Acta Chemica Scandinavica B 32 (1978) 588—592

Bitterness in Aqueous Extracts of Apricot Kernels

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When intact apricot kernels are steeped in cold water, considerable quantities of soluble matter are extracted along with the bitter, cyanogenic glycosides prunasin and, notably, amygdalin. Endogeneous emulsin catalyzes the hydrolysis of these two glycosides to glucose and mandelonitrile, the latter reversibly dissociating into benzaldehyde and hydrogen cyanide. Attempts at recovery of the extracted matter by concentration of the aqueous solution at temperatures below 40 °C are invariably accompanied by the production of new bitterness, not attributable to the original cyanogenic glycosides. Evidence is presented of the *de novo*-synthesized bitter principle being benzyl β-D-glucopyranoside; its formation is discussed.

(*R*)-Mandelamide, not previously recorded as a constituent of living matter, has been isolated from concentrated aqueous extracts of apricot pits.

Gas chromatography, combined with electron-impact and chemical-ionization mass spectrometry of per(trimethylsilyl) derivatives of various glycosides, including amygdalin and prunasin, proved a powerful analytical tool for work of the type here discussed.

Among the raw materials employed for the industrial production of marzipan, apricot kernels play an important role. In the first step of an industrial process, the intact pits are steeped in cold water to more or less remove their bitterness, attributable to amygdalin (*1*) and, to a minor extent, prunasin (*2*). During the extraction, the endogeneous enzyme system, emulsin, catalyzes the hydrolysis of *1* and *2* to glucose and (*R*)-mandelonitrile (*3*), the latter reversibly dissociating, spontaneously or enzyme-catalyzed, into benzaldehyde and hydrogen cyanide. The aqueous extract from the above process, henceforth designated 'the bitterwater', contains substantial quantities of organic dry matter. Recycling of this, however desirable in the industrial marzipan production, is hampered by its extreme bitterness, a problem that prompted the present study, aimed at elucidating the chemical character of the bitter principle and clarifying its formation.

A sample of bitterwater from the industrial process, collected shortly after the extraction was initiated and briefly boiled in order to arrest enzymic reactions, was analyzed. Amygdalin (*1*), prunasin (*2*), mandelonitrile (*3*), benzaldehyde, glucose, and sucrose were found to be present (see Experimental). After adding sweet almond emulsin to the solution and keeping it overnight at room temperature the original glycosides *1* and *2*, and with them all bitterness, had vanished whereas the sucrose was unaffected. On concentration of the solution *in vacuo*, at temperatures not exceeding 40 °C, bitterness reappeared; at the same time, benzyl alcohol was encountered as a novel constituent of the solution. To be sure, no obvious glycosidic progenitor of the latter was observed in the bitterwater before the enzyme was added. In order to isolate the bitter principle, the emulsin-treated and extensively concentrated bitterwater was extracted with a series of solvents, ranging in polarity from light petroleum to butanol. In the appropriate extracts (see Experimental) the following compounds were identified, after isolation or by spectroscopic characterization: benzyl benzoate (*4*), (*R*)-mandelamide (*5*), butyl β-D-glucopyranoside (*6*), and benzyl β-D-glucopyranoside (*7*). The two glucosides possessed an intensely bitter taste, in accord with literature reports.[1] The origin of the non-bitter amide (*5*), constituting

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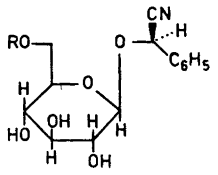
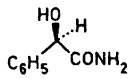


1, R=β-D-glucopyranosyl
2, R=H



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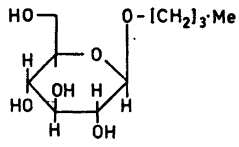

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at least 0.3 % of the dry matter in the bitterwater and formerly unknown as a product of natural derivation, has not been traced during the present studies. Whether hydration of a glycosidic progenitor of (*R*)-mandelonitrile (*3*), alternatively, stereospecific addition of water to *3* itself, is in fact a natural process hence remains an open question. Equally undecided is the origin of the ester (*4*), isolated from the emulsin-treated bitterwater. Emulsin-catalyzed β-glucoside synthesis from glucose and alcohols, the reverse of the enzymic hydrolysis, is a well-known and occasionally useful synthetic reaction. Thus, in the present case, the butylglucoside (*6*) could obviously originate from the butanol used in the extraction procedure * whereas the analogous formation of the benzylglucoside (*7*) demands involvement of benzyl alcohol of endogenous origin. That the industrial bitterwater is indeed a β-glucoside-synthesizing system was ascertained by (i) the production of (*E*)-cinnamyl β-D-glucopyranoside,[3] identified by its spectroscopical characteristics, when cinnamyl alcohol was added to the system, (ii) the absence of butylglucoside (*6*) when contact of the bitterwater with butanol was avoided, and (iii) the annihilation of all capacity for glucoside synthesis after brief heating of the bitterwater to 90 °C.

In a concentrate of emulsin-treated bitterwater, kept out of contact with butanol and other alcohols, benzyl β-D-glucopyranoside (*7*) seems to account for the total bitterness. Importantly, the glucoside could not be detected in fresh bitterwater, rich in amygdalin and prunasin, but was gradually produced, at the expense of the two cyanogenic glycosides, on storage and concentration of the emulsin-treated bitterwater at temperatures not exceeding 40 °C.

The initial product of the enzymic hydrolysis of amygdalin (*1*), proceeding *via* prunasin (*2*), is (*R*)-mandelonitrile (*3*), recognized in the bitterwater. It is conceivable, though unproven, that in the present system, *3*, competitively with its reversible dissociation, enters into

* In a similar case, butyl β-D-glucopyranoside, isolated from a butanol extract of *Amanita muscaria*, was considered to be an artefact.[3]

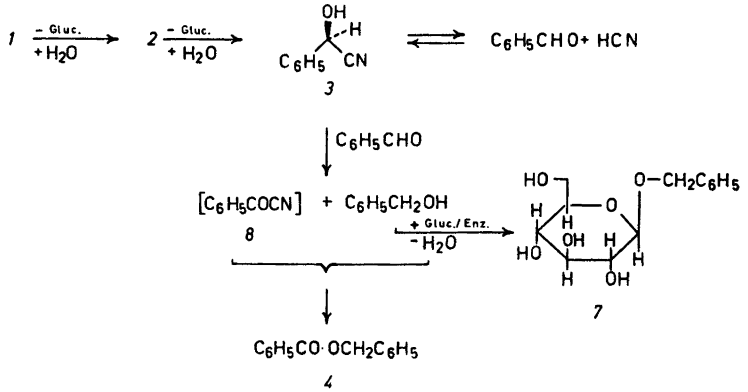

Table 1. Electron impact mass spectra of per(trimethylsilylated) glycosides recorded after gas chromatographic inlet.

Glycoside	Formula No.	M·+	Diagnostically important peaks (*m/e*) [a]
Prunasin	*2*	583 [b]	478[M−15−90 [c]], 234[PhCH(CN)OCH=$\overset{+}{O}$SiMe$_3$], 232, 116[PhCH(CN)+], 106[PhCHO·+], 105[PhCO+]
Amygdalin	*1*	961 [b]	829[M−PhCH(CN)O·], 232, 116, 106, 105
Cinnamyl β-D-glucopyranoside		584 [b]	479[M−15−90 [c]], 263, 117[PhCH=CHCH$_2$+] (base peak)
Benzyl β-D-glucopyranoside	*7*	558 [b]	453[M−15−90 [c]], 209[PhCH$_2$OCH=$\overset{+}{O}$SiMe$_3$] [d], 91[PhCH$_2$+] [e]
Butyl-β-D-glucopyranoside	*6*	524 [b]	419[M−15−90 [c]], 175[BuOCH=$\overset{+}{O}$SiMe$_3$] [f]
Sucrose		918 [b]	437, 289, 257, 169, 131 [g]

[a] Numerous additional peaks arising from the sugar moieties, hence of less diagnostic value (*e.g.* *m/e* 451, 361, 333, 331, 305, 291, 243, 233, 231, 217, 204, 191, 189, and 147), were consistently observed, yet with widely varying relative intensities. [b] Not observed. [c] Ninety mass units correspond to $Me_3Si{\cdot}OH$. [d] Found: 209.1014; Calc. 209.0998. [e] Found: 91.0549; Calc. 91.0548. [f] Found 175.1144; Calc. 175.1154. [g] *cf.* Ref. 6.

Table 2. Chemical ionization mass spectra of per(trimethylsilyl) glycoside derivatives.

Glycoside	Formula No.	Molecular weight	Reactant gasses	Quasi-molecular ions and diagnostically important peaks (*m/e*) [a]
Prunasin [b]	*2*	583	Isobutane + pyridine [c]	663[M+(79+1) [d]], 591[663−72 [e]]
Amygdalin [b]	*1*	961	Isobutane + ammonia [f]	979[M+(17+1) [g]], 962[M+1], 907 [979−72 [e]], 451, 379[361+(17+1)], 361
Cinnamyl-β-D-glucopyranoside [b]		584	Isobutane + pyridine [c]	664[M+(79+1) [d]], 592[664−72 [e]]
Benzyl β-D-glucopyranoside [j]	*7*	558	Isobutane Ammonia	559[M+1], 543[M−15], 453[M−15−90 [h]], 209 [i], 91 [i] 576[M+(17+1) [g]], 468[576−90 [h]]
Butyl-β-D-glucopyranoside [j]	*6*	524	Isobutane Ammonia	525[M+1], 509[M−15], 419[M−15−90 [h]], 175 [i] 542[M+(17+1) [g]], 452[542−90 [h]]

[a] Several additional peaks, arising from the sugar moieties and hence of less diagnostic value (*e.g.* *m/e* 451, 361 and 271), were consistently observed. [b] Direct inlet. [c] Pyridine used as solvent in the silylation reaction. [d] Corresponding to the molecular weight of protonated pyridine. [e] Corresponding to the molecular weight of $Me_2Si{=}CH_2$. [f] Ammonia produced in the silylation reaction. [g] Corresponding to protonated ammonia. [h] Corresponding to the molecular weight of Me_3SiOH. [i] *m/e*-Values identical with those observed in the electron impact spectra (*cf.* Table 1). [j] Introduced through the gas chromatograph.

a redox reaction with benzaldehyde to give benzoyl cyanide (*8*) and benzyl alcohol, the latter undergoing enzyme-catalyzed glucosylation to the bitter-tasting benzylglucoside (*7*). In this context, it may be of interest that benzoyl cyanide (*8*), together with mandelonitrile (*3*), were recently reported as a pair of cyanogenic constituents of the defensive secre-

tion of the centipede *Geophilus vittatus*; benzaldehyde and benzoic acid were other components present in the same secretion.[4]

During the present work mass spectrometry played a pre-eminent role. Extension of the previously reported technique for mass-spectrometric analysis of aliphatic cyanohydrin glucosides as per(trimethylsilyl) derivatives[5] to their aromatic counterparts was unexceptional. Diagnostically important peaks observed in the electron-impact mass spectra are listed in Table 1 along with similar peaks from a few simple glucopyranosides and sucrose, similarly derivatised. Useful, but only partly conclusive information could be extracted from the data (*cf.* Table 1) in view of the absence of molecular ions. In the chemical ionization mode, however, with ammonia, or isobutane, combined with pyridine or ammonia, as the reactant gasses, quasimolecular ions of considerable intensity were invariably observed (Table 2). In the same table, a number of fragment ions of diagnostic interest are presented. After the conclusion of the present work, the molecular weight (M = 961) of hepta(trimethylsilyl)-amygdalin was confirmed by chemical ionization mass spectrometry, with ammonia as the reactant gas.[7]

EXPERIMENTAL

Gas chromatography of silicon-free volatiles was performed on a 10 % Carbowax column maintained at 130 °C. The per(trimethylsilyl)-derivatives were chromatographed on a 2 % SE-30 column with a temperature gradient of 10 °C/min within the range 100 – 300 °C. Electron-impact mass spectra were recorded on a Perkin-Elmer model 270 mass spectrometer attached to a gas chromatograph, equipped with the columns specified above. Temperatures were: 250 °C (injector), 280 °C (transfer lines and separator), 200 °C (ion source). Carrier gas: He, flow rate, 40 ml/min. Ionization energy: 70 eV. CI-Mass spectra were recorded either on a JEOL D-300 mass spectrometer with isobutane or ammonia as reactant gasses (Table 2; *6*, *7*) or on a Varian MAT 311A mass spectrometer, with isobutane, in combination with pyridine or ammonia, as reactant gasses (Table 2; *1*, *2*, and the cinnamylglucoside).

^{1}H NMR spectra were recorded on a 90 MHz Bruker HX-90 E instrument.

Analysis of fresh bitterwater. A sample of bitterwater, withdrawn from the industrial process shortly after the extraction had been started, was heated briefly to its boiling point. After cooling, the solution was extensively extracted with ether.

After removal, through a short column, of most of the ether from the dried extract, an aliquot of the residue was subjected to gas chromatographic analysis. The only major, volatile component was benzaldehyde.

An aliquot (10 ml) of the ether-extracted, aqeueous phase was freeze-dried and the residue dissolved in anhydrous pyridine (1 ml), to which hexadimethyldisilazane (100 µl) and trimethylchlorosilane (50 µl) were added with careful exclusion of moisture. After standing for 20 min at 20 °C, 20 µl aliquots of the pyridine solution were injected into the gas chromatograph. Distinct peaks, in the ratio 1:70:3, with retention times of 16, 20, and 28 min, respectively, were observed, additional to large peaks of per(trimethylsilyl)-derivatives of α- and β-glucose, emerging with retention times of 11 and 12 min. The identity of the 16 and 28 min peaks as per(trimethylsilyl) derivatives of prunasin and amygdalin, respectively, was ascertained upon GC/MS-comparison with authentic specimens (Tables 1 and 2). The 20 min peak, according to its MS-characteristics (*cf.* Table 1), represents per(trimethylsilyl)-sucrose, confirmed by MS comparison with an authentic specimen.

No peak was detectable at the site of authentic per(trimethylsilyl) benzyl β-D-glucopyranoside (retention time: 17 min).

Analysis of bitterwater after emulsin hydrolysis. A briefly boiled and ether-extracted bitterwater sample (400 ml), prepared as described above, was treated with sweet almond emulsin (50 mg, 'Sigma', 5 units/mg) and left overnight at room temperature.

GC/MS-Analysis of a concentrated ether extract disclosed its contents of benzaldehyde and benzyl alcohol, with retention times of 2 and 9 min, respectively, identified upon comparison with authentic specimens. Preparative TLC on silica gel plates (C_6H_6:EtOAc, 9:1) brought about separation into benzaldehyde (16 mg), benzyl alcohol (13 mg,) and mandelonitrile (17 mg), the latter identified by its ^{1}H NMR-spectrum ($CDCl_3$, δ 7.4 (5 H, s), 5.4 (1 H, d), and 3.4 (1 H, d, exchangeable in D_2O).

In the non-bitter, aqueous extract no trace of prunasin or amygdalin could be detected after freeze-drying, silylation, and GC/MS-analysis; the derivatised sucrose, however, remained unchanged.

Production and isolation of the bitter principle. A sample of bitterwater was treated with exogeneous emulsin; after standing at 20 °C overnight, the solution was concentrated *in vacuo*, at a bath temperature not exceeding 40 °C, to a thin syrup which was sequentially extracted with: (i) light petroleum; (ii) ether; (iii) ethyl acetate; (iv) butanol. The light petroleum extract contained, according to GC/MS-analysis, a series of hydrocarbons which were not subjected to further studies. GC/MS-

Analysis of the ether extract showed that it contained substantial quantities of benzyl benzoate (*4*), in addition to benzaldehyde and several unidentified, minor components. Concentration of the ethyl acetate extract afforded a nicely crystalline product (amounting to 0.2 g from a portion of bitterwater containing 70 g of dry matter). Two recrystallizations from EtOAc afforded colourless needles, m.p. 121 °C, $[\alpha]_D^{21}$ −73° (*c* 1.8, Me_2CO). The rotation, the MS with characteristic peaks at *m/e* 151 (M^+), 134, 108, 107 (base peak), 105, 90, 79, and 77, and the ^1H NMR spectrum (in MeCN), with signals at δ 7.2 (5H, s), 5.8–7.0 (2H, broad), 4.9 (1H, d), and 4.4 (1H, d, exchangeable in D_2O) unambiguously define the crystalline product as (−)-mandelamide [reported:[8] m.p. 122–122.5 °C; $[\alpha]_D^{14.4}$ −73.1° (*c* 1.64, Me_2CO)], possessing (*R*)-configuration (*5*) in view of its synthetic derivation from (−)-mandelic acid[8] of established (*R*)-configuration.[9]

All bitterness remained, however, in the aqueous phase, but could be removed upon exhaustive extraction with butanol. The concentrated butanol extract, chromatographically separable into two bitter-tasting components on silica gel plates (EtOAc:EtOH, 8:1), was freeze-dried, silylated, and subjected to GC/MS-analysis. Whereas the EI-mode (Table 1) provided useful but hardly conclusive information as to the identity of the two major components, the CI-spectra, with their strong quasi-molecular ions (Table 2) revealed the identity of the two fractions as the per(trimethylsilylated) butyl and benzyl β-D-glucopyranosides (*6*) and (*7*). Comparative GC/MS-analysis, performed on the derivatised, authentic glucosides, served to ascertain this diagnosis.

A control experiment, performed as described above but omitting the butanol extraction, revealed benzyl β-D-glucopyranoside as the sole, bitter-tasting product of the ether-extracted, aqueous solution.

Enzymic synthesis of cinnamyl β-D-glucopyranoside. A mixture of non-heated bitterwater (25 ml) and cinnamyl alcohol (0.5 g) was set aside at 20 °C for 40 h. The ether-extracted aqueous phase was freeze-dried, and the residue was chromatographed (silica gel plates; ethyl acetate:ethanol, 8:1) to give a purified fraction (10 mg), identified as cinnamyl β-D-glucopyranoside through its ^1H NMR spectrum and MS characteristics, the latter recorded in the EI (Table 1) as well as in the CI-mode after per(trimethylsilylation) (Table 2).

Only one reference to the cinnamylglucoside has been found in the literature, *viz.* to its production from glucose and cinnamyl alcohol, catalyzed by emulsin.[3]

Acknowledgements. The authors are grateful to Dr. J. Møller, Odense University, Denmark, and to the JEOL company, Tokyo, for help in recording of the CI-mass spectra. Thanks are due also to Dr. L. Dalgaard, The Royal Agricultural and Veterinary University, Denmark, for accurate mass measurements. The Perkin-Elmer mass spectrometer was kindly placed at our disposal by the Danish National Science Research Council.

The authors are grateful to the Danish Fancy Food Group A/S, Odense, Denmark, for the gift of the industrial bitterwater used in the present investigation.

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Received April 18, 1978.