Mechanism of α-Hydroxy-α-Amino Acid Formation in the Biosynthesis of Peptide Ergot Alkaloids

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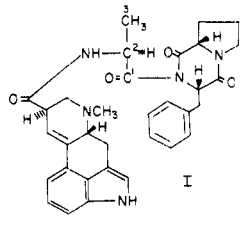
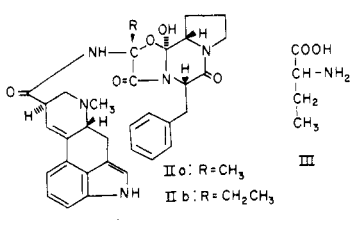
(*R,S*)-2-Amino[3-^{13}C,3-D_2]butyric acid was synthesized and fed to cultures of *Claviceps purpurea*. Mass spectral analysis of the resulting peptide ergot alkaloid ergostine (IIb) showed the presence of ^{13}C as well as two atoms of deuterium, ruling out a 2,3-dehydro amino acid intermediate in the formation of the α-hydroxy-α-amino acid moiety of the peptide.

Most ergot alkaloids of the peptide type, e.g., ergotamine (IIa), contain a unique cyclol structure resulting from the modification of the amino acid adjacent to the lysergyl moiety into an α-hydroxy-α-amino acid. This modification is thought to take place after the assembly of the lysergyl tripeptide, possibly at the stage of the corresponding prolyl lactam (I), as a late step in the biosynthesis, followed only



I



IIa: R = CH_3
IIb: R = CH_2CH_3

III

by, possibly nonenzymatic, closure of the cyclol ring.[1] Three plausible mechanisms can be envisioned for this conversion of the α-amino into the α-hydroxy-α-amino acid moiety (Figure 1): (a) dehydrogenation to the 2,3-dehydro amino acid followed by addition of water, (b) dehydrogenation to the imino acid followed by hydration, and (c) replacement of H-α with OH by a direct hydroxylation. In this paper we report results which exclude one of these possibilities.

Results and Discussion

It has been shown[2] that analogues of the peptide ergot alkaloids can be produced by adding analogues of one of the amino acids making up the peptide moiety to cultures of *Claviceps purpurea*. For example, when 2-aminobutyric acid (III) is added to a culture of the ergotamine-producing *C. purpurea* strain 275 FI, the resulting alkaloid mixture contains ergostine (IIb) in addition to IIa. Ergostine, although a natural product,[3] is not a normal constituent of this fermentation; it is produced entirely from the added III as evidenced by the fact that labeled III is converted to IIb without isotopic dilution.[4] We used this system to probe the mechanism of the α-hydroxylation reaction.

(*R,S*)-2-Amino[3-^{13}C,3-D_2]butyric acid was synthesized by the reaction sequence shown in Figure 2 in an overall yield of 17% based on sodium [1-^{13}C]acetate. The absence of a detectable signal for the methylene protons or their ^{13}C satellites in the proton NMR spectrum of the product indicated that, within the limits of detection (~4%), the compound was completely deuterated at C-3. The isotopic composition of the material as determined by mass spectral analysis of the methyl ester is shown in Table I (experiment 1).

Two feeding experiments were carried out with the labeled 2-aminobutyric acid. In the first of these, 80 mg of the precursor was added to two stationary cultures of *C. purpurea* strain PCCE1[5] on day 8 of the fermentation, and the cultures were harvested 4 days later. They had produced 28.9 mg of alkaloid containing approximately 10% ergostine plus ergostinine. The ergostine was isolated from the mixture by repeated preparative TLC and subjected to mass spectrometric analysis. The fragmentation pattern observed closely paralleled that reported for ergotamine and related peptide ergot alkaloids.[6] The peaks corresponding to the fragment ions at *m/e* 328 (peptide fragment - H)[7] and *m/e* 300 (peptide fragment - H - CO) were isotopically shifted by 3 mass units, whereas the fragment ions *m/e* 244 (prolyl - phenylalanyl lactam) and *m/e* 267 (lysergic acid amide) were unchanged. This pattern indicates the presence of the label exclusively in the α-hydroxy-α-amino acid portion of the molecule. The quantitative isotopic composition of the ergostine is shown in Table I (experiment 1). Compared to the precursor, the ergostine shows a slight apparent increase in the abundance of the species containing two, one, or no atoms of isotope, which may be partly due to some metabolic exchange of deuterium and partly may merely be an artifact of the measurement.[8] However, the predominance of the P + 3 species, containing ^{13}C and two atoms of deuterium, clearly indicates that both hydrogens of the methylene group of 2-aminobutyrate have been retained during the transformation into the α-hydroxy-α-aminobutyric acid moiety of ergostine.

In the second experiment, a mixture of the labeled 2-aminobutyric acid with about 3.6 parts of nonlabeled material was used as the precursor for the biosynthesis of ergostine. The purpose of this experiment was to check

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(5) J. A. Anderson, I.-S. Kim, P. Lehtonen, and H. G. Floss, *J. Nat. Prod.*, **42**, 271 (1979).
(6) D Voigt, S. Johne, and D. Gröger, *Pharmazie*, **29**, 697 (1974).
(7) The appearance of the P + 3 isotopic species for this ion indicates that this fragment cannot have resulted by loss of a proton from C-3 of the α-hydroxy-α-amino acid moiety, as tentatively suggested by Voigt et al.[6]
(8) Due to scarcity of material the ergostine spectra showed more background and impurities than those of the 2-aminobutyric acid.

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Table I. Mass Spectroscopic Analysis of Carbon-13 and Deuterium Labeled 2-Aminobutyric Acid and Ergostine Biosynthesized from It

compd analyzed	increase in mass no. over parent peak	method of ionization: EI peak analyzed, m/e	EI isotopic abundance, %	CI peak analyzed, m/e	CI isotopic abundance, %	av isotopic comp, %
		Experiment 1				
(*R,S*)-2-amino-[3-^{13}C,3-D_2]butyric acid (as methyl ester)	0	58	4.9	118	2.0	3.5, $^{13}C_0D_0$
	1	59	2.3	119	1.8	2.1, $^{13}C_1D_0$ or $^{13}C_0D_1$
	2	60	9.7	120	9.7	9.7, $^{13}C_1D_1$ or $^{13}C_0D_2$
	3	61	73.9	121	76.1	74.9, $^{13}C_1D_2$
	4	62	9.2	122	10.4	9.8, $^{13}C_1D_3$ (?)
ergostine	0	328	5.9	329	14.0	10.0, $^{13}C_0D_0$
	1	329	16.2	330	10.1	13.2, $^{13}C_1D_0$ or $^{13}C_0D_1$
	2	330	20.3	331	22.1	21.2, $^{13}C_1D_1$ or $^{13}C_0D_2$
	3	331	51.1	332	47.3	49.1, $^{13}C_1D_2$
	4	332	6.5	333	6.5	6.5, $^{13}C_1D_3$ (?)
		Experiment 2				
(*R,S*)-2-amino-[3-^{13}C,3-D_2]butyric acid and unlabeled (*R,S*)-2-aminobutyric acid (as methyl ester)	0	58	81.9	118	74.6	78.2, $^{13}C_0D_0$
	1	59	1.9	119	5.5	3.7, $^{13}C_1D_0$ or $^{13}C_0D_1$
	2	60	0.5	120	3.0	1.8, $^{13}C_1D_1$ or $^{13}C_0D_2$
	3	61	15.7	121	16.9	16.3, $^{13}C_1D_2$
ergostine	0	328	80.4	329	83.5	81.9, $^{13}C_0D_0$
	1	329	4.1	330	4.2	4.1, $^{13}C_1D_0$ or $^{13}C_0D_1$
	2	330	8.3	331	3.6	6.0, $^{13}C_1D_1$ or $^{13}C_0D_2$
	3	331	7.2	332	8.7	8.0, $^{13}C_1D_2$

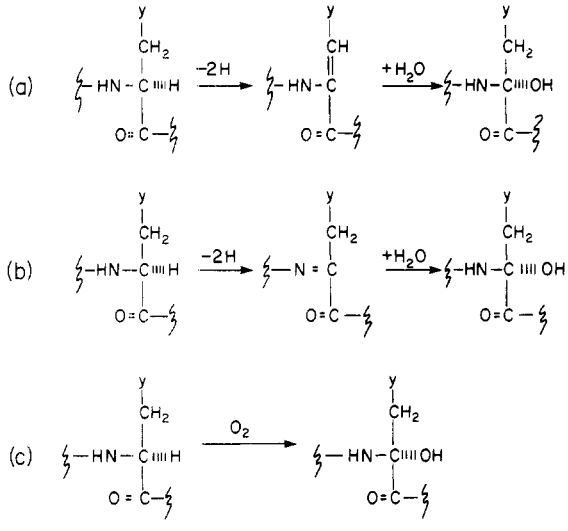

Figure 1. Three possible mechanisms for the conversion of the α-amino into the α-hydroxy-α-amino acid moiety of peptide ergot alkaloids.

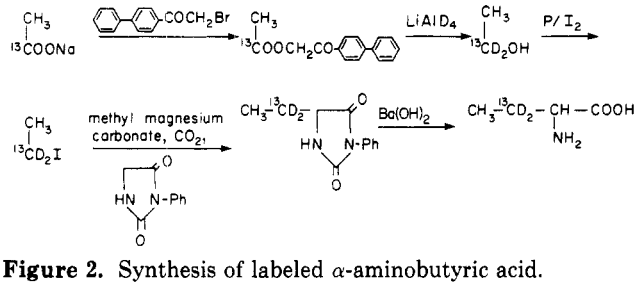

Figure 2. Synthesis of labeled α-aminobutyric acid.

for a possible intermolecular recycling of a hydrogen during the introduction of the α-hydroxy group. If the hydrogen removed from C-3 of the aminobutyrate moiety in the dehydrogenation step were reutilized subsequently in the addition of a hydrogen to C-3 and if such a process were intermolecular, the deuterium atom removed from C-3 of a labeled molecule would statistically recombine with labeled and unlabeled species. This would produce a marked reduction of the P + 3 ($^{13}C_1D_2$) and the P ($^{13}C_0D_0$) species in the product and a concomitant increase in the P + 2 ($^{13}C_1D_1$) and P + 1 ($^{13}C_0D_1$) species. The experiment was carried out with shake cultures of *C. purpurea* strain 275 FI,[9] with 200 mg of the precursor mixture, and produced 1.48 g of alkaloid containing 5.6% ergostine and ergostinine. The ergostine isolated from part of the alkaloid mixture by preparative TLC was analyzed by mass spectrometry, as was a sample of the precursor, with the results shown in Table I (experiment 2). The presence of a significant P + 3 peak for the ion at *m/e* 328 in the ergostine spectrum again indicates predominant retention of both deuterium atoms from the methylene group of the precursor. A slight increase in the P + 2 peak and a decrease in the P + 3 peak again suggests some loss of deuterium by metabolic exchange. The isotopic composition observed for the ergostine differs greatly from that calculated for intermolecular recycling of one deuterium atom (P, 65.9%; P + 1, 17.4%; P + 2, 13.1%; P + 3, 3.6%), indicating that such a process does not take place to any significant extent.

The results thus indicate that the formation of the α-hydroxy-α-amino acid moiety during peptide ergot alkaloid biosynthesis does not proceed via a 2,3-dehydro amino acid intermediate.[10] This rules out one of the three mechanisms outlined for the introduction of the α-hydroxy group of the α-hydroxy-α-amino acid portion of these alkaloids (mechanism a, Figure 1). A distinction between the other two mechanisms (b and c, Figure 1) cannot be made at this point; experiments with $^{18}O_2$ and/or $H_2{}^{18}O$ should distinguish between these two possibilities.

Experimental Section

General Methods. Proton NMR spectra were recorded in D_2O on a Varian EM-360 spectrometer. Mass spectra were obtained on a Du Pont 21-492 BR instrument using the direct-inlet system and either electron impact (70 eV) or chemical (isobutane, 0.5

(9) H. G. Floss, G. P. Basmadjian, M. Tcheng, C. Spalla, and A. Minghetti, *Lloydia*, 34, 442 (1971).

(10) This conclusion would only be invalid if a completely intramolecular recycling of a hydrogen at C-3 were to take place. Such a possibility is considered to be very remote.

torr) ionization. The isotopic composition of labeled samples was calculated from the mass spectra as described by Biemann.[11] Sodium [1-^{13}C]acetate and $LiAlD_4$ were purchased from Merck Sharp and Dohme.

Culture Conditions and Feeding Experiments. *Claviceps purpurea* strains PCCE1 and FI 275 were cultivated as described previously.[5,12] For experiment 1, two 100-mL stationary cultures of strain PCCE1 were grown to day 8 in production medium.[5] To each was then added aseptically 40 mg of (*R,S*)-2-amino[3-^{13}C,3-D_2]butyrate. Four days later the cultures were filtered, and both medium and mycelium were made alkaline with NH_4OH and extracted with chloroform to give 22.5 and 6.8 mg of alkaloid, respectively, from the two cultures. The combined extracts were partitioned between 2% succinic acid and chloroform and then subjected to preparative TLC (silica gel G; chloroform/methanol 95:5). Ergostine was eluted from the plate with chloroform/methanol (1:1), the eluate was evaporated to dryness, and the residue was partitioned between 2% succinic acid/ether. If necessary, the TLC was repeated, and the chloroform solution was then taken to dryness. For mass spectrometry the residue was dissolved in methanol, filtered through a Teflon membrane filter, and evaporated in melting point capillaries. The conditions for experiment 2 were as described earlier.[9] In this case 200 mg of precursor was used for 20 cultures, and ergostine was isolated from the precipitated crude alkaloids[9] (1.48 g, 72.9% ergotamine/ergotaminine, 5.6% ergostine/ergostinine, 21.5% others) by preparative TLC of a 50-mg aliquot as described above. Unlabeled ergostine for identification and as a reference for mass spectrometry was produced with strain PCCE1, adding 50 mg of (*S*)-2-aminobutyrate/100 mL of culture: EI mass spectrum, *m/e* (relative intensity) 209 (59), 221 (39, M − peptide fragment − CONH), 244 (76, Phe-Pro lactam), 267 (100, lysergic acid amide), 328 (43, peptide fragment − H); CI mass spectrum, *m/e* (relative intensity) 210 (23), 223 (11), 245 (99), 279 (25), 294 (23), 329 (100, peptide fragment).

Synthesis of (*R,S*)-2-Amino[3-^{13}C,3-D_2]butyric Acid. *p*-Phenylphenacyl [1-^{13}C]Acetate. A solution of 2 g (24.4 mmol) of sodium [1-^{13}C]acetate (91% ^{13}C enriched) in 25 mL of H_2O was acidified to pH 6.5 with dilute HCl and added to a solution of 7.03 g (25.6 mmol) of *p*-phenylphenacyl bromide in 250 nmL of acetone. The mixture was refluxed for 3 h and then concentrated to 50–75 mL in a rotary evaporator. CH_2Cl_2 (125 mL) was added to dissolve a yellow precipitate which had formed and to extract residual organic material from the aqueous phase. The organic layer was then separated, dried over $MgSO_4$, and evaporated to dryness, and the residue was dried under vacuum over P_2O_5. Recrystallization from methanol gave 3.28 g of product, mp 104–106 °C; two additional, slightly less pure crops of material were obtained from the mother liquor. The combined yield was 5.02 g (81%).

[1-^{13}C,1-D_2]Ethanol. A 5-g (19.7 mmol) sample of phenylphenacyl [1-^{13}C]acetate was dissolved in 30 mL of triethylene glycol diethyl ether (Aldrich), and the solution was stirred magnetically and cooled to 3–4 °C, while 1.24 g (29.7 mmol) of $LiAlD_4$ (99.5% deuterium enriched) was added in portions over a period of 1.5 h. After being stirred for another 4 h at room temperature under an argon atmosphere, the mixture was again cooled to 4–5 °C in an ice–salt bath, and the complex was decomposed by dropwise addition of 21.6 mL of ethylene glycol monophenyl ether. The ethanol was then distilled into a trap cooled with 2-propanol/dry ice by passing a slow stream of argon through the magnetically stirred reaction mixture, which was heated in an oil bath at 70 °C. Four fractions of 0.34, 0.36, 0.14, and 0.1 g were collected after 6, 7, 11, and 8 h of gas flow. The combined yield was 0.94 g (95%).

Iodo[1-^{13}C,1-D_2]ethane. The combined first three fractions of [1-^{13}C,1-D_2]ethanol (0.84 g, 17.1 mmol) were dissolved in 20 mL of *n*-octane and added to a mixture of 0.14 g of red phosphorus and 2.17 g (17.1 mmol) of iodine in 20 mL of *n*-octane. The reaction mixture was heated for 1 h in an oil bath at 100 °C with stirring in a flask fitted with a reflux condenser cooled with dry ice/acetone. After cooling to room temperature, the dark brown-red solution was decanted, washed with solutions of 5% NaOH and 3% $Na_2S_2O_3$, and with water, and dried over K_2CO_3. The solution was then distilled through a 10-cm Vigreux column, and the fraction distilling between 70 and 125 °C was collected in a receiver cooled in an ice–salt bath. A yield of 5.17 g of distillate, containing 1.53 g (56%) of iodoethane as determined by GLC (based on the density of the distillate, a yield of 71% is calculated), was obtained.

3-Phenyl-5-[1′-^{13}C,1′-D_2]ethylhydantoin.[13] A solution of 2 M methylmagnesium carbonate[14] (12.2 mL, 24.4 mmol) in dimethylformamide was stirred at 80 °C under a slow stream of CO_2 and dried by passage through a $CaCl_2$ tower for 1 h. The CO_2 was then replaced by argon, 2.14 g (12.2 mmol) of 3-phenylhydantoin was added in one portion, and the solution was stirred for 1.5 h at 80 °C. The flask was then equipped with a reflux condenser cooled with dry ice/acetone, and the solution of iodo[1-^{13}C,1-D_2]ethane (1.53 g, 9.6 mmol) in octane from the previous step was added dropwise. Stirring was continued for 4 h at 80 °C and 1 h at 110 °C. After cooling off, the reaction mixture was poured on 25 g of ice and 6.5 mL of concentrated HCl, and after 12 h at 0 °C the precipitate was collected by filtration: yield 1.2 g (60%); mp 108–110 °C (pure unlabeled compound, mp 115 °C).

(*R,S*)-2-Amino[3-^{13}C,3-D_2]butyric Acid. A mixture of 3.1 g of $Ba(OH)_2 \cdot 8H_2O$, 1.2 g (5.8 mmol) of 3-phenyl-5-[1′-^{13}C,1′-D^2]ethylhydantoin, and 15 mL of H_2O was heated in a sealed tube in an oil bath at 150–160 °C for 1 h. After the mixture cooled barium carbonate was removed by filtration, 0.6 of $(NH_4)_2CO_3$ was added, and the mixture was boiled for 15 min. After filtration the solution was evaporated to dryness, and the crude product (0.5 g) was recrystallized by dissolving it in 1 mL of hot water, filtering, and adding 2 mL of methanol. Cooling in an ice bath gave pure crystalline product: 0.4 g, 65%; mp >200 °C dec; NMR (D_2O) δ 2.1 (d, $J_{^{13}CCH}$ = 4.6 Hz, CH_3), 4.9 (d, $J_{^{13}CCH}$ = 4.4 Hz, CH); EI mass spectrum (for methyl ester), *m/e* (relative intensity) 117 (2, M^+), 88 (8), 74 (9), 58 (100, $C_3H_8N^+$); CI mass spectrum, *m/e* (relative intensity) 118 (100, M + H^+).

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Registry No. *p*-Phenylphenacyl [1-^{13}C]acetate, 73377-65-8; sodium [1-^{13}C]acetate, 23424-28-4; *p*-phenylphenacyl bromide, 135-73-9; [1-^{13}C,1-D_2]ethanol, 63459-49-4; iodo[1-^{13}C,1-D_2]ethane, 73377-66-9; 3-phenyl-5-[1′-^{13}C,1′-D_2]ethylhydantoin, 73377-67-0; 3-phenylhydantoin, 2221-13-8; (*R,S*)-2-amino[3-^{13}C,3-D_2]butyric acid, 73377-68-1; ergostine, 2854-38-8; methyl (*R,S*)-2-amino[3-^{13}C,3-D_2]-butyrate, 73377-69-2; methyl (*R,S*)-2-aminobutyrate, 2483-62-7.

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