Biosynthesis of Ergot Alkaloids: Origin of the Oxygen Atoms in Chanoclavine-I and Elymoclavine

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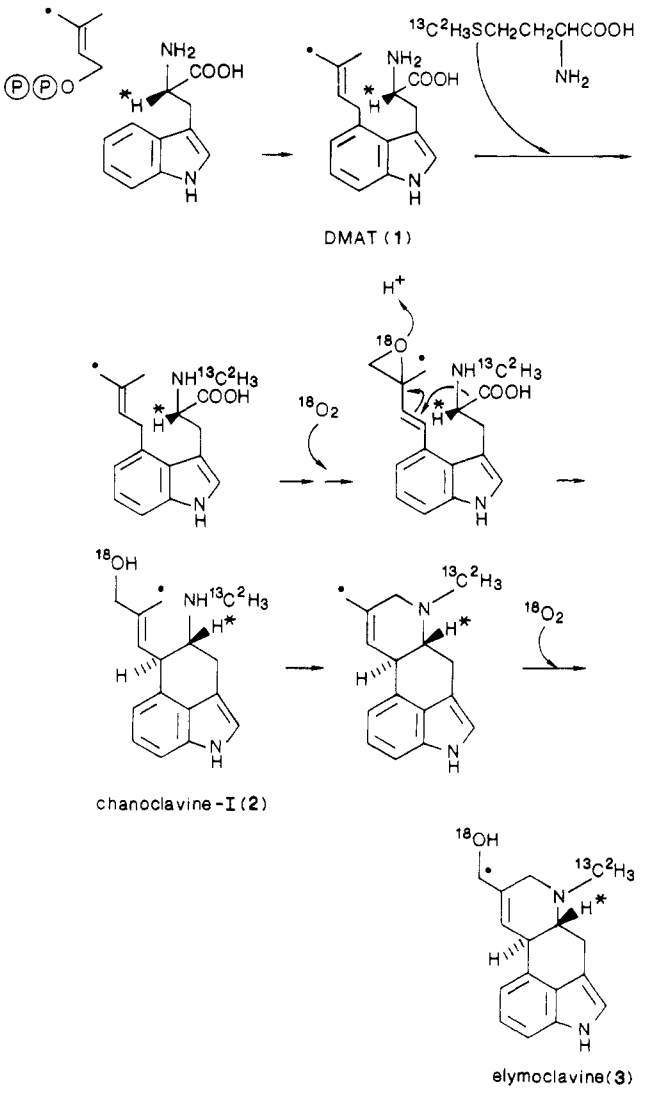
Incubation of cultures of *Claviceps* sp. strain SD 58 in an atmosphere of $^{18}O_2$ gas and GC-MS analysis of the resulting alkaloids showed that the oxygen atoms of both elymoclavine (**3**) and chanoclavine-I (**2**) are derived from molecular oxygen. The mechanistic implications of this finding are discussed.

Despite extensive investigations[1,2] several aspects of the biosynthesis of ergot alkaloids from the precursors L-tryptophan, dimethylallyl pyrophosphate, and the methyl group of methionine have remained enigmatic. One of these is the mechanism by which ring C of the ergoline system is closed. It has been generally hypothesized[1] that this process involves modification of the isoprenoid side chain of the first pathway intermediate, 4-(γ,γ-dimethylallyl)tryptophan (DMAT, **1**) to generate a carbocation equivalent at the benzylic position and formation of a carbanion at the α-carbon of the alanine side chain. Formation of this carbanion was thought to result from pyridoxal phosphate (PLP)-catalyzed decarboxylation of the amino acid. However, PLP catalysis of the decarboxylation was ruled out when it was found[3-5] that methylation of the amino nitrogen precedes the decarboxylation. On the other hand, decarboxylation must occur prior to or simultaneous with C-ring closure, because the α-hydrogen of L-tryptophan is retained and gives rise to H-5 of the ergolines.[6] While the results on the sequence of the methylation and decarboxylation steps do not rule out catalysis of decarboxylation and carbanion formation via a simpler Schiff's base (e.g., with the carbonyl group of a pyruvate moiety in an enzyme), e.g., a pathway as shown in Scheme I, they do suggest that mechanistic alternatives should be considered seriously.

A principally different mechanism would be cyclization via a potential carbanion at the benzylic carbon and a carbocation at the α-carbon. A plausible version of such a mechanism is illustrated in Scheme II. Oxidative decarboxylation of amino acids to generate the postulated imine is well precedented in alkaloid biochemistry.[7,8] A distinguishing feature of the mechanism shown in Scheme II and other variants of it, relative to Scheme I, is the origin of the oxygen in the tricyclic intermediate, chanoclavine-I (**2**).[9] Whereas most other plausible mechanisms would predict that the oxygen of **2**, like that of elymoclavine (**3**),[10] is introduced by an oxygenase, and should thus originate from atmospheric oxygen, Scheme II points to water as the source of the chanoclavine oxygen.[11]

Scheme I[a]



[a] • denotes position of an isotopic label from C-2 of mevalonic acid. * denotes position of a tritium label from H-α of L-tryptophan.

The origin of the oxygen atoms of chanoclavine-I (**2**) and elymoclavine (**3**) had been investigated 20 years ago. The alkaloids isolated from cultures of the ergot fungus grown in medium made up with $H_2{}^{18}O$ had negligible ^{18}O enrichment.[13] However, the ^{18}O enrichment of the medium

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(11) C-ring closure must lead to an oxygenated product, because deoxychanoclavine-I has been ruled out as a biosynthetic intermediate.[12]
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Scheme II

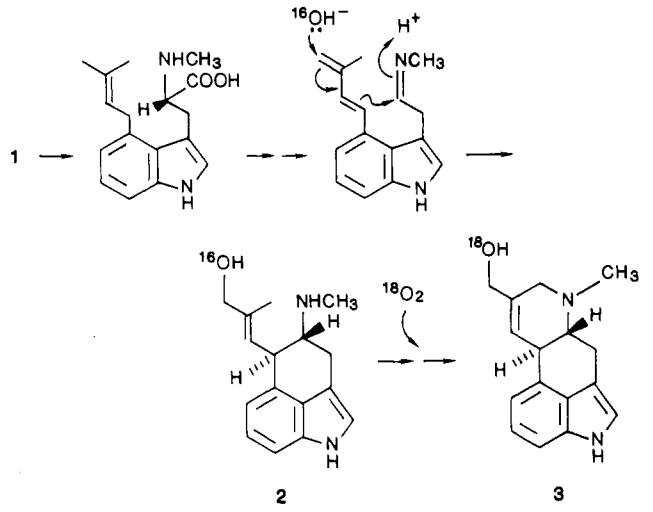

Table I. Incorporation of Label from $^{18}O_2$ and L-[$^{13}C^2H_3$]Methionine into Elymoclavine (3) by Replacement Cultures of *Claviceps* sp. strain SD 58 (specific incorporation values are reproducible to ±3%)

	isotopic species/relative abundance				
		3 from L-[$^{13}C^2H_3$]-methionine and $^{16}O_2$ (sample 1)		**3** from L-[$^{13}C^2H_3$]-methionine and $^{18}O_2$ (sample 2)	
ion (m/z)	unlabeled sample				
253	100%	unlabeled	5405	unlabeled	1417
254	49%		2897		1124
255	7%		697	^{18}O	4048
256			364		2066
257		$^{13}C^2H_3$	2891	$^{13}C^2H_3$	919
258			1412		530
259			197	$^{18}O + {}^{13}C^2H_3$	1945
260			13		970
261			0		144

Specific Incorporation

sample 1: $^{13}C^2H_3$: $\dfrac{i_{257}}{i_{253}+i_{257}} = 35\%$

sample 2: $^{13}C^2H_3$: $\dfrac{i_{257}+i_{259}}{i_{253}+i_{255}+i_{257}+i_{259}} = 34\%$

^{18}O: $\dfrac{i_{255}+i_{259}}{i_{253}+i_{255}+i_{257}+i_{259}} = 72\%$

was low in these experiments, and attempts to grow the cultures under an $^{18}O_2$ gas atmosphere were unsuccessful, resulting in loss of alkaloid production. Hence, the tentative conclusion that the oxygens of chanoclavine-I and elymoclavine must originate from molecular oxygen rests only on negative evidence. In view of the enhanced mechanistic significance of this issue resulting from the findings on the point of methylation in the biosynthetic sequence, we decided to reexamine the origin of these oxygen atoms from atmospheric oxygen using more advanced methodology available today.

Mycelia from 6-day-old shake cultures of *Claviceps* sp. strain SD 58 were washed and replaced into phosphate buffer, pH 7.3, containing L-tryptophan, D,L-mevalonic acid, and L-[$^{13}C^2H_3$]methionine (99 atom % ^{13}C, 98 atom % ^2H). These replacement cultures were then closed with a rubber septum and incubated with shaking at 25 °C for 2 days under an atmosphere of either $^{16}O_2/N_2$ or $^{18}O_2/N_2$. The alkaloids were extracted from the medium, purified by solvent partitioning, and separated by TLC. The bands of **2** and **3** were eluted and the alkaloids subjected to GC-MS analysis. The results are summarized in Tables I and II. The mass spectrum of **3** shows a base peak at m/z 253 corresponding to the $(M - H)^+$ ion. In the sample derived from [$^{13}C^2H_3$]methionine in $^{16}O_2$ 35% of this peak is displaced to m/z 257, indicating substantial intact incorporation of the methyl group of methionine. The sample of **3** produced in an atmosphere of $^{18}O_2$ shows the most prominent peak at m/z 255 and another intense peak at m/z 259, corresponding to about 72 atom % ^{18}O enrichment in this material. The specific incorporation of methionine, calculated from the intensities of the peaks at m/z 257 and 259, is 34%. The results show very clearly that the oxygen atom of **3** is derived from atmospheric oxygen, consistent with its introduction by a P_{450}-containing mixed function oxygenase.[10]

Table II. Incorporation of Label from $^{18}O_2$ and L-[$^{13}C^2H_3$]Methionine into Chanoclavine-I (2) by Replacement Cultures of *Claviceps* sp. strain SD 58 (specific incorporation values are reproducible to ±3%)

	isotopic species/relative abundance				
		2 from L-[$^{13}C^2H_3$]-methionine and $^{16}O_2$ (sample 3)		**2** from L-[$^{13}C^2H_3$]-methionine and $^{18}O_2$ (sample 4)	
ion (m/z)	unlabeled sample				
237	85%[a]	unlabeled	362	unlabeled	395
238			147		173
239			47		48
240			24		28
241		$^{13}C^2H_3$	247	$^{13}C^2H_3$	215
242			88		86
243			19		20
256	66%[a]	unlabeled	383	unlabeled	86
257			77		34
258			26	^{18}O	265
259			18		54
260		$^{13}C^2H_3$	238	$^{13}C^2H_3$	46
261			37		23
262				$^{18}O + {}^{13}C^2H_3$	152
263					26

Specific Incorporation

sample 3:

$^{13}C^2H_3$: $\dfrac{i_{241}}{i_{237}+i_{241}}$ or $\dfrac{i_{260}}{i_{256}+i_{260}} = 40\%, 38\%$

sample 4:

$^{13}C^2H_3$: $\dfrac{i_{241}}{i_{237}+i_{241}}$ or $\dfrac{i_{260}+i_{262}}{i_{256}+i_{258}+i_{260}+i_{262}} = 35\%, 36\%$

^{18}O: $\dfrac{i_{258}+i_{262}}{i_{256}+i_{258}+i_{260}+i_{262}} = 76\%$

[a] Base peak, m/z 183.

The results on **2** are equally clear-cut. The mass spectrum of **2** shows prominent peaks at m/z 256 and 237, the molecular ion and a fragment ion arising by loss of H_2O and $H^\bullet$ from the molecular ion. In the sample of **2** generated in $^{16}O_2$ mass spectral peaks at m/z 241 and 260 indicate a 38–40% specific incorporation of the methyl group of methionine. Similarly, in the sample generated in $^{18}O_2$ the intensity of the peak at m/z 241 indicates 35% enrichment by the methionine methyl group. In the mo-

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lecular ion cluster the prominent peaks are displaced to m/z 258 and 262, corresponding to 76 atom % ^{18}O enrichment of **2**. This result demonstrates unequivocally that the oxygen atom of **2** also originates from molecular oxygen; it confirms the conclusion of the earlier work[13] and places it on a much firmer experimental basis.

On the basis of the data presented above, mechanisms for C-ring closure as the one shown in Scheme II can be excluded. It seems therefore likely after all that the formation of ring C of the ergot alkaloids proceeds by a mechanism involving a potential carbocation at the benzylic carbon and a potential carbanion at C-α, but modified to account for the fact that the process must take place after methylation of the amino group. Scheme I shows a plausible reaction sequence which is consistent with all the experimental data.

Experimental Section

Claviceps sp. strain SD 58[14,15] was grown for 6 days in shake culture at 25 °C in 500-mL Erlenmeyer flasks containing 100 mL of medium NL 406.[15] The cultures were then filtered aseptically, and the mycelia were washed with sterile water and resuspended in 100 mL of 1/15 M phosphate buffer, pH 7.3. This process was repeated once, and the mycelia were then suspended in 100 mL of 1/15 M phosphate buffer, pH 7.3, containing 5 mg of L-tryptophan, 10 mg of D,L-mevalonic acid, and 5 mg of L-[$^{13}C^2H_3$]-methionine (synthesized from $^{13}C^2H_3I$, 99% ^{13}C, 98% ^2H, MSD Isotopes). The flasks were closed with a rubber septum, evacuated through a needle, filled with nitrogen, and evacuated again. They were than connected through a needle to a flask containing ^{18}O-oxygen gas (1 L, 97.8 atom % ^{18}O, MSD Isotopes). After pressure equilibration, the culture flasks were brought to atmospheric pressure with nitrogen and incubated for 2 days at 25 °C with shaking. Parallel cultures were treated in the same way with $^{16}O_2$ gas. Two flasks were used for each experiment.

The alkaloids were extracted with 1:2 2-propanol–chloroform from the culture filtrate made alkaline with ammonium hydroxide. The extract was evaporated to dryness in vacuo and partitioned between 2% aqueous succinic acid and CH_2Cl_2. The aqueous phase was washed twice with CH_2Cl_2, made alkaline with ammonium hydroxide, and extracted 3 times with CH_2Cl_2. The extract was dried over Na_2SO_4 and evaporated in vacuo, and the residue was subjected to preparative TLC (SIL G-25 UV_{254}, 0.25 mm, Macherey-Nagel, developed with 150:40:40:1 $CHCl_3$–MeOH–*t*-BuOH–NH_4OH). The bands of **2** (R_f 0.23) and **3** (R_f 0.69) were scraped off and eluted with 100:50:1 $CHCl_3$–MeOH–NH_4OH, and the alkaloids were analyzed by GC-MS (Hewlett-Packard 5970A GC-mass spectrometer, SPB-5 capillary column 0.25 mm × 15 m; flow rate 1.0 mL/min; temperature program: 60 °C for 4 min, then 10°/min to 295 °C, retention times, **2**, 22.3 min; **3**, 24 min).

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Registry No. **2**, 2390-99-0; **3**, 548-43-6.

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Diastereoselective and Enantioselective Total Synthesis of the Hepatoprotective Agent Clausenamide†

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The diastereoselective total synthesis of the naturally occurring hepatoprotective agent clausenamide (3-hydroxy-5-(α-hydroxybenzyl)-1-methyl-4-phenylpyrrolidin-2-one) is described, starting from ethyl cinnamate and diethyl acetamidomalonate. The enantioselective total synthesis of optically pure (+)-clausenamide is presented. The synthesis is based on a novel method for the preparation of optically pure (2*S*,3*S*)-3-phenylglutamic acid.

In Chinese folk-medicine the aqueous extract of the leaves of the plant *Clausena lansium* (lour) skeels is held to be an efficacious liver protecting agent and is used in cases of acute and chronic viral hepatitis. Scientists at the Institute of Materia Medica of the Chinese Academy of Medical Sciences in Peking[1] have studied this plant extract and have isolated as the main component of the extract γ-lactam 1[2] called clausenamide, occuring at about 4 × 10^{-2}% on the basis of dry leaf weight (Figure 1).

Clausenamide showed a marked hepatoprotective effect against chemical toxins, such as carbon tetrachloride and thioacetamide, in initial tests. In addition, **1** was observed to have an inducing effect on cytochrome P450, which is of course essential for the metabolisation of xenobiotics.

Larger quantities of clausenamide were then required for more detailed pharmacological studies. As only 3.8 g of the natural product can be isolated from 10 kg of dried leaves, a total synthesis of clausenamide seemed to be a desirable alternative.

Structure. According to X-ray analysis (Figure 2), clausenamide has the 3*S**,4*R**,5*R**,7*S** relative configuration and, most surprisingly, is a racemate. This naturally reduces the synthetic problem quite drastically.

Retrosynthetic Analysis. According to the retrosynthetic approach (Scheme I) high stereoselectivity should be expected in the construction of the hydroxybenzyl unit in clausenamide from the aldehyde function, due to the inducing effect of the neighboring C(4)-phenyl ring. It should be possible to introduce the hydroxy group by base-induced oxidation. The C(4)-phenyl ring should also exert a benevolent influence here upon the stereoselectivity and direct the hydroxy group into the trans position. A

† Dedicated to Prof. U. Schoellkopf on the occasion of his 60th birthday.

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(2) For the sake of clarity, the descriptors for the other antipode are omitted.

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