Synthesis of Isotopically Labelled 3-Amino-2-phenylpropionic Acid and Its Role as a Precursor in the Biosynthesis of Tenellin and Tropic Acid

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A synthesis of [3-^{13}C]- and [3-^{14}C]-3-amino-2-phenylpropionic acids **7a** and **7b** is described. The incorporation of this amino acid into tenellin **1** from *Beauveria bassiana* (Bals.) Vuill. and into tropic acid **10** moiety of the tropane alkaloids hyscoyamine **8** and scopolamine **9** from *Datura innoxia* was studied but proved unsuccessful in each case.

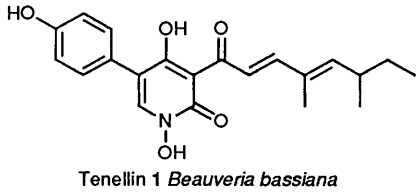
Tenellin **1** is a bright yellow metabolite elaborated by the fungus *Beauveria Bassiana* (Bals.) Vuill.[1] It belongs to a group of four fungal metabolites, which include bassianin **2**,[2] ilicicolin-H **3**[3] and funiculosin **4**,[4] that possess an interesting substituted 2-pyridone ring system. The biosynthetic origin of the 2-pyridone ring has been investigated for tenellin **1**[5,6] and ilicicolin **3**[7] and these studies have demonstrated that the ring has its origin from the L-2-aminopropionoid moiety of L-phenylalanine and the terminal acetate subunit of a polyketide chain. The aryl substituent at C-5 clearly derives intact from L-phenylalanine and both branching methyl groups of the polyketide moieties in tenellin and ilicicolin-H are donated by the *S*-methyl group of L-methionine.

The most interesting feature of the biosynthesis of these metabolites is an apparent intramolecular rearrangement of L-phenylalanine to provide the requisite structural framework of the 2-pyridone ring. In the definitive experiment carried out on tenellin **1**,[8] the labelled carbons of DL-[1,3-$^{13}C_2$]-phenylalanine came together and were incorporated intact at C-4 and C-5. A dominant homonuclear ^{13}C–^{13}C coupling was observed between these carbons in the resultant ^{13}C NMR spectrum and was indicative of an intramolecular rearrangement of the original L-phenylalanine skeleton. L-Phenylalanine and not L-tyrosine is the more efficient precursor to tenellin **1** (and ilicicolin-H **3**) suggesting that aryl hydroxylation may take place after condensation of the amino acid and polyketide moieties. On the basis of these observations pathway **A** of Scheme 1 was proposed[6] which suggested an initial condensation to a tetramic acid **5** followed by oxidation to a quinomethine such as **6**. Subsequent ring expansion and rearomatisation would generate the required structural skeleton for tenellin.

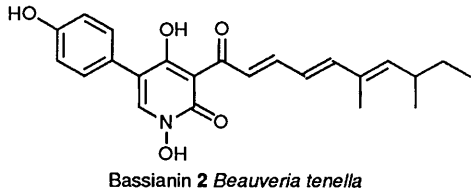
We entertained an alternative possibility, pathway **B**, for the generation of the 2-pyridone ring system of tenellin **1** which is consistent with all the experimental evidence afforded to date. The key feature of this pathway involves a direct rearrangement of L-phenylalanine, to 3-amino-2-phenylpropionic acid **7**, prior to condensation with the polyketide fragment. Although there is no precedence for such a L-phenylalanine mutase, tentative support for this hypothesis was supplied by the discovery of Leete that there is an apparent rearrangement of L-phenylalanine occurring during the biosynthesis of the tropic acid moiety of the tropane alkaloids hyoscyamine **8** and scopolamine **9**, in plants of *Datura innoxia*.[9,10,11] These studies have demonstrated a stereospecific migration of the carboxy group of L-phenylalanine from C-2 to C-3 and evidence for the back migration of the 3-*pro*-*S* hydrogen atom to C-2 in going towards tropic acid **10** (see Scheme 2). No intermediates between L-phenylalanine and tropic acid **10** have been identified. At the outset we reasoned that an enzyme mediating the rearrangement of L-phenylalanine to a specific enantiomer of 3-amino-2-phenylpropionic acid **7** would unify both the pathway to the fungal 2-pyridones and that to tropic acid in plants. We set out to establish the validity of this hypothesis by investigating the role of DL-3-amino-2-phenylpropionic acid **7** in the biosynthesis of tenellin **1** and tropic acid **10**.

Results

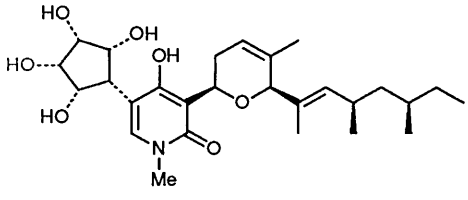
We needed to prepare isotopically enriched **7** and selected to synthesise DL-[3-^{13}C]- and [3-^{14}C]-3-amino-2-phenylpropionic acids (**7a** and **7b** respectively) as shown in Scheme 3 for the biosynthetic experiments. The isotopic labels were introduced by treatment of benzyl chloride with either potassium [^{14}C]- or [^{13}C]-cyanide to generate either benzyl [1-^{14}C]- or [1-^{13}C]-cyanide **11**. Treatment of **11** with butyl-



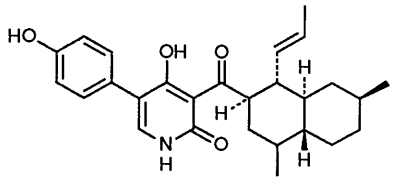
Tenellin **1** *Beauveria bassiana*



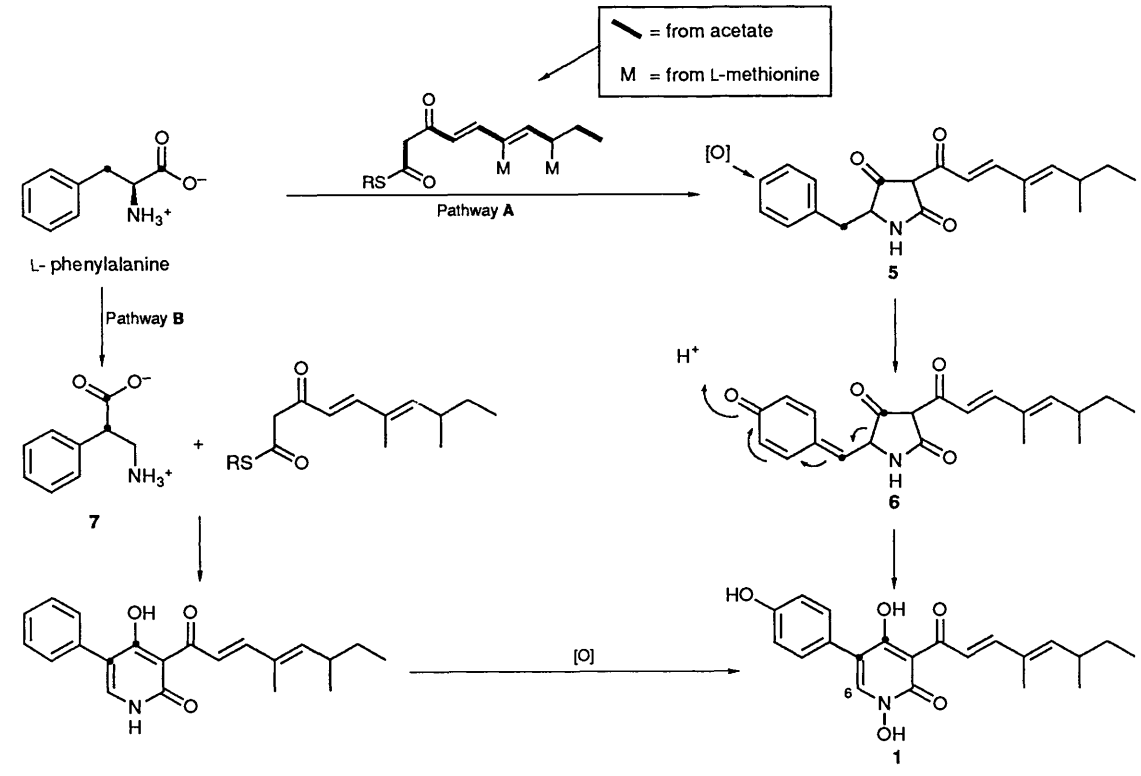
Bassianin **2** *Beauveria tenella*



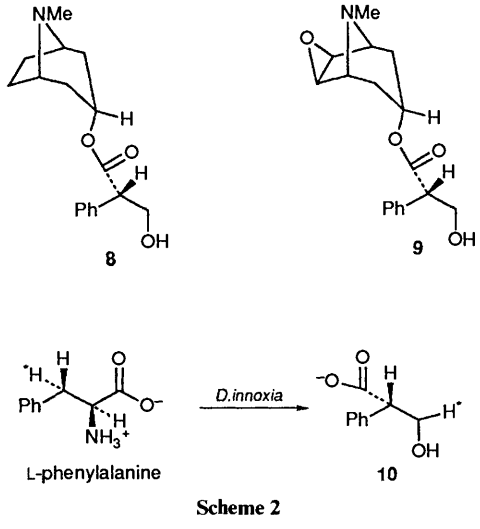
Funiculosin **3** *Penicillium funiculosum*



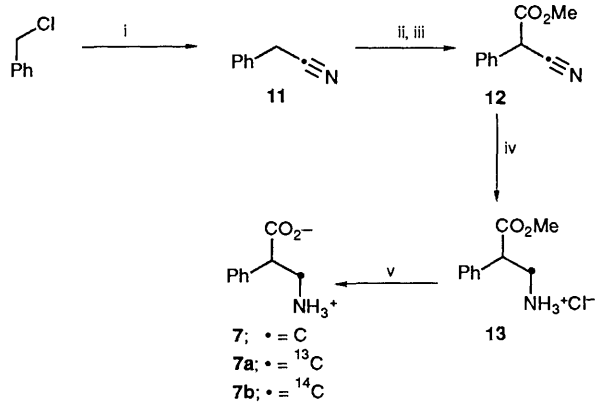
Ilicicolin H **3** *Cylindrocladium ilicocola*



Scheme 1



Scheme 2



Scheme 3 *Reagents and Conditions:* i, [^{13}C]- or [^{14}C]-KCN, 18-crown-6, acetonitrile, 12 h, 18 °C; ii, BuLi in THF, then solid CO_2 and acidify with 10% H_2SO_4; iii, CH_2N_2; iv, H_2–PtO_2 in EtOH–$CHCl_3$ (10:1), 2.5 atm, 18 h, 18 °C; v, NaOH then H^+-Dowex

lithium followed by quenching of the generated anion with carbon dioxide, gave phenylcyanoacetic acid, which was converted into its methyl ester **12** prior to isolation. Catalytic hydrogenation of **12** in ethanol containing chloroform (10%) provided ideal reduction conditions as the amine hydrochloride **13** was formed *in situ* and could be isolated as a white crystalline solid in good yield. Basic ester hydrolysis followed by ion exchange chromatography then afforded the desired labelled amino acid.

DL-[3-^{13}C]-3-Amino-2-phenylpropionic acid **7a** was introduced into cultures of *Beauveria bassiana* at a final concentration of 2 mmol dm^{-3} just prior to tenellin production. A trace of L-[U-^{14}C]phenylalanine (specific activity 1.7 μCi mmol^{-1}) was added simultaneously as an internal reference. The absolute incorporation into tenellin **1** from L-[U-^{14}C]phenylalanine was 3.4%, however analysis of the resultant ^{13}C NMR spectrum of recovered tenellin **1** showed no enrichment of the signal corresponding to C-6. In order to eliminate the possibility that **7a** was unable to penetrate the fungal cells, equimolar amounts of unlabelled **7** and L-phenylalanine were added, in a separate experiment, to a culture of *B. bassiana*. After 4 days incubation, and at the onset of tenellin **1** production, the cells were harvested and repeatedly washed. Ethanolic extraction of the cells, followed by analytical chromatography, revealed that the two predominant cellular amino acids were the exogenously added 3-amino-2-phenylpropionic acid **7** and L-phenylalanine. It would appear therefore that there was no impediment to the cellular accumulation of **7a** during the biosynthetic experiment.

We then addressed the role of **7** as an intermediate in tropic acid **10** biosynthesis. DL-[3-^{14}C]-3-Amino-2-phenylpropionic acid **7b** (80 mg, 7.97 μCi/mmol dm^{-3}) was fed to plants of *Datura innoxia* by the wick method. After a 4 week period

>99% of the radioactivity had been taken up by the plants. On harvesting, 46% of this activity was detectable in the aqueous extract. The organic extract, containing the alkaloids, had only 0.26% of the original activity present and on purification and isolation of hyoscyamine **8** and scoploamine **9** as their hydrochloride salts, the residual activity was negligible.

Discussion

Clearly we have to discount **7** as a *bona fide* intermediate in tenellin **1** and tropic acid **10** biosynthesis. The identity of the true intermediates on both of these pathways remains an intriguing problem. There are many possible scenarios for intermediates between L-phenylalanine and tropic acid **10**. For the fungal 2-pyridones however, the fact that the nitrogen atom of L-phenylalanine is retained[6,7] from L-phenylalanine limits the number of possibilities, and those intermediates on pathway **A** of Scheme 1 emerge as likely candidates. It should be stated however that these results do not preclude the possibility that a derivative of L-phenylalanine, such its coenzyme-A ester, could be involved in a rearrangement to the coenzyme-A ester of **7**. It is noteworthy that those mutase enzymes which mediate a carboxy group migration, *e.g.* methylmalonyl-CoA mutase[12] and isobutyryl-CoA mutase[13] utilise coenzyme-A esters. The fungal and plant systems may be able to activate the α-amino acid to this status but be devoid of an acyl-CoA synthetase activity capable of generating the coenzyme-A ester of the β-amino acid.

Experimental

General.—^{1}H NMR spectra were recorded on either a Bruker AC 250 (250 MHz) machine operating at 250.133 MHz or a Varian VXR 400S (400 MHz) instrument operating at 399.952 MHz. ^{13}C NMR spectra were recorded on a Varian VXR 400S (400 MHz) spectrometer operating at 100.577 MHz. Chemical shifts are quoted in ppm relative to TMS [$(CH_3)_4Si$] in $CDCl_3$ and H_2O in D_2O, all coupling constants are in Hz. IR spectra were recorded on Perkin-Elmer 577 and 377 grating spectrophotometers, samples being embedded in a KBr disc for solids or neat between KBr plates for liquids. Mass spectra were obtained using a VG Analytical 7070E mass spectrometer operating at 70 eV. All solvents were distilled and dried before use. Specific activities for ^{14}C compounds were obtained from samples dissolved in 'Ecoscint A' scintillation solution using a Packard 2000CA liquid scintillation analyser. A Beckman J2-21M/E centrifuge was used for all centrifugations. Physical and spectrometric properties of ^{14}C-labelled compounds were identical to the unlabelled materials.

Benzyl Cyanide **11**.—Potassium cyanide (1.0 g, 15.4 mmol) was added to a solution of benzyl chloride (distilled, b.p. 22–24 °C, 0.005 mbar, 1.94 g, 15.3 mmol) and 18-crown-6 (0.3 g, 1.1 mmol) in acetonitrile (6 cm^3) and stirred for 12 h at 18 °C. Methylene dichloride (50 cm^3) was added, and the suspension filtered. The filtrate was washed with water (2 × 50 cm^3), dried ($MgSO_4$) and evaporated under reduced pressure to yield a yellow oil (*ca.* 2g) which was distilled (b.p. 46 °C, 0.1 mbar) to yield benzyl cyanide as a clear colourless oil (1.68 g, 14.4 mmol, 93.8%); $\delta_H(CDCl_3)$ 7.30 (5 H, m, Ph) and 3.66 (2 H, s, CH_2); ν_{max}(neat)/cm^{-1} 3100, 3078, 3042, 2975, 2925, 2250, 1605, 1500, 1456 and 1418.

Benzyl [1-^{13}C]*Cyanide*.—Potassium [^{13}C]cyanide (1.0 g, 15.2 mmol, 99 atom% ^{13}C) was used to prepare benzyl [1-^{13}C]-cyanide (1.62 g, 13.7 mmol, 90.3%) by the method described above; $\delta_H(CDCl_3)$ 7.30 (5 H, m, Ph) and 3.66 (2 H, d, J_{2H-13C}, 10, CH_2).

Benzyl [1-^{14}C]*cyanide*. 1.0 g (15.4 mmol) of potassium [^{14}C]cyanide (specific activity 9.47 μCi/mmol) was used to make benzyl[1-^{14}C]cyanide, (1.48 g, 12.6 mmol, 82.1%) by the method described above.

Methyl Phenylcyanoacetate **12**.—Butyllithium in pentane (1.7 mol dm^{-3}; 10 ml) was added to a solution of benzyl cyanide (1.68 g, 14.4 mmol) in tetrahydrofuran (THF) (20 cm^3) at −78 °C and the solution stirred for 5 min; after this it was quenched with an excess of solid carbon dioxide in THF (50 cm^3). The white suspension was allowed to warm to room temperature, acidified with 10% sulphuric acid and the free carboxylic acid extracted into methylene dichloride. The organic extract was dried ($MgSO_4$) and treated with an excess of an ethereal solution of diazomethane and the solvent removed under reduced pressure to afford **12** after distillation as a clear yellow oil (1.07 g, 6.11 mmol, 42.5%); (b.p. 78–80 °C, 0.08 mbar); $\delta_H(CDCl_3)$ 7.37 (5 H, m, Ph), 4.78 (1 H, s, CH) and 3.71 (3 H, s, Me); ν_{max}/cm^{-1} 3080–3020, 2962, 2260, 1755, 1600, 1500, 1458 and 1438.

Methyl [3-^{13}C]*Phenylcyanoacetate*. Benzyl[1-^{13}C]cyanide (1.62 g, 13.7 mmol) was used without further purification to prepare methyl [3-^{13}C]phenylcyanoacetate (0.92 g, 5.2 mmol, 38.2%) by the method described above.

Methyl [3-^{14}C]*phenylcyanoacetate*. Benzyl[1-^{14}C]cyanide (1.48 g, 12.6 mmol) was used to afford [3-^{14}C]methylphenylcyanoacetate by the method described above (0.84 g, 4.8 mmol, 38.1%).

Methyl 3-Amino-2-phenylpropionate Hydrochloride **13**.—A solution of **12** (1.07 g, 6.11 mmol), in ethanol (50 cm^3) and chloroform (5 cm^3) was shaken under H_2 (2.5 atm) with PtO_2 (200 mg) for 18 h. The catalyst was filtered off and the solvent evaporated under reduced pressure to give a cream solid which was washed with ethyl acetate, collected by filtration and air dried to afford **13** as a white crystalline solid (1.12 g, 5.20 mmol, 85.1%); $\delta_H(D_2O)$ 7.34–7.22 (5 H, m, Ph), 4.02 (1 H, t, J_{2-3ab} 7.6, 2-H), 3.59 (3 H, s, Me), 3.53 (1 H, dd, J_{2-3a} 7.2, J_{gem} 13.2, 3-H_A) and 3.29 (1 H, dd, J_{2-3b} 7.2, J_{gem} 13.2, 3-H_B); ν_{max}/cm^{-1} 3450br, 3300–2500br, 1734, 1605, 1590, 1495, 1460, 1440 and 1410; *m/z* (CI) 180 (M + 1, 35.3%) and 122 (1.6%).

Methyl [3-^{13}C]-3-*Amino-2-phenylpropionate Hydrochloride*.—Methyl [3-^{13}C]-phenylcyanoacetate (0.92 g, 5.2 mmol) prepared as above was used to prepare methyl [3-^{13}C]-3-amino-2-phenylpropionate hydrochloride (0.45 g, 2.08 mmol, 40%).

Methyl [3-^{14}C]-3-*Amino-2-phenylpropionate Hydrochloride*.—Methyl [3-^{14}C]phenylcyanoacetate (0.84 g, 4.8 mmol) prepared as above was used to afford the title compound (0.9 g, 4.18 mmol, 87.0%).

3-*Amino-2-phenylpropionic Acid* **7**.—A solution of **13** (1.12 g, 5.2 mmol) in potassium hydroxide (1.2 mol dm^{-3}; 50 cm^3) was stirred for 12 h and then acidified to pH 7 before purification by ion exchange chromatography (DOWEX 50X8-200 resin). Aqueous washings containing the amino acid (ninhydrin test) were evaporated under reduced pressure to afford *compound* **7** as a white powder (0.5 g, 3.03 mmol, 58.3%) m.p. 223.7–223.9 °C (EtOH–H_2O) (lit.,[14] 222–224 °C) $\delta_H(D_2O$–$D_2SO_4)$, 7.26 (5 H, m, Ph), 3.56 (1 H, t, J_{2-3ab} 7.6, 2-H), 3.19 (1 H, dd, J_{2-3a} 7.6, J_{gem} 12.4, 3-H_A) and 2.99 (1 H, dd, J_{2-3b} 7.6, J_{gem} 12.8, 3-H_B); $\delta_C(D_2O$–$D_2SO_4)$, 40.95 (C-3), 48.36 (C-2), 128.16 (C-6, C-8),* 128.77 (C-7), 129.48 (C-5, C-9),* 134.17 (C-4) and 174.52 (C-1); ν_{max}/cm^{-1} 3420, 3200–2400br, 2200, 1660–

* Assignments may be interchanged.

1490br, 1452 and 1440; *m/z* (CI) 166 (M + 1, 74.7%) (Found: C, 65.05; H, 7.02; N, 8.33. $C_9H_{11}NO_2$ requires C, 65.44; H, 6.71; N, 8.48%).

[3-^{13}C]-3-*Amino-2-phenylpropionic Acid* **7a**.—Methyl [3-^{13}C]-3-amino-2-phenylpropionate hydrochloride (0.45 g, 2.08 mmol) prepared as above was used to generate [3-^{13}C]-3-amino-2-phenylpropionic acid (0.2 g, 1.2 mmol, 57.7%); $\delta_C(D_2O)$, 42.310 (s, 99% enriched, C-3), 51.40 (d, J_{CC} 37, C-2), 127.90 (C-6, C-8),* 128.12 (C-7), 129.22 (C-5, C-9),* 137.22 (C-4) and 178.25 (C-1), $\delta_H(D_2O)$ 7.25 (5 H, m, Ph), 3.648 (1 H, dt, J_{2-3ab} 7.3, J_{2-13C} 6.4), 3.32 (1 H, ddd, J_{2-3a} 7.3, J_{gem} 12.8, J_{3a-13C} 145.7) and 3.142 (1 H, ddd, J_{2-3b} 7.3, J_{gem} 12.8, J_{3b-13C} 145.7); *m/z* (CI) 167 (M + 1, 51%) and 123 (11%).

[3-^{14}C]*Amino-2-phenylpropionic Acid* **7b**.—Methyl [3-^{14}C]-3-amino-2-phenylpropionate hydrochloride (0.9 g, 4.18 mmol) prepared as above was used to afford [3-^{14}C]-3-amino-2-phenylpropionic acid (0.48 g, 2.91 mmol, 69.6%, 7.97 μCi/mmol).

Growth of B. bassiana (*Bals.*) *Vuill. and Isolation of Tenellin.*—Primary cultures of *B. bassiana* (Bals.) Vuill. (obtained from the CBS culture collection strain No. 110.25), were initiated from frozen stock by innoculation (4 cm^3) of sterilised D-mannitol medium[6] (50 cm^3) in 250 cm^3 Erlenmeyer flasks. The culture was incubated for 4 d on an orbital shaker (27 °C at 200 rpm). This primary culture was used to innoculate (2 cm^3) a production culture of the same medium (50 cm^3) rotating under the same conditions. Labelled compounds were filter sterilised and administered 72 h after innoculation. The cells were harvested by centrifugation (14 000 rpm, 4 °C, 60 min) 7 d after innoculation and were exhaustively extracted into acetone in a Soxhlet apparatus. The extract was evaporated under reduced pressure to afford a red–brown slurry which was dissolved in methylene dichloride (200 cm^3). The orange organic solution was washed with brine (3 × 100 cm^3) to remove sugars, dried ($MgSO_4$) and evaporated under reduced pressure to yield a tan solid. The solid was washed with pentane to remove lipids and crude tenellin collected by filtration as a light brown powder which was then recrystallised from methanol; δ_C([$^2H_6$]-DMSO) 11.79 (C-14), 12.39 (C-16), 19.91 (C-15), 29.40 (C-13), 34.63 (C-12), 105.89 (C-3), 110.84 (C-5), 115.01 (C-3′, C-5′), 122.68 (C-1′), 123.08 (C-8), 130.26 (C-2′, C-6′), 132.60 (C-10), 140.22 (C-6), 149.83 (C-9), 151.04 (C-11), 156.89 (C-4′), 157.49 (C-2), 173.03 (C-4) and 193.77 (C-7).

Feeding of [3-^{13}C]-3-*Amino-2-phenylpropionic Acid* **7a** *to* B. bassiana.—Compound **7a** (100 mg) and L-phenylalanine (0.25 mg) were dissolved in deionised water (12 cm^3). The solution was spiked with L-[U-^{14}C]phenylalanine (2.62 μCi) and administered to six 50 cm^3 cultures of *B. Bassiana* such that the final concentration of **7a** was 2 mmol dm^{-3}. Tenellin (250 mg), isolated by the method described above, was recrystallised to constant activity (3.60 × 10^{-4} μCi mg^{-1}) from methanol.

Amino Acid Analysis.—L-Phenylalanine (5 mg) and 3-amino-2-phenylpropanoic acid **7** (5 mg) were added to a four day old production culture (50 cm^3) of *B. Bassiana*. The culture was incubated for 4 d and then the cells were harvested and washed thoroughly with deionised water. A suspension of the cells in 70% ethanol solution (200 cm^3) was homogenised and then shaken overnight. Filtration and evaporation under reduced pressure to a volume of 5 cm^3 afforded a solution suitable for TLC. TLC was performed using 0.2 mm silica coated plates, eluted with a solution of propanol–ammonia (7:3) and developed by spraying the chromatographs with a 0.2% solution of ninhydrin in ethanol. L-Phenylalanine and **7** were identified against reference samples.

Feeding of [3-^{14}C]-3-*Amino-2-phenylpropionic Acid* **7b** *to* D. innoxia.—**7b** (80 mg, 1.08 × 10^5 dpm/mg) was administered by the wick method to 10, 3-month old, *D. innoxia* plants growing in soil in a greenhouse. Four weeks later the plants were harvested (fresh wt. 460 g) and chopped up in a mixture of chloroform (3 dm^3), diethyl ether (400 cm^3) and concentrated ammonia (100 cm^3). The two layers obtained after filtering were assayed for radioactivity. The aqueous ammoniacal layer contained 46%, and the organic layer only 0.26%, of the original activity. The organic solvents were evaporated, and the tropane alkaloids purified as their hydrochloride salts as previously described.[15] The level of radioactivity in the purified salts was negligible.

* Assignments may be interchanged.

Acknowledgements

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