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Leaf coumarins of *Angelica archangelica*[1]

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Coumarin derivatives are abundant in leaves of *Angelica archangelica*, and differ in kind from those of the roots. Angelicin, bergapten, imperatorin, isopimpinellin, xanthotoxin, oxypeucedanin, and five unidentified coumarins were isolated from leaves. A time study of their development was carried out. Methods for the isolation of leaf neutral coumarins are described.

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Introduction

Many plants of the family *Umbelliferae* are known to accumulate coumarin derivatives in their roots and seeds (1). The species *Angelica archangelica* L. has been particularly well studied in this respect. Späth and Pesta (2) found the main root coumarin to be osthol (**2** in Fig. 1), accounting for nearly 0.2% of the dry weight. Among the many other coumarins subsequently isolated from this source were osthenol and angelicin (**3**) (2); imperatorin (**8**), bergapten (**5**), umbelliprenin, xanthotoxol, and xanthotoxin (**6**) (3); oxypeucedanin (**9**), oreoselone, and archangelicin (4). The seeds contain a similarly complex array of coumarins (5).

The study of plant leaf coumarins has received less attention, and presents certain difficulties. The main problem is the separation of the ether-soluble neutral coumarins from the chlorophylls, waxes, and other constituents of similar solubility. Occasionally the appreciable solubility of neutral coumarins in hot water has been used to effect the separation directly (6), but more usually an alcohol extract has been made and treated with aqueous alkali for several hours to open the lactone ring and give coumarinate salts, other components then being removed by ether extraction. The coumarin derivatives can themselves be ether-extracted subsequent to their regeneration on acidification of the medium. Washing the final ether extract with carbonate leaves a lactone fraction. In the presence of chlorophyll, which is degraded to dark products by alkali, the isolation of such a lactone fraction is not usually clean-cut and may have to be done twice (7). A more serious objection to the use of alkali is the loss of ester linkages, which are not uncommon in *Angelica* coumarins. For example, ostruthol is transformed to oxypeucedanin.

We report in this paper a general method of leaf treatment which avoids entirely the use of alkali, yet affords a fraction from which neutral coumarins may be conveniently isolated and purified. We have used the technique with several umbellifers, but the case of *A. archangelica*, with its interesting biosynthetic implications, is alone described below.

Methods and Results

Fresh leaves were homogenized in boiling methanol and the hot filtrate adjusted to 60% aqueous methanol, then washed twice with Skelly B (*n*-hexane) to remove chlorophylls and other fatty materials. The Skelly B was back-extracted once with 60% methanol and this was added to the main alcoholic solution. This procedure was shown to retain more than 98% of the xanthotoxin and bergapten in the aqueous part while removing about 70–80% of the total weight of the original methanol extract. Concentration of the alcohol solution resulted in an aqueous suspension; this was extracted overnight with ether to remove the coumarins present.

The ether extract was chromatographed on silicic acid, eluting with 1:1 Skelly B:ether. This solvent mixture is slightly more polar than that used successfully for *Citrus* coumarins by Stanley and Vannier (8), and somewhat less solublizing than the system of Floss and Mothes (9), which was not entirely successful with *Pimpinella* coumarins. The fractions collected were allowed to evaporate overnight to allow deposition of crystalline material.

The first crystalline sample, long needles

[1]NRCC No. 10670.

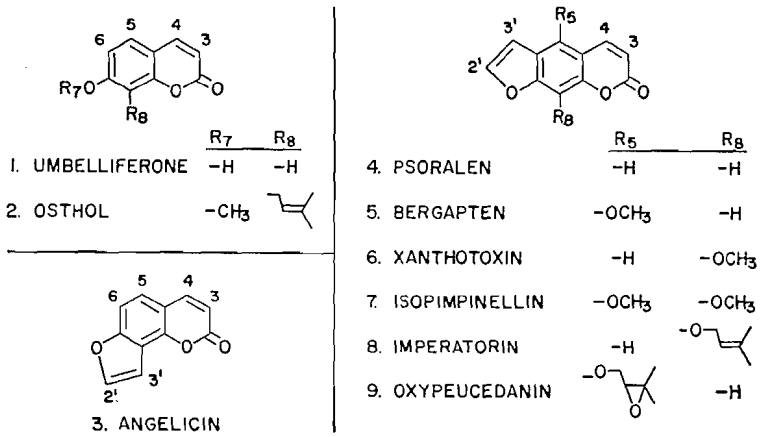

FIG. 1. Some coumarins mentioned in the text.

melting at 97.5–99.5°, was a lactone having an ultraviolet (u.v.) spectrum suggestive of a xanthotoxol derivative. The nuclear magnetic resonance (n.m.r.) spectrum indicated the presence of five aromatic protons: two were typical coumarin pyrone ring doublets ($J = 10$ c.p.s.); two others were typical furan ring doublets ($J = 2 \sim 2.5$ c.p.s.); the fifth was a singlet. All had very nearly the same chemical shifts as those of xanthotoxin. A dimethylallyl group was also evident. The material thus appeared to be imperatorin, lit. m.p. 102° (10). A mixed melting point with an authentic sample (m.p. 96–99°) was undepressed, and the u.v. and n.m.r. spectra of the standard were superimposable upon those of the isolated compounds. Chromatography on paper and examination of fluorescence properties confirmed the identity.

The second group of crystals, m.p. 188–190° with sublimation, also showed lactonic properties. A u.v. maximum near 306 mμ suggested a bergaptol or isobergaptol derivative. The n.m.r. spectrum showed the H_4 signal at $\delta > 8.0$, indicating substitution at the 5-position. The existence of 5 appropriate aromatic protons and a single aromatic methoxyl group suggested bergapten, lit. m.p. 188–189° with sublimation (11), as the identity of the material. A mixed melting point with authentic material was undepressed and direct comparison of u.v., n.m.r., fluorescence, and chromatographic data for isolated and authentic material showed no differences between the two.

The third crystalline deposit, a lactone melting at 144–145°, had the u.v. spectrum of a xanthotoxol derivative. The n.m.r. spectrum, indicating a monomethoxy furocoumarin, was identical with that of xanthotoxin (lit. m.p. 145–146° (12)), and an authentic sample of xanthotoxin had the same chromatographic and fluorescence properties. A mixed melting point was undepressed.

The fourth batch of crystals, melting at 147–149°, gave an absorption maximum at 311 mμ. A furocoumarin with two methoxy groups, one in the 5-position ($\delta > 8.0$ for H_4) was indicated by the n.m.r. spectrum. Since all the pyrone and furan ring protons appeared to be present in the spectrum, the only structures possible, assuming the usual 7-oxy-substitution, were pimpinellin (5,6-dimethoxyangelicin) and isopimpinellin (7), lit. m.p. 148–151° (13). A mixed melting point with an authentic sample of the latter was undepressed, and the u.v. and n.m.r. spectra of the isolated and authentic samples were identical. The fluorescence and chromatographic properties were also those of isopimpinellin. This coumarin, while known in other *Angelica* species, does not seem to have been reported previously in *A. archangelica.*

From the mother liquors of the bergapten fractions, another coumarin was isolated by gas–liquid chromatography (g.l.c.). Collection and recrystallization from alcohol gave crystals melting at 137–138°. Analysis by n.m.r. revealed six protons, all aromatic. The u.v. and n.m.r. spectra of angelicin (lit. m.p. 138–139.5° (2)) were identical with those of the isolated sample, and a mixed melting point showed no depression.

Co-chromatography confirmed the identification.

Leaves of plants from different seed sources gave differing amounts of the above coumarins. The plants examined in the work described here contained relatively large amounts of xanthotoxol derivatives, in contrast to another strain which afforded primarily bergaptol derivatives. Examination of this latter strain showed the presence of all the coumarins described above, and in addition demonstrated the presence of a lactone (m.p. 140–142°) eluting from silicic acid along with isopimpinellin. Its u.v. and n.m.r. spectra strongly suggested it to be racemic oxypeucedanin (lit. m.p. 142–143° (14)). The optically active (+)-isomer, which was available for comparison, gave identical u.v. and n.m.r. spectra.

Study of the non-crystalline fractions from silicic acid was carried out by g.l.c. In this way several other leaf coumarins were detected, but because of their scarcity, none was identified. These included three compounds U_1, U_2, and U_3 eluting with isopimpinellin and having identical xanthotoxol ether u.v. spectra; a compound U_4 eluting with bergapten and having a u.v. spectrum like osthol or herniarin but with chromatographic properties unlike those of any reference compound available; and a compound U_5 eluting just before bergapten in very small quantities, believed (on the basis of its chromatographic behavior) to be psoralen (**4**).

It might be noted here that g.l.c. analyses demonstrated the absence of other simple furocoumarins such as isobergapten (5-methoxyangelicin), sphondin (6-methoxyangelicin), pimpinellin (5,6-dimethoxyangelicin), and phellopterin (5-methoxy-8-dimethylallyloxypsoralen). Neither could osthol be detected, a point to be discussed further below.

Examination of the leaf water-solubles by acid hydrolysis and re-extraction with ether were carried out to test for the presence of furocoumarin glycosides, such as occur in some plants (e.g. ref. 15). No neutral coumarins were liberated by the hydrolysis, but umbelliferone (**1**) was found. It appeared to be present in unhydrolyzed extracts principally as the glucoside, skimmin.

Although osthol could be readily detected in root extracts, we were unable to find it in the leaves. This and the quantitative variation in coumarins from different seed strains prompted us to assay growing *A. archangelica* shoots and roots at various stages of development. This was done by direct g.l.c. of prepared coumarin-rich fractions, with estimation of the detected coumarins by comparison with standard area curves. The results for the seed strain whose component coumarins have just been described are shown in Figs. 2 and 3. The two outstanding features of these results are the selective accumulation of osthol in the roots and the early appearance of leaf coumarins. The data clearly show that the coumarins in the leaves undergo no great qualitative changes during the maturation of the

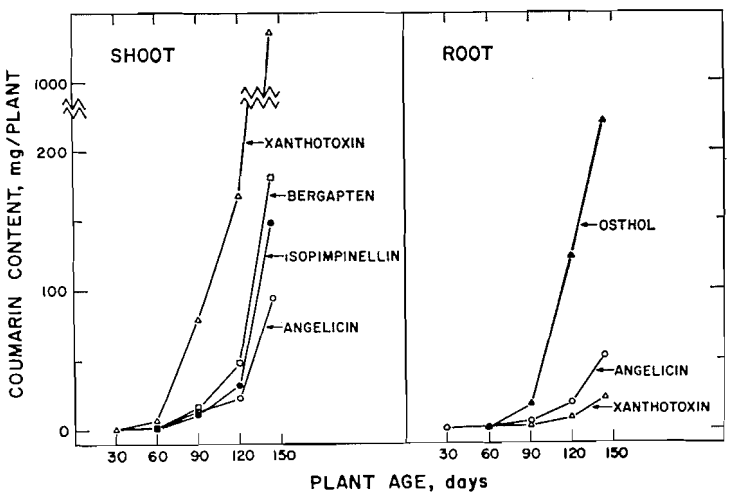

FIG. 2. Development of coumarins in *Angelica archangelica*; total content of some coumarins in shoots and roots.

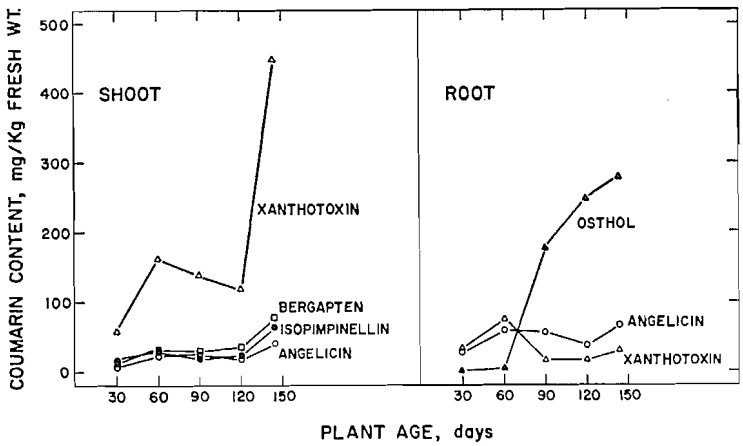

FIG. 3. Development of coumarins in *Angelica archangelica*; concentration of some coumarins in shoots and roots.

plant; they are apparently synthesized simultaneously and in more or less fixed ratios one to another. Moreover, their appearance ahead of, or coincident with the root coumarins suggests the leaves as an independent site of furocoumarin formation.

The situation in the roots is different. Here the main coumarins are angelicin and xanthotoxin in the first two months, but after this time the xanthotoxin concentration falls and osthol begins to accumulate, becoming, as reported in the literature, the main coumarin of the mature roots. How this accumulation comes about is impossible to say at this time, as the biosynthetic pathways leading to osthol and to the furocoumarins are not known with certainty. However, as osthol was not detected in the leaves, it seems probable that it is formed in the roots.

Experimental

A. archangelica was grown from seed in the greenhouse and the plants maintained hydroponically. A constant day length of 14 h was imposed.

Fresh leaves (1720 g, i.e. 300 g dry weight) of three month old plants, trimmed free of petioles and stems, were boiled and homogenized in 3 l methanol. The alcohol solution was adjusted to contain 60% methanol by concentration, then was washed with two 400 ml portions of Skelly B. This petroleum ether was backwashed with 60% methanol and the alcoholic solutions combined; methanol was removed from the orange solution and the aqueous residue continuously extracted overnight with ether. A small amount of white precipitate was filtered off before the extract was concentrated to the point of dryness.

A 4 × 35 cm column of silicic acid (Mallinckrodt, 100 mesh, used as received. Other brands gave less sharp separations.) was prepared by slurrying 200 g powder in about 1 l of 1:1 ether:Skelly B (60–80°), pouring this into the column, and allowing settling until the meniscus approached the top of the silicic acid. The ether extract, dissolved in about 50 ml of the same solvent mixture, was carefully poured upon the top of the column and washed in with a little extra solvent. Two hundred 15 ml fractions were collected and allowed to stand for a day or two to permit the ether to evaporate and thus bring about the slow crystallization of the chief coumarins. These were then filtered off, the appropriate fractions combined, and each crystalline substance recrystallized from ethanol.

Imperatorin

Fractions 41–47 deposited needles of imperatorin, m.p. 97.5–99.5°; λ_{max}(EtOH) 221, 248, 264 (shoulder), and 298 mμ. The n.m.r. spectrum (Varian HA-100, solvent deuterochloroform, internal standard tetramethylsilane) showed signals at δ = 6.32 (H_3) and 7.71 (H_4), both doublets having J = 10 c.p.s.; at 7.31 (H_5), 7.64 ($H_{2'}$), and 6.77 ($H_{3'}$), for a total of 5 aromatic protons. The assignments of aromatic signals here and below follow those of Sheinker *et al.* (16) and Batterham and Lamberton (17). The later work of Reisch *et al.* (18) has also been considered. Other signals, present at 4.97 (CH_2), 5.58 (unsaturated CH), and 1.74 (vinyl methyls), were typical for a 3,3-dimethylallyl substituent. Authentic imperatorin was identical in all respects. The yield was 170 mg; abundance = 0.57 mg/g dry leaves.

Bergapten

Fractions 54–62 gave bergapten, m.p. 188–190° with sublimation; λ_{max}(EtOH) 221, 247, 257, 266, and 306 mμ. The n.m.r. spectrum showed signals at δ = 6.25 (H_3) and 8.14 (H_4) (both doublets, J = 10 c.p.s.); at 7.12 (H_8), 7.59 ($H_{2'}$), and 7.02 ($H_{3'}$). A single aromatic methoxyl group was present at δ = 4.25. Authentic bergapten was identical in all respects. The yield was 46 mg; abundance = 0.15 mg/g.

Angelicin

The bergapten mother liquors were concentrated to

small volume and a little more bergapten was filtered off. Gas–liquid chromatography of the remaining solution showed several components, the major one emerging, after about 5 min. This was collected and recrystallized to colorless needles, m.p. 137–138°; λ_{max}(EtOH) 218, 242 (shoulder), 248, and 297 mμ. The n.m.r. showed only 6 protons, all in the aromatic region, at $\delta = 6.36$ (H_3), 7.78 (H_4), both $J = 10$ c.p.s.; 7.40 (H_5), 7.38 (H_6), 7.67 ($H_{2'}$), and 7.12 ($H_{3'}$). Authentic angelicin was identical in all respects. Estimated leaf content = 42 mg; abundance = 0.14 mg/g.

Xanthotoxin

Fractions 67–81 gave crystals of xanthotoxin, m.p. 144–145°; λ_{max}(EtOH) 221, 242 (shoulder), 247, 260 (shoulder), and 297 mμ. The n.m.r. signals were located at $\delta = 6.32$ (H_3) and 7.72 (H_4), both doublets of $J = 10$ c.p.s.; at 7.31 (H_5), 7.65 ($H_{2'}$), 6.78 ($H_{3'}$), and 3.25 (aromatic OCH_3). Authentic xanthotoxin was identical in all respects. The yield was 260 mg; abundance = 0.87 mg/g.

Isopimpinellin

Fractions 82–88 contained isopimpinellin, deposited as very pale yellow needles, m.p. 147–149°. (In some plants the amount of xanthotoxin present was so large relative to isopimpinellin that the latter was best purified in small quantities by g.l.c.) λ_{max}(EtOH) 221, 240, 247, 266–270 (broad), and 311 mμ. The n.m.r. signals, located at $\delta = 6.25$ (H_3) and 8.08 (H_4), both doublets of $J = 10$ c.p.s., and at 7.59 ($H_{2'}$) and 6.96 ($H_{3'}$), doublets of $J = 2$ c.p.s., showed the presence of unsubstituted pyrone and furan rings. Two aromatic methoxyl groups were indicated by a six-proton signal at $\delta = 4.14$. Authentic isopimpinellin was identical in all respects to the isolated material. Estimated leaf content = 34 mg; abundance = 0.11 mg/g.

Oxypeucedanin

Fractions 80–90 from one strain of plants deposited this racemic compound as prisms, m.p. 140–142°. The u.v. spectrum, with λ_{max}(EtOH) 221, 247, 257, 266, and 305 mμ, indicated a bergapten-type nucleus. The n.m.r. signals were seen at $\delta = 6.24$ (H_3) and 8.15 (H_4), doublets of $J = 10$ c.p.s.; 7.11 (H_8), 7.57 ($H_{2'}$), and 6.92 ($H_{3'}$) for a total of 5 aromatic protons. In addition there were multiplets centered at $\delta = 4.49$ (CH_2) and 3.20 (saturated CH) and sharp three-proton singlets at 1.40 and 1.32 (aliphatic CH_3). Although no authentic racemic sample was available for comparison, (+)-oxypeucedanin gave the same spectra. Abundance in the strain = 0.46 mg/g.

Unidentified Coumarins

U_1, U_2, and U_3 from fractions 82–88 emerged from the gas chromatograph at retention times of 6.2, 6.6, and 8.0 (relative to angelicin = 1.0). All had u.v. maxima at 221, 242 (shoulder), 248, 261 (shoulder), and 298 mμ, suggesting xanthotoxol derivatives.

U_4 from fractions 54–62, relative retention time 3.9, had λ_{max}(EtOH) 218, 247, 256, and 320 mμ, indicating a coumarin without a furan ring. No available coumarin derivative co-chromatographed (e.g. osthol, relative retention time (r.r.t.) 2.6 and herniarin, r.r.t. 1.08).

U_5 from fractions 48–56 was not always present. Its retention times relative to angelicin under several conditions of g.l.c. exactly matched those of psoralen, (r.r.t. = 1.15 under stated g.l.c. conditions).

Development of Coumarins in the Plant

Plants of the same strain were taken 30, 60, 90, 120, and 144 days after germination and separated into weighed root and shoot portions. Both portions were worked-up as above, but the ether-soluble fractions were analyzed directly by g.l.c. The same conditions were used for these analyses as for those above: a 0.25 in. × 10 ft copper column, packed with 5% silicone (SE-30) on acid-washed Chromosorb W-DMCS support (70–80 mesh), was operated at 205°. Injector and detector temperatures were 252 and 264° respectively. A helium flow of 60 ml/min was employed. Imperatorin, most phenolic coumarins, and many complex neutral coumarins do not pass through the g.l.c. column under these conditions, but other simple neutral coumarins could be separated and quantitated in this way. Details of this method and its further applications will appear elsewhere.

Osthol

This coumarin was found at a retention time of 2.6 relative to angelicin in the gas chromatograph. It was collected and crystallized to m.p. 82–84° (lit. m.p. 84° (2)); λ_{max}(EtOH) 219, 247, 256, and 320 mμ indicated the absence of a furan ring. The n.m.r. spectrum showed doublets at $\delta = 6.19$ (H_3), 7.59 (H_4), 7.28 (H_5), and 6.82 (H_6), altogether 4 aromatic protons. Other signals were present at $\delta = 3.52$ (CH_2), 5.23 (unsaturated CH), 3.90 (aromatic OCH_3), 1.83, and 1.67 (vinyl methyls). Authentic osthol gave identical spectra. Finally, a mixed melting point of the sample with authentic osthol showed no depression.

Acknowledgments

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