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# 3-Hydroxy-3-phenylpropanoic acid is an intermediate in the biosynthesis of benzoic acid and salicylic acid but benzaldehyde is not

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**Abstract.** Stable-isotope-labelled ($^2H_6$,$^{18}O$) 3-hydroxy-3-phenylpropanoic acid, a putative intermediate in the biosynthesis of benzoic acid (BA) and salicylic acid (SA) from cinnamic acid, has been synthesized and administered to cucumber (*Cucumis sativus* L.) and *Nicotiana attenuata* (Torrey). Analysis of the products by gas chromatography-mass spectrometry revealed incorporation of labelling into BA and SA, but not into benzaldehyde. In a separate experiment, 3-hydroxy-3-phenylpropanoic acid was found to be a metabolite of phenylalanine, itself the primary metabolic precursor of BA and SA. These data suggest that cinnamic acid chain shortening is probably achieved by $\beta$-oxidation, and that the proposed "non-oxidative" pathway of side-chain degradation does not function in the biosynthesis of BA and SA, in cucumber and *N. attenuata*.

**Key words:** Benzaldehyde – Benzoic acid – 3-Hydroxy-3-phenylpropanoic acid – Phenylpropanoid metabolism – Salicylic acid

## Introduction

The phenylpropanoid pathway leads to a large number of plant secondary metabolites, many of which are stress-induced (Dixon and Paiva 1995). Notable phenylpropanoids include the coumarins, furanocoumarins, flavonoids, isoflavonoids, and lignin. Compounds belonging to the above groups incorporate all nine carbon atoms from cinnamic acid into their respective skeletons, but a number of phenylpropanoids are produced by shortening of the cinnamate $C_3$ side-chain. Of these, perhaps the most important are the derivatives of benzoic acid (BA). Salicylic acid (SA), produced by hydroxylation of BA (León et al. 1993, 1995; Yalpani et al. 1993), has attracted considerable attention because of its role in the onset of systemic acquired resistance in pathogen-infected plants. Pathogen attack results in an increase of endogenous SA in both infected and uninfected leaves, leading to broad-spectrum systemic resistance (Malamy et al. 1990; Métraux et al. 1990). At present there is some disagreement as to whether SA is the translocated signal responsible for activating systemic acquired resistance in uninfected parts of the plant (Vernooij et al. 1994; Shulaev et al. 1995). However, there is little doubt that the presence of SA is essential for transduction of the signal for systemic acquired resistance (Ryals et al. 1996).

Given the importance of SA in disease resistance, the biosynthesis of SA may be a significant control point in plant defence. Despite its simple structure, and ubiquitous occurrence in the Angiospermae, several aspects of SA biosynthesis remain unclear (Lee et al. 1995). Foremost among these is the mechanism of side-chain degradation responsible for producing BA from cinnamic acid. Two basic routes have been proposed (Ryals et al. 1996; Fig. 1), one analogous to fatty acid $\beta$-oxidation (a retro Claisen condensation, in chemical terms), and the other a so-called "non-oxidative" pathway proceeding via benzaldehyde (**3**, Fig. 1) (a retro aldol condensation). The name given to this latter route is somewhat unsatisfactory, since although the cleavage reaction is non-oxidative, the overall pathway contains an oxidative step (Fig. 1). Evidence for the "non-oxidative" pathway comes from the observation that isotopically labelled benzaldehyde is converted into BA and SA when externally applied to tobacco (*Nicotiana tabacum*) plants (Ribnicky et al. 1997). This route can also be compared with the biosynthesis of 4-hydroxybenzoic acid from 4-hydroxycinnamic acid (*p*-coumaric acid). Here, 4-hydroxycinnamic acid is

Dedicated to Professor E. David Morgan on the occasion of his 70th birthday

Abbreviations: BA = benzoic acid; PLE = pig liver esterase; PPTS = pyridinium *p*-toluene sulfonate; SA = salicylic acid; RP-HPLC-MS = reverse phase-high performance liquid chromatography-mass spectrometry

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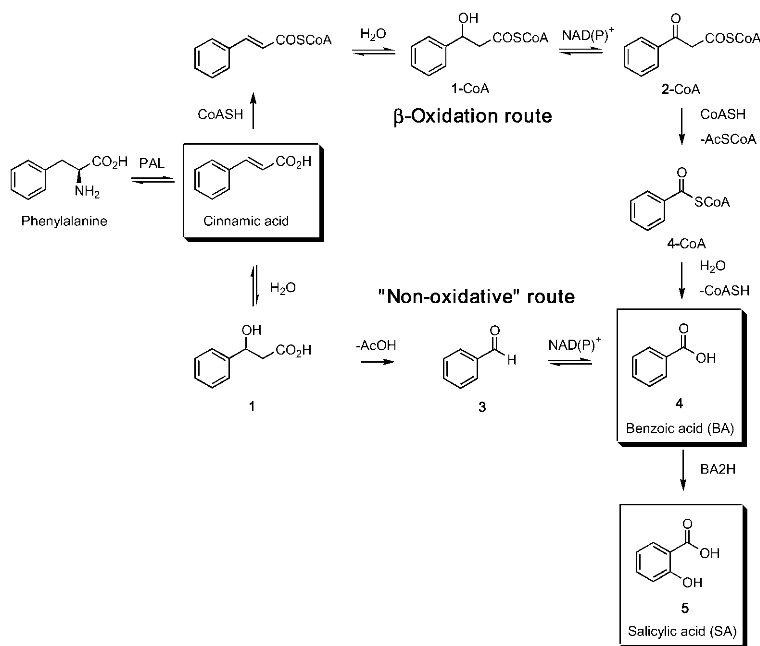


**Fig. 1.** The two proposed pathways leading to BA and SA in plants. *BA2H*, benzoic acid 2-hydroxylase; *PAL*, phenylalanine ammonia-lyase

converted to 4-hydroxybenzaldehyde, by the action of a hydratase/lyase, before oxidation to 4-hydroxybenzoic acid (for *Lithospermum erythrorhizon*, see Yazaki et al. 1991 and Löscher and Heide 1994; for *Daucus carota*, see Schnitzler et al. 1992; and for *Pseudomonas fluorescens*, see Mitra et al. 1999). Thus, the process is analogous to the potential "non-oxidative" route shown in Fig. 1.

Evidence for the $\beta$-oxidation route originates from the observation that acetyl-CoA and ATP stimulate the formation of SA from cinnamic acid in cell-free extracts of *Quercus pedunculata* (Alibert and Ranjeva 1971). This is endorsed by recent work on tobacco (*N. tabacum*), which shows that while labelling from phenylalanine is incorporated into cinnamic acid, BA and SA, none is present in endogenous benzaldehyde (Ribnicky et al. 1998). This suggests that the aldehyde is not an intermediate between cinnamic acid and BA, and that incorporation of externally applied benzaldehyde (vide supra) may be a misleading result.

To the best of our knowledge no one has investigated the role of 3-hydroxy-3-phenylpropanoic acid (**1**, Fig. 1) in the biosynthesis of BA and SA, despite the fact that it is a key intermediate in both the proposed $\beta$-oxidation and "non-oxidative" pathways (Ryals et al. 1996; Ribnicky et al. 1998; Knaggs 1999). Here we communicate our recent findings that **1** does play a role in BA and SA biosynthesis, but that benzaldehyde does not.

## Materials and methods

### *Plant rearing and treatment*

Seeds of cucumber (*Cucumis sativus* L. cv. Delikateß; Julius Wagner, Heidelberg, Germany) and *Nicotiana attenuata* Torrey (a gift from Prof. Ian Baldwin) were cultivated in a growth chamber with a 32 °C/27 °C day/night and 16-h daylight cycle, with 65% humidity. Cucumber plants were grown individually until two mature leaves had developed (about 14 d). The stems were then cut, and placed in aqueous solutions (15 ml tap water) of [$^2H_5$]phenylalanine (0.6 mM) or [$^2H_6$,$^{18}O$]-**1** (0.5 or 5.0 mM). *Nicotiana attenuata* plants were grown in a tray, sprayed with liquid smoke [condensed smoke from hickory wood (House of Herbs, Passaic, N.Y. USA), 10 ml/per 1 water]. The young seedlings were replanted after 14 d of growth and allowed to grow individually for a further 14 d. Cut stems were then placed in aqueous solutions (15 ml tap water) of [$^2H_5$]phenylalanine (0.6 mM) or [$^2H_6$,$^{18}O$]-**1** (0.5 mM). Plants treated with phenylalanine were extracted after 2 d, whilst those treated with [$^2H_6$,$^{18}O$]-**1** were extracted after 4 d. All plants were maintained under light during the treatment period.

### *Synthesis*

*General.* Deuterated benzaldehyde, $^{18}O$-labelled water and [$^2H_5$]-L-phenylalanine were purchased from Cambridge Isotope Laboratories Inc. (Andover, Mass., USA). Pig liver esterase (PLE) was purchased from Sigma, as a suspension in $(NH_4)_2SO_4$ (3.2 M, pH 8). Nuclear magnetic resonance (NMR) spectroscopy was performed using a Bruker Avance DRX 500 (Bruker Analytik, Rheinstetten, Germany) at 500 and 125 MHz for $^1H$ NMR and $^{13}C$ NMR, respectively. Infrared (IR) spectra were measured on a Bruker Equinox 55 spectrometer (Bruker Optik, Ettlingen, Germany), with solid samples pressed into KBr discs and liquids prepared as thin films. Low- and high-resolution (HR) electron impact mass spectrometry (EI-MS) was performed on a Micromass MasSpec (Micromass, Manchester, UK).

*2-[2,3,4,5,6-$^2H_5$]Phenyl[2-$^2H$]-[1,3]dioxolane.* To a stirred suspension of 20 mg polymer-bound pyridinium *p*-toluene sulfonate (PPTS) in anhydrous benzene (50 ml), ethylene glycol (0.63 g, 10.16 mmol) and [$^2H_6$]-**3** (1.0 g, 8.93 mmol) were added. The reaction mixture was heated at 95 °C for 4 h in a Dean-Stark water-separating apparatus equipped with a condenser. Whilst still hot, the PPTS was removed by filtration, and the filtrate was dried (anhydrous $MgSO_4$) to give, after evaporation of the solvent, 1.09 g of pure 2-[2,3,4,5,6-$^2H_5$]phenyl[2-$^2H$]-[1,3]dioxolane (78.3% yield). $^1H$ NMR ($CDCl_3$, ppm): 4.12 (2 H, dd), 4.03 (2 H, dd); $^{13}C$ NMR ($CDCl_3$, ppm): 138.1 (s), 129.1 (t), 128.3 (t), 126.5 (t), 103.8 (t), 65.7

(s); IR (film, $cm^{-1}$): 3516, 2957, 2888, 2272, 2117, 1469, 1394, 1330, 1251, 1208, 1075, 1038, 1006; EI-MS (relative intensity): 156 ($M^+$, 69), 155 (20), 154 (99), 153 (5), 124 (14), 110 (57), 98 (14), 97 (28), 96 (51), 94 (26), 85 (7), 84 (42), 83 (5), 82 (45), 74 (65), 68 (7), 66 (12), 56 (6), 54 (31), 52 (11), 46 (32), 43 (5), 42 (10); HR-MS: found 156.1055, calculated for $C_9H_4{}^2H_6O_2$ 156.1057.

*[$^2H_6$,$^{18}O$]Benzaldehyde ([$^2H_6$,$^{18}O$]-**3**)*. Non polymer-bound PPTS (15 mg) was added to a solution of 2-[2,3,4,5,6-$^2H_5$]phenyl[2-$^2H$]-[1,3]dioxolane (1.0 g, 6.41 mmol) in $H_2{}^{18}O$ (1.0 g, 50 mmol) and tetrahydrofuran (4 ml), in a 25-ml round-bottomed flask, fitted with a condenser. After stirring at 50 °C for 4.5 h, the reaction was cooled, and extracted with diethyl ether (3 × 10 ml). The combined organic phase was dried (anhydrous $MgSO_4$), and the solvent was removed by distillation to give 0.66 g of [$^2H_6$,$^{18}O$]-**3** (90.3% yield). After EI-MS analysis, this was immediately used in the next stage. EI-MS (relative intensity) 114 ($M^+$, 90), 113 (11), 112 (100), 111 (7), 110 (9), 84 (10), 83 (5), 82 (67), 56 (6), 54 (24), 52 (12).

*Ethyl 3-[$^{18}O$]hydroxy-3-[2,3,4,5,6-$^2H_5$]phenyl[3-$^2H$]propanoate ([$^2H_6$,$^{18}O$]-**1**-ethyl ester)*. A mixture of [$^2H_6$,$^{18}O$]-**3** (0.66 g, 5.79 mmol) and trimethyl borate (1.50 g, 14.4 mmol) in absolute tetrahydrofuran (2.0 ml) was added slowly to zinc granules (0.647 g, 10 mmol) with stirring. Ethyl bromoacetate (1.65 g, 10 mmol) was added dropwise and the resulting mixture was stirred at room temperature for 16 h. The reaction was quenched by the addition of glycerol (1.65 ml) and concentrated ammonium hydroxide (1.65 ml), and the product was extracted with diethyl ether (3 × 10 ml), dried (anhydrous $MgSO_4$) and evaporated to dryness. The crude product was purified by silica column chromatography, the product being eluted with 40:60 (v/v) ethyl acetate:light petroleum (40–60 °C). After evaporation of the solvent, 0.41 g of pure [$^2H_6$, $^{18}O$]-**1**-ethyl ester was obtained (35.1% yield). $^1H$ NMR ($CDCl_3$, ppm): 4.18 (2 H, q), 3.28 (1 H, bs), 2.74 (1 H, d), 2.72 (1 H, d), 1.25 (3 H, t); $^{13}C$ NMR ($CDCl_3$, ppm): 172.3 (s), 142.3 (s), 128.0 (t), 127.2 (t), 125.2 (t), 69.8 (t), 60.8 (s), 43.2 (s), 14.1 (s); IR (film, $cm^{-1}$): 3468, 2979, 1729, 1373, 1330, 1192, 1091, 1033; EI-MS (relative intensity): 202 ($M^+$, 45), 200 (12), 157 (7), 127 (8), 116 (8), 115 (100), 114 (16), 113 (30), 110 (14), 108 (5), 88 (19), 85 (67), 84 (8), 83 (8), 82 (23), 60 (18), 54 (13). HR-MS: found 202.1362, calculated for $C_{11}H_8{}^2H_6O_2{}^{18}O$ 202.1362.

*3-[$^{18}O$]Hydroxy-3-[2,3,4,5,6-$^2H_5$]phenyl[**3**-$^2H$]propanoic acid ([$^2H_6$,$^{18}O$]-**1**)*. To a solution of [$^2H_6$,$^{18}O$]-**1**-ethyl ester (80 mg, 0.396 mmol) in 0.01 M phosphate buffer (10 ml, $K_2HPO_4$/$KH_2PO_4$, pH 7), PLE solution [26.6 µl, 100 units (1 unit will hydrolyse 1.0 µmol of ethyl butyrate per min at pH 8.0 and 25 °C)] was added. The mixture was sonicated for 15 min, and then allowed to stir for 14 h at room temperature. After extracting with diethyl ether (5 ml), the aqueous phase was placed in an ultrafiltration concentrator and centrifuged at 10 600 *g* for 20 min. The filtrate was adjusted to pH 8 with 5% NaOH solution and extracted with diethyl ether (5 ml). The aqueous phase was then acidified with concentrated HCl (to pH 1) and extracted with diethyl ether (3 × 10 ml). The combined ether extracts were dried (anhydrous $MgSO_4$) and the solvent evaporated to give 26.2 mg of [$^2H_6$, $^{18}O$]-**1** (38.0% yield). $^1H$ NMR ($CD_3OD$, ppm): 2.63 (1 H, d), 2.60 (1 H, d); $^{13}C$ NMR ($CD_3OD$, ppm): 173.9 (s), 143.9 (s), 127.9 (t), 126.5 (t), 125.5 (t), 70.5 (t), 43.9 (s); IR (KBr disc, $cm^{-1}$): 3189, 2928, 2628, 1709, 1408, 1320, 1199, 1088, 930; EI-MS (relative intensity): 174 ($M^+$, 17), 172 (5), 115 (61), 114 (33), 113 (27), 112 (27), 111 (13), 110 (100), 109 (20), 108 (25), 107 (6), 99 (9), 85 (66), 84 (15), 83 (27), 82 (54), 81 (31), 80 (9), 66 (6), 56 (8), 55 (12), 54 (35), 53 (13), 52 (18), 46 (6), 45 (10), 44 (51), 43 (22), 42 (20), 41 (7); HR-MS: found 174.1047, calculated for $C_9H_4{}^2H_6O_2{}^{18}O$ 174.1049. The abundance of $^{18}O$ was found to be 76% by MS.

*Ethyl 3-oxo-3-[2,3,4,5,6-$^2H_5$]phenylpropanoate ([$^2H_5$]-**2**-ethyl ester)*. To a cooled stirring solution of ethyl 3-chloro-3-oxopropanoate (1.0 g, 6.64 mmol) in [$^2H_6$]-benzene (5.0 g, 59.5 mmol), finely-powdered aluminium chloride (1.30 g, 9.75 mmol) was slowly added. The flask was then heated to 50 °C for 2 h. The resultant mixture was cooled, and water (10 ml) was added. The product was extracted with diethyl ether (3 × 15 ml) and the organic phase washed with $H_2SO_4$ (10%), and then with $NaHCO_3$ solution. After removal of the solvent, 1.01 g of pure [$^2H_5$]-**2**-ethyl ester was obtained (82.1% yield). Both $^1H$ and $^{13}C$ NMR spectra showed signals for the keto and enol forms of [$^2H_5$]-**2**-ethyl ester. For simplicity, only the keto signals are reported. $^1H$ NMR ($CDCl_3$, ppm): 4.23 (2 H, q), 3.98 (2 H, s), 1.26 (3 H, t); $^{13}C$ NMR ($CDCl_3$, ppm): 192.5 (s), 167.7 (s), 135.9 (s), 133.4 (t), 128.3 (m), 61.3 (s), 46.0 (s), 14.3 (s); IR (film, $cm^{-1}$): 2982, 1740, 1686, 1603, 1523, 1414, 1381, 1304, 1244, 1182, 1146, 1097, 1028; EI-MS (relative intensity): 197 ($M^+$, 27), 152 (10), 151 (8), 138 (5), 137 (49), 136 (7), 111 (18), 110 (100), 109 (39), 108 (5), 96 (7), 83 (8), 82 (53), 80 (5), 69 (9), 54 (18), 52 (5); HR-MS: found 197.1099, calculated for $C_{11}H_7{}^2H_5O_3$ 197.1100.

*3-Oxo-3-[2,3,4,5,6-$^2H_5$]phenylpropanoic acid ([$^2H_5$]-**2**)*. The preparation of [$^2H_5$]-**2** was carried out in a similar manner to the preparation of [$^2H_6$,$^{18}O$]-**1**, with PLE, using [$^2H_5$]-**2**-ethyl ester (80 mg, 0.407 mmol) as the starting material. The reaction gave 43.0 mg of [$^2H_5$]-**2** (63.3% yield). $^1H$ NMR ($CD_3OD$, ppm): 3.21 (2H, s); $^{13}C$ NMR ($CD_3OD$, ppm): 193.7 (s), 169.9 (s), 135.9 (s), 132.7 (t), 127.7 (m), signal for C2 obscured by solvent; IR (KBr disc, $cm^{-1}$): 3030, 2690, 1625, 1605, 1461, 1228, 1187, 1065; EI-MS (relative intensity): 169 ($M^+$, 48), 168 (15), 152 (8), 151 (13), 137 (23), 136 (7), 126 (6), 125 (59), 124 (20), 111 (19), 110 (100), 109 (71), 108 (17), 107 (6), 96 (14), 95 (11), 94 (5), 87 (9), 83 (35), 82 (83), 81 (47), 80 (16), 76 (6), 69 (14), 65 (5), 55 (7), 54 (46), 53 (15), 52 (21), 45 (6), 44 (40), 43 (16), 42 (15); HR-MS: found 169.0786, calculated for $C_9H_3{}^2H_5O_3$ 169.0787.

*Ethyl 3-hydroxy-3-[2,3,4,5,6-$^2H_5$]phenylpropanoate ([$^2H_5$]-**1**-ethyl ester)*. To a stirred solution of [$^2H_5$]-**2**-ethyl ester (250 mg, 1.27 mmol) in ethanol (95%, 5 ml), was added a solution of sodium borohydride (16 mg, 0.4 mmol in 1 ml water). After stirring at room temperature for 3 h, concentrated ammonium hydroxide (2 ml) was added, and this was stirred for a further hour. After addition of water (2 ml), the product was extracted with diethyl ether (3 × 10 ml), and the combined ether extracts were washed with HCl (5%), water and dried ($Na_2SO_4$). The solvent was evaporated to give 225.4 mg of [$^2H_5$]-**1**-ethyl ester (89.2% yield). $^1H$ NMR ($CDCl_3$, ppm): 5.10 (1 H, m), 4.15 (2 H, q), 3.18 (1 H, bs), 2.66 (2 H, m), 1.25 (3 H, t); $^{13}C$ NMR ($CDCl_3$, ppm): 172.4 (s), 142.5 (s), 128.1 (m), 127.1 (m), 125.3 (m), 70.3 (s), 61.2 (s), 43.0 (s), 15.5 (s); IR (film, $cm^{-1}$): 3457, 2984, 2936, 2910, 1729, 1368, 1166, 1022; EI-MS (relative intensity): 199 ($M^+$, 30), 198 (9), 197 (12), 179 (7), 171 (19), 170 (8), 164 (6), 152 (10), 151 (8), 139 (29), 138 (12), 137 (53), 136 (15), 125 (10), 124 (6), 113 (9), 112 (86), 111 (46), 110 (100), 109 (48), 108 (13), 107 (8), 97 (5), 96 (7), 95 (10), 94 (4), 88 (13), 85 (7), 84 (53), 83 (37), 82 (46), 81 (28), 80 (18), 73 (8), 69 (14), 68 (5), 57 (8), 55 (11), 54 (18); HR-MS: found 199.1251, calculated for $C_{11}H_9{}^2H_5O_3$ 199.1257.

*3-Hydroxy-3-[2,3,4,5,6-$^2H_5$]phenylpropanoic acid ([$^2H_5$]-**1**)*. The preparation of [$^2H_5$]-**1** was carried out as previously described for [$^2H_6$, $^{18}O$]-**1**, using [$^2H_6$]-**1**-ethyl ester (80 mg, 0.402 mmol). The reaction gave 43.0 mg of [$^2H_6$]-**1** (62.5% yield). $^1H$ NMR ($d_6$-dimethyl sulfoxide, ppm): 12.11 (1 H, bs), 5.40 (1 H, bs), 4.95 (1 H, m), 3.17 (2 H, m); $^{13}C$ NMR ($d_6$-dimethyl sulfoxide, ppm): 172.3 (s), 144.9 (s), 127.8 (t), 126.7 (t), 125.6 (t), 69.4 (s), 44.6 (s); IR (KBr disc, $cm^{-1}$): 3292, 2931, 2649, 1703, 1416, 1400, 1272, 1187, 1059, 1011; EI-MS (relative intensity): 171 ($M^+$ 70), 170 (29), 169 (6), 157 (14), 149 (14), 139 (22), 138 (10), 137 (7), 125 (6), 112 (100), 111 (86), 110 (65), 109 (46), 108 (20), 107 (17), 96 (14), 95 (11), 94 (5), 87 (9), 83 (35), 82 (83), 81 (47), 80 (16), 76 (6), 69 (14), 65 (5), 55 (7), 54 (46), 53 (15), 52 (21), 45 (6), 44 (40), 43 (16), 42 (15); HR-MS: found 171.0941, calculated for $C_9H_5{}^2H_5O_3$ 171.0940.

*Extraction of benzaldehyde, BA and SA*

Following incubation, plants (in batches of five) were ground in liquid nitrogen for 5 min and transferred to a round-bottomed flask (100 ml), while still frozen. The flask was sealed with a small piece of aluminium foil and the fibre of a solid-phase microextraction (SPME) device (Pawliszyn 1997) exposed to the headspace of the plant tissue. The flask was then placed in a water bath (30 °C) and left for 30 min before the SPME device was withdrawn and placed in the injection port of a gas chromatograph-mass spectrometer for analysis of benzaldehyde. Solvent extraction of benzaldehyde was achieved by grinding the plant tissue in a mixture of 50 mM aqueous citric acid (45 ml) and acetone (100 ml). After filtration, and evaporation of the acetone, the aqueous layer was partitioned with ether (3 × 100 ml) and the ether solution analysed by GC-MS for the presence of benzaldehyde. Extraction of BA and SA then followed the protocol of Baldwin et al. (1997), developed for jasmonic acid analysis. The resulting methyl ester derivatives were analysed by GC-MS directly (vide infra).

*Extraction of 3-hydroxy-3-phenylpropanoic acid*

[$^2H_5$]-3-Hydroxy-3-phenylpropanoic acid was extracted from plants treated with [$^2H_5$]phenylalanine using a modification of the protocol of Baldwin et al. (1997). Samples were not derivatised with diazomethane, but analysed as free acids by liquid chromatography-mass spectrometry (LC-MS) (vide infra).

*Gas chromatography–mass spectrometry*

A Micromass MasSpec double-focusing magnetic sector mass spectrometer (geometry EBE) connected to a Hewlett Packard HP6890 II gas chromatograph, equipped with an AT-5 (Alltech, Unterhaching, Germany) non-polar (5% phenyl, 95% methyl polysiloxane) capillary column (30 m × 0.25 mm × 0.25 μm), was used for GC-MS. For analysis of methyl benzoate and methyl salicylate the injection port of the gas chromatograph was operated at 220 °C and the transfer line at 280 °C. The oven temperature was programmed to rise from 30 °C to 280 °C at 8 °C $min^{-1}$, with an initial and final isothermal time of 2 min. For benzaldehyde analysis, a similar program was employed, but with an initial isothermal time of 5 min. Helium was used as a carrier gas at 1 ml $min^{-1}$ and samples were injected in the splitless mode. Typically, 5% of the total extract from five plants was analysed. Mass spectra were measured in EI mode at 70 eV, with a source temperature of 200 °C, and an acceleration voltage of 8 kV. In the full-scan mode the instrument was scanned between $m/z$ 40 and $m/z$ 300 at 1 scan $s^{-1}$. Selective ion monitoring (SIM) was performed by acceleration voltage scanning. Unit mass spectra were recorded at a resolution of 700, and accurate mass spectra at a resolution of 5000. The B/E-linked scanning spectra were measured using a collision cell in the first field free region of the mass spectrometer. Helium was used as the collision gas and the collision energy was 8 keV. In all cases perfluorokerosene (Aldrich, Deisenhofen, Germany) was used as a calibration gas.

*High-performance liquid chromatography–mass spectrometry*

A Hewlett-Packard series 1100 HPLC system connected to a Quattro II quadrupole-hexapole-quadrupole tandem mass spectrometer (Micromass) was used. Samples were analysed with atmospheric pressure chemical ionization (APCI) employing the Micromass Z-spray source. MassLynx 3.1 (Micromass) was used for data recording and analysis. The HPLC system was operated at a column temperature of 30 °C with a SUPELCOSIL LC-18 reversed-phase HPLC column (Supelco, Deisenhofen, Germany). Analyses were carried out with a gradient of $H_2O$ + 0.1% trifluoroacetic acid and methanol at a constant flow rate of 0.5 ml $min^{-1}$, starting with 25% methanol (5 min isocratic), which was increased to 90% methanol over a range of 10 min and then kept isocratic for 3 min. Analyses with APCI were performed in positive mode with a probe temperature of 400 °C and a source block temperature of 150 °C. Nitrogen (300 l $h^{-1}$) served as APCI sheath and drying gas, while argon was used as collision gas for selective reaction monitoring (SRM) experiments at a pressure of $2 \times 10^{-3}$ mbar. The Z-spray source was operated on conditions optimized by injection of standard solutions with a corona pin voltage of 3.50 kV and cone voltage of 14 V. Ion transitions for SRM (collision energy 14 eV) were selected by MS/MS experiments (product ion scans) with reference compounds. Extract samples were prepared for HPLC-MS by re-dissolving the residue in 1 ml methanol. This was concentrated to approx. 100 μl by partially evaporating the methanol with argon. Injection volumes were 20 μl in each case, using an autosampler. Compounds were identified by co-injection with authentic references.

## Results and discussion

*Synthesis of 3-[$^{18}O$]hydroxy-3-[2,3,4,5,6-$^2H_5$]phenyl[3-$^2H$]propanoic acid ([$^2H_6$,$^{18}O$]-1)*

Determination of the involvement of 3-hydroxy-3-phenylpropanoic acid (**1**) (or its CoA thioester) in the biosynthesis of BA and SA required the synthesis and administration of an isotopically labelled probe. Earlier, unpublished work in our laboratory had demonstrated partial dehydration of exogenously applied [$^2H_5$]-**1** to yield [$^2H_5$]cinnamic acid. Since the latter is a known precursor of BA and SA, administration of [$^2H_5$]-**1** could not be used to demonstrate the role of **1** in BA and SA biosynthesis. To overcome this problem, the synthesis of a metabolic probe possessing an $^{18}O$-labelled hydroxy group, which would be lost on dehydration to cinnamic acid, was designed. Incorporation of $^{18}O$ into the two chain-shortened acids would then confirm **1** as a real intermediate en route to BA and SA. Besides $^{18}O$, five deuterium atoms on the phenyl ring were employed to provide a substantial difference in molecular mass, simplifying mass-spectroscopic analysis of any products exhibiting incorporation. In addition, we chose to place a deuterium atom on C3, since retention of this label in benzaldehyde (as the aldehydic hydrogen) would provide evidence for the "non-oxidative" route, while loss of the label would support a $\beta$-oxidation type pathway (see Fig. 1). The structure and synthesis of [$^2H_6$,$^{18}O$]-**1** are shown in Fig. 2. [$^2H_6$]Benzaldehyde ([$^2H_6$]-**3**) was converted to its acetal by treatment with ethylene glycol and a catalytic amount of immobilised PPTS. Hydrolysis of the acetal in a 1:4 mixture $H_2{}^{18}O$ and tetrahydrofuran gave [$^2H_6$,$^{18}O$]-**3**, which, after extraction and removal of solvent, was immediately used for a Reformatsky reaction with ethyl bromoacetate. Hydrolysis of the resulting $\beta$-hydroxyester using PLE at pH 7 resulted in the desired (±)-$\beta$-hydroxy acid, [$^2H_6$,$^{18}O$]-**1**, with 76% $^{18}O$ (under the conditions employed no kinetic resolution of enantiomers was detected). The presence of a small amount of $^{16}O$ in the probe was of little consequence, as the dehydration to [$^2H_6$]cinnamic acid, expected in vivo, would inevitably reduce any potential

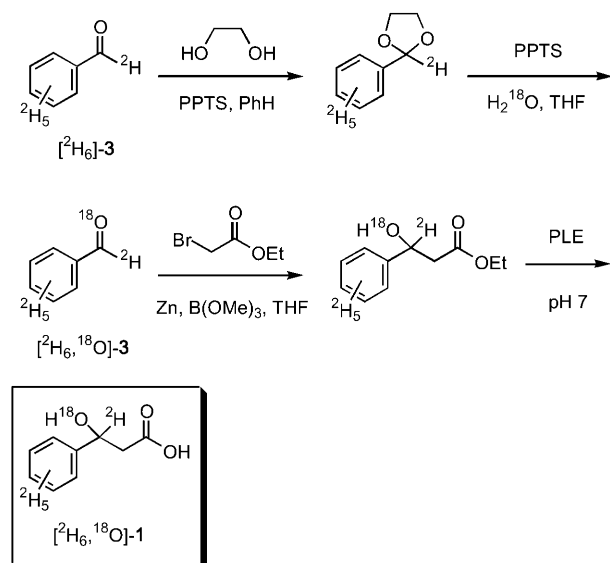


**Fig. 2.** Synthesis of 3-[$^{18}$O]hydroxy-3-[2,3,4,5,6-$^{2}H_5$]phenyl[3-$^{2}$H] propanoic acid ([$^{2}H_6$,$^{18}$O]-**1**). *PPTS*, pyridinium *p*-toluene sulfonate; *PhH*, benzene; *THF*, tetrahydrofuran; *PLE*, pig liver esterase; *Et*, ethyl; *Me*, methyl

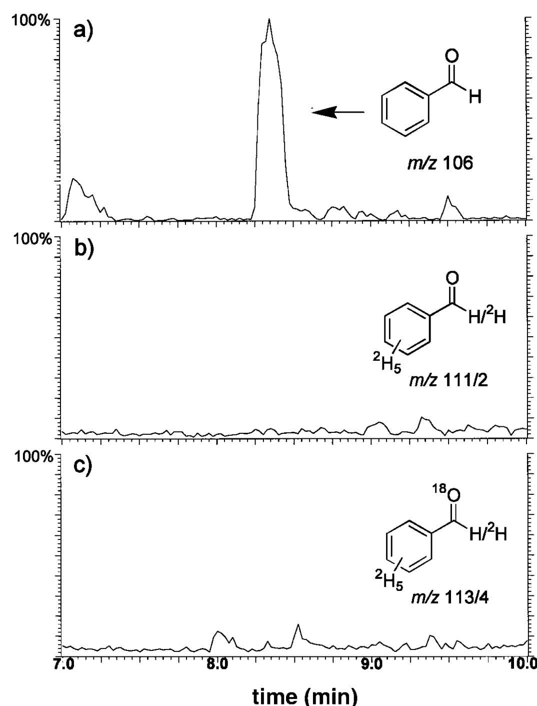


**Fig. 3a–c.** Ion chromatograms of the molecular ions of possible benzaldehyde isotopomers, extracted from cucumber plants following treatment with [$^{2}H_6$,$^{18}$O]-**1**. Unlabelled aldehyde (**a**) was the only detected product; there was no detection of labelled aldehydes (**b, c**)

enrichment of $^{18}$O in BA and SA. Overall, this route represented a relatively efficient (four steps, 10% overall yield) and cost-effective method for incorporating an expensive isotope, like $^{18}$O, into a biosynthetic probe.

### *Incorporation of [$^{2}H_6$,$^{18}$O]-1 into BA and SA*

To investigate the role of **1** in the biosynthesis of BA and SA, [$^{2}H_6$,$^{18}$O]-**1** (0.5 mM in water) was administered to 2-week-old seedlings of cucumber (*Cucumis sativus*) and 4-week-old seedlings of *Nicotiana attenuata* by uptake through the stem. Following an incubation period of 4 d the plants were ground in liquid nitrogen and the headspace analysed for benzaldehyde (**3**), using solid-phase microextraction (SPME) and GC-MS. Figure 3 shows ion chromatograms for the molecular ions of benzaldehyde isotopomers from the headspace GC-MS analysis. Unlabelled **3** gave a clear peak (Fig. 3a), but there were no detectable amounts of either [$^{2}H_6$]-**3**/[$^{2}H_5$]-**3** (Fig. 3b) or [$^{2}H_6$,$^{18}$O]-**3**/[$^{2}H_5$,$^{18}$O]-**3** (Fig. 3c). Experiments using a 10-fold higher concentration of [$^{2}H_6$,$^{18}$O]-**1** did result in the production of small quantities of [$^{2}H_5$]-**3** and possibly traces of [$^{2}H_5$,$^{18}$O]-**3** but, crucially, the aldehydic hydrogen remained unlabelled. Thus, it is unlikely that benzaldehyde is produced directly from $\beta$-hydroxy acid **1**, as both the $^{18}$O and $^{2}$H on C3 should be retained in a retro aldol condensation. Solvent extraction of the aldehyde from plant tissue gave identical results to headspace analysis by SPME, and a similar result was obtained for both *C. sativus* and *N. attenuata*.

Following benzaldehyde analysis, BA and SA were extracted from the plant tissue using a method developed for the analysis of endogenous jasmonic acid (Baldwin et al. 1997). Conversion of BA and SA to their methyl esters, by treatment with diazomethane, was performed prior to GC-MS analysis (only the results for BA are presented in detail, as it is a known precursor of SA). High-resolution-selective ion monitoring (HR-SIM) was employed in order to detect unlabelled methyl benzoate (MW = 136.052), [$^{2}H_5$] methyl benzoate (MW = 141.084) and [$^{2}H_5$,$^{18}$O]methyl benzoate (MW = 143.088) specifically. Figure 4 shows the resulting chromatograms. The unlabelled ester was the most abundant form (set to 100% intensity, Fig. 4a), but clear peaks were also visible for the [$^{2}H_5$]- and [$^{2}H_5$,$^{18}$O]-isotopomers (19% and 7%, Fig. 4b and c, respectively). The presence of [$^{2}H_5$,$^{18}$O]BA (**4**) clearly demonstrates that **1** (or its CoA thioester) is converted to BA (and subsequently to SA) with retention of the labelled oxygen (Fig. 5). From the size of the [$^{2}H_5$]methyl benzoate peak, it is also clear that a considerable amount of $^{18}$O is lost from the probe prior to incorporation. This latter result can be explained by the expected dehydration of [$^{2}H_6$,$^{18}$O]-**1** in planta (vide supra), followed by rehydration with $H_2{}^{16}O$ (Fig. 5). It is probable that reduction of BA, by an oxidoreductase, is the principal route to benzaldehyde seen in the previous experiment (Fig. 5). Such a sequence explains the total absence of $^{2}$H from the aldehydic position, and the very low labelling of **3** in general.

Although a small shift to shorter GC retention time was observed for the two methyl benzoate isotopomers containing a [$^{2}H_5$]phenyl ring (Fig. 4), co-elution prevented clean mass spectra of labelled and unlabelled benzoate from being recorded. To overcome this

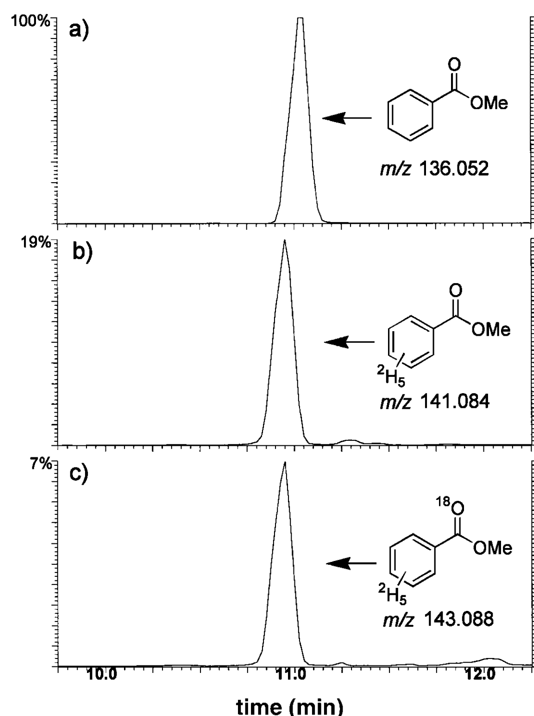


**Fig. 4a–c.** High resolution-selective ion monitoring (HR–SIM) chromatograms of the molecular ions of possible BA isotopomers (analysed as their methyl esters), extracted from cucumber plants following treatment with $[^2H_6,^{18}O]$-**1**. **a** Detection of unlabelled BA (methyl ester). Incorporation is clearly visible (**b, c**)

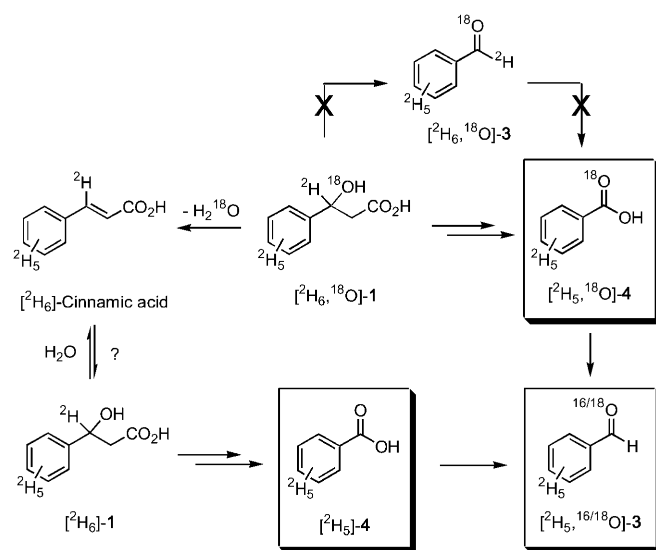


**Fig. 5.** The proposed metabolism of $[^2H_6,^{18}O]$-**1** in planta, explaining the absence of labelling in benzaldehyde (**3**) and the mixture of labelling in BA (**4**)

problem, product ion linked-scanning mass spectrometry was performed on the three individual molecular ions (*m*/*z* 136, 141 and 143) using high-energy (8 keV) collision-induced dissociation, with helium as the collision gas. Figure 6a shows the result for unlabelled methyl benzoate, with the molecular ion at *m*/*z* 136

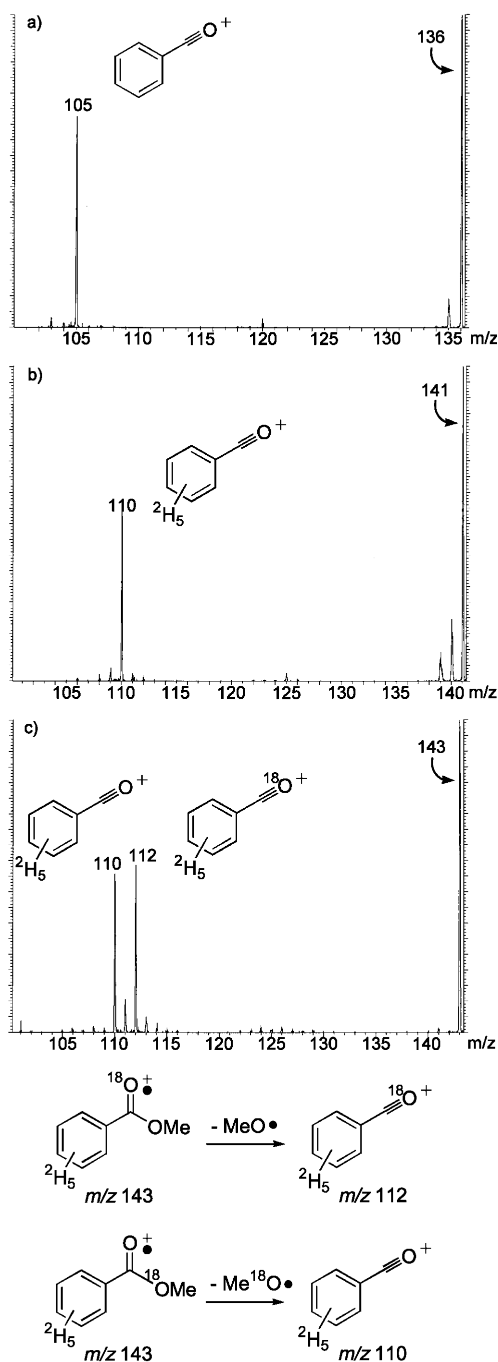


**Fig. 6a–c.** Product ion linked-scanning mass spectra (using high-energy collision-induced dissociation) of the molecular ions of (**a**) unlabelled methyl benzoate (*m*/*z* 136), (**b**) $[^2H_5]$methyl benzoate (*m*/*z* 141), and (**c**) $[^2H_5,^{18}O]$methyl benzoate (*m*/*z* 143). The fragmentation scheme below the mass spectra explains the presence of both *m*/*z* 112 and *m*/*z* 110 in (**c**)

fragmenting to give the benzoyl ion $[C_6H_5CO]^+$, at *m*/*z* 105, as the major species. An analogous result was obtained for $[^2H_5]$methyl benzoate (molecular ion at *m*/*z* 141), with the labelled benzoyl ion $[C_6{}^2H_5CO]^+$ appearing at *m*/*z* 110 (Fig. 6b). However, fragmentation of *m*/*z* 143, the molecular ion of $[^2H_5,^{18}O]$methyl benzoate, yielded a pair of benzoyl ions at *m*/*z* 112 and 110 (ratio 1:1, Fig. 6c). The former arises due to $^{18}O$-labelling on

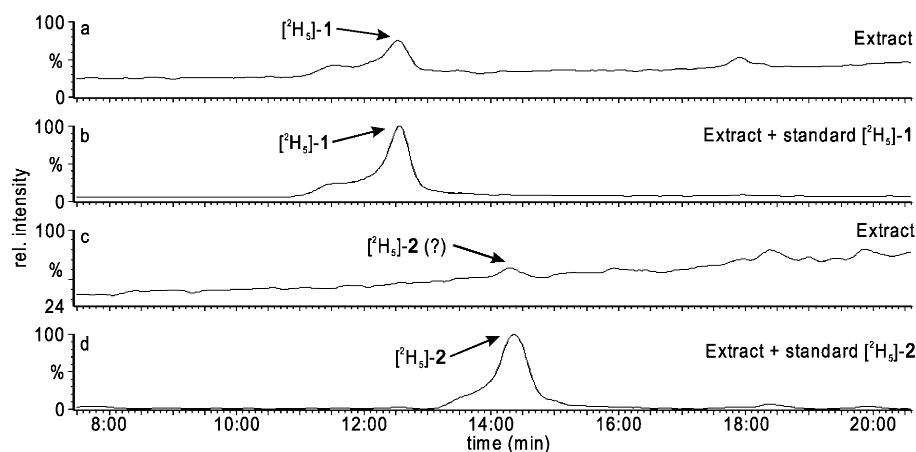


**Fig. 7a–d.** Analysis of tobacco extracts by RP-HPLC-MS following treatment with [$^2H_5$]phenylalanine. **a, b** Selective reaction monitoring chromatograms for the transition *m/z* 186 → *m/z* 154 (specific for [$^2H_5$]-**1**). **c, d** Selective reaction monitoring chromatograms for the transition *m/z* 170 → *m/z* 110 (specific for [$^2H_5$]-**2**)

the carbonyl oxygen of the ester functionality, giving $[C_6{}^2H_5C^{18}O]^+$. In contrast, the ion at *m/z* 110 is caused by the presence of $^{18}O$ labelling on the methoxy oxygen, which is lost in the fragmentation *m/z* 143 → *m/z* 110 (Fig. 6). Although Fig. 5 shows that the $^{18}O$ from [$^2H_6$,$^{18}O$]-**1** becomes the carbonyl oxygen of BA ([$^2H_5$,$^{18}O$]-**4**), spontaneous inter-conversion of the two carboxylate oxygens, in solution (through deprotonation), leads to the observed mixture. Thus, the result seen in Fig. 6c is to be expected.

### *Detection of 3-hydroxy- and 3-oxo-3-phenylpropanoic acid (1 and 2) as metabolites of phenylalanine*

While the previous experiments have demonstrated that 3-hydroxy-3-phenylpropanic acid (**1**) is converted to BA (and subsequently to SA, data not shown) by cucumber and *N. attenuata*, we also wished to determine whether **1** is a product of phenylalanine metabolism. Such a demonstration would provide further evidence to support the role of **1** in BA/SA biosynthesis, since phenylalanine is the key primary metabolite in the pathway (Fig. 1). To this end, ten young *N. attenuata* plants were treated with [$^2H_5$]phenylalanine for 48 h. Following extraction, using the same procedure employed for BA and SA (but without methylation), the free carboxylic acids (extraction conditions were sufficient to hydrolyse any CoA thioesters) were analysed by RP-HPLC-MS. An HPLC-based method was preferred over GC because of its ability to cope with the high levels of impurity in the sample. An atmospheric pressure chemical ionisation (APCI) source was employed, and both HPLC and MS conditions optimised using standard [$^2H_5$]-**1** and its keto analogue [$^2H_5$]-**2**. The standards were prepared by Friedel Crafts acylation of [$^2H_6$]benzene with ethyl 3-chloro-3-oxopropanoate, giving ethyl [$^2H_5$]-**2**. Reduction with $NaBH_4$ yielded ethyl [$^2H_5$]-**1**, and mild hydrolysis of the two esters with PLE produced the corresponding free acids. Because of the crude nature of the plant extract, and the expected low concentration of **1** and **2**, selective reaction monitoring (SRM) MS was used to provide highly specific detection of the acids (*m/z* 186 → *m/z* 154 for [$^2H_5$]-**1** and *m/z* 170 → *m/z* 110 for [$^2H_5$]-2). The resulting chromatograms are shown in Fig. 7. The extract sample gave a small but clear peak at the same retention time as that of authentic [$^2H_5$]-**1** (Fig. 7a), and co-injection of the standard confirmed the identification (Fig. 7b). Therefore, hydroxy acid **1** is, indeed, a metabolite of phenylalanine. In addition, a very small peak was detected that appeared to co-elute with authentic keto acid [$^2H_5$]-**2** (Fig. 7c extract, Fig. 7d co-injection with standard). Due to the low intensity of this signal, a conclusive identification could not be obtained (repeat experiments also failed to give absolute confirmation). However, this may represent detection of another crucial intermediate in the β-oxidation route (Fig. 1). As with the hydroxy acid **1**, such an intermediate would probably be present as a CoA thioester, prior to extraction.

*In summary*, 3-hydroxy-3-phenylpropanoate **1** has been shown to be a biosynthetic precursor of BA and SA, by treatment of cucumber and *N. attenuata* with a labelled probe. The lack of labelling in benzaldehyde, in particular the absence of $^{18}O$ and $^2H$ on C1, suggests that the aldehyde is not an intermediate between **1** and BA. Therefore, the "non-oxidative route" (Fig. 1) would not appear to function in the biosynthesis of BA and SA, and these metabolites are most likely produced by a β-oxidation type degradation (Fig. 1). Our observation that the hydroxy acid is a product of phenylalanine metabolism, as well as a precursor of BA and SA, provides further evidence for the role of **1** in the pathway. The tentative detection of keto acid **2** also supports the existence of a β-oxidation type pathway. However, since the analogous degradation of 4-hydroxycinnamic acid to 4-hydroxybenzoic acid is known to proceed via 4-hydroxybenzaldehyde (Yazaki et al. 1991; Schnitzler et al. 1992; Mitra et al. 1999), it seems that at least two mechanisms of side-chain shortening exist in the phenylpropanoid pathway in plants – determined, perhaps, by substitution (hydroxylation) of the phenyl ring.

Work is currently in progress to investigate this pathway further, and to determine the absolute configuration of natural **1**.

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