# 3-Hydroxy-5,6-epoxy-β-ionol β-D-Glucopyranoside and 3-Hydroxy-7,8-dihydro-β-ionol β-D-Glucopyranoside: New $C_{13}$ Norisoprenoid Glucoconjugates from Sloe Tree (*Prunus spinosa* L.) Leaves

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After isolation of a glycosidic extract obtained from sloe tree (*Prunus spinosa* L.) leaves by Amberlite XAD-2 adsorption and methanol elution followed by hydrolysis with a commercial pectinase enzyme, HRGC and HRGC–MS analyses revealed the occurrence of 41 aglycons. Benzaldehyde, benzyl alcohol, and benzoic acid were found as major constituents. In addition, a number of $C_{13}$ norisoprenoids, i.e. isomeric oxoactinidols, 3-hydroxy-7,8-dehydro-β-ionone, 3-oxo-α-ionol, 3-hydroxy-7,8-dihydro-β-ionol, 3-hydroxy-5,6-epoxy-β-ionol, 3-hydroxy-β-ionone, and vomifoliol were also present in the milligram per kilogram range. By using prefractionation of the glycosidic leaf extract by rotation locular countercurrent chromatography (RLCC), subsequent acetylation and liquid chromatographic purification, two glycoconjugates were isolated in pure form whose structures were elucidated by LC–MS and NMR analyses to be β-D-glucopyranosides of 3-hydroxy-5,6-epoxy-β-ionol and 3-hydroxy-7,8-dihydro-β-ionol, respectively.

## INTRODUCTION

In the course of our studies about the aroma compounds and their bound forms in various *Prunus* species (Krammer et al., 1991), we also came upon sloe tree (*Prunus spinosa* L.) whose fruits are esteemed for the production of sloe gin (Franke, 1989). A preliminary screening of free and bound volatiles of fruits did not exhibit remarkable results, but the study of leaves revealed a high amount of bound aroma substances including a number of $C_{13}$ norisoprenoids. Thus, we investigated the glycoconjugated aroma compounds from sloe leaves in detail. This paper concerns the results obtained during this study.

## EXPERIMENTAL PROCEDURES

**Chemicals.** All commercial chemicals used were of analytical grade quality. Solvents were redistilled before use.

**Plant Material.** Leaves were plucked in September 1991 from wild sloe trees (*P. spinosa* L.) grown in the Würzburg area.

**Isolation of a Glycosidic Extract.** After the mixing of 1.1 kg of leaves with 1200 mL of methanol and maceration of the mixture (adjusted to pH 7) at ambient temperature overnight, a clear extract was obtained by centrifugation (5000*g*, 30 min). Methanol was removed under reduced pressure (rotavapor). The aqueous residue was extracted 3 times with 100 mL of pentane and subsequently diethyl ether to separate chlorophyll and free volatiles, respectively, and then applied to an Amberlite XAD-2 column (25 × 900 mm; 10 mL/min) (Günata et al., 1985). After the mixture was washed with 3000 mL of distilled water, a glycosidic extract was obtained by eluting with 1 L of methanol. The methanol eluate was concentrated under reduced pressure to dryness (rotavapor) and redissolved in 25 mL of 0.2 M citrate phosphate buffer (pH 5) (yield, 11 g). Remaining volatiles were removed by diethyl ether extraction.

**Rotation Locular Countercurrent Chromatography (RLCC).** Four milliliters of the glycosidic extract (1.76 g) was subjected to a prefractionation using RLCC. The apparatus (Eyela RLCC, Tokyo Rikakikai Co.) was operated in the ascending mode, employing a solvent system made from the two phases produced by mixing $CHCl_3$–MeOH–$H_2O$ (7:13:8) with the more dense, less polar layer used as stationary phase. The flow rate was 1 mL/min, rotation speed 80 rpm, slope 25°. Fifty 10-mL fractions were separated and were analyzed by TLC using $SiO_2$ $GF_{254}$ (Merck, Darmstadt) as stationary and the less polar RLCC layer as mobile phase (detection, 254 nm and vanillin/$H_2SO_4$). RLCC fractions 1–23, 24–27, 28–31, 32–35, 36–39, and 40–50 were pooled, resulting in combined fractions I–VI, respectively. The presence of an aglycon moiety in these fractions was checked by (i) enzymatic and (ii) acid hydrolysis.

**Acetylation of RLCC Fraction IV.** The combined fractions IV of six RLCC separations were concentrated under reduced pressure to dryness. The dry residue was acetylated by stirring with 5 mL of acetic anhydride in 5 mL of pyridine at ambient temperature overnight. After addition of 10 mL of ice–water, extraction was performed 3 times with 100 mL of diethyl ether. The organic phase was extracted with 100 mL of 0.1 N HCl, neutralized with distilled water, dried over anhydrous sodium sulfate, and concentrated in vacuo to 1 mL.

**Flash Chromatography.** The acetylated glycosidic fraction IV was subjected to flash chromatography (Still et al., 1978) using a 15- × 250-mm glass column filled with silica gel (0.032–0.063 mm; Merck). Elution with diethyl ether under $N_2$ pressure (20 mL/min) led to separation of 25 10-mL fractions. After check by TLC (cf. above) fractions 4–10 were combined and concentrated in vacuo to 1 mL for subsequent HPLC purification. Fractions 1–3 and 11–25 were discarded.

**Preparative HPLC.** The combined fractions 4–10 obtained by flash chromatography were subjected to preparative HPLC using a 16- × 250-mm $SiO_2$ Lichrospher 60 column (5 μm; Knauer, Berlin). Elution was performed with diethyl ether (10 mL/min), yielding 10 fractions (detection, 200 nm).

**Deacetylation.** After the addition of 0.5 mg of acetylated glycoside to a solution of 25 mg of sodium methylate in 5 mL of methanol and stirring overnight, 100 mg of Dowex 50-WX8 (20–50 mesh, $H^+$ form) was added. After 30 min, the exchanger was filtered off, the solvent was removed under reduced pressure (rotavapor) to dryness, and the residue was taken up in 5 mL of distilled water.

**Enzymatic Hydrolysis.** (*a*) *Analysis of Aglycons.* In a typical experiment, a nonselective pectinase (300 μL of Rohapect D5L; Röhm, Darmstadt) and a standard (50 μg of phenyl β-D-glucoside; Serva, Heidelberg) were added to the glycosidic extract (300 mg), and the mixture was incubated at 37 °C overnight. The liberated aglycons were extracted with diethyl ether, and the dried (anhydrous $Na_2SO_4$), filtered, and concentrated (Vigreux column, 45 °C) extract was subjected to HRGC and HRGC–MS

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analyses. In the same manner, blank tests without addition of enzyme were carried out.

(*b*) *Hydrolysis of Glycosides 4b and 5b.* Two hundred micrograms of the deacetylated glycoside was dissolved in 50 mL of 0.2 M citrate phosphate buffer (pH 5.0) and the solution incubated with 10 mg of emulsin (Serva, Heidelberg) at 37 °C overnight. The liberated aglycon was extracted with diethyl ether; the organic phase was dried over anhydrous sodium sulfate and carefully concentrated to approximately 0.2 mL by a Vigreux column (45 °C) for subsequent HRGC and HRGC–MS analysis.

**Acid Hydrolysis.** A solution of 200 μg of deacetylated glycoside in 100 mL of distilled water (pH 2.5) was subjected to simultaneous distillation–extraction (SDE) (Schultz et al., 1977) over 2 h. The organic phase was dried over anhydrous sodium sulfate and carefully concentrated to approximately 0.2 mL by a Vigreux column (45 °C) for subsequent HRGC and HRGC–MS analysis.

**Capillary Gas Chromatography (HRGC).** (*a*) A Carlo Erba Fractovap 4160 gas chromatograph with FID equipped with a J & W fused silica DB-Wax capillary column (30 m × 0.259 mm i.d., film thickness 0.25 μm) was used. Split injection (1:50) was employed. The temperature program was 3 min isothermal at 50 °C, raised from 50 to 220 °C at 4 °C/min. The flow rate for the carrier gas was 1.8 mL/min He and for the makeup gas 30 mL/min $N_2$; for the detector gases the flow rates were 30 mL/min $H_2$ and 300 mL/min air. Injector and detector temperatures were kept at 220 °C.

(*b*) A Hewlett-Packard 5890 Series II gas chromatograph with FID equipped with a J & W fused silica DB-5 capillary column (30 m × 0.259 mm i.d., film thickness 0.25 μm) was used. Split injection (1:20) was employed. The temperature program was 1 min isothermal at 60 °C, raised from 60 to 280 °C at 5 °C/min. The flow rate for the carrier gas was 1.5 mL/min He and for the makeup gas 30 mL/min $N_2$; for the detector gases the flow rates were 30 mL/min $H_2$ and 300 mL/min air. Injector and detector temperatures were kept at 220 °C.

**Capillary Gas Chromatography–Mass Spectrometry (HRGC–MS).** A Varian Aerograph 3300 gas chromatograph with split injector (1:20) was combined by direct coupling to a Finnigan MAT 44 mass spectrometer with PCDS data system. The same types of columns as mentioned above for HRGC were used. The conditions were as follows: temperature programs, (DB-Wax) 3 min isothermal at 50 °C, raised from 50 to 220 °C at 4 °C/min and kept at 220 °C for 10 min; (DB-5) 60–300 °C at 5 °C/min; carrier gas flow, 1.5 mL/min He; temperature of ion source and all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 0.8 mA; mass range, 41–250. Volumes of 1 μL were injected.

Results of qualitative analyses were verified by comparison of HRGC retention ($R_i$) and mass spectral data with those of authentic reference substances. Quantitative HRGC determinations were carried out by means of standard added (cf. above) without consideration of extraction yields ($F$ = 1.0).

**Liquid Chromatography–Mass Spectrometry (LC–MS).** Thermospray LC–MS was performed with a Finnigan MAT 4500 system equipped with a Finnigan thermospray interface at 70 eV using 0.1 mM $NH_4Ac$ (10% MeOH). Injection was made by bypass. A flow rate of 1.3 mL/min and a vaporization temperature of 110 °C was used. Mass range was 100–700.

**Direct Chemical Ionization Mass Spectrometry (DCI–MS).** DCI–MS was carried out with a Finnigan MAT 90 mass spectrometer at 70 eV using ammonia as reactant gas, ion source temperature and pressure of 150 °C and 8 × $10^{-5}$ mbar, respectively, as well as a temperature gradient of 400 °C/min. Mass range was 60–900.

**Nuclear Magnetic Resonance (NMR).** NMR spectra were recorded on Bruker WM 400 (400 MHz) and AC 200 (200 MHz) spectrometers with $CDCl_3$ as solvent and $Me_4Si$ as internal standard.

## RESULTS AND DISCUSSION

In the aglycon fraction of sloe tree (*P. spinosa* L.) leaves obtained by enzymatic hydrolysis (Rohapect D5L) of a methanolic eluate yielded from XAD-separated glycosidic fraction (Günata et al., 1985) 41 aroma substances were identified by HRGC and HRGC–MS (Humpf, 1992). Among the total amount of 143 mg/kg, benzaldehyde (68 mg/kg), benzyl alcohol (11 mg/kg), and benzoic acid (22 mg/kg) were found as major constituents, but a number of more interesting $C_{13}$ norisoprenoids was also present in the mg/kg range. These aglycons and their quantities are summarized in Table I. Their structures are outlined in Figure 1.

**Table I. $C_{13}$ Norisoprenoids Identified in the Leaves of Sloe Tree by HRGC and HRGC–MS after Enzymatic Hydrolysis (Rohapect D5L) of a Methanolic Eluate Obtained from XAD-Separated Fraction**

| $R_i$[a] | | | |
|---|---|---|---|
| sample | reference | compound | amount, mg/kg |
| 1585 | 1595 | oxoactinidol, isomer I (**1A**) | 0.4 |
| 1615 | 1622 | oxoactinidol, isomer II (**1B**) | 0.5 |
| 1655 | 1660 | 3-hydroxy-7,8-dehydro-β-ionone (**2**) | 0.8 |
| 1661 | 1664 | 3-oxo-α-ionol (**3**) | 4.0 |
| 1681 | 1683 | 3-hydroxy-7,8-dihydro-β-ionol (**4**) | 3.9 |
| 1690 | 1692 | 3-hydroxy-5,6-epoxy-β-ionol (**5**) | 4.0 |
| 1705 | 1707 | 3-hydroxy-β-ionone (**6**) | 1.5 |
| 1805 | 1798 | vomifoliol (**7**) | 1.6 |

[a] Linear retention index on a J&W DB-5 fused silica capillary column (30 m × 0.259 mm i.d., df = 0.25 μm).

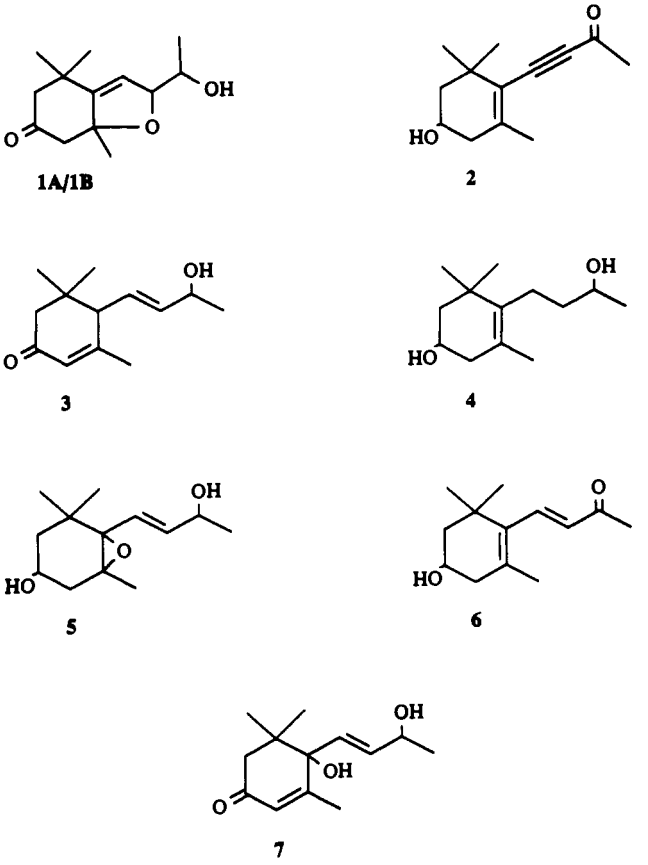



**Figure 1.** Structures of $C_{13}$ norisoprenoids identified in the aglycon fractions from sloe tree leaves: **1A/1B**, isomeric oxoactinidols; **2**, 3-hydroxy-7,8-dehydro-β-ionone; **3**, 3-oxo-α-ionol; **4**, 3-hydroxy-7,8-dihydro-β-ionol; **5**, 3-hydroxy-5,6-epoxy-β-ionol; **6**, 3-hydroxy-β-ionone; **7**, vomifoliol.

Due to the high amounts of $C_{13}$ norisoprenoids occurring in sloe tree leaf, this material was a convenient source for the isolation and characterization of glycoconjugates. RLCC preseparation of a glycosidic extract provided RLCC fractions 32–35, in which **4** and **5** were detected as major hydrolytic products after enzymatic treatment with Rohapect D5L. After acetylation of these combined fractions (IV), subsequent flash chromatography on silica gel allowed

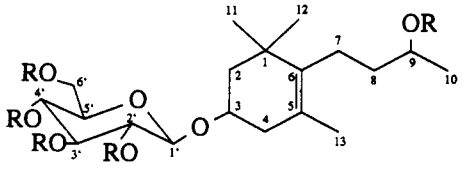
Table II. $^1$H NMR Spectral Data of Isolated Compound 4b ($CDCl_3$, 200 MHz, Coupling Constants in Hertz, δ Relative to TMS)

| δ | signal | J | atom |
|---|---|---|---|
| 1.01/1.02 | 6 H, 2s | | $H_3C11/H_3C12$ |
| 1.22 | 3 H, d | 6.3 | $H_3C10$ |
| 1.6 | 3 H, s | | $H_3C13$ |
| 1.6–1.8 | 2 H, m | | $H_2C2$ |
| 1.8–2.4 | 6 H, m | | $H_2C4/H_2C7/H_2C8$ obscured |
| 2.00–2.07 | 15 H, 5s | | $H_3$ acetates (5×) |
| 3.69 | 1 H, ddd | 9.6/5.2/2.5 | H C5′ |
| 3.91 | 1 H, m | | H C3 |
| 4.11 | 1 H, dd | 12.2/2.5 | H C6a′ |
| 4.25 | 1 H, dd | 12.2/5.2 | H C6b′ |
| 4.62 | 1 H, d | 8.0 | H C1′ |
| 4.87 | 1 H, sextet | 6.3 | H C9 |
| 4.95 | 1 H, dd | 9.4/8.0 | H C2′ |
| 5.06 | 1 H, dd | 9.6/9.4 | H C4′ |
| 5.21 | 1 H, dd | 9.4/9.4 | H C3′ |

Table III. $^{13}$C NMR Spectral Data of Isolated Compound 4b ($CDCl_3$, 50 MHz, δ Relative to TMS)

| δ | DEPT | atom | δ | DEPT | atom |
|---|---|---|---|---|---|
| 19.49 | $CH_3$ | C 13 | 71.59 | CH | C 2′ |
| 19.6–20.5 | $CH_3$ | $CH_3CO$ (5×) | 71.76 | CH | C 5′ |
| 21.2 | $CH_3$ | C 10 | 72.93 | CH | C 3′ |
| 23.85 | $CH_2$ | C 7 | 73.44 | CH | C 9 |
| 28.37[a] | $CH_3$ | C 11 | 99.47 | CH | C 1′ |
| 29.41[a] | $CH_3$ | C 12 | 123.73 | CH | C 5 |
| 36.25 | $CH_2$ | C 8 | 137.18 | CH | C 6 |
| 37.5 | C | C 1 | 169.38 | C | $CH_3CO$ |
| 38.65 | $CH_2$ | C 4 | 169.55 | C | $CH_3CO$ |
| 45.83 | $CH_2$ | C 2 | 170.47 | C | $CH_3CO$ |
| 62.29 | $CH_2$ | C 6′ | 170.78 | C | $CH_3CO$ |
| 68.71 | CH | C 4′ | 170.95 | C | $CH_3CO$ |
| 71.29 | CH | C 3 | | | |

[a] Interchangeable values.



R = H 4a

R = Ac 4b

Figure 2. Structure of the glucoconjugate 4a.

the separation of two major glycosides, which were finally obtained in pure forms by preparative HPLC on silica gel.

The $^1$H and $^{13}$C NMR data of the isolated glycoside 4a are represented in Tables II and III. From these data the isolated compound was identified as sugar conjugate from 4, i.e. the pentaacetate of 3-hydroxy-7,8-dihydro-β-ionol β-D-glucopyranoside (4b), with the glucose moiety attached to carbon 3 (Figure 2). The data recorded by thermospray LC–MS were also in accordance with structure 4b; only one peak was registered, i.e. $m/z$ 602 $(M + NH_4)^+$, indicating a molecular mass of 584. Deacetylation of the isolated compound and subsequent treatment with β-glucosidase (emulsin) led to the liberation of aglycon 4.

The $^1$H and $^{13}$C NMR data of the second isolated glycoside 5a are represented in Tables IV and V. From these data the isolated compound was identified as glycoconjugate from 5, i.e. the pentaacetate of 3-hydroxy-5,6-epoxy-β-ionol β-D-glucopyranoside (5b), with the glucose moiety attached to carbon 3 (Figure 3). The data obtained by thermospray LC–MS supported the structure 6b; only one peak was recorded, i.e. $m/z$ 616 $(M + NH_4)^+$, indicating a molecular mass of 598. This value was confirmed by DCI-MS (Table VI).

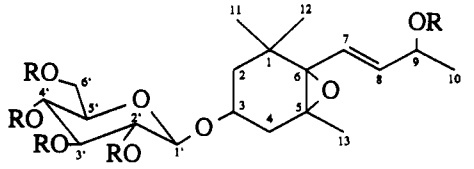
Table IV. $^1$H NMR Spectral Data of Isolated Compound 5b ($CDCl_3$, 400 MHz, Coupling Constants in Hertz, δ Relative to TMS)

| δ | signal | J | atom |
|---|---|---|---|
| 0.92/1.1 | 6 H, 2s | | $H_3C11/H_3C12$ |
| 1.16 | 3 H, s | | $H_3C13$ |
| 1.31 | 3 H, d | 6.4 | $H_3C10$ |
| 1.6–1.8 | 2 H, m | | $H_2C2$ |
| 2.00–2.07 | 15 H, 5s | | $H_3$ acetates (5×) |
| | 1 H, m | | H C4a (obscured) |
| 2.27 | 1 H, ddd | 14.4/5.0/1.5 | H C4b |
| 3.67 | 1 H, ddd | 9.9/5.5/2.4 | H C5′ |
| 3.8 | 1 H, m | | H C3 |
| 4.11 | 1 H, dd | 12.2/2.4 | H C6a′ |
| 4.23 | 1 H, dd | 12.2/5.5 | H C6b′ |
| 4.53 | 1 H, d | 8.0 | H C1′ |
| 4.93 | 1 H, dd | 9.6/8.0 | H C2′ |
| 5.03 | 1 H, dd | 9.9/9.5 | H C4′ |
| 5.18 | 1 H, dd | 9.6/9.5 | H C3′ |
| 5.36 | 1 H, quintet d | 6.4/1.1 | H C9 |
| 5.65 | 1 H, dd | 15.5/6.4 | H C8 |
| 5.88 | 1 H, dd | 15.5/1.1 | H C7 |

Table V. $^{13}$C NMR Spectral Data of Isolated Compound 5b ($CDCl_3$, 100 MHz, δ Relative to TMS)

| δ | DEPT | atom | δ | DEPT | atom |
|---|---|---|---|---|---|
| 19.8 | $CH_3$ | C 13 | 71.49 | CH | C 2′ |
| 20.75–20.43 | $CH_3$ | $CH_3CO$ (5×) | 71.76 | CH | C 5′ |
| 21.33 | $CH_3$ | C 10 | 72.86 | CH | C 3′ |
| 24.81[a] | $CH_3$ | C 11 | 73.19 | CH | C 9 |
| 29.08[a] | $CH_3$ | C 12 | 99.95 | CH | C 1′ |
| 34.76 | C | C 1 | 127.11 | CH | C 7 |
| 37.66 | $CH_2$ | C 4 | 133.41 | CH | C 8 |
| 44.25 | $CH_2$ | C 2 | 169.21 | C | $CH_3CO$ |
| 62.24 | $CH_2$ | C 6′ | 169.45 | C | $CH_3CO$ |
| 65.89 | C | C 5 | 170.27 | C | $CH_3CO$ |
| 68.58 | CH | C 4′ | 170.31 | C | $CH_3CO$ |
| 69.60 | C | C 6 | 170.64 | C | $CH_3CO$ |
| 70.31 | CH | C 3 | | | |

[a] Interchangeable values.



R = H 5a

R = Ac 5b

Figure 3. Structure of the glucoconjugate 5a.

Table VI. MS Spectral Data of 5b Obtained by DCI-MS

| fragment | interpretation |
|---|---|
| 616 | $M + NH_4^+$ |
| 539 | M – 59 (Ac) |
| 331 | 162 (hexose – $H_2O$) + 4 × 42 (acetyl) + 1 |
| 208 | aglycon – $H_2O$ |
| 169 | 331 – (2 × HAc) – $CH_2CO$ |

Neither glucoside, 4a or 5a, has been described as yet. The low amounts of glucosides isolated (3 mg and 2.5 mg of 4a and 5a, respectively) were not sufficient to elucidate their stereochemistry ($2^2$ for 4a and $2^3$ for 5a).

The aglycon 4 is an ubiquitous natural compound that has been detected in various fruit species (Winterhalter and Schreier, 1988; Krammer et al., 1991; Humpf and Schreier, 1991; Wintoch et al., 1991). The epoxy diol 5 has also been found in nature (Takagi et al., 1978; Behr et al., 1979). From our SDE studies carried out at pH 2.5 it can be concluded that 4 does not play a role as flavor precursor. While with 4 no volatile formation was observed, diol 5, however, yielded a number of volatiles under

**Table VII. Volatiles Formed from 5a at pH 2.5 under SDE Conditions**

| $R_I$[a] sample | $R_I$[a] reference | compound |
|---|---|---|
| 1710 | 1712 | 1,1,6-trimethyl-1,2-dihydronaphthalene (9) |
| 1920 | 1922 | actinidol, isomer I (10A) |
| 1932 | 1936 | actinidol, isomer II (10B) |
| 2115 | *b* | 3,4-dehydro-5,6-epoxy-β-ionol (11) |
| 2212 | *c* | 2-(3-hydroxybut-1-enyl)-2,6,6-trimethyl-cyclohex-3-enone (12) |

[a] Linear retention index on a J&W fused silica DB-Wax capillary column (30 m × 0.259 mm i.d., df = 0.25 μm). [b] Postulated compound due to its MS data similar to 5. [c] Tentatively evaluated by MS data provided by Dimitriadis et al., 1985.

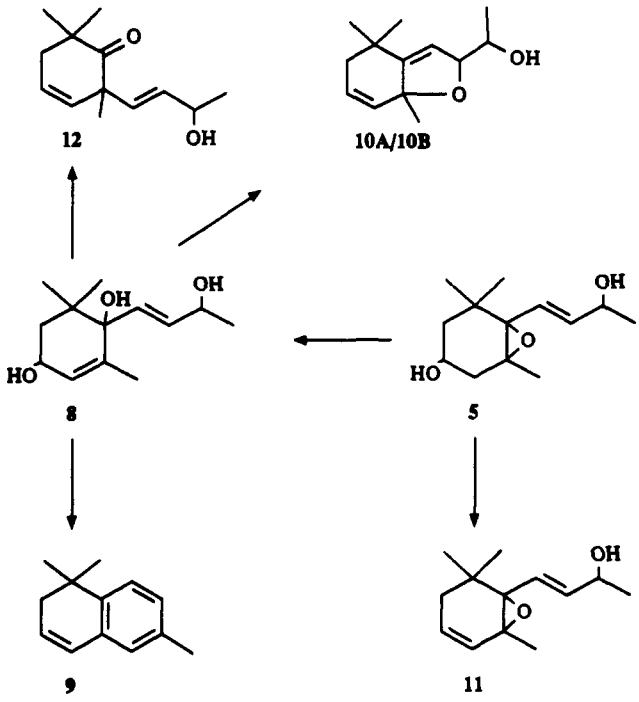



**Figure 4.** Acid-catalyzed degradation of aglycon 5 (SDE, pH 2.5).

SDE conditions at acidic pH. From the distribution of products formed (Table VII), acid-catalyzed ring opening leading to triol 8 can be postulated as initial step of reactions, from which the subsequent formation of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (9), the isomeric actinidols (10A/10B), and the hydroxy dienone 12 from 8 has been previously described (Strauss et al., 1986) (Figure 4). The acid-catalyzed formation of 3-hydroxy-actinidols has been earlier reported (Behr et al., 1979); under SDE conditions at pH 2.5 their further dehydration to the actinidols 10A/10B is a likely process. Compounds 10A/10B are known constituents of *Actinidia polygama* leaves (Sakan et al., 1967) and aged wines (Dimitriadis et al., 1985). TDN is a well-known off-flavor compound in wine (Simpson, 1978); several precursors have been described (Strauss et al., 1986; Winterhalter, 1991; Humpf et al., 1991).

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