CELL-FREE CONVERSION OF 4-γ,γ-DIMETHYLALLYLTRYPTOPHAN TO 4-[4-HYDROXY-3-METHYL-Δ^2-BUTENYL]-TRYPTOPHAN IN *CLAVICEPS PURPUREA* PRL 1980

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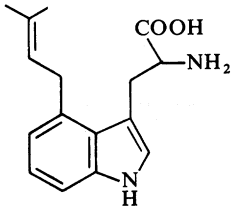
Key Word Index—*Claviceps* sp.; Clavicipitaceae; ergot; enzymatic study; ergot alkaloids; 4-dimethylallyltryptophan; 4-[4-hydroxy-3-methyl-Δ^2-butenyl]-tryptophan.

Abstract—The conversion of 4-γ,γ-dimethylallyltryptophan to 4-[4-hydroxy-3-methyl-Δ^2-butenyl]-tryptophan was catalyzed by the 60–80% ammonium sulphate fraction from *Claviceps purpurea* PRL 1980. The conversion was stimulated by NADPH. Two major unidentified products in the incubation mixture were not significantly incorporated into elymoclavine when they were added to cultures of *C. purpurea* PRL 1980.

INTRODUCTION

Although 4-γ,γ-dimethylallyltryptophan (DMAT) (**1**) has been established as the first intermediate in ergot alkaloid biosynthesis [1, 2], the next compound in the pathway has not been determined. Both *cis-trans* isomers of 4-[4-hydroxy-3-methyl-Δ^2-butenyl]-tryptophan (HODMAT) (**2**) were converted to elymoclavine but not to agroclavine [3]. HODMAT is therefore not on the main pathway in which agroclavine is converted to elymoclavine [4, 5]. HODMAT was isolated from cultures of *C. purpurea* PRL 1980 [6]. The production of HODMAT indicates an alternate pathway for biosynthesis of elymoclavine which does not include agroclavine as an intermediate. We report the formation of HODMAT from DMAT in an $(NH_4)_2SO_4$ fraction from *C. purpurea* PRL 1980 and the cofactor requirement for the conversion.



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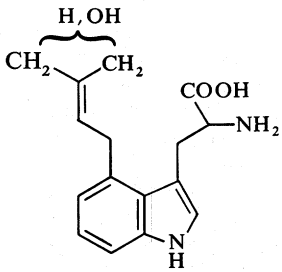

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RESULTS AND DISCUSSION

The HODMAT produced from DMAT with the 60–80% $(NH_4)_2SO_4$ fraction comigrated with reference HODMAT in the two Sil G solvent systems used for the PLC and in the polyamide TLC system. The conversion of DMAT to HODMAT was 0.2% (Table 1). The NADPH-generating system increased the conversion three to four fold. The stimulation of conversion by NADPH addition suggests that the hydroxylation involves a mixed function oxygenase. NADPH-dependent conversion of agroclavine to elymoclavine was previously observed in the 60–80% $(NH_4)_2SO_4$ fraction from *C. purpurea* PRL 1980 [5].

After TLC of the incubation mixture with $CHCl_3$–MeOH–HOAc (10:5:1), two prominent Van Urk's positive spots were observed below DMAT. The lower R_f compound X was fluorescent. The higher R_f compound Y was not. Conversion of DMAT at pH 6.5 was 25% to X and 12% to Y. X and Y were isolated by PLC and fed

Table 1. Conversion of 4-γ,γ-dimethylallyltryptophan (sidechain 3-^{14}C) to 4-[4-hydroxy-3-methyl-Δ^2-butenyl]-tryptophan with 60–80% $(NH_4)_2SO_4$ fraction from *Claviceps purpurea* PRL 1980*

Additions	cpm 3-day culture	cpm 5-day culture
	47	52
NADPH-generating system	147	193
Liver concentrate	189	278

*The incubation mixture contained 0.15 μCi ^{14}C-DMAT (1.0 μCi/mg), 2 mg liver concentrate, and in the NADPH-generating system 5 μmol NADP, 5 μmol glucose-6-phosphate and 0.02 units of glucose-6-phosphate dehydrogenase, in 3.9 ml 0.1 M NaPi pH 7. Protein conc 3 mg/ml for the 3-day culture and 5.7 mg/ml for the 5-day culture. Half of the sample was spotted on the Cheng-Chin polyamide sheet for counting.

to *C. purpurea* PRL 1980. The specific incorporation was 0.38% into elymoclavine for both compounds. The specific incorporation of DMAT into elymoclavine in the same experiment was 26%. X and Y are therefore apparently not intermediates in alkaloid biosynthesis.

The conversion of DMAT to HODMAT demonstrated here plus the *in vivo* conversion of HODMAT [3] comprises an alternate pathway to elymoclavine which does not include agroclavine. An alternate pathway from chanoclavine I to elymoclavine has been previously suggested from cell-free studies [7]. The existence of alternate routes increases the number of possible intermediates between DMAT and elymoclavine. This indicates either a correspondingly larger number of enzymes involved or the ability of the enzymes to act on more than one substrate. The contributions of the alternate pathways to the biosynthesis of elymoclavine will be difficult to ascertain until the intermediates between DMAT and chanoclavine I in the main pathway have been determined.

EXPERIMENTAL

Culture conditions for *C. purpurea* PRL 1980 [8], method of synthesis of DMAT (sidechain 3-^{14}C) [9], and method of prepn of the 60–80% $(NH_4)_2SO_4$ fraction [5] were as previously described. Liver concentrate (catalog no. 202-20) was from Sigma. After the cell-free incubation HODMAT was purified with Dowex 50 cation exchange resin, PLC on Sil G with MeAc–isoPrOH–NH_4OH (9:7:5), and then PLC on Sil G with $CHCl_3$–MeOH–HOAc (10:8:5) with 10% formamide added. The sample was then cospotted with reference HODMAT and developed on a Cheng-Chin polyamide sheet with 80% HCO_2H–H_2O (1:2) and a radioautogram was made. The sheet was then either sprayed with Van Urk's reagent or the radioactive HODMAT spot was cut out into 0.5% 2,5-diphenyloxazole and the radioactivity measured with a liquid scintillation counter.

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ENZYMATIC HYDROLYSIS OF α-CHACONINE AND α-SOLANINE

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; enzymes; steroid glycoalkaloids; α-chaconine; α-solanine.

Several authors have reported on the hydrolysis of potato (*Solanum tuberosum*) glycoalkaloids by enzymes of potato sprouts, blossoms and foliage and the isolation of partial hydrolysis products of α-chaconine and α-solanine [1–6]. We wish to report the results of our studies using enzyme preparations from potato sprouts and dormant tubers (Table 1).

We have confirmed a previous report of the apparently anomalous (non-stepwise) hydrolysis of α-chaconine by an enzyme mixture prepared from potato sprouts [1]. This enzyme mixture removed the rhamnose substituent at the 2-position of the glucose residue in α-chaconine and converted the β_2-chaconine thus produced to solanidine without the demonstrable production of γ-chaconine (the glucoside of solanidine, that would result from the hydrolysis of both rhamnose residues). From α-solanine, this same enzyme mixture first produced β-solanine (by removal of rhamnose), then γ-solanine (the galactoside of solanidine resulting from the loss of glucose from β-solanine), and finally solanidine. Our results confirm the presence of rhamnosidase, glucosidase and galactosidase activities in the enzyme mixture from sprouts.

Our enzyme preparation from dormant tubers produced β_1-chaconine (the 2-rhamnosylglucoside of solanidine), β_2-chaconine, γ-chaconine and solanidine from α-chaconine but only β-solanine and solanidine from α-solanine. This is the first report of the stepwise hydrolysis of α-chaconine and the apparently anomalous (non-stepwise) hydrolysis of α-solanine by potato tuber enzymes.

We also studied the action of these enzyme preparations on β_2-chaconine isolated from dried potato blossoms and on β_1-chaconine and γ-chaconine obtained by partial acid hydrolysis of α-chaconine. Both enzyme mixtures hydrolyzed the β-chaconines and

Table 1. Action of enzyme preparations from potato sprouts and tubers on glycoalkaloids

Enzyme source	Substrate: I α-Chaconine (α-C) Incubation at 37° (hr)	Found by TLC α	β1	β2	γ	S*	II α-Solanine (α-S) Incubation at 37° (hr)	Found by TLC α	β	γ	S	III γ-Chaconine (γ-C) Incubation at 37° (hr)	Found by TLC γ	S	IV β2-Chaconine (β2-C) Incubation at 37° (hr)	Found by TLC β2	γ	S	V β1-Chaconine (β1-C) Incubation at 37° (hr)	Found by TLC β1	γ	S
Sprouts	0	+	−	−	−	−	0	+	−	−	−	0	+	−	0	+	−	−	0	+	−	−
	0.02	+	−	+	−	+	0.5	+	+	−	−	3.0	−	+	0.1	+	−	tr	0.1	−	−	+
	0.25	−	−	+	−	+	3.0	+	+	−	+				17	+	−	+	2.0	−	−	+
	1.0	−	−	+	−	+	18	+	+	+	+				120	+	−	+	5.0	−	−	+
	96	−	−	tr	−	+																
Tubers	0	+	−	−	−	−	0	+	−	−	−	0	+	−	0	+	−	−	0	+	−	−
	1	+	+	−	−	−	3	+	+	−	−	1	+	−	22	−	+	+	1	+	+	+
	3	+	+	tr	−	−	17	+	+	−	tr	5	+	+	72	−	−	+	5	+	+	+
	28	+	+	+	+	tr	24	+	+	−	+	22	−	+					22	−	−	+
	50	−	+	−	+	+	50	+	+	−	tr											

*S = Solanidine.
tr = trace.

produced solanidine from γ-chaconine. In the hydrolysis of the β-chaconines by the enzyme preparation from dormant tubers, the intermediate γ-chaconine appeared as expected. However, with the enzyme preparation from sprouts, no detectable γ-chaconine resulted from the hydrolysis of β_2-chaconine or, suprisingly, from β_1-chaconine.

EXPERIMENTAL

Enzyme prepns were obtained from chlorophyll-free sprouts of *S. tuberosum* L. cv Wauseon and dormant tubers of cv Kennebec. Sprouts or tubers were minced and pressed out through linen with the addition of a small amount of H_2O. Proteins were separated from the expressed juice by precipitating with $(NH_4)_2SO_4$ (0.6 satn) and centrifuging at 14600 *g* for 60 min at 4° [1]. After dialysis against H_2O, the enzyme prepns were stored at 4° with the addition of a little toluene. Enzymatic hydrolysis of glycoalkaloids was carried out in buffer (pH range 4–7) in sealed glass capillaries [7]. Following incubation of enzyme prepns with glycoalkaloid substrates, products were separated on Si gel TLC plates which were developed with the lower layer of MeOH–$CHCl_3$–1% NH_4OH (2:2:1) and then visualized with I_2 vapor. α-Solanine, α-chaconine and β_2-chaconine were isolated from extracts of potato blossoms by precipitation from aq. soln made alkaline with NH_4OH at 70–80°, followed by column chromatography on dry Al_2O_3 [8]. Fractions were monitored by TLC on Si gel plates using Boll's solvent [9], the lower layer of EtOH–$CHCl_3$–1% NH_4OH (2:2:1). Glycoalkaloids isolated by this procedure were identified on the basis of the products of their hydrolysis, IR spectra, R_f comparison with authentic compounds on TLC plates, GLC retention values and MS of permethyl derivatives [10].

β_1-chaconine and γ-chaconine were isolated from a partial acid hydrolysate of α-chaconine by prep TLC.

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THE ABSOLUTE CONFIGURATION OF THE NEW AMINO ACID 2-AMINO-4-METHYL-HEX-5-ENOIC ACID FROM A NEW GUINEA *BOLETUS**

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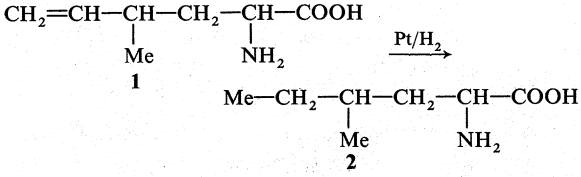
Key Word Index—*Boletus*; Boletaceae; amino acids; absolute configuration.

Abstract—The absolute configuration of the 2-amino-4-methyl-hex-5-enoic acid isolated from *Boletus* was shown to be 2*S*, 4*S*, by an unambiguous synthesis of its dihydro derivative from 2*S*-(−)-2-methylbutan-1-ol.

We have previously reported the isolation of 2-amino-4-methyl-hex-5-enoic acid (**1**) [1, 2] and shown that the asymmetric center at carbon-2 was *S* but could not establish the configuration at carbon-4 by physicochemical methods. We have now synthesized (2*S*,4*S*)-2-amino-4-methyl hexanoic acid (**2**) unambiguously from 2(*S*)-methylbutan-1-ol and acetylamino malonate [3, 4], followed by resolution of the carbon-2 center of the acetylaminomethylhexanoic acid by hog kidney acylase I [5, 6]. The synthetic product was identical in all respects with the dihydro compound (**2**) obtained from the natural product (**1**) by catalytic hydrogenation. As catalytic hydrogenation of the Δ5 double bond at room temperature will not change the chirality at C-4, the absolute configuratio of **1** is established as (2*S*,4*S*)-2-amino-4-methyl-hex-5-enoic acid.



The unambiguous synthesis of sterically pure **2** also establishes the stereochemistry of the naturally occurring amino acid, homoisoleucine, which had been isolated by Fowden and Smith [7] from *Aesculus californica*. Fowden *et al.* [8] had already proposed the 2*S*,4*S* configuration for homoisoleucine on the basis of a comparison of its solubility, ORD and CD with those of isoleucine.

EXPERIMENTAL

Mps are uncorr. Optical rotations were determined on an ETL-NPL automatic polarimeter.

Hydrogenation of 2-amino-4-methyl-hex-5-enoic acid (**1**). 0.1 g **1** over Pt required one mole of hydrogen (15.4 ml) and gave (2*S*, 4*S*) 2-amino-4-methylhexanoic acid (**2**) (0.1 g) mp 240–246°; *m/e* 146, MH^+ from CI; $[\alpha]_D^{22}$ +34.3° (*c* 0.385 in HOAc).

(2S,4S)-2-*Amino-4-methylhexanoic acid.* Refluxing 2*S*-(+)-1-bromo-2-methylbutane (4.5 g, prepared from 2*S*-(−)-2-methylbutan-1-ol) with ethyl acetamidomalonate (5.4 g) and NaOEt (0.6 g) in EtOH [3, 4] gave a mixture of (2*S*,4*S*) and (2*R*,4*S*) ethyl 2-acetamido-2-carbethoxy-4-methylhexanoate as a light yellow oil (5.6 g). Alkaline hydrolysis (10% NaOH, 2 hr), followed by refluxing (2 hr) of the acidified soln yielded a mixture of (2*S*,4*S*) and (2*R*,4*S*)-2-acetamido-4-methylhexanoic acid (2.3 g), mp 190–191° from H_2O, $[\alpha]_D^{27}$ +5.1° (*c*, 2.86 in MeOH). The CI (isobutane) MS showed peaks at *m/e* 188, MH^+, 142 (M^+-COOH), 130 (M^+-C_4H_9), 99 (M^+-COOH—MeCO), 57 $C_4H_9^+$. An aq. soln of the above acid mixture was adjusted to pH 7 with ammonia and hydrolysed at 38° with powdered hog kidney acylase I (12 mg) [5, 6] overnight. The soln was acidified to pH 5, filtered, passed through a Zeocarb 225 cation exchange resin column in the H^+ form, and the column washed with H_2O. The aq. eluates from the column were combined and evapd to dryness. Recrystallization of the solid from H_2O gave (2*R*,4*S*) 2-acetamido-4-methylhexanoic acid (0.4 g), mp 192°. $[\alpha]_D^{30}$ +22.3° (*c*, 6.27 in MeOH). Found: C, 58.0; H, 9.0; N, 7.3%; $C_9H_{17}NO_3$ requires: C, 57.7; H, 9.1; N, 7.5%. The amino acid was eluted from the column with 2N ammonia, and the soln concd to *ca* 200 ml. Cooling of the soln gave the lustrous, colourless crystals of (2*S*,4*S*)-2-amino-4-methyl-hexanoic acid (0.5 g), mp 240–244°. Found: C, 57.9; H, 10.4; N, 9.6%; $C_7H_{15}NO_2$ requires C, 57.9; H, 10.4; N, 9.6%. $[\alpha]_D$ +35.4° (*c*, 0.41 in glacial AcOH) and $[\alpha]_D^{22}$ +25.7° (*c*, 1.0 in 5 N HCl). The CI (isobutane) MS showed peaks at *m/e* 146 MH^+, 100 (M^+-COOH), 74 (M^+-C_5H_{11}) and 57 $C_4H_9^+$, identical with the acid obtained on hydrogenation of **1** (MS, GLC, TLC, mp, mmp). This is in agreement with data recently obtained by Bernasconi *et al.* [9] who used α-chymotrypsin for the resolution of the C-2 centre. The 2*S* configuration of our amino acid was also confirmed by GLC of its *N*-TFA-*L*-prolyl methyl ester derivative [10].

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* Part 3 in the series 'Constituents of a New Guinea *Boletus*'. For Part 2 see ref. [2].