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SESQUITERPENE LACTONES OF THE CAPITATE GLANDULAR TRICHOMES OF *HELIANTHUS ANNUUS*

OTMAR SPRING, THOMAS BENZ* and MARTIN ILG*

Universität Tübingen, Institut für Biologie I; *Institut für Organische Chemie, Auf der Morgenstelle, 7400 Tübingen, F.R.G.

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Key Word Index—*Helianthus annuus*; Asteraceae; sesquiterpene lactones; glandular trichomes.

Abstract—A chemical analysis of the resinous content of mechanically collected capitate glandular trichomes from the leaf surface of sunflower established the existence of six sesquiterpene lactones by HPLC separations. In addition to the known heliangolides niveusin C, 15-hydroxy-3-dehydrodesoxyfruticin and argophyllin B, three new germacranolides of the niveusin A-type were isolated and characterized. A simple and quick technique for the extraction, chromatographic separation and quantification of sesquiterpene lactones from plant material is discussed with special regard to their *in vivo* occurrence within the plant.

INTRODUCTION

Previous investigations of the sesquiterpene lactones from sunflower, *Helianthus annuus* L., reported the existence of six germacranolides (1–6) [1–3]. The extraction and purification technique used for structural determination of these compounds followed the standard procedures, applied in many sesquiterpene lactones papers during the past 25 years. Although these methods comprise a multitude of preparation steps which might modify the plant products, the results obtained were generally accepted to represent the natural situation within the plant.

In a more recent study, however, it has been shown that the sesquiterpene lactones of sunflower are localized within the cuticular globe of a special type of glandular hair at the leaf surface [4, 5]. Thus it appeared appropriate to modify the extraction procedure in order to specifically examine the trichome constituents. Extracts of mechanically collected glandular trichomes with methanol or methylene chloride in fact revealed six peaks (A–F) in the elution diagram of the HPLC analysis (Fig. 1). However, only three (B, D, F) were identical with known sesquiterpene lactones from *H. annuus*. Structural elucidation of the remaining three substances is described in this paper.

RESULTS AND DISCUSSION

Structural elucidation

Methylene chloride extracts of air dried leaves of *H. annuus* afforded six sesquiterpene lactones, which were shown to derive from the subcuticular globe of glandular trichomes at the leaf surface, by comparison of HPLC analysis with extracts from mechanically collected trichomes. ^1H and ^{13}C NMR spectroscopic data as well as MS of three compounds, purified by HPLC separations, were identical to those from the known sesquiterpene

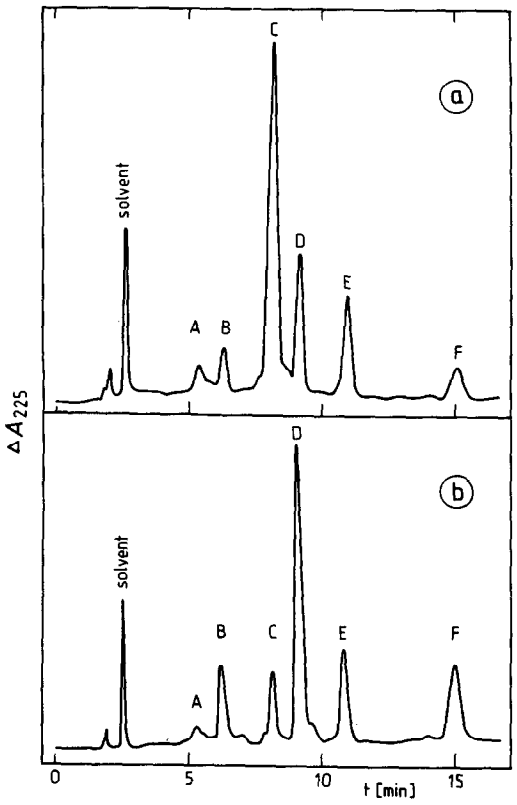


Fig. 1. HPLC analysis of a methanolic extract of 15 capitate glandular trichomes from *H. annuus* (a) plants grown five weeks in the botanical garden; (b) plants grown five weeks in a greenhouse under natural light conditions. Solvent MeOH–H_2O (1:1); Hypersil ODS 5 μm, 4 × 250 mm column, flow 1 ml/min, A_{255}.

lactones niveusin C (**1**; Fig. 1 peak F), 15-hydroxy-3-dehydrodesoxyfruticin (**2**; peak D) and argophyllin B (**6**; peak B) [1–3, 6].

Fraction E afforded a mixture of two sesquiterpene lactones, which were separated on TLC to give **4a** and **4c**. The spectroscopic data of compound **4a** were almost identical with those of the furanoheliangolide 4,5-dihydroniveusin A (**4**), previously reported to derive from sunflower [3]. However, the $[M]^+$ signal of FD mass spectra indicated a M_r of 410 ($C_{21}H_{30}O_8$) instead of the expected 396 ($C_{20}H_{28}O_8$). NMR spectra afforded an additional methyl signal at δ 3.37 (3 protons intensity) and δ 58.6, supporting the existence of a methoxyl group. Acetylation of **4a** with acetic anhydride–pyridine gave the monoacetate (**4b**) which was analysed by GC–MS and showed characteristic signals at *m/z* 451 ($[M]^+ - H$), 420 ($[M]^+ - MeOH$), 392 ($[M]^+ - AcOH$), 391 (451 − AcOH). As acetylation indicated a single primary hydroxyl group and since the H-1 signal of **4a** was shifted upfield [7] compared to **4**, we suggested the methoxyl group was located at C-1. Analogous configuration of the R^1O-group in position C-1 and that of 4,5-dihydroniveusin A in a recent publication [3] might be suggested from the similarity of the coupling constants (9.9, 7.8; 9.0, 8.8 Hz). Moreover, it was impossible to determine the stereochemistry at C-4 since the multiplet formed by couplings with H-5a/H-5b and H-15 did not allow analysis of coupling constants.

In addition, TLC separations of fraction E afforded the isomer **4c** (R_f 0.32 in CH_2Cl_2–Me_2CO–EtOAc, 5:4:1) which was partly transformed into **4a** (R_f 0.41) after purification so that spectrometric investigations became difficult. In ^{13}C NMR spectra of the mixture, the signals of C-4′ and C-5′ were doubled and new signals occurred at δ 28.7 (C-14) and 62.5 (C-15) in a ratio of *ca* 1:2 compared to the corresponding signals of **4a** (δ 25.8, C-14; δ 65.6, C-15). The ^1H NMR spectra were identical in all parts with those of compound **4a** and mass spectrometric measurements accounted for the same formula $C_{21}H_{30}O_8$. From these results we suggest that **4c** is the keto form of **4a**, although we could observe only a weak ^{13}C NMR signal for C-3 at δ 207.5 in the mixed sample.

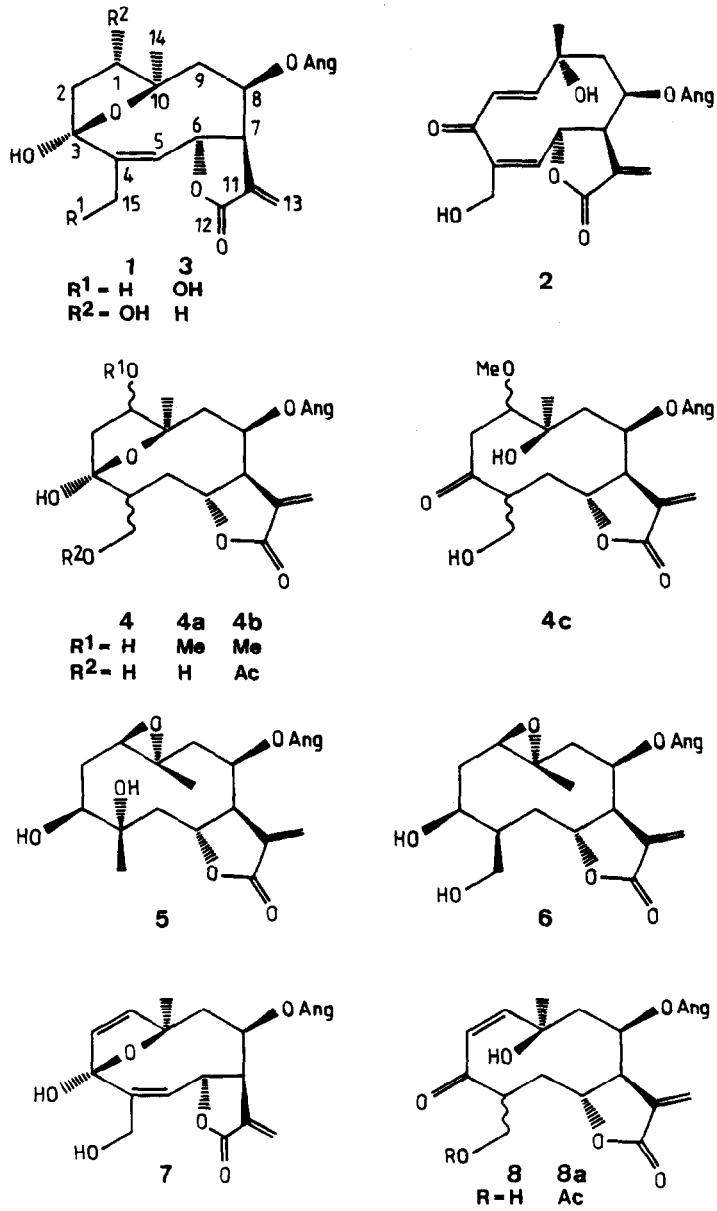
The NMR-spectroscopic data of **7** (Fig. 1 peak C) resembled niveusin A [6] and were also identical in most parts with those of liatrin, the major lactone (SQL) from *Liatris chapmanii* [8, 9]. However, in contrast to the latter the mass spectrum of **7** showed the highest signals at *m/z* 358 ($[M]^+ - H_2O$), 340 ($[M]^+ - 2H_2O$) and 276 ($[M]^+ - C_5H_8O_2$) and gave the typical base peak at *m/z* 83 (rel. intens. 100%) of a C_5 unsaturated ester side chain. This was shown to be an angelic ester by its typical ^1H NMR signals at δ 6.12 (H-3′), 1.95 (H-4′; Me) and 1.81 (H-5′; Me). The existence of two proton signals at δ 4.40 (*d*) and 4.13 (*d*) together with the characteristic ^{13}C NMR signal at δ 66.2 provided evidence for a C-15-OH residue. The assignment of the residual signals was established by decoupling experiments and in comparison with the data of the very similar compounds liatrin [8], niveusin A and B [6], thus supporting the structure of **7** (1,2-anhydridoniveusin A, $C_{20}H_{24}O_7$).

The ^1H and ^{13}C NMR patterns of **8** indicated the known skeletal elements of an α-methylene-γ-lactone between C-7 and C-6 as well as an angelic ester side chain cleaved at C-8. On irradiation at δ 2.91 the two doublets for H-13a (δ 6.38) and H-13b (δ 5.78) changed into sharp singlets, thus confirming that this signal could be assigned to H-7; at the same time the signal of the multiplet at δ 5.20 (H-8) and the doublet of doublets at δ 4.61 (H-6) sharpened. Irradiation at H-8 (δ 5.20) changed the doublet of doublets at δ 2.83 (H-9a) and δ 1.32 (H-9b, *J* 15.8 Hz) into doublets. Coupling of the H-6 signal (δ 4.61) with two double doublets at δ 2.46 and 1.77 confirmed the presence of H-5a and H-5b, respectively. Irradiation at the latter two signals sharpened part of a multiplet at δ 2.80, which could be assigned to H-4. The presence of two protons and a hydroxyl group at C-15 was concluded from a two proton signal at δ 4.11 (H-15a, H-15b) and the ^{13}C NMR signal at δ 65.3. However, the stereochemistry at C-4 could not be deduced because of the overlapping signals at H-4. At the olefinic part of the ^1H NMR spectrum the assignment of only two protons remained at δ 5.58 and 6.61 ($J = 10.8$). Irradiation at each of these signals changed them into a sharp singlet. As the decoupling experiments at H-8 and H-9a, b ascertained that C-10 was fully substituted the only possibility for the double bond was between C-1 and C-2. This was confirmed by the lack of further coupling from irradiation at H-4 and H-2, indicating that the C-3 signal had to be quaternary. As the acetylation of **8** gave a monoacetate (**8a**), we suggest that its structure is 1,2-anhydrido-4,5-dihydroniveusin A, ($C_{20}H_{26}O_7$) instead of the hemiketal form of the molecule. This was further supported by the lack of a typical C-signal at around δ 105 instead of which a very weak signal at 195.6 was observed for C-3.

Natural occurrence of sesquiterpene lactones within sunflower trichomes

HPLC separations of glandular trichome extracts gave no indication for the existence of 4,5-dihydroniveusin A (**4**). Thus the question arises, whether **4a** is formed from another substance in the extract due to the use of methanol as solvent [cf. 7, 10]. Hence glandular trichomes were collected and extracted in methyl cyanide (35% in H_2O) and directly analysed in the same solvent by HPLC separation. The elution diagram again showed six peaks, the fifth of which had identical retention time and UV absorption as a methanol-treated reference sample of **4a** run in 35% methyl cyanide. From this experiment we conclude that 1-methoxy-4,5-dihydroniveusin A is a natural product of the sunflower glandular trichomes.

Furthermore, previous investigations on sesquiterpene lactones from sunflower afforded two additional compounds, niveusin B (**3**) [2, 3] and argophyllin A (**5**) [3]. We did not observe these compounds in the HPLC-elution diagrams of extracts from glandular trichomes. There might be several reasons for this discrepancy. Either the amount of these compounds is so low in comparison to the other six sesquiterpene lactones so that we failed to identify their signals, or both substances are artefacts of the previously employed purification procedures. According to our observations the lactones are well protected against oxidation or other forms of degradation as long as they are located within the cuticular space of the glandular trichomes. Thus HPLC-patterns of freshly collected hairs are not significantly different from those of air-dried material (up to one year and more), neither in the quality nor in the quantity of the compounds. On the other hand, purified samples of the sesquiterpene lactones stored in solvents are relatively unstable, even at low temperatures. $T_{1/2}$ of **2** was found to



be 14 days at 20° and 58 days when kept at −15°, respectively. Reactions of the compounds with solvents, especially with alcohol [7, 10] were observed as well as intramolecular alterations such as isomerization or epimerization [11, 12]. In many cases the chosen preparation steps may influence these processes. It therefore is very difficult to give information about the real constitution of the sesquiterpene lactone content of the plant *in vivo*. As the time between the destruction of the cellular compartment (in which the sesquiterpene lactones are located) and the HPLC separation of the sample is less than 20 min by the use of the method described here, we have minimized the environmental influence and expect that this form of analysis most likely represents the natural situation within the glandular trichome.

With respect to the biosynthesis of the sunflower sesquiterpene lactones the high similarity of the compounds isolated so far implies, that all derive from a precursor of the niveusin A type, although this molecule has not yet been isolated from *H. annuus*. Some observations concerning the relative quantity of specific compounds in various plants emphasize that environmental influences such as exposure to light may be responsible for the above mentioned processes. Thus it was demonstrated previously [13], that plants cultivated under low irradiation (5 W/m^2) contain almost identical amounts of compounds **1** and **2** (ratio 2:3), whereas high irradiation (100 W/m^2) leads to a large increase in compound **2** compared with **1** (ratio 1:4.8). Moreover, we observed that glandular trichomes from greenhouse plants contain relatively higher amounts of **2** than of **4a/c** and **7**, whereas in plants grown outdoors the latter compounds are

dominant (Fig. 1). Whether such alterations are triggered enzymatically or are the result of direct photooxidative processes is still unclear.

EXPERIMENTAL

^1H and ^{13}C NMR spectra were measured at 400 and 100.6 MHz, respectively, in $CDCl_3$ with TMS as int. standard. Hypersil ODS (5 μm Shandon; analytical column 250 × 4 mm; prep. column 250 × 20 mm; solvent MeOH/H_2O 1:1, A_{225}) was used for HPLC separation. TLC was done on precoated Silica gel 60 GF_{254}. MS were recorded either by FDMS (50°) or by EIMS at 70 eV ionization. GC was performed with a Carlo Erba HR GC 5300 Mega Series; column Chrompack WCOT fused silica, 25 m; temp. progr. 100–280°, 10°/min; FID. GC/MS was measured on a LKB 9000 analyzer GC/MS unit; column SE 30, 22 m; temp. progr. 100–280°, 10°/min.

Extraction and isolation. For determination in glandular trichomes. Capitate glandular trichomes were collected as described previously [4] from the leaf surface of fresh and air-dried sunflower (*Helianthus annuus* L. cv. giganteus; Fa. Benary, Han.-Münden, F.R.G.), grown in the Botanic Garden of the University of Tübingen. For HPLC analysis of the resinous content at least 10 trichomes were incubated in MeOH. After centrifugation (3000 *g*, 3 min) an aliquot of the supernatant was analysed and gave 6 peaks (A–F, Fig. 1) in the HPLC elution diagram at 225 nm. TLC separations (CH_2Cl_2–Me_2CO–EtOAc, 5:4:1) of the same extract revealed 5 spots between R_f 0.28 and 0.55 which contained the 6 substances A–F as documented by HPLC. The use of other solvents (e.g. $CHCl_3$, CH_2Cl_2, Me_2CO, MeCN) for the extraction of the trichomes afforded no additional compounds.

For spectroscopic measurements: Air-dried and ground leaves (140 g) of sunflower were extracted with CH_2Cl_2 (3 × 200 ml, 30 min) at room temp. The filtered extract was concd *in vacuo* to give 1.8 g of dark brown syrup. The extract was dissolved in CH_2Cl_2, adsorbed to silica gel 60 (10 g) and successively eluted in a glass filter G_4 under red. pres. with fr. 1–4 (CH_2Cl_2, 100 ml), fr. 5–10 (CH_2Cl_2–MeOH 19:1, 150 ml), fr. 11–12 (MeOH, 50 ml). The SQL containing fraction 5–10 were combined, evap. *in vacuo*, redissolved in MeOH and further purified by prep. HPLC as described above. Fractions of the six peaks A–F (deriving from capitate glandular trichomes as identified by selective preparation on analyt. HPLC) were collected from several separations and dried under red. pres. Sample A gave 25.6 mg of (**8**), sample B gave 27.4 mg of (**6**), sample D contained 10.6 mg of (**2**) and sample F afforded 16.2 mg of (**1**). The two remaining samples (C, E) rearranged during handling and had to be rechromatographed on TLC (CH_2Cl_2–Me_2CO–EtOAc, 5:4:1). Sample C afforded 36 mg of compound (**7**) and sample E gave 29.2 mg of (**4a**) and 12.3 mg of a mixture of (**4a**/**4c**).

Niveusin C (**1**). ^1H NMR spectra were identical to those of previous reports [6].

15-Hydroxy-3-dehydrodesoxyfruticin (**2**). ^1H NMR data were identical to those of an authentic sample [2].

1-Methoxy-4,5-dihydroniveusin A (**4a**). ^1H and ^{13}C NMR data of (**4a**) showed very high similarity to those of previously reported 4,5-dihydroniveusin A [3] despite an additional methyl signal at δ 3.37 (H-1″)) and δ 58.6 (C-1″). $C_{21}H_{30}O_8$, for $[M]^+$ found *m/z* 410 (FDMS); EIMS 70 eV *m/z* (rel. int.): 392 $[M-H_2O]^+$ (4), 374 (2), 360 (2), 261 (21), 83 (100), 55 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3360 (OH), 1760 (lactone), 1710 (ester), 1600, 1150.

The ^1H NMR spectrum of the isomer **4c** was identical to that of **4a**. ^{13}C NMR data of **4c** deduced from a mixture of **4a** plus **4c** by elimination of the typical signals of the hemiketal form: δ 77.6 *d* (C-1), 37.6 *t* (C-2), 207.5 *s* (C-3), very slight signal), 43.3 *d* (C-4), 31.9 *t* (C-5), 86.7 *d* (C-6), 46.7 *d* (C-7), 66.3 *d* (C-8), 35.6 *t* (C-9), 81.6 *s* (C-10), 136.9 *s* (C-11), 169.5 *s* (C-12), 119.2 *t* (C-13), 28.7 *q* (C-14), 62.5 *t* (C-15), 166.8 *s* (C-1′), 127.2 *s* (C-2′), 139.2 *d* (C-3′), 15.8 *q* (C-

Table 1. ^1H NMR spectra of compounds **4a**, **7** and **8**

H	**4a**	**7**	**8**
1	3.93 *dd* (9.9, 7.8)	5.76 *d* (5.6)	6.61 *dd* (10.8, 2.2)
2a	2.36 *dd* (13.5, 7.7)	6.34 *d* (5.6)	5.58 *d* (10.8)
2b	2.11 *dd* (13.5, 9.8)	—	—
4	2.26 *m*	—	2.80 *m* (4.5)
5a	2.23 *dd* (15.6)	5.86 *d* (6.6)	2.46 *dd* (14.9, 4.5)
5b	2.05 *m*	—	1.77 *dd* (14.9, 2.4)
6	4.59 *ddd* (10.3, 6.5)	5.96 *dd* (6.7, 3.3)	4.61 *dd* (4.6, 2.2)
7	4.51 *ddd* (10.3, 2.5)	3.51 *dd* (3.4)	2.91 *m*
8	5.73 *ddd* (9.2, 7.1, 2.5)	5.14 *dd* (3.4)	5.20 *m*
9a	1.76†	2.37 *dd* (16.6, 3.6)	2.83 *dd* (15.8, 4.8)
9b	1.85†	2.37 *dd*	1.32 *dd* (15.8, 4.0)
13a	6.14 *d* (3.3)	6.31 *d* (2.5)	6.38 *d* (2.3)
13b	5.38 *d* (3.0)	5.71 *d* (2.2)	5.78 *d* (2.0)
14*	1.55 *s*	1.40 *s*	1.48 *s*
15a	3.65 *m*	4.40 *br d* (12.7)	4.11 *m*
15b	3.65 *m*	4.13 *d* (12.7)	4.11 *m*
3′	6.07 *q* (7.2, 1.5)	6.12 *q* (7.2, 1.5)	6.10 *q* (7.3, 1.5)
4′*	1.92 *dq* (7.2, 1.5)	1.95 *dq*	1.97 *dq* (7.2, 1.5)
5′*	1.77 *dq* (1.5)	1.81 *dq* (1.5)	1.85 *dq* (1.5)
1″*	3.37 *s*		

Run at 400 MHz in $CDCl_3$ with TMS as an int. standard. Coupling constants are given in (Hz).

*Intensity three protons.

†Obscured signal.

Table 2. ^{13}C NMR spectra of compounds **4a**, **7** and **8**

C	4a	7	8
1	77.5 *d*	141.3 *d*	143. 9 *d*
2	37.6 *t*	140.0 *d*	126.7 *d*
3	106.6 *s*	108.6 *s*	195.6 *s**
4	43.3 *d*	134.6 *s*	37.4 *d*
5	32.0 *t*[a]	126.7 *d*	33.9 *t*
6	86.7 *d*	76.5 *d*[a]	75.8 *d*[a]
7	46.7 *d*	47.8 *d*	48.3 *d*
8	66.3 *d*	73.9 *d*[a]	73.8 *d*[a]
9	35.6 *t*[a]	43.6 *t*	43.8 *t*
10	81.7 *s*	87.7 *s*	69.0 *s*
11	136.9 *s*	138.6 *s*	137.1 *s*
12	169.5 *s*	169.5 *s*	169.8 *s*
13	119.2 *t*	124.5 *t*	125.1 *t*
14	25.8 *q*	31.1 *q*	19.6 *q*
15	65.6 *t*	66.2 *t*	65.3 *t*
1′	166.8 *s*	166.1 *s*	166.8 *s*
2′	127.3 *s*	126.7 *s*	126.9 *s*
3′	139.2 *d*	140.9 *d*	140.5 *d*
4′	15.9 *q*	15.8 *q*	15.8 *q*
5′	20.5 *q*	20.5 *q*	20.4 *q*
1″	58.6 *q*		

Run at 100.6 MHz in $CDCl_3$ with TMS as an int. standard.

*Very small signal.

[a]Assignments interchangeable.

4′), 20.1*q* (C-5′), 58.6*q* (C-1″); run in $CDCl_3$ with TMS as an intern. standard. $C_{21}H_{30}O_8$, for $[M]^+$ found *m/z* 410 (FDMS).

Acetylation of (**4a**). 14 mg (**4a**) were acetylated with Ac_2O (2 ml) and pyridine (1 ml) for 24 hr at room temp. according to [14]. After prep. TLC (CH_2Cl_2–Me_2CO–EtOAc, 5:4:1) 2.7 mg of the monoacetate (**4b**) was obtained as colourless oil. GC–MS afforded signals at *m/z* (rel. int.): 451 (1), 420 (2), 392 (2), 391 (2), 360 (1), 309 (3), 292 (3), 291 (3), 83 (100).

Argophyllin B (**6**). ^{1}H and ^{13}C NMR as well as MS spectra were identical with previously published data [14]. The so far missing data for the protons at C-2, C-4 and C-5 could be established by single frequency decoupling: δ 1.87 (*dd*, H-4), 2.01 (*m*, H-5a and H-5b), 2.30 (*ddd*, H-2b) and 2.34 (*ddd*, H-2a).

1,2-*Anhydridoniveusin A* (**7**) (the hemiketal form of **2**). ^{1}H and ^{13}C NMR data: Tables 1, 2. $C_{20}H_{24}O_7$, EIMS 70 eV *m/z* (rel. int.): $[M]^+$ 376 (not seen), 358 (19), 340 (2), 276 (5), 275 (11), 258 (72), 83 (100), 55 (100). IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3400, 1760, 1715, 1645, 1600, 1280, 1140, 1020, 905.

1,2-*Anhydrido*-4,5-*dihydroniveusin A*. (**8**). ^{1}H and ^{13}C NMR data: Tables 1, 2. $C_{20}H_{26}O_7$, for M^+ found *m/z* 378 (FD spectra); EIMS 70 eV *m/z* (rel. int.): 360 $[M-H_2O]^+$ (2), 342 (1.5), 260 (2), 242 (2.5), 83 (100), 55 (80).

Acetylation of **8**. 5 mg (**8**) were acetylated as described above. After prep. TLC (CH_2Cl_2–Me_2CO–EtOAc, 5:4:1) 1.5 mg of the monoacetate (**8a**) was obtained as colourless oil EIMS 70 eV *m/z* (rel. int.): $[M]^+$ 420 (not seen), 402 (0.8), 319 (1.6), 302 (0.7), 260 (1.6) 243 (4), 83 (100), 55 (76), 43 (86).

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