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BIOSYNTHESIS OF THE COUMARINS—V.
PATHWAYS OF UMBELLIFERONE FORMATION IN *HYDRANGEA MACROPHYLLA**

STEWART A. BROWN

Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan

G. H. N. TOWERS† and D. CHEN‡

Department of Botany, McGill University, Montreal, Quebec, Canada

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Abstract—^{14}C-labelled *p*-coumaric and *o*-coumaric acids, when administered to cut shoots of *Hydrangea macrophylla* Ser., were both converted with good efficiency to umbelliferone. No randomization of ^{14}C could be detected when the umbelliferone formed from *o*-coumaric acid was chemically degraded. Trapping experiments showed that both coumaric acids can be converted to umbelliferone via umbellic (2,4-dihydroxycinnamic) acid, which is itself an extremely efficient umbelliferone precursor when fed in labelled form. When cinnamic acid-β-^{14}C was administered to *H. macrophylla* with non-radioactive *o*-coumaric acid, no ^{14}C could be detected in the recovered *o*-coumaric acid, although *p*-coumaric acid, umbelliferone, and hydrangetin (7-hydroxy-8-methoxycoumarin) were radioactive. The results establish the existence of a pathway: cinnamic acid→*p*-coumaric acid→umbellic acid→umbelliferone in *Hydrangea*, but the normal participation of *o*-coumaric acid in umbelliferone biosynthesis cannot be considered proved. The results of one experiment indicate that cinnamic and *p*-coumaric acids are also precursors of hydrangetin.

IN THE preceding paper of this series[1] evidence from tracer studies was presented which showed that herniarin (7-methoxycoumarin) can be synthesized by lavender plants from *p*-coumaric acid whereas *o*-coumaric acid-^{14}C was not utilized as a precursor to any significant degree. These results were in harmony with an earlier proposal[2] that plants normally synthesize 7-hydroxylated coumarins from *p*-coumaric acid, and coumarin itself from *o*-coumaric acid, cinnamic acid being a common precursor.

It was also demonstrated that highly efficient conversion of labelled *p*-methoxycinnamic acid could be carried out by lavender plants, but that umbellic (2,4-dihydroxycinnamic) acid, although utilized with fair efficiency, was a much poorer precursor than was *p*-methoxycinnamic. On the basis of this evidence, the major biosynthetic pathway was inferred to be:

Cinnamic acid → *p*-Coumaric acid → *p*-Methoxycinnamic acid → → Herniarin

In more recent studies,[3] 2-hydroxy-4-methoxycinnamic acid-^{14}C has also been shown to be a very efficient herniarin precursor. The above studies are most reasonably interpreted on the

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† Present address: Atlantic Regional Laboratory, National Research Council, Halifax, Nova Scotia, Canada.

‡ Present address: University of Connecticut, Storrs, Connecticut, U.S.A.

[1] S. A. BROWN, *Phytochemistry*, **2**, 137 (1963).

[2] S. A. BROWN, *Z. Naturforsch.* **15b**, 768 (1960).

[3] S. A. BROWN, *Abstracts*, 3rd Annual Meeting, Plant Phenolics Group of North America, Toronto, 1963, p. 17.

assumption that the lavender enzyme which introduces the *ortho* hydroxyl substituent is more active on a substrate in which the *para* hydroxyl is methylated rather than free.

A simpler case is that of the widely distributed umbelliferone (7-hydroxycoumarin) in which the complication of an O-methyl group is absent. It appeared probable that umbellic acid would be efficiently utilized for umbelliferone synthesis. Since previous work[4] on *Hydrangea macrophylla* Ser. had established the occurrence of umbelliferone in this species, it was chosen for biosynthetic experiments. The presence of 7-hydroxy-8-methoxycoumarin, for which the trivial name hydrangetin is now proposed, offered the possibility of investigating the formation of this compound simultaneously.

Isotope Competition and Trapping Experiments with Cinnamic Acid-^{14}C

Preliminary studies were made in which cinnamic acid-β-^{14}C was administered to *H. macrophylla* shoots simultaneously with a non-radioactive compound; *p*-coumaric, *o*-coumaric, ferulic, or caffeic acids, or coumarin. After administration, various compounds were isolated by paper chromatography of the plant extracts. *p*-Coumaric acid, umbelliferone, and hydrangetin were radioactive after each feeding. *o*-Coumaric acid, on the other hand, could not be detected either by radioautography, fluorescence, or colour reaction on any chromatograms except in the case where *o*-coumaric acid had been fed. Here, although an *o*-coumaric acid spot was easily detected, no radioactivity was found by radioautography. Trouble encountered with badly streaked chromatograms, which made interpretation of some results uncertain, led to the abandonment of this approach in favour of direct comparisons of ^{14}C-labelled potential precursors. However, the conversion of cinnamic acid-β-^{14}C to umbelliferone while *o*-coumaric acid remained non-radioactive was in accord with the fact that *p*-coumaric, but not *o*-coumaric acid, was involved in the biosynthesis of lavender herniarin.[1]

Comparisons of Labelled Compounds as Precursors

Table 1 shows the results of an experiment in which labelled cinnamic acid, *p*-coumaric acid, and coumarin were compared as precursors of umbelliferone and hydrangetin. Umbelliferone was readily formed from both cinnamic and *p*-coumaric acids, but because the dose

TABLE 1. FORMATION OF UMBELLIFERONE AND HYDRANGETIN FROM ^{14}C-LABELLED PHENYLPROPANOID PRECURSORS

Compound administered	Specific activity (μc/mmole)	Amount administered* (mmoles)	Compound isolated†	Specific activity (μc/mmole)	Dilution value
Coumarin-2-^{14}C	120	$3{\cdot}3\times10^{-2}$	U	0·16	750
			H	0·19	620
p-Coumaric acid-α-^{14}C	68	$5{\cdot}9\times10^{-2}$	U	5·2	13
			H	0·92	74
Cinnamic acid-β-^{14}C	905	$3{\cdot}6\times10^{-3}$	U	23·7	38
			H	11·5	79

* The extracted dry weight of the plant in these experiments was roughly 4 g.
† U: Umbelliferone, H: Hydrangetin.

ratios (μmoles/g extracted dry weight) differed by about one order of magnitude for these two precursors, the dilution values (38 and 13 respectively) are not quantitatively comparable.

[4] B. A. BOHM, R. K. IBRAHIM and G. H. N. TOWERS, *Can. J. Biochem. Physiol.* **39**, 1389 (1961).

There was a measurable incorporation of ^{14}C from coumarin, but the dilution was almost 60 times that of *p*-coumaric acid, so that direct hydroxylation of coumarin does not appear to be a major pathway in umbelliferone formation. The pattern of utilization in the biosynthesis of hydrangetin was similar. The specific activity of the umbelliferone formed from cinnamic or *p*-coumaric acid was 2 and 6 times as high, respectively, as that of the hydrangetin formed by the same plant. These findings are in keeping with the idea that umbelliferone can be converted to hydrangetin in *H. macrophylla*, and confirm an earlier direct feeding experiment.[4]

The results of a second series of tracer experiments (Expts. 1, 2 and 3) are collected in

TABLE 2. INCORPORATION OF *o*-COUMARIC, *p*-COUMARIC, AND UMBELLIC ACIDS INTO UMBELLIFERONE BY *Hydrangea macrophylla*

				Umbelliferone isolated	
Expt.*	Compound administered	Specific activity (μc/mmole)	Dose (μmole/g extd. dry wt.)	Specific activity (μc/mmole)	^{14}C-Dilution
1.	*o*-Coumaric acid-(COOH)-^{14}C	48·2	19	1·74	27·6
			30	0·37	130·0
	Umbellic acid-(COOH)-^{14}C	52·6	25	46·5	1·1
			28	32·9	1·6
2.	*o*-Coumaric acid-(COOH)-^{14}C	48·0	30	1·46	32·8
	p-Coumaric acid-α-^{14}C	48·0	27	1·67	28·7
	Umbellic acid-(COOH)-^{14}C	157·0	31	53·0	3·0
3.	*o*-Coumaric acid-(COOH)-^{14}C	48·0	33	1·01	47·5
	p-Coumaric acid-α-^{14}C	48·0	32	0·95	50·5
	Umbellic acid-(COOH)-^{14}C	157·0	33	92·0	1·7

* Expt. 1 was done at Montreal in summer 1962. Expts. 2 and 3 at Saskatoon in summer and autumn 1963.

Table 2. In these experiments both *o*-coumaric and *p*-coumaric acids were compared with umbellic acid as precursors of umbelliferone. As predicted, umbellic acid was utilized very well for umbelliferone synthesis. In fact, it is by far the most efficient precursor yet tested, with dilution values from just over 1 to 3. An unexpected result was the almost identical utilization of *o*-coumaric and *p*-coumaric acids in the experiments where a direct comparison was made. This is in direct contrast to the results of the investigations on herniarin,[1] and to the results reported in the preceding section, and constitutes evidence for the existence of a new pathway, by which 7-oxygenated coumarins are formed from administered *o*-coumaric acid.

Degradation of Umbelliferone Formed from o-*Coumaric Acid*

The feeding of *o*-coumaric acid-[COOH]-^{14}C was repeated on a larger scale and the umbelliferone (recovered as its acetate) was degraded as shown in Fig. 1. The specific

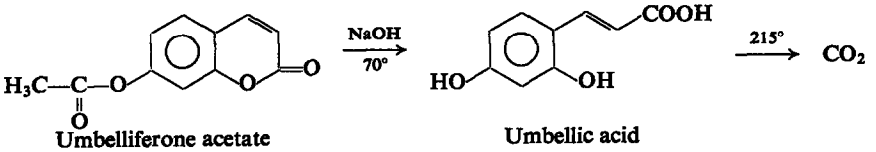

FIG. 1. DEGRADATION OF UMBELLIFERONE.

activity of the evolved carbon dioxide (385 mμc/mmole) was 105% of the specific activity of the umbelliferone acetate (368 mμc/mmole). The direct conversion of *o*-coumaric acid to umbellic acid without randomization was thus established.

Conversion of o- *and* p-*Coumaric Acids to Umbellic Acid*

While the above experiments provided evidence that *o*- and *p*-coumaric acids and umbellic acid are all effective precursors of umbelliferone, evidence was also required whether or not umbellic acid is an intermediate in the conversion of the two monohydroxycinnamic acids to this coumarin. This question was investigated by means of a trapping technique, in which either *o*- or *p*-coumaric acid labelled with ^{14}C was fed to *Hydrangea* leaves along with non-radioactive umbellic acid, and the latter was then recovered after one hour's metabolism. Owing to the rapid metabolism of umbellic acid only 6–11% of the administered compound was recovered from the plant.

TABLE 3. INCORPORATION OF *o*-COUMARIC AND *p*-COUMARIC ACIDS INTO UMBELLIC ACID AND UMBELLIFERONE BY *H. macrophylla* IN TRAPPING EXPERIMENTS

Labelled compound administered	Expt.	Specific activity (μc/mmole)	Dose (μmoles/g dry wt.)	Umbellic acid recovered		Umbelliferone recovered	
				Sp. act. (μc/mmole)	Dilution of ^{14}C	Sp. act. (μc/mmole)	Dilution of ^{14}C
o-Coumaric acid-(COOH)-^{14}C (+umbellic acid*)	1	141	32	F† 1·19	120	0·59	240
				B† 1·18	120	0·38	370
	2	120	32	F 0·33	360	0·55	220
				B 1·01	120	0·70	170
p-Coumaric acid-α-^{14}C (+umbellic acid*)	1	164	31	F 0·46	360	0·20	840
				B 1·63	101	0·28	590
	2	111	30	F 0·11	1000	2·3	48
				B 0·29	380	0·80	140

* An equimolar amount of non-radioactive umbellic acid was given with each labelled compound.
† F = free, B = bound (see text).

The results of two such experiments are shown in Table 3. In all cases the recovered umbellic acid (isolated after conversion to umbelliferone; see Experimental), after exhaustive purification, contained radioactivity, and most of the ^{14}C dilutions fell in the 100–400 range. These values compare favourably with those obtained in the conversion of *o*- and *p*-coumaric acids to umbelliferone itself (Table 2), when allowance is made for the diluting effect of the simultaneously administered inactive umbellic acid. The results of the trapping experiment thus provide a clear indication that *H. macrophylla* leaves possess the enzymes necessary to convert both *o*- and *p*-coumaric acids to umbellic acid.

There is no clear relationship evident between the incorporation of ^{14}C into the free and into the bound forms of umbellic acid and umbelliferone (Table 3), and interpretation of the data is made difficult by the danger of artifact production in working up the plant material.[5] More needs to be learned about the nature of the bound material before any speculation even is warranted.

Although in five cases in Table 3 the specific activity of umbelliferone is lower than that of umbellic acid, as would have been expected if umbellic acid is the major precursor of

[5] F. A. HASKINS and H. J. GORZ, *Crop Sci.* **1**, 320 (1961).

umbelliferone, this relation is reversed in the other three cases. In at least the two experiments with *p*-coumaric acid-α-^{14}C the differences are so large that it is difficult to doubt their reality. Such anomalous results could be explained by unequal rates of transport in the plant of *p*-coumaric and umbellic acids, during the short period of metabolism allowed; this might in some cases destroy the homogeneity of the administered mixture and permit much of the labelled *p*-coumaric acid to be converted to umbelliferone in the absence of a large diluting pool of umbellic acid. There is also the possibility, admittedly not easy to visualize, that a second major route exists, not involving umbellic acid, for the conversion of *p*-coumaric acid to umbelliferone. The weight of the evidence at present, however, points to the participation of umbellic acid in the normal scheme.

DISCUSSION

The results of the studies reported in this paper provide proof that *H. macrophylla* can utilize exogenously supplied *o*- and *p*-coumaric acids about equally well to synthesize umbellic acid, which is then rapidly and efficiently converted to umbelliferone. The marked contrast to herniarin biosynthesis, in which *o*-coumaric acid is not appreciably utilized,[1] is striking, and makes it necessary to re-open the question whether exclusive use of *p*-coumaric acid is the general rule in the elaboration of 7-oxygenated coumarins. More of these coumarins must be studied before this question can be resolved. The current findings nevertheless do provide further support for the hypothesis that *p*-coumaric acid is a general intermediate in the biosynthesis of coumarins which possess either a free or substituted 7-hydroxyl.

What is not yet clear is whether both pathways to umbellic acid are normally utilized by the *Hydrangea* plant. From the efficient conversion of both these precursors when exogenously supplied we can infer the presence of the enzymes required for their metabolism to umbelliferone, and this might be regarded as *prima facie* evidence that both pathways are normally used. But we know nothing of specificities, and one enzyme could conceivably catalyse both reactions. The negative result mentioned above—the lack of demonstrable *o*-coumaric acid formation from cinnamic acid—would suggest that inability to *ortho*-hydroxylate cinnamic acid may prevent the synthesis of *o*-coumaric acid in this species, and hence its participation in the production of umbelliferone, under normal circumstances. The failure of Ibrahim[6] to find *o*-coumaric acid in *Hydrangea* extracts provides indicative, but not conclusive, evidence in support of this idea. The fact that Ibrahim did find *p*-coumaric acid, together with the present results, unequivocally establishes this compound to be an intermediate in the scheme, but caution is still needed in ascribing a similar role to the *ortho* isomer.

A major unresolved question in umbelliferone biosynthesis is the role of glycosides. Very little free umbelliferone appears to exist in *H. macrophylla*, and only after acidic or basic hydrolysis, or treatment with a β-glucosidase, can the greater part of the umbelliferone be recovered. By analogy to coumarin and herniarin,[7] some of it may be present as *cis*-2-glucosyloxy-4-hydroxycinnamic acid (*cis*-GHC), but the possibility of sugar attachment on the *para* hydroxyl is an additional (or alternative) possibility here. The release of some umbellic acid by hydrolysis with emulsin, in the trapping experiments, is consistent with the formation of *trans*-GHC as an intermediate, but again, attachment of a sugar molecule on the *para* hydroxyl is not ruled out. It is planned to investigate these points in an extension of the present study.

[6] R. K. IBRAHIM, Ph.D. Thesis, Department of Botany, McGill University, 1961.
[7] S. A. BROWN, *Lloydia*, **26**, 211 (1963).

Little can be concluded at present about the biosynthesis of hydrangetin, as it was formed in insufficient amounts for study in the later experiments. It appears that the coumarin pattern in *H. macrophylla* may be a function of stage or conditions of growth. Such a phenomenon has been suggested for coumarins of *Phellopterus littoralis* Benth.[8] The results of the one experiment conducted suggest that both cinnamic and *p*-coumaric acids are hydrangetin precursors, an expected finding in view of known pathways.

EXPERIMENTAL

Cultivation of Plants

H. macrophylla plants were obtained commercially and propagated through cuttings. Several methods of cultivation were attempted, but the most satisfactory was to grow the plants in a greenhouse in pots containing expanded mica (Vermiculite), top-watered at intervals with "California" nutrient medium,[9] during the summer and autumn. Under these conditions, milligram quantities of umbelliferone were synthesized by the time the plants approached the flowering stage. The earlier experiments were done on younger plants, which contained considerably less of the compound.

Preparation of ^{14}C-labelled Compounds

Earlier papers have contained procedures for the preparation of coumarin-2-^{14}C,[10] *o*-coumaric acid-[COOH]-^{14}C,[10] *p*-coumaric acid-α-^{14}C,[11] and umbellic acid-[COOH]-^{14}C.[1] Cinnamic acid-β-^{14}C was purchased from Merck Sharp & Dohme, Montreal.

Administration of Labelled Compounds

The compounds investigated were administered in solution through the cut ends of stems or petioles. In the isotope competition and trapping experiments with cinnamic acid-β-^{14}C, 0·2 mg (5 μc) of this compound in 2·5 ml of water was administered to 30 $\pm$ 5 g fresh weight of the plant shoots, with a metabolic period of 30 hr in the light being allowed after adsorption. The labelled cinnamic acid was fed either alone or in solution with 5 μmoles of a non-radioactive compound: coumarin, *o*-coumaric acid, *p*-coumaric acid, caffeic acid, or ferulic acid. In the experiments recorded in Table 1 the compounds fed were dissolved in 25 ml of water and absorbed over a period of 24 hr through the cut ends of stems. Only about half of each solution was taken up by this method. In later experiments the volume fed was reduced to 3 or 4 ml, and complete absorption in as short a period as 1 hr was achieved in this way. In the experiments of Table 2, metabolism was allowed to proceed an additional 24 hr after uptake was complete. In the trapping experiments with umbellic acid, detached leaves were employed, and absorption was through the cut ends of the petioles. In these experiments much shorter metabolic periods were employed—about 2 hr after absorption commenced.

Isolation of the Coumarins

The plant material was cut into boiling ethanol (about 6 parts v/w is convenient) and disintegrated in a motor-driven homogenizer. The mixture was then filtered and the solid residue washed with ethanol or 80% ethanol.

In the isotope competition and trapping experiments with labelled cinnamic acid, the

[8] C.-H. YANG and S. A. BROWN, *Can. J. Chem.* **40**, 383 (1962).
[9] C. ELLIS and M. W. SWANEY, *Soilless Growth of Plants*, 2nd ed. revised and enlarged by T. EASTWOOD, Reinhold Publishing Corp., New York (1947).
[10] S. A. BROWN, G. H. N. TOWERS and D. WRIGHT, *Can. J. Biochem. Physiol.* **38**, 143 (1960).
[11] S. A. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **34**, 769 (1956).

residue after removal of the solvent was hydrolysed for 2 hr with 50 ml of 2 N hydrochloric acid under reflux on the steam bath. An ether extract of the hydrolysate was back-extracted with bicarbonate solution and the acid fraction thus obtained was submitted to two-dimensional chromatography on Whatman No. 1 paper, with benzene–acetic acid–water (9:7:3) as the first solvent, and 2% aqueous formic acid as the second.

In the experiments of Table 1, the combined filtrate and washings were dried under an air jet, and the residue was hydrolysed as just described. The hydrolysate was continuously extracted with ether, and the concentrated ether extract was chromatographed on sheets of Whatman 3MM paper in 2% formic acid. Bands corresponding to umbelliferone and hydrangetin were detected by their characteristic fluorescence under u.v. irradiation, excised, and eluted with cold ethanol. Upon removal of the solvent in a jet of air the extracted material was sublimed *in vacuo* at a temperature of ca. 205° for umbelliferone and ca. 100° for hydrangetin. The sublimates were dissolved in ethanol, the solutions were each made up to a known volume, and the absorption at 325 mμ was determined. Concentrations were determined from a standard curve. Two portions of the stock solutions were dried and the residues oxidized to carbon dioxide for ^{14}C analysis.

In the experiments of Table 2 a slightly different isolation procedure was used. The original ethanol–water solution was concentrated until the chlorophyll and fatty material had precipitated, and this was removed by filtration and the residue washed with warm water. In the first experiment the acid hydrolysis described above was preceded by overnight hydrolysis in N potassium hydroxide, at room temperature. In Experiments 2 and 3 both the acidic and basic hydrolyses were replaced by hydrolysis with 0·1% emulsin overnight, also at room temperature. In all cases ether extraction and paper chromatography in 1% aqueous acetic acid followed. In these studies insufficient hydrangetin was present for isolation. The material in the umbelliferone band was extracted into boiling methanol, sublimed at ca. 170°, < 1 Torr, and gas-chromatographed as the acetate,[12] usually after the addition of carrier. The eluate from the gas chromatograph was given a final purification by recrystallization from water or, better, high-boiling ligroin containing a little benzene.

In the trapping experiments it was necessary to recover umbellic acid, a compound difficult to purify because of its rather labile character. This difficulty was evaded by conversion of umbellic acid to the more tractable umbelliferone. An acid fraction was recovered from the ether extract by extraction into 5% sodium bicarbonate. This bicarbonate solution in turn was exhaustively ether-extracted until no characteristic blue fluorescence of umbelliferone was visible in the ether under u.v. illumination. After acidification, the liberated acids were recovered by a second continuous ether extraction. This acid fraction, dissolved in methanol, was irradiated under a u.v. lamp for 24 hr in a quartz tube. The methanol was removed, the residue taken up in 5% sodium bicarbonate, and the solution extracted continuously with ether to remove umbelliferone formed by the irradiation. In Experiment 1, Table 3, a preliminary sublimation at 80°, < 1 Torr, was used to remove any coumarin formed from *o*-coumaric acid by the irradiation. In Experiment 2 the ether extract was chromatographed on paper in 1% acetic acid and the umbelliferone band eluted with boiling alcohol. In both cases, vacuum sublimation at 170° allowed recovery of the umbelliferone. A small portion of the sublimate was analysed fluorometrically for umbelliferone,[13] with exciting and fluorescence wavelengths of 380 and 460 mμ, respectively. Final purification was as described above, for the experiments of Table 2.

[12] S. A. Brown and J. P. Shyluk, *Anal. Chem.* **34**, 1058 (1962).
[13] S. H. Vannier and W. L. Stanley, *J. Assoc. Offic. Agr. Chemists*, **41**, 432 (1958).

Degradation of Umbelliferone

Umbelliferone acetate, recovered from the gas chromatograph, was heated with alkali to cleave the ester, open the lactone ring, and effect *cis-trans* isomerization.[1] The umbellic acid recovered from this treatment was thermally decarboxylated as previously described for *o*-coumaric acid.[10] A bath temperature of 215° was used in the decarboxylation, and no precautions against loss by sublimation were necessary. A carbon dioxide yield of 92%, based on umbellic acid, was obtained.

Radioautography

Chromatograms to be radioautographed were left for 16 days in contact with a sheet of Kodak No-Screen X-ray film (35 × 43 cm), which was then developed by standard procedures.

Measurement of Radioactivity

Organic compounds to be analysed were oxidized by the Van Slyke-Folch method to carbon dioxide, which was counted in a Dynacon Model 6000 condenser electrometer incorporating an ion chamber (Nuclear-Chicago Corp.).

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Note added in press—The results of the isotope competition and trapping experiments with cinnamic acid-^{14}C are in accord with the findings of Billek and Kindl,[14] who reported that *p*-coumaric acid-α-^{14}C (and glucose-G-^{14}C) gave rise to radioactive spots of umbelliferone and hydrangetin on paper chromatograms.

Since the submission of this paper, Austin and Meyers[15] have also reported the efficient conversion of *trans-p*-coumaric acid to umbelliferone by *H. macrophylla*. The *cis* form of *p*-coumaric acid was less efficient, and *trans*-cinnamic acid less still. Their findings have also provided strong evidence that spirolactones do not participate in the formation of 7-oxygenated coumarins.

[14] G. BILLEK and H. KINDL, *Monats. Chem.* **93**, 85 (1962).
[15] D. J. AUSTIN and M. B. MEYERS, *Tetrahedron Letters* **14**, 765 (1964).