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CYANOGENIC GLYCOSIDES FROM *SAMBUCUS NIGRA*

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Abstract: A new cyanogenic glycoside, 2*S*-β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranosylmandelonitrile, has been isolated from the leaves of *Sambucus nigra* along with the already known sambunigrin, prunasin, holocalin and the acetyl derivative of the latter. The structures were elucidated by chemical and physical methods.

Key Words: *Sambucus nigra*, cyanogenic glycosides, allelopathy, NMR.

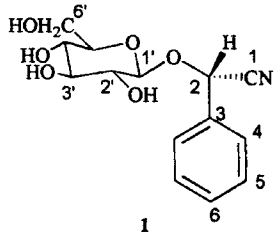
INTRODUCTION

Sambucus nigra L. is a noxious weed, widely spread in the Southern Italy, which causes large crops losses. In a previous study of this Caprifoliacea, Jensen and Nielsen[1] reported the presence of cyanogenic glucosides and these compounds are considered among the strongest allelochemicals[2] as they not only produce hydrogen cyanide by hydrolysis but also benzoic acids by oxidation of benzaldehyde or hydroxybenzaldehydes formed during hydrolysis.

In a study of the role of allelopathy in the detrimental effects of *S. nigra* on the growth of crops, we have reinvestigated the plant and in this paper a chemical study of the cyanogenic components is reported.

RESULTS AND DISCUSSION

The MeOH extract of frozen leaves of samples collected in Agerola (Italy) was partitioned between EtOAc and H_2O. The aqueous layer was chromatographed on Amberlite and the MeOH eluate was chromatographed on Sephadex LH-20 to give, after repeated chromatographic processes, cyanogenic glycosides **1** – **5**.



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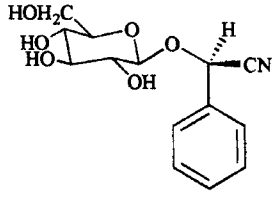

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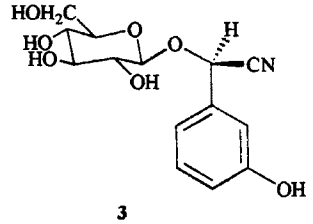

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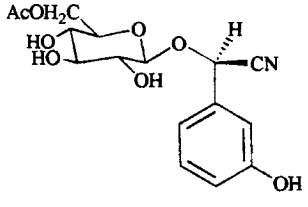

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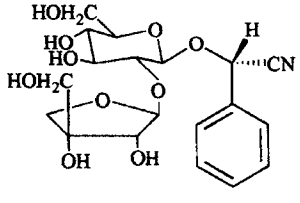

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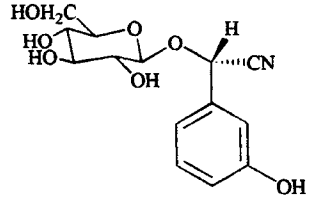

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Compounds **1**, **2** and **3** were identified as the already known sambunigrin[3], prunasin[4] and holocalin[5] respectively on the basis of their physical features.

The structure of 6'-acetylholocalin was attributed to compound **4**. Its ^{1}H NMR spectrum showed the H-2 singlet at δ 5.68 and the H-6' double doublet protons downfield shifted at δ 4.39 and 4.23 in addition to the acetyl signal at δ 2.11. Present in the ^{13}C NMR spectrum were the signals of the acetyl group at δ 170.5 and 21.3 and the C-6' carbon shifted at δ 66.4. The chemical shift of the H-2 proton suggested the *R* configuration. At the moment it is not possible to establish if **4** is a true metabolite of the plant or an artefact arising during the extraction processes by transesterification.

The distribution of cyanogenic glucosides in *S. nigra* seems to be rather irregular: Jensen and Nielsen,[1] in a study of six samples of the plant collected in separate localities, found that sambunigrin (**1**) was present in all the collections while its epimer prunasin (**2**) as well as the *m*-hydroxyderivative epimers holocalin (**3**) and zierin (**6**)[4] were present in only two collections. In the sample collected in Agerola we have found no zierin (**6**), only small amounts of epimeric sambunigrin (**1**) and prunasin (**2**) and large amounts of holocalin (**3**) alomg with its acetyl derivative **4**.

Recently Nahrstedt and Schwind[6] have shown that phenylalanine is the biogenetic precursor of the aglycone of cyanogenic glycosides. Enzymatic hydroxylation occurs in a subsequent step in the pathway to *m*-hydroxyphenylacetonitriles. In this way zierin (**6**) derives from sambunigrin (**1**). In the samples collected in Italy are present both the *R* and *S* epimers at C-2, prunasin (**2**) and sambunigrin (**1**) respectively, but only holocalin (**3**) and it is reasonable that in the plant stereospecific catalytic hydroxylation occurs.

Besides the glucosides **1** - **4** a new glycoside was isolated to which was attributed the structure 2*S*-β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranosylmandelonitrile (**5**). Positive FAB mass spectrometry gave a pseudomolecular ion at *m/z* 428 [M + 1] and fragments at *m/z* 295 and 133,

indicating a diglycoside formed by a hexose and a pentose. Acid hydrolysis of **5** gave D-glucose and D-apiose as sugar components, identified by GC analysis.[7] Methylation of **5** by a modified Hakomori procedure,[8] and subsequent hydrolysis with TFA gave a mixture which, after reduction with $NaBD_4$ and acetylation, was GC-MS analyzed.[9] Analysis gave 1,2,5-tri-O-acetyl-3,4,6-tri-O-methylglucitol and 1,4-di-O-acetyl-2,3,5-tri-O-methylapiitol in 1:1 ratio, according to a disaccharide chain with the structure D-apiofuranosyl-(1→2)-D-glucopyranoside. The ^1H and ^{13}C resonances of **5** were attributed by a combination of COSY, HOHAHA, DEPT, HMQC and HMBC experiments. The ^1H NMR spectrum, besides the H-2 signal as a singlet at δ 6.02 and the five aromatic protons H-4 – H-8 as two multiplets centred at δ 7.43 and 7.62 from the aglycone moiety showed the glucose H-1' as a doublet at δ 4.73, the H-2' as a triplet at δ 3.56, the H-3' as a double doublet at δ 3.44, the overlapped H-4' and H-5' as a multiplet at δ 3.37 and the H-6' protons as two double doublets at δ 3.66 and 3.93. The apiose H-1'' appeared as a narrow doublet at δ 5.38, the H-2'' as a doublet at δ 3.98, the H-4'' as two doublets at δ 3.94 and 3.65 and the H-5'' as a singlet at δ 3.67. The downfield shift of the H-2' of the glucose as well as that of the corresponding C-2' at δ 79.0 was in agreement with the link between the moieties while the couplings of the H-1' of glucose and H-1'' of apiose justified the β configuration at both the anomeric carbons. Finally the quite similar chemical shifts of the H-2 proton in **5** and **1** justified the *S* configuration at C-2.

Compound **5** is the third cyanogenic glycoside bearing an apiose moiety after xeranthin[10] and oxyanthin.[11] Xeranthin is a trisaccharide with the apiose 1→4 linked to glucose while oxyanthin is a disaccharide like **5**. However in oxyanthin the apiose is 1→6 linked to glucose and (*R*)-mandelonitrile, instead of (*S*)-mandelonitrile is the aglycone.

EXPERIMENTAL

Amberlite XAD-4 (20 – 60 mesh, Fluka) and Sephadex® LH-20 (Pharmacia biotech) were used for column chromatography. Merck Hibar Lichrosorb RP-18 (5 μm, 250 × 4 mm i.d.) and Lichrosorb RP-18 (7 μm, 250 × 10 mm i.d.) columns were used for analytical and preparative HPLC purposes. GC and GC-MS analyses were carried using a Supelco SP-2330 capillary column (30 m × 0.25 mm i.d.). EIMS spectra were obtained on a Kratos MS-80 spectrometer at 70 eV; FABMS were recorded on a VG ZAB 2SE apparatus. ^1H and ^{13}C NMR experiments were carried out in CD_3OD on a Bruker AM 400 spectrometer. UV spectra were recorded in EtOH on a Shimadzu UV 1204 spectrometer.

Plant material

Plants of *Sambucus nigra* were collected on July in Agerola (Naples) and were identified by prof. G. Aliotta of II University of Naples. A voucher specimen is deposited at the Botanical Garden of the University Federico II of Naples.

Extraction, isolation and characterization

The air dried and powdered plant material (4.1 Kg) was sequentially extracted with light petrol, EtOAc and MeOH exhaustively. The MeOH extract (370 g) was treated with H_2O (2 L) and EtOAc (1 L). The aqueous layer, concd at 500 mL, was chromatographed on Amberlite XAD-4 and the MeOH fraction was partioned by chromatography on Sephadex LH-20. The fraction eluted with H_2O was rechromatographed on Sephadex LH-20 using MeOH – $CHCl_3$ – hexane (4 : 2 : 1) as eluent to give crude **5** which was purified by reverse phase C-18 HPLC (MeOH – MeCN – H_2O 17 : 3 : 30). The fraction eluted with H_2O – MeOH (3 : 1) was rechromatographed on Sephadex LH-20 (MeOH – $CHCl_3$ – hexane 4 : 2 : 1) to give a mixture of **1**, **2** and **4** which was resolved by reverse phase C-18 HPLC (A: 2% AcOH, B: MeOH – 2% AcOH (8 : 2); gradient A : B from 17 : 3 to 7 : 3 in 25 min). The fraction eluted with H_2O – MeOH (2 : 1) was rechromatographed on Sephadex LH-20 using MeOH – $CHCl_3$ – hexane (4 : 2 : 1) as eluent to give crude **3** (220 mg). An aliquot of the crude product was purified by reverse phase C-18 HPLC (A: 2% AcOH, B: MeOH – 2% AcOH (8 : 2); gradient A : B from 8 : 2 to 3 : 1 in 10 min).

Sambunigrin (**1**) (6 mg): $[\alpha]_D$ –75° (c 0.6 in EtOH); EIMS *m/z* 295 $[M]^+$, 163, 133; UV λ_{max} nm 278; ^1H NMR δ 7.61 (2H, m, H-4 and H-6), 7.44 (3H, m, H-

5, H-6, H-7), 6.05 (1H, s, H-2), 4.69 (1H, d, J = 7.8 Hz, H-1'), 3.96 (1H, dd, J = 1.4 and 11.2 Hz, H-6'), 3.70 (1H, dd, J = 5.5 and 11.2, H-6'), 3.44 (1H, ddd, J = 1.4, 5.5 and 9.8 Hz, H-5'), 3.40 (1H, dd, J = 9.1 and 9.2 Hz, H-3'), 3.28 (2H, m, H-2' and H-4'); ^{13}C NMR δ 119.4 (C-1), 69.5 (C-2), 135.8 (C-3), 131.1 (C-4), 129.9 (C-5), 131.9 (C-6), 129.9 (C-7), 131.1 (C-8), 103.0 (C-1'), 75.7 (C-2'), 79.5 (C-3'), 72.5 (C-4'), 78.9 (C-5'), 63.7 (C-6').

Prunasin (**2**) (4 mg): $[\alpha]_D$ –31° (c 0.4 in EtOH); EIMS *m/z* 295 $[M]^+$, 163, 133; UV λ_{max} nm 278; ^{1}H NMR δ 7.60 (2H, m, H-4 and H-6), 7.45 (3H, m, H-5, H-6, H-7), 5.91 (1H, s, H-2), 4.26 (1H, d, J = 7.6 Hz, H-1'), 3.93 (1H, dd, J = 1.1 and 11.9 Hz, H-6'), 3.72 (1H, dd, J = 5.8 and 11.9, H-6'), 3.44 (1H, ddd, J = 1.4, 5.5 and 9.8 Hz, H-5'), 3.40 (1H, dd, J = 9.1 and 9.2 Hz, H-3'), 3.28 (2H, m, H-2' and H-4'); ^{13}C NMR δ 119.4 (C-1), 69.5 (C-2), 136.1 (C-3), 130.8 (C-4), 129.6 (C-5), 131.6 (C-6), 129.6 (C-7), 130.8 (C-8), 103.0 (C-1'), 75.7 (C-2'), 79.5 (C-3'), 72.5 (C-4'), 78.9 (C-5'), 63.7 (C-6').

Holocalin (**3**) (210 mg): $[\alpha]_D$ –61° (c 1.1 in EtOH); EIMS *m/z* 311 $[M]^+$, 163; UV λ_{max} nm 278; ^{1}H NMR δ 7.26 (1H, dt, J = 1.0 and 8.1 Hz, H-7), 7.04 (1H, ddd, J = 1.0, 1.8 and 8.1 Hz, H-8), 7.00 (1H, d, J = 1.8 Hz, H-4), 6.86 (1H, ddd, J = 1.0, 1.8 and 8.1 Hz, H-6), 5.82 (1H, s, H-2), 4.28 (1H, d, J = 7.9 Hz, H-1'), 3.92 (1H, dd, J = 1.2 and 12.0 Hz, H-6'), 3.71 (1H, dd, J = 6.0 and 12.0, H-6'), 3.35 – 3.20 (4H, m, H-2' - H-5'); ^{13}C NMR δ 120.4 (C-1), 69.2 (C-2), 136.9 (C-3), 116.6 (C-4), 160.2 (C-5), 118.9 (C-6), 132.1 (C-7), 120.9 (C-8), 102.7 (C-1'), 75.7 (C-2'), 79.2 (C-3'), 72.4 (C-4'), 78.8 (C-5'), 63.7 (C-6').

6'-Acetylholocalin (**4**) (2 mg): $[\alpha]_D$ –47° (c 0.2 in EtOH); EIMS *m/z* 353 $[M]^+$, 311 and 149; UV λ_{max} nm 278; ^{1}H NMR δ 7.29 (1H, dt, J = 1.1 and 8.0 Hz, H-7), 7.03 (1H, ddd, J = 1.1, 1.8 and 8.0 Hz, H-8), 7.01 (1H, d, J = 1.8 Hz, H-4), 6.87 (1H, ddd, J = 1.1, 1.8 and 8.0 Hz, H-6), 5.68 (1H, s, H-2), 4.39 (1H, dd, J = 1.2 and 12.0 Hz, H-6'), 4.36 (1H, d, J = 7.9 Hz, H-1'), 4.23 (1H, dd, J = 6.0 and 12.0, H-6'), 3.50 – 3.38 (4H, m, H-2' - H-5'), 2.11 (3H, s, Me); ^{13}C NMR δ 119.7 (C-1), 69.7 (C-2), 138.2 (C-3), 117.4 (C-4), 161.0 (C-5), 119.8 (C-6), 133.0 (C-7), 121.6 (C-8), 104.0 (C-1'), 75.9 (C-2'), 80.0 (C-3'), 71.6 (C-4'), 79.5 (C-5'), 66.4 (C-6') 170.5 (CO), 21.3 (Me).

2S-β-D-apio-D-furanosyl-(1→2)-β-D-glucopyranosylmandelonitrile (**5**) (8 mg): $[\alpha]_D$ –73° (c 0.7 in EtOH); FAB MS *m/z* 428 $[M + 1]^+$, 295 and 133; UV λ_{max} nm 278; ^{1}H NMR δ 7.62 (2H, m, H-4 and H-8), 7.43 (3H, m, H-5, H-6, H-7), 6.02 (1H, s, H-2), 4.73 (1H, d, J = 7.6 Hz, H-1'), 3.93 (1H, dd, J = 2.1 and 12.1, H-6'), 3.66 (1H, dd, J = 5.8 and 12.1 Hz, H-6'), 3.56 (1H, t, J = 8.9 Hz, H-2'), 3.44 (1H, dd, J = 7.7 and 8.9 Hz, H-3'), 3.37 (2H, m, H-4' and H-5'),

5.38 (1H, d, J = 2.1 Hz, H-1"), 3.98 (1H, d, J = 2.1 Hz, H-2"), 3.94 (1H, d, J = 9.6 Hz, H-4"), 3.67 (2H, s, H-5"), 3.65 (1H, d, J = 9.6 Hz, H-4"); ^{13}C NMR δ 118.9 (C-1), 69.0 (C-2), 135.5 (C-3), 130.3 (C-4), 129.1 (C-5), 131.1 (C-6), 129.1 (C-7), 130.3 (C-8), 101.3 (C-1'), 79.0 (C-2'), 79.0 (C-3'), 72.0 (C-4'), 78.7 (C-5'), 63.2 (C-6'), 111.3 (C-1"), 79.0 (C-2"), 81.3 (C-3"), 76.1 (C-4"), 66.9 (C-5").

Acid hydrolysis of **5** *and identification of monosaccharides.*
A sample of **5** (3 mg) in EtOH (0.3 mL) was heated at 100 °C for 2 hr with 7% H_2SO_4 (0.5 mL). The reaction mixt was extracted with EtOAc and the aq. layer neutralized on Amberlite IRA-401 and evapd in *vacuo*. Reverse phase HPLC (MeCN – H_2O 4 : 1) gave the mixture of monosaccharides which was dissolved in pyridine (0.1 mL) was treated with L-cysteine Me ester HCl (4 mg, 0.2 mL). After 1hr at 60 °C, pyridine was removed and TMS-imidazole (0.5 mL) was added to the residue, and the mixt. kept at 60 °C for another hr. The *n*-hexane extract was analysed by GC and compared with authentic samples of D-glucose and D-apiose derivatives.

Permethylation of **5**.
Glycoside **5** (3 mg) was permethylated by a modification of the Hakamori procedure and the product was hydrolysed with TFA (2M, 1 mL containing inositol 100 μg mL^{-1}) at 120 °C for 1 hr. The methylated product was reduced with $NaBD_4$, acetylated and analysed by GC-MS on SP-2330 with flow rate 0.8 mL min^{-1}, using He as carrier gas with the temp. programme: 170 °C for 3 min, 170 ° → 240 ° at 4° for min, 240 ° for 10 min.

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