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POLYOXYGENATED STEROID SOPHOROSIDES FROM THE DEFENSE GLANDS OF *CHRYSOLINA QUADRIGEMINA*

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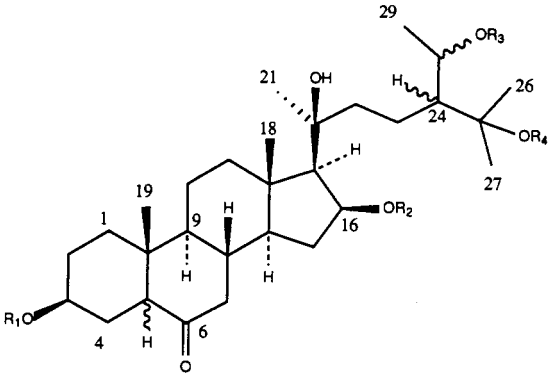
ABSTRACT.—The defensive secretion of the chrysomelid beetle *Chrysolina quadrigemina* contains six new steroid polyoxygenated sophorosides, **1**, **2**, **4**, **5**, **7**, and **8**, that were isolated as three pairs of 5α and 5β isomers. The structures of these compounds were determined by comparing the spectroscopic properties of the diastereomeric mixtures with those of closely related compounds isolated from other *Chrysolina* species.

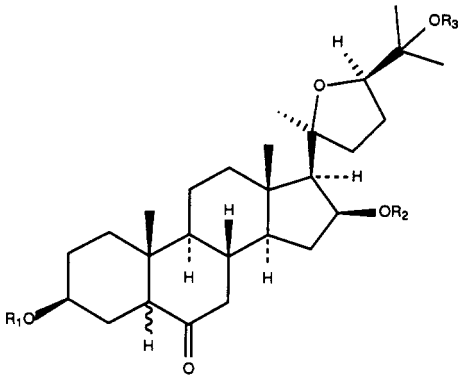
Chrysomelid beetles belonging to the sub-tribe Chysolinina are characterized by their capacity to biosynthesize cardenolides, which are found in defense glands opening at the surface of the pronotum and elytra (1,2). However, in contrast to this general trend, several *Chrysolina* species feeding exclusively on *Hypericum* produce original polyoxygenated steroid glycosides. Till now, nine of these compounds have been isolated from four species of the genus *Chrysolina* and their structures determined by spectroscopic methods (3,4). We have now studied the composition of the defense secretions of yet another species that feeds on *Hypericum: Chrysolina quadrigemina* Suffrian (Coleoptera, Chrysomelidae).

Tlc [SiO_2 plates, eluent CH_2Cl_2-MeOH (8:2)] of the defense secretion (44 mg from 770 individuals) collected by "milking" the beetles (1) showed the presence of two major and one minor spots. Repetitive Si gel flash chromatographies afforded three homogeneous fractions: CQ1 (5.0 mg), CQ2 (15.0 mg), and CQ3 (3.0 mg), in order of increasing polarity. The ^{1}H-nmr spectra of these fractions (see below) showed that they were still mixtures of two closely related compounds that could not be separated from each other. Structure determinations were thus realized on these mixtures.

The major compound present in fraction CQ2 (about 90%) was shown to have structure **1** on the basis of its spectral properties ($[M+Na]^+$ at *m/z* 883 in positive ion fabms; fragment ions at *m/z* 682 $[M+H-OH-162]^+$, 664 $[M+H-OH-162-H_2O]^+$, 520 $[M+H-OH-2\times162]^+$, 502 $[M+H-OH-2\times162-H_2O]^+$; $[M-H]^-$ at *m/z* 859 in negative ion fabms; ir ν C=O 1730 and 1705 cm^{-1}, ν C-O 1274 cm^{-1}; ^{1}H nmr see Table 1). These data are compatible with molecular formula $C_{43}H_{72}O_{17}$ for **1**, the presence of two hexose moieties, and a $C_{31}H_{52}O_7$ steroid aglycone. Moreover, except for the presence of only one acetyl group, the ^{1}H-nmr spectrum of **1** was very similar to that of triacetate **3**, isolated from *Chrysolina brunsvicensis* and *Chrysolina geminata* (4). This suggested the presence in **1** of the familiar β-sophorose moiety **9** and the 6-oxo-5β-stigmastane skeleton, which are characteristic of this group of beetles. The acetyl group of **1** was easily located at C-28 of the stigmastane skeleton on the basis of the characteristic signal (4) of the H-28 (δ 5.32, dq, $J = 6.5$ and 3.0 Hz), whereas the presence of hydroxyl groups at C-20 and C-25 was deduced from the chemical shifts of the Me-21 (δ 1.31, s), Me-26 and Me-27 (δ 1.24 and 1.29, s) groups. The last OH group was placed at C-16β, because the Me-18 group (δ 1.14) underwent a strong deshielding arising from a 1,3-Van der Waals interaction with a C-16β substituent (5,6). This assignment was further supported by comparison of the ^{1}H-nmr spectrum of octaacetyl-**1** [**1a**], with that of **3a**, allowing inter alia identifica-



			R_1	R_2	R_3	R_4
1	5β-H	β-sophorose	H	Ac	H
1a	5β-H	β-acetylsophorose	Ac	Ac	H
2	5α-H	β-sophorose	H	Ac	H
2a	5α-H	β-acetylsophorose	Ac	Ac	H
3	5β-H	β-sophorose	Ac	Ac	Ac
3a	5β-H	β-acetylsophorose	Ac	Ac	Ac
7	5β-H	β-sophorose	H	H	H
8	5α-H	β-sophorose	H	H	H



			R_1	R_2	R_3
4	5β-H	β-sophorose	H	Ac
4a	5β-H	β-acetylsophorose	Ac	Ac
5	5α-H	β-sophorose	H	Ac
5a	5α-H	β-acetylsophorose	Ac	Ac
6	5α-H	β-sophorose	Ac	H
6a	5α-H	β-acetylsophorose	Ac	H

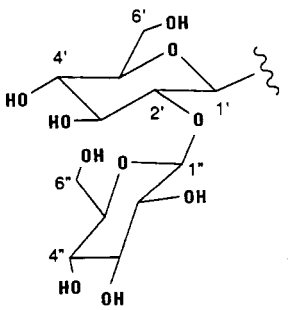


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TABLE 1. ^{1}H-nmr Data of Natural Glycosides **1**, **4**, **5**, **7**, and **8** (CD_3OD, TMS, δ, *J* in Hz, 250 MHz).

Proton	Compound				
	1	**4**	**5**	**7**	**8**
Me-18	1.14 (s)	1.10 (s)	1.10 (s)	1.12 (s)	1.12 (s)
Me-19	0.87 (s)	0.87 (s)	0.77 (s)	0.87 (s)	0.77 (s)
Me-21	1.31 (s)	1.36 (s)	1.36 (s)	1.29 (s)	1.29 (s)
Me-26	1.24 (s)[a]	1.45 (s)[a]	1.45 (s)[a]	1.25 (s)[a]	1.25 (s)[a]
Me-27	1.29 (s)[a]	1.54 (s)[a]	1.54 (s)[a]	1.29 (s)[a]	1.29 (s)[a]
Me-29	1.25 (d, 6.5)	—	—	1.24 (d, 6.5)	1.24 (d, 6.5)
H-3	4.08 (bs)	4.08 (bs)	[b]	4.13 (bs)	[b]
H-5	2.56 (dd, 12.3, 4.2)	2.57 (dd, 12.3, 4.2)	[b]	2.56 (dd, 12.3, 4.2)	[b]
H-28	5.32 (dq, 6.5, 3.0)	—	—	[b]	[b]
H-1′	4.43 (d, 7.5)	4.43 (d, 7.5)	4.53 (d, 7.7)	4.43 (d, 7.5)	4.52 (d, 7.7)
H_a-6′	3.85 (dd, 12.5, 2.0)	3.86 (dd, 12.5, 2.0) [b]	3.86 (dd, 12.5, 2.0) [b]	3.85 (dd, 12.5, 2.0) [b]	3.85 (dd, 12.5, 2.0) [b]
H_b-6′	3.68 (dd, 12.5, 5.0)				
H-1″	4.66 (d, 7.5)	4.67 (d, 7.5)	4.58 (d, 7.7)	4.68 (d, 7.5)	4.58 (d, 7.7)
H_a-6″	3.85 (dd, 12.5, 2.0)	3.86 (dd, 12.5, 2.0) [b]	3.86 (dd, 12.5, 2.0) [b]	3.85 (dd, 12.5, 2.0) [b]	3.85 (dd, 12.5, 2.0) [b]
H_b-6″	3.68 (dd, 12.5, 5.0)				
OAc	2.01 (s)	1.97 (s)	1.97 (s)	—	—

[a]Assignments are interchangeable.
[b]Overlapped by other signals.

tion of the 16-C*H*-OAc signal at δ 5.41 (dt, $J = 6.1$ and 4.2 Hz) (4). The ^{1}H nmr of CQ2 also allowed us to detect the presence of about 10% of the 5α-isomer of **1** which displayed small but characteristic signals (4) at δ 0.77 for the Me-19 group and at δ 4.53 ($J = 7.7$ Hz) and δ 4.57 ($J = 7.7$ Hz) for the anomeric protons of the sophorose moiety and which is accordingly formulated as **2**.

Fraction CQ1 showed spectral properties ($[M+Na]^+$ at *m/z* 837 in positive ion fabms; fragment ions at *m/z* 755 $[M+H-HOAc]^+$, 653 $[M+H-162]^+$, 593 $[M+H-HOAc-162]^+$, 473 $[M+H-2\times162-H_2O]^+$, 431 $[M+H-HOAc-2\times162]^+$; $[M-H]^-$ at *m/z* 813 in negative ion fabms; ^{1}H nmr see Table 1) indicating that it is a 1:1 mixture of two $C_{41}H_{66}O_{16}$ steroid glycosides epimeric at C-5 (H-5α: Me-19 at 0.77; H-5β: Me-19 at δ 0.87). Comparison of the fab mass spectrum and ^{1}H-nmr spectrum of fraction CQ1 with those of **6** isolated from *Chrysolina varians* (4) revealed that the two compounds differed from **6** only by the position of the acetyl group on the steroid skeleton. This group was located at C-25 in the two epimers (Me-26 and Me-27: δ 1.45 and 1.54), instead of C-16β as in **6** (Me-26 and Me-27: δ 1.14 and 1.16). It follows that the two epimers of fraction CQ1 have structures **4** and **5**. These assignments were fully confirmed by comparison of the ^{1}H-nmr and mass spectra of the mixture of the acetyl derivatives **4a** and **5a** with those of **6a** (4).

The more polar fraction CQ3 ($[M+Na]^+$ at *m/z* 841 in positive ion fabms; fragment ions at 801 $[M+H-H_2O]^+$, 639 $[M+H-H_2O-162]^+$, 477 $[M+H-H_2O-2\times162]^+$, $[M-H]^-$ at *m/z* 817 in negative ion fabms, compatible with the molecular formula $C_{41}H_{70}O_{16}$; ^{1}H nmr see Table 1), is again a mixture of 5β and 5α epimers of a stigmastane steroid linked to a sophorose **9**, but in an approximately 3:1 ratio. Comparison of the molecular formula and ^{1}H-nmr spectrum of these epimers

with those of **1** suggested that they are identical to **1** except for the presence of a C-28 acetyl group in **1**. Consequently, the two epimers of fraction CQ3 were assigned structures **7** and **8**.

The defense secretion of *C. quadrigemina* also contains ethanolamine, as is the case for all the *Chrysolina* so far examined.

This study confirms that the production of closely related polyoxygenated steroid sophorosides seems to be a characteristic feature of the *Chrysolina* beetles feeding on *Hypericum*. It is thus intriguing that in a previous study, we reported that *Chrysolina didymata*, also feeding on this plant, contains the cardenolide sarmentogenin (7). However, only a few specimens of that species were available at that time, thus precluding a thorough study. We hope to settle that question in the near future.

C. varians is classified in the subgenus *Sphaeromela*, whereas the other species feeding on *Hypericum* are classified in the subgenus *Hypericia* (8,9). Many *Chrysolina* feed on Lamiaceae, considered to represent the primitive host plant of the genus, and in the course of speciation several shifts of host plant occurred (9). Our results point to a close relationship between the members of the two subgenera feeding on *Hypericum*, further suggesting that the shift on *Hypericum* has occurred only once in a common ancestor of both subgenera. Indeed, if *C.* (*Sphaeromela*) *varians* only produces cholestane derivatives, and *C.* (*Hypericia*) *brunsvicensis*, *Chrysolina* (*Hypericia*) *hyperici* and *C.* (*Hypericia*) *geminata* exclusively produce stigmastane derivatives, *C.* (*Hypericia*) *quadrigemina* incorporates both types of skeletons in its defense secretion. The close relationship between the species of the two subgenera is further indicated by the fact that polyoxygenated steroid sophorosides were, till now, only found in those species. Moreover, the production of these compounds does not seem to be influenced by the host plant (4).

One species of *Hypericia*, *Chrysolina lepida*, is reported in France, Spain, and Algeria to feed on a plant in the Asteraceae (*Microlonchus salmanticus*) and not on *Hypericum*. This may represent a more primitive condition within the *Chrysolina*, inasmuch as plants in the Asteraceae are common food plants within the genus. A study of the defense secretion of *C. lepida* could shed some light on the evolution of chemical defense in this group of insects.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Medium pressure chromatography (flash) was performed on Macherey-Nagel Kieselgel 60 (0.04–0.063 mm). Tlc analyses were performed on Macherey-Nagel Si gel plates [eluent CH_2Cl_2-MeOH (8:2) for the glycosides and CH_2Cl_2-EtOAc (8:2) for the acetates]. The compound were visualized by spraying with a 2 N H_2SO_4 solution of ceric sulfate. The ^{1}H-nmr spectra were run on a Bruker WM250 in CD_3OD (glycosides) or $CDCl_3$ (acetates) with TMS as internal standard, and are reported in Table 1 and below. Eims and fabms were obtained on Micromass VG 7070 and VG 70S spectrometers, respectively. The ir spectra were recorded on a Bruker IFS25 Fourier transform spectrometer.

COLLECTION AND REARING OF THE INSECTS.—Beetles were collected in Davis and Chico (California) on *Hypericum calycinum* and reared in Brussels on either *H. calycinum* or *Hypericum perforatum*. Voucher specimens are located at the University of Brussels in the collection of J.M. Pasteels. Tlc analyses demonstrated that the composition of the defense secretion did not depend on the food plant.

ISOLATION OF FRACTIONS CQ1, CQ2, AND CQ3.—The defense secretions were obtained by "milking" 770 individuals of *C. quadrigemina* on bits of filter paper which were stored in MeOH. The filter papers were repeatedly extracted with CH_2Cl_2-MeOH (1:1), and the organic extract were combined and evaporated under reduced pressure, yielding 44 mg of material. Repetitive flash column chromatography [eluent CH_2Cl_2-MeOH (9:1→7:3)] afforded three fractions homogeneous in tlc: CQ1 (5.0 mg), CQ2 (15.0 mg), and CQ3 (3.0 mg), in order of increasing polarity.

ACETYLATION REACTIONS.—The acetylation of the glycosides was performed as previously described (4), and the acetates were purified by Si gel flash chromatography using CH_2Cl_2-MeOH (99:1→95:5) as eluent.

Compounds **1a** *and* **2a**.—Eims *m/z* [M − 2 × H_2O × HOAc]$^+$ 1040, [M − 2 × H_2O − 2 × HOAc − Me]$^+$ 1025; 619, 543, 525, 483, 465, 405, 381, 331, 289, 271, 229, 169; ^{1}H nmr 0.90 (3H, s, Me-19), 1.12 (3H, s, Me-18), 1.21 and 1.25 (2 × 3H, s, Me-26 and Me-27), 1.26 (3H, d, J = 6.5, Me-29), 1.31 (3H, s, Me-21); OAc 1.99 (6H, s), 2.01 (3H, s), 2.02 (3H, s), 2.03 (3H, s), 2.06 (6H, s), 2.08 (6H, s), 3.63 (1H, m, H-5′), 3.66 (1H, m, H-5″), 3.73 (1H, dd, J = 9.1, 7.5, H-2′), 3.96 (1H, bs, H-3), 4.07 (1H, dd, J = 12.3, 2.8, H_a-6″), 4.09 (1H, dd, J = 12.3, 2.8, H_a-6′), 4.24 (1H, dd, J = 12.3, 5.1, H_b-6″), 4.33 (1H, dd, J = 12.3, 5.3, H_b-6′), 4.45 (1H, d, J = 7.5, H-1′); 4.75 (1H, d, J = 7.8, H-1″), 4.95 (1H, dd, J = 9.6, 9.6, H-3′), 5.01 (1H, dd, J = 9.5, 7.8, H-2″); 5.15 (3H, m, H-4′, -3″, -4″), 5.20 (1H, m, H-28), 5.40 (1H, dt, J = 6.1, 4.2, H-16).

Compounds **4a** *and* **5a**.—Eims *m/z* [M − Me]$^+$ 1135, [M − HOAc]$^+$ 1090, [M − 2 × HOAc]$^+$ 1030, 619, 515, 455, 413, 395, 371, 331, 289, 271, 229, 169; ^{1}H nmr 0.76 [3H, s, Me-19 (5α-H)], 0.89 [3H, s, Me-19 (5β-H)], 1.03 (3H, s, Me-18), 1.21 (3H, s, Me-21), 1.45 (3H, s) and 1.52 (3H, s) (Me-26 and Me-27); 1.99 to 2.07 (9-OAc); 3.70 (4H, m, H-24, -2′, -5′, -5″), 3.95 [1H, bs, H-3 (5β-H)]; 4.44 [1H, d, J = 7.5, H-1′ (5β-H)]; 4.57 [1H, d, J = 7.5, H-1′ (5α-H)], 4.76 [1H, d, J = 7.5, H-1″ (5β-H)], 4.82 [1H, d, J = 7.5, H-1″ (5α-H)], 5.37 (1H, m, H-16).

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