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PRODUCTION OF HYDROXYPHENYLETHANOL GLYCOSIDES IN SUSPENSION CULTURES OF *SYRINGA VULGARIS*

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Key Word Index—*Syringa vulgaris*; Oleaceae; lilac; cell cultures; biosynthesis; hydroxyphenylethanol glycosides; salidroside; verbascoside; caffeic acid.

Abstract—Cell suspension cultures of *Syringa vulgaris* accumulate up to 16% of their dry wt as a mixture of hydroxyphenylethanol glycosides. The main component is the caffeoyl ester, verbascoside (acteoside). Tyrosine and tyramine are efficient biosynthetic precursors of the 4-hydroxy- and 3,4-dihydroxyphenylethanol moieties of these glycosides.

INTRODUCTION

Ester and amide derivatives of caffeic acid occur widely in the plant kingdom [1, 2]. They are believed to be part of the chemical defence mechanisms used by plants to resist pathogen attack, particularly in the hypersensitive response [3], and have been shown to be capable of inactivating virus particles [4], perhaps by oxidative coupling to viral coat proteins [5]. Despite the importance of these compounds in the biochemistry of plant cells, relatively little is known about their formation, storage and mobilization. For such metabolic studies, *in vitro* cultured cells offer many advantages over intact plants and we have, therefore, established cultures of several species reported to contain various caffeoyl derivatives. Cultures of *Syringa* species have proven to accumulate large amounts of hydroxyphenylethanol glycosides, dominated by a group of caffeoyl ester derivatives.

RESULTS AND DISCUSSION

Juvenile stem explants of *Syringa vulgaris L.*, *S. lacinata*, *S. meyeri*, *S. tomentosa*, *S. amurensis*, *S. patula* and *S. reticulata* all rapidly gave rise to undifferentiated light-brown callus on B5 medium. TLC screening of soluble phenolics showed that calli of all species were producing qualitatively similar patterns of phenolic metabolites and the *S. vulgaris* cultures were, therefore, selected for further study. Suspension cultures of *S. vulgaris* displayed the growth and verbascoside production patterns shown in Fig. 1.

Cells harvested after 10 days growth and extracted with 70% ethanol yielded a mixture of phenolic glycosides which were separated into 'catechol' and 'non-catechol' fractions by lead salt precipitation. The major 'non-catechol' glycoside was purified by CC on polyamide-6 powder and crystallized. The mp, spectral and chromatographic properties of the glucoside were all identical to those of salidroside (**1**), a compound previously reported from several plant sources, including *S. vulgaris* [6–8].

The 'catechols' fraction contained at least four compounds which fluoresced light-blue under long wave-

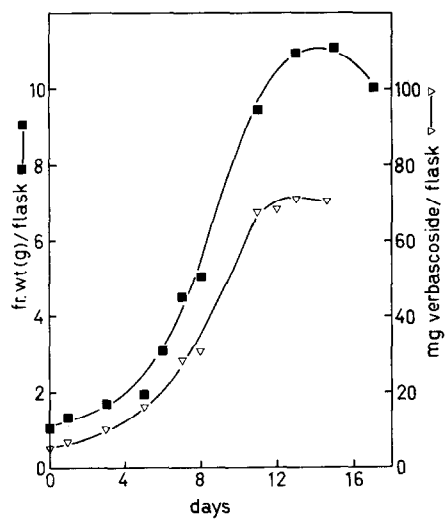
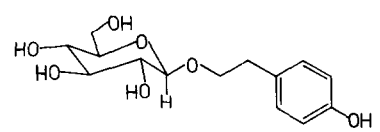


Fig. 1. Culture growth and verbascoside production. The contents of duplicate 250 ml (55 ml medium) flasks were harvested by filtration on the days indicated. The tissue fr. wt and glycoside content were determined as in the Experimental.



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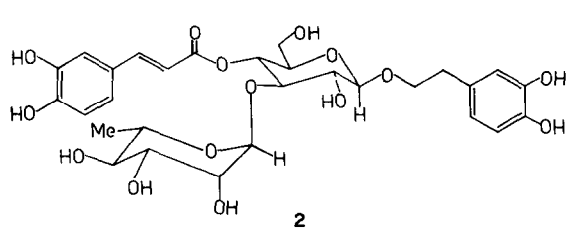

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length UV (yellow with ammonia). The main component (> 95% of the mixture) was purified by polyamide-6 CC, PC and gel permeation CC but could not be crystallized. Base hydrolysis yielded caffeic acid and an unconjugated catechol glycoside; total acid hydrolysis yielded equimolar glucose and rhamnose plus caffeic acid and a water-soluble unconjugated catechol derivative. Prior methylation of the intact glycoside allowed a better post-hydrolysis recovery of the catechol moiety, which was isolated as 3,4-dimethoxyphenylethanol. These data were consistent with the structure proposed for verbascoside (acteoside) (2), a caffeoyl ester previously isolated from *S. vulgaris* blossoms [6], *Conandrum ramoidioides* [9], *Clerodendron myricoides* [10], *Verbascum sinuatum* [11] and *Orobanche rapum-genistae* [12]. The chromatographic and spectral properties of the culture-derived glycoside were identical both to those reported for verbascoside [6, 12] and to those of the main caffeoyl derivative detected in *S. vulgaris* blossoms in our laboratory.

Caffeoyl esters of this type have been found in a number of taxonomically related species [2] but have not previously been reported from cultured plant cells. *S. vulgaris* suspension cultures synthesize large quantities of verbascoside (12–16% of cell dry wt) (Fig. 1) and this production is independent of the stage of growth of the culture. Salidroside accumulation parallels that of verbascoside at a molar ratio of *ca* 1:4 (data not shown). The level and pattern of production of verbascoside are very similar to those reported for another caffeoyl ester, rosmarinic acid, in *Coleus blumei* suspension cultures [13, 14].

Radiotracer studies

The biosynthesis of the 4-hydroxyphenylethanol (tyrosol) and 3,4-dihydroxyphenylethanol moieties of salidroside and verbascoside was examined by supplying potential radioactive precursors to linear-phase suspension cultures (Table 1). While [^{14}C]phenylalanine was efficiently incorporated into the caffeoyl moiety of verbascoside it was not an efficient precursor of either hydroxyphenylethanol unit. [^{14}C]Tyrosine and [^{14}C]tyramine, however, were both efficient precursors of tyrosol and 3,4-dihydroxyphenylethanol (Table 1). These results are not necessarily inconsistent with the reported conversion of *p*-hydroxyphenylacetaldehyde oxime to tyrosol in *Aubretia* spp. [15] since, in that case, the oxime was proposed to be hydrolysed to the corresponding aldehyde before reduction to the primary alcohol. Oxidation of tyramine to *p*-hydroxyphenylacetaldehyde in the *Syringa* cells is also a plausible sequence and we have been able to detect the latter reaction when tyramine is incubated with crude extracts of *S. vulgaris* cultured cells [Ellis, B. E., unpublished observations]. In rose petals, on the other hand, phenylethanol has been reported to be synthesized from phenylalanine via cinnamic acid [16].

The dihydroxy precursors DOPA and dopamine were much less efficiently incorporated into verbascoside than were the corresponding monohydroxy compounds. This might indicate that 3-hydroxylation is normally preceded by both decarboxylation and deamination, but intracellular compartmentation could also be a factor. Labelled tyrosol and salidroside administered to the cells were only poorly incorporated into verbascoside, although label could be recovered in the salidroside fraction in each case. Simple phenols and phenolic glycosides supplied exogenously can be metabolized in an artefactual manner, however, and thus diverted from the normal *in vivo* reaction sequence. Determination of the reaction network by which phenylalanine, tyrosine, glucose and rhamnose are ultimately converted to verbascoside will, therefore, probably require isolation of the appropriate enzymes and analysis of their substrate preferences.

EXPERIMENTAL

Cultures. Surface-sterilized juvenile stem segments (University of Guelph Arboretum) were set out on B5 medium [17] (0.1 mg/l. 2,4-dichlorophenoxyacetic acid; 0.1 mg/l. kinetin). Calli were transferred to liquid medium to initiate suspension cultures. Cultures established in Münster, West Germany, produced results similar to those from the University of Guelph lines.

Chemicals. L-[U-^{14}C]Phenylalanine, L-[U-^{14}C]tyrosine, [2-^{14}C]tyramine, [2-^{14}C]dopamine and L-[3-^{14}C]DOPA were obtained from Amersham, Toronto. Labelled DOPA and dopamine were purified by PC, *n*-BuOH–EtOH–H_2O (4:1:1), immediately before use. [^{14}C]Salidroside was produced by incubation of *Syringa vulgaris* cells with [2-^{14}C]tyramine for 4 hr followed by extraction and purification of the labelled glucoside. [^{14}C]Tyrosol was produced by incubation of [2-^{14}C]tyramine (10 Ci; 0.5 mol) and $NADPH_2$ (2 mol) with a crude protein extract of *S. vulgaris* cells (0.05 M KH_2PO_4, pH 7.5, 0.5 ml) for 3 hr at 30°. Et_2O extraction of the acidified reaction mixture

Table 1. Incorporation of aromatic substrates into salidroside and verbascoside during a 4 hr incubation with *S. vulgaris* suspension cultures

Substrate administered	Sp. act. of purified product (dpm/μmol)		
	Salidroside	Verbascoside	Caffeoyl moiety of verbascoside
L-[U-^{14}C]Phenylalanine (0.1 μmol; 0.67 μCi)	trace	1.1×10^4	1.1×10^4
L-[U-^{14}C]Tyrosine (0.1 μmol; 0.5 μCi)	5.9×10^4	1.5×10^4	—
[2-^{14}C]Tyramine (0.1 μmol; 0.5 μCi)	4.8×10^4	1.1×10^4	N.D.
[2-^{14}C]Dopamine (0.1 μmol; 0.5 μCi)	—	2.4×10^3	N.D.
L-[3-^{14}C]DOPA (0.1 μmol; 0.5 μCi)	—	6.4×10^2	N.D.
[^{14}C]Salidroside (0.3 μmol; 1 μCi)	1.4×10^4	3.1×10^2	N.D.
[^{14}C]Tyrosol (0.04 μmol; 0.13 μCi)	3.7×10^4	9.2×10^2	N.D.

N.D., Not determined.

yielded [^{14}C]tyrosol which was purified by SG-TLC, $CHCl_3$–MeOH (9:1).

Growth measurements. Suspension cultures were vacuum filtered on Miracloth discs (Calbiochem) and the tissue fr. wt measured. The cells were lyophilized to determine dry wt and then extracted 30 min with boiling 70% EtOH. The A of the filtered extract at 330 nm was measured and the verbascoside concn calculated using log $\varepsilon_{330} = 4.28$ [6].

Isolation of verbascoside and salidroside from S. vulgaris *suspension cultures.* Lyophilized cells (90 g) were refluxed in 70% EtOH for 2 hr. The concd, filtered extract was treated with 20% aq. PbOAc until no further ppt appeared. The Pb salt ppt was washed with H_2O and 70% EtOH, resuspended in H_2O and decomposed with H_2S. After removal of PbS by filtration, the filtrate was concd *in vacuo* to 10 ml ('catechols fraction'). The supernatant from the original Pb salt ppt was treated with H_2S to remove excess Pb ions, filtered and concd to *ca* 10 ml *in vacuo* ('non-catechols fraction').

The 'catechols fraction' was fractionated on a column (30 × 5 cm) of polyamide-6 powder (Woelm) using a step gradient of EtOH in H_2O. The main ester was eluted with 50% EtOH, further purified by PC, *n*-BuOH–EtOH–H_2O (4:1:1), and CC on a column (30 × 1.5 cm) of Biogel P-2, eluting with H_2O. The purified product would not crystallize from H_2O or EtOH and was ultimately lyophilized to yield a white amorphous solid (λ_{max}^{EtOH} nm: 330, 285 sh). Base hydrolysis (1 M NaOH under N_2, 18 hr) followed by acidification and Et_2O extraction yielded caffeic acid, identified by chromatographic and spectral comparison with the authentic compound. The aq. phase contained one phenolic compound ($\lambda_{max}^{H_2O}$ nm: 285) which reacted with $FeCl_3$ (black) and diazotized *p*-nitroaniline (yellow) oversprayed with 1 M NaOH (sky blue).

Acid hydrolysis (1 M HCl, 100°, 1 hr) of the purified ester followed by exhaustive Et_2O extraction yielded caffeic acid plus small amounts of an unconjugated catechol compound (colour reactions and UV spectrum as above). The aq. phase contained glucose and rhamnose, identified by chromatographic comparison with the standard compounds. Their relative amounts were determined according to ref. [18].

After methylation of the purified ester, acid hydrolysis and Et_2O extraction yielded 3,4-dimethoxycinnamic acid and 3,4-dimethoxyphenylethanol (UV, MS, TLC comparison with the authentic compound prepared by $LiAlH_4$ reduction of 3,4-dimethoxyphenylacetic acid).

The ^{1}H and ^{13}C NMR spectra (400 MHz; D_2O) of the purified ester correspond very well with lit. data for verbascoside [12].

The 'non-catechols fraction' was eluted from a 30 × 5 cm column of polyamide-6 powder with H_2O. The main component absorbing at 280 nm was free of contaminants as judged by TLC and was crystallized from *n*-PrOH (mp 155–157°, lit. 159–160° [19] $\lambda_{max}^{H_2O}$ nm: 272). Acid (1 M HCl, 100°, 1 hr) or enzymic (glucosidase, 0.01 M NH_4OAc pH 6.5, 25°, 18 hr) hydrolysis yielded glucose and 4-hydroxyphenylethanol (TLC, MS, UV comparison with the authentic compound produced by $LiAlH_4$ reduction of 4-hydroxyphenylacetic acid).

Biosynthetic studies. Radioactive substrate dissolved in 0.2 ml H_2O was added to 10 ml suspension culture and shaken (120 rpm) at 25° for 4 hr. The cells were removed by filtration and extracted with boiling 70% EtOH. The concd (*ca* 3 ml) extract was loaded onto a 1 × 15 cm column of polyamide-6 powder. Elution with 50 ml H_2O removed salidroside and subsequent elution with 50 ml 50% EtOH removed verbascoside. These compounds were further purified by PC, *n*-BuOH–EtOH–H_2O (4:1:1); 10% HOAc; *n*-BuOH–HOAc–H_2O (4:1:2.2). Molar concns were determined by UV spectrophotometry and radioactivity by liquid scintillation counting. Where necessary, verbascoside samples were hydrolysed with 1 M NaOH (N_2, 18 hr), acidified and extracted with Et_2O to recover the caffeic acid moiety. The latter was purified by cell. TLC, *n*-BuOH–HOAc–H_2O (4:1:2.2).

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