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16. The water-soluble solids loosely described as peptides obtained from these and other experiments are probably partial hydrolysis products of cyanide polymers possessing segments of several kinds (*4*) including **3**, **4**, and **5**. More relevant to the proposed model are high-pressure liquid chromatography experiments now in progress with nonaqueous solvents to separate the various polymeric structures obtained from HCN polymers synthesized in solvents other than water.
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Mandelonitrile β-Glucuronide: Synthesis and Characterization

Abstract. *Mandelonitrile β-glucuronide, the compound patented as Laetrile®, has been synthesized from rabbit liver uridine diphosphate-glucuronosyl transferase immobilized on beaded sepharose, has been analyzed by thin-layer chromatography, nuclear magnetic resonance, and gas chromatography-mass spectrometry, and has been tested for cytotoxicity and mutagenic activity with* Salmonella typhimurium *strains TA 98 and TA 100. Several commercial laetrile preparations contained no glucuronide; they contained amygdalin and neoamygdalin instead. Mandelonitrile, mandelonitrile glucuronide, and a mixture of amygdalin and neoamygdalin were each found to be mutagenic.*

Significant structural differences exist between mandelonitrile glucuronide (**1**), originally patented (*1*) as Laetrile®, and amygdalin (**2**) (see Fig. 1), the mandelonitrile β-*d*-gentiobioside, reported (*2*) to be the major component of Mexican preparations marketed as laetrile.

Laetrile® (**1**) was originally reported (*1*) to be obtained from amygdalin by a multistep chemical synthesis. *R*-Amygdalin (**2**) is commercially derived from a variety of natural sources, including apricot pits (*2*), and it has also been synthesized chemically (*3*).

Considerable confusion exists (*2*) concerning the relation between the structure and nomenclature of these two compounds. We undertook to obtain physicochemical data on **1** and **2** which would permit the reliable identification of these compounds in products found on the market.

Although authentic amygdalin (**2**) was available commercially, we had to develop a synthesis of mandelonitrile glucuronide (**1**) in our own laboratories. The synthesis of **1** described in the original patents (*1*) has not been reproduced (as revealed by our search of the chemical literature); to us, a more feasible approach to this synthesis appeared to be through the Koenigs-Knorr reaction that has provided other glucuronides on a large scale (*4*). With this reaction we were successful in producing the glucuronide of benzyl alcohol, but not the glucuronide of mandelonitrile (**1**). However, the β-glucuronide of *dl*-mandelonitrile was easily prepared from UDP-glucuronosyl transferase (E.C. 2, 4, 1, 17) immobilized on beaded sepharose. The technique for this procedure has been described (*5*).

Conjugation of racemic mandelonitrile was expected to lead to two glucuronides, mandelonitrile glucuronide and a similar structure that is epimeric at the benzyl center. For characterization by gas chromatography–mass spectrometry (GC-MS), the product mixture was converted by standard methods to tetrakis (trimethylsilyl) derivatives. The gas chromatogram contained two peaks of equal height whose mass spectra were virtually identical. The GC retention times of the two tetrakis trimethylsilylated diastereomeric epimers (*6*) are given in Table 1. Each of the mass spectra contained molecular ions (M) at mass 597, accompanied by M − 15 ions of mass 582, peaks characteristic of persilylated glucuronides (*7*) at *m/e* 375, 333, 217, and 204, and peaks at *m/e* 116 and 133, corresponding to the ionized aglycon moiety. The product mixture was also converted to the tris(trimethylsilyl) methyl ester derivatives and analyzed by GC-MS. However, trifluoroacetylation did not provide satisfactory derivatives. The nuclear magnetic resonance spectrum of the product of enzymatic conjugation measured in [$^2H_6$]dimethyl sulfoxide exhibited absorptions at δ 7.49 (singlet, phenyl protons), 5.95, and 5.99 (chiral benzyl proton in the two diasteromers) and 2.72 to 4.93 (multiplet, glucosiduronic protons) (*8*). The two products were designated as the expected glucuronides of mandelonitrile (**1**) epimeric about the chiral benzyl center. The patent literature (*1*) appears to specify the epimer with the *R* configuration at the benzyl carbon as Laetrile®.

Commercial amygdalin (**2**) (*9*) was also converted to its per (trimethylsilyl) derivative for analysis by GC-MS. As indicated in Table 1, the retention time of the derivatized amygdalin (**2**) was quite

Table 1. Retention data for laetrile, amygdalin, and related samples. The TLC systems consisted of butanol, benzene, water, and methanol (2:1:1:1.25). Silica gel 60 F-254 plates (E. E. Merck) were used. The GC-MS system consisted of the DuPont Dimaspec 321 GC-MS; 3 percent OV-101, 1.3-m column temperature programming 200° to 300°C at 4° per minute; helium at 30 ml/min; and samples derivatized with BSTFA (Pierce Chemical Co.) in 1 percent trimethyl chlorosilane and pyridine. Other instruments were used with similar results.

Sample	TLC (R_F)	GC-MS* (retention)*
Mandelonitrile	0.91	
Benzyl alcohol	0.91	
Benzyl alcohol glucuronide	0.49	0.71
Mandelonitrile glucuronides (**1**)	0.50 0.50	0.78 0.81
R-Amygdalin (**2**)	0.55	1.90
Commercial laetrile		
R-amygdalin	0.55	1.90
Neoamygdalian	0.55	1.83

*The ratio of retention time of the sample to the retention time of (tetrakis)trimethylsilated *p*-nitrophenol glucuronide. Mandelonitrile and benzyl alcohol were eluted in the solvent peak.

Table 2. Mutagenic activities on *S. typhimurium* (strains TA 100 and TA 98).

S_9 treatment*	Compounds per plate (nmole)	Revertants per nanomole	
		TA 100	TA 98
	Mandelonitrile		
	22.5	0.31	
	67.5	0.22	0.16
	225	0.24	0.14
	22.5	0.44	1.15
	67.5	0.40	1.08
	225	0.45	0.87
	22.5		0.27
	67.5	0.32	0.33
	225	0.28	0.39
	Mandelonitrile glucuronide		
	9.6	0	0
	28.8	0	0
	96	0	0
Aroclor	9.6	0.31	
	28.8	0.24	0.21
	96	0.19	0.24
Phenobarbital	9.6	0	0
	28.8	0	0
	96	0	0

*Livers were induced as indicated before preparation of microsomal fraction S_9 used in the mutagen test.

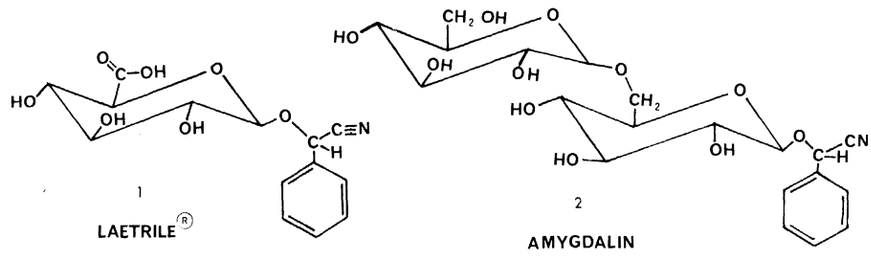

Fig. 1. The structures of Laetrile® (**1**) and *R*-amygdalin (**2**).

distinct from the retention times of synthetic Laetrile® and its epimer. The molecular weight of hepta(trimethylsilyl)-amygdalin was confirmed as 961 by chemical ionization mass spectrometry, with ammonia as the reagent gas (*10*). Attempts to form the trifluoroacetyl derivative of **2** led to pentakis (trifluoroacetyl) glucose. Although molecular ions were not present in the electron impact spectrum of heptatrimethylsilylated **2** introduced through the GC, peaks characteristic of the aglycon and saccharide sequence were present, including those at *m/e* 116, 133, 451, and 493. Other intense peaks in the spectrum occur at *m/e* 147, 204, 217, 291, 305, and 361, characteristic of silylated sugars. The nuclear magnetic resonance (NMR) spectrum (in [$^2H_6$]dimethyl sulfoxide) of amygdalin contains absorptions at δ 7.52 (singlet, phenyl protons), 5.98 (singlet, chiral benzyl proton), 3.13 (singlet, glucosyl hydroxyl, and water protons), and 2.74 to 5.40 (multiplet, glucosyl protons) (*8*). Laetrile® (**1**) and amygdalin (**2**) can also be easily distinguished from each other by thin-layer chromatography (TLC) (Table 1).

After the reference materials had been characterized, a sample of commercial laetrile was analyzed by TLC, GC-MS, and NMR. The preparation contained no mandelonitrile glucuronide (**1**). Rather, the two major constituents detected were amygdalin and an isomer of amygdalin. This isomer had a GC retention time (Table 1) similar to that of **2** and a mass spectrum identical to that of amygdalin (**2**). The NMR spectrum of the commercial sample was also identical to that of amygdalin, except that, instead of the signal at δ 5.98, assigned to the benzyl proton, two peaks of roughly equal size appeared at δ 5.98 and 6.06. On the basis of this information, the isomer was assigned as epimeric about the chiral benzyl center. The characterization of neoamygdalin was further supported when amygdalin was treated with 29 percent ND_3 in D_2O. The benzyl proton exchanged with deuterium, and the NMR signal at δ 5.89 (in D_2O) disappeared. When amygdalin (**1**) was treated briefly with 29 percent NH_4OH at room temperature and the solvent was changed to [$^2H_6$]dimethyl sulfoxide, the singlet at δ 5.98 became a 1:1 doublet at 5.98 and 6.06, supporting the conclusion that epimerization occurred at the benzyl carbon under these mild basic conditions.

Laetrile® was also obtained from two sources which submitted samples in partial support of two petitions. These samples were analyzed by NMR. On the basis of the NMR spectra, particularly the doublets at δ 5.98 and 6.06 discussed above, these two samples appeared to contain amygdalin (**2**) and neoamygdalin, but not mandelonitrile glucuronide (**1**).

Because no other successful synthesis of mandelonitrile glucuronide (**1**) has been reported since the original issuance of the Laetrile® patents, we felt obliged to carry out a minimal assessment of the biological activity of our synthetic product. We tested the mutagenic activity of our diastereomeric product with *Salmonella typhimurium* strains TA 98 and TA 100 (*11*). We also tested the parent aglycon, racemic mandelonitrile. Both compounds released cytotoxic HCN under the conditions of the incubation, and it was necessary to use an incubator whose atmosphere was continuously renewed. Otherwise the tests were run by a modification of the procedures of Ames *et al.* (*12*) as described (*11*). The aglycon mandelonitrile exhibited mutagenic activity in both strains (Table 2), which was enhanced by a microsomal fraction of rat liver, especially when the latter had been induced by Aroclor. The glucuronide (Table 2) showed mutagenic activity in both strains only in the presence of Aroclor-activated microsomes. This result may indicate hydrolysis to mandelonitrile.

Amygdalin was also found to be mutagenic in host-mediated assays with both tester strains (TA 100 and TA 98) (Table 3). The mutagenic activity of the urine is usually increased by treatment with mixture of β-glucuronidase and arylsulfatase.

The mutagenic activities exhibited by mandelonitrile, mandelonitrile glucuronide, and amygdalin against the bacterial strains fall within the normal range of activity exhibited by mutagens, carcinogens, and many chemotherapeutic agents (*13*).

Note added in proof: Nahrstedt (*14*) has examined the NMR spectra of amygdalin, isomerized in H_2O, D_2O, and ammonia, run in CCl_4 as the TMS derivatives. He observed two singlets at 5.50 and 5.57 S for the methine protons in the *R*- and *S*- configurations, respectively. The material isomerized in D_2O gave no signal in this region because of deuterium exchange during isomerization.

Table 3. Mutagenic activity of amygdalin administered orally to mice. Immediately after the administration of amygdalin to 6- to 8-week-old female mixed Swiss Webster mice (CF-1), 3×10^9 organisms of the tester strain were injected intraperitoneally; after 6 hours, they were retrieved and assayed as described (*11*). The values represent the number of revertants per 10^8 bacteria in excess of controls. The urines were obtained from mice kept in metabolism cages and were collected in containers surrounded by wet ice for 24 hours, beginning immediately after the administration of amygdalin. The mutagenic activities of 0.25- and 0.5-ml portions of urines were assayed without prior concentration. All determinations were conducted in duplicate and in the presence of a rat liver microsomal fraction (S_9) (*12*). Figures indicate revertants in excess of controls (urines before treatment) excreted per mouse over 24 hours. Without, indicates activity before incubation with the mixture of β-glucuronidase and arylsulfatase; with, indicates activity after incubation with β-glucuronidase-arylsulfatase mixture.

Single oral dose of amygdalin (mg/kg)	Host-mediated assay		Mutagenic activity of urines			
			TA 100		TA98	
	TA 100	TA 98	Without	With	Without	With
125			186	285	69	81
250	44 ± 5	36 ± 3	447	665	156	143

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3β-Hydroxy-5α-cholestan-6-one: A Possible Precursor of α-Ecdysone Biosynthesis

Abstract. *The conversion of cholesterol into 3β-hydroxy-5α-cholestan-6-one has been demonstrated to occur in prothoracic glands of last instar larvae of the silkworm,* Bombyx mori. *Incubation of glands containing radiolabeled 3β-hydroxy-5α-cholestan-6-one results in the disappearance of this sterol from the glands and the concomitant appearance of radiolabeled α-ecdysone in the medium. The observations suggest that the sterol is an intermediate in the synthesis of α-ecdysone.*

Since the elucidation of the structures of α- and β-ecdysone (*1*), a number of synthetic cholesterol derivatives have been studied as possible intermediates in the conversion of cholesterol to ecdysone (*2*). However, no attempts have been made to identify the intermediates of α-ecdysone synthesis in the prothoracic gland, which is a major site of α-ecdysone production (*3, 4*). Organ culture of prothoracic glands facilitates biosynthetic studies of this type, and has been used in this investigation of α-ecdysone synthesis in the prothoracic gland of the silkworm, *Bombyx mori*.

We have demonstrated that *Bombyx* prothoracic glands produce a large amount of α-ecdysone when the glands are cultivated in medium containing hemolymph (*3*). This, together with related reports (*5, 6*) and the observed presence of cholesterol in hemolymph (*7*), has led to the general assumption that cholesterol is a precursor of ecdysone synthesis. To further substantiate the role of cholesterol in ecdysone synthesis, prothoracic glands (day 9, fifth instar) were cultured in Wyatt's insect culture medium in the presence and in the absence of cholesterol. The glands were maintained in culture for 5 days before α-ecdysone was extracted from the medium and bioassayed by Ohtaki's method (*8*). The results (Table 1) indicated that inclusion of cholesterol, emulsified with Tween 80, in the culture medium enhanced ecdysone production four- to fivefold to levels similar to those obtained in pure hemolymph. However, when the glands were cultured in medium to which only the emulsifying agent Tween 80 was added, a similar enhancement was observed. These data suggest that the major precursors for ecdysone synthesis are contained within the gland, and no extraneous supply is required during the 5-day incubation period. The role of Tween 80 in activating ecdysone synthesis is not understood.

The extent of cholesterol incorporation from the medium into α-ecdysone was investigated by incubating 30 pairs of prothoracic glands in Wyatt's medium containing [4-^{14}C]cholesterol (4.125×10^7 dis/min, 380 nmole) emulsified with Tween 80. After 5 days, α-ecdysone was extracted from the medium and purified by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) (*9*). The amount of α-ecdysone recovered was 7.6 nmole, estimated by the ultraviolet absorbance of the HPLC fraction (*10*). This represented 2 percent of the total cholesterol (380 nmole) available in the medium. The radioactivity associated with the α-ecdysone fraction was 3.4×10^3 dis/min, indicating conversion of 0.0082 percent of the labeled cholesterol into α-ecdysone. From these data it can be calculated that only 0.4 percent of the total α-ecdysone produced was derived from the cholesterol provided in the medium. Thus the major precursor of α-ecdysone was not the cholesterol contained within the incubation medium.

Table 1. Effect of cholesterol and Tween 80 on the synthesis of α-ecdysone by prothoracic glands. Glands were cultured in Wyatt's medium containing cholesterol (100 μg/ml) emulsified with Tween 80 (20 μg in 1 percent ethanol), or only Tween 80 (20 μg/ml in 1 percent ethanol). The amount of hormone is expressed as nanograms of α-ecdysone per pair of glands.

Medium	Gland cultures (pairs)	Amount of hormone
Wyatt's	23	15
Wyatt's + hemolymph*	22	81
Wyatt's + cholesterol + Tween 80	28	70
Wyatt's + Tween 80	28	67

*See (*3*).

When last instar larvae were injected with labeled cholesterol, an unknown labeled sterol was found to accumulate in the prothoracic glands. Incubation of such glands resulted in the disappearance of this compound from the glands and the concomitant appearance of labeled α-ecdysone in the culture medium (Fig. 1). The radioactivity associated with the α-ecdysone fraction was 118 dis/min per gland (*11*), which was 4 percent of that present in the labeled sterol fraction of the glands (2960 dis/min per gland) before incubation. This conversion ratio far exceeded that (0.0082 percent) obtained with cholesterol in the medium, and identified the unknown sterol as a possible precursor of α-ecdysone.

The unknown sterol was purified and chemically identified by a combination of chromatographic and mass-spectrometric procedures. Prothoracic glands (400) were removed from larvae (day 9, fifth instar) and extracted with chloroform and methanol (2:1), and the extract was applied to TLC plates (the developing solvent was benzene and acetone, 2:1). The labeled unknown steroid (*12*) was put through the procedure at the same time as a marker. The fraction corresponding to the unknown sterol was eluted and subjected to HPLC together with the labeled marker; it was then collected and analyzed by gas chromatography–mass spectrometry (GC-MS). The presence of peaks at 402 (M), 387 (M−15), 384 (M−18), 369 (M−15−18), 289 (M−113), and 271 (M−113−18) indicated that the unknown sterol has a cholesterol skelton with two oxygen functions. The unknown compound was silylated with trimethylsilylimidazole at