POTENTIAL ANTITUMOR AGENTS: A CYTOTOXIC CARDENOLIDE FROM *CORONILLA VARIA*[1]

JOHNNY A. HEMBREE, C.-J. CHANG, JERRY L. MCLAUGHLIN, GARNET PECK and JOHN M. CASSADY[2]

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907

ABSTRACT.—Hyrcanoside has been previously isolated as a cytotoxic and antitumor cardenolide of the seeds of *Coronilla varia* L. (crown vetch). Reextractions detected cytotoxic activity attributable to another cardenolide which was isolated and crystallized after repeated chromatographic manipulations. Spectral analyses (pmr, ^{13}C nmr, and ms) suggested that the compound was deglucohyrcanoside. Partial enzymatic hydrolysis of hyrcanoside provided reference compound which was identical (tlc, ms).

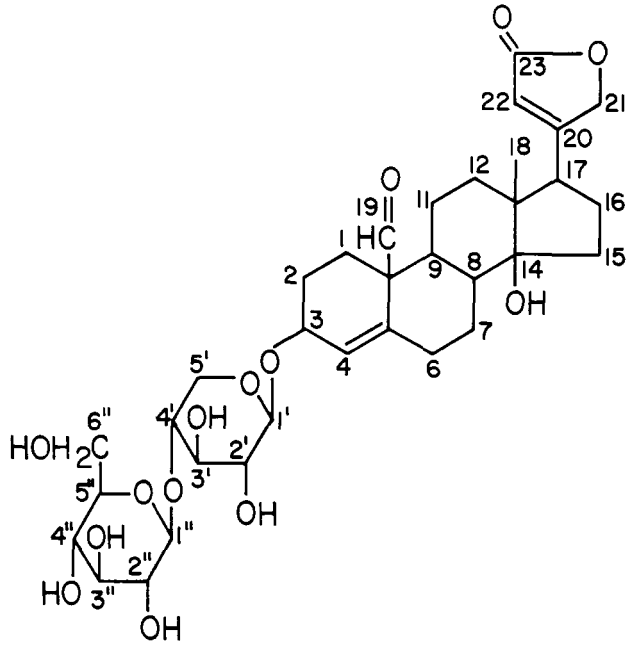
Coronilla varia L. (var. Penngift) (Fabaceae) was previously investigated for its antitumor activity, and hyrcanoside (**1**), obtained by means of an activity-directed isolation, was found to be the principle active component (1). Additional cytotoxic cardenolides were detected while isolating larger quantities of hyrcanoside. Therefore an investigation regarding their identity and activity was initiated.

The powdered seed material was extracted with 95% ethanol followed by 50% ethanol. The 50% ethanol solubles were subsequently partitioned between chloroform-ethanol (8.5:1.5) and water. The water solubles were partitioned further with chloroform-ethanol (2:1) and then with chloroform-ethanol (1:1). The respective organic residues were tested for P388 and 9KB activity (table 2) (2). The 9KB activity was found to concentrate in the choloform-ethanol (2:1) soluble fraction (ED_{50} 1.8×10^{-1} $\mu g/ml$). Tlc indicated that the chlorform-ethanol (2:1) soluble fraction contained the largest proportion of hyrcanoside (**1**) and another cardenolide, substance A. Column chromatography of the fraction on silica gel yielded both a hyrcanoside-enriched fraction and a fraction enriched with substance A. Further chromatography on silica gel, columns and plates, provided 70 mg of pure substance A.

Pmr (1, 3, 4), ^{13}C nmr (5, 6) and ms (7–9) analysis of substance A suggested a monoglycosidic cardenolide very similar to hyrcanoside (**1**). Prior phytochemical studies of *Coronilla* species containing cardenolides having the hyrcanogenin nucleus suggested that substance A was deglucohyrcanoside (**2**) (10, 11). Identification of substance A as deglucohyrcanoside (**2**) was confirmed by a step-wise enzymatic hydrolysis of a sample of semi-pure hyrcanoside (**1**) with β-amylase (11, 12). The chloroform-ethanol solubles thus obtained were analyzed by tlc. Hyrcanoside (**1**, R_f 0.26) along with two other Kedde positive components (R_f 0.58 and 0.76, respectively) were visualized in the mixture with no other contaminants indicated by $uv_{254-366}$. The two high-running cardenolides were isolated by preparative tlc and recrystallized from aqueous methanol. Melting points corresponded to literature values for deglucohyrcanoside (**2**) and the aglycone, hyrcanogenin (11). CIms showed the expected fragmentation patterns for the two cardenolides (9). Substance A co-chromatographed (tlc) with deglucohyrcanoside (**2**) obtained enzymatically. In addition, these two compounds showed identical CIms fragmentation patterns.

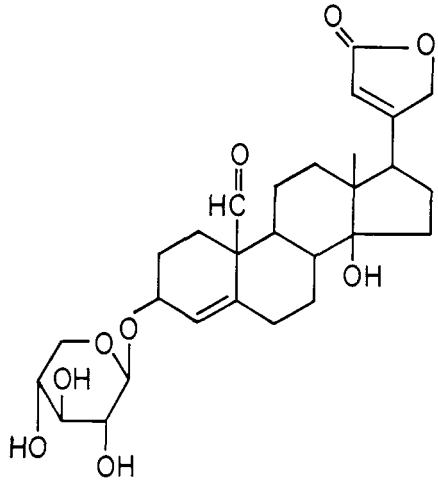
[1]Part 8 in the series Potential Antitumor Agents. Previous paper: J. A. HEMBREE, C.-J. CHANG, J. L. MCLAUGHLIN, G. PECK, and J. M. CASSADY, *Lloydia*, **41**, 491 (1978).

[2]To whom correspondence should be addressed.



1, *Hyrcanoside*

A comparison of the ^{13}C nmr spectral data of substance A with that of hyrcanoside (**1**) and its hexaacetate further established their relationship. The spectral assignments were made through use of proton coupled and decoupled spectra, consideration of chemical shifts, and comparison to the spectra of structurally related compounds (5, 6, 13–15). Comparison of the ^{13}C nmr data of hyrcanoside (**1**) and deglucohyrcanoside (**2**) (table 1) with that of synthetic hyrcanoside hexaacetate allowed assignment of all of the glycone carbon atoms. The only significant difference between the ^{13}C nmr spectra of the two cardenolides was, as



2, Deglucohyrcanoside

expected, in that portion representing the glycone carbons (δ102.87–61.21). Additionally, the anomeric carbons, C–1′ and C–1″, were observed upon acetylation to be shifted upfield, along with C–3 of the hyrcanogenin nucleus (14, 17, 18).

The stability of xylose and glucose, derived by enzymatic degradation of hyrcanoside, to acid hydrolysis has previously ascertained their existence in the

TABLE 1. *^{13}C Nmr assignment for deglucohyrcanoside and* hyrcanoside.[a]

Carbon atom	Deglucohyrcanoside	Hyrcanoside
1	27.92[b]	27.61[b]
2	27.50[b]	27.46[b]
3	73.16[c]	73.32[c]
4	116.41	116.48
5	137.98	138.10
6	28.05[b]	27.92[b]
7	26.36	26.39
8	31.52[d]	31.59[d]
9	49.86	49.89
10	53.25	53.25
11	21.32	21.29
12	32.64[d]	31.68[d]
13	49.17	49.18
14	83.28	83.29
15	28.05[b]	28.05[b]
16	27.81[b]	27.92[b]
17	48.90	48.91
18	15.54	15.55
19	204.79	204.82
20	176.17	176.17
21	73.23	73.16
22	126.62	126.59
23	173.85	173.87
1′	102.87	102.60
2′	73.16[c]	73.23[c]
3′	76.75	76.64[e]
4′	69.63	77.09
5′	65.70	63.69
1″		101.80
2″		72.80
3″		76.48[e]
4″		70.24
5″		74.84
6″		61.21

[a]Spectra were obtained in DMSO-d_6 from a Varian FT 80 NMR Spectrometer. Values are presented in ppm (δ) relative to TMS (δ=0).

[b,c,d,e]May be reversed in vertical series.

pyranose ring form (16). The xylose and glucose carbons were assigned by consideration of their relative electron densities and by correlation to literature values (5, 18, 19). Anomeric C–1′ of deglucohyrcanoside was determined at δ102.87 due to the deshielding effects of the two geminal electron withdrawing oxygens. Additionally, the $^1J(^{13}C–^1H)$ value of C–1′ for **2** of 157.8 Hz indicated that the oxygen functionality exists in the β-position since the $^1J(^{13}C–^1H)$ for the α-anomer would lie nearer 177 Hz (20). C–2′ and C–4′ of **2** were assigned at δ73.16 and 69.63, respectively, due to their relative electron densities, as C–2′ is positioned α to anomeric C–1′ with the deshielding effects of two oxygens and the single oxygen at C–3′. These assignments agree with α- and β-carbon effects observed in a study

utilizing deuterium substitution (20). The assignment of C-4' was confirmed when peak displacement to δ77.09 was observed on comparison of deglucohyrcanoside (**2**) to hyrcanoside (**1**). Also, relatively little displacement for C-4' was noted (Δδ-1.28 ppm) in going from hyrcanoside to its hexaacetate. The downfield shift of the C-4' of xylose, on comparison of **2** to **1**, also provided proof for the C-1"→C-4' linkage of glucose to xylose. Historically this has been assumed by analogy (11, 21). The peak (5) centered at δ76.75 was assigned to C-3'.

The assignment of C-5' to the peak centered at δ65.7 (t, $J=147.4$ Hz) in **2** and C-5" to the peak centered at δ74.84 in **1** provided additional evidence for the existence of xylose and glucose in the pyranose ring form in both **1** and **2**. The existence of xylose in the furanose ring form, which was a possibility, would have necessitated the appearance of C-5' approximately 4-5 ppm further upfield (17-19). By analogy the assignment of C-2" (δ72.80), C-3" (δ76.48), and C-4" (δ70.24) was straighforward for **1**. C-6" was assigned to the peak centered at δ61.21 due to its expected electron density when compared to C-5' and its observed multiplicity. C-5' was assigned further downfield than C-6" due to its decreased electron density resulting from close proximity to the pyranose oxygen and the presence of β- and γ-glycosidic oxygens.

Table 2 contains the biological testing data obtained for both **1** and **2**. Both compounds were cytotoxic against 9KB cells; however, only hyrcanoside (**1**) showed activity in P388. Further testing of **1** in the tumor panel demonstrated little activity except in the C-6 mouse colon tumor system.

TABLE 2. *Antitumor and cytotoxic activity of* **1**, **2**, *and fractions from* Coronilla varia.

	9KB ED_{50} (μg/ml)	P388 % T/C at mg/kg	LL, C8, B1, LE	C6 % T/C at mg/kg
Hyrcanoside (NSC 256926) (**1**)	1.0	133 at 1.25	inactive	169 at 0.31
	0.7			
	0.1			143 at 2.5
Deglucohyrcanoside (Substance A, NSC 281266) (**2**)	0.52	inactive	—	—
	0.02	up to 8		
$CHCl_3$-EtOH Solubles				
(8.5:1.5)	10	—	—	—
(2:1)	0.18	—	—	—
(1:1)	0.7	—	—	—

EXPERIMENTAL[3]

[3]All mps are uncorrected. The pmr and ^{13}C nmr were obtained in DMSO-d_6 with TMS as the relative standard. Chemical ionization mass spectra (CIms) were obtained with isobutane at 70 eV from a solid probe. Tlc on silica gel F-254 was performed using chloroform-ethanol (2:1) as the developing solvent, unless otherwise indicated. Visualization of the cardenolides was accomplished *via* their positive Kedde's reaction (2% 3,5-dinitrobenzoic acid followed by 5% KOH in methanol as spray reagents) with which a violet color was obtained. Preparative tlc was performed as silica gel (2 mm) using the above solvent system, unless otherwise indicated. Cardenolide bands were located on preparative tlc plates by marginal spraying with the Kedde spray reagent. Fractions and compounds were tested against the P388 lymphocytic leukemia system in the mouse and Eagles 9KB nasopharyngeal carcinoma cell culture system according to established protocols (2). In the P388 system an effective response is obtained when a minimal percentage increase in the median survival time of treated animals over control animals (% T/C) results in a % T/C≥130. In the 9KB cell culture system results are expressed as that dose which inhibits growth to 50% of control growth (ED_{50}). An ED_{50} of 4 μg/ml and 20 μg/ml are considered effective for pure compounds and crude plant fractions, respectively. The P388 antitumor testing was performed by RALTECH Scientific Services, Inc. while the 9KB cytotoxicity testing of samples was performed by the Purdue Cancer Center Cell Culture Laboratory.

EXTRACTION AND ISOLATION.—Powdered seed (14.8 kg) was extracted with 46 liters of 95% ethanol and then with 50 liters of 50% ethanol by percolation. The 50% ethanol-soluble residue (1.714 kg) was partitioned between water and three respective chloroform-ethanol solutions (8.5:1.5, 2:1, and 1:1). The chloroform-ethanol (2:1) soluble residue (21.3 g) was subjected to column chromatography on silica gel (Silicar-7, 70–270 mesh, Mallinckrodt) by elution with chloroform through chloroform-methanol (1:1). A hyrcanoside (**1**) enriched fraction, 5.41 g, eluted with chloroform-methanol (4:1), and a substance A enriched fraction, 2.67 g, eluted with chloroform-methanol (9:1) were obtained. The hyrcanoside (**1**) fraction was partially purified by lpcc on silica gel (10–20 μ, Quantum Industries) by elution with chloroform-ethanol (4:1) to yield semi-pure hyrcanoside (2.57 g). Lpcc of the fraction containing substance A on silica gel (10–20 μ, Quantum Industries) provided an enriched fraction (0.82 g) that was eluted with chloroform-methanol (95:5). The fraction was rechromatographed with chloroform-methanol (98:2), and substance A (0.652 g) was obtained contaminated with a slightly lower overlapping component as evidenced by tlc. Further purification was provided by multiple preparative tlc on silica gel (30 mg/20 cm x 20 cm x 2 mm layer) developed with chloroform-ethanol (1:1) and by elution of the top third portion of the Kedde positive band with the developing solvent. Recrystallization from aqueous methanol gave 70 mg of pure substance A.

SUBSTANCE A (**2**, DEGLUCOHYRCANOSIDE, NSC 281266).—Mp 197–200° (water-methanol); ir (KBr): 2600–3060 (OH), 2850 (C*H*O), 1740 (CHO), 1714 (lactone CO), 1625 and 1612 cm^{-1} (C=C); pmr (60 MHz, DMSO-d_6): 0.8 (3H, s, H-18), 5.73 (1H, bs, H-4), 5.93 (1H, bs, H-22), 9.83 (1H, s, H-19): 3 exchangeable hydrogens with D_2O. ^{13}C nmr (table 2); CIms: *m/e* 369 (aglycone, 100), 351 (A-HOH, 89), 341 (A-CO, 18), 333 (A-2 HOH, 15), 323 (A-CO+HOH, 20) and 133 (xylose-HOH, 36): Field Desorption ms (FDms): $[M+H]^-$ *m/e* 519 (0.63), 368 (aglycone, 100).

MODIFIED STEP-WISE ENZYMATIC HYDROLYSIS.—β-Amylase (Sigma) (0.5 g) in 20 ml of water was added to semi-pure hyrcanoside (**1**, 0.5 g) in 50 ml of water and incubated at 47° with continuous stirring for 7 min. Work-up in the usual way yielded the chloroform-ethanol (2:1) soluble residue (151 mg). Preparative tlc yielded small quantities of deglucohyrcanoside (R_f 0.58, mp 197–200°; CIms: *m/e* 369 (97), 341 (100) 323 (46) and 133 (49), and hyrcanogenin (R_f 0.76, mp 227–230°; CIms: *m/e* 369 (61), 351 (33), 341 (100), and 323 (40).

ACKNOWLEDGMENTS

The authors thank Dr. Robert Perdue, USDA, for authenticating and supplying the plant material and Dr. David Brent and Mr. Gerald L. Peele, The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, N.C. 27709, for FDms data. The cytotoxicity testing was provided by Dr. Linda Jacobsen, Purdue Cell Culture Laboratory, Purdue Cancer Center. This investigation was supported by contract N01-CM-62091 awarded by the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD to Purdue University.

Received 17 January 1979.

LITERATURE CITED

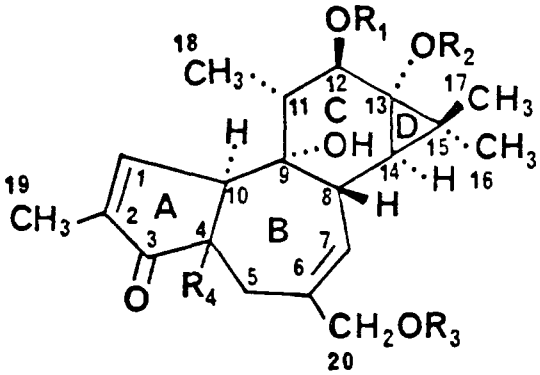
1. M. WILLIAMS and J. M. CASSADY, *J. Pharm. Sci.*, **65**, 912 (1976).
2. R. I. GERAN, N. H. BREENBERG, M. N. MACDONALD, A. M. SCHUMACHER and B. J. ABBOTT, *Cancer Chemother. Rep.*, Part 3, **3**, 1 (1972).
3. G. V. KAPADIA, *J. Pharm. Sci.* **58**, 1555 (1969).
4. R. W. DOSKOTCH, M. Y. MALIK, C. D. HUFFORD, S. N. MALIK, J. E. TRENT and W. KUBELKA, *J. Pharm. Sci.*, **71**, 570 (1972).
5. E. BREITMAIER and W. VOELTER, ^{13}C NMR Spectroscopy, in *Monographs in Modern Chemistry*," Vol. 5, H. G. Ekel, ed., Verlag Chemie, Weinheim/Bergstr., p. 1 1974.
6. L. F. JOHNSON and W. C. JANKOWSKI, "*Carbon-13 NMR Spectra*," John Wiley and Sons, New York, 1972, p. 494.
7. H. BUDZIKIEWICZ, C. DJERASSI and D. H. WILLIAMS, "*Mass Spectrometry of Organic Compounds*," Holden-Day, Inc., San Francisco, 1964, p. 64.
8. P. BROWN, F. R. BRUSCHWEILER, G. R. PETTIT and T. REICHSTEIN, *J. Amer. Chem. Soc.*, **92**, 4470, 1970.
9. B. BLESSINGTON and I. M. MORTON, *Org. Mass Spectrum.*, **3**, 95 (1970).
10. R. B. BAGIROV and N. F. KOMISSARENKO, *Khim. Prir. Soedin.*, **2**, 251 (1966).
11. N. F. KOMISSARENKO, *Khim. Prir. Soedin.*, **5**, 141 (1969).
12. P. I. GVOZDYAK, N. F. KOMISSARENKO and D. G. KOLESNIKOV, *Med. prom. SSSR*, no. 12, 12 (1960).
13. K. TORI, H. ISHII, Z. W. WOLKOWSKI, C. CHACHATY, M. SANGARE, F. PIRIOU and G. LUKACS, *Tetrahedron Letters*, 1077 (1973).
14. K. TORI, T. T. THANG, M. SANGARE and G. LUKACS, *Tetrahedron Letters*, 717 (1977).
15. J. W. BLUNT and J. B. STROTHERS, *Org. Mag. Res.*, **9**, 439 (1977).
16. A. HUNGER and T. REICHSTEIN, *Helv. Chim. Acta*, **35**, 1073 (1952).

17. R. G. S. RITCHIE, N. CYR, B. KORSCH, H. J. KOCH and A. S. PERLIN, *Can. J. Chem.*, **53**, 1424 (1975).
18. K. BOCK and C. PEDERSEN, *J. Chem. Soc.*, Perkin II, 293 (1974).
19. K. BOCK and C. PEDERSEN, *Acta Chem. Scand.*, B2g, 258 (1975).
20. P. A. J. GORIN and M. MAZUREK, *Canadian J. Chem.*, **53**, 1212 (1975).
21. T. REICHSTEIN and E. WEISS, *Advances in Carbohydrate Chemistry*, **17**, 65 (1962).
22. C. S. HUDSON and J. M. JOHNSON, *J. Org. Chem.* **37**, **2748** (1951).

ERRATA

A. Douglas Kinghorn: Characterization of an irritant 4-deoxyphorbal diester from *Euphorbia tirucalli*. Vol. **42**, No. 1, p. 113.

R_2 for structure **2** should be –H instead of –OH. The structure should be positioned as follows:



	R_1	R_2	R_3	R_4
1	$-COCH^{1'}(Z)=CH^{2'}-CH^{3'}(E)=CH^{4'}CH_2^{5'}CH_2^{6'}CH_3^{7'}$	$-COCH_3$	–H	β-H
2	$-COCH^{1'}(Z)=CH^{2'}-CH^{3'}(E)=CH^{4'}CH_2^{5'}CH_2^{6'}CH_3^{7'}$	–H	–H	α-H
3	$-COCH_3$	$-COCH_3$	$-COCH_3$	α-H

L. H. Zalkow, R. N. Harris, III, and N. I. Burke: The lower terpenoids of *Isocoma wrightii*. Vol. **42**, No. 1, pp. 96–102.

The correct spelling for the compound represented by structure **2** is modhephene.