Insecticidal Constituents from the Argentine Liverwort *Plagiochila bursata*

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The new 2,3-secoaromadendrane **1**, together with the known compounds plagiochilines A and M (**2** and **3**, resp.), fusicogigantone A (**4**), and 1,4-dimethylazulene (**5**) were isolated from an Argentine collection of the liverwort *Plagiochila bursata*. Structures were elucidated by extensive 1D- and 2D-NMR studies. Compounds **2** and **4**, incorporated to the larval diet at 100 μg per g of diet, reduced the larval growth of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) by 66±29% and 25±8% and produced 55 and 75% larval mortality at early instars and 20 and 25% pupal mortality, respectively. Treatment with compound **2** also produced abdomen and wing malformation in adults leading to impossibility to mate.

Introduction. – Species of the genus *Plagiochila* are among the most common bryophytes in tropical forests [1] forming extended large cushions on soil, rocks, and trees. Over 500 species of *Plagiochila* have been described from tropical America [2]. Although they are rich sources of various types of secondary metabolites, only a few have been chemically investigated [3].

Some species of *Plagiochila* contain 2,3-secoaromadendrane-type sesquiterpenoids such as plagiochiline A, which has a potent hot taste [4][5] and also exhibits a strong insect antifeedant effect against the African army worm (*Spodoptera exempta*) [6]. Based on their chemical constituents, *Plagiochila* species have been classified into eight chemo-types. Those of type I contain 2,3-secoaromadendranes; type II, bibenzyls; type III, cuparanes and isocuparanes; type IV, bibenzyls, cuparanes, and isocuparanes; type V, gymnomitranes and bicyclogermacrane; type VI, bicyclogermacrane and spathulenol; type VII, pinguisanes, and type VIII, sesquiterpene lactones. Members of type I are further subdivided into more highly evolved or more primitive species, depending on the degree of oxidation and acetylation of the 2,3-secoaromadendrane-type sesquiterpenois [7].

The only previous publication on the chemistry of *P. bursata*, collected in Panama, reported the presence of β-barbatene and *ent*-spathulenol [8]. As part of our ongoing chemical investigation of South American liverworts [9–13], we decided to analyze a *P. bursata* collection from the northwestern Argentine rain forests where this species is

widespread. We report herein the isolation and identification of three 2,3-secoaromadendrane-type sesquiterpenoids **1**–**3**, one fusicocane-type diterpenoid, fusicogigantone A (**4**), and 1,4-dimethylazulene (**5**), therefore, *P. bursata* is a member of chemotype I.

Finally, compounds **2**–**5** were evaluated for their antifeedant and insecticidal capacity on the lepidopteran *Spodoptera frugiperda*, a pest that causes serious damages in corn crops in Argentina.

Results and Discussion. – *Compounds.* The air-dried plant material was extracted with Et_2O. After solvent removal, the extract was chromatographed on silica gel to yield fractions that were further processed by *Sephadex LH-20* to get rid of pigments. Final purification of compounds was accomplished by preparative HPLC of the fractions, on normal and reversed phase.

GC/MS of non-polar fractions showed the presence of the common plant constituents β-barbatene [8], ergosterol, globulol, phytol, sitosterol, squalene, spathulenol, and stigmasterol. Linalyl acetate and oct-1-en-3-yl acetate, earlier isolated from the liverworts *Wiesnerella denudata* and *P. ovalifolia* [3], respectively, were also detected by GC/MS. The blue constituent, 1,4-dimethylazulene (**5**), could be isolated and identified by its MS and NMR features in comparison to those of an authentic sample. It had previously been found in *Plagiochila longispina*, *P. micropterys*, *Macrolejeunea pallescens*, and *Calypogeia* species [14].

The known 2,3-secoaromadendranes plagiochiline A (**2**), previously isolated from many other *Plagiochila* species [15–20], and plagiochiline M (**3**), only found in the liverwort *Heteroscyphus planus* [21], as well as the diterpenoid fusicogigantone A (**4**), previously reported from the liverworts *Pleurozia gigantea* and *Plagiochila adianthoides* [22][23], were also isolated from the Et_2O extract. Identification of the mentioned constituents was accomplished by their HR-EI-MS and 600 MHz NMR data in comparison to those reported in the literature. In addition, a new compound, **1**, was isolated from the present Argentine collection and identified as a 2,3-secoaromadendrane-type sesquiterpenoid. Compound **1** showed a molecular-ion peak at *m*/*z* 438, and its HR-EI-MS spectrum was in agreement with the molecular formula $C_{22}H_{30}O_9$ which accounted for eight degrees of unsaturation. FT-IR indicated the presence of ester CO groups (1746 cm^{-1}) and C=C bonds (1640 cm^{-1}). The UV spectrum showed an intense band at 235 nm (ε 8589) assigned to an α,β-unsaturated CO group. The ^{1}H- and ^{13}C-NMR spectral data (*Table 1*) indicated that **1** was structurally related to plagiochilines A and M (**2** and **3**, resp.). Two signals at δ(H) 0.55 (*dd*) and 0.69 (*ddd*) were assigned to H-atoms on a cyclopropane ring with two Me groups attached to it, as can be inferred by the ^{1}H-NMR signals at δ(H) 0.99 (*s*, 3 H) and 1.12 (*s*, 3 H), assigned to Me(13) and Me(12), respectively. In the HMBC spectrum (*Table 1*), cross-peaks were observed between the *singlet* at δ(H) 0.99 and the signals of C(6), C(7), C(11), and C(12), and the *singlet* at δ(H) 1.12 ppm and the signals of C(6), C(7), C(11), and C(13). The *gem*-dimethyl cyclopropane located at C(6) and C(7) of the secoaromadendrane skeleton in compound **1** is a structural feature shared with plagiochilines A and M (**2** and **3**, resp.). Additionally, the presence of a methyl ester could be inferred from the Me signal at δ(C) 51.3 in the ^{13}C-NMR that correlated with the *singlet* at δ(H) 3.74 in the HMQC spectrum (supporting information). The location of the methyl ester was evident by the cross-peak between the H-atom signals of this Me group, H–C(3),

Table 1. *^{1}H-, ^{13}C-, and HMBC-NMR Data of* **1** (600 MHz, $CDCl_3$)

	δ(H)	δ(C)	HMBC (H→C)
H−C(1)	2.71 (br. *d*, *J* = 10)	43.7	
H−C(2)	6.83 (*d*, *J* = 10)	92.2	C=O of *Ac*O−C(2)
H−C(3)	7.36 (*s*)	151.5	2, 4, 5, 15
C(4)		113.9	
H−C(5)	2.61 (*dd*, *J* = 10, 3)	29.2	1, 2, 3, 4, 6, 15
H−C(6)	0.55 (*dd*, *J* = 10, 9)	30.8	4, 8, 11, 13
H−C(7)	0.69 (*ddd*, *J* = 10, 9, 6)	23.3	5, 6
H_α−C(8)	1.47 (br. *d*, *J* = 13)	18.8	
H_β−C(8)	1.84–1.94 (*m*)[a])		
H_α−C(9)	1.84–1.94 (*m*)[a])	31.4	
H_β−C(9)	2.15–2.20 (*m*)		
C(10)		83.8	
C(11)		19.9	
Me(12)	1.12 (*s*)	15.8	6, 7, 11, 13
Me(13)	0.99 (*s*)	28.7	6, 7, 11, 12
H−C(14)	4.37 (*d*, *J* = 11)	65.4	C=O of *Ac*O−C(14), 1, 9, 10
H′−C(14)	4.88 (*d*, *J* = 11)		C=O of *Ac*O−C(14), 1, 9, 10
*Ac*O−C(10)	2.00 (*s*)	21.8, 169.6	Me of *Ac*O−C(10) with C=O of *Ac*O−C(10)
*Ac*O−C(14)	2.07 (*s*)	20.8, 170.3	Me of *Ac*O−C(14) with C=O of *Ac*O−C(14)
*Ac*O−C(2)	2.18 (*s*)	21.2, 169.1	Me of *Ac*O−C(2) with C=O of *Ac*O−C(2)
MeO	3.74 (*s*)	51.3	15
C(15)		166.2	

[a]) Overlapping signals.

and H−C(5) and the ^{13}C signal at δ(C) 166.2 assigned to C(15) of the secoaromadendrane backbone, in the HMBC spectrum (*Table 1*). Moreover, a signal at δ(C) 65.4, which correlated in the HSQC spectrum with the signals at δ(H) 4.37 and 4.88, evidenced the presence of the typical *AB* system of a CH_2 group attached to an ester chain which was assigned to CH_2(14) (*Fig. 1*). The molecule contains three AcO residues, detected by their Me signals at δ(H) 2.00, 2.07, and 2.18 and the CO signals at δ(C) 169.6, 170.3, and 169.1. As shown in *Table 1*, in the HMBC spectrum, the correlations between H−C(2) and the CO signal of one AcO group (δ(C) 169.1), between H−C(14) and H′−C(14) and the CO signal of another AcO group (δ(C) 170.3), and between H−C(9) and the signal of C(10) that carries the third AcO group, permitted to locate the AcO groups in the molecule. The relative configuration, as

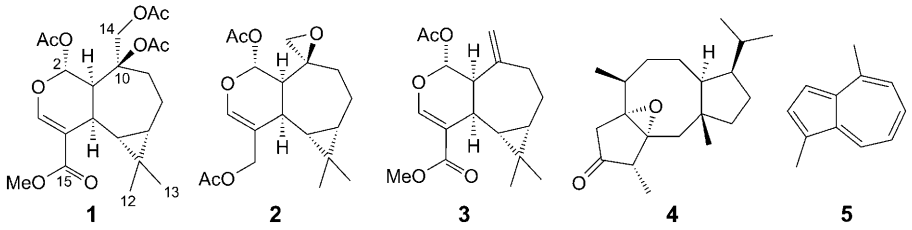

Fig. 1. *Chemical structures of the isolated compounds*

depicted, was established by the NOESY key correlations shown in *Fig. 2*. Cross-peaks were detected between H–C(1) and H–C(5) and H–C(14), as well as between H–C(5) and H–C(14) and H′–C(14), and between H–C(8), α-oriented, and H–C(14) and H′–C(14), indicating that all the mentioned H-atoms lie on the same side of the molecule. In addition, correlations were observed between the signals of H–C(6) and H–C(2) and H–C(7), but no correlations were detected between these H-atoms and the ones located on the α-side of the molecule indicating that H–C(2), H–C(6), and H–C(7) are β-oriented.

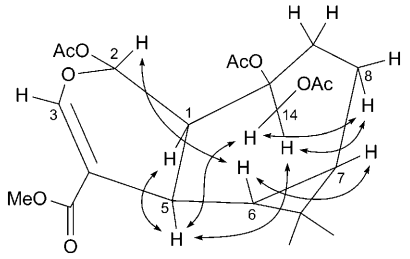

Fig. 2. *Key NOESY correlations for compound* **1**

Larval Diet Choice Test. No significant modification in the feeding preferences were detected when compounds **2**–**5** were incorporated to the larval diet of *Spodoptera frugiperda* at 100 μg per g of diet as indicated by the values of FR_{50} (feeding ratio) of 0.93 ± 0.23, 0.99 ± 0.13, 0.86 ± 0.18, and 0.95 ± 0.17, respectively, calculated for the treatments with compounds **2**–**5**, respectively. Compound **1** underwent rapid decomposition after incorporation to the larval diet, therefore its effects could not be evaluated.

Toxic Effects on Spodoptera frugiperda *Larvae.* Compounds **2**, **4**, and **5** were incorporated to the larval diet of *S. frugiperda* at 100 μg per g of diet. Insects consumed equal amounts of treated and control diets (no significant differences in the values of the consumption rate (*CR*) for control and treatments). However, a determination of the larval growing rate (*GR*) during 14 days, starting with second instar larvae, showed that in the mentioned period, compounds **2** and **4** reduce larval growth by 66 ± 29 and $25 \pm 8\%$, respectively, compared to control. Our results also showed that compounds **2** and **4** produced a small but significant increment in the duration of the larval period ($24 \pm 1\%$ and $21 \pm 1\%$ longer than the control, resp.). In addition, they produced 55 and 75% larval mortality at early instars and 20 and 25% pupal mortality, respectively. Treatment with compound **2** also produced abdomen and wing malformation in adults leading to impossibility to mate.

Secoaromadendrane-type sesquiterpenoids are rare in nature and their biological activity is almost unknown. Unexpectedly, the pungent secoaromadendrane **2**, previously reported as a strong antifeedant on *S. exempta* [6] displayed no alterations on the feeding behavior of *S. frugiperda*. However, at 100 ppm, it seriously affected the development of *S. frugiperda* larvae producing large mortality rates. The present is the first report on the bioactivity of fusicogigantone A (**4**), which, according to our results, has shown to be a strong insecticide against *S. frugiperda*.

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Experimental Part

General Experimental Procedures. TLC: silica gel (*Kieselgel 60 F_{254}*, *Merck*) with hexane/AcOEt (1:1 and 4:1); *Godin* reagent followed by heating at 120° was used for detection. Column chromatography (CC): silica gel *60* (SiO_2; 70–230 mesh, *Merck*) and *Sephadex LH-20* (CH_2Cl_2/MeOH 1:1). Prep. HPLC: *Gilson M 45* pump system, *Ultrasphere Beckman Silica* and reversed-phase *Beckman C_8* and *C_{18}* columns (25 × 1 cm) were used. GC-MS Analysis: *Hewlett-Packard 5890* series instrument; with an *HP-5MS* (30 m × 0.25 mm i.d. × 0.25 μm) column with temp. programming from 50°, then 50–280° at 5° min^{-1}, and finally isothermal at 280° for 15 min. $[\alpha]_D$: *JASCO DIP-1000* polarimeter with $CHCl_3$ as solvent. UV Spectra: *Shimadzu UV-160A* spectrophotometer. IR Spectra: *Shimadzu Type CVT-04* spectrophotometer by the diffuse reflectance method. NMR Spectra: *Varian Unity 200*, a *Varian Gemini 200* (200 MHz), or a *Varian Unity 600* (600 MHz) spectrometer, in $CDCl_3$ as solvent. EI-MS and HR-EI-MS: *JEOL JMS AX-500* spectrometer.

Plant Material. Plagiochila bursata was collected in Yala, Jujuy, Argentina. A voucher specimen (A. Bardon 21) has been deposited with the Abteilung Systematische Botanik, Albrecht-von-Haller Institut, D-Göttingen.

Extraction and Isolation. The air-dried (whole plant) material (227 g) was extracted with Et_2O (2 l) during 15 d at r.t.

The Et_2O extract (3.5 g) was chromatographed on SiO_2 with a gradient solvent system of hexane/AcOEt to give twelve fractions. From *Fr. II* (180 mg), 1,4-dimethylazulene was identified as the major compound by GC-MS. After a HPLC separation (*Ultrasphere Beckman Silica* Column, hexane, 1 ml min^{-1}) 18.5 mg (t_R 31 min) of this compound were isolated. *Fr. IV* (7 mg) and *V* (1.5 mg) showed *β*-barbatene and squalene as the main constituents by GC-MS. From *Fr. VI* (308 mg), 1-octen-3-yl acetate and linalyl acetate were identified by CG-MS.

HPLC (*Ultrasphere Beckman Silica* column; hexane/AcOEt 95:5, 1.2 ml min^{-1}) of *Fr. VII* (203 mg) gave 13.8 mg (t_R 24 min) of fusicogigantone A (**4**) and a mixture which was further purified by HPLC (*Ultrasphere Beckman Silica* column; hexane/AcOEt 92:8) to give 1.4 mg (t_R 54 min) of plagiochiline M (**3**). From *Fr. VIII* (41 mg) and *IX* (81 mg) spathulenol; from *Fr. X* (126 mg) globulol and phytol and from *Fr. XI* (399 mg) ergosterol, stigmasterol, and sitosterol were idendified by GC/MS. HPLC (*Beckman C18*; MeOH/H_2O 7:3, 1.2 ml min^{-1}) of *Fr. XII* gave 6.7 mg (t_R 36 min) of plagiochiline A (**2**) and 6.1 mg of the new 2,3-secoaromadendrane-type sesquiterpenoid **1** (t_R 62 min).

*Methyl (4*R*,4a*R*,5*S*,7a*S*,8a*S*,8b*R*)-4,5-Bis(acetyloxy)-5-[(acetyloxy)methyl]-4a,5,6,7,7a,8,8a,8b-octahydro-8,8-dimethyl-4*H*-cyclopropa[3,4]cyclohepta[1,2-*c*]pyran-1-carboxylate* (**1**). $[\alpha]_D^{25} = +64.2$ ($c = 1.20$, $CHCl_3$). UV ($CHCl_3$): 235 (8589). FT-IR (KBr): 1746, 1713, 1640, 1437, 1370, 1306, 1250. ^{1}H- and ^{13}C-NMR: see *Table 1*. EI-MS: 438 (7, M^+), 379 (17), 336 (14), 335 (12), 197 (50), 139 (100), 137 (20), 91 (15), 43 (92). HR-EI-MS: 438.1887 (M^+, $C_{22}H_{30}O_9^+$; calc. 438.4682).

Insects. Larvae from *S. frugiperda* were obtained from our laboratory colonies. The colonies had not been previously exposed to insecticides. Larvae fed on an artificial diet that consisted of a mixture of yeast (3 g), beans boiled and milled (250 g), wheat germ (12.5 g), agar agar (12.5 g), ascorbic acid (1.5 g), methyl 4-hydroxybenzoate (1.5 g), formaldehyde (4 ml of a 38 % H_2O soln.), and H_2O (500 ml).

Larval Diet Choice Test. A portion of artificial diet was mixed with acetone, and after solvent removal *in vacuo*, this portion was employed as control diet. Another portion was mixed with an acetone soln. of pure test compounds (treatment), in order to give 100 μg of treatment per g of diet. After evaporation of the solvent, 120 mg of control and the same amount of treated diet were placed in a glass tube. Between the two diet portions, a larva was introduced in the tube. The larva was allowed to choose the diet, and, when 50% of control diet had been eaten (around 48 h after starting the bioassay), the remaining diets (control and treated) were weighed. The experiment was carried out in 20 replicates.

Results of the choice test were then reported by the feeding ratio $FR_{50} = T/C$ [24] where C and T represent the amounts eaten of control and treated diets, resp.

Toxicity Bioassay. A portion of the artificial diet was impregnated with acetone and, after solvent removal, this portion was employed as control diet. Another portion was impregnated with an acetone soln. of pure test compounds in order to leave 100 μg of compound per g of diet. After evaporation of the solvent, control and treated diets were placed in test tubes (20 replicates for treated and 20 for control). Second instar larvae of homogeneous size and accurately weighted were placed in each tube and kept at 27° until emergence of the 1st generation of adults. Fourteen days after the beginning of the experiment, the larval weight was determined again, in order to record the larval growth rate (GR). The weight of the diet provided to larvae was also determined in order to determine the consumption rate (CR) [25].

Determination of GR *and* CR. $GR = (A - B)/t$, is the average of the larval weight increment per day where A = final larval weight, B = initial larval weight, and t = period of evaluation (14 d). $CR = D/t$, is the average of larval diet consumed per day, where D = weight of food eaten during the experimental period ($t = 14$ d).

Statistical Analysis. The results are reported as mean ± SEM. The differences in the mean values were evaluated by analysis of variance (ANOVA). The *Tukey* test was used for all pairwise multiple comparisons of groups. In all statistical analysis, $P > 0.05$ was considered not significant.

Supporting Information Available. CI-MS, ^{1}H- and ^{13}C-NMR, ^{1}H,^{1}H-COSY, HSQC, HMBC, and NOESY spectra of compound **1** are available upon request from the corresponding author.

REFERENCES

[1] S. R. Grandstein, in 'Biodiversity and conservation of neotropical montane forests', Eds. S. P. Churchill, H. Balslev, E. Forero, J. L. Luteyn, The New York Botanical Garden, New York, 1995, p. 321.
[2] J. Heinrichs, S. R. Grandstein, R. Grolle, *J. Hattori Bot. Lab.* **1998**, *85*, 1.
[3] Y. Asakawa, in 'Progress in the Chemistry of Organic Natural Products', Eds. W. Herz, G. W. Kirby, R. E. Moore, W. Steglich, C. Tamm, Springer, Wien, 1995, Vol. 65, p. 1.
[4] Y. Asakawa, in 'Physiology and Biochemistry Development of Bryophytes', Eds. R. N. Chopra, S. C. Bhatla, CRC Press, Florida, 1990, p. 259.
[5] Y. Asakawa, in 'Bryophytes: Their Chemistry and Chemical Taxonomy', Eds. H. D. Zinsmeister, R. Mues, Oxford University Press, Oxford, 1990, p. 369.
[6] Y. Asakawa, M. Yoyota, T. Takemoto, I. Kubo, K. Nakanishi, *Phytochemistry* **1980**, *19*, 2147.
[7] Y. Asakawa, *J. Hattori Bot. Lab.* **1988**, *64*, 97.
[8] J. Spörle, Ph.D. Thesis, Universität des Saarlandes, 1990.
[9] Y. Asakawa, *Phytochemistry* **2001**, *56*, 297.
[10] M. Toyota, A. Bardón, N. Kamiya, S. Takaoka, Y. Asakawa, *Chem. Pharm. Bull.* **1997**, *45*, 2119.
[11] A. Bardón, N. Kamiya, M. Toyota, Y. Asakawa, *Phytochemistry* **1999**, *51*, 281.
[12] A. Bardón, N. Kamiya, M. Toyota, S. Takaoka, Y. Asakawa, *Phytochemistry* **1999**, *52*, 1323.
[13] A. Bardón, G. Bovi Mitre, N. Kamiya, M. Toyota, Y. Asakawa, *Phytochemistry* **2002**, *59*, 205.
[14] U. Siegel, R. Mues, R. Dönig, T. Eicher, M. Blechschmidt, H. Becker *Phytochemistry* **1992**, *31*, 1671.
[15] F. Nagashima, E. Nishioka, K. Kameo, C. Nakagawa, Y. Asakawa, *Phytochemistry* **1991**, *30*, 215.
[16] Y. Asakawa, H. Inoue, in 'Studies on Cryptogams in Southern Peru', Ed. H. Inoue, Kenseisha, Tokyo, 1984, p. 117.
[17] Y. Asakawa, H. Inoue, in 'Studies on Cryptogams in Southern Peru', Ed. H. Inoue, Tokai University Press, Tokyo, 1987, p. 119.
[18] Y. Fukuyama, Y. Asakawa, *Phytochemistry* **1991**, *30*, 4061.
[19] Y. Fukuyama, M. Toyota, Y. Asakawa, *Phytochemistry* **1988**, *27*, 1425.
[20] F. Nagashima, Y. Sari, M. Tori, Y. Asakawa, S. Huneck, *Phytochemistry* **1993**, *34*, 1341.
[21] T. Hashimoto, I. Nakamura, M. Tori, S. Takaoka, Y. Asakawa, *Phytochemistry* **1995**, *38*, 119.
[22] Y. Asakawa, X. Lin, M. Tori, K. Kondo, *Phytochemistry* **1990**, *29*, 2597.
[23] S. Valcic, V. Huch, M. Veith, H. Becker, *Phytochemistry* **1995**, *40*, 199.

[24] X. F. Bellés, F. Camps, J. Casas, J. Lloria, A. Messeguer, M. D. Piulachs, F. J. Sánchez, *Pestic. Biochem. Physiol.* **1988**, *32*, 1.
[25] C. S. Shea, J. T. Romeo, *Fla. Entomol.* **1991**, *74*, 10.

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