryness. The samples were then taken up in a small amount of methanol and evaporated in melting point capillaries for mass spectral analysis.

The amount of total alkaloid formed was determined colorimetrically with p-dimethylaminobenzaldehyde[37,38] and is expressed as milligrams of elymoclavine (mol wt 254).

Labeled Precursors. (3R,S)-Mevalonic-2-¹⁴C acid, (3R,4R)-mevalonic-4-t acid, and NaBH₄-t were obtained from Amersham-Searle, Inc. and sodium acetate-2-¹³C and acetic-d_4 acid from Prochem Ltd. Chanoclavine-I-¹⁴C has been biosynthesized from tryptophan-¹⁴C using Claviceps paspali strain Li 342/SE 156[11] and was a gift from Professor Gröger, Halle. Mevalonic-4-d_2 acid dibenzylethylenediamine salt (mp 120°) was synthesized by the method of Cornforth, et al.,[13] from methyl bromoacetate which in turn had been prepared from acetic-d_4 acid.

Mevalonic-3′,4-d_5 Acid. 1,1-Dimethoxybutan-3-one (39.6 g) (Chemische Fabrik Hüls, Marl, Germany), 60 ml of D₂O (99.7% D), and 3 g of potassium carbonate were heated to 80° for 8 hr with protection from atmospheric moisture. The mixture was then extracted with ether, the ether evaporated, and the exchange repeated twice. Extensive decomposition took place during the exchange as evidenced by the appearance of a red color and a crystalline product in the reaction mixture. After the third exchange the product was distilled (71–72°, 14 Torr) and combined with that from a second identical run to yield 15.7 g of material which by ¹H nmr analysis contained ∼100% D in the methylene group and 86.5% D in the C-methyl group. This material was subjected to one more exchange with 40 ml of D₂O and 2 g of potassium carbonate at 80° for 15 hr. Extraction and distillation gave 3.95 g of 1,1-dimethoxybutan-3-one-2,4-d_5, which according to the mass spectrum contained 78.2% D₅, 13.3% D₄, 6.4% D₃, and less than 1% each of D₂, D₁, and D₀ species. Reformatzki reaction of 3.8 g of this acetal with ethyl bromoacetate according to Cornforth, et al.,[13] gave 2.175 g of 3-hydroxy-3-methyl-5,5-dimethoxyvaleric acid ethyl ester (bp 69–73°, 10⁻³ Torr, 34.3% yield), which was converted into mevalonic-3′,4-d_5 acid dibenzylethylenediamine salt (mp 121–121.5°, 19.4% yield) as described by Eggerer, et al.[7] Analysis by mass spectrometry as the lactone indicated the presence of 82.2% D₅, 8.9% D₄, and 8.9% D₃ species and essentially no di-, mono-, or undeuterated species. Averaged with the figures for the 1,1-dimethoxybutan-3-one the mevalonic acid was assumed to consist of about 80% D₅, 11% D₄, 7% D₃, and <1% each of D₂, D₁, and D₀ species.

Mevalonic-2-¹³C Acid.[14] Sodium acetate-2-¹³C (0.5 g = 6 mmol, 86.8% enrichment) was converted into ethyl acetate-2-¹³C by the method of Ropp.[39] This was then distilled under vacuum into a suspension of lithium amide in 100 ml of liquid ammonia (prepared from 173 mg of lithium and 25 mg of Fe(NO₃)₃·6H₂O),

which was cooled with liquid nitrogen. The mixture was then placed in a bath at −40° and stirred for 30 min. One gram of 1,1-dimethoxybutan-3-one was then added and the reaction mixture was stirred at −40° for another 2 hr. Following neutralization with 2.4 g of NH₄Cl the ammonia was evaporated while 200 ml of ether was added, followed by 30 ml of H₂O. The ether and the aqueous layer were separated and the aqueous phase extracted continuously with ether for 4 hr. The combined ether extracts were evaporated and then dried further at 10⁻² Torr to give 646 mg (50% yield) of ethyl 5,5-dimethoxy-3-methyl-3-hydroxyvalerate-2-¹³C as a yellowish oil. The structure of this intermediate and the presence and location of the ¹³C were confirmed by ¹H nmr. The ester acetal was hydrolyzed with a solution of 1 g of Ba(OH)₂·8H₂O in 12 ml of H₂O for 4 hr at 40°, the excess Ba²⁺ was then removed by addition of Dry Ice and after filtration the solution was evaporated to dryness under vacuum. The residue and 375 mg of NaBH₃CN[40] were dissolved in 5 ml of methanol containing a drop of methyl orange indicator, and 2 N HCl was added dropwise with stirring to maintain the red color of the indicator. After 15 min 35 ml of 0.1 N citrate buffer, pH 2.7, was added and stirring was continued for 48 hr at room temperature. Two milliliters of 5 N HCl were then added and the solution was saturated with NaCl and continuously extracted with ether for 5 hr. The ether extract was dried with Na₂SO₄ and evaporated to dryness. The residue was converted to the dibenzylethylenediamine salt of mevalonic-2-¹³C acid by the method of Eggerer, et al.[7] The yield by titration was ∼100% and as crystalline product (due to losses) 10.3%, mp 123°.

Mevalonic-3′-d_3 Acid.[22,23] To a mixture of 38 g of dihydropyran and 30 mg of p-toluenesulfonic acid was added slowly with cooling 18 g of acetic-d_4 acid. After 1 hr at room temperature, this mixture was diluted with an equal volume of ether and added dropwise with stirring and ice cooling to a Grignard solution prepared from 60.5 g of magnesium turnings and 181.5 g of freshly distilled allyl bromide in 750 ml of dry ether. The reaction was stirred for 1 hr at room temperature, then cooled and treated with ice and saturated NH₄Cl solution, and the aqueous phase was extracted four times with ether. The ether extract was washed with dilute NaHCO₃ solution and water, dried with Na₂SO₄, and distilled in vacuo. After removal of the volatile materials, the fraction distilling at 54–71° (10 mm) was collected and redistilled at atmospheric pressure (bp 151–155°) to give 23.0 g of methyldiallylcarbinol (63.3%). This material was dissolved in 150 ml of methanol, ozonized for 10 hours with ice cooling, and then treated in portions with 10 g of NaBH₄ with good cooling. Water was added and the mixture was washed through a column of 250 g of Dowex 50 H⁺. The effluent and washings were evaporated in a rotary evaporator; methanol was added to the residue several times and evaporated again. In the last evaporation the temperature was raised to near 100°. The residue consisted of 12.4 g of 3-methylpentane-1,3,5-triol (50.6%). The product was dissolved in 80 ml of glacial acetic acid and added slowly with stirring and slight water cooling to 13.6 g of CrO₃ in 800 ml of glacial acetic acid and 17 ml of H₂O. The mixture was left for 15 hr at room temperature; 35 ml of methanol and 1.7 l. of water were then added and the solution was extracted continuously with ether for 2 days. The extract was evaporated on a rotary evaporator and benzene was added to the residue several times and evaporated again. The residue was taken up in 50% ethanol and titrated with 0.4 N Ba(OH)₂; the equivalent amount of dibenzylethylenediamine sulfate was added as a saturated aqueous solution and the barium sulfate was removed by centrifugation. After evaporation of the solution to dryness the residue was crystallized from methanol–ether to give 1.3 g of crude DBED–mevalonate (5.2%). Repeated recrystallization gave pure material of mp 124–126°.

Chanoclavine-I-17-t. Seventy milligrams of chanoclavine-I was dissolved in 1 ml of acetic anhydride at room temperature and the solution was immediately evaporated to dryness on a rotary evaporator. The residue was taken up in 2 ml of ethanol, evaporated again, and then chromatographed on a column of 10 g of Al₂O₃ (Brockmann) with chloroform containing 0.5% methanol as the developing solvent. The fractions containing N-acetylchanoclavine-I were combined and evaporated to give 51.3 mg (by colorimetry) of product. The latter was dissolved in 10 ml of anhydrous acetone and stirred with 490 mg of MnO₂[41] for 5 hr at

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(38) M. J. Smith, Pub. Health Rep., 45, 1466 (1950).
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(40) R. F. Borch, M. D. Bernstein, and H. D. Durst, J. Amer. Chem. Soc., 93, 2897 (1971).
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Table IX. ^{1}H Nmr Spectral Data of Chanoclavine-I-aldehyde

Compd	C–CH$_3$	N–CH$_3$	4-CH$_2$, 5-CH	10-CH	CH$_2$–OH	9-HC=C—	Arom H	Indole H	O=CH
Chanoclavine-I	2.1 (br, s)	2.67 (s)	3.1–3.9 (m)	4.50 (m)	4.42 (br, s)	5.94 (m)	7–7.7 (m)	11.6 (m)	
Chanoclavine-I-aldehyde	2.08 (br, s)	2.51 (s)	2.8–3.6 (m)	4.40 (m)		6.75 (m)	6.8–7.7 (m)	11.7 (m)	9.74

room temperature. After filtration, the solution was evaporated and the residue crystallized from chloroform–hexane to give 48.8 mg of slightly impure N-acetylchanoclavine-I-aldehyde,[9] which was used without further purification in the next step.

The aldehyde (48.8 mg) was dissolved in 2 ml of tetrahydrofuran (freshly distilled over NaBH$_4$), and 10 μl of 1.25 M aqueous NaBH$_4$ solution was added, followed 30 min later by 100 mCi of tritiated NaBH$_4$ (16 Ci/mmol, 6.25 μmol). The tritiated material was added as a solid and the ampoule containing it was then rinsed with an aliquot of the reaction mixture and then with 0.1 ml of H$_2$O. The combined solutions were kept for 1.5 hr, when 100 μl of 1.25 M NaBH$_4$ (unlabeled) was added. After another hour 2 ml of H$_2$O were added, the mixture was extracted with ether, and the ether extract was dried over Na$_2$SO$_4$ and evaporated to dryness. The residue was chromatographed on a column of 20 g of Al$_2$O$_3$ (Brockmann), which was eluted first with chloroform and then with chloroform containing 0.5% methanol. As before, the fractions containing N-acetylchanoclavine-I were combined and evaporated to dryness to give 95 mCi of oily product which crystallized in the freezer. Thin-layer chromatography at this point indicated the presence of a single radioactive component, which coincided in R$_f$ with the only van Urk-positive spot on the chromatograms and with authentic N-acetylchanoclavine-I. In view of the very poor yield in the next step, later preparations of chanoclavine-I-17-t involved the direct reduction of chanoclavine-I-aldehyde (see below) rather than the acetyl derivative by exactly the same technique.

A portion of the N-acetylchanoclavine-I-17-t (76 mCi = 1.67 × 10^{11} dpm) was heated with 1.5 ml of ethanol and 0.5 ml of 10 N aqueous KOH in an evacuated, sealed tube for 2 hr at 170°. The black reaction mixture was diluted with water and extracted with chloroform. Tlc of the extract showed that most of the radioactivity was still associated with unreacted N-acetylchanoclavine-I, some with a new less polar compound, and only very little with chanoclavine-I. To separate the latter from starting material, the chloroform solution was extracted with 2% aqueous succinic acid solution, the aqueous phase made alkaline with NH$_4$OH, and the alkaloid reextracted into ether. Thin-layer chromatography at this point showed about equal amounts of radioactivity associated with N-acetylchanoclavine-I and chanoclavine-I. The latter was then purified to radiochemical homogeneity by successive preparative layer chromatography on silica gel H (Merck), first with tert-butyl alcohol–CHCl$_3$ 1:3 in an ammonia atmosphere and second with CHCl$_3$–ethanol 1:1 as developing solvents. The final yield of chanoclavine-I-17-t was 5 × 10^8 dpm (0.3%).

Chanoclavine-I-aldehyde. Chanoclavine-I (32 mg), 450 mg of MnO$_2$,[41] and 150 ml of acetone were refluxed for 1.5 hr when tlc indicated that all of the chanoclavine-I had been reacted. The manganese dioxide was filtered off, the pale yellow solution evaporated to dryness, and the residue subjected to preparative layer chromatography (silica gel H, CHCl$_3$–methanol 7:3). The band at R$_f$ 0.5 (blue–green color with p-dimethylaminobenzaldehyde) was eluted with methanol to give 24.5 mg (76%) of chanoclavine-I-aldehyde: average yields range from 50–80%; mp 119–123° (from CHCl$_3$–hexane); uv (ethanol) λ$_{max}$ (log ε) 227 (4.57), 276 nm (3.91); ir absorptions (CHCl$_3$) 2.87 (N–H, medium), 5.92 μ (C=O strong); ORD (c 0.003, pyridine) [α]$_{320}$ −52.6° (maximum, negative Cotton effect); mass spectrum m/e (composition, intensity) 254.1403 (100) (calcd for C$_{16}$H$_{18}$N$_2$O: 254.1419), 237 (C$_{16}$H$_{17}$N$_2$, 50), 236 (C$_{16}$H$_{16}$N$_2$, 54), 235 (C$_{16}$H$_{15}$N$_2$, 80), 194 (C$_{13}$H$_{12}$N, 48), 168 (50), 167 (C$_{12}$H$_9$N, 46), 155 (C$_{11}$H$_9$N, 54), 154 (C$_{11}$H$_8$N, 62); ^{1}H nmr spectrum (pyridine-d$_5$) the ^{1}H nmr data for chanoclavine-I-aldehyde and, for comparison, chanoclavine-I are shown in Table IX.

For the preparation of chanoclavine-I-aldehyde-17-t and -^{14}C-17-t the correspondingly labeled chanoclavine samples were oxidized as above. In the case of the double labeled material, chanoclavine-I of T/^{14}C = 3.50 gave aldehyde of T/^{14}C = 1.78, confirming that all the tritium was present at C-17.

Nonlabeled chanoclavine-I used as starting material was obtained in two ways. The first samples were isolated from the fermentation broth of Claviceps paspali strain Li 342/SE 156[11] and were a gift

from Professor Gröger, Halle. Later, further quantities were prepared chemically from elymoclavine by conversion to N-methylsecoelymoclavine,[12] which was then demethylated with diethyl azodicarboxylate as described by Fehr[9] to give chanoclavine-I in about 20–30% overall yield.

Degradation of Elymoclavine. Degradations to determine the amount of tritium present at C-7 of elymoclavine were carried out as described earlier.[5]

General Techniques. ^{1}H nmr spectra were recorded on a Jeol MH 60, a Varian XL-100, or a Varian HR-220 nmr spectrometer using tetramethylsilane as internal standard, ir spectra on a Perkin-Elmer Infracord, uv spectra on a Beckman 124, and ORD spectra on a Cary 60 instrument. Low resolution mass spectra were obtained on a Hitachi RMU-6 and a CEC 2110 spectrometer. The latter was also used for the high resolution spectra. For quantitative isotope analyses, several slow scans of the molecular ion region were usually recorded at low electron voltage. Radioactivity determinations were done by liquid scintillation counting in a Beckman LS 100 scintillation counter, using PPO and dimethyl-POPOP in toluene as scintillator solution and internal standardization as the means of determining counting efficiencies. Radioactivity on chromatograms was located by scanning in a Packard Model 7401 radiochromatogram scanner. Indolic compounds on chromatograms were visualized by spraying with van Urk's spray reagent (1 g of p-dimethylaminobenzaldehyde in 10 ml of H$_2$O and 20 ml of concentrated HCl).

Calculation of Isotope Composition from Mass Spectral Data. The actual isotope composition of deuterium- and/or ^{13}C-labeled compounds was calculated from the intensities of the molecular ion and its isotope satellites of the labeled compound and of the corresponding unlabeled compound, using Biemann's formulas[42] for the correction for natural isotope abundances. Biemann's formula for the correction in the presence of an M − 1 peak did not work for elymoclavine in experiments 1–4; it gave large negative values for some of the isotopic species, possibly because of the presence of deuterium in positions from which a hydrogen might be eliminated (C-7). This problem was not encountered in experiments 6 and 7. To overcome the difficulty, all peaks were first corrected for the natural ^{13}C, ^{15}N, and ^{18}O content, using the theoretical values. Correction for the P − 1 contribution then involved a series of approximations in which the ratio of (P − 1)/P was varied, starting from the observed I_{253}/I_{254} ratio, until the intensity at 253 (M − I) became 0. The values derived in this way may be only approximate, because the method of calculation includes the assumption that all peaks of the molecular ion region give a P − 1 peak in the same ratio, which may not be true in view of the presence of deuterium at C-7.

Estimations of the expected deuterium distributions in tetracyclic ergolines in experiments 1–4 are based on the observed distribution in chanoclavine-I from the same experiment, on the experimentally determined tritium retentions, and on the knowledge (from the deuterium distribution in the precursor) that all chanoclavine-I molecules containing any deuterium will carry deuterium at C-9. For the case of the simple exchange at C-9, the fraction of each species corresponding to the tritium retention is retained and the rest is converted to the P − 1 species. For example, in experiment 1 (53% tritium retention) 28.4% D$_3$ give 15.1% D$_3$ and 13.3% D$_2$, 12.4% D$_2$ produce 6.6% D$_2$ and 5.8% D$_1$, 1.8% D$_1$ give 1.0% D$_1$ and 0.8% D$_0$. Therefore, the predicted distribution in the tetracyclic ergolines is 15.1% D$_3$, 13.3% + 6.6% = 19.9% D$_2$, 5.8% + 1.0% = 6.8% D$_1$, and 57.4% + 0.8% = 58.2% D$_0$ species. To correct for the loss of one hydrogen from C-17, all the D$_3$ species in chanoclavine-I are converted to D$_2$ species. However, only half of the D$_2$ species will give rise to D$_1$ species whereas the other half remains D$_2$, because in the D$_2$ molecules only one of the two methylene hydrogens is replaced by deuterium and there is an almost equal chance of losing H or D. The D$_1$ species remain unchanged because they are only labeled at C-9. The resulting distribution

(42) K. Biemann, "Mass Spectrometry, Organic Chemical Applications," McGraw-Hill, New York, N. Y., 1962, pp 224–227.

is then used to correct for exchange or intermolecular transfer. Predicting the distribution for the intermolecular transfer involves the following process (example experiment 1): (a) remove one atom of D from all deuterated species to give 28.4% D_2 + 12.4% D_1 + 59.2% D_0 and 42.6% D which is being transferred, (b) exchange 47% of the deuterium which has been removed for H (the hydrogen being transferred is now 22.6% D and 77.4% H), (c) recombine this H/D mixture statistically with the mixture of acceptor molecules from a to give 28.4% × 22.6% = 6.4% D_3, 28.4% × 77.4% = 22.0% D_2, 12.4% × 22.6% = 2.8% D_2, 12.4% × 77.4% = 9.6% D_1, 59.2% × 22.6% = 13.4% D_1, and 59.2% × 77.4% = 45.8% D_0, or a predicted distribution of 6.4% D_3, 24.8% D_2, 23.0% D_1, and 45.8% D_0. This prediction is obviously only very approximate since it assumes that the final ratio of labeled to unlabeled chanoclavine-I molecules is the same as that throughout the experiment. The prediction of the deuterium distribution for the case of intermolecular transfer and loss of one hydrogen from C-17 involves the same process using the figures obtained after correcting chanoclavine-I for loss of one hydrogen from C-17. Alternatively, the figures for the intermolecular transfer case can be used and corrected for loss of one atom of deuterium, giving a somewhat different set of numbers. Obviously, the number of assumptions inherent in these estimations is too great to allow prediction of accurate figures and the range covered by these two modes of calculation is therefore given.

In experiments 6 and 7 the prediction of the range of double labeled molecules expected is based on the following consideration. The minimum value follows from the assumption that the isotope distribution in chanoclavine-I was constant throughout the experiment. Single labeled chanoclavine-I species consist of ^{13}C and

D molecules in a ratio of 43:50, i.e., in experiment 6 of 19.4% ^{13}C and 22.6% D. With a tritium retention of 65%, of the deuterium present 7.9% will exchange and the remaining 14.7% will statistically combine with 80.6% ^{12}C and 19.4% ^{13}C molecules to give 2.9% molecules containing ^{13}C and D. The other extreme follows from the assumption that all the labeled alkaloid is formed at a different time than unlabeled alkaloid from endogenous mevalonate. In this case there is no dilution of the precursor and therefore 65% of the deuterium of mevalonate (i.e., 32.5% D) would be distributed between 57% ^{12}C and 43% ^{13}C molecules to give 43% × 32.5% = 14% molecules containing ^{13}C + D.

Isolation, Gas Chromatography-Mass Spectrometry, and Structures of New Alkaloids from Erythrina folkersii Krukoff and Moldenke and Erythrina salviiflora Krukoff and Barneby[1]

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Abstract: We have developed a method for routine analysis of the Erythrina alkaloids to facilitate chemotaxonomic studies on species of Erythrina. The crude alkaloid mixtures are resolved and identified as their trimsyl (trimethylsilyl) derivatives by combined gas chromatography-mass spectrometry. We report here the salient gas chromatographic and mass spectral characteristics of the trimsyl derivatives of alkaloids from E. folkersii Krukoff and Moldenke and E. salviiflora Krukoff and Barneby. We also describe the isolation of three new Erythrina alkaloids—erysoline (5), erythravine (8), and erysosalvine (12)—and the isolation of erysotine (11) and erysotinone (17) for the first time from a natural source. Two more new Erythrina alkaloids-erysoflorinone and erysosalvinone (16 and 18)—have been characterized by gas chromatography-mass spectrometry as their trimsyl derivatives.

The Erythrina alkaloids[2] have been the subject of active investigation in recent years with respect to biosynthesis,[3] identification of known and new alkaloids,[4] mass spectrometric behavior,[5] and total syn-

thesis.[6] Our own efforts have been directed toward a

(1) Presented in part at the 19th Annual Conference on Mass Spectrometry and Allied Topics, Atlanta, Ga., May 1971, Paper F7, and at the 8th International Symposium on the Chemistry of Natural Products, IUPAC, New Delhi, India, Feb 1972, Abstract A31.

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