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Cyanogenic and non-cyanogenic pyridone glucosides from *Acalypha indica* (Euphorbiaceae) ☆

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ABSTRACT

Seven cyanopyridone derivatives and one corresponding *seco* compound have been isolated from a methanolic extract of the inflorescences and leaves of *Acalypha indica* L. (Euphorbiaceae). The absolute configuration of the main cyanogenic glucoside acalyphin, (−)-(5*R*,6*S*)-5-cyano-5-β-D-glucopyranosyloxy-6-hydroxy-4-methoxy-1-methyl-2(5,6-dihydro)-pyridone, was deduced from an X-ray crystallographic study. In addition, the 6*R*-epimer of acalyphin, epiacalyphin, and the corresponding pair of *N*-demethyl derivatives were isolated. The corresponding amide of acalyphin and a 1′,2′-glucosyl-fused epiacalyphin amide were isolated from air-dried material. Structural elucidation was performed by means of ^{1}H and ^{13}C NMR-spectra, chiroptical methods such as CD-spectroscopy and optical rotation. Two further corresponding derivatives, an aromatized compound and an open-chain structure, were isolated from the aqueous phase.

1. Introduction

Acalypha indica L. (Euphorbiaceae), Indian copperleaf, is an annual tropical weed indigenous to the Indian peninsula, the southern districts of China and South Africa (Hagers Handbuch, 1998). The aerial parts and the roots are traditionally used in folk medicine as an expectorant, against asthma and pneumonia, as an emetic, a purgative and an anthelminthic (Hagers Handbuch, 1998; Siregar, 2001). Various extracts showed antimicrobial and estrogenic activity (Hiremath et al., 1993, 1999). The plant has been described in homoeopathic pharmacopoeias (Homöopathisches Arzneibuch, 2000). Cyanogenesis of *A. indica* has been long known (Rimington and Roets, 1937), but it was not before 1982, that the (main) cyanogenic compound was shown to be a cyanopyridone glucoside which was named acalyphin (**1**) (Fig. 1) (Nahrstedt et al., 1982). Acalyphin (**1**) represents a new biogenetic type of cyanogenic glucoside that apparently derives from nicotinic acid metabolism.

Cyanogenesis, the ability of living organisms to liberate cyanide upon tissue disruption, is widespread amongst plants. More than 60 different cyanogenic glycosides are known from higher plants and insects (Lechtenberg and Nahrstedt, 1999). Cyanogenic glucosides are α-hydroxynitriles which are stabilized by β-linked sugar chains, usually formed of D-glucose. In plants, cyanogenic glycosides are usually accompanied by enzymes (β-glucosidase, hydroxynitrile lyase) which finally lead to a liberation of hydrogen cyanide when the tissue is disrupted.

Interestingly, the cyanopyridone glucoside acalyphin (**1**) and similar nitrile structures (without glucosidation) such as ricinine (Waller and Nowacki, 1978), mallorepine (Hikino et al., 1978), ricinidine (Ganguly, 1970) and nudiflorine (Mukherjee and Chatterjee, 1966) have been isolated only from members of the subfamily Acalyphoideae (Acalypheae) within the Euphorbiaceae. Nicotinic acid and nicotinic acid amide were shown to be the biogenetic precursors of ricinine, which indicates a close connection of this type of compound to the pyridine nucleotide (NAD) cycle (Johnson and Waller, 1974).

Because the configuration at C-5 and C-6 of the cyanopyridone ring of (**1**) was not established by Nahrstedt et al. (1982), the aim of this work was to isolate and purify sufficient amount of (**1**) for

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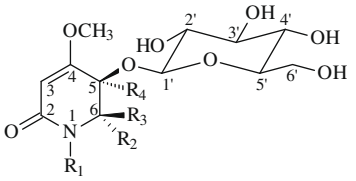

(**1**): $R_1 = CH_3$; $R_2 = OH$; $R_3 = H$; $R_4 = CN$; [5*R*, 6*S*]
(**2**): $R_1 = CH_3$; $R_2 = H$; $R_3 = OH$; $R_4 = CN$; [5*R*, 6*R*]
(**3**): $R_1 = H$; $R_2 = OH$; $R_3 = H$; $R_4 = CN$; [5*R*, 6*S*]
(**4**): $R_1 = H$; $R_2 = H$; $R_3 = OH$; $R_4 = CN$; [5*R*, 6*R*]
(**5**): $R_1 = CH_3$; $R_2 = OH$; $R_3 = H$; $R_4 = CONH_2$; [5*R*, 6*S*]

Fig. 1. Structures of cyanopyridone derivatives.

X-ray analysis as well as the search for related compounds with a pyridine nucleus.

2. Results and discussion

Chromatography of the methanolic extract of the freeze-dried aerial parts of *A. indica* on TLC plates, using the picrate-sandwich method (Brimer et al., 1983) or anisaldehyde-H_2SO_4 for detection, showed one main cyanogenic zone (R_f-value: 0.39), which was related to the reference acalyphin (**1**). In addition, some weak cyanogenic zones appeared below the acalyphin spot (R_f: 0.20–0.32). The parallel examined fresh plant material showed an additional, weak cyanogenic zone with an R_f-value of 0.59.

Quantitative examination of different organs of *A. indica* indicated the inflorescences and leaves containing the highest concentrations of (**1**) (roots 0.055%, stems 0.033%, inflorescences and leaves 0.35%, seeds: not detectable; all on fresh weight basis) by enzymatic liberation of HCN as well as by GC-determination of acalyphin (**1**) as a TMSi derivate. GC-monitoring of the crude methanolic extract of dried (lyophylized) material using an *N*-selective detector showed several *N*-positive signals with retention times close to the peak of (**1**).

The extract was partitioned between dichloromethane and water. The HPLC-chromatogram of the aq. phase showed several peaks with similar UV-spectra, predominantly the peak of (**1**). The aq. phase was further purified by MLCCC (multi-layer coil countercurrent chromatography) yielding fractions I–VIII. For detection of cyanogenic and nitrogen containing compounds, the fractions were monitored by TLC, *N*-selective GC and HPLC. Positive fractions were purified chromatographically by MPLC (Europrep C-18) and prep. HPLC as a final purification (RP-8-Polaris®). Eight cyanopyridone derivatives were isolated from the aq. phase, namely acalyphin (**1**) as a main compound, epiacalyphin (**2**), noracalyphin (**3**), epinoracalyphin **(4)**, acalyphin amide (**5**), epiacalyphin amide cycloside **(6)** and two minor products, *ar*-acalyphidone (**7**) and *seco*-acalyphin (**8**). All compounds yielded D-glucose, when hydrolysed and examined after chiral derivatization by means of capillary zone electrophoresis (Noe and Freissmuth, 1995). ^{1}H NMR coupling constants of the anomeric protons with a ^3J value between 7.5 and 8.3 Hz revealed a β-glucosidic linkage of all sugars (Table 1) (Nahrstedt, 1981).

The molecular weight of (**1**) of 360.2 g/mol was deduced from the positive ESI-MS at *m*/*z* 360.3 $[M]^+$ and *m*/*z* 383.2 $[M+Na]^+$ and the negative ESI-MS at *m*/*z* 359.2 $[M-H]^-$ and *m*/*z* 719.2 $[2M-H]^-$. The ^{1}H-/^{13}C-data and NOE-experiments were consistent with the published data for (**1**) Nahrstedt et al. (1982). 2D-NMR-experiments (HSQC, HMBC) allowed to assign all glucose signals (Table 1) in contrast to (Nahrstedt et al., 1982). The optical rotation of (**1**) was determined to be levorotary at −140°.

After crystallization of (**1**) in a mixture of water and *n*-BuOH, the absolute stereochemistry of the carbons as 5*R*,6*S* was deduced from an X-ray crystallographic study (Fig. 2), by assigning the model to be the enantiomer having the known configuration of D-glucose. The asymmetric unit of the crystal contains three molecules of acalyphin (**1**), only one of which is illustrated in Fig. 2. It also contains three water molecules, one of which is disordered about a twofold axis, thus the crystal is the 5/6 hydrate. The conformations of the three independent molecules are quite similar, having some variation in the linkage of the N-containing ring to the glucose ring. Torsion angles about the two C–O bonds in this linkage show a maximum variation of about 13° over the three molecules. The conformations of the rings themselves show less variation, with the glucose ring having a chair conformation and the pyridone ring exhibiting the sofa conformation, in which one atom lies out of the plane of the other five. In this case, the out-of-plane atom is the C atom carrying the OH group, and its deviation from the plane ranges from 0.579(2) to 0.612(3) Å over the three independent molecules. Thus, acalyphin (**1**) is (−)-(5*R*,6*S*)-5-cyano-5-β-D-glucopyranosyloxy-6-hydroxy-4-methoxy-1-methyl-2(5,6-dihydro)pyridone.

The DAD-UV-spectrum of compound **(2)** showed an absorption at 223 nm as did (**1**) ($\log\varepsilon$ 4.07 (**1**); $\log\varepsilon$ 4.10 (**2**); MeOH) and a shoulder at 255 nm whereas the retention during HPLC differed for ca. 20 min. The molecular mass was confirmed to be 360.2 g/mol by positive ESI-MS (*m*/*z* 361.2 $[M+H]^+$, *m*/*z* 383.1 $[M+Na]^+$). The ^{1}H NMR of **(2)** in MeOH-d_4 showed great similarity to that of (**1**) (Table 1) with a slight downfield shift of the anomeric glucose proton from (**1**) to (**2**) of 0.11 ppm. In the ^{13}C NMR spectrum in MeOH-d_4 the signals of the cyanopyridone ring in (**2**) slightly differed from those in (**1**); in particular the resonance of C-5 showed an upfield-shift of $\Delta\delta$ almost 4 ppm. These data indicated (**2**) to be an isomer of (**1**) with configuration change at C-5 and/or C-6 of the cyanopyridone ring. Contrary to (**1**), compound (**2**) showed dextrorotation with a value of +31°. Since it was not possible to obtain a crystalline sample of (**2**) for an X-ray study, circular dichroism (CD) spectra of (**1**) and (**2**) were compared. Acalyphin (**1**) showed a strong negative CD absorption with a molar circular dichroism of $\Delta\varepsilon$ −7.35 at 249 nm, while compound (**2**) exhibited a strong positive CD effect at 246 nm with a molar CD of $\Delta\varepsilon$ +6.65. For related *cis*-diol *N*-methyl-2-pyridones Boyd et al. (2002) discovered a strong positive CD absorption at lower (210–250 nm) and a weaker negative CD effect at longer wavelengths (235–300 nm). Compared to the 5*R*,6*S* configuration of (**1**) compound (**2**) should have a 5*R*,6*R* configuration; however, this observation has to be treated cautiously as (**1**) and (**2**) are attached to a β-configured D-glucose, which might cause additional chiroptical effects. An additional argument for the 5*R*,6*R* configuration of (**2**) is the fact that (**2**) partly isomerizes into (**1**) in aq. solution obviously via an open-chain aldehyde intermediate as observed for dihydrodiol-*N*-methyl-2-pyridones (Modyanova and Azerad, 2000; Boyd et al., 2002) and similar vicinal diols (Boyd and Sheldrake, 1998) from the unstable *cis*-diol to the more stable *trans*-diol configuration. Compound (**2**) thus is (+)-(5*R*,6*R*)-5-cyano-5-β-D-glucopyranosyloxy-6-hydroxy-4-methoxy-1-methyl-2(5,6-dihydro)pyridone named epiacalyphin (**2**) (Fig. 1).

Compounds (**3**) and (**4**) were isolated from the more polar MLCCC fraction VI and showed consistent UV-spectra with a λ_{max} at 221 nm, comparable to compounds (**1**) and (**2**). The M_r of (**3**) was 346 (pos. ESI-MS: $[M+Na]^+$ = 369; $[2M+Na]^+$ = 715), with a difference of 14 in comparison to (**1**). Likewise the ^{1}H NMR spectrum of (**3**) was very similar to that of (**1**) with the striking difference

Table 1
^{1}H and ^{13}C NMR data of cyanopyridone derivatives (**1**)–(**8**) (all spectra measured in MeOH-d_4, (**7**)*, (**8**)* in DMSO-d_6, for numbering of (**8**) see Fig. 6).

	(**1**) Acalyphin	(**2**) Epiacalyphin	(**3**) Noracalyphin	(**4**) Epinoracalyphin	(**5**) Acalyphin amide	(**6**) Epiacalyphin amide cycloside	(**7**)* *ar*-Acalyphidone	(**8**)* *seco*-Acalyphin
H-3	5.31 (*s*)	5.28 (*s*)	5.28 (*s*)	5.34 (*s*)	5.45 (*s*)	5.37 (*s*)	5.81 (*s*)	3.52 (*s*)
H-6	5.29 (*s*)	5.29 (*s*)	5.22 (*s*)	5.46 (*s*)	5.36 (*s*)	4.95 (*s*)	7.41 (*s*)	–
N-CH_3	3.01 (*s*)	3.02 (*s*)	–	–	2.93 (*s*)	3.78 (*s*)	3.29 (*s*)	2.59 (*d*, 4.3)
O-CH_3	3.82 (*s*)	3.82 (*s*)	3.84 (*s*)	3.85 (*s*)	3.74 (*s*)	3.02 (*s*)	3.73 (*s*)	3.82 (*s*)
N–H	–	–	–	–	–	–	–	7.91 (*d*, 4.3)
H-1′	4.71 (*d*, 7.7)	4.83 (*d*, 7.5)	4.73 (*d*, 7.8)	4.64 (*d*, 8.3)	4.50 (*d*, 7.8)	4.39 (*d*, 7.8)	4.57 (*d*, 7.5)	4.35 (*d*, 7.8)
H-2′	3.20 (*dd*, 7.7/8.9)	3.32 (*m*, 7.5)	3.20 (*dd*, 7.8/8.9)	3.24 (*m*, 8.3)	3.38 (*dd*, 7.8/9.1)	3.25 (*dd*, 7.8/8.9)	3.14 (*ddd*, 5.4/7.5/9.8)	3.03 (*m*, 7.8)
H-3′	3.37 (*m*, 8.9)	3.39 (*m*)	3.37 (*m*, 8.9)	3.35 (*m*)	3.28 (*m*, 9.1)	3.50 (*dd*, 8.9/10.5)	3.19 (*ddd*, 2.1/5.7/13.2)	3.17 (*m*)
H4′	3.30 (*m*)	3.38 (*m*)	3.31 (*m*)	3.30 (*m*)	3.33 (*m*)	3.41 (*m*)	3.08 (*m*)	3.04 (*m*)
H5′	3.34 (*m*, 2.0/5.1)	3.37 (*m*, 2.0/4.5)	3.30 (*m*, 1.5/5.1)	3.31 (*m*, 2.0/5.0)	3.10 (*m*, 2.2/5.2/7.8)	3.43 (*m*, 1.9/4.8)	3.21 (*ddd*, 2.2/9.8/13.1)	3.15 (*m*, 1.4/5.6)
H6′	H-6A: 3.90 (*dd*, 2.0/11.9)	H-6A: 3.88 (*dd*, 2.0/12.2)	H-6A: 3.89 (*dd*, 1.5/11.9)	H-6A: 3.89 (*dd*, 2.0/12.0)	H-6A: 3.78 (*dd*, 2.2/12.0)	H-6A: 3.88 (*dd*, 1.9/12.2)	H-6A: 3.67 (*ddd*, 2.1/5.7/12.0)	H-6A: 3.64 (*dd*, 1.4/11.0)
	H-6B: 3.67 (*dd*, 5.1/11.9)	H-6B: 3.75 (*dd*, 4.5/12.2)	H-6B: 3.68 (*dd*, 5.1/11.9)	H-6B: 3.69 (*dd*, 5.0/12.0)	H-6B: 3.65 (*dd*, 5.2/12.0)	H-6B: 3.72 (*dd*, 4.8/12.2)	H-6B: 3.42 (*m*, 2.1/5.7/12.0)	H-6B: 3.42 (*dd*, 5.6/11.0)
C-2	166.00	166.93	167.83	166.63	167.11	168.13	160.99	166.90
C-3	97.01	96.20	96.60	97.03	100.33	98.42	96.50	33.20
C-4	161.12	161.79	162.80	162.79	163.85	163.73	160.82	161.67
C-5	78.77	75.07	78.33	78.58	81.75	79.62	132.03	112.86
C-6	84.64	85.55	78.27	82.33	84.19	88.06	126.43	114.74
N-CH_3	32.93	32.66	–	–	28.02	33.62	55.88	25.85
O-CH_3	57.79	57.79	57.65	57.58	57.19	57.21	36.11	56.73
R_4 (CN)	115.06	115.58	115.40	115.60	172.64	169.73	–	(=C6: 114.74)
C-1′	102.09	102.89	102.33	102.23	100.13	95.89	101.81	104.48
C-2′	74.27	74.42	74.45	74.41	75.02	78.85	73.26	72.92
C-3′	77.68	77.66	77.90	77.68	78.12	74.22	76.70	76.14
C-4′	70.99	70.61	71.15	71.09	70.94	71.38	69.84	69.55
C-5′	78.30	78.08	78.42	78.41	78.59	79.91	77.03	77.25
C-6′	62.37	61.79	62.47	62.46	62.14	62.24	60.87	60.92

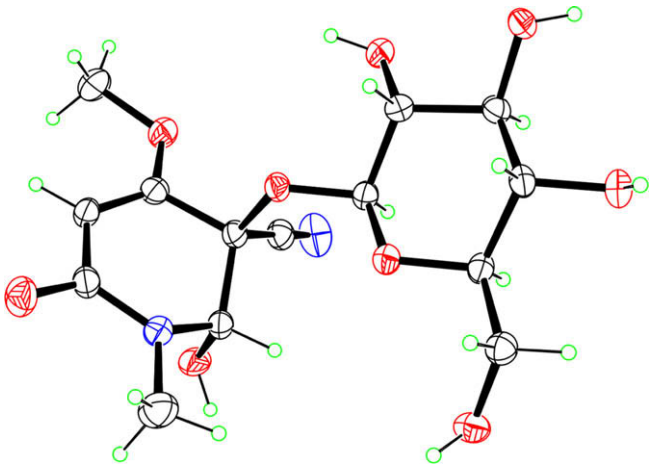

Fig. 2. ORTEP-illustration of acalyphin (**1**).

that the *N*-methyl signal at δ 3.0 ppm was missing. The ^{13}C NMR spectrum exhibited 13 carbon resonances again with the *N*-CH_3 group (δ ca. 33 ppm) missing. The data indicated that (**3**) is an *N*-demethyl analogue of (**1**). The previously unknown configuration of *N*-demethyl acalyphin (**3**) was deduced from the chiroptical properties: the optical rotation was −138° and therefore very similar to the levorotary value of acalyphin (−140°). The CD spectrum showed a strong negative CD effect at 247 nm with a molar CD of $\Delta\varepsilon$ −15.91. Thus, compound (**3**) is (−)-(5*R*,6*S*)-5-cyano-5-β-D-glucopyranosyloxy-6-hydroxy-4-methoxy-2(5,6-dihydro)pyridone named noracalyphin (**3**) (Fig. 1).

The ^{1}H and ^{13}C NMR spectra of (**4**) showed close similarity to those of (**3**); however, almost all proton and carbon signals were doubled; one set of signals of both spectra in fact was identical to that of compound (**3**) (see Table 1). Differences particularly remarkable in the ^{1}H NMR of (**4**) are the shift of H-6 of 0.24 ppm to lower field and that of the anomeric glucose proton of 0.9 ppm to higher field in comparison to (**3**). Assignment of the ^{13}C signals was facilitated by the help of 2D-NMR experiments. Thus, C-6 of (**4**) undergoes a downfield shift of $\Delta\delta$ 4 ppm, indicating a deshielding of this carbon in comparison to (**3**). A second recording of the ^{1}H NMR spectrum of sample (**4**) after three weeks resulted in a proton spectrum completely identical to noracalyphin **(3)**. These data indicate a by and by transformation of compound (**4**) to noracalyphin **(3)** in the solvent used for NMR analysis. From these results compound (**4**) very likely is (5*R*,6*R*)-5-cyano-5-β-D-glucopyranosyloxy-6-hydroxy-4-methoxy-2(5,6-dihydro)pyridone, epinoracalyphin (Fig. 1) which easily isomerizes to the stable *trans*-configuration of (**3**) (because of the small amount of (**3**) additional studies could not be performed).

Compound (**6**) was isolated from MLCCC fraction VI and compound (**5**) from the more polar fraction VII. Their UV-spectra exhibited common similarity with an λ_{max} at 219 nm (both logε 3.6; MeOH). Surprisingly neither (**5**) nor (**6**) showed a positive cyanide test (Feigl/Anger test (Tantisewie et al., 1969) or the sandwich method with an enzyme preparation for hydrolysis lacking specific activity (Brimer et al., 1983)).

The ^{13}C NMR spectrum of compound (**5**) in MeOH-d_4 exhibited 14 signals, of which 13 carbons were very similar to the resonances of acalyphin (**1**). However, the typical CN group at δ 115 ppm (Hübel et al., 1981) was missing, instead a carbonyl resonance at ca. δ 172.6 ppm was present. In comparison to NMR data of prunasin and its corresponding amide (Nahrstedt and Rockenbach, 1993; Sendker and Nahrstedt, in press) the ^{13}C spectrum of (**5**) pointed to the corresponding amide of **(1)**. Likewise, the ^{1}H NMR spectrum of (**5**) in MeOH-d_4 was similar to that of (**1**); however, when recorded in DMSO-d_6, the two diagnostic *N*-attached protons of the carboxy amide group appeared at δ 7.7 and δ 7.4 ppm. The M_r of compound (**5**) was 378 (pos. ESI-MS: $[M+Na]^+$ = 401; $[2M+Na]^+$ = 779) and thus compatible with an amide structure of (**5**). The optical rotation of −43° and the strong negative CD effect of $\Delta\varepsilon$ −3.97 at 244 nm provided evidence for the 5*R*,6*S* configuration. Thus compound (**5**) is (−)-(5*R*,6*S*)-5-carbamoyl-5-β-D-glucopyranosyloxy-6-hydroxy-4-methoxy-1-methyl-2(5,6-dihydro)-pyridone that was named acalyphin amide (Fig. 1).

Compound (**6**) afforded NMR spectra similar to those of acalyphin amide (**5**). The ^{13}C NMR spectrum in MeOH-d_4 showed 14 signals, including the characteristic carbonyl amide resonance at δ 169.7 ppm. A striking difference was the atypical downfield shift of the glucose carbon C-2′, whereas C-3′ was shifted upfield (Table 1). The ^{1}H NMR of (**6**) in DMSO-d_6 was consistent to the spectrum of acalyphin amide (**5**). Interestingly the H-6 resonated as a sharp signal, though in aprotic solvents a proton bound to a secondary alcoholic function normally appears broad (Nahrstedt et al., 1982). The optical rotation value of +89° and a strong positive CD effect of $\Delta\varepsilon$ +12.62 at 246 nm pointed to a 5*R*,6*R* configuration and thus the structure of epiacalyphin amide for compound (**6**). However, the expected rearrangement to the more stable *trans*-configured acalyphin amide (**5**) could not be observed. The positive ESI-MS showed signals at *m*/*z* 383 $[M+Na]^+$ and 743 $[2M+Na]^+$, corresponding to a relative molecular mass of 360, which was 18 less than the structure of (**5**). After crystallization of compound (**6**), its structure was solved by an X-ray study. Both oxygen-containing six-membered rings in (**6**) have approximate chair conformations, with endocyclic torsion angle magnitudes in the range 52.2(7)–63.5(7)° for the dioxane ring and 46.8(8)–65.8(7)° for the tetrahydropyran ring. As in (**1**), the pyridone ring approximates the sofa conformation, with the C atom at the ring junction adjacent to N lying 0.557(6) Å out of the best plane of the other five atoms. The

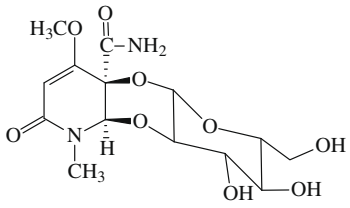

Fig. 3. Absolute configuration of epiacalyphin amide cycloside (**6**).

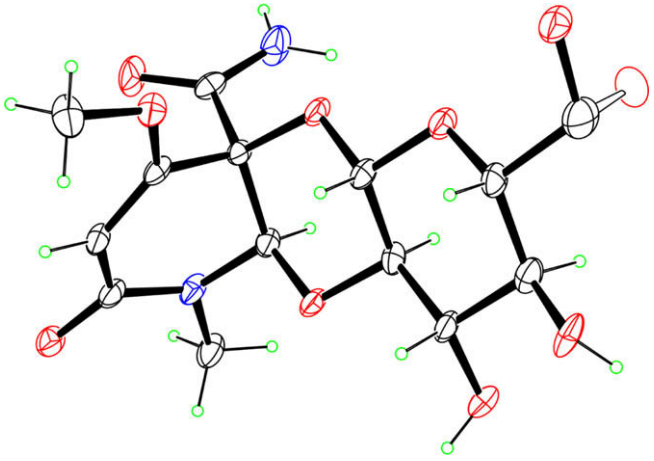

Fig. 4. ORTEP-illustration of epiacalyphin amide cycloside (**6**) showing both disordered conformations of the 6-CH_2OH of the glucosyl.

sofa is slightly distorted, however, and the five atoms defining the plane deviate from it by an average of 0.067 Å. As seen in Fig. 4, the hydroxymethyl group is disordered into two conformations, differing by the sign of the rotation with respect to the tetrahydropyran ring. The O(ring)–C–C–OH torsion angle is +59(1)° for the OH group drawn with solid lines and −55(1)° for that drawn in outline. OH hydrogen atoms were not located. The OH group of the *n*-butanol solvent molecule is similarly disordered. The ORTEP-illustration of (**6**) shows a 1′,2′-glucosyl-fused pyridone with a 5*R*,6*R* configuration (Fig. 4). Compound (**6**) thus is (+)-(5*R*,6*R*)-5-carbamoyl-6,5-(2′,1′-*O*-β-D-glucopyranosyloxy)-4-methoxy-1-methyl-2(5,6-dihydro)pyridone, epiacalyphin amide *cycloside* (Fig. 3). Examples for similar structures with a glucosyl-fused cycloside skeleton showing a 1,4-dioxane structure are cardiac glycosides of the Asclepiadaceae (Roeske et al., 1976) or pilosidine, a norlignan glucoside (Palazzino et al., 2000).

Compounds (**7**) and (**8**) were obtained in small amounts during the extraction and isolation of acalyphin (**1**). The M_r of (**7**) was 317 (pos. ESI-MS: $[M+Na]^+ = 340.2$, $[2M+Na]^+ = 657.1$; neg. ESI-MS: $[M-H]^- = 316.2$, $[2M-H]^- = 633.4$). Cyanogenesis could not be detected (Feigl/Anger test (Tantisewie et al., 1969), sandwich method (Brimer et al., 1983)). A survey of the ^{1}H NMR spectrum in DMSO-d_6 showed resonances of a β-D-glucose, an *N*-Me (δ 3.29 ppm), an *O*-Me group (δ 3.73 ppm) and two singlets at δ 5.81 and δ 7.41 ppm, the latter being shifted to the characteristic downfield aromatic area. The ^{13}C NMR showed 13 carbons but no nitrile group at ca. δ 115 ppm (Hübel et al., 1981); two new resonances in the aromatic region at δ 126.4 and 132.0 ppm appeared. 2D-NMR experiments allowed assignment of all signals. In correspondence with the molecular mass of 317 the NMR data pointed to the elimination of HOCN (cyanic acid) from structure (**1**) and introduction of a double bond from C-5 to C-6. Thus (**7**) represents an aromatized, energy-low system named *ar*-acalyphidone (**7**, 5-β-D-glucopyranosyloxy-4-methoxy-1-methyl-2-pyridone (Fig. 5). Compound (**7**) showed little levorotation of −26° and a weak CD effect of Δε −0.73 at 236 nm.

Compound (**8**) was detected in the sandwich test by liberation of HCN at a main zone at R_f 0.40. The M_r of (**8**) was shown to be 332 (pos. ESI-MS: $[M+H]^+ = 333.1$, $[M+Na]^+ = 355.2$, $[2M+Na]^+ = 687.5$; neg. ESI-MS: $[M+Cl]^- = 367.2$, $[2M+COO]^- = 377.3$). An ESI-MS/MS experiment of signal $[M+Na]^+ = 355.2$ *m*/*z* (positive mode) indicated the typical daughter fragments of hexoses ($[M\text{-glucose}+Na]^+ = 193.1$; $[\text{glucose-}H_2O+Na]^+ = 185$). The 13 resonances of the ^{13}C NMR spectrum in DMSO-d_6 could be assigned to glucose, a CN group (δ 114.7 ppm), an *N*-Me (δ 25.9 ppm), an *O*-Me (δ 56.7 ppm), a carbonyl (δ 166.9 ppm), a >C-OR (δ 161.7 ppm) and a cyanohydrin (>**C**-CNOR) group (δ 112.9 ppm). Carbon C-6 of the original pyridone ring was missing, whereas a weak resonance of C-3 was observed upfield at δ 33.2 ppm. The ^{1}H NMR in MeOH-d_4 confirmed the presence of a glucose and the singlets of the *N*-Me and *O*-Me group. In DMSO-d_6 doublets of an *N*H– at δ 7.9 ppm and of the *N*-Me group at δ 2.6 ppm were observed, both with coupling constants of 4.3 Hz. These data ascertained compound (**8**) to be an open-chain structure. 2D-NMR experiments allowed assignment of all signals and confirmed the open-chain

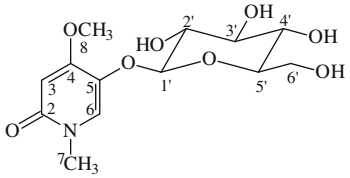

Fig. 5. Structure of *ar*-acalyphidone (**7**).

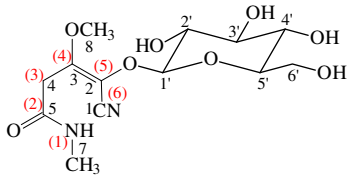

Fig. 6. Structure of *seco*-acalyphin (**8**), red numeration used for NMR data.

structure. However, the NMR data were in agreement with two possible structures differing in the position of the double bond either from C-3 to C-4 or C-4 to C-5. Unambiguous structure elucidation was achieved with a phase sensitive, corrected HSCQ experiment showing C-3 is a methylene group. Proton NOE difference spectra of (**8**) indicated the stereochemically preferred *Z*-configuration: Irradiation on the *N*H caused a weak enhancement of H-3 and the *N*-Me group, but not of the anomeric proton H-1′. Thus compound (**8**) is a butyro nitrile, (*Z*-2-β-D-glucopyranosyloxy-3-methoxy-4-(*N*-methylcarbamoyl-)but-2-en nitrile, Fig. 6) named *seco*-acalyphin. Structurally similar cyanogenic glucosides with an α,β unsaturated nitrile function are acacipetalin, an artifact of isomerization formed from proacacipetalin (Ettlinger et al., 1977) and the naturally occurring triglochinin (Lechtenberg and Nahrstedt, 1999).

The question arises which of the compounds (**1**)–(**8**) are genuine, which may be artifacts. It is obvious that acalyphin **(1)** as the main cyanogenic constituent in fresh and lyophilized leaves is genuine. Epiacalyphin (**2**), though at low concentrations of around 0.02–0.03%, is always present in freshly lyophilized leaves; the ratio of (**1**): (**2**) decreases from ca. 200:1 in the apical young expanding leaves to 50:1 in the old basal leaves. It seems that hydroxylation at carbon 6 of the cyanopyridone nucleus is not stereospecific and that the glucosyltransferase which transfers the glucose moiety to the 5C-OH of the 5,6-dihydroxy-cyanopyridone nucleus, is more specific for its 5,6-*trans*- than for the 5,6-*cis*-OH form. If this assumption is correct, (**2**) is also genuine. From this view, also the nor-compounds (**3**) and (**4**) should be genuine because *N*-demethylation and *N*-methylation reactions are well known in cyanopyridone metabolism (Waller and Nowacki, 1978). However, for both epimeric pairs we cannot role out that each pair isomerizes at least to a certain extend *via* an open-chain-aldehyde (see also above) with (**1**) and (**3**) as more stable mother compounds.

It was shown recently that amides corresponding to cyanogenic glycosides are formed under the influence of reactive oxygen species during the drying and/or senescence process of the plant material (Sendker and Nahrstedt, in press). Thus (**5**) and (**6**) detectable only in air-dried material and with the carboxyamide group instead of the nitrile function, should be artifacts produced during pre/post-senescence reactions. Also, (**7**) and (**8**) may be artifacts, although an enzymatically catalysed formation cannot be excluded at the present state of investigations.

The occurrence of the previously reported alkaloids acalyphamide, aurantiamide, succinimide (Talapatra et al., 1981) and flindersin (Taufiq-Yap et al., 2000) in *A. indica* could not be confirmed in the examined plant material although isolation procedures were carried out according to the published data.

The present data underline the important cyanopyridone metabolism in genera of the Acalyphoideae. Up to now, cyanogenic cyanopyridones have been found only in *A. indica* whereas other species such as *A. hispida* Burm. and *A. wilkesiana* Muell. Arg. were not cyanogenic in the Feigl/Anger and sandwich-picrate test (Tantisewie et al., 1969; Brimer et al., 1983). However, the entire genus *Acalypha* consists of circa 430 species, most of which have yet not been examined for cyanogenesis or cyanopyridones.

3. Experimental

3.1. General

Acalyphin as a reference originated from the collection of A.N. ^1H and ^{13}C spectra were recorded on a Varian Mercury plus spectrometer at 400 and 100 MHz, respectively. Chemical shifts are given in ppm relative to the NMR solvents (MeOH-d_4: δ scale, 3.30 and 49.00 ppm; DMSO-d_6: δ scale, 2.49 and 39.50 ppm), coupling constants in Hz. ESI mass spectra were run on a Waters-Micromass Quattro LC Z, UV-spectra on a Shimadzu UV-Visible Recording Spectrophotometer UV-260, optical rotation values on a Perkin–Elmer 341 polarimeter ($[\alpha]_D^{20} = \alpha/c \times l$) in [deg × ml/g × dm], conc. of measured solution in g/100 ml). CD spectra were recorded on a Jasco J-600 polarimeter and molar CD values ($\Delta\varepsilon = \Theta/32980 \times c \times l$ in [mdeg × l/mol × cm], conc. of measured solution in g/100 ml).

3.2. Measurement of hydrogen cyanide

Plant tissues, extracts and purified compounds were tested for cyanogenesis by means of the Feigl/Anger test, using an unspecific enzyme preparation with β-glucosidase, β-glucuronidase and esterase activity (Röhm enzyme El No. 1-77, Röhm GmbH Darmstadt) for hydrolysis (Tantisewie et al., 1969; Brinker and Seigler, 1989). Extracts and fractions were monitored for cyanogenic compounds by TLC (see Section 3.3) using the cyanide-specific sandwich-picrate method (Brimer et al., 1983) and Röhm enzyme as spray reagent. Sheets of Polygram® Ionex-25 SB-Ac, 0.25 mm (Machery Nagel, Düren) were employed for detection.

For quantitative determination of HCN according to the Aldridge method (Aldridge, 1944), Conway chambers and the Röhm enzyme were applied; HCN was determined photometrically using the ABS method (Nahrstedt, 1977).

3.3. Chromatographical methods

3.3.1. TLC

Silica 60 F_{254} 0.25 mm (Merck 5554) plates were employed using EtOAc–Me_2O–CH_2Cl_2–MeOH–H_2O (20:15:6:5:4, v/v) as a solvent system. The bands were located under UV_{254} and visualized with anisaldehyde-H_2SO_4 spray reagent. R_f-values were for (**5**): 0.20, (**3**)/(**4**): 0.32, (**6**): 0.28, (**1**)/(**2**): 0.39, (**7**): 0.14, (**8**): 0.40 (0.49).

3.3.2. GC

The TMS-derivatized sample was analyzed on an OV-225 column (30 m × 0.25 mm × 0.25 μm) on an HP 6890 Gas Chromatograph by programming from 160°, 1 min isothermal, to 260 °C at 5 °C per min; 29 min isothermal. He (1.0 ml min^{-1} at room temperature) total flow 7.4 ml min^{-1}; injector 200 °C; split ratio 1:4; split flow 4.0 ml min^{-1}; injection volume 1 μl; FID/PND 300 °C. R_t values in min were TMSi-(**3**): 21.9, TMSi-(**4**): 25.1, TMSi-(**1**): 26.1, TMSi-(**8**): 26.9, TMSi-(**2**): 28.0, TMSi-(**5**): 29.5 min, TMSi-(**7**): 34.2, TMSi-(**6**): 35.4 min.

TMSi cyanopyridone derivatives of (**3**)–(**6**) were unstable and exhibited several peaks beside the main signal during GC analysis; thus a GC quantification was impossible.

3.3.3. Derivatization

For persilylation the sample (ca. 1 mg) was dissolved in dry pyridine (25 μl) and BSTFA (60 μl) and TMCS (15 μl) were added; the solution was used for GC after 1 h at room temperature (1–5 μl).

3.3.4. MLCCC

Multi-layer coil countercurrent chromatography was performed using an Ito Multi-layer coil separator-extractor (P.C. Inc., Potomac, Maryland, USA) with a total column volume of 375 ml (I.D. 1.6 mm, 800 rpm, flow 1.0 ml min^{-1}), a Knauer HPLC 64 pump and a 10 ml Rheodyne 502 injector.

3.3.5. MPLC

Samples were chromatographed on Besta Europrep C-18, 100 Å, 18–32 μm, using an open 36 × 500 mm column, hooked up to a 14 × 80 mm precolumn, a Besta E 100 pump (Besta Technik GmbH) and a 10 ml Rheodyne 5020 injector. Mobile phase consisted of a water (Aqua Millipore®)-methanol gradient, produced by two vessels connected with a teflon tube.

3.3.6. Prep. HPLC

Final chromatography was performed with a system consisting of two Waters 515 HPLC pumps connected with a Waters Pump Control Module, Uni-*f*-lows Degasys DG-2410 degaser, 1 ml Rheodyne 7125 injector, Waters 2487 Dual λ Absorbance detector, Varian Polaris®-C-8-A 250 × 21.2 I.D. (5 μm) column, hooked up to a Varian Polaris®-C-8-A 50 × 21.2 I.D. (5 μm) guard column and Waters Millenium^{32} software. Detection was at 230 nm; injection volume was 1 ml.

3.3.7. Analytical HPLC

The HPLC system consisted of a Waters Multisolvent Delivery System 600, 20 μl Rheodyne7125 injector, Waters 996 Photodiode Array detector and Waters Millenium^{32} software. Analytical HPLC was used for characterizing extracts and fractions and carried out on a reverse phase column (Varian, Polaris® C-8-A, 5 μm, 250 × 4.6 mm) using methanol (A) and water (B) in a linear gradient (0.5 ml min^{-1}, 0 min – 100% B, 40 min – 100% A). Detection was at 230 nm. R_t values in min were (**5**) 6.8, (**3**) 9.1, (**6**) 9.9, (**4**) 11.1, (**1**) 12.8, (**7**) 22.2 min, (**8**) 26.1, (**2**) 29.1 min.

3.4. Plant material

The plant material was grown from seeds originally obtained from India. It was cultivated in the greenhouse of the Institute of Pharmaceutical Biology and Phytochemistry in Muenster, where a voucher specimen (PBMS 188) has been deposited.

3.5. Extraction of plant material and isolation

Fresh plant material (inflorescences and leaves, 850 g) was ground in liquid N_2, carefully freeze-dried and the powder (163 g) exhaustively extracted under ice-cooling with MeOH using an Ultra Turrax. The extract was evaporated to dryness, suspended in H_2O and partitioned with CH_2Cl_2 to remove chlorophyll. The lyophilized aq. phase (24.05 g) was then subjected to MLCCC with *n*-BuOH/*n*-PropOH/H_2O (2:1:3), using the upper layer as mobile phase. Fractionation was monitored by TLC, HPLC and/or the presence of *N*-positive compounds checked by GC analysis with an *N*-selective detector. *N*-Positive fractions V (460–654 ml, 2.35 g), VI (655–729 ml, 2.10 g) and VII (730–768 ml, 1.39 g) of the resulting 10 fractions were further purified by MPLC (Europrep C-18).

Using a water–methanol gradient (8 ml min^{-1}, 10% MeOH (1.0 l) → 20% (1.0 l), then 50% MeOH (1.0 l) → 100% MeOH (1.0 l) MeOH), MPLC-purification of conc. fraction V yielded (**1**) as the major compound (V_b: 551–810 ml, 2.36 g), which after a final MPLC (same system) yielded 1.34 g acalyphin (**1**). After evaporation of the MPLC fractions, compounds (**7**)–(**8**) (V_c: 811–950 ml, 20.4 mg) and (**2**) (V_d: 951–1410 ml, 63.6 mg) were finally purified by prep. HPLC on RP-8-Polaris® material, using methanol (A) and water (B) in a linear gradient (5 ml min^{-1}, 0 min – 100% B, 20 min–100% B → 30 min 20% A, 40 min – 100% A; (**7**): 37.2 min, 6 mg; (**8**): 41.9 min, 9 mg; (**2**): 43.3 min, 36.3 mg).

Purification of fractions VI and VII was also carried out by MPLC (7 ml min^{-1}, 3% MeOH (1.0 l) → 10% (1.0 l), then 50% MeOH (1.0 l) → 100% MeOH (1.0 l) MeOH). Fraction VI exhibited concentration of three cyanopyridones (**3**), (**6**) (VI_b: 606–745 ml, 95 mg) and (**4**) (VI_c: 746–860 ml, 60 mg), which were then chromatographed by prep. HPLC on RP-8-Polaris® material (5.0 ml min^{-1}, 100% Aqua Millipore®, isocratic; (**3**): 24.5 min, 20 mg; (**6**): 25.1 min, 7 mg; (**4**): 27.3 min, 2.0 mg). After MPLC (VII_b: 516–601 ml, 32 mg) and prep. HPLC (20.2 min, 13 mg), conc. fraction VII yielded (**5**). Purity of all compounds was shown by TLC-, GC- and HPLC-examination. (The Polaris® material allowed an elution with 100% water and excellently separated the polar fractions.)

3.6. Capillary zone electrophoresis

D-configuration of glucose as the sugar of (**1**)–(**8**) was confirmed by capillary zone electrophoresis after derivatization (Noe and Freissmuth, 1995) using D- and L-glucose as standards on a P/ACE 5010 (Beckman Instruments) with a 70/77 cm × 50 μm capillary and 50 mM $Na_2B_4O_7$ with 4.4 M acetonitrile (pH 10.3) as buffer; migration times of D- and L-glucose were 16.5 and 16.3 min, respectively. Configuration of the glycosidic bond was determined by ^{1}H NMR.

3.7. Crystallization of compounds (*1*) and (*6*)

Crystals of (**1**) were obtained by suspending 35 mg acalyphin in some water, then adding *n*-BuOH. After a few weeks of storage at 5 °C small particles were visible; microscopic examination exhibited hexagonally shaped crystals. In case of (**6**), 7 mg epiacalyphin amide cycloside were dissolved in few MeOH, then a surplus of $CHCl_3$ and some drops of BuOH were added. After some weeks of storage at 5 °C very small particles were detected whose microscopic examination showed small hexagonal crystals.

3.8. Xray experimental

The structures of the 5/6 hydrate of acalyphin (**1**) and of the *n*-butanol solvate of epiacalyphin amide cycloside (**6**) were determined, using data collected at low temperature with Mo Kα radiation on a Nonius KappaCCD diffractometer. Crystal data for (**1**): $C_{14}H_{20}N_2O_9 \cdot 5/6H_2O$, monoclinic space group C2, $a = 14.880(2)$, $b = 8.995(2)$, $c = 38.897(6)$ Å, $\beta = 90.506(5)°$, $V = 5206.0(16)$ Å^3, $Z = 12$, $T = 100$ K, $R = 0.033$ for 5628 observed data of 6315 unique data having $2\theta < 55.0°$; crystal data for (**6**): $C_{14}H_{20}N_2O_9 \cdot C_4H_9OH$, monoclinic space group C2, $a = 19.419(5)$, $b = 8.234(2)$, $c = 13.093(4)$ Å, $\beta = 102.10(2)°$, $V = 2047.0(10)$ Å^3, $Z = 4$, $T = 105$ K, $R = 0.075$ for 1285 observed data of 1951 unique data having $2\theta < 50.2°$. The X-ray crystallographic data can be found in supplementary publications CCDC-697194 and CCDC-697195, available from the Cambridge Crystallographic Data Centre.

3.9. Quantitative determination of nitrile glucosides

GC method: Lyophilized plant tissue or single leaves were exhaustively extracted using an Ultra Turrax with ice-cooled MeOH, filtered and evaporated to dryness. After evaporation, 25 μl of pyridine, containing the internal standard prunasin (2.0 mg/ml), 60 μl BSTFA and 15 μl TMCS (see Section 3.3 GC) were added. The vial was closed, kept at room temperature for 1 h and 1 μl injected for GC analysis. Linearity was established for the tissue quantification in the range of 25–100 μg acalyphin/100 μl (c_{aca}/c_{ISTD} = 0.5–2.0; $r^2 = 0.998$). For the estimation of single leaves a calibration curve for larger amounts of acalyphin was established in the range of 200–500 μg/100 μl (c_{aca}/c_{ISTD} = 4.0–10.0; $r^2 = 0.996$), for minor quantities of epiacalyphin in the range of 1–5 μg/100 μl (c_{aca}/c_{ISTD} = 0.02–0.1; $r^2 = 0.999$). Results were calculated with the help of response factors, using acalyphin as reference.

HPLC method: Quantitative determination of compounds (**1**)–(**4**) was performed under the conditions described above for analytical HPLC (see Section 3.3) using a reverse phase column (Merck, LiChrospher® 100, Rp-18, 5 μm, 250 × 4 mm plus guard column – LiChrospher® 100, Rp-18, 5 μm, 4 × 4 mm) and methanol (A) and water (B) in a linear gradient (0.5 ml min^{-1}, 0 min – 3% A, 10 min – 3% A, 40 min – 65% A, 45 min – 100% A). Detection was at 220 nm. Acalyphin (**1**) obtained in this work was taken for calibration, prunasin (Sigma–Aldrich) as an internal standard.

3.10. Acalyphin (*1*)

1.34 g, white powder, $[\alpha]_D^{20}$ −140° (MeOH; c 1.0); $\Delta\varepsilon_{249}$ −7.35 (H_2O; c 0.002); λ_{max}^{MeOH}: 223 (4.1); ^{1}H NMR (400 MHz, MeOH-d_4): see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4): see Table 1; ESI-MS (MeOH), pos. mod., *m*/*z* (rel. int.): 360.3 $[M]^+$ (100), 383.2 $[M+Na]^+$ (45), 743.0 $[2M+Na]^+$ (46); neg. mod., *m*/*z* (rel. int.): 359.2 $[M-H]^-$ (52), 719.2 $[2M-H]^-$ (43).

3.11. Epiacalyphin (*2*)

36 mg, white powder, $[\alpha]_D^{20}$ +31° (MeOH; c 1.0); $\Delta\varepsilon_{246}$ +6.65 (H_2O; c 0.002); λ_{max}^{MeOH}: 223 (4.1); ^{1}H NMR (400 MHz, MeOH-d_4): see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4): see Table 1; ESI-MS (MeOH), pos. mod., capillary 1.15 kV, cone 31 V, extractor 2 V, *m*/*z* (rel. int.): 361.2 $[M+H]^+$ (15), 383.1 $[M+Na]^+$ (100).

3.12. Noracalyphin (*3*)

20 mg, white powder, $[\alpha]_D^{20}$ −138° (MeOH; c 1.0); $\Delta\varepsilon_{247}$ −15.91 (MeOH; c 0.004); λ_{max}^{MeOH}: 221 (4.0); ^{1}H NMR (400 MHz, MeOH-d_4): see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4): see Table 1; ESI-MS (MeOH), pos. mod., *m*/*z* (rel. int.): 369 $[M+Na]^+$ (100), 715 $[2M+Na]^+$ (42).

3.13. Epinoracalyphin (*4*)

2 mg, white powder; ^{1}H NMR (400 MHz, MeOH-d_4): see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4): see Table 1; ESI-MS (MeOH), pos. mod., *m*/*z* (rel. int.): 399 $[M+OCH_3-H+Na]^+$ (100), 369 $[M+Na]^+$ (35).

3.14. Acalyphin amide (*5*)

13 mg, white powder, $[\alpha]_D^{20}$ −43° (MeOH; c 0.3); $\Delta\varepsilon_{244}$ −3.97 (MeOH; c 0.004); λ_{max}^{MeOH}: 219 (3.7); ^{1}H NMR (400 MHz, MeOH-d_4): see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4): see Table 1; ESI-MS (MeOH), *m*/*z* (rel. int.): 401 $[M+Na]^+$ (100), 779 $[2M+Na]^+$ (37).

3.15. Epiacalyphin amide cycloside (*6*)

7 mg, white powder, $[\alpha]_D^{20}$ +89° (MeOH; c 0.1); $\Delta\varepsilon_{246}$ −12.62 (MeOH; c 0.006); λ_{max}^{MeOH}: 219 (3.7); ^{1}H NMR (400 MHz, MeOH-d_4): see Table 1; ^{13}C NMR (100 MHz, MeOH-d_4): see Table 1; ESI-MS (MeOH), capillary 1.05 kV, cone 9 V, extractor 1 V, *m*/*z* (rel. int.): 360.4 $[M]^+$ (82), 383.2 $[M+Na]^+$ (100).

3.16. ar-Acalyphidone (*7*)

6 mg, white powder, $[\alpha]_D^{20}$ −26° (H_2O; c 0.3); $\Delta\varepsilon_{236}$ −0.73 (MeOH; c 0.02); λ_{max}^{MeOH}: 209 (4.5), 289 (3.6); ^{1}H NMR (400 MHz, DMSO-d_6): see Table 1; ^{13}C NMR (100 MHz, DMSO-d_6): see Table 1; ESI-MS (MeOH), pos. mod., *m*/*z* (rel. int.): 340.2 $[M+Na]^+$ (35), 657.1 $[2M+Na]^+$ (100), neg. mode: 316.2 $[M-H]^-$ (14), 633.4 $[2M-H]^-$ (100).

3.17. seco-Acalyphin (**8**)

9 mg, yellowish powder, $[\alpha]_D^{20}$ −51° (MeOH; c 0.2); λ_{max}^{MeOH}: 240 (4.0); ^{1}H NMR (400 MHz, DMSO-d_6): see Table 1; ^{13}C NMR (100 MHz, DMSO-d_6): see Table 1; ESI-MS (MeOH), pos. mod., capillary 1.05 kV, cone 9 V, extractor 1 V, *m*/*z* (rel. int.): 355.2 $[M+Na]^+$ (52), 356.2 $[MD_1+Na]^+$ (70), 357.2 $[MD_2+Na]^+$ (100), neg. mode: 367.2 $[M+Cl]^-$ (22), 368.2 $[MD_1+Cl]^-$ (32), 369.2 $[MD_2+Cl]^-$ (45).

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