Elaboration of the 6,7,8 Oxygenation Pattern in Simple Coumarins: Biosynthesis of Puberulin in *Agathosma puberula* Fourc.[1]

Stewart A. Brown*, D. E. A. Rivett**, and H. Joan Thompson*

* Department of Chemistry, Trent University, Peterborough, Ont., Canada K9J 7B8.
** Department of Chemistry, Rhodes University, Grahamstown 6140, South Africa.

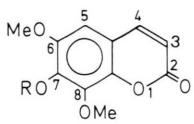
Z. Naturforsch. **39 c**, 31–37 (1984); received July 11, 1983

Biosynthesis, Puberulin, Coumarins, *Agathosma puberula*

Umbelliferone and scopoletin are well utilized as precursors of puberulin, a 7-O-prenyl ether of isofraxidin elaborated by *Agathosma puberula* Fourc. (Rutaceae). As ferulic, sinapic, and caffeic acids were all more poorly utilized than 4′-hydroxycinnamic (*p*-coumaric) acid, the partial biosynthetic route: 4′-hydroxycinnamic acid ⇢ umbelliferone → aesculetin → scopoletin ⇢ puberulin is suggested as the major pathway. Prenyl ether formation apparently occurs at or beyond the scopoletin stage. The implication that ferulic acid does not participate in the formation of scopoletin from 4′-hydroxycinnamic acid is at variance with the known role of ferulic acid as a scopoletin precursor in tobacco.

Introduction

The naturally occurring coumarins, of which well over 800 have now been described [1], are characterized by a variety of oxygenation patterns on the benzopyrone nucleus. The vast majority bear an oxygen function at C-7 (*cf.* Structure 1), and the largest single category is oxygenated at this position only – 335 compounds in a recent listing [1]. As for those coumarins oxygenated at more than one carbon, examination of a tabulation published by Murray *et al.* [1] reveals the distribution of types shown in Table I. In addition to these, other patterns of oxygenation involving the lactone ring, such as the 4,7 pattern of the coumestans, have been recognized.



1

a R = CH_2-CH = CMe_2

b R = H

Structure 1

Oxygenation pattern	Number of coumarins
5,7	287
7,8	66
6,7	53
5,7,8	28
6,7,8	22
5,6,7	17
5,6,7,8	1

Table I. Distribution of natural coumarins according to oxygenation pattern, as reported up to 1980.

Biosynthetically, coumarins in several categories of the Table have been investigated, and from those studies distinct differences have emerged. The 7-hydroxyl of umbelliferone is, without doubt, introduced at the phenylpropanoid acid stage, before lactone ring formation, and the resulting 4′-hydroxycinnamic acid is then 2′-hydroxylated [1]. There is very good evidence also that the 6,7-dioxygenated simple coumarin, scopoletin, derives in at least one species from the analogously oxygenated ferulic acid. This is indicated by a number of tracer studies [1], and an enzyme which hydroxylates ferulic acid at position 2′ has been reported from *Hydrangea macrophylla* [2]. This 6-hydroxylation (coumarin numbering), therefore, also can occur before elaboration of the coumarin nucleus.

Other oxygenation patterns have also been studied, three of which are represented among the linear furanocoumarins: bergapten (5,7), xanthotoxin (7,8), and isopimpinellin (5,7,8). In all these instances there is no evidence for introduction of oxygenation at C-5 or C-8 prior to elaboration of the lactone ring [1], and, in fact, it would appear

Abbreviation: TMC, 6,7,8-trimethoxycoumarin.

[1] The studies reported here were supported by grants from the Natural Sciences and Engineering Research Council of Canada and the South African Council for Scientific and Industrial Research.

Reprint requests to Prof. S. A. Brown.

0341-0382/84/0100-0031 $ 01.30/0

that the principal pathway to these coumarins involves hydroxylation of psoralen, which possesses a fully formed furanocoumarin nucleus, oxygenated at C-7 only. However, oxygenation of an intermediate 4′,5′-dihydrofuranocoumarin, marmesin, at positons 5 or 8 can also take place in some species [3–5]. Among the angular furanocoumarins, Steck and Brown [6] showed that angelicin (C-7 only) is a very efficient precursor of isobergapten (5,7), sphondin (6,7), and pimpinellin (5,6,7) in *Heracleum lanatum*. It is, therefore, highly probable that there is also no 5- or 6-hydroxylation in furanocoumarins until the lactone ring has been synthesized. However, this is in clear contrast to the above-mentioned case of scopoletin, whose oxygenation pattern is that of sphondin.

Of the 22 coumarins bearing the 6,7,8 pattern, none has yet been investigated biosynthetically. We have chosen for the study reported here to examine the biosynthesis of puberulin (**1a**) in *Agathosma puberula* Fourc., from the aerial parts of which this coumarin, a prenyl ether of isofraxidin (**1b**) was isolated earlier [7].

Experimental

Preparation of labelled substrates

[2-^{14}C]Hydroxycinnamic Acids – [2-^{14}C]-Labelled 4′-hydroxycinnamic, caffeic, ferulic, and sinapic acids were prepared from [2-^{14}C]malonic acid (New England Nuclear, Boston, USA) essentially according to the procedure of Vorsatz [8]. In a typical synthesis, 0.5 mmol of the appropriate aldehyde (4-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, 3-methoxy-4-hydroxybenzaldehyde, or 3,5-dimethoxy-4-hydroxybenzaldehyde, respectively) was dissolved in 0.3 ml of anhydrous pyridine, to which was added 0.5 mmol of the labelled malonic acid and 10 µl of aniline. The reaction mixture was heated at 55 °C in an oil bath for ~ 7 h. Carrier malonic acid (0.5 mmol) was then added, and the heating continued a further 7 h. Dilution with water and acidification with HCl yielded in each case crystals of the cinnamic acid derivative, which was recrystallized from hot water. Radiochemical yields in the 70–80% range were recorded.

[2-^{14}C]Umbelliferone. This was prepared by a cyanoacetate condensation from K^{14}CN as previously described [9].

[^{14}CH$_3$]Scopoletin. The synthesis of Braymer *et al.* [10] was adapted to the labelling of the O-methyl carbon of scopoletin. 7-O-Benzylaesculetin (167 mg, 0.625 mmol) in 3 ml of dry acetone was stirred under reflux for 4 h with 77 mg of (^{14}CH$_3$)$_2$SO$_4$ (15 MBq/mmol) and 250 mg of anhydrous potassium carbonate. A further 77 mg of carrier dimethyl sulphate was then added, and refluxing was continued for another 4 h. Removal of the solvent gave a quantitative yield (based on the coumarin) of benzyloxyscopoletin, which was recrystallized from aqueous methanol to yield two crops of crystals totalling 170 mg. This intermediate was debenzylated by heating 6 h at 100 °C in 2 ml of glacial acetic acid + 1 ml of concentrated HCl. The product was decolorized with charcoal in methanol and sublimed at 170 °C, < 1 Torr, to provide 115 mg of scopoletin. A final recrystallization from 50% ethanol gave a yield of 91 mg, m. p. 205–208 °C.

Administration of labelled substrates to A. puberula

Plant shoots were collected on the farm "Upper Gletwyn" east of Grahamstown, usually from a single bush, and the cut ends immediately plunged into water. Immediately before the feeding, the ends were recut at an angle under water with sharp secateurs. About 0.02 mmol of the labelled substrate was weighed into a small test tube (fusion tube), and in the case of acids a slight excess of sodium carbonate was added. After the addition of 1 ml of water the mixture was warmed to effect solution. The phenols, scopoletin and umbelliferone, were dissolved in 1 ml of water and 3 drops of 0.02 M NaOH with warming. The freshly cut ends of the shoots (15–25 g) were dipped into the substrate solutions and the shoots exposed either to sunlight or to a 200 W lamp placed 30 cm away in a fume hood through which a gentle stream of air was flowing. The ratio of molar dose to shoot rate was kept reasonably constant. The solution was taken up by the shoots during 2 h. The shoots were then allowed to absorb two rinses of water, placed in an excess of tap water, and allowed to metabolize for 2–3 days.

Processing of treated plants

The shoots were transferred to a Soxhlet apparatus and extracted for two days with methanol. The solvent was removed under reduced pressure, the residue dissolved in 40 ml of warm

chloroform, and shaken with 2 × 20 ml of 4% aqueous NaOH to remove acids and phenols. The chloroform extract was evaporated and the residue refluxed 1 h with methanol containing 2% sulphuric acid (40 ml) to convert puberulin to isofraxidin. Ten per cent NaOH was added and the pH carefully adjusted to 9–10 with the aid of Merck test papers. At more alkaline pH green pigments are later extracted with the isofraxidin and make purification difficult. The methanol was removed under reduced pressure, the mixture made up to about 40 ml with water, and heated on the steambath for a few minutes to ensure complete solution of isofraxidin. The mixture was cooled in ice, filtered through Celite and the residue washed with water. The filtrate (90 ml) was transferred to an efficient liquid-liquid extractor and extracted with ether for 1 h, the light green extract being discarded. The aqueous layer was cooled in ice, acidified with 50% H_2SO_4 (8 ml) and extracted for 1 h with ether. The ether extract was evaporated, the residue dissolved in warm ethyl acetate (30 ml) and shaken with 4 ml of aqueous $NaHCO_3$, then 2 × 2 ml of water, and dried over anhydrous sodium sulphate. The solution was poured through a column of neutral alumina which was washed with the same solvent (30 ml); the eluate and rinse were then combined and evaporated. When the crude crystalline residual isofraxidin was very green it was decolorized with charcoal in ethanol; otherwise it was recrystallized from ethyl acetate-hexane (1–2 ml).

Final purification of isofraxidin was via its derivative, TMC. The isofraxidin was dissolved in 1–2 ml of acetone with an excess of anhydrous potassium carbonate and stirred for 2 h at room temperature. Methyl iodide (0.1 ml) was then added, and the mixture was heated at reflux with stirring until the yellow colour disappeared or faded to very light. The solvent was removed in an air jet, the residue treated with a few millilitres of water, and the aqueous mixture was twice extracted with ethyl acetate. The residue from evaporation of the solvent was slowly sublimed at 100 °C, < 1 Torr, to give colourless crystals, m.p. 103–105 °C (literature m.p. 104 °C [1]). This derivative was finally recrystallized to constant specific activity (2 ×) from hexane-ethyl acetate, in some cases after the addition of carrier TMC.

In the final series of experiments reported here, difficulty was encountered in getting isofraxidin to crystallize. The crude isolate was therefore chromatographed on 20 × 20 cm chromatoplates of silica gel G, 1 mm thick, developed in chloroform-acetone, 9:1, repeated if necessary. The bands corresponding to isofraxidin were extracted into hot acetone in Soxhlet extractors and the solvent was evaporated to give crude, usually crystalline isofraxidin. These residues were converted to TMC as described above.

Degradation of TMC

TMC from isofraxidin recovered after feeding [$^{14}CH_3$]scopoletin in Series 6, Table II, was demethylated with hydriodic acid [11]. The purified sample (9.6 mg) was dissolved by warming with a few crystals of phenol in a 5 ml flask with a sidearm. Hydriodic acid (sp. gr. 1.7, 2 ml) was added, and the mixture gently refluxed under an air condenser for 2 h, during which the apparatus was swept with a slow stream of nitrogen. The gas stream was passed through a scrubber containing equal parts of 5% sodium thiosulphate and 5% cadmium sulphate to remove iodine and hydrogen iodide, and then through sintered glass into a test tube containing the cold 5% ethanolic solution of triethylamine previously described [12]. Sweeping was terminated when the reaction flask had cooled. After standing overnight at room temperature in the stoppered tube, the solution was evaporated to dryness with an air jet in a tared scintillation counting vial, and the residue of hygroscopic triethylmethylammonium iodide formed from the evolved methyl iodide was dried in a vacuum oven and weighed prior to counting.

Aqueous solutions of this quaternary iodide react with Bray's scintillation counting solution [13] to yield free iodine, which causes essentially total quenching. To avoid this, the salt was dissolved in an excess of 2% sodium thiosulphate solution, and water to a total volume of 1.5 ml was added. Slow addition of Bray's solution yielded a clear, colourless counting medium.

Measurement of Carbon-14

All samples were analysed in a Beckman model 7000 scintillation counter after solution in methanol; except as noted above, a toluene-based counting medium was employed.

Results and Discussion

In Table II are collected the results of six series of feeding experiments in which six labelled substrates were administered to *A. puberula*. In Table III, for ease of comparison, the dilution values from Table II are expressed relative to those of a reference standard, 4′-hydroxycinnamic acid (taken as 100), a known intermediate in the biosynthesis of 7-oxygenated coumarins. Caffeic, ferulic, and sinapic acids were tested to ascertain whether there was any evidence for additional hydroxylation of the benzene ring in the 3′ and 5′ positions (corresponding to the 6 and 8 positions of isofraxidin)

Table II. Incorporation of ^{14}C from labelled substrates into the coumarin moiety of puberulin.

Date of feeding	Substrate fed [a]	Sp. activity of isofraxidin [MBq/mmol]	^{14}C dilution	Conditions of feeding
Series 1				
1981 12 04	4′-hydroxy-cinnamic acid	1740	7 020	Sunlight outdoors
	Ferulic acid	716	15 400	
	Sinapic acid	154	52 100	
	Scopoletin	3080	1 900	
Series 2				
1982 01 20	4′-hydroxy-cinnamic acid	1400	8 700	Sunlight outdoors
	Ferulic acid	134	82 000	
	Sinapic acid	268	29 900	
	Scopoletin	1750	3 700	
Series 3				
1982 03 15	4′-hydroxy-cinnamic acid	1490	8 170	200 W incandescent lamp
	Caffeic acid	965	13 100	
	Umbelliferone	91	26 000	
Series 4				
1982 05 13	4′-hydroxy-cinnamic acid	119	103 000	200 W incandescent lamp
	Ferulic acid	82	130 000	
	Sinapic acid	35	230 000	
	Caffeic acid	65	190 000	
	Scopoletin	308	19 300	
	Umbelliferone	62.5	37 600	
Series 5				
1982 11 23	4′-hydroxy-cinnamic acid	872	14 000	Greenhouse
	Ferulic acid	435	25 300	
	Sinapic acid	714	11 200	
	Caffeic acid	210	59 900	
	Scopoletin	[b]	[b]	
	Umbelliferone	697	3 360	
Series 6				
1983 02 08	4′-hydroxy-cinnamic acid	713	17 100	Greenhouse
	Sinapic acid	157	51 100	
	Caffeic acid	270	46 700	
	Scopoletin	583	10 200	
	Umbelliferone	748	3 130	

[a] Specific activities (MBq/mmol): 4′-hydroxy-cinnamic acid 12.2, ferulic acid 11.0, caffeic acid 12.6, sinapic acid 8.02, umbelliferone 2.34, scopoletin 5.95. Labelling position was C-2 except for scopoletin, which was methyl-labelled.
[b] Sample lost.

Table III. Relative ^{14}C dilution values after administration of ^{14}C-labelled substrates to shoots of *A. puberula*. For each series listed in Table II, the ^{14}C dilution in the conversion of 4′-hydroxycinnamic acid to puberulin is arbitrarily set = 100.

Substrate	Series Number					
	1	2	3	4	5	6
Ferulic acid	219	942	–	129	181	–
Sinapic acid	742	343	–	223	80	299
Caffeic acid	–	–	160	188	428	273
Scopoletin	27	43	–	19	[a]	60
Umbelliferone	–	–	316	37	24	18

[a] Sample lost.

preceding the reactions leading to lactone ring formation. If such were the case, better utilization of one or more of these substrates compared to 4′-hydroxycinnamic acid would have been expected, as evidenced by lower carbon-14 dilutions. In fact, these dilutions were higher in almost all cases, and none was lower (to a degree inconsistent with normal biological variation) than was that of 4′-hydroxycinnamic acid. These results, therefore, provide no support for any hypothesis that the oxygenation pattern of isofraxidin, beyond that at C-7, is established before the coumarin nucleus is formed.

In contrast, scopoletin, in all experiments where it was tested, and umbelliferone in three out of the four experiments done, exhibited markedly lower carbon-14 dilutions than that of 4′-hydroxycinnamic acid, strongly suggesting that these two coumarins lie on the biosynthetic pathway between 4′-hydroxycinnamic acid and isofraxidin. These findings are entirely consistent with the current concept that 7-oxygenated coumarins originate from 4′-hydroxycinnamic acid via 2′-hydroxylation to yield umbelliferone, considered to be the parent compound of all coumarins with 7-oxygenation.

The role of scopoletin merits further discussion. If one disregards the anomalously high relative dilution for umbelliferone in Series 3 of Table III, this substrate appears to be somewhat more efficiently utilized for isofraxidin biosynthesis than is scopoletin, even though the latter, being presumably a closer intermediate to isofraxidin, would be expected to undergo less dilution. However, it must be emphasized that umbelliferone was labelled in its skeleton with carbon-14, whereas scopoletin was not, bearing label only in the O-methyl carbon. As O-demethylation of coumarins is a known phenomenon [14], it seems probable that some of the label in administered scopoletin was lost, with the possibility of higher-than-expected dilution values. The reported dilution values for scopoletin should be regarded as maxima, and as such are not inconsistent with a position for scopoletin beyond umbelliferone on the route to isofraxidin. Even without this complication, discrepancies of this sort have long been recognized.

The label in the isofraxidin formed from [$^{14}CH_3$]scopoletin in Series 6 was entirely localized in the methoxyl carbons. A portion of the TMC prepared from this isofraxidin was demethylated with hydriodic acid and the evolved methyl iodide trapped as a quaternary iodide. Recovery of the radioactivity was quantitative (98%), and it appears reasonable to assume that it was confined to the methoxyl group at C-6.

A further point worthy of mention is that the origin of scopoletin via umbelliferone implied by the current observations is at variance with a biosynthetic route now generally accepted for tobacco [1], where there is good evidence that the oxygenation pattern is complete at the cinnamic acid stage, before the coumarin nucleus is elaborated, and that ferulic acid is an intermediate in the pathway. The consistently higher dilutions noted for ferulic acid in our experiments, by comparison to 4′-hydroxycinnamic, are a contraindication of ferulic acid's role in the biosynthesis of scopoletin in *A. puberula*, en route to isofraxidin. It is of interest that Tsang and Ibrahim [15] have described the isolation from tobacco cell suspension cultures of an O-methyltransferase which has high activity against caffeic acid, but which also O-methylates aesculetin, among a number of other substrates, but not phenolic glucosides. They have proposed an alternative pathway to scopoletin in tobacco via caffeic acid and aesculetin. It would appear that our understanding of scopoletin formation in tobacco would benefit from yet more study.

In the early series of experiments reported here, percent incorporation of carbon-14 into isofraxidin was calculated on the basis of the crude recovered isofraxidin. Unfortunately it became apparent as the work progressed that there were uncontrolled variables in the isolation procedure, whose nature has still not been established, that sometimes led to large variations (by a factor as large as 7) in the

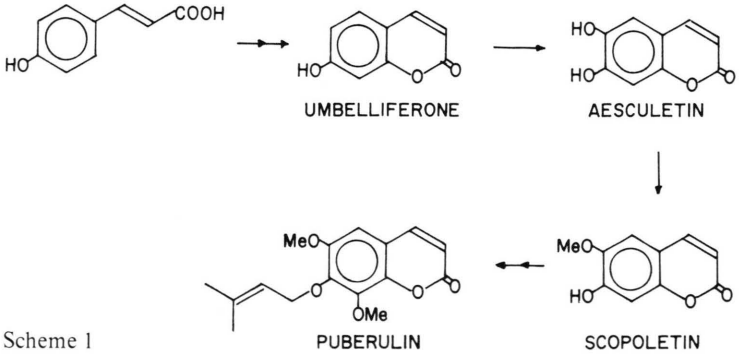
weights of isofraxidin recovered. Inasmuch as shoots from the same bush were usually employed in each series of feedings, such variations can hardly be explained by biological variability. Because of these discrepancies we have not reported per cent incorporations. However, in the case of umbelliferone and scopoletin, values of the order of 0.2–0.5% have been observed, which may be compared with the 0.01% generally accepted [16] as representing the lower limit of significant incorporation. As implied above, these values for scopoletin can be regarded as minima, if the effective dose has been lowered by O-demethylation *in vivo*. The fact that no dilution of carbon-14 much lower than 2000 has been recorded for any precursor clearly reflects the large amount of preformed puberulin, sometimes exceeding 100 mg in 20 g, in the shoots of the experimental plants. In the experiments of Series 4, as well as in some preliminary experiments not reported here, dilutions were much higher overall than in the other feedings, presumably because of a low rate of puberulin synthesis in the late autumn.

Present indications from the work reported above are, therefore, that puberulin (and by inference other coumarins of this oxygenation pattern) is formed via the partial biosynthetic pathway shown in Scheme 1. Aesculetin, although it has not yet been tested, must be assumed to be the intermediate in the conversion of umbelliferone to scopoletin. Steck [17] was unable to show incorporation of this intermediate into scopoletin by tobacco, but as the route in that species is evidently via ferulic acid, the involvement of aesculetin would not be expected there. In this context it is also germane to recall the inability of Satô and Hasegawa [18] to demonstrate the formation of aesculetin from caffeic acid in *Cichorium intybus*, and to raise again the possibility that its derivation in that species may be, instead, via umbelliferone.

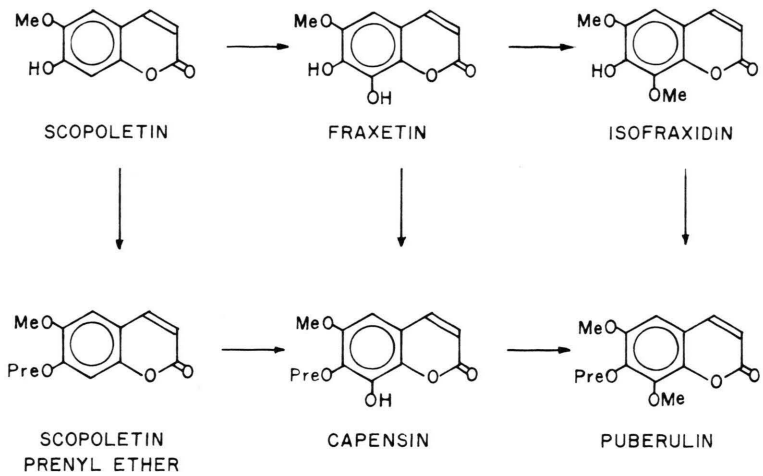
The pathway beyond scopoletin

The sequence of reactions beyond scopoletin is still open to speculation. In the present study we have not directly addressed the question of the stage at which prenylation of the 7-hydroxyl group of isofraxidin occurs to yield puberulin. Our results do, however, have some bearing on this question. The relatively high incorporations of carbon-14 from scopoletin suggest that this is the earliest compound in the biosynthetic sequence likely to be prenylated, as prenylation earlier in the pathway would imply non-participation of scopoletin itself. (We assume that the prenylating enzyme system is specific.) The hypothetical metabolic grid shown in Scheme 2 summarizes the three possible routes from scopoletin to puberulin. As all four potential intermediates are naturally occurring [1], there is no *prima facie* reason for favouring any of them, but we plan further study in this area.

Of interest in the present context are earlier studies by Towers and his associates [19, 20] on the origin of an 8-oxygenated coumarin, hydrangetin (7-hydroxy-8-methoxycoumarin) in *H. macrophylla*. Although they are in each case based on the results of only one feeding experiment, and therefore unconfirmed, data obtained by these workers suggest that the pathway to hydrangetin proceeds from 4′-hydroxycinnamic acid via umbelliferone. If these findings are valid, they and the data from our experiments support a theory that, whether or not C-6 is oxygenated, position 7 is hydroxylated at the cinnamic acid stage, but position 8 not until after



Scheme 1



Scheme 2 Pre = Prenyl

elaboration of the coumarin nucleus. This is quite in harmony with the sequence of events in linear furanocoumarin biosynthesis, previously discussed, where there is convincing evidence [1] that formation of the benzopyrone structure precedes oxygenation at C-8. The principle would thus seem to be a general one. In *A. puberula* our results indicate that oxygenation at C-6, as well as C-8, takes place on the coumarin.

It remains to be shown whether the analogous situation known to exist in the case of 5-oxygenated furanocoumarins [1] prevails as well in simple coumarins. Two of us (S. A. B. and H. J. T.) are currently exploring this question.

[1] R. D. H. Murray, J. Méndez, and S. A. Brown, The Natural Coumarins: Occurrence, Chemistry, and Biochemistry, John Wiley & Sons Ltd., Chichester, U. K. 1982.
[2] H. Kindl, Hoppe-Seylers Z. Physiol. Chem. **352,** 78 (1971).
[3] G. Caporale, F. Dall'Acqua, A. Capozzi, S. Marciani, and R. Crocco, Z. Naturforsch. **26b,** 1256 (1971).
[4] G. Caporale, G. Innocenti, A. Guiotto, P. Rodighiero, and F. Dall'Acqua, Phytochemistry **20,** 1283 (1981).
[5] F. Dall'Acqua, A. Capozzi, S. Marciani, and G. Caporale, Z. Naturforsch. **27b,** 813 (1972).
[6] W. Steck and S. A. Brown, Can. J. Biochem. **48,** 872 (1970).
[7] N. Finkelstein and D. E. A. Rivett, Phytochemistry **15,** 1080 (1976).
[8] F. Vorsatz, J. Prakt. Chem. **145,** 265 (1936).
[9] S. A. Brown, Phytochemistry **2,** 137 (1963).
[10] H. D. Braymer, M. R. Shetlar, and S. H. Wender, Biochim. Biophys. Acta **44,** 163 (1960).
[11] A. Steyermark, Quantitative Organic Microanalysis, Chap. 16, Academic Press, New York 1961.
[12] S. A. Brown and R. U. Byerrum, J. Amer. Chem. Soc. **74,** 1523 (1952).
[13] G. A. Bray, Anal. Biochem. **1,** 279 (1960).
[14] S. A. Brown and S. Sampathkumar, Can. J. Biochem. **55,** 686 (1977).
[15] Y.-F. Tsang and R. K. Ibrahim, Phytochemistry **18,** 1131 (1979).
[16] E. Leete, in T. A. Geissman, Biosynthesis, A Specialist Periodical Report, **Vol. 1,** p. 159, The Chemical Society, London 1972.
[17] W. Steck, Can. J. Biochem. **45,** 1995 (1967).
[18] M. Satô and M. Hasegawa, Phytochemistry **11,** 657 (1972).
[19] B. Bohm, R. K. Ibrahim, and G. H. N. Towers, Can. J. Biochem. **39,** 1389 (1961).
[20] S. A. Brown, G. H. N. Towers, and D. Chen, Phytochemistry **3,** 469 (1964).