Pergamon

0031-9422(95)00837-3

Phytochemistry, Vol. 42, No. 1, pp. 45–49, 1996

0031-9422/96 $15.00 + 0.00

PRESENCE OF CARDENOLIDES AND URSOLIC ACID FROM OLEANDER LEAVES IN LARVAE AND FRASS OF *DAPHNIS NERII**

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(*Received in revised form* 23 *October* 1995)

Key Word Index—*Daphnis nerii*; Lepidoptera; Sphingidae; *Nerium indicum*; oleander; Apocynaceae; cardenolide; adynerin; oleandrin; ursolic acid.

Abstract—Cardenolide glycosides from larvae of the sphingid moth *Daphnis nerii* reared on oleander leaves, and those from their frass, were examined by HPLC and then isolated preparatively. Most of the cardenolide triosides in the leaves were detected as their corresponding monosides in the larval and frass extracts. From the frass, adynerin was obtained in large amounts, along with nine cardenolide monosides. A decrease of oleandrin due to deacetylation and an increase of adynerin in the frass are discussed.

INTRODUCTION

Since the 1970s, a sphingid moth, *Daphnis nerii* L., has become established in the Okinawa Islands of Japan. Although the larvae of *D. nerii* feed on cardenolide-rich oleander leaves (*Nerium oleander* L., *N. indicum* Mill.) in both Japan and their native Europe, Rothschild *et al.* [1–3] reported that they do not store cardenolides. However, during our investigations of the cardenolide glycosides and pregnane glycosides present in oleander [4–11], we found that cardenolide glycosides are present in the larvae of *D. nerii*. Hence, we describe here a comparative analysis of the cardenolide glycosides in final-instar larvae of *D. nerii* and their frass, and a comparison with those of the original oleander leaves.

RESULTS

The methanolic extracts of larvae (D), frass (F) and one of the two sources of fresh leaves (L-a) were compared by HPLC (Fig. 1). In order to confirm each peak in HPLC and to know approximate amounts of the glycosides, the methanolic extracts from D, F and L-a were subjected to column chromatography, along with the other sample of leaves (L-b). Major cardenolide glycosides were isolated preparatively and identified by ^{1}H NMR and by comparison with authentic glycosides. Along with cardenolides, ursolic acid was obtained from D, F and L-a,b and rutin from F and L-a,b, but not from D (Table 1, Fig. 2).

While most of the glycosides in L-a,b were observed as triosides [5–7], along with small amounts of monosides, the predominant glycoside among them was oleandrin gentiobioside (**1**) [5–7]. On the other hand,

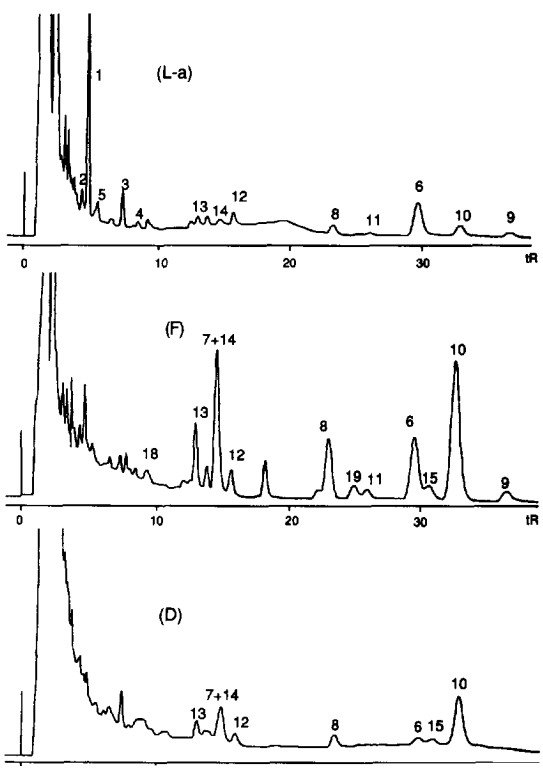

Table 1. Concentrations (mg $g^{-1} \times 10$) of cardenolides, ursolic acid and rutin in frass (F), larvae (D) and leaf (L-a, L-b) samples

		Frass (F) (41.6 g)	Larvae (D) (61.2 g)	Leaves (L-a)* (88.0 g)	Leaves (L-b) (400 g)
	Ursolic acid	235.6	3.6	80.7	161.0
	Rutin	14.9	(−)	18.2	17.5
Triosides	Oleandrin gentiobioside (**1**)			9.1	11.8
	Odoroside A gentiobioside (**2**)			0.1	0.1
	Adynerin gentiobioside (**3**)			2.5	2.9
	Δ^{16}-Adynerin gentiobioside (**4**)			0.9	1.2
	Oleaside E (**5**)			0.6	1.6
Monosides	Oleandrin (**6**)	4.1	(+)	0.7	0.5
	Deacetyloleandrin (**7**)	7.2	(+)		
	Odoroside A (**8**)	2.4	(+)	0.1	0.5
	Δ^{16}-Adynerin (**9**)	6.0		1.1	1.5
	Adynerin (**10**)	18.3	0.3	1.3	0.7
	Oleaside A (**11**)	0.2			0.1
	Nerigoside (**12**)	1.0	(+)		0.3
	Neriaside (**13**)	1.9	(+)		
	8β-Hydroxyodoroside A (**14**)†	1.4	(+)		
	5α-Adynerin (**15**)	0.5	(+)		0.03
	Δ^{16}-Adynerigenin digitaloside (**16**)				0.1
	Oleandrigenin sarmentoside (**17**)				0.1
Cardenolides	Oleandrigenin (**18**)				0.1
	Δ^{16}-Adynerigenin (**19**)				0.1
Pregnanes and	Neridienone A				0.7
sterol	6,7-Dihydroneridienone A				0.4
	Cholesterol		1.6		

* Isolation was carried out non-quantitatively.

† HPLC peaks are duplicates of each other.

no triosides but monosides were isolated from D and F, and adynerin (**10**) [12] was the predominant monoside. Besides **10**, nine monosides were isolated from F, but isolation of the glycosides from D was unsuccessful except for 2 mg of 10.

The HPLC patterns of D and F were similar, and the peak of oleandrin (**6**) was low, particularly in D, at almost the same level as that of 5α-adynerin (**15**), one of the minor components in the leaves (Fig. 1). Instead of the decrease of **6**, deacetyloleandrin (**7**) was one of the major peaks in D and F, although the peak was duplicated with 8β-hydroxyodoroside A (**14**), as a result of splitting of the 16-*O*-acetyl group in the oleandrigenin moiety, along with deglucosylation, during the digestive process. Δ^{16}-Adynerin (**9**) was obtained from F, but not detected in HPLC of D.

Based on the amounts of ursolic acid from L-b and F, the weight of fresh leaves eaten by larvae during feeding was calculated as 60.9 g, of which 48.6 mg was **6** and 15.2 mg was **10**. Since 18 mg of **6** and 76 mg of **10** were isolated from F, and their structures confirmed by NMR and direct comparisons with authentic samples, the amount of **10** in the frass appears to be unusually large. Based on the peak height corresponding to 2 mg of **10** in D, the whole amount of bioactive cardenolide glycosides including **6**, **7**, **8** and

DISCUSSION

Based on the previous work by Rothschild *et al.*, *D. nerii* has been considered to be free from cardenolide glycosides of oleander leaves [1–3]. However, the presence of small amounts of cardenolide glycosides was confirmed in the larvae of *D. nerii* in this study. Among the cardenolide glycosides, **10** was isolated as a dominant glycoside as in the case of *Aphis nerii* feeding in the phloem of oleander [1]. Although there is no proof that cardenolides from D were not present in the alimentary canal when the larvae were soaked in ethanol, rutin was isolated in a large amount from L-a,b and F, but not observed in D. In addition, a difference of cardenolide patterns between D and F was shown, particularly on the peak heights of **6** and **9** in HPLC. Since larvae are believed to excrete the gut content and not eat before pupation, the possibility that cardenolide glycosides from D in this study were stored in the larvae cannot be excluded.

TLC and HPLC of D and F apparently showed the strong activities of esterase as well as of glucosidase in the digestive system as in other small phytophagous animals such as snails. Deacetylation of oleandrin seems to reduce autotoxicity, while potential toxicity against predators may also be weakened. Deglucosylation to produce oleandrin from its tioside may work

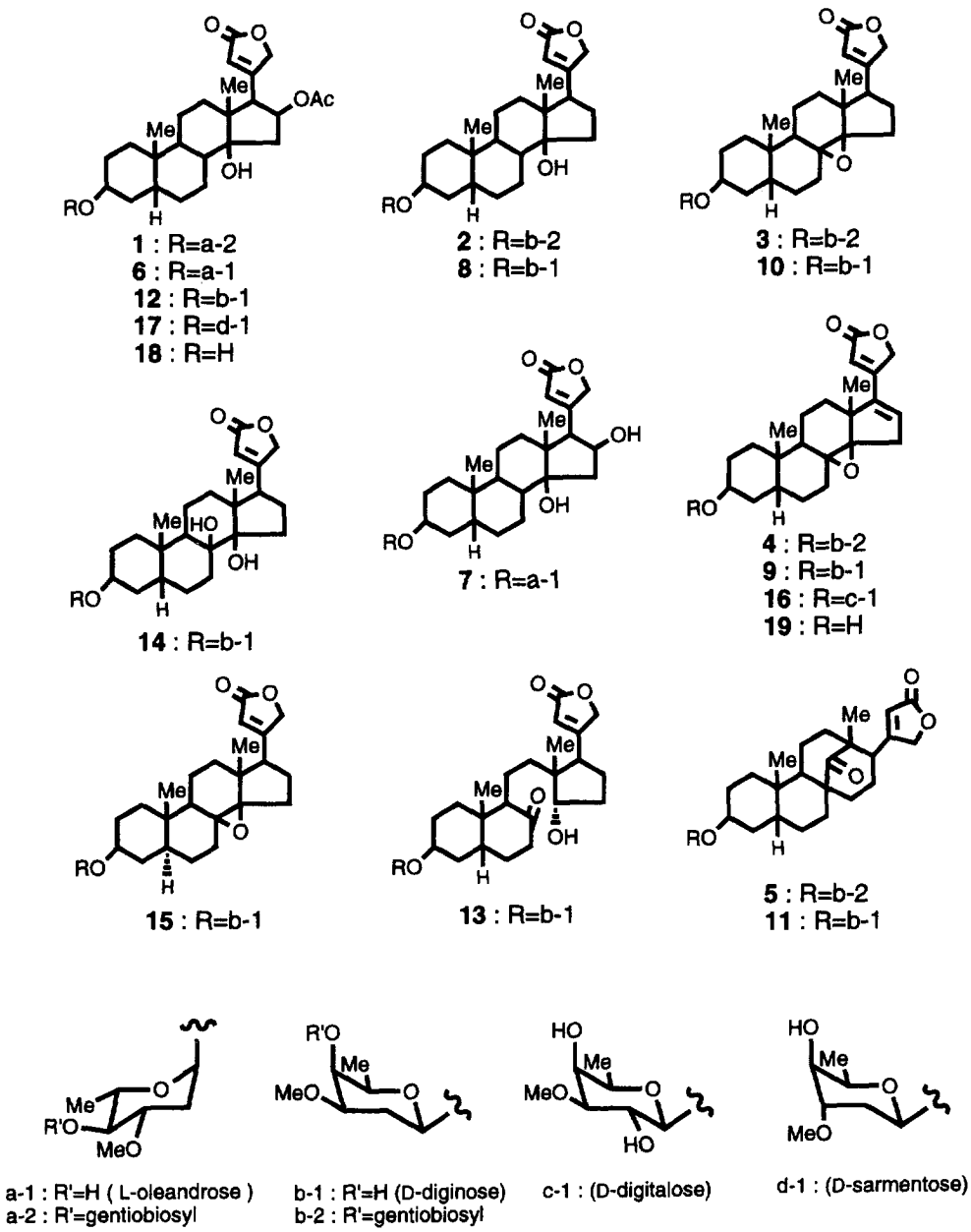

Fig. 2. Cardenolide glycosides from oleander leaves.

cardenolide glycosides from the leaves are passing through the alimentary canal with bioactive forms during their growth, on the basis of the isolation of cardenolides from the frass.

Only three monosides, adynerin, strospeside and odoroside H, were isolated from *A. nerii* feeding on oleander, but no oleandrin or its homologues, the principal glycoside in oleander leaves, were obtained [1]. Since the aphid sucks sap from phloem of leaves or soft stems, the difference in cardenolide patterns between larvae of *D. nerii* and *A. nerii* may suggest such as **6** are not present in the stem and root bark in which 16-*O*-acetylstrospeside (neritaloside), odoroside H and uzarigenin β-D-digitaloside are present as major glycosides along with their glucosides [11, 13].

EXPERIMENTAL

General. ^{1}H NMR: 400 MHz in pyridine-d_5 with TMS as int. standard. For TLC and silica gel CC, the following solvent systems were applied: 1, $CHCl_3$–MeOH–H_2O (7:2:1–7:3:1, bottom layer); 2. EtOAc–

1.5 ml min^{-1}, 30% MeCN–H_2O, Capcell pak C18 SG120 (4.6 × 250 mm), UV 220 nm.

Insect and plant materials. *D. nerii* was collected at the campus of Ryukyu University in November 1992 and identified by the Museum, Faculty of Agriculture, Ryukyu University. The fresh leaves of *N. indicum* were collected from the trees on which the larvae fed, in November 1992 (88 g) (L-a) and March 1993 (400 g) (L-b).

Preparation of HPLC samples. 35 2nd or 3rd instar larvae of *D. nerii* were captured early in November 1992, and reared on the leaves of *N. indicum* in a container placed in a room for 1–2 weeks with artificial light in daytime. Fresh oleander leaves with stems were supplied during feeding by wrapping the end of stems with moistened cotton. Room temp. was kept at 18–23°. 11 live larvae at final-instar (w 61.2 g as total), immediately before pupation where the body colour turned dark, were soaked in EtOH (400 ml) for 1 week and filtered. The larval bodies were further homogenized with MeOH and filtered. The EtOH and MeOH filtrates were combined together and concd *in vacuo* (D). Among the remaining larvae, 3 became pupae, one of which emerged in the following spring. Other larvae were extracted with MeOH after 2 weeks feeding and used for the preparatory check of the procedure. The frass (dry w: 41.6 g), obtained from 35 larvae reared on the oleander leaves, was collected and percolated with MeOH and filtered. The residue was again homogenized with MeOH and filtered. The MeOH soln was combined and concd *in vacuo* (F). The fresh leaves of *N. indicum* collected at Ryukyu University (L-a: 88 g, L-b: 400 g) were percolated with MeOH. Portions of the MeOH extracts from larvae, frass and leaves (L-a) were dissolved in 80% MeCN and filtered on a Sep-Pak C_{18} cartridge. The filtrates were subjected to HPLC (Fig. 1).

Isolation of cholesterol, ursolic acid and adynerin from the larvae. After evapn of solvent, the larval extract was suspended in 50% MeOH and partitioned with C_6H_6. The C_6H_6 extract (1.45 g) was then subjected to CC with C_6H_6–Me_2CO (10:1–4:1). The 1st fr. was purified on a Sephadex LH-20 column with $CHCl_3$ and the eluate was crystallized from $CHCl_3$–MeOH to give cholesterol (10 mg). The 2nd fr. was purified on a silica gel column with solvent 1 (10:1:5) to give ursolic acid (22 mg). The following fr. showing positive staining to Kedde's reagent was subjected to CC on a Sephadex LH-20 with $CHCl_3$, followed by silica gel CC with solvent 3 (1:1) to give **10** (2 mg).

Isolation of cardenolides, ursolic acid and rutin from the frass. The MeOH extract of F was suspended on 50% MeOH and partitioned with C_6H_6 to separate the C_6H_6 fr. (extract: 4.37 g) and 50% MeOH fr. (extract: 3.42 g). The C_6H_6 extract was crystallized from MeOH to give ursolic acid (536 mg). The mother liquor fr. was subjected to CC on Sephadex LH-20 and eluted with silica gel CC with solvents 3, 1 and 4, cardenolide glycosides (shown in Table 1), **6–15**, were isolated. From fr. 2, 444 mg of urselic acid (980 mg as total) was obtained. The 50% MeOH fr., after partition with C_6H_6, was subjected to Diaion HP-20 CC and the column was eluted with H_2O, 25–100% MeOH, successively. From 100% MeOH eluate, **7** (20 mg) was isolated after silica gel CC with solvent 1 (10:1:2). Cardenolides, ursolic acid and rutin were crystallized to give prisms.

Oleandrin (**6**). Mp 251–253°, ^{1}H NMR: 0.90, 1.10 (3H each, *s*, H-18, 19), 1.60 (3H, *d*, J = 6 Hz, H-6′), 1.86 (3H, *s*, 16-OAc), 3.40 (1H, *d*, J = 9 Hz, H-17), 3.47 (3H, *s*, 3′-OMe), 3.58 (1H, *td*, J = 9, 4 Hz, H-4′), 3.96 (1H, *m*, H-3′), 4.09 (1H, *br s*, H-3α), 4.17 (1H, *m*, H-5′), 5.18 (1H, *d*, J = 3 Hz, H-1′), 5.23, 5.43 (1H each, *dd*, J = 18, 2 Hz, H-21a,b), 5.68 (1H, *s*, 14-OH), 5.71 (1H, *td*, J = 9,2 Hz, H-16), 6.34 (1H, *br s*, H-22), 6.77 (1H, *d*, J = 4 Hz, 4′-OH).

Odoroside A (**8**). Mp 201–205°, ^{1}H NMR: 0.91, 1.02 (3H each, *s*, H-18, 19), 1.55 (3H, *d*, J = 6 Hz, H-6′), 2.80 (1H, *dd*, J = 9, 4 Hz, H-17), 3.39 (3H, *s*, 3′-OMe), 3.42 (1H, *ddd*, J = 12, 4, 3 Hz, H-3′), 3.56, (1H, *qd*, J = 6, 1 Hz, H-5′), 3.90 (1H, *br s*, H-4′), 4.33 (1H, *br s*, H-3α), 4.75 (1H, *dd*, J = 10, 2 Hz, H-1′), 5.03, 5.31 (1H each, *dd*, J = 18, 2 Hz, H-21a,b), 5.21 (1H, *s*, 14-OH), 6.13 (1H, *br s*, H-22).

Adynerin (**10**). Mp 205–215°, ^{1}H NMR: 0.82, 1.11 (3H each, *s*, H-18, 19), 1.56 (3H, *d*, J = 6 Hz, H-6′), 3.40 (3H, *s*, 3′-OMe), 3.45 (1H, *ddd*, J = 12, 5, 3 Hz, H-3′), 3.59 (1H, *qd*, J = 6, 1 Hz, H-5′), 3.92 (1H, *d*, J = 3 Hz, H-4′), 4.36 (1H, *br s*, H-3α), 4.79 (1H, *dd*, J = 10, 2 Hz, H-1′), 4.82, 4.93 (1H each, *dd*, J = 18, 2 Hz, H-21a,b), 6.06 (1H, *br s*, H-22).

5α-Adynerin (**15**). Mp 225–232°, ^{1}H NMR: 0.81, 0.95 (3H each, *s*, H-18, 19), 1.60 (3H, *d*, J = 6 Hz, H-6′), 3.42 (3H, *s*, 3′-OMe), 3.49 (1H, *ddd*, J = 12, 5, 3 Hz, H-3′), 3.65 (1H, *qd*, J = 6, 1 Hz, H-5′), 3.94 (1H, *br s*, H-4′), 3.98 (1H, *m*, H-3α), 4.79, 4.90 (1H each, *dd*, J = 16, 2 Hz, H-21a,b), 4.87 (1H, *dd*, J = 9, 2 Hz, H-1′), 6.05 (1H, *br s*, H-22).

Deacetyloleandrin (**7**). Mp 217–220°, ^{1}H NMR: 0.91, 1.14 (3H each, *s*, H-18, 19), 1.60 (3H, *d*, J = 6 Hz, H-6′), 3.28 (1H, *d*, J = 8 Hz, H-17), 3.57 (1H, *t*, J = 9 Hz, H-4′), 3.95 (1H, *m*, H-3′), 4.06 (1H, *br s*, H-3α), 4.16 (1H, *m*, H-5′), 4.99 (1H, *td*, J = 8, 1 Hz, H-16), 5.17 (1H, *d*, J = 3 Hz, H-1′), 5.54, 5.68 (1H each, *dd*, J = 18, 1 Hz, H-21a,b), 6.24 (1H, *br s*, H-22).

Ursolic acid. Mp 264–268°(dec), ^{1}H NMR: 0.91, 1.03, 1.07, 1.24, 1.25 (3H each, *s*, H-23–27), 0.97, 1.02 (3H each, *d*, J = 6 Hz, H-29, 30), 2.64 (1H, *d*, J = 11 Hz, H-18), 3.46 (1H, *dd*, J = 10, 6 Hz, H-3α), 5.50 (1H, *br s*, H-12).

Rutin. Mp 190–200°.

Isolation of cardenolides, ursolic acid and rutin from fresh leaves. The leaves were collected in November 1992 (88 g) (L-a) and March 1993 (400 g) (L-b) from

principally in same manner as described for the frass. Yields of cardenolides, ursolic acid and rutin are presented in Table 1.

Acknowledgements—This work was supported by a Grant-in-Aid for Scientific Research (C) (No. 05680517) from the Ministry of Education, Science and Culture. We thank Ms Y. Iwase for NMR operation.

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