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PATHWAYS OF SALICYL ALCOHOL AND SALICIN FORMATION IN *SALIX PURPUREA* L.

M. H. ZENK

Botanisches Institut der Universität München, Germany

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Abstract—Salicyl alcohol (saligenin) was shown to be formed in leaf tissue of *Salix purpurea* from phenylpropanoid compounds rather than from acetate or glucose. β-labelled *o*-coumaric acid proved to be the best precursor of this alcohol, while salicylic acid and its β-glucoside was not reduced to saligenin or salicin. In contrast, benzoic acid and benzyl alcohol were incorporated into salicin. By means of competition experiments and direct isolation it could be shown that salicylaldehyde and its *o*-glucoside (helicin) are intermediates in the formation of salicin in this species. The results establish the existence of a pathway: cinnamic acid→*o*-coumaric acid→(salicyloyl-CoA ?)→salicylaldehyde→helicin→salicin, and: benzoic acid (benzyl alcohol)→benzaldehyde→salicylaldehyde→helicin→salicin. The report that saligenin is not a direct precursor of salicin was confirmed using labelled salicyl alcohol. It was incorporated into *o*-hydroxybenzyl-β-D-glucoside which does not occur naturally in *Salix*.

INTRODUCTION

BENZOIC acids [1–4] and benzaldehydes [5] are formed in higher plants from the correspondingly substituted cinnamic acids presumably by β-oxidation.[6,7] In order to get more information on the biosynthesis of these aromatic C_6–C_1 compounds, especially on the mechanism of reduction,[7] we have now studied the biosynthesis of salicyl alcohol (*o*-hydroxy benzylalcohol, saligenin). This alcohol occurs in members of the Salicaceae in rather large amounts[8] as the phenol glucoside, salicin, which was the first glucoside found in nature.[9] The phenolic glucosides of Salicaceae have recently been comprehensively investigated and reviewed by Thime.[10]

Salicin biosynthesis is also of interest because it was shown that salicyl alcohol (XII, Fig. 1) is not the direct precursor of salicin in higher plants, but is instead converted to *o*-hydroxybenzyl-β-D-glucoside[11] (XIII). Also this latter compound, and not salicin as was formerly claimed,[12] is formed when salicyl alcohol is incubated in the presence of enzymes from wheat germ and uridine diphosphate glucose (UDPG). Pridham and Saltmarsh[11] have proposed therefore that free salicyl alcohol cannot be the direct precursor of salicin, and that it is probable that salicylic acid-β-glucoside[13] is reduced to salicin. These questions have now been investigated using ^{14}C-labelled precursors.

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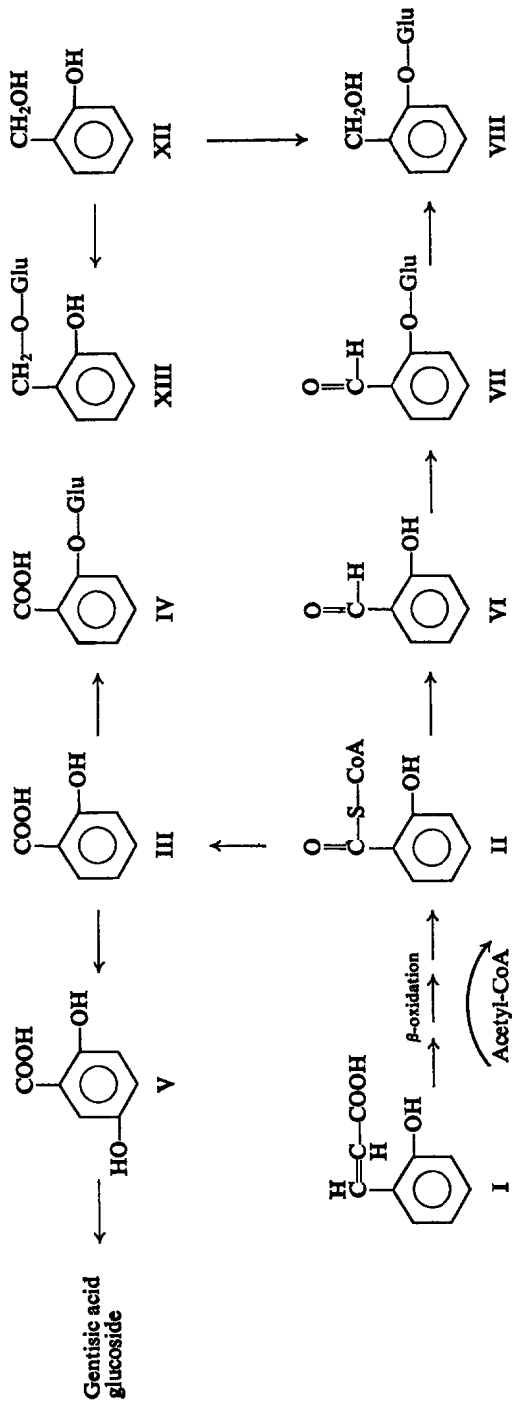

FIG. 1. SUGGESTED SCHEME OF THE BIOSYNTHESIS OF SALICIN IN *S. purpurea*.

RESULTS

Comparison of Labelled Compounds as Precursors of Salicyl Alcohol and Salicin

The first question arising is whether salicyl alcohol itself is formed in *Salix* via the acetate-, shikimate-, or phenylalanine pathway. Feeding experiments (Table 1) show clearly that phenylalanine is a precursor of this benzyl alcohol while neither acetate nor glucose is incorporated. It seems clear therefore that the biosynthesis of salicyl alcohol involves the cinnamic acid pathway as in the case of the benzoic acids and of vanillin in higher plants. This is further confirmed by the finding that cinnamic acid is readily incorporated into saligenin. By analogy with the biosynthetic schemes of other C_6–C_1 compounds[7] we would assume that a cinnamic acid with the same substitution pattern as the end product would give the highest incorporation rate. This is indeed the case. As shown in Table 1 *o*-coumaric acid is incorporated to about 22 per cent.

TABLE 1. INCORPORATION OF POTENTIAL PRECURSORS INTO SALICYL ALCOHOL BY *S. purpurea*

Compound administered*	Specific activity (cpm/ μmole × 10^6)	Radio-activity taken up by leaves (cpm × 10^6)	Salicyl alcohol isolated			
			μmole/g fresh wt.	Specific activity (cpm/ μmole)	Incorporation %	^{14}C-dilution
Acetate-2-^{14}C	0·52	1·29	44·0	0	0	∞
Glucose-6-^{14}C	1·05	2·63	41·5	0	0	∞
Phenylalanine-β-^{14}C	1·22	1·57	45·6	346	1·00	3540
Cinnamic acid-β-^{14}C	0·16	0·34	41·0	486	5·92	323
o-Coumaric acid-β-^{14}C	0·51	0·96	49·5	4400	22·5	117
Salicylic acid-(COOH)-^{14}C	0·57	1·43	44·0	77	0·237	7429
Benzoic acid-(COOH)-^{14}C	0·58	1·43	45·0	2470	7·72	232
Benzyl alcohol-(CH_2OH)-^{14}C	0·40	0·91	44·25	1970	9·6	203

* The compounds were supplied in 2×10^{-4} M solution (a total of 2·5 μmoles/g fresh weight).

The next question is whether salicin and hence salicyl alcohol can be formed by reduction of salicylic acid or its phenolic glucoside. Application of carboxyl-labelled salicylic acid gave an incorporation of only 0·2 per cent, 100 times less than the incorporation rate of *o*-coumaric acid. Salicylic acid was predominantly converted to salicylic acid-β-D-glucoside. In very young leaves of *S. purpurea* and *S. viminalis* there was a conversion of up to 30 per cent of the salicylic acid administered to a gentisic acid glucoside. This gentisic acid glucoside was found also to occur naturally; the position of the glucose on the acid molecule has not yet been determined. In no case a significant incorporation (higher than 0·3 per cent) of salicylic acid to salicyl alcohol was observed. This indicates strongly that the reduction of salicylic acid or its glucoside cannot be the major route of biosynthesis of salicin in Salicaceae as previously suggested.[11]

Surprisingly, however, in contrast to salicylic acid, benzoic acid was well incorporated (8 per cent) into salicyl alcohol (Table 1). Since salicylic acid was found not to be an intermediate in the biosynthesis of saligenin, we have to assume that *ortho*-hydroxylation of benzoic acid was not the first step, but rather that reduction of benzoic acid to benzaldehyde or benzyl alcohol precedes it. To test this possibility, benzyl alcohol was fed to the *Salix* tissue and again a rather high rate of incorporation (10 per cent) was observed. If benzyl alcohol

were directly *ortho*-hydroxylated to give salicyl alcohol we would not expect to find salicin but rather *o*-hydroxybenzyl glucoside in the tissue, as discussed in the introduction. To solve this question the nature of the isomeric glucosides of salicyl alcohol which were formed after feeding different precursors to *Salix* tissue were investigated. Table 2 and Fig. 1 show the results of this experiment. Cinnamic and benzoic acid as well as benzyl alcohol yield only, or in the case of benzyl alcohol predominantly (99 per cent), salicin while labelled salicyl alcohol is incorporated only 46 per cent into salicin, 54 per cent being found in the isomeric glucoside with the free phenolic group. In a different plant tissue, *Helianthus annuus*, almost 100 per cent of the salicyl alcohol administered was incorporated into *o*-hydroxybenzyl glucoside. These results indicate that neither the hydroxylation of benzoic acid nor of benzyl alcohol can be the first step in the conversion of these compounds to salicin. Thus we have to assume that in the tissue of Salicaceae, benzoic acid is reduced and benzyl alcohol is oxidized to benzaldehyde which is *ortho*-hydroxylated to salicylaldehyde. This aldehyde in turn can be glucosylated to salicylaldehyde glucoside (helicin) which occurs in *Spiraea*[14] and is also found after feeding salicylaldehyde to leaf disks.[15] Helicin could then be easily reduced further to salicin, a route

TABLE 2. DISTRIBUTION OF RADIOACTIVITY FROM DIFFERENT PRECURSORS IN THE TWO ISOMERIC SALICYL ALCOHOL GLUCOSIDES

Precursor	% in Salicin	% in *o*-Hydroxybenzyl glucoside
Salix		
Cinnamic acid	100	0
Benzoic acid	100	0
Benzyl alcohol	98·8	1·2
Salicyl alcohol	46·4	53·6
Helianthus		
Salicyl alcohol	2·2	97·8

which has already been proposed in salicin biosynthesis.[11] Salicylaldehyde on the other hand could also be formed by reduction of salicyloyl-CoA an intermediate in the β-oxidation of *o*-coumaric acid. The aldehyde would then be glucosylated by UDPG as mentioned above. This pathway (probably the major one, as judged from the high incorporation rate of *o*-coumaric acid) would parallel almost exactly the suggested biosynthetic route of vanillin and glucovanillin in *Vanilla* bean tissue.[5]

Isotope Competition

In order to establish the role of salicylaldehyde in salicin biosynthesis an isotope competition experiment was conducted by feeding labelled benzoic acid in the presence of different amounts of unlabelled salicylaldehyde. If salicylaldehyde were an intermediate we would expect it to affect the incorporation rate of benzoic acid into salicin and, as shown in Fig. 2, this was found to be the case. In the presence of one and a half times the amount of salicylaldehyde, ^{14}C-benzoic acid conversion to salicin is inhibited by about 50 per cent; with five times

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more salicylaldehyde present in the incubation mixture the formation of labelled salicin is blocked completely. Besides salicin, there are two unidentified compounds present in the water-soluble neutral glucoside fraction into which benzoic acid is incorporated. Their synthesis is much less affected by salicylaldehyde (Fig. 2). However, the uptake of benzoic acid by the leaf tissue is also strongly inhibited by salicylaldehyde, probably by competing for the same carrier systems. These results strengthen the assumption that free salicylaldehyde is an intermediate in the biosynthesis of salicin.

Isolation of Radioactive Helicin after Supplying ^{14}C-Benzoic Acid

If the assumption is correct, that salicylaldehyde and helicin are intermediates in the conversion of *o*-coumaric- and benzoic acid to salicin, there must be a certain stationary pool of helicin in the leaf tissue. The pool size of helicin inside the cell is probably extremely small. However, attempts were made using ^{14}C-benzoic acid to detect labelled helicin if present. *Salix* leaf disks were incubated with ^{14}C-benzoic acid for 8 hr and the glucoside fraction isolated. Unlabelled carrier helicin was added and β-glucosidase (emulsin) added. After 14 hr, the incubation mixture was acidified, steam distilled and the aldehydes precipitated as 2,4-dinitrophenylhydrazones. The hydrozone fraction contained 321 c/min in the sample which was treated with emulsin. In contrast, the control experiment without hydrolytic cleavage of the glucosides contained no radioactivity in spite of a small amount of hydrazone formation. The labelled hydrazone fraction was subjected to thin-layer chromatography in two solvents whereupon 60 per cent of the original radioactivity present could be found in the zone of authentic salicylaldehyde-2,4-dinitrophenylhydrazone. This would mean that about 0·027 per cent of the benzoic acid is incorporated into helicin and that the pool size of helicin inside the cell is in the order of 3×10^{-7} M. These findings, together with the isotope competition experiments, strongly support the assumption that helicin is also an intermediate in the formation of salicin.

DISCUSSION

The results of the studies reported here provide evidence that benzyl alcohols like the aromatic acids and aldehydes of the C_6–C_1 group are derived from the phenylpropane metabolism in higher plants. As summarized in Fig. 3, the major pathway of biosynthesis of

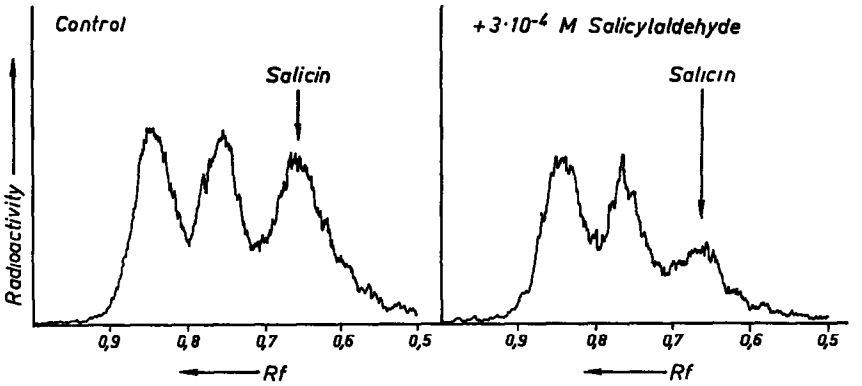

FIG. 3. SCANNING RECORD OF PAPER CHROMATOGRAMS OF THE GLUCOSIDE FRACTION OF *S. purpurea* LEAVES FED WITH A 2×10^{-4} M SOLUTION OF BENZOIC ACID-(COOH)-^{14}C IN THE ABSENCE (CONTROL) AND PRESENCE OF 3×10^{-4} M UNLABELLED SALICYLALDEHYDE.

salicyl alcohol involves deamination of phenylalanine to cinnamic acid,[16] the latter being *ortho*-hydroxylated to *o*-coumaric acid (I). *o*-Coumaric acid must be β-oxidized in the manner shown for benzoic acids[1–4] and vanillin.[5] This β-oxidation most probably follows the classical way of removing a C_2 fragment from the side chain of the phenylacrylic acids. The proof that this C_2-unit is indeed acetate was obtained by degrading aspartic and glutamic acid after feeding *p*-coumaric acid carboxyl-^{14}C to wheat tissue[6] and of the malonic acid moiety of malonyl-D-tryptophan after feeding cinnamic acid (α-^{14}C) and D-tryptophan simultaneously to *Salix* leaves.[7] The β-oxidation of *o*-coumaric acid should give rise to salicyloyl-CoA (II) which may be an important branching point. The thiolester could be enzymatically hydrolysed to salicylic acid (III), which is immediately converted to salicylic acid β-glucoside (IV), or III could be *meta*-hydroxylated to give gentisic acid (V) which is also stored as a glucoside. Salicyloyl-CoA (II) could, however, also be reduced to salicylaldehyde and the latter converted to helicin (VII) in exactly the same manner as it was suggested for vanillin biosynthesis.[5] Helicin can then be reduced further to salicin (VIII). Furthermore benzoic acid (IX) which arises from phenylalanine by β-oxidation of cinnamic acid,[17, 7] was shown to be most probably reduced first to benzaldehyde (X), while benzyl alcohol (XI) was oxidized to benzaldehyde. We believe that this compound is *ortho*-hydroxylated to give salicylaldehyde (VI). The reduction of benzoic acid to benzaldehyde must also involve the activation of this compound, most probably via a CoA-thiolester or an activated intermediate as found in the reduction of veratric acid by fungi.[18] There were two reasons for assuming that salicylaldehyde is an intermediate in the biosynthesis of salicin: (1) there could hardly be a one-step reduction of an activated C_6–C_1 compound directly to the alcohol and (2) Pridham and Saltmarsh[11] made the important finding, that in some plants salicyl alcohol (XII) is not converted to salicin but rather to *o*-hydroxybenzyl β-glucoside (XIII). The evidence for a participation of salicylaldehyde (VI) and helicin (VII) in the biosynthesis of salicin has now substantially been increased by isotope competition experiments and by the isolation of radioactive helicin after supplying ^{14}C-benzoic acid. Since salicylic acid and its glucoside are definitely not reduced to a major extent to salicin, the existence of an activated intermediate in the β-oxidation of *o*-coumaric acid such as salicyloyl-CoA has become more likely. This thiolester could directly be reduced to the aldehyde.

EXPERIMENTAL

Cultivation of Plants

Salix purpurea was propagated by cuttings from a single plant. The cuttings were grown in pots in a growth chamber at 21° and 80 per cent humidity receiving a 15 hr day. The light was supplied by banks of five Philips fluorescent lamps (white), giving 4500 lx at the upper level of the pots. The plants were allowed to grow about 45 cm in height and the youngest ten to fifteen leaves were used. It was important to grow the plants under completely standardized conditions since otherwise very large differences are noticed in salicin content and in the plant's ability to convert precursors. *Helianthus annuus* was grown under the same conditions.

^{14}C-Labelled Compounds

Acetate-2-^{14}C, benzoic acid (COOH)-^{14}C, benzyl alcohol (CH_2OH)-^{14}C, benzaldehyde (CHO)-^{14}C, glucose-2-^{14}C, salicylic acid (COOH)-^{14}C were purchased from the Radio-

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[18] M. H. ZENK and G. G. GROSS, *Z. Pflanzenphysiol.* **53**, 356 (1965).

chemical Centre, phenylalanine-β-^{14}C from Calbiochem. *o*-Coumaric acid-β-^{14}C was a gift from Professor Grisebach and Dr. Vollmer. Cinnamic acid β-^{14}C was prepared from benzaldehyde (CHO)-^{14}C[19]. Salicyl alcohol (CH_2OH)-^{14}C was synthesized according to the following procedure:

8 mg salicylic acid (COOH)-^{14}C (0·1 mc) in 4 ml dry ethyl ether were added dropwise to a vigorously stirred suspension of 30 mg $LiAlH_4$ in 3 ml dry ether over a 10 min period. An additional 4 ml of ether were added and the mixture heated to reflux for 20 min. After cooling, 2 ml water were added to decompose excess AlH_4 and the mixture acidified with 2 ml 2 N H_2SO_4. The ether layer was separated from the aqueous phase which was extracted with additional ether and the combined ether extracts evaporated to dryness. The residue was taken up in alcohol and subjected to preparative descending chromatography using benzene: glacial acetic acid: H_2O = 4:2:1 as solvent. The zone (R_f: 0·36) containing the salicyl alcohol was eluted and rechromatographed ascending in isopropanol: NH_3: H_2O = 8:0·1:1·9 (R_f: 0·90). The labelled salicyl alcohol was radiochemically pure as shown by chromatography in three different solvents. The yield was better then 75 per cent based on ^{14}C-salicylic acid; spec. activity was 1·55 μc/μmole.

Administration of Labelled Compounds

The compounds investigated as precursors were administered to uniform leaf squares of about 5 mm side length as previously reported.[5] 1 gm of tissue was shaken gently (sixty-four strokes per min) in the dark in 200-ml Erlenmeyer flasks containing 12·5 ml of a 2×10^{-4} M solution of the labelled precursor, carefully adjusted to the pK of the compound under investigation; the incubation medium was buffered with 10^{-2} M citrate-phosphate buffer of the corresponding pH value. If not otherwise mentioned incubation proceeded for 24 hr at 21°.

Isolation of Glucosides

The thoroughly washed leaf tissue was extracted with a total of 50 ml 80 per cent ethanol at reflux. The alcoholic extract was evaporated to dryness and the residue extracted with boiling water. The insoluble residue was centrifuged off, washed with additional water and the combined clear heavily coloured aqueous extract (20 ml) was passed through a column of polyamide (Durethan BK; 8 × 1 cm). The column was washed with water, a total of 50 ml of clear colourless eluate was collected. The eluate was taken to dryness *in vacuo*, the residue dissolved in 50 per cent ethanol and streaked on Schleicher and Schüll 2043b chromatography paper. The chromatograms were developed ascending in butanol: ethanol: H_2O = 40:11:19. Salicin and *o*-hydroxybenzyl glucoside had identical R_f values of 0·66 in this solvent. These glucosides cannot be separated satisfactorily by paper chromatography,[11] but the separation is achieved easily by paper electroendosmosis. Whatman No. 1 paper was wetted with 0·02 N NaOH, a voltage of 13 V/cm was applied for 5 hr with a refrigerated electrophoresis apparatus "Mini 65" from Vetter and Hormuth, Heidelberg. Salicin moved towards the cathode while *o*-hydroxybenzyl glucoside moved towards the anode (see Fig. 1). Salicin was located by spraying with Millons reagent[20] and its isomer by use of diazotized sulfanilic acid.

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[20] H. Thime, *Pharmazie* 19, 471 (1964).

Isolation of Salicyl Alcohol

In those cases where the specific activity of the salicyl alcohol had to be determined, 3 ml of sweet almond emulsin[21] were added, to the polyamide eluate mentioned above. After hydrolysis for 20 hr the incubation mixture was acidified with 2 ml 2 N H_2SO_4, the precipitated protein centrifuged off and the supernatant exhaustively extracted with 3 × 80 ml ether. The combined ether extracts were taken to dryness and the residue chromatographed descending with benzene:glacial acetic acid:H_2O=4:2:1 as solvent. The solvent was allowed to drip from the end of the paper for about 3 hr. The salicyl alcohol band was eluted from the paper with methanol and was radiochemically pure as checked by thin-layer chromatography in different solvents. The concentration of salicyl alcohol was determined by its absorbance at 275 nm ($\epsilon_{275}=2{\cdot}25\times10^3$[cm^2/m-mole]). In each case the total u.v. absorption spectrum of the isolated salicyl alcohol was recorded to confirm its purity.

Preparation of Glucosides

Helicin (m.p., 174–175°) was prepared according to Robertson and Waters [22] by Mr. G. Schindler in this laboratory. *o*-Hydroxybenzyl β-D-glucoside was prepared biosynthetically by feeding a 10^{-2} M aqueous solution of salicyl alcohol to *Helianthus* hypocotyls through the cut ends. After standing for 10 days in the greenhouse the plants were worked up as described for the isolation of glucosides. The compound crystallized on concentrating the polyamide eluate. After recrystallization from water-saturated ethyl acetate, the colourless needles showed a m.p. of 67–69°.

Measurement and Detection of Radioactivity

^{14}C-Labelled compounds were counted at infinite thickness on aluminium planchettes in a methane-gasflow counter FH 411 (Frieseke and Hoepfner, Erlangen). Radioautograms were prepared from chromatograms by contact with Agfa-X-ray paper. Chromatograms in some cases were also scanned with a paper strip counter (Fa. Berthold, Wildbad).

Thin-Layer Chromatography

The 2,4-dinitrophenylhydrazone of salicylaldehyde was chromatographed on 250 μ layers of Kieselgel GF_{254} (Merck) with benzene:petrolether (30–70°)=3:1. The plates were developed twice to obtain better separation (salicylaldehyde-2,4-dinitrophenylhydrazone, R_f=0·6). This band was scraped off and rechromatographed on another plate with ethylacetate:ligroin=1:2; as solvent and again developed twice (R_f=0·5).

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Note added in proof: *o*-Hydroxybenzyl β-D-glucoside was recently found to occur in flowers of *Filipendula ulmaria* and termed isosalicin (H. THIME, *Pharmazie* **21**, 123 (1966)).

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[22] A. ROBERTSON and R. B. WATERS, *J. Chem. Soc.* 2729 (1930).