

# A New Acyclic Diterpene Glycoside from *Nicotiana attenuata* with a Mild Deterrent Effect on Feeding *Manduca sexta* Larvae

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To investigate the role of secondary metabolites in the feeding behavior of *Manduca sexta* larvae feeding on *Nicotiana attenuata*, an aqueous acetone extract of the aerial parts of the plant was subjected to feeding-performance bioassay-guided fractionation. We isolated three 20-hydroxygeranyllinalool glycosides from the leaves of *N. attenuata*, which acted as mild deterrents to the feeding herbivore *M. sexta*. One of the diterpenoid glycosides, attenoside (**3**), is a novel natural product. The structures of the compounds were determined using APCI mass spectrometry and 1- and 2D-NMR spectroscopy.

**Key words:** Hydroxygeranyllinalool Diterpene Glycosides, *Nicotiana attenuata*, *Manduca sexta*, Solanaceae, Lepidoptera

## Introduction

*Manduca sexta* (Lepidoptera, Sphingidae) is an oligophagous insect living on different plants of the family solanaceae, including *Nicotiana attenuata*. The performance of *M. sexta* larvae on three plants, *N. attenuata*, *N. bigelovii*, and *N. clevelandii*, has been investigated [1]. The larvae gained increasing amounts of weight when they fed on the following plants in the order: *N. clevelandii* > *N. bigelovii* > *N. attenuata* [1]. Rutin, diterpene glycosides (DTGs), volatiles, nicotine, chlorogenic acid, and trypsin inhibitors (TPIs) were measured in the above plants [1,2]. DTGs were not detected in *N. clevelandii* and only small amounts were found in the second-most palatable plant, *N. bigelovii*. Finally, *N. attenuata* has the highest concentrations of DTGs among the above plants [1]. These observations prompted us to investigate the role of DTGs in the feeding performance of *Manduca sexta*.

The acyclic diterpenoid (3*S*,6*E*,10*E*,14*Z*)-20-hydroxygeranyllinalool was isolated from *N. sylvestris* more than twenty years ago [3]. The rarely occurring acyclic DTGs with geranyllinalool diterpenoid skeletons were subsequently separated from *Capiscum* plants and their structures elucidated using FAB mass spectrometry, and 1- and 2D-NMR spectroscopy [4, 5]. Two hydroxygeranyllinalool DTGs containing five sugar moieties were separated from *N. tabacum* [6].

Lyciumoside IV, 3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranosyl-20-hydroxygeranyllinalool-20-*O*- $\beta$ -D-glucopyranoside (compound **1**) and Lyciumoside II, 3-*O*- $\beta$ -D-glucopyranosyl-20-hydroxygeranyllinalool-20-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside (compound **2**) were previously isolated from *Lycium chinese* (solanaceae) [7,8]. Two hydroxygeranyllinalool glycosides with antibiosis effects on the larvae of tobacco budworm (*Heliothis virescens*) were isolated from a resistant tobacco line [9]. The presence of the DTGs was shown to be the reason plants are able to resist the herbivores [9].

To investigate the role of secondary metabolites on the feeding behavior of *M. sexta* that fed on *N. attenuata*, we subjected an aqueous acetone extract of the aerial parts of the plant to bioassay-guided fractionation based on an agar-disk feeding bioassay [10]. Although some non-host secondary metabolites with deterrent effects on the larvae of *M. sexta* have been reported, but to the best of our knowledge the effects of the DTGs on larval feeding behavior of the larvae have not been previously reported [11, 12]. We isolated three DTGs (**1–3**) from leaves of *N. attenuata*, which acted as mild feeding deterrents on the larvae of *M. sexta*. To the best of our knowledge attenoside, 3-*O*- $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranosyl-20-hydroxygeranyllinalool-20-*O*- $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside (com-

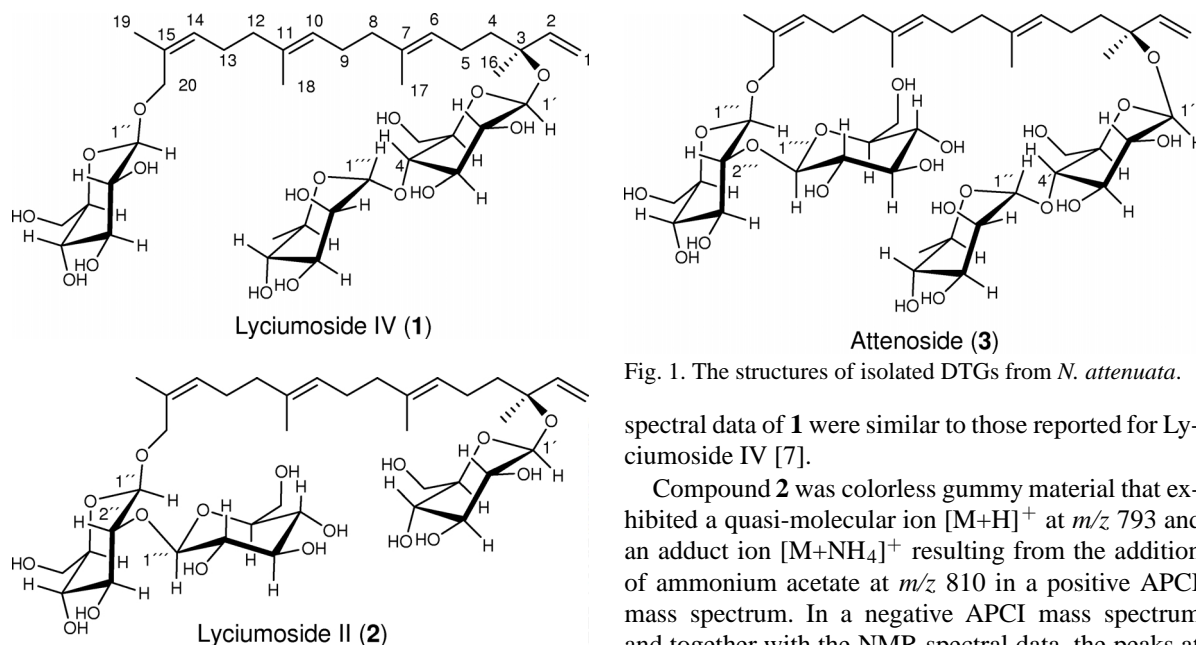


Fig. 1. The structures of isolated DTGs from *N. attenuata*.

spectral data of **1** were similar to those reported for Lyciumoside IV [7].

Compound **2** was colorless gummy material that exhibited a quasi-molecular ion  $[M+H]^+$  at  $m/z$  793 and an adduct ion  $[M+NH_4]^+$  resulting from the addition of ammonium acetate at  $m/z$  810 in a positive APCI mass spectrum. In a negative APCI mass spectrum and together with the NMR spectral data, the peaks at  $m/z$  850  $[M+OAc-H]^-$  and 791  $[M-H]^-$  suggested the molecular formula:  $C_{38}H_{64}O_{17}$ . The elemental composition of  $[M-H]^-$  was confirmed when a peak was detected at  $m/z$  791.4090 ( $C_{38}H_{63}O_{17}$ , calcd. 791.4065). The  $^1H$  NMR and  $^{13}C$  NMR spectral data for compound **2** were similar to those reported for Lyciumoside II [8].

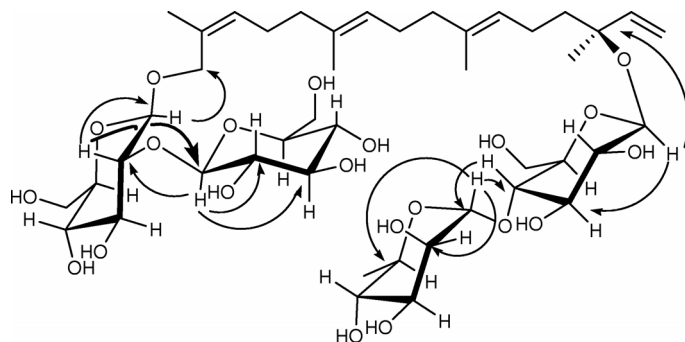
Compound **3** (attenoside) was also obtained as a colorless gummy material whose negative APCI-MS spectral data showed a quasi-molecular ion  $[M-H]^-$  peak at  $m/z$  937. The ion and the adduct peak at  $m/z$  996  $[M+OAc-H]^-$ , together with the NMR spectral data, suggested the molecular formula  $C_{44}H_{74}O_{21}$  for the molecule. In tandem mass spectra, peaks at  $m/z$  791  $[M-rham.]^-$ , 775  $[M-gluc.]^-$ , 629  $[M-gluc.-rham.]^-$ , 612  $[M-2Xgluc.]^-$ , and 466  $[M-2Xgluc.-rham.]^-$ , derived from the parent ion at  $m/z$  937, suggested the presence of both rhamnose and glucose moieties in the molecule. The elemental composition of its quasi-molecular ion  $[M-H]^-$  peak at  $m/z$  937.4699,  $C_{44}H_{73}O_{21}$  (exact mass: 937.4644), was determined using HR-ESI-TOF mass spectrometry in negative mode. The signals at  $\delta = 5.23$  (1H, *dd*,  $J = 1, 18$  Hz, H-1<sub>trans</sub>), 5.20 (1H, *dd*,  $J = 1, 11$  Hz, H-1<sub>cis</sub>), and 5.92 (1H, *dd*,  $J = 11, 18$  Hz, H-2) presented a terminal double bond in the  $^1H$  NMR spectrum. The other signals at  $\delta = 5.11$  (2H, *dt*,  $J = 1.0, 7.5$  Hz, H-6, H-10) and 5.37 (1H, *bt*,  $J = 7.5$  Hz, H-14) were due to three more tri-substituted double bonds. Three methyl signals at

pound **3**) is a new natural product, isolated here for the first time from *N. attenuata*.

## Results and Discussion

The water-soluble layer of the aqueous acetone extract was subjected to RP-18 flash column chromatography, resulting in 30 fractions. The fractions were tested for their dietary attractiveness to the larvae (see Experimental Section). The fractions containing phenolics (*e.g.*, chlorogenic acid) showed feeding stimulants, while those rich in DTGs deterred feeding. After one week chlorogenic acid in  $0.5 \times 10^{-3}$  M had stimulated feeding by 15%, while compounds **1** and **3** in a concentration of  $10^{-2}$  M and when applied in 500  $\mu g$ / diet disks had deterred larval feeding by 10–20%. For each experiment the number of replicates was 12–15. The feeding index was calculated from the following equation:  $FI = (T-C)/(T+C) \times 100$ , where T and C represent the weight (in mg) of the larvae that fed on treated and control diet disks, respectively.

Compound **1** was a colorless gummy material whose negative HR-ESI-TOF mass spectrum showed a quasi-molecular  $[M-H]^-$  ion peak at  $m/z$  775.4172 ( $C_{38}H_{63}O_{16}$ , exact mass: 775.4116). The molecule may lose rhamnose, glucose, and rutinose moieties to form peaks at  $m/z$  629, 613, and 467, respectively recorded in the APCI-MS spectrum.  $^1H$  and  $^{13}C$  NMR

Fig. 2. Important HMBC spectral data for **3**.

$\delta = 1.59$  (6H, *s*, H-17, H-18), 1.78 (3H, *s*, H-19) and 1.37 (3H, *s*, H-16) confirmed the presence of three methyls connected to the olefinic carbons and one to a quaternary carbon atom [3]. Signals at  $\delta = 4.35$  (2H, *d*,  $J = 7.5$  Hz, H-1', H-1''), 4.62 (1H, *d*,  $J = 8.5$  Hz, H-1'''), and 4.85 (1H, *d*,  $J = 3$  Hz, H-1'') suggested the presence of four sugar moieties, three with  $\beta$ - and one with  $\alpha$ -glycosyl linkage. The chemical shifts and coupling constants in the  $^1\text{H}$  NMR spectra of the compound were very similar to those observed for compounds **1** and **2** and for those published in the literature [7, 8]. In the APT  $^{13}\text{C}$  NMR spectra, 20 signals were recorded for the diterpenoid part of the molecule, which in turn matched those signals observed in the spectra of the related diterpene glycosides [7, 8]. The APT  $^{13}\text{C}$  NMR spectrum also showed 24 signals in the sugar region with four anomeric signals at  $\delta = 99.4$  (*d*, C-1'), 102.8 (*d*, C-1''), 101.3 (*d*, C-1'''), and 104.8 (*d*, C-1'''). The signals for three hydroxymethylene at  $\delta = 62.2$ , 62.9 and 62.7 (*t*, C-6', 6'', 6'''), together with the methyl signal at  $\delta = 17.9$  (*q*, C-6'') and the chemical shifts for the sugars moieties (Table 1), were compatible with the presence of three glucoses and one rhamnose in the molecule [4, 7, 8]. The connectivity and type of glycosidation in positions C-3 and C-20 are the same for **1** ( $\alpha$ -L-rhamnopyranosyl-(1 $\rightarrow$ 4)- $\beta$ -D-glucopyranoside) and **2** ( $\beta$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-glucopyranoside) respectively.

The connectivity of the sugars to the aglycone and to each other was deduced with the help of HMBC as well as  $^{13}\text{C}$  NMR data. In the  $^{13}\text{C}$  NMR spectrum the signal of C-2''' of the glucose was shifted to  $\delta = 82.1$  in contrast to that for non-substituted C-2''' ( $\delta = 75.5$ ) of the glucose at C-20 in **1**. In compound **3**, the chemical shift for C-4' is  $\delta = 79.8$ , while it is 71.4 for non-substituted glucose moiety in **2**. In the HMBC spectrum the cross peaks between H-1' and C-3 and between H-1''' and C-20 suggested the *O*-glycosylation

at C-3 and C-20, respectively. H-1'' of the rhamnose correlated with C-4' of the glucose at C-3 position. A correlation between H-1''' of the external glucose with C-2'' of the inner glucose at C-20 together with other correlations in the HMBC spectrum (Fig. 2) established the structure of attenoside (**3**). The stereochemistry for the double bonds and C-3 was concluded by comparing  $^{13}\text{C}$  NMR spectral data to the related compounds [4, 7, 8].

Although the water-soluble layer of the plant extract contained feeding stimulant, its constituents displayed opposing characteristics: the phenolic constituents of the plant seem to stimulate *M. sexta* to feed, while the diterpene glycosides deter this insect from feeding. However in our study the deterrent effects of DTGs on *M. sexta* are not as pronounced as those of DTGs extracted from tobacco, on tobacco budworm [9]. But the feeding deterrent effect of the diterpenes of the plant may explain the low performance of the larvae on *N. attenuata* compared to the other *Nicotiana* species mentioned above.

## Experimental Section

### General procedure

$^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data,  $^1\text{H}$ - $^1\text{H}$  COSY, ROESY, HMQC, and HMBC experiments were measured on a Bruker Avance DRX 500 NMR. APCI-MS spectra were recorded on a Thermo Finnigan LCQ Deca XP spectrometer coupled to an Agilent 1100 HPLC. HR-ESI-MS spectra were recorded using a microTOF ESI-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany). The optical rotations were recorded on a JASCO DIP-1030 digital polarimeter. Flash column chromatographies were performed on Merck LiChroprep RP-18 (40–63  $\mu\text{m}$ ) and LiChroprep Diol (25–40  $\mu\text{m}$ ) as stationary phases. Analytical TLC experiments were performed on Merck silica gel 60 F<sub>254</sub>, DIOL F<sub>254</sub> HPTLC and RP-18 WF<sub>254</sub> HPTLC pre-coated glass plates. Preparative TLC was performed on Merck silica gel 60 F<sub>254</sub>

Table 1. <sup>1</sup>H NMR (500 MHz), and <sup>13</sup>C NMR (125 MHz) data for attenoside (**1**) in CD<sub>3</sub>OD (*J* in Hz in parentheses).

Position	δ <sub>H</sub> (Hz)	δ <sub>C</sub>
1	5.23 <i>dd</i> (1, 18); 5.20 <i>dd</i> (1, 11)	115.8
2	5.92 <i>dd</i> (11, 18)	144.4
3	–	81.5
4	1.59 <i>m</i>	42.7
5	2.05 <i>m</i>	23.6
6	5.11 <i>dt</i> (1.0, 7.5)	125.8
7	–	135.9
8	1.99 <i>m</i>	40.9
9	2.08 <i>m</i>	27.6
10	5.11 <i>dt</i> (1.0, 7.5)	125.8
11	–	135.5
12	1.99 <i>m</i>	40.7
13	2.17 <i>m</i>	27.3
14	5.37 <i>br.t</i> (7.5)	131.1
15	–	132.6
16	1.37 <i>s</i>	23.3
17	1.59 <i>s</i>	16.1
18	1.59 <i>s</i>	16.2
19	1.78 <i>s</i>	21.9
20	4.26 <i>d</i> (11.5); 4.31 <i>d</i> (11.5)	68.4
Gluc. C-3		
1'	4.35 <i>d</i> (7.5)	99.4
2'	3.22 <i>m</i>	75.3
3'	3.42 <i>m</i>	77.0
4'	3.52 <i>m</i>	79.8
5'	3.26 <i>m</i>	76.5
6'	3.74 <i>m</i>	62.2
Rham. C-3		
1''	4.85 <i>d</i> (3.0)	102.8
2''	3.82 <i>m</i>	72.5
3''	3.62 <i>m</i>	72.3
4''	3.39 <i>m</i>	73.8
5''	3.95 <i>dddd</i> (6, 6, 6, 10)	70.7
6''	1.27 <i>d</i> (6)	17.9
Gluc. (inner) C-20		
1'''	4.35 <i>d</i> (7.5)	101.3
2'''	3.49 <i>m</i>	82.1
3'''	3.55 <i>m</i>	77.9
4'''	3.64 <i>m</i>	71.4
5'''	3.36 <i>m</i>	78.2
6'''	3.82 <i>dd</i> (2.5, 12); 3.67 <i>dd</i> (5, 12)	62.9
Gluc. (outer) C-20		
1''''	4.62 <i>d</i> (8.5)	104.8
2''''	3.24 <i>m</i>	75.9
3''''	3.26 <i>m</i>	77.8
4''''	3.26 <i>m</i>	71.6
5''''	3.24 <i>m</i>	78.2
6''''	3.61 <i>dd</i> (5.0, 12.0); 3.71 <i>dd</i> (2.5, 12.0)	62.7

pre-coated glass plates. The HPLC columns were Luna 5μ C18 (2) 100A for DTGs analysis and Nucleosil 100-5 NH<sub>2</sub> RP for sugar analysis.

#### Plant materials, insects, and extraction procedures

The plant materials and the larvae of the insects were prepared as described previously [13, 14]. Fifty plants of *N. at-*

*tenuata*, which originated in Utah, were grown under hydroponic culturing [14]. From the aerial parts of the plant in the beginning of the flowering stage, 280 g were extracted with 80% acetone (2 l) for one week. The extract was partitioned between water and dichloromethane (DCM) to afford polar and non-polar fractions of the extract.

#### Feeding bioassay

The bioassay procedure was developed according to that previously reported for willow beetles, with some modifications [10]. To determine the deterrent effect of DTGs, we used an artificial diet based on the recipes presented in the literature [15]. Twenty-five ml of the hot liquefied diet was poured into a 12 × 12 cm dish. After a few minutes the agar layer solidified and was cut into 1 cm circles. Each disk was put in a plastic vessel (6 cm Ø) with a lid. One series of disks (12 replicates) was treated with 50 μl solutions of the test compound in water and the other was treated with only solvent as the control. One newly hatched larva of *M. sexta* was released on the diet inside the dish. The insects were kept in a growth chamber (Snijders Scientific) at 28 °C, relative humidity 60%, and 16 h light and 8 h dark. The larvae were weighed after one week.

#### Purification of the water-soluble part of the aqueous acetone extract of *N. attenuata*

The water-soluble layer part (29 g) of the aqueous acetone extract was subjected to LiChroprep® RP-18 (40–63 μm, 100 g) flash column chromatography using methanol and water as the mobile phase (from 5% to 100% methanol v/v). The fractions were monitored by reversed-phase HPLC. The HPLC column was eluted with acetonitrile (solvent B) and 0.25% H<sub>3</sub>PO<sub>4</sub> in distilled water (solvent A) as follows: 0–6 min, 0–12% of B; 6–10 min, 12–18% of B; 10–30 min, 18–58% of B; 30–35 min, 58–80% of B; 35–45 min, 100% of B and 45–50 min, 100–0% of B. Compound **1** (1.0 g) was obtained in an almost pure form from the twenty-fifth fraction of the RP-18 column, eluted with 70% methanol. Fractions eluted with 60% methanol (506 mg) were mixed and subjected to flash chromatography using Diol stationary phase (30 g). The column was eluted with chloroform by increasing the polarity to pure methanol. The purity of the fractions was examined by TLC on silica gel. Compounds **2** (10 mg) and **3** (37 mg) were further purified by silica gel PTLC using a solution of chloroform: methanol: water (7 : 3 : 0.5) as the mobile phase.

#### LC-MS analysis of the chemical constituents of the extract

In order to identify the chemical constituents of the active fractions, the fractions were subjected to LC-MS analysis. For the HPLC coupled to mass spectrometer, the type

of column and LC conditions used were those described for the analytical HPLC. One percent acetic acid in water and acetonitrile were used as solvents A and B, respectively. The flow rate of the LC pump was 1 ml/min. Atmospheric pressure chemical ionization was used as the ionization source for MS. The MS parameters were as follows: sheath gas, 79 units; auxiliary gas, 5 units; vaporizer temperature 460 °C; needle discharge current 5  $\mu$ A; capillary temperature 180 °C; capillary voltage 21 V. The known compounds – nicotine, chlorogenic acid, and rutin – were identified using their HPLC retention indices and their mass spectra.

**Compound 1:** colorless gummy material (1.0 g).  $-\alpha_D^{25} - 25.6^\circ$  (MeOH, *c* 1.250); –MS (negative ion APCI-MS):  $m/z = 775$  [M-H] $^-$ , 629 [M-rham.] $^-$ , 613 [M-gluc.] $^-$ , and 467 [M-gluc.-rham.] $^-$ . –MS (HR-ESI-TOF):  $m/z = 775.4172$  [C<sub>38</sub>H<sub>63</sub>O<sub>16</sub>, calcd. 775.4116].  $^1\text{H}$  and  $^{13}\text{C}$  NMR (400, 100 MHz respectively, in CD<sub>3</sub>OD) spectral data are similar to those reported in the literature [7].

**Compound 2:** colorless gummy material (10 mg).  $-\alpha_D^{25} - 12.8^\circ$  (MeOH, *c* 0.460); –MS (positive ion APCI-MS):  $m/z = 810$  [M+ NH<sub>4</sub>] $^+$ , 793 [M+H] $^+$ . –MS (negative ion APCI-MS):  $m/z = 850$  [M+OAc-H] $^-$ , 791 [M-H] $^-$ . –MS (HR-ESI-TOF):  $m/z = 791.4090$  [C<sub>38</sub>H<sub>63</sub>O<sub>17</sub>, calcd. 791.4065].  $^1\text{H}$  and  $^{13}\text{C}$  NMR (500, 125 MHz respectively, CD<sub>3</sub>OD) spectral data are similar to those reported in the literature [8].

**Compound 3:** colorless gummy material (37 mg).  $-\alpha_D^{25} - 24.6^\circ$  (MeOH, *c* 1.450). –MS (negative ion APCI-MS):  $m/z = 996$  [M+OAc-H] $^-$ , 937 [M-H] $^-$ . –MS (HR-ESI-TOF):  $m/z = 937.4699$  [C<sub>44</sub>H<sub>73</sub>O<sub>21</sub>, calcd. 937.4644]. For NMR data see Table 1.

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