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DIRECTED BIOSYNTHESIS OF UNNATURAL ALKALOIDS IN *DOLICHOTHELE SPHAERICA*

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Abstract—Using appropriate precursors, the two unnatural alkaloids 4(5)-[*N*-isocaproylaminomethyl]imidazole and 3-[2-*N*-isovalerylaminoethyl]pyrazole were produced by *Dolichothele sphaerica*. The former compound represents an unnatural alkaloid formed by the simultaneous introduction of two unnatural precursors, namely isocaproic acid and 4(5)-aminomethylimidazole. The latter compound represents an aberrant alkaloid formed by the introduction of a precursor of different heterocyclic entity, 3-aminoethylpyrazole.

INTRODUCTION

UNNATURAL or aberrant alkaloids are analogs of naturally occurring alkaloids that are produced when unnatural compounds, closely related to the natural precursors, are introduced into plants.

The cactus *Dolichothele sphaerica* yields a novel imidazole alkaloid named dolichotheline (*N*-isovalerylhistamine) (**1**).[1,2] This monosubstituted amide appears to arise biosynthetically by means of a mechanism involving the condensation of histamine and isovaleric acid.[3] The cactus was then considered suitable for carrying out experiments to determine whether the enzyme(s) responsible for this linkage could also yield aberrant alkaloids following the administration of selected precursors to the plant. Preliminary studies on the production of unnatural analogs of dolichotheline resulted in the formation of two aberrant alkaloids,[4] 4(5)-[*N*-isovalerylaminomethyl]imidazole (**2**) following the administration of 4(5)-aminomethylimidazole to the plant and *N*-isocaproylhistamine (**3**) following the administration of isocaproic acid.

All studies on the production of aberrant alkaloids have been limited to the biosynthetic conversion of a structurally modified, single, precursor into a correspondingly modified unnatural alkaloid.[5–9] The objectives of our current investigation were 2-fold: (a) to test the simultaneous administration of two unnatural precursors, namely isocaproic

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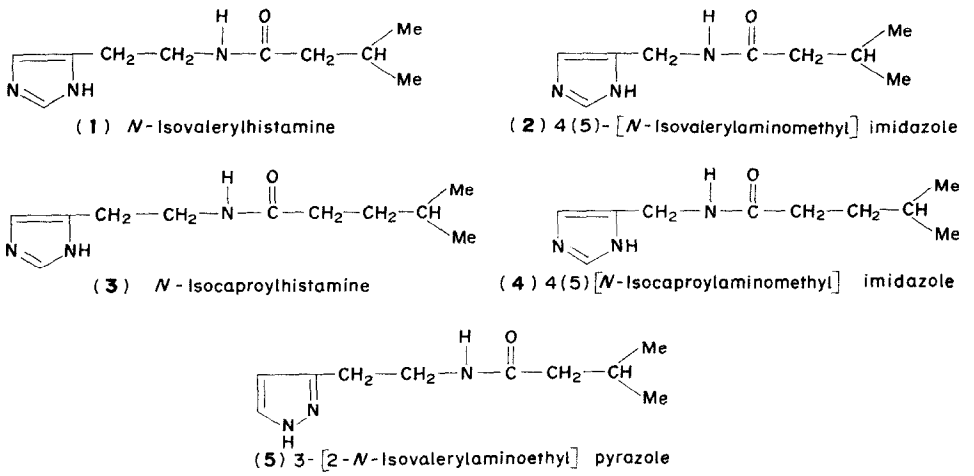
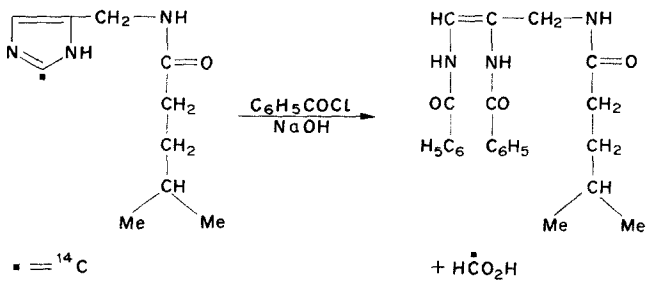


SCHEME 1. UNNATURAL ALKALOIDS OF *Dolichothele sphaerica*.

acid and 4(5)-aminomethylimidazole, in the expectation that the corresponding aberrant alkaloid 4(5)-[*N*-isocaproylaminomethyl]imidazole (**4**), incorporating both precursors would be formed; (b) to test a completely different heterocyclic entity, 3-aminoethylpyrazole (the pyrazole isomer of histamine), as a precursor of its corresponding aberrant alkaloid, 3-[2-*N*-isovalerylaminoethyl]pyrazole (**5**).

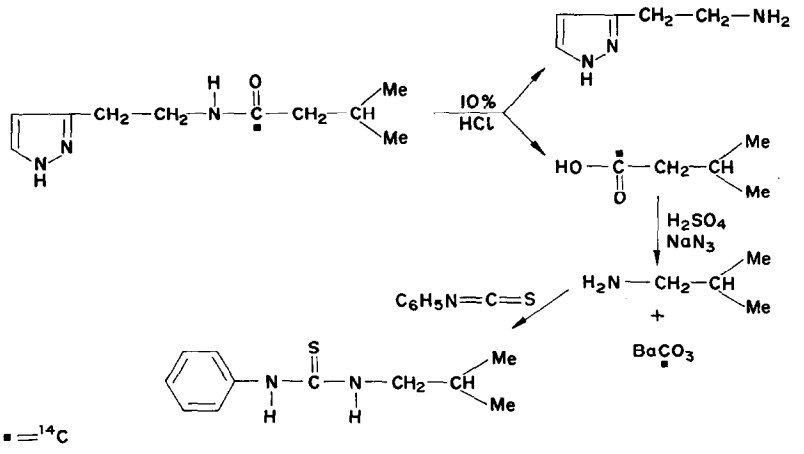


SCHEME 2. DEGRADATION OF **4**.

RESULTS AND DISCUSSION

Since the quantities of the expected unnatural alkaloids were small, authentic samples of the required dolichotheline analogs were synthesized to serve both as standards and as carriers during the extraction procedure. All precursors were aseptically injected into the cactus plants. The radioactive analogs were separated from dolichotheline by preparative TLC, recrystallized to constant specific activity, a single derivative prepared as a further check of the radiopurity, and degraded to localize the label.

The data obtained from *in vivo* studies on the production of aberrant alkaloids are summarized in Table 1. They indicate that the combination of isocaproic acid and 4(5)-aminomethylimidazole-[2-^{14}C ring] was incorporated to the extent of 14·12% into **4**, while the combination of 3-aminoethylpyrazole and isovaleric acid-[1-^{14}C] was incorporated to the extent of 1·81% into **5**. Calculations of per cent injected activity recovered



SCHEME 3. DEGRADATION OF **5**.

as dolichotheline analog are based on the 500 mg of the analog added as carrier. Since the actual amount of analog formed is less than 1 mg, the error made in calculating percent incorporation based on a total yield of 500 mg is very small.

TABLE 1. UNNATURAL PRECURSORS TESTED

Experiment	Precursor	Position of label	Sp. act. mCi/mM	Amount injected μCi	mg	% Injected activity recovered as dolichotheline analog
1	4(5)-Aminomethyl-imidazole DI-HCl	2-^{14}C (ring)	0·71	66·73	16	14·12
	Isocaproic acid (sodium salt)				16	
2	3-Aminoethyl-pyrazole DI-HCl				20	
	Isovaleric acid (sodium salt)	1-^{14}C	52·4	81	0·2	1·81

The isolated alkaloid **4**, when benzoylated, yielded nearly inactive 1,2-dibenzoylamino-3-isocaproylaminopropene-1. This degradation showed that greater than 99% of the activity resided in the predicted position.

When **5** obtained from plants injected with isovaleric acid-[1-^{14}C] and 3-aminoethylpyrazole was hydrolyzed, the activity resided in the isovalerate portion of the molecule. Decarboxylation by the Schmidt reaction provided carbonate which contained greater than 96% of the activity present in the isovaleric acid. The remaining isobutylamine, isolated as isobutylphenylthiourea, was essentially inactive.

It is interesting to note the large percent incorporation obtained when 4(5)-aminomethylimidazole and isocaproic acid were used in combination as compared to when they were introduced separately (1·27 and 0·81% respectively).[4] Perhaps both the amine and acid precursors must be present in sufficient quantity to promote or induce the activity of the enzyme(s) involved.

The positive result obtained with 3-aminoethylpyrazole as the precursor suggests the possibility that other properly substituted heterocyclic nuclei, such as thiazoles and triazoles, could prove to be successful precursors also.

The present study indicates the versatility of the enzyme system that catalyzes the condensation of histamine and isovaleric acid. It also illustrates two previously unreported techniques relative to the production of aberrant alkaloids in higher plants. These include the simultaneous incorporation of two unnatural precursors into an unnatural alkaloid and the incorporation of a heterocyclic entity, completely different from the natural precursor, into its corresponding unnatural alkaloid. In addition, the present investigation points out the potential application of the techniques utilized in the preparation of analogs of biologically active natural products that are difficult to synthesize. Furthermore, such experimentation can reveal the specificity of enzymes involved in the biosynthesis of natural products in cases when these enzymes may not, for technical reasons, be accessible for study in the free state.

EXPERIMENTAL

Preparation and administration of labeled test compounds. Isovaleric acid-[1-^{14}C] was purchased from a commercial source. 4(5)-Aminomethylimidazole-[2-^{14}C] ring was prepared by the method of Pyman.[10,11] This synthesis was initially accomplished using nonradioactive material. The product displayed an IR spectrum identical with that of 4(5)-aminomethylimidazole dihydrochloride and showed no m.p. depression when mixed with authentic 4(5)-aminomethylimidazole dihydrochloride. Diaminoacetone dihydrochloride (100 mg) was added to a hot solution of 66 mg potassium thiocyanate-[^{14}C] in 0·2 ml H_2O, and the mixture was heated on a steam bath for 30 min. The resulting crystalline product was filtered, added to 5 ml hot H_2O, and filtered again. The filtrate was added to 900 mg $FeCl_3$ in 10 ml H_2O, and the mixture was digested for 30 min on the steam bath. Three ml of 10% aq. Na_2CO_3 was added, followed by a hot soln of 400 mg picric acid in 10 ml 100° H_2O. The mixture was boiled with a little charcoal and filtered. On cooling 102 mg of 4(5)-aminomethylimidazole-[2-^{14}C ring] dipicrate separated, m.p. 210–212° [lit.[11] m.p. 212°]. The dihydrochloride was prepared by treating the picrate with 5 ml 6 M HCl, removing the picric acid with four successive 5 ml portions C_6H_6, evaporating the acidic aqueous solution to dryness, and recrystallizing the residue from MeOH–H_2O, yielding 26 mg, m.p. 241–243° [lit. m.p. 244°[10], 246–247°[12]]. Plants of *D. sphaerica* were maintained in a greenhouse. The test compounds were injected into the plants by the method previously described.[4] Specific activities and amounts injected are shown in Table 1.

Preparation of dolichotheline analogs. 4(5)-[*N*-Isocaproylaminomethyl]-imidazole (**4**). 4(5)-Aminomethylimidazole was synthesized under conditions previously reported.[4] This amine (1 g) and 4-methylvaleryl chloride (1·5 g) were refluxed gently for 20 min. The reaction mixture was then cooled, dissolved in 10 ml 1 M HCl, and extracted with Et_2O (×3). The aq. soln was made alkaline with conc NH_4OH to pH 9·5 and extracted with hot $CHCl_3$ in a liquid–liquid extractor for 24 hr. The $CHCl_3$ extract was dried and evaporated to yield the crude **4**. It was recrystallized from MeOH–Et_2O, yielding 1·192 g, m.p. 124° (Found: C, 61·30; H, 8·64; N, 21·35. $C_{10}H_{17}N_3O$ requires: C, 61·51; H, 8·77; N, 21·52%). 3-[2-*N*-Isovalerylaminoethyl]pyrazole (**5**). 3-Aminoethylpyrazole dihydrochloride was obtained from a commercial source. This amine (1·84 g) was dissolved in 3 ml H_2O and subsequently basified to pH 11 with 10% NaOH. The soln was then overlain with C_6H_6 to which 1·21 g isovalerylchloride was added and the entire mixture was refluxed for 1 hr, while maintaining the pH at >10. When cool, the reaction mixture was acidified with 1 M HCl and the C_6H_6 layer was removed. The aq. soln was extracted with Et_2O (×2) and then basified with conc. NH_4OH to pH 9·0 and extracted with hot $CHCl_3$ in a liquid–liquid extractor for 24 hr. The $CHCl_3$ extract was dried and evaporated; the syrupy residue of **5** could not be induced to crystallize. As a consequence, the picture was formed and recrystallized from H_2O, yielding 613 mg, m.p. 136° (Found: C, 45·06; H, 4·72; N, 19·75. $C_{16}H_{20}N_6O_8$ requires: C, 45·28; H, 4·75; N, 19·8%). If strongly basic conditions are not maintained throughout the course of the above synthesis, the major product formed is the diamide 1-isovaleryl-3-[2-*N*-isovaleryl-aminoethyl]pyrazole, m.p. 76° (Found: C, 64·41; H, 8·85; N, 15·25. $C_{15}H_{25}N_3O$ requires: C, 64·48; H, 9·01; N, 15·04%).

Isolation of aberrant alkaloids. Three weeks after the injection of the precursors, the plants were removed from the greenhouse and sliced, dried in an oven at 45° for 96 hr and ground. In both cases, 500 mg of the

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[12] TURNER, R. A., HAEBNER, C. F. and SCHOLZ, C. R. (1949) *J. Am. Chem. Soc.* **71**, 2801.

appropriate nonradioactive dolichotheline analog was added to the ground material. The method used for the extraction and isolation of dolichotheline and its unnatural analogs was identical to that previously reported for the isolation of dolichotheline.[2] Any radioactivity residing in the unnatural analogs was monitored using TLC and radiochromatogram scanning. The radioactive analogs were separated from dolichotheline by preparative TLC.

Separation of **4** *from dolichotheline.* A mixture (637 mg) of **4** and dolichotheline was dissolved in a small vol. MeOH and applied (100 mg/plate) to a 2 mm layer of silica gel GF_{254}, 20 × 20 cm plates. The plates were then subjected to multiple development (×3) in $CHCl_3$–EtOH–1 M HCl (8 : 2 : 0·1). The R_f for dolichotheline was 0·39; for **4**, it was 0·61. The silica gel scraped off the plates was extracted with MeOH (3 × 100 ml). The MeOH extract was reduced to dryness and dissolved in 15 ml H_2O; the pH was adjusted to 9·5 with conc. NH_4OH, and the soln was extracted with hot $CHCl_3$ in a liquid–liquid extractor for 24 hr. The $CHCl_3$ extract was dried and evaporated to yield 211 mg of **4**, m.p. 123–124°.

Separation of **5** *from dolichotheline.* A mixture (600 mg) of **5** and dolichotheline was separated by a method similar to that employed for the separation of **4** and dolichotheline. The preparative plates in this case were developed 2 × using the above solvent system. The R_f for dolichotheline was 0·25; for **5**, it was 0·68. The $CHCl_3$ extract when evaporated yielded a syrupy residue of **5** which was converted to the picrate, m.p. 136°.

Degradation of **4** (*Scheme* 2). The amide (200 mg) was added to a cold soln of 1 g NaOH in 5 ml H_2O. This was followed by the addition of 0·5 ml PhCOCl and 2 drops C_6H_6. The mixture was stirred vigorously for 2 hr with cooling, after which the solid product was filtered, washed with a little H_2O, and dried. The 1,2-dibenzoylamino-3-isocaproylaminopropene-1 was recrystallized from EtOH–H_2O, yielding 172 mg, m.p. 165°. (Found: C, 70·24; H, 6·83; N, 10·77. $C_{23}H_{27}N_3O_3$ requires: C, 70·20; H, 6·92; N, 10·68%). Using an identical procedure, 40 mg of the separated radioactive amide was reacted with PhCOCl to yield 20 mg 1,2-dibenzoylamino-3-isocaproylaminopropene-1, m.p. 164–165°.

Degradation of **5** (*Scheme* 3). **5** was hydrolyzed with 10% HCl, and the resulting 4(5)-aminoethylpyrazole and isovalerate were recovered as the dihydrochloride and sodium salts, respectively, by identical procedures described for the hydrolysis of dolichotheline.[3] The sodium isovalerate was further degraded by means of a Schmidt decarboxylation essentially as described by Phares.[13] The sodium isovalerate and NaCl mixture (300 mg) was dissolved in cold 100% H_2SO_4 (3 ml) in a 25 ml pear-shaped flask and powdered NaN_3 (250 mg) was added in small amounts, with shaking, to the chilled soln. The flask was immediately connected to two bead towers in series, the first containing 10 ml 5% $KMnO_4$ in 0·5 M H_2SO_4 and the second containing 10 ml 1 M CO_2-free NaOH. A slow stream of N_2 was forced through the solution while the flask was heated to 60–65° for 6 hr. The bead tower containing the NaOH was then washed down with CO_2-free H_2O, and 2 ml satd. $BaCl_2$ was added. The resulting precipitate was centrifuged and the supernate was decanted. The $BaCO_3$ was resuspended in H_2O and again centrifuged. This process was repeated with EtOH and the carbonate was finally dried in a desiccator. To recover the isobutylamine, the contents of the reaction flask were basified to pH 12 with 20% NaOH and the isobutylamine was extracted 3 × with equal portions of Et_2O. The isobutylamine was then reacted with phenylisothiocyanate to form isobutylphenylthiourea.[14]

Radioactive assay. The method employed for the preparation of samples for scintillation counting was described previously.[15,16] The aberrant products were recrystallized to constant sp. act., and a single derivative of each product was also counted as a check of the radiopurity. The two degradation products were assayed to localize the label. The $BaCO_3$ (0·5 mg) was assayed as a suspension in scintillation fluid containing a thixotropic gel (5 g Cab-O-Sil/1,000 ml of scintillation cocktail). All samples were counted in a liquid scintillation system at a preset 2σ statistical counting error of 1%. The external standard ratio method was used to determine losses due to quenching. All counts were corrected for counter efficiency.

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