Biosynthetic Capacity of *Stachys* Seedlings for Verbascoside and Related Caffeoyl Derivatives

Claude Andary

Laboratoire de Botanique et Cryptogamie, Faculté de Pharmacie, 34060 Montpellier Cedex, France

Ragai K. Ibrahim

Department of Biology, Concordia University, 1455, De Maisonneuve Blvd. West, Montreal, Quebec, Canada H3G 1M8

Z. Naturforsch. **41c,** 18–21 (1986); received June 4/August 8, 1985

Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Stachys, Labiatae, Verbascoside, Stachyoside, Caffeoyl Derivatives

The caffeoyl derivatives of *Stachys* leaves (Labiateae) were identified as chlorogenic acid, verbascoside and stachyoside. The latter compound, reported here for the fist time, was shown to be a verbascoside derivative containing an extra glucose residue attached to C-2, C-3 or C-4 of rhamnose.

Cotyledonary leaves of *Stachys* accumulate high levels (8–16 μmol/g dry tissue) of the three caffeoyl derivatives; all of which decrease significantly in amount during plant growth. Intact organs of the seedling were shown to efficiently incorporate the label from different precursors into caffeoyl derivatives. The biosynthesis of both verbascoside and stachyoside from labelled precursors revealed that the caffeoyl moiety was exclusively labelled from phenylalanine or cinnamic acid, whereas the 3,4-dihydroxyphenylethanol moiety was labelled from tyrosine, and better from tyramine.

Introduction

Verbascoside, poliumoside and other related caffeoyl derivatives are well represented in the order Tubiflorae, especially the Labiatae [1–4]. These compounds have been used as taxonomic markers [4, 5] and are known for their pharmacological activity [6]. They are characterized by having caffeoyl and 3′,4′-dihydroxyphenylethyl moieties, both of which are linked to β-D-glucose by ester (C-4) and glucosidic (C-1) linkages, respectively (Fig. 1). Other sugars, such as rhamnose, may be attached to C-2, C-3 or C-6 of the glucosyl residue, as in other caffeoyl derivatives [3, 7]. The complete structure of verbascoside has recently been elucidated [7] and methods for its hydrolysis and identification of the hydrolytic products have been developed [1, 8, 9].

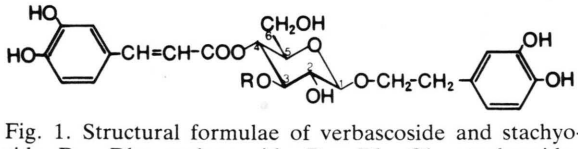

Fig. 1. Structural formulae of verbascoside and stachyoside. R = Rha, verbascoside; R = Rha-Glu, stachyoside.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0100–0018 $ 01.30/0

Despite the accumulation of caffeoyl derivatives in high concentration in various tissues, relatively little is known of their biosynthesis *in vivo,* or their mobilization within the plant. Very recently, Ellis [10] reported on the production and biosynthesis of verbascoside and hydroxyphenylethyl glycosides in cell cultures of *Syringa vulgaris.* Although *in vitro* cultured tissues have been successfully used in some metabolic studies [11], they may not reflect the actual metabolic state occurring *in vivo.* Intact plants, on the other hand, offer the advantage to study the capacity of different organs for the biosynthesis and translocation of these significant metabolites.

We wish to report in this paper on the accumulation of verbascoside and related compounds in different organs of *Stachys* seedlings and their ability to synthesize these compounds *in vivo* from labelled phenolic precursors. Furthermore, a new caffeoyl derivative of *Stachys* has been isolated and identified as stachyoside.

Materials and Methods

Plant material

Seeds of *Stachys albens* Gray, *S. rigida* Nutt. Ex. Benth. and *S. lanata* Jacq. were obtained from the Biosystematics Research Institute, Ottawa. They

were germinated in one cm-thick layer of vermiculite on top of potting soil, under glass-house conditions.

Chemicals

L-[U-^{14}C]phenylalanine, L-[U-^{14}C]tyrosine, *trans*-[2-^{14}C]cinnamic acid and [2-^{14}C]tyramine were purchased from Amersham, Oakville, Ontario. Stachyoside was isolated from *S. lanata* using the method reported for poliumoside [3]. Verbascoside and its hydrolysis products were from our laboratory collection [1, 7].

Labelling experiments

Excised cotyledons, hypocotyls or roots were vacuum infiltrated, then floated on aqueous solutions of the labelled precursors for four hours at room temperature. Expanded leaves near the terminal bud were administered the label through the petiole and water was added when necessary. The tissues were successively washed with water before extraction with 95% MeOH and concentration to a final volume of 0.5 ml.

Chromatography and identification of metabolites

The methanolic extracts were chromatographed on silica or cellulose TLC plates in EtOAc-HCOOH-H_2O (10:2:3, v/v/v) or 2% aqueous HOAc, respectively. The different caffeoyl derivatives were characterized by their fluorescence in ultraviolet light (366 nm), their colour with diazotized *p*-nitroaniline [1] and Neu's (2-aminoethyl diphenyl borinate) [12] reagents, cochromatography with authentic samples [3, 7] and finally, by autoradiography on X-ray film. The specific activities of the labelled metabolites were determined by measuring their absorbance at 330 nm, followed by liquid scintillation counting. Absorbance values were transformed to nmoles using previously prepared calibration curves [9]. The estimation of caffeoyl derivatives during growth of *S. albens* was carried out on the methanolic extracts of cotyledonary leaves and leaves obtained from 4-, 8- and 16-leaf growth stage. The methanolic extracts were spotted quantitatively on cellulose HPTLC plates in parallel with different concentrations of caffeoyl derivatives. Individual compounds were quantified by a direct spectrofluorometric method [9].

The incorporation of label from different precursors into either of the phenolic moieties of verbascoside (Fig. 1) was determined by acid hydrolysis of the latter and chromatography of the hydrolysis products on silica gel HPTLC plates (Merck, Darmstadt) in EtOAc-HCOOH-H_2O (10:2:3, v/v/v) [8]. The hydrolysis products were identified as previously described [1, 8] before being autoradiographed.

Results and Discussion

Identification of the caffeoyl derivatives of Stachys spp.

The pattern of caffeoyl derivatives of the three species examined, *S. albens, S. rigida* and *S. lanata,* was found to be qualitatively similar. It consisted of three major phenolic constituents: verbascoside, chlorogenic acid and another caffeoyl derivative which seemed related to verbascoside. The two former compounds were identified by their UV absorption spectra, fluorescence in UV light (366 nm) before and after treatment with Neu's reagent [1], R_f values in three solvent systems (Table I) and cochromatography with reference compounds.

The other caffeoyl derivative was characterized by total acid hydrolysis which yielded equimolar caffeic acid, 3,4-dihydroxyphenylethanol, rhamnose and two molecules of glucose. Partial and micro-scale acid hydrolysis was carried out for different time periods (5–30 min) on silica gel HPTLC [8], in parallel with verbascoside, and using the intermediate

Table I. Characteristics of the caffeoyl derivatives of *Stachys*.

Caffeoyl derivative	UV maxima [nm]	R_f values[a] in solvents 1	2	3	Fluorescence in UV light (+ Neu's reagent)[b]
Chlorogenic acid	(300), 331	0.48	0.65	0.95	blue
Verbascoside	(293), 333	0.62	0.70	0.67	yellowish
Stachyoside	(293), 332	0.70	0.52	0.75	yellowish

[a] Solvent 1, 2% aq. HOAc on cellulose TLC; solvent 2, EtOAc-HCOOH-H_2O (10:2:3) on silica gel TLC; solvent 3, 0.05% aq. HOAc on cellulose TLC.
[b] 2-Aminoethyl diphenyl borinate.

hydrolytic fragments of the latter as reference compounds. The fact that this caffeoyl derivative yielded verbascoside and glucose after 5-min hydrolysis, suggests that the latter sugar is attached to C-2, C-3 or C-4 of rhamnose and was identified as stachyoside (Fig. 1). The presence of an extra glucose residue conforms with its polarity in three solvent systems (Table I). The complete structural elucidation of this new compound will be reported elsewhere.

Accumulation of caffeoyl derivatives during growth of S. albens

The results (Table II) show that the cotyledonary leaves accumulate high levels of the three caffeoyl derivatives: chlorogenic acid, verbascoside and stachyoside. However, there was a significant decrease in all constituents with increasing age of *S. albens*, suggesting their translocation to other organs and/or their turn-over within the plant.

Table II. Accumulation of caffeoyl derivatives during growth of *S. albens*.

Growth stage	Metabolite concentration [μmol/g dry tissue][a]		
	Chlorogenic acid	Verbascoside	Stachyoside
Cotyledons	16.0	12.7	8.0
4-leaf stage	8.6	4.2	0.63
8-leaf stage	6.0	3.5	0.8
16-leaf stage	6.2	3.6	1.0

[a] Determined as described in the Methods section.

Biosynthetic capacity of different organs of Stachys seedlings

Seedlings of *S. albens* and *S. rigida* were separated to their component organs and administered [2-^{14}C]cinnamic acid and [2-^{14}C]tyramine at 20 °C for 3–4 h. The results (Table III) show that intact organs of the seedling can incorporate the label of either precursor into verbascoside with almost equal efficiency; thus indicating their capacity for the biosynthesis of caffeoyl derivatives. Furthermore, the incorporation of label of cinnamic acid into verbascoside increased 4–10-fold with growth of the root, hypocotyl or cotyledon of *S. albens* (Table III).

Biosynthesis of caffeoyl derivatives from phenolic precursors

The biosynthesis of caffeic acid and 3,4-dihydroxyphenylethanol moieties was examined in young leaves of *S. albens* which were administered labelled precursors. Both radioactive verbascoside and stachyoside were further hydrolysed [8] and autoradiographed in order to localize the label in either phenolic moiety. The results (Table IV) show that all phenolic precursors used were efficiently incorporated into both verbascoside and stachyoside. However, tyramine was the most efficient precursor of both metabolites, possibly due to its better uptake, as compared with either phenylalanine or tyrosine. Furthermore, these two amino acids are also utilized in protein synthesis. It is interesting to note that the specific activity of chlorogenic acid was usually lower

Table III. Incorporation of [2-^{14}C]cinnamic acid and [2-^{14}C]tyramine into verbascoside by different organs of *Stachys* seedlings.

Stachys sp. (age)	Substrate	Specific activity of verbascoside[a] [dpm/μmol]			
		Root	Hypocotyl	Cotyledon	Bud
S. albens (10 days old)	[2-^{14}C]cinnamic acid (2 μCi, 0.67 μmol)	7.1×10^4	1.2×10^4	4.6×10^4	–[b]
S. albens (15 days old)	[2-^{14}C]cinnamic acid (3 μCi, 1.0 μmol)	4.1×10^5	1.9×10^5	3.8×10^5	2.5×10^5
S. rigida (15 days old)	[2-^{14}C]tyramine (5 μCi, 1.5 μmol)	1.8×10^5	–[c]	3.8×10^5	2.9×10^5

[a] After isolation and activity determination as described in the Methods section.
[b] Terminal bud not yet developed.
[c] No significant hypocotyl growth.

Table IV. Incorporation of phenolic precursors into caffeoyl derivatives of *S. albens* leaves.

Precursor	Specific activity [dpm/μmol][a] in		
	Chlorogenic acid	Verbascoside	Stachyoside
[2-^{14}C]cinnamic acid (2 μCi, 0.67 μmol)	5.6×10^4	7.2×10^4	1.43×10^5
L-[U-^{14}C]phenylalanine (2 μCi, 0.2 μmol)	3.6×10^4	4.7×10^4	7.95×10^4
L-[U-^{14}C]tyrosine (2 μCi, 0.2 μmol)	–	2.5×10^4	3.77×10^4
[2-^{14}C]tyramine (2 μCi, 1.6 μmol)	–	1.3×10^5	2.45×10^5

[a] After isolation and activity determination as described in the Methods section.

than that of verbascoside or stachyoside. This seems to suggest that the two latter compounds derive their caffeoyl moiety from a different metabolic pool than that of chlorogenic acid.

The fact that the specific activity of stachyoside was consistently higher than that of verbascoside (Table IV) indicates that the former compound is an end metabolite and suggests that further glucosylation of the latter to stachyoside is a final step in the biosynthetic sequence.

Examination of the hydrolysis products of both verbascoside and stachyoside revealed that the caffeoyl moiety was, as expected, exclusively labelled from phenylalanine or cinnamic acid, whereas the 3,4-dihydroxyphenylethanol moiety was labelled from tyrosine, and better from tyramine. These results are consistent with those reported for verbascoside in *Syringa vulgaris* cell culture [10], except for the higher specific activity obtained with intact tissues of *Stachys*.

Apart from the enzymatic steps leading to the formation of the caffeoyl moiety [13], nothing is known of the enzymes involved in the biosynthesis of verbascoside or stachyoside. Their isolation, characterization and establishing the sequence of assemblage of these molecules should be a challenging problem.

Acknowledgements

This work was supported in part by operating grants (to RKI) from the Natural Sciences and Engineering Research Council of Canada and the Department of Education, Government of Quebec, for which we are grateful. Part of this work was carried out in the context of a France-Quebec scientific exchange. We wish to thank Mr. Derek Monro for the generous gift of seeds of *Stachys spp.*

[1] C. Andary, Thèse Doct. (Etat) Pharm., Montpellier, France (1975).
[2] O. Sticher and M. F. Lahloub, Planta Med. **45,** 157 (1982).
[3] C. Andary, R. Wylde, A. Heitz, J. P. Rascol, and J. L. Roussel, Phytochemistry **24,** 362 (1985).
[4] J. B. Harborne, Z. Naturforsch. **21b,** 604 (1966).
[5] C. Andary, J. P. Rascol, S. Puech, J. L. Roussel, and G. Privat, Can. J. Botany, in press.
[6] C. Andary, G. Privat, P. Chevallet, H. Orzalesi, J. J. Serrano, and M. Boucard, Il Farmaco **35,** 1 (1980).
[7] C. Andary, R. Wylde, C. Laffite, G. Privat, and F. Winternitz, Phytochemistry **21,** 1123 (1982).
[8] C. Andary, J. L. Roussel, J. P. Rascol, and G. Privat, J. Chromatogr. **303,** 312 (1984).
[9] C. Andary, J. Pellecuer, I. Soediro, and G. Privat, Pharm. Mediter. **12,** 237 (1978).
[10] B. Ellis, Phytochemistry **22,** 1941 (1983).
[11] E. J. Staba, in: Plant Tissue Culture as a Source of Biochemicals (E. J. Staba, ed.), p. 59, CRC Press (1980).
[12] R. Neu, Naturwissenschaften **43,** 82 (1956).
[13] G. G. Gross, in: The Biochemistry of Plants, **Vol. 7** (E. E. Conn, ed.), p. 301, Academic Press, New York 1981.