Bargetzi, J. P., Sampath Kumar, K. S. V., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1468.
Bargetzi, J. P., Thompson, E. O. P., Sampath Kumar, K. S. V., Walsh, K. A., and Neurath, H. (1964), *J. Biol. Chem. 239*, 3767.
Bradshaw, R. A., Babin, D. R., Nomoto, M., Srinivasan, N. J., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 3859.
Cox, D. J., Bovard, F. S., Bargetzi, J.-P., Walsh, K. A., and Neurath, H. (1964), *Biochemistry 3*, 44.
Davies, R. C., Riordan, J. F., Auld, D. S., and Vallee, B. L. (1968), *Biochemistry 7*, 1090.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 124*, 404.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Gross, E., and Witkop, B. (1961), *J. Am. Chem. Soc. 83*, 1510.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Lipscomb, W. N., *et al.* (1968), *Brookhaven Symp. Biol. 21*, 24.
McClure, W. O., Neurath, H., and Walsh, K. A. (1964), *Biochemistry 3*, 1897.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Peterson, E. A., and Sober, H. A. (1959), *Anal. Chem. 31*, 857.
Pétra, P. H., Bradshaw, R. A., Walsh, K. A., and Neurath, H. (1969), *Biochemistry 8*, 2762.
Pétra, P. H., and Neurath, H. (1969), *Biochemistry 8*, 2466.
Sampath Kumar, K. S. V., Clegg, J. B., and Walsh, K. A. (1964b), *Biochemistry 2*, 1728.
Sampath Kumar, K. S. V., Walsh, K. A., and Neurath, H. (1964a), *Biochemistry 3*, 1726.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.
Smith, E. L., and Stockell, A. (1954), *J. Biol. Chem. 207*, 501.
Walsh, K. A., Ericsson, L. H., and Neurath, H. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1339.
Whitaker, J. R., Menger, F., and Bender, M. L. (1966), *Biochemistry 5*, 386.

Monoterpene Biosynthesis. I. Occurrence and Mevalonoid Origin of Gentiopicroside and Loganic Acid in *Swertia caroliniensis**

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ABSTRACT: Gentiopicroside (**1**), a secoiridoid monoterpene glucoside, and loganic acid (**2**), a new naturally occurring iridoid monoterpene glucoside, have been isolated from the same higher plant, *Swertia caroliniensis*. These congeners have been characterized by chemical and spectral means. Double-resonance nuclear magnetic resonance studies corroborated a recently revised structure **1** for gentiopicroside, isomeric to the previously designated one **1a**. Loganic acid has been identified by methylation to loganin (**3**), and acetylation to pentaacetate derivatives. Tracer experiments with acetate-2-^{14}C, mevalonate-2-^{14}C (**6**), and mevalonate-2-^{3}H have established the mevalonoid origin of the aglucone moieties of the glucosides. Conversion of mevalonate-labeled gentiopicroside into gentianine (**4**) has revealed all of the radioactivity occurs in the aglucone. Decarboxylation of the gentianine has afforded barium carbonate from C-11 with 20% of the total activity. Loganic acid derived from mevalonate-2-^{3}H-2-^{14}C has been converted to 7-oxologanin (**5**) with the loss of 63% of total tritium. Kuhn–Roth oxidation of the methyl ester (loganin (**3**)) of loganic acid which was derived from mevalonate-2-^{14}C has afforded acetic acid from C-10 and C-8 possessing only 0.7% of the total radioactivity. These results are consistent with a biogenetic scheme wherein mevalonate (**6**) is converted *via* geranyl pyrophosphate (**7**) into loganic acid (**2**) which then undergoes ring cleavage to afford gentiopicroside (**1**) (Scheme I).

Gentiopicroside (**1**) and loganic acid (**2**) belong to a class of highly oxygenated monoterpene glucosides referred to as secoiridoids and iridoids, respectively. (For a review of the iridoids (cyclopentanoid monoterpenes), see Bobbitt and Segebarth, 1969.) Chemical studies on this group of plant metabolites has led to the recognition of a structural resemblance to the nontryptamine-derived moiety of the indole alkaloids (Thomas, 1961; Wenkert 1962) and subsequent tracer work has implicated them in indole alkaloid biosynthesis. Scott and collaborators were first to succeed in incorporating mevalonate-2-^{14}C into vindoline (**12**) in studies with *Vinca rosea* (Money *et al.*, 1965). His group and Arigoni's found that the distribution of label from mevalonate-2-^{14}C (**6**) in the *Aspidosperma* alkaloid suggested that its C_{10} segment was of cyclopentanoid monoterpenoid origin (McCapra *et al.*, 1965; Goeggel and Arigoni, 1965). The first cyclopentanoid mono-

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terpene intermediate identified was loganin (**3**) (Battersby *et al.*, 1966). Further work has revealed that the secoiridoids, secologanin (**9**), and sweroside (**10**) are also precursors for major indole alkaloids of the *Rauwolfia*, *Yohimbé*, *Strychnos*, and *Cinchona* genera. Leete (1969) has written a detailed chronicle of these developments exclusive of the sweroside work (Inouye *et al.*, 1968b, 1969b).

Plumieride was the first iridoid monoterpene demonstrated to be of mevalonoid origin (Yeowell and Schmid, 1964), but until recently no definitive biogenetic data for the secoiridoids had been available. Previous studies in higher plants revealed that acetate-1-^{14}C could be utilized in the synthesis of gentiopicroside (**1**) (Floss *et al.*, 1964) and swertiamarin (Sugii and Hashimoto, 1958) but the fate of the label was not determined. Proof of the monoterpenoid nature of gentiopicroside (**1**) and loganic acid (**2**) is presented in this report. Shortly after our preliminary communication (Coscia and Guarnaccia, 1967) analogous findings on gentiopicroside biosynthesis were published (Inouye *et al.*, 1967).

Materials and Methods[1]

Labeled Precursors. Radioactive substrates were purchased from Amersham-Searle Corp., Chicago, Ill. Labeled mevalonolactone was taken up in an equivalent amount of 0.01 N sodium hydroxide just prior to feeding and this solution was used without heating.

Radioassay. Samples were counted in a liquid-scintillation spectrometer (Packard Models 4000 or 3380) using an aqueous-scintillation fluid (Kinard, 1957). Barium carbonate was suspended in a Cab-O-Sil system which gave efficiencies comparable with the aqueous system (80%). Radioactive compounds were recrystallized until a specific activity with standard deviation of no more than 2.5% was realized in three successive recrystallizations.

Incorporation. Young sterile plants were collected in the wild in Southern Illinois from April through August. Healthy specimens were potted and placed in the Missouri Botanical Garden greenhouses until used. The plants ranged from 15 to 20 cm in length from the apex of the taproot to the basal rosette of 4 leaves and weighed 1–10 g. Initially plants were fed acetate-2-^{14}C hydroponically in 150 ml of nutrient solution (Ellis and Swaney, 1938) containing a commercial fungicide (Panodrench, Morton Chemical Co.). Although *Swertia* plants could be kept alive for a few weeks in this solution, the hydroponic method was abandoned when it was found mevalonate-2-^{14}C was not incorporated into the monoterpenoids.

The cotton wick technique (Gear and Spenser, 1963) was then adopted and proved to give good per cent incorporation of mevalonate-2-^{14}C. These feeding experiments were conducted in the laboratory on potted plants. Using a needle, a piece of unmercerized cotton thread was drawn through the stem 1–2 mm above the root issue (transition zone). The end of the wick was immersed in the solution of 25–50 μCi of radioactive tracer and within a few hours the solution was absorbed. This was followed by several successive washings and after all the water was taken up, the plant was harvested anywhere from 1 to 15 days after the feeding. All organs of the plant were radioactive with the highest activity in the leaves. Incorporations for gentiopicroside ranged between 0.02 and 0.11%, whereas those for loganic acid were between 0.08 and 1.2%. The limiting factor in these experiments appeared to be the condition of the plant. Healthy transplanted specimens, which readily adapted to the new environment, gave the best results. Per cent incorporation was calculated on the basis of total radioactivity present in the monoterpene glucoside divided by the total activity administered. Assuming that only the 3*R* isomer of racemic mevalonate-2-^{14}C is utilized by *Swertia* (as in *Vinca rosea*, Battersby *et al.*, 1968c), the total precursor activity is calculated to be one-half of that administered.

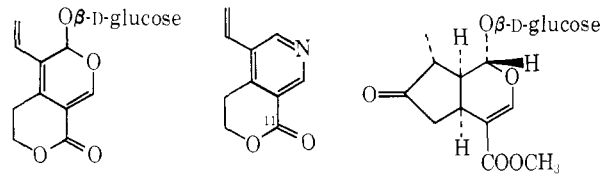
Isolation and Characterization of Gentiopicroside (**1**). The lability of gentiopicroside necessitates isolation from fresh plant tissue or plants which have been kept frozen. The diced plant material was homogenized in a Waring blender in 100 parts of either ethyl acetate or methanol. In large scale isolations, sugars[2] were obtained as crystalline residues from the methanol. After filtration and concentration of the filtrate, the brown syrupy residue was chromatographed on a column of silica gel G eluted with ethyl acetate–methanol (4:1) or chloroform–methanol (4:1). Thin-layer chromatography on silica gel G plates, developed with chloroform–methanol (4:1), was used to assay fractions for gentiopicroside (R_F 0.55). Compounds were detected with either iodine or sulfuric acid, the latter producing a characteristic blue-violet color. The amorphous hydrated gentiopicroside isolated in this manner melted at 118–121°. Recrystallization from mixtures of ethyl acetate–benzene–methanol (9:9:2) gave anhydrous crystalline gentiopicroside (**1**): mp 190°; $[\alpha]_D^{25}$ −217.6° (*c* 1, MeOH); ultraviolet spectra λ_{max}^{EtOH} at 247 (sh) (log ϵ 3.84), 255 (sh) (3.93) and 270 nm (3.97); infrared spectra λ_{max}^{Nujol} at 2.83, 2.89, 3.06, 5.84, 5.96, 6.20, 10.74, and 12.95 μ. *Anal.* Calcd for $C_{16}H_{20}O_9$ (356.32): C, 53.93; H, 5.66. Found: C, 54.39; H, 5.66. The mass spectrum of the trimethylsilyl derivative of gentiopicroside aglucone was *m/e* (relative intensity) 266 (M^+ 86%) and 176 (M − 90 100%).[3]

[1] All solvents used were reagent grade quality and were redistilled. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Silica gel was obtained from E. Merck, A.G. (Darmstadt, Germany). Analytical thin layer plates were prepared on glass at a 0.25-mm thickness whereas for preparative work a 0.75-mm thickness was used. Silica gel columns were eluted under nitrogen at a pressure of 2–5 psi. The absorbent to mixture ratio was 200:1 in these columns. All solvent mixtures are expressed in v/v ratios. Infrared spectra were determined with a Perkin-Elmer Model 21 spectrophotometer. For ultraviolet spectra, a Cary Model 14 recording spectrophotometer was used. Nuclear magnetic resonance spectra (60 and 100 MHz) were determined in $CDCl_3$ at ambient temperatures in Varian A-60, A-60A, and HA-100 spectrometers; chemical shifts are reported in δ (parts per million), using tetramethylsilane at δ 0.00 as an internal standard. Specific rotations were determined using a Rudolph photoelectric spectropolarimeter. Elemental analyses were performed by Galbraith Labs., Knoxville, Tenn.

[2] The plant contains substantial amounts of sucrose (3–4%) and a number of xanthones. The latter may be responsible for the alleged febrifugal properties of the *Gentianaceae* family (Stout and Balkenhol, 1969).

[3] The trimethylsilyl derivatives of gentiopicroside and loganic acid were prepared by the method of Sweeley *et al.* (1963) and subjected to gas chromatography–mass spectrometry. Using the LKB Model 9000, the mass spectra of the derivatives of **1** and **2** were obtained, but molecular ions were not observed. The highest detected peak under the gas chromatography conditions and at 70 eV was 15 mass units less in most cases. The trimethylsilyl derivatives of the aglucones of gentiopicroside and loganic acid gave the expected molecular ions and diagnostic fragmentation patterns. The detailed spectra and mode of fragmentation for these and related monoterpenoids will be reported elsewhere.

CHART I



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Acetylation of the hydrous or anhydrous form of gentiopicroside in equal amounts of pyridine and acetic anhydride at room temperature yielded the tetraacetate: mp 140°; $[\alpha]_D^{25}$ −159.5 (*c* 1, chloroform); ultraviolet spectra λ_{max}^{EtOH} at 248 (sh) (log ε 3.86), 254 (sh) (3.89), and 270 nm (3.94); infrared spectra $\lambda_{max}^{CHCl_3}$ at 5.70, 5.80, and 6.18 μ. *Anal.* Calcd for $C_{24}H_{28}O_{13}$ (524.46): C, 54.96; H, 5.38. Found: C, 54.66; H, 5.26.

Gentianine (**4**) (Chart I). To 100 mg of gentiopicroside tetraacetate dissolved in 10 ml of methanol, 10 ml of concentrated ammonium hydroxide was added. After refluxing for 2 hr the solvent was removed and the residue was refluxed for 0.5 hr in 3 ml of 16% hydrochloric acid. After ether extraction of the cooled aqueous solution, it was brought to pH 8 with ammonium hydroxide. The basic solution was extracted with chloroform, this organic phase was washed with water and dried, and the solvent was removed to afford a yellow semicrystalline solid. Chromatography on silica gel G with benzene–ethyl acetate (1:4) was followed by recrystallization from benzene–ethyl acetate–hexane: yield 15 mg; mp 81°; ultraviolet spectra λ_{max}^{EtOH} at 218 (log ε 4.42), 245 (sh) (3.93), and 285 (sh) nm (3.18); infrared spectra $\lambda_{max}^{CHCl_3}$ at 5.80, 6.30, 6.35, and 6.75 μ; nuclear magnetic resonance spectra at δ 3.12 (triplet C-6-H), 4.63 (triplet C-7-H), 5.75 (octet C-10-H, AB of ABX system, J_{AB} = 1.5 Hz), 6.90 (quartet C-8-H of ABX system, J_{AX} = 17.5 Hz, J_{BX} = 11 Hz), 8.91 (singlet C-3-H), and 9.23 (singlet C-1-H). *Anal.* Calcd for $C_{10}H_9NO_2$ (175.18): C, 68.56; H, 5.18; N, 8.00. Found: C, 68.83; H, 5.38; N, 7.84 (Canonica *et al.*, 1962; Plat *et al.*, 1963).

Gentianine Decarboxylation. In a typical experiment 20 mg of gentianine (**4**) was neutralized with 0.12 ml of 1 N sodium hydroxide by gentle warming in a tube from a wet combustion apparatus. Calcium chloride (11 mg) was dissolved in this solution which was then carefully evaporated to dryness. A saturated barium hydroxide solution was introduced into the other arm and the wet combustion apparatus was assembled. The tube containing base was frozen with acetone–Dry Ice. The system was evacuated and thereupon the solution of base was allowed to slowly warm to room temperature. Upon heating the gentianine salt to 375°, carbon dioxide evolved and after 20–40 min precipitation of barium carbonate appeared to be complete. In this manner 8.9 mg (40%) of barium carbonate was obtained. Without gentianine, no measurable barium carbonate was observed. A crude yellow oil (8 mg) remaining in the pyrolysis tube was chromatographed on silica gel G with benzene–methanol (95:5). The purified material (1 mg) possessed the same R_F as an authentic sample of isoquinoline; addition of methyl iodide gave crystals, mp 159°, lit. mp 159°.

Isolation and Characterization of Loganic Acid. The methanol extract obtained by homogenization of the plant material as described above was utilized in essentially three different procedures for the isolation of loganic acid. (a) The free glucoside was isolated by column chromatography (silica gel, particle size less than 0.08 mm), the eluting solvent being chloroform–methanol–water (49:49:2). Subsequently it was found that preparative thin-layer chromatography on acid-washed silica gel $HF_{254+366}$ with chloroform–methanol (1:1) was faster for small amounts. (b) The free glucoside was also obtained by means of ion exchange chromatography on Dowex 1-X8 (200–400 mesh) formate form (40–80 × the theoretical milliequivalents). After removal of sugars and other neutral substances by exhaustive washing with water, 0.1 M formic acid displaced the loganic acid. These columns were assayed by ultraviolet spectral studies as well as thin-layer chromatography (R_F 0.52). In the latter silica gel G plates were developed with chloroform–methanol–water (45:45:10). Upon spraying with sulfuric acid, loganic acid as well as loganin produced a characteristic deep violet color. The amorphous loganic acid was precipitated from acetone–benzene–methanol mixtures: mp 126–130 and 167–170°; ultraviolet spectra λ_{max}^{EtOH} at 233 nm (log ε 4.02); infrared spectra λ_{max}^{Nujol} at 3.05, 5.94, and 6.14 μ. The mass spectrum of the trimethylsilyl derivative of the aglucone of loganic acid was *m/e* 430 (M^+ 3.7%), and 197 (94%).[3] (c) The residue from the crude methanol extract was acetylated with equal amounts of pyridine and acetic anhydride at room temperature for about 12 hr. After removal of excess reagent by coevaporation with benzene, the product was chromatographed on silica gel G columns eluted with chloroform–benzene–methanol (45:45:10). Thin-layer chromatography assay of the acid pentaacetate was carried out with the same solvent system (R_F 0.5). Recrystallization from methanol gave plates of loganic acid pentaacetate; mp 168°; ultraviolet spectra λ_{max}^{EtOH} at 230 nm (log ε 4.02, hypsochromic shift in base); $[\alpha]_D^{25}$ −70.7 ± 0.5° (*c* 1, $CHCl_3$); infrared spectra $\lambda_{max}^{CHCl_3}$ at 2.87, 5.7, 5.95 (sh), 6.11, and 7.98 μ; nuclear magnetic resonance spectra at δ 1.05 (doublet C-10-H, J = 6 Hz), 4.2 (double doublet C-6′-H), 7.42 (doublet C-3-H, J = 1 Hz). *Anal.* Calcd for $C_{26}H_{34}O_{15}$ (586.43): C, 53.23; H, 5.84. Found: C, 53.16; H, 5.75.

Loganin (**3**). Loganic acid (**1**) was converted into loganin by treatment of a methanolic solution with ethereal diazomethane. Loganin was purified by preparative thin-layer chromatography on silica gel $HF_{254+366}$ plates with chloroform–methanol (4:1) as solvent. Recrystallization from acetone afforded pure loganin (**3**): mp and mmp 222–224°; ultraviolet spectra λ_{max}^{EtOH} at 235 nm (log ε 4.03); infrared spectra λ_{max}^{Nujol} at 2.87, 3.08, 5.84, and 6.08 μ. Acetylation of loganin or methylation of loganic acid pentaacetate gave loganin pentaacetate: mp and mmp 138–140°; $[\alpha]_D^{25}$ −75.7° (*c* 1, $CHCl_3$); ultraviolet spectra λ_{max}^{EtOH} at 232 nm (log ε 4.03); infrared spectra $\lambda_{max}^{CHCl_3}$ at 5.7, 5.84, and 6.09 μ; nuclear magnetic resonance spectra at δ 7.32 (doublet C-3-H, J = 1.5 Hz), 4.2 (double doublet C-6′-H), 3.72 (singlet OMe), and 1.00 (doublet C-10-H, J = 6 Hz).

7-Oxologanin Tetraacetate. To a stirred solution of 15 mg of loganin in 15 ml of acetone, 0.045 ml of a solution of chromic acid (700 mg of CrO_3 in 5 ml of H_2O and 0.6 ml of concentrated H_2SO_4) were added. Stirring was continued for 3 min at room temperature. After addition of ~200 mg of sodium bicarbonate, the reaction mixture was evaporated to one-third of the initial volume, and the precipitate was filtered and washed thoroughly with acetone–methylene chloride (1:1). After evaporation of part of the solvent, the mixture was purified by

preparative thin-layer chromatography (chloroform–methanol, 4:1) to 10 mg of crystalline material **5**: mp 194–197°; ultraviolet spectra λ_{max}^{EtOH} at 234 nm (log ϵ 4.02); infrared spectra λ_{max}^{Nujol} at 2.80–3.30, 3.24, 5.73, 5.95, 6.19, and 7.69 μ.

7-Oxologanin (**5**) was acetylated in the usual way. Purification by thin-layer chromatography (benzene–ethyl acetate, 1:1) yielded 7-oxologanin tetraacetate (10 mg) which was crystallized from ethanol–methylene chloride: mp 107–109 and 145–147°; $[\alpha]_D^{25}$ −147 ± 1° (*c* 1, $CHCl_3$); ultraviolet spectra λ_{max}^{EtOH} at 234 nm (log ϵ 4.00); infrared spectra $\lambda_{max}^{CHCl_3}$ at 5.69, 5.75, 5.87, and 6.10 μ; nuclear magnetic resonance at δ 1.13 (doublet C-10-H, J = 10 Hz), 1.88, 1.98, 2.03, 2.06 (4 CH_3CO), 2.57 (multiplet C-6-H), 3.67 (O-Me), 4.3 (multiplet, C-6′-H), and 7.35 (doublet C-3-H, J = 2.0 Hz). *Anal.* Calcd for $C_{25}H_{32}O_{14}$ (556.51): C, 53.95; H, 5.80. Found: C, 53.78; H, 5.85.

Results

Distribution. The higher plant chosen for these studies was *Swertia caroliniensis* (Walt) Kuntze (also known as *Frasera caroliniensis*), a member of the *Gentianaceae* indigenous to Missouri and Illinois (Steyermark, 1963). Gentiopicroside (**1**) is a major constituent of the plant roots (yield 2.5% on a fresh weight basis) whereas loganic acid (**2**) was found in lesser amounts (0.16%) in this tissue. The aerial parts, *i.e.*, stem and leaves, contained a greater distribution of loganic acid (0.86%) than of gentiopicroside (0.3%).

Revision of Gentiopicroside Structure. In the identification of gentiopicroside (**1**), both the free glucoside and its tetraacetate exhibited the same physical and spectral properties previously reported (Korte, 1954; Pesonen and Ramstad, 1956; Canonica *et al.*, 1961). However, these were not completely consistent with the accepted structure **1a** of gentiopicroside. The following chemical and spectral data support a revised structure **1** which is isomeric with the former.

In the ultraviolet spectrum of this amaroid the interaction of cross-conjugated chromophores engenders a 271-nm λ_{max} with shoulders at 251 and 247 nm. Both sweroside (**10**) and tetrahydrogentiopicroside (Canonica *et al.*, 1961) possess absorption maxima at 246 nm. The 271-nm absorption was lower than expected for the previously postulated structure **1a** containing the incorporated homoannular diene. Since cross-conjugation effects can give rise to hypsochromic as well as bathochromic shifts as large as 30 nm (Scott, 1964), either a transoid or a homoannular diene system was compatible with the ultraviolet spectra. Careful catalytic hydrogenation of the vinyl group affords a dihydrogentiopicroside with the same ultraviolet spectra (Inouye *et al.*, 1968c).

The proton magnetic resonance spectra of gentiopicroside and its tetraacetate provided definitive evidence for the revised structure. The 60-MHz spectrum of gentiopicroside tetraacetate exhibited a doublet[4] at δ = 7.37 ppm (J = 1.5 Hz) characteristic for the enol ether C-3 proton of the iridoids and secoiridoids (Briggs *et al.*, 1963) (Figure 1). Four distinct acetate methyl group signals can be observed; three of these have chemical shifts from 2.02 to 2.12 ppm (secondary alcohol esters) whereas the fourth, the C-6′ primary acetate, occurred at 1.97 ppm. These shifts were consistent with a β-D-glucose configuration wherein the acetyl groups occupy equatorial positions (Hall, 1964). Most of the remaining downfield monoterpene protons were obscured by those of the glucose and in the upfield region only a two-proton multiplet and two single hydrogen multiplets were discerned between 2.15 and 4.5 ppm. The former structure **1a** should integrate for seven protons between 2 and 4.5 ppm (C-6, 7, 5′, and 6′).

Assignment of these four upfield protons was achieved by utilization of double-resonance 100-MHz nuclear magnetic resonance spectroscopy. Using the frequency sweep technique it was possible to ascribe the 1H multiplet centered at 3.75 ppm and the 2H multiplet at 4.2 ppm to C-5′ and C-6′ hydrogens of glucose (see Figure 1). Coupling constants were consistent with treating these hydrogens as an ABX system. As seen in Figure 1, upon irradiation at δ = 3.75 ppm, the pair of doublets of doublets at 4.2 collapsed into a pair of doublets (J_{AB} = 12.5 Hz) characteristic of the nonequivalent hydrogens at C-6′. Alternatively irradiation of the protons of C-6′ afforded a doublet at 3.75 ppm representing the A doublet of an AX *trans* diaxial spin–spin coupling of C-5′ and C-4′ protons ($J_{4'5'}$ = 9 Hz).

Upon irradiation at 3.27 ppm, a doublet at δ = 5.40 ppm (C-1-H, $J_{1,9}$ = 2.3 Hz) collapsed to a sharp singlet and the doublet of doublets of doublets ascribed to C-8-H (5.47–5.82 ppm, $J_{8,9}$ = 7, $J_{8,10}$ = 9.5, and $J_{8,10'}$ = 17.5 Hz) becomes a doublet of doublets ($J_{8,10}$ = 9.5 and $J_{8,10'}$ = 17.5 Hz). If the C-8-H multiplet was irradiated (at 5.75 ppm), the 3.27-ppm double doublet collapsed into a broadened unsymmetrical singlet (with a width of 4 Hz at half-peak height) instead of an expected doublet (J = 2.3 Hz) which would represent the spin–spin interaction of the protons at C-9 and C-1 (Figure 1). This was assumed to be attributable to the occurrence at 5.40 ppm of an observable side band of the irradiating field giving rise to a partial decoupling of the C-9 and C-1 protons.

The high-resolution nuclear magnetic resonance spectra of gentiopicroside in either deuteriopyridine or dimethyl sulfoxide-*d* did not lend itself to a definitive analysis.

Identification of Loganic Acid. Characterization of loganic acid (**2**) was less difficult. Acetylation and/or methylation provided compounds which had physical properties consistent with those reported for loganin derivatives (Merz and Lehmann, 1957; Birch and Grimshaw, 1961; Sheth *et al.*, 1961). Noteworthy is the typical iridoid uv chromophore at 230 nm (log ϵ 4.2). A hypsochromic shift in base suggested the acidic nature of **2** as did pH measurements on the pure compound and infrared spectra ($\lambda_{max}^{CHCl_3}$ 5.95 μ for loganic acid pentaacetate). Upon careful methylation of pure loganic acid, with diazomethane, loganin (**3**) was the only product.

Other characteristics of the methyl cyclopentanoid monoterpenes were observed in the nuclear magnetic resonance spectrum of loganic acid pentaacetate, *i.e.*, a slightly broadened singlet at δ = 7.45 ppm (C-3-H) and a three-hydrogen doublet at 1.05 ppm (C-10-H, J = 6 Hz). Kuhn–Roth oxidation of loganin (**3**) afforded 1 mole of acetic acid and Jones oxidation gave the previously described 7-oxologanin (**5**) (Sheth *et al.*, 1961; Battersby *et al.*, 1969b).

Finally loganin (**3**) and loganin pentaacetate derived from loganic acid exhibited identical chromatographic properties and no depression in mixture melting points with authentic samples kindly provided by Professor J. Wolinsky.

Biosynthesis. In initial tracer studies, acetate-2-^{14}C was fed

[4] A possibility which cannot be excluded is that this signal is a poorly resolved quartet arising from long-range coupling of C-3-H with protons of C-6 as well as C-7 or C-9 (see Figure 1).

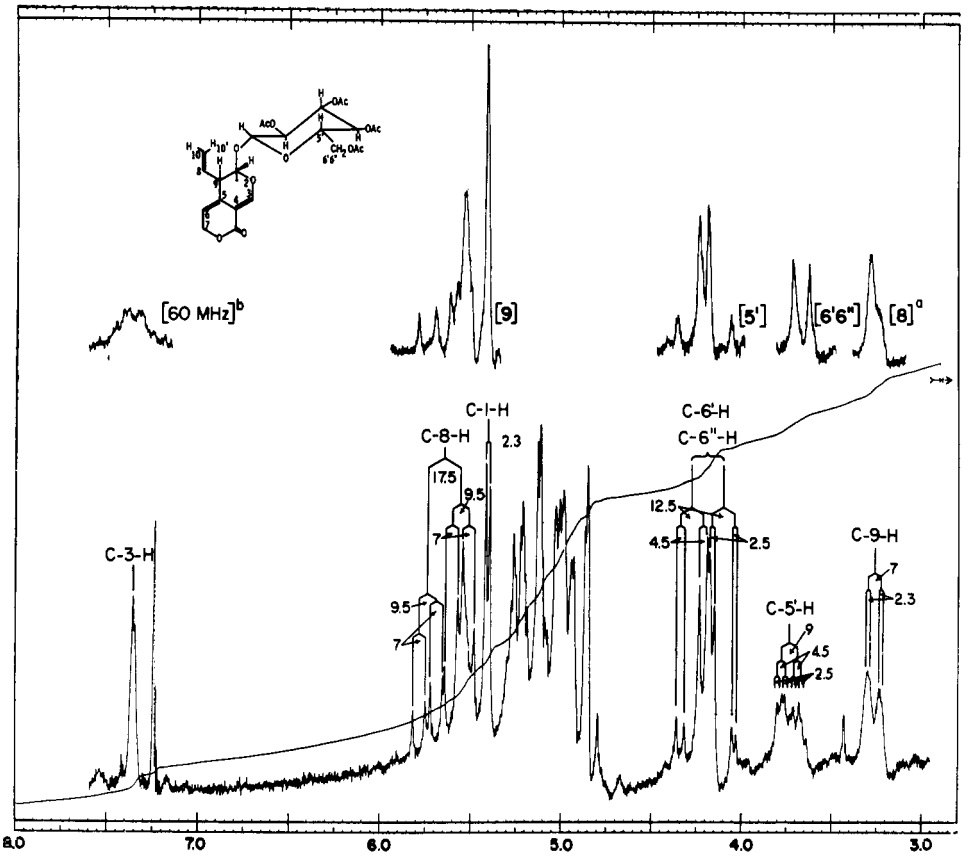


FIGURE 1: 100-MHz nuclear magnetic resonance spectra of gentiopicroside tetraacetate. (a) Boldface numerals in brackets represent the proton irradiated to effect the depicted perturbation. (b) The 60-MHz signals of C-3-H was measured at a sweep width of 100 Hz.

TABLE I: Distribution of Radioactivity in Monoterpene Glucosides.

	Sp Act. (μCi/mole)	% Rel Act.
Acetate-2-^{14}C derived		
Gentiopicroside tetraacetate	9.46	100
Gentianine (**4**)	5.86	62
Mevalonate-2-^{14}C derived		
Gentiopicroside tetraacetate	130	100
Gentianine (**4**)	130	100
$BaCO_3$ from C-11 of **4**	26	20
Loganin (**3**)	55.9	100
CH_3COOH from C-10 and C-8 of **3**	0.41	0.7

hydroponically to young *Swertia* plants and the isolated gentiopicroside (0.01% incorporation) was characterized as its tetraacetate. Degradation to gentianine (**4**) which retained the aglucone moiety resulted in recovery of 62% of the total radioactivity indicating labeling of carbohydrate and monoterpene (Table I).

Upon mevalonate-2-^{14}C administration to young specimens of *Swertia* by the cotton wick technique, loganic acid of higher specific activity than gentiopicroside was obtained (Coscia and Guarnaccia, 1968). Degradation of the tetraacetate of **1** to gentianine (**4**) revealed that all of the radioactivity was in the aglucone (Table I). Decarboxylation of the calcium salt of gentianine gave a barium carbonate containing 20% of the total radioactivity (Scheme I). Identical results were reported by Inouye and collaborators (1967).

Mevalonate-2-^{14}C-labeled loganic acid was crystallized to constant specific activity most expediently as its methyl ester loganin (**3**). After Kuhn–Roth oxidation of this loganin (**3**), the isolated acetic acid was purified as 1-acetamidonaphthalene (Leete *et al.*, 1965). As seen in Table I only 0.7% of the total activity was found in the acetate which represents carbons 8 and 10. This is in agreement with theory (Scheme I) and indicates little general randomization of label had occurred.

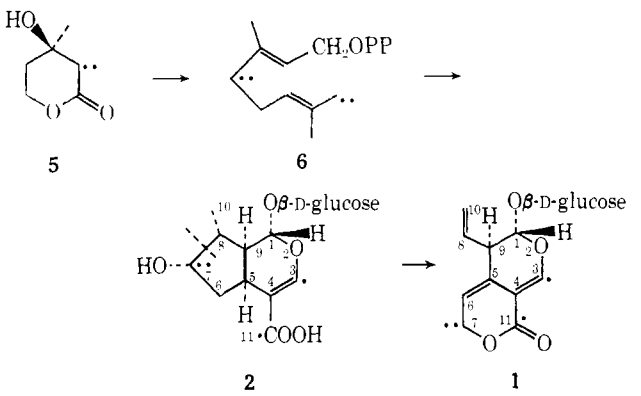
TABLE II: Distribution of Radioactivity in Mevalonate-2-^{3}H-2-^{14}C-Labeled Loganic Acid.

	Sp Act. (μCi/mole)		^{3}H/^{14}C ratio	
	^{3}H	^{14}C	Obsd	Theor
Mevalonate-2-^{3}H-2-^{14}C (**6**)			17	17
Loganin (**3**)	98.7	14.8	6.7	5.7
7-Oxologanin tetraacetate	36.1	14.5	2.5	1.4

After administration of mevalonate-2-^{14}C-2-^{3}H, loganic acid was isolated, recrystallized to constant activity as loganin (**3**), and subjected to Jones oxidation. The product, 7-oxologanin (**5**), characterized as its tetraacetate, retained ^{14}C but lost 63% of its tritium (Table II).

Repetition of this oxidation with inactive loganin in the presence of tritium oxide did not yield a radioactive 7-oxologanin (**5**). Hence enolization of the 7-ketone did not occur and the possibility of a loss of tritium from C-6 and C-8 instead of C-7 during oxidation was obviated. Comparison of ^{3}H/^{14}C ratios with theoretical values (Table II) also supported specific labeling of loganic acid as in Scheme I (see Discussion).

SCHEME I[a]



[a] The dots indicate the fate of label from C-2 of mevalonolactone.

Discussion

Inouye and coworkers (1968c) recently revised the gentiopicroside structure and configuration on the basis of chemical and spectral data. Concurrently, our biogenetic data prompted us to consider this alternate structure **1** (Guarnaccia *et al.*, 1969) and the herein reported double-resonance studies corroborate the new assignments.

The distribution of label in gentiopicroside and loganic acid derived from radioactive mevalonate was similar to that observed for cyclopentanoid monoterpenes (Yeowell and Schmid, 1964; Hüni *et al.*, 1966) and the indole alkaloids (McCapra *et al.*, 1965; Goeggel and Arigoni, 1965; Battersby *et al.*, 1966). As outlined in Scheme I, 2 moles of mevalonate (**6**) undergo conversion to geranyl pyrophosphate (**7**) which is subsequently oxidized and cyclized to a cyclopentanoid monoterpene. Randomization of the terminal dimethyl groups (either as methyl groups or in a higher oxidation state; Yeowell and Schmid, 1964; Auda *et al.*, 1967) occurred presumably after geranyl pyrophosphate formation (Guarnaccia *et al.*, 1969). Whether such equilibration of terminal dimethyl groups is age dependent or can occur for a fraction of the molecules in the intermediate pool (Auda *et al.*, 1967; Regnier *et al.*, 1968) was not answered in these studies. Measurement of ^{3}H/^{14}C ratios of loganic acid derived from mevalonate-2-^{3}H-2-^{14}C revealed a deviation from a theoretical value (Table II) calculated on the basis of a randomization mechanism (Scheme I). Since this increase was sustained in 7-oxologanin tetraacetate, either an enrichment of tritium occurred at C-3 after equilibration of C-3 and C-11 (Scheme I) or equilibration of C-3 and C-11 occurred for only a fraction of the molecules in the pool.[5] The possibility of no equilibration of C-3 and C-11 is excluded by our results in the decarboxylation of gentianine (Coscia and Guarnaccia, 1967; Inouye *et al.*, 1967).

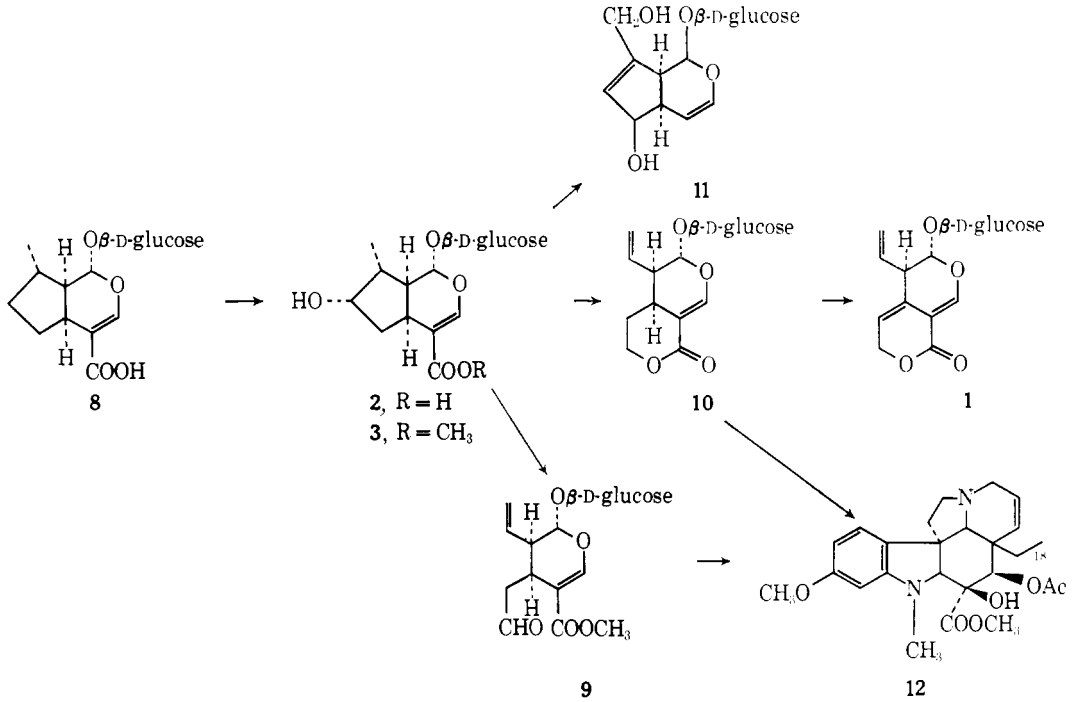
An isotope effect which could account for the higher ratio may have occurred through reversal of isopentenyl pyrophosphate–dimethylallyl pyrophosphate isomerization. The possibility of such a reversibility has been reported (Holloway and Popják, 1968) and could lead to tritium enrichment in dimethylallyl pyrophosphate. A small isotope effect may also occur in the oxidation of the terminal dimethyl groups to the aldehydo oxidation state. There is precedent for a tritium isotope effect during biological oxidation of methyl groups (Light, 1965).

Gentiopicroside is a common constituent of plants of the *Gentianaceae* (Canonica *et al.*, 1961), and its occurrence in *S. caroliniensis* was not unanticipated. The discovery of loganic acid as a natural product in plants, however, is unprecedented. Cooccurrence of iridoid and secoiridoid monoterpenes is biogenetically predictable and recently such congeners have also been demonstrated in *Menyanthes trifoliata* (Loew *et al.*, 1968; Battersby *et al.*, 1968a) as well as *V. rosea* (Battersby *et al.*, 1968b). Interestingly, loganin or loganic acid is the cyclopentanoid monoterpene in all of these cases and a more general role for these compounds as precursors of various iridoids and secoiridoids may be envisaged. An attractive hypothesis would have 7-desoxyloganic acid (**8**) (Inouye *et al.*, 1969a) hydroxylated to loganic acid (**2**) (Scheme II) and related acids[6] such as monotropein (Inouye *et al.*, 1964) and

[5] In related mechanism studies we have found 95–100% of the total tritium in loganic acid biosynthesized from mevalonate-2-^{3}H to be at carbons 3 and 7 (Coscia *et al.*, 1969).

[6] Implicit in the proposal of the intermediacy of the cyclopentanoid monoterpenecarboxylic acids is that they may also exist as CoA or similar derivatives.

SCHEME II



paederosidic acid (Inouye *et al.*, 1968a). By methylation of the corresponding acid, the methyl esters such as genipin (Djerassi *et al.*, 1961), verbenalin (Büchi and Manning, 1962), and loganin (**3**) would be formed. The methyl group of methionine served as a precursor for the *O*-methyl group of loganin (**3**) (Brechbühler-Bader *et al.*, 1968), genipin (C. J. Coscia and R. Guarnaccia, unpublished observations, 1967), and verbenalin (Hüni *et al.*, 1966).

Alternatively decarboxylation of corresponding carboxylic acids would provide a route to the C_9 cyclopentanoid monoterpenes such as aucubin (**11**) (Scheme II). Inouye *et al.* (1969a) has provided support for this hypothesis by labeling a 7-desoxyloganic acid (**8**), feeding it to various plants, and demonstrating direct conversion of this acid **8** into loganin (**3**), aucubin (**11**) (Scheme II), asperuloside, and verbenalin.

Finally, loganic acid (**2**) and loganin (**3**) can be transformed to secoiridoids as well as indole alkaloids (Scheme II). Thus loganic acid (**2**) in *S. caroliniensis* (Guarnaccia *et al.*, 1969) as well as loganin (**3**) in *Swertia petiolata* (Gröger and Simchen, 1969) and in *Gentiana triflora* (Inouye *et al.*, 1969a) serve as precursors of gentiopicroside (**1**) presumably *via* sweroside (**10**) (Scheme II) (Inouye *et al.*, 1968b). Both loganin (**3**) and sweroside (**10**) can also serve as indole alkaloid precursors. In *V. rosea* singly and doubly labeled loganin is transferred to indole alkaloids with the *O*-methyl group retained (Battersby *et al.*, 1966; Loew and Arigoni, 1968). This has been shown to occur *via* secologanin (**9**) which can condense with tryptamine to enter into the alkaloid series (Battersby *et al.*, 1969a) (Scheme II). Sweroside (**10**) is also present in *V. rosea* (Battersby *et al.*, 1969a) and a C-10-^{14}C-labeled sweroside has been converted into vindoline-18-^{14}C (**12**) (11% incorporation; Inouye *et al.*, 1968b) in this plant, and to reserpine and quinine in other plants (Inouye *et al.*, 1969b). It appears then that a common pathway exists for these monoterpenoids and location of the bifurcation is under investigation.

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References

Auda, H., *et al.* (1967), *J. Amer. Chem. Soc. 89*, 2416.
Battersby, A. R., Burnett, A. R., Knowles, G. D., and Parsons, P. G. (1968a), *Chem. Commun.* 1277.
Battersby, A. R., Burnett, A. R., and Parsons, P. G. (1968b), *Chem. Commun.* 1280.
Battersby, A. R., Burnett, A. R., and Parsons, P. G. (1969a), *J. Chem. Soc.*, 1187.
Battersby, A. R., *et al.* (1966), *Chem. Commun.* 888, 890.
Battersby, A. R., *et al.* (1968c), *Chem. Commun.*, 951.
Battersby, A. R., Hall, E. S., and Southgate, R. (1969b), *J. Chem. Soc.*, 721.
Birch, A. J., and Grimshaw, J. (1961), *J. Chem. Soc.*, 1407.
Bobbitt, J. M., and Segebarth, K. P. (1969), Cyclopentanoid Terpene Derivatives, New York, N. Y., Marcel Dekker.
Brechbühler-Bader, S., Coscia, C. J., Loew, P., von Szczepanski, Ch., and Arigoni, D. (1968), *Chem. Commun.*, 136.
Briggs, L. H., Cain, B. F., LeQuesne, P. W., and Shoolery, J. N. (1963), *Tetrahedron Letters*, 69.
Büchi, G., and Manning, R. E. (1962), *Tetrahedron 18*, 1049.
Canonica, L., Pelizzoni, F., and Jommi, G. (1962), *Gazz. Chim. Ital. 92*, 298.
Canonica, L., Pelizzoni, F., Manitto, P., and Jommi, G. (1961), *Tetrahedron 16*, 192.

Coscia, C. J., Botta, L., and Guarnaccia, R. (1969), *Arch. Biochem. Biophys.* (in press).
Coscia, C. J., and Guarnaccia, R. (1967), *J. Amer. Chem. Soc. 89*, 1280.
Coscia, C. J., and Guarnaccia, R. (1968), *Chem. Commun.*, 138.
Djerassi, C., *et al.* (1961), *J. Org. Chem. 26*, 1192.
Ellis, C., and Swaney, M. (1938), Soilless Growth of Plants, New York, N. Y., Reinhold.
Floss, H.-G., Mothes, U., and Rettig, A. (1964), *Z. Naturforschg. 19b*, 1106.
Gear, J. R., and Spenser, I. D. (1963), *Can. J. Chem. 41*, 783.
Goeggel, H., and Arigoni, D. (1965), *Chem. Commun.*, 538.
Gröger, D., and Simchen, P. (1969), *Z. Naturforschg. 24b*, 356.
Guarnaccia, R., Botta, L., and Coscia, C. J. (1969), *J. Amer. Chem. Soc. 91*, 204.
Hall, L. D. (1964), *Advan. Carbohydrate Res. 19*, 51.
Holloway, P. W., and Popják, G. (1968), *Biochem. J. 106*, 835.
Hüni, J. E. S., Hiltebrand, H., Schmid, H., Gröger, D., Johne, S., and Mothes, K. (1966), *Experientia 22*, 656.
Inouye, H., Arai, T., and Miyoshi, Y. (1964), *Chem. Pharm. Bull. (Tokyo) 12*, 888.
Inouye, H., Inouye, S., Shimokawa, N., and Okigawa, M. (1968a), *Tetrahedron Letters*, 683.
Inouye, H., Ueda, S., Aoki, Y., and Takeda, Y. (1969a), *Tetrahedron Letters*, 2351.
Inouye, H., Ueda, S., and Nakamura, Y. (1967), *Tetrahedron Letters*, 3321.
Inouye, H., Ueda, S., and Takeda, Y. (1968b), *Tetrahedron Letters*, 3453.
Inouye, H., Ueda, S., and Takeda, Y. (1969b), *Tetrahedron Letters*, 407.
Inouye, H., Yoshida, T., Nakamura, Y., and Tobita, S. (1968c), *Tetrahedron Letters*, 4429.
Kinard, F. E. (1957), *Rev. Sci. Instruments 28*, 293.
Korte, F. (1954), *Chem. Ber. 87*, 512.
Leete, E. (1969), *Accounts Chem. Res. 2*, 59.
Leete, E., Gregory, H., and Gros, E. G. (1965), *J. Amer. Chem. Soc. 87*, 3475.
Light, R. J. (1965), *Arch. Biochem. Biophys. 112*, 163.
Loew, P., and Arigoni, D. (1968), *Chem. Commun.*, 137.
Loew, P., von Szczepanski, Ch., Coscia, C. J., and Arigoni, D. (1968), *Chem. Commun.*, 1276.
McCapra, F., Money, T., Scott, A. I., and Wright, I. G. (1965), *Chem. Commun.*, 537.
Merz, K. W., and Lehmann, H. (1957), *Arch. Pharm. 290*, 543.
Money, T., Wright, I. G., McCapra, F., and Scott, A. I. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 901.
Pesonen, S., and Ramstad, E. (1956), *J. Amer. Pharm. Assoc. 45*, 522.
Plat, M., Koch, M., Bouquet, A., LeMen, J., and Janot, M. (1963), *Bull. Soc. Chim. France*, 1302.
Regnier, F. E., Waller, G. R., Eisenbraun, E. J., and Auda, H. (1968), *Phytochemistry 7*, 221.
Scott, A. I. (1964), Interpretation of UV Spectra of Natural Products, Oxford, Pergamon, p 251.
Sheth, K., Ramstad, E., and Wolinsky, J. (1961), *Tetrahedron Letters*, 394.
Steyermark, J. A. (1963), Flora of Missouri, Ames, Iowa, Iowa State University, p 1191.
Stout, G. H., and Balkenhol, W. J. (1969), *Tetrahedron 25*, 1947.
Sugii, M., and Hashimoto, Y. (1958), *Bull. Inst. Res. Kyoto Univ. 36*, 127.
Sweeley, C. C., Bentley, R., Makita, M., and Wells, W. W. (1963), *J. Amer. Chem. Soc. 85*, 2497.
Thomas, R. (1961), *Tetrahedron Letters*, 544.
Wenkert, E. (1962), *J. Amer. Chem. Soc. 84*, 98.
Yeowell, D. A., and Schmid, H. (1964), *Experientia 20*, 250.