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Antitumoral and antileishmanial dioncoquinones and ancistroquinones from cell cultures of *Triphyophyllum peltatum* (Dioncophyllaceae) and *Ancistrocladus abbreviatus* (Ancistrocladaceae)

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Ancistroquinone C
Ancistroquinone D
Ancistroquinone E
Ancistroquinone F

ABSTRACT

From the methanolic extracts of solid callus cultures from two species of the closely related palaeotropical plant families Dioncophyllaceae and Ancistrocladaceae seven new natural naphthoquinones were isolated, dioncoquinones A (**4**) and B (**5**) from *Triphyophyllum peltatum*, and ancistroquinones B (**6**), C (**7**), D (**9**), E (**10**), and F (**12**) from *Ancistrocladus abbreviatus*. Their structures were elucidated by spectroscopic, chemical, and computational methods. Furthermore, the already known naphthoquinones plumbagin (**2**), droserone (**3**), malvone A (**8**), and nepenthone A (**11**) were found in the extract of *A. abbreviatus*. Dioncoquinones A (**4**) and B (**5**) showed good – and specific – activity against *Leishmania major*, while they were not active against other protozoic parasites. Moreover, treatment with **4** and **5** strongly induced apoptosis in human tumor cells derived from two different B cell malignancies, B cell lymphoma and multiple myeloma, without any significant toxicity towards normal peripheral mononuclear blood cells.

1. Introduction

Tropical lianas of the small palaeotropic families Dioncophyllaceae and Ancistrocladaceae, among them *Triphyophyllum peltatum* (Hutch. & Dalz.) Airy Shaw and *Ancistrocladus abbreviatus* Airy Shaw from West Africa, are the only plant species known to produce naphthylisoquinoline alkaloids (Bringmann and Pokorny, 1995; Bringmann et al., 1998a), like, e.g., dioncophylline A (**1a**, see Fig. 1) (Bringmann et al., 1990a,b). These remarkable natural products are characterized by their unprecedented structures, usually with rotationally hindered and thus stereogenic biaryl axes, by their promising antiprotozoal bioactivities (e.g., against *Plasmodium*, *Leishmania*, and *Trypanosoma* species) (Bringmann et al., 2006, 2003a; François et al., 1997), and by the unprecedented biosynthetic origin of both, the isoquinoline and the naphthalene portions, from each six acetate units (Bringmann et al., 2000a). This unique biosynthesis of acetogenic isoquinoline alkaloids is, however, extremely susceptible to all sorts of chemical, physical, or biotic stress, which easily blocks the transamination step in the biosynthesis of the tetrahydroisoquinoline moiety (Bringmann and Feineis, 2001), so that only the corresponding naphthoquinones, plumbagin (**2**) and droserone (**3**), are formed. These are produced as phytoalexins (Bringmann and Feineis, 2001) by many other, related plant families, like, e.g., Nepenthaceae, Droseraceae, Plumbaginaceae, and Drosophyllaceae (Bringmann et al., 1998b), but have also been found to possess antitumoral (Devi et al., 1999; Kapadia et al., 1997; Morello et al., 1995) and antimalarial

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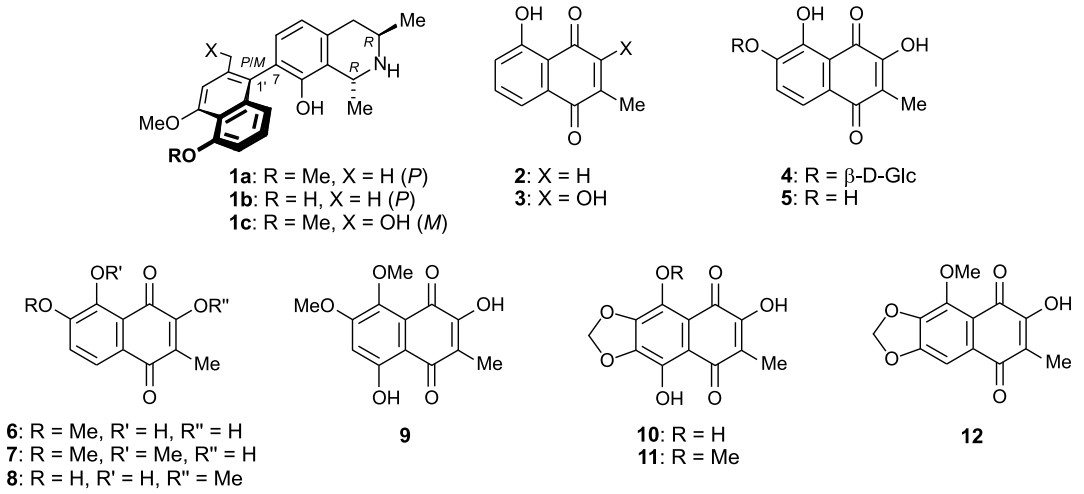


Fig. 1. Natural products from *Triphyophyllum peltatum* and *Ancistrocladus abbreviatus*: selected structures previously reported (**1**–**3**) and the naphthoquinones **4–12** described in this paper.

(Likhitwitayawuid et al., 1998) effects. After early experiments using ^{14}C-labelled precursors (Durand and Zenk, 1971), the acetogenic origin of these secondary metabolites has more recently been established by feeding experiments with ^{13}C-labelled acetate (Bringmann et al., 2000b, 1998b) resulting in the first proof of the so-called F folding mode (Thomas, 2001) of the intermediate polyketide chain (i.e., with two intact acetate derived C_2 units in the first ring) in higher plants. These biosynthetic experiments were possible only by the establishment of cell cultures of these sensitive tropical lianas (Bringmann et al., 2000b, 1999a), thus providing an *in vitro* system that reliably produces both, the alkaloids and the naphthoquinones. And still, the cultivation of these plants as sterile callus cultures imposes stress to the cells, eventually resulting in a largely increased formation of **2** and **3** (Bringmann et al., 2000b, 1999a).

A systematic change of the cultivation procedure now led to improved solidification of the medium, hence exposing the cells to aerobic and thus oxidative conditions, which favored the formation of further, structurally related new naphthoquinones. In this paper, we thus report on the isolation and structural elucidation of nine naphthoquinones, among them the already known natural products malvone A (**8**) (Veshkurova et al., 2006) and nepenthone A (**11**) (Cannon et al., 1980), while compounds **6** and **7** had already been described in the course of the total synthesis of ancistroquinone (Govindachari et al., 1971) and – in the case of **6** – as a degradation product of rubromycins (Brockmann and Zeeck, 1970), but had as yet not been found as natural products; they were henceforth named ancistroquinones B (**6**) and C (**7**). The remaining five new substances were named dioncoquinones A (**4**) and B (**5**) and ancistroquinones D (**9**), E (**10**), and F (**12**) – according to their Dioncophyllaceous and Ancistrocladaceous producers, respectively. They were only found in the cell cultures, but not in freshly prepared extracts of the whole plants, thus evidencing their stress-induced biosynthetic origin.

2. Results and discussion

The cell cultures of *T. peltatum* and *A. abbreviates* were initially established using the MS medium (Murashige and Skoog, 1962), but with a fifth of the concentration of macro elements and with Gelrite as the solidifying agent. Due to the low concentration of divalent cations in this composition, the medium liquefied rapidly, so that the calli sank into the medium. Therefore, the concentration of calcium and magnesium ions (necessary for gelling) was stepwise increased, eventually leading to an improved solidity of the medium. As a consequence, the cell cultures were exposed to more aerobic and thus oxidative conditions, which favored the formation of further new and higher oxygenated naphthoquinones.

After 6–8 weeks of cultivation the calli were removed from the medium, lyophilized, ground, and exhaustively extracted with CH_2Cl_2/MeOH 1:1. The resulting extract was filtered and submitted to preparative HPLC and repeated column chromatography, which, in the case of *T. peltatum*, permitted the isolation of three known naphthylisoquinoline alkaloids – dioncophylline A (**1a**) (Bringmann et al., 1990a, 1990b), 5′-*O*-demethyldioncophylline A (**1b**) (Bringmann et al., 1998c), and habropetaline A (**1c**) (Bringmann et al., 2003b) – and three substances with UV spectra of naphthoquinones.

The most polar of these three substances from *T. peltatum* was found to possess a molecular formula of $C_{17}H_{18}O_{10}$ as revealed from the number of signals in the ^{13}C NMR spectrum and from HRESIMS. In contrast to all other naphthoquinones described herein, the compound was not soluble in $CDCl_3$ and therefore DMSO-d_6 was used as the NMR solvent. Its ^{1}H NMR spectrum exhibited two signals of chelated hydroxy functions (11.41 and 10.86 ppm,

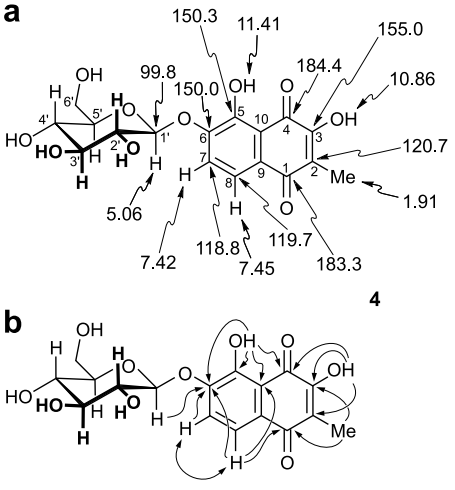
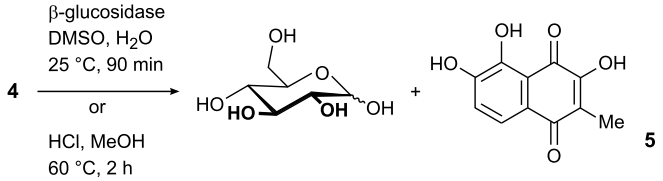


Fig. 2. Selected NMR data (DMSO-d_6) of dioncoquinone A (**4**): ^{1}H and ^{13}C NMR shifts (δ in ppm) (a), and HMBC (single arrows) and ^{1}H,^{1}H-COSY (double arrows) interactions (b) relevant for the structural elucidation.

exchangeable with D_2O), both next to the same quinoid carbonyl (184.4 ppm), as evidenced by HMBC correlations with C-4 (see Fig. 2). The signal at 11.41 ppm was attributed to HO-5 by additional HMBC couplings to C-5, C-6, and C-10. This assignment was in agreement with the high-field shift of the ^{1}H NMR signal of HO-5, as compared to other chelated hydroxy groups of β-unsubstituted naphthoquinones (Brockmann and Zeeck, 1970; Moore and Scheuer, 1966). Although the ^{1}H NMR signal of the second OH group in that region (10.86 ppm) might thus again hint at a similar *peri*-position next to a carbonyl function, this OH group was, by its HMBC interactions with C-2, C-3, and C-4, clearly assigned to be located at C-3. It thus showed an unexpectedly large down-field shift in contrast to other 3-hydroxy-1,4-naphthoquinones (Moore and Scheuer, 1966), which, however, had been measured in $CDCl_3$, where they did not exhibit such an intramolecular hydrogen bonding. The resonance of a methyl group at 1.91 ppm and thus at a δ value smaller than 2.1 ppm (Brockmann and Zeeck, 1970), showed that it should be attached to the quinoid ring (and not to the aromatic one). This and the almost equal chemical shifts of two neighboring aromatic protons (7.42 and 7.45 ppm, ^3J = 8.6 Hz) and the presence of three phenolic, and thus quaternary, carbon atoms (155.0, 150.3, and 150.0 ppm) in ^{13}C NMR hinted at an 8-oxygenated droserone derivative, although this was in disagreement with an HMBC interaction of one aromatic proton with C-1, which rather indicated an oxygen substitution at C-6 (Fig. 2b). That the additional oxygen function (as compared to the substitution pattern of droserone, **3**) was linked to a glucopyranosyl residue, became evident from the characteristic signals in ^{1}H and ^{13}C NMR, from ^{1}H,^{1}H-COSY and HMBC interactions, and from the fragmentation pattern in EIMS. The configuration at the anomeric sugar carbon atom C-1′ (and hence also the attachment to the aglycon) was unambiguously assigned to be β by the large coupling constant from H-1′ to H-2′ ($^3J_{1'/2'}$ = 7.4 Hz), evidencing a bis-axial arrangement of H-1′ and H-2′ in the six-membered ring.

Treatment of the glucoside with methanolic HCl at 60 °C or with a commercial β-glucosidase at room temperature (Scheme 1) led to complete deglucosidation, delivering D-glucose (identical to an authentic sample by ^{1}H NMR, $[\alpha]_D$, m.p., and co-chromatography on TLC) along with a new peak in both, TLC and RP-HPLC. The physical, chromatographical, and spectroscopic data of the obtained aglycon were identical in all respects with those of the second naphthoquinone, compound **5**, from the extract of *T. peltatum* (see below).

Surprisingly the ^{1}H NMR spectrum of the aglycon **5** (see Fig. 3a) in $CDCl_3$ showed the presence of only one OH signal in the region of chelated hydroxy groups (11.22 ppm). The second hydroxy function resonated at 7.04 ppm and its position at C-3 was deduced from HMBC interactions with C-2, C-3, and C-4. The large high-field shift of this signal (7.04 ppm), as compared to that of the glucoside **4** (10.86 ppm), was shown to be due to a solvent effect: By using DMSO-d_6 as the NMR solvent (like in the case of **4**), the signal of HO-3 was shifted to 10.70 ppm, i.e., to a value similar to that of **4** (see above). Additionally, a third, new hydroxy group was observed, obviously again not chelated (6.04 ppm). The signals of the two aromatic protons now appeared largely separated from



Scheme 1. Enzymatic or acid-catalyzed transformation of **4** into **5**.

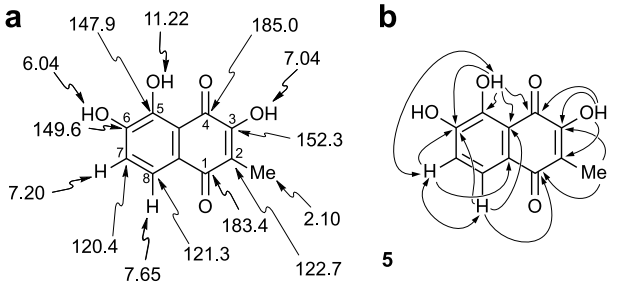


Fig. 3. Selected ^{1}H and ^{13}C NMR shifts (δ values in ppm) (a), and HMBC (single arrows) and ^{1}H,^{1}H-COSY (double arrows) interactions (b) of dioncoquinone B (**5**).

each other (7.65 and 7.20 ppm). The down-field shift of the first of these signals (7.65 ppm) indicated the presence of a proton in a *peri*-position (Moore and Scheuer, 1966). This finding was further corroborated by an HMBC coupling of this proton to C-1. Further HMBC correlations – HO-3/C-2, H_3C-2/C-1, H-8/C-1, H-8/C-6, HO-5/C-6, HO-5/C-4, HO-3/C-4 – evidenced a 3,5,6-trihydroxy substitution pattern.

Still, although all data were thus in agreement with structure **5**, the less probable possibility of a likewise imaginable 8-substituted analog (structure not shown, Budzianowski, 2000) could not be ruled out entirely. Quantum chemical ^{1}H and ^{13}C NMR simulations, however, clearly evidenced structure **5** and excluded the alternative structure.

A further confirmation of the structure of **5** was obtained from its selective conversion into the dimethylether 7 by using an excess of CH_2N_2 in Et_2O (see Section 3). NOE correlations in the series {OCH_3-5–OCH_3-6–H-7–H-8} together with the results of the calculations and the HMBC couplings unambiguously confirmed the 3,5,6-trioxygenation pattern, thus eventually assigning **5** to be 3,5,6-trihydroxy-2-methyl-1,4-naphthoquinone and hence **4** to be its 6-*O*-β-glucopyranosyl derivative. Since no naphthoquinones with these constitutions had been described before, compounds **4** and **5** were new natural products and were given the names dioncoquinones A (**4**) and B (**5**), according to their isolation from Dioncophyllaceae plants. Finally, the least polar of the three naphthoquinones from the cell cultures of *T. peltatum* was easily identified as the known droserone (**3**), which had already been isolated from cell cultures of this plant earlier (Bringmann et al., 2000b).

The occurrence of the new naphthoquinones **4** and **5** in cell cultures of *T. peltatum* encouraged us to look for similar compounds in cultures of the related Ancistrocladaceae plant species *A. abbreviatus*. The dried and ground calli were extracted with CH_2Cl_2/MeOH 1:1 and the filtered extract resolved by preparative HPLC and repeated column chromatography, leading to a total of 10 substances. Three of them were easily identified as plumbagin (**2**), droserone (**3**), and dioncoquinone B (**5**) by comparison of their physical and spectroscopic data with those reported in the literature (Bringmann et al., 2000b, 1998b) and by coelution with authentic natural material. Interestingly, dioncoquinone A (**4**) could not be detected in the crude extract, although its aglycon **5** was the predominant peak in the HPLC chromatogram.

The molecular formula of the first, and most polar, compound from *A. abbreviatus* was $C_{12}H_{10}O_5$, as deduced from HRESIMS and the number of ^{13}C NMR signals. In addition to the characteristic quinone carbonyl signals (184.9 and 183.4 ppm), three signals of phenolic, quatenary carbon atoms (153.0, 152.7, and 151.7 ppm) were observed in ^{13}C NMR (see Fig. 4a).

^{1}H NMR again suggested the presence of a 3,5-dihydroxy-2-methyl-1,4-naphthoquinone derivative, as indicated by the signal of a methyl group attached to the quinoid ring (2.01 ppm), two

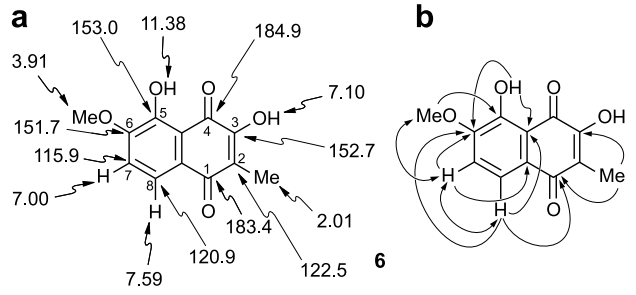


Fig. 4. Ancistroquinone B (**6**): Selected ^{1}H and ^{13}C NMR shifts ($CDCl_3$, δ values in ppm) (a), and HMBC (single arrows) and $^{1}H,^{1}H$-COSY (double arrows) interactions (b).

signals of aromatic protons (7.00 and 7.59 ppm), and the existence of one chelated hydroxy function and one in a β-position (11.38 and 7.10 ppm, respectively). Moreover, the signal of one methoxy group resonated at 3.91 ppm; $^{1}H,^{1}H$-COSY interactions in the series {OCH_3-6–H-7–H-8} established its location at C-6 (see Fig. 4b). From these data and from the analysis of the HMBC couplings the compound was confirmed to be 3,5-dihydroxy-6-methoxy-2-methyl-1,4-naphthoquinone, which had previously been described only as a synthetic side product (Govindachari et al., 1971) and as a degradation product of rubromycins (Brockmann and Zeeck, 1970), but had never before been found in nature. Due to its close structural analogy to ancistroquinone (3,6-dihydroxy-5-methoxy-1,4-naphthoquinone), previously isolated from *A. heyneanus* Wall. (Govindachari et al., 1971), it was henceforth named ancistroquinone B.

Moreover, two further compounds with a close structural relationship to **6** according to ^{1}H and ^{13}C NMR were isolated. In the first of these two substances (i.e., the second from *A. abbreviatus*), the presence of an additional methoxy group as compared to **6** was indicated by the occurrence of two three-proton singlets (3.87 and 3.89 ppm) in ^{1}H NMR, which was corroborated by two corresponding signals in ^{13}C NMR (56.2 and 61.2 ppm, respectively) and by its molecular formula $C_{13}H_{12}O_5$, as deduced from HRESIMS. The interpretation of all $^{1}H,^{1}H$-COSY and HMBC correlations permitted unequivocal attribution of this compound to be 3-hydroxy-5,6-dimethoxy-2-methyl-1,4-naphthoquinone, i.e., structure **7**.

The physical and spectroscopic data of **7** were identical to those obtained in the course of the derivatization of **5** and also to those reported in the literature (Govindachari et al., 1971). Again, this substance had as yet been described only as a synthetic intermediate (Govindachari et al., 1971); it was henceforth named ancistroquinone C.

The other compound related to **6**, i.e., the third naphthoquinone isolated from *A. abbreviatus*, possessed the same molecular formula $C_{12}H_{10}O_5$ according to HRESIMS. The analysis of its ^{1}H and ^{13}C NMR data easily identified it as a regioisomer of **6**, differing only by the position of the *O*-methyl group. 2D NMR experiments (HMQC, HMBC, and NOESY) led to the elucidation of the constitution to be 5,6-dihydroxy-3-methoxy-2-methyl-1,4-naphthoquinone. This compound, called malvone A (**8**), is a known phytoalexin, previously isolated from *Malva sylvestris*, its physical and spectroscopic data were identical to those reported earlier (Veshkurova et al., 2006).

The fourth naphthoquinone from *A. abbreviatus*, which was eluted in the less polar HPLC region, gave an empirical formula of $C_{13}H_{12}O_6$, from the number of signals in ^{13}C NMR and from HRESIMS. This implied the presence of a fourfold oxygenated 1,4-naphthoquinone, which was confirmed by the observation of four signals of quatenary carbon atoms at 160.9, 160.0, 153.7, and 145.9 ppm in ^{13}C NMR (see Fig. 5a). That two of these oxygen functions were methylated was revealed by 2 three-proton signals in ^{1}H NMR (3.94 and 3.89 ppm), while the signals of the free hydroxy groups resonated at 13.44 ppm (down-field shifted and hence in the 8-position) (Moore and Scheuer, 1966) and 7.64 ppm. The only aromatic proton gave rise to a singlet at 6.70 ppm, thus it had to be located in one of the two non-*peri* positions, i.e., at C-6 or C-7. NOESY interactions of this proton with HO-8 and with OCH_3-6 (but not with the second OCH_3-group, as expected for H-6) in conjunction with a weak long-range HMBC coupling to C-1 (see Fig. 5b) established its position to be at C-7. NOESY and HMBC correlations thus clearly evidenced a 3,8-dihydroxy-5,6-dimethoxy substitution and established the compound to possess the structure **9** shown in Fig. 6. This new natural product was named ancistroquinone D.

The fifth naphthoquinone derivative from *A. abbreviatus* exhibited a strikingly simple ^{1}H NMR spectrum: only four singlets, due to the presence of two chelated hydroxy functions in positions 8 and 5 (12.94 and 11.48 ppm, respectively), one methyl group on the quinone ring (2.07 ppm) and a methylenedioxy group (see

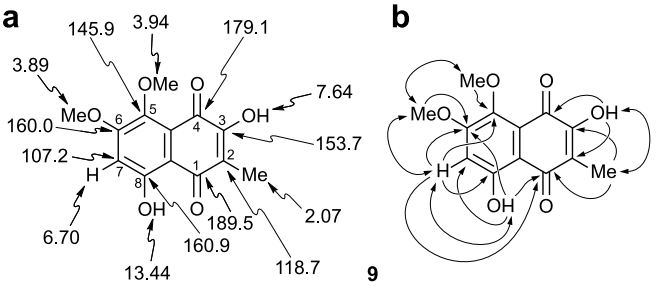


Fig. 5. Selected ^{1}H and ^{13}C NMR data ($CDCl_3$, δ values in ppm) (a), and NOESY (double arrows) and HMBC (single arrows) interactions (b) of ancistroquinone D (**9**).

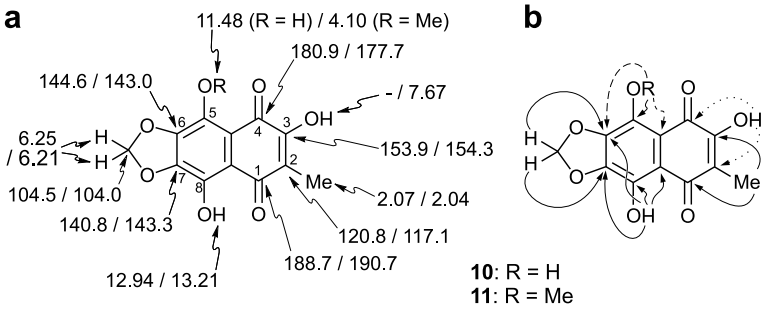


Fig. 6. Selected NMR data of **10**/**11**: ^{1}H and ^{13}C NMR shifts ($CDCl_3$, δ values in ppm) (a), HMBC (dashed arrows only for **10**; dotted arrows only for **11**) interactions (b) relevant for the constitution.

Fig. 6a), as less frequently found in naphthoquinones (Cannon et al., 1980; Thomson, 1997). The high degree of oxygenation (seven of eight possible positions) was demonstrated both, by ^{13}C NMR and by HRESIMS, resulting in the assignment of the molecular formula to be $C_{12}H_8O_7$. The interpretation of all HMBC couplings (see Fig. 6b) revealed that the methylene group bridges the two oxygens at C-6 and C-7, thus leading to a structural assignment of the compound as **10**, i.e., as 3,5,8-trihydroxy-2-methyl-6,7-methylenedioxy-1,4-naphthoquinone. The signal of the hydroxy group at C-3 was not observed, probably due to exchange phenomena. This new substance was named ancistroquinone E; it might also be addressed as the 5-*O*-demethyl analog of the known (Cannon et al., 1980) naphthoquinone nepenthone A (**11**), which likewise occurs in the cell cultures (see below).

A further, sixth naphthoquinone showed a chromatographical behavior similar to that of **10** and was also found to be structurally closely related according to ^1H and ^{13}C NMR. With an empirical formula of $C_{13}H_{10}O_7$ determined by HRESIMS, its ^1H NMR differed from that of **10** essentially only by the occurrence of a characteristic signal of a methoxy group (4.10 ppm), combined with the disappearance of the signal of the chelated hydroxy group at C-5 (see Fig. 6a). HMBC experiments (see Fig. 6b) confirmed this compound to correspond to the structure **11**, i.e., to the known compound nepenthone A and all physical and spectroscopic data agreed well with those reported earlier (Cannon et al., 1980).

The seventh naphthoquinone possessed a molecular formula of $C_{13}H_{10}O_6$ as revealed from the number of signals in the ^{13}C NMR spectrum and from HRESIMS. Like in the cases of **10** and **11**, the ^1H NMR spectrum showed the characteristic two-proton singlet of a methylenedioxy group (6.11 ppm), accompanied by signals of a methyl group on the quinone ring (2.02 ppm), of a methoxy group (4.14 ppm), of an aromatic proton (7.39 ppm), and of a non-chelated hydroxy proton (7.67 ppm, exchangeable with D_2O) (see Fig. 7a). HMBC correlations (see Fig. 7b) – H_3C-2/C-1, H-8/C-1, H-8/C-6, H-8/C-7, and OCH_2O/C-6 and C-7 – clearly showed the aromatic proton to be located at C-8 and the methylenedioxy group to bridge C-6 and C-7. Finally, the non-*peri* character of the free hydroxy group could only be interpreted by an attachment to C-3, although no 2D NMR cross peaks were observed for this proton due to line broadening. Hence the compound was attributed the new structure of 3-hydroxy-5-methoxy-2-methyl-6,7-methylenedioxy-1,4-naphthoquinone (**12**) and was given the name ancistroquinone F.

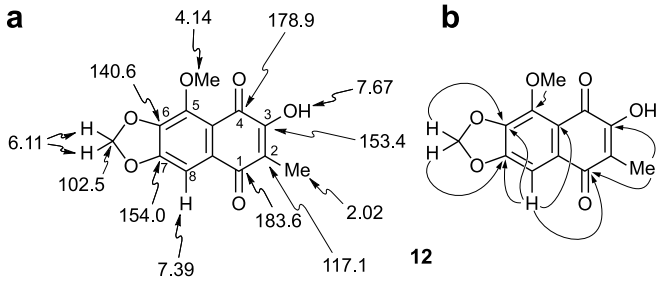


Fig. 7. Selected NMR data of **12**: ^1H and ^{13}C NMR shifts ($CDCl_3$, δ values in ppm) (a) and HMBC interactions (b) determining the constitution.

The known activities of several naphthoquinones against the pathogens belonging to the genera *Plasmodium*, *Leishmania*, and *Trypanosoma* (see, e.g., Likhitwitayawuid et al., 1998; Morello et al., 1995) encouraged us to screen the isolated naphthoquinones against these parasites. All of the compounds **4**-**12** exhibited moderate to good activities (Table 1) against *Leishmania major*, i.e., the pathogen of visceral leishmaniasis, with dioncoquinones A (**4**) and B (**5**) being the most active, whereas only weak or no antiplasmodial or antitrypanosomal activities were observed against the pathogens of malaria tropica, *Plasmodium falciparum*, and of African sleeping sickness, *Trypanosoma brucei rhodesiense*, or against *T. cruzi* (Chagas' disease).

In view of the reported antitumoral properties of some naphthoquinones (see, e.g., Devi et al., 1999; Kapadia et al., 1997; Morello et al., 1995), compounds **4**–**12** were also tested against human tumor cells of two different B cell malignancies, B cell lymphoma and multiple myeloma. B cell lymphoma (also known as B non-Hodgkin lymphoma) is a neoplasm of the immature B cell, whereas multiple myeloma is a neoplasm of the terminally differentiated B cell (plasma cell). Compounds **4** and **5** showed significant antitumoral activities (Fig. 8a and b), while the other compounds tested were virtually inactive. Of note, these effective concentration ranges of the compounds **4** and **5** were similar to those of melphalan, a well-known DNA-alkylating agent routineously used in standard chemotherapeutic regimens for B-cell lymphoma and multiple myeloma (Table 2).

Normal mononuclear cells isolated from the peripheral blood of healthy donors, by contrast, were not affected by compounds **4** and **5** (Fig. 8c) but were driven into apoptosis by melphalan (data not shown). This selective antitumoral activity of the compounds **4** and **5** *in vitro* is promising for their further development as anticancer agents.

The discovery of five new, highly oxygenated naphthoquinones from *T. peltatum* and *A. abbreviatus* demonstrates the remarkable ability of these two small plant families to produce a broad variety of structurally interesting secondary metabolites. From a biosynthetic point of view, the naphthoquinones **4**-**12** are apparently closely related to their likewise acetogenic (Durand and Zenk, 1971) less oxygenated analogs, **2** and **3**, which originate from six acetate units, constituting the first proven example (Bringmann et al., 2000b, 1998b) of a mode F type polyketide folding (Thomas, 2001) in higher plants. Remarkable is their stress-induced

Table 1
Growth inhibition of different protozoic parasites by compounds **4–12** at a concentration of 0.8 μg ml^{-1}

	Growth inhibtion (%)								
	4	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Plasmodium falciparum (strain: K1) Standard: artemisinine[a] 75.2[b]	0.0	1.1[e]	5.5	9.6	0.0	0.0	0.0	2.1	0.0
Trypanosoma cruzi Standard: benznidazole[c] 57.6[b]	2.5	3.8	16.1	0.0	9.6	0.0	0.0	0.0	17.1
T. b. rhodesiense Standard: melarsoprol[a] 40.0[b]	0.0	0.0	0.0	0.0	0.0	2.0	0.0	6.3	0.0
L. donovani Standard: miltefosine[d] 53.0[b]	49.6	79.2	30.4	0.0[f]	15.2	11.4	19.6	12.6	0.3

[a] 0.003 μg/ml.
[b] Growth inhibition (%).
[c] 0.2 μg/ml.
[d] 0.22 μg/ml.
[e] 97.4% at a concentration of 4.8 μg/ml.
[f] 88.4% at a concentration of 4.8 μg/ml.

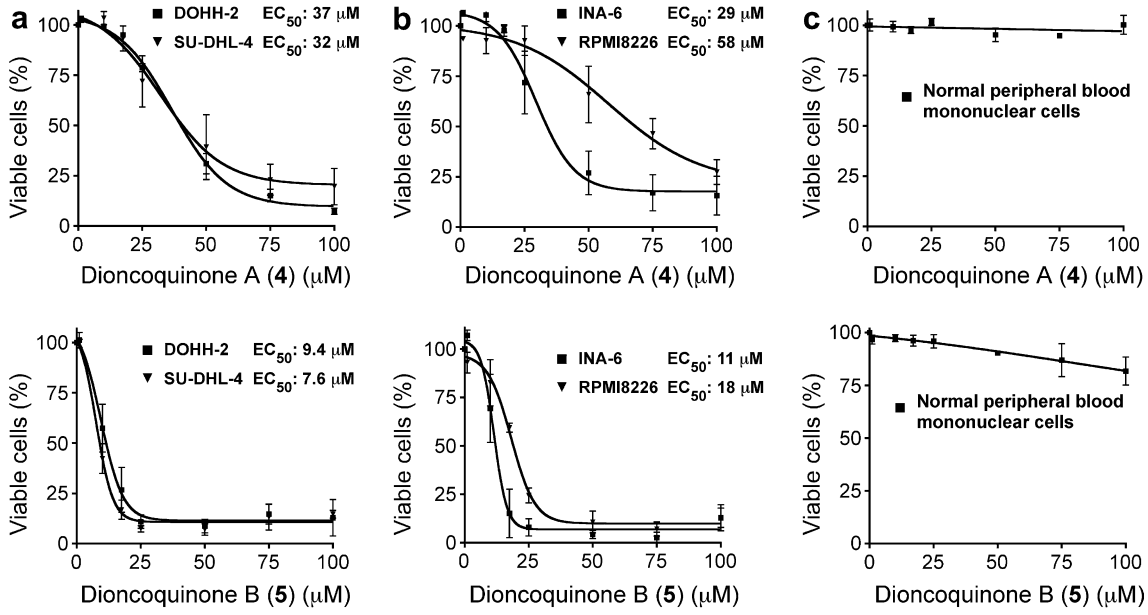


Fig. 8. Antitumoral properties of dioncoquinones A and B (compounds **4** and **5**). Human B cell tumor cells of two different B cell tumor entities, B cell lymphoma (a) and multiple myeloma (b), or normal peripheral mononuclear blood cells (c) were treated with different concentrations either of dioncoquinone A (upper panel) or dioncoquinone B (lower panel) for 3 d. The viable fractions of the treated cells were determined by annexin V-FITC/PI staining. Error bars indicate the range of values derived from three independent experiments.

Table 2
EC_{50} values (μM) of B cell lymphoma and multiple myeloma cell lines and PBMCs treated with compounds **4** and **5** and with melphalan

	4	**5**	Melphalan
B cell lymphoma cell line: DOHH-2	37	9.4	1
B cell lymphoma cell line: SU-DHL-4	32	7.6	4
Multiple myeloma cell line: INA-6	29	11	2
Multiple myeloma cell line RPMI-8226	58	18	2
Peripheral blood mononuclear cells (PMBCs)	Not reached	Not reached	3

formation in – otherwise naphthylisoquinoline producing – Ancistrocladaceae and Dioncophyllaceae plants (Bringmann and Feineis, 2001), but also in Nepenthaceae (Rischer et al., 2002). The first isolation of malvone A (**8**) and nepenthone A (**11**) from an Ancistrocladaceae plant species again emphasizes the close phylogenetic connection to other naphthoquinone-producing families (Malvaceae, Nepenthaceae, Droseraceae, Plumbaginaceae, Drosophyllaceae) (Bringmann et al., 1998b).

3. Experimental

3.1. General

Melting points were obtained on a Stuart Scientific SMP10 instrument and are uncorrected. UV spectra were taken on a Varian Cary 50 spectrophotometer, IR spectra on a Jasco FT/IR-410 spectrometer, and optical rotations on a Jasco P-1020 polarimeter. ^{1}H NMR (600 MHz, 400 MHz) and ^{13}C NMR (150 MHz, 100 MHz) were recorded on a Bruker DMX 600 or on an AMX 400, using $CDCl_3$ (δ 7.26 and 77.01), CD_3OD (δ 3.30 and 49.15), and DMSO-d_6 (δ 2.50 and 39.51) as solvents and internal ^{1}H and ^{13}C standards. Proton-detected, heteronuclear correlations were analyzed using HMQC (optimized for $^1J_{HC}$ = 145 Hz) and HMBC (optimized for $^nJ_{HC}$ = 7 Hz). EIMS (70 eV), HREIMS (70 eV), and HRESIMS were determined on Finnigan MAT 8200, Finnigan MAT 90, and Bruker microTOF instruments, respectively. For analytical TLC, silica gel precoated glass plates (60 F_{254}, Merck) were used and flash chromatography was carried out on silica gel (0.063 mm, Merck). HPLC (preparative): SymmetryPrep C_{18}, 19 × 300 mm; 7 Vμm (Waters); flow 12 ml/min; solvent (A) CH_3CN (0.05% trifluoroacetic acid), (B) H_2O (0.05% trifluoroacetic acid); using the following gradient: 0 min 5% A, 30 min 70% A, 35 min 100% A, 40 min 100% A, 41 min 5% A, 46 min 5% A. Diazald was purchased from Aldrich and β-glucosidase (from almonds) from Fluka. Organic solvents were dried and distilled prior to use.

3.2. Cell material

Seeds from *T. peltatum* were obtained from the Parc de Taï, Ivory Coast. Plants of *A. abbreviatus* were cultivated in the Botanical Garden of the University of Würzburg (see also Bringmann et al., 1993). Voucher specimens of both plant species have been deposited in the Herb. Bringmann, University of Würzburg [Nos. 2, 35, and 36 (*T. peltatum*) and No. 32 (*A. abbreviatus*)].

3.3. Tissue cultures

The shoot cultures of *T. peltatum* were established as previously reported (Bringmann et al., 1999b). Callus cultures were induced from stem segments transferring them to modified Murashige and Skoog medium (Murashige and Skoog, 1962) – with full strength of micro elements and organics but 1/5 of macro elements except for Ca^+ and Mg^+ (using 1/4). The basic medium was supplemented with 2 mg/l 6-benzylaminopurine, 0.1 mg/l 1-naphthalene acetic acid, 3% sucrose, 0.25% Gelrite (Roth). The cultures were kept under fluorescent light with a 14-h photoperiod at 51 μM/m s photosynthetically active radiation at 24 ± 2 °C. For maintains and propagation, the calli were transferred to fresh solid medium every 2 months for a couple of years.

A. abbreviatus callus cultures were established as described before (Bringmann et al., 1999a). The calli were cultivated on a modified Murashige and Skoog medium, with full strength of micro elements and organics but 1/5 of macro elements except for Ca^+ and Mg^+ (using 1/4) but supplemented with 0.5 mg/l of 2,4-dichlo-

rophenoxyacetic acid, 0.5 mg/l 6-benzylaminopurine, 0.5 mg/l 1-naphthalene acetic acid, 3% sucrose, 0.25% Gelrite, 100 mg/l glutathione, and 4.5 mg/l poly(vinylpolypyrrolidone). The cultures were kept in darkness at 24 ± 2 °C and monthly subcultured for at least 6 months.

3.4. Extraction and isolation

6.87 g (in the case of *T. peltatum*) and 8.04 g (in the case of *A. abbreviatus*) of lyophilized cell material were grounded and extracted with CH_2Cl_2:MeOH (1:1). The extracts were concentrated *in vacuo* to give 1.49 g and 1.83 g of a crude residue, respectively, which were dissolved in MeOH and directly submitted to preparative HPLC. In the case of *T. peltatum* this yielded 18.5 mg of compound **4** (retention time 15.4 min) and 9.6 mg of compound **5** (retention time 21.6 min). In the case of *A. abbreviatus* the resolution gave 11.0 mg of compound **2** (retention time 32.3 min), 4.9 mg of compound **3** (retention time 23.6 min), 10.9 mg of compound **5** (retention time 24.0 min), 12.0 mg of compound **6** (retention time 24.3 min), 5.6 mg of compound **7** (retention time 25.2 min), 4.4 mg of compound **8** (retention time 26.0 min), 5.2 mg of compound **9** (retention time 28.8 min), 4.5 mg of compound **10** (retention time 29.3 min), 5.9 mg of compound **11** (retention time 28.4 min), and 6.4 mg of compound **12** (retention time 26.0 min). Compounds **9**-**12** were further purified by flash column chromatography on SiO_2.

3.5. Dioncoquinone A (*4*)

Yellow crystals; m.p. 189 °C; $[\alpha]_D^{20}$ +67.2 (MeOH:DMSO 1:1; *c* 0.05); UV (MeOH:DMSO 1:1) λ_{max} ($\log\varepsilon$) nm: 413 (0.34), 295 (0.75), 255 (1.37), 206 (1.72); IR (KBr) ν_{max} cm^{-1}: 3405 (m), 2923 (m), 2853 (w), 1638 (s), 1458 (s), 1365 (m), 1262 (s), 1074 (s), 842 (w), 641 (w); ^{1}H NMR (600 MHz, DMSO-d_6): δ 1.91 (3H, *s*, CH_3-2), 3.18 (1H, *d*, *J* = 8.5 Hz, H-4′), 3.29–3.31 (1H, *m*, H-5′), 3.30–3.32 (1H, *m*, H-2′), 3.38 (1H, *dd*, *J* = 7.6, 1.9 Hz, H-3′), 3.47 (1H, *dd*, *J* = 11.9, 5.8 Hz, H-6′), 3.68 (1H, *dd*, *J* = 11.9, 1.9 Hz, H-6′), 5.06 (1H, *d*, *J* = 7.4 Hz, H-1′), 7.42 (1H, *d*, *J* = 8.6 Hz, H-7), 7.45 (1H, *d*, *J* = 8.6 Hz, H-8), 10.86 (1H, *s*, OH-3), 11.41 (1H, *s*, OH-5); ^{13}C NMR (150 MHz, DMSO-d_6]: δ 8.7 (CH_3-2), 60.4 (C-6′), 69.4 (C-4′), 73.0 (C-5′), 76.5 (C-2′), 77.2 (C-3′), 99.8 (C-1′), 114.0 (C-10), 118.8 (C-7), 119.7 (C-8), 120.7 (C-2), 125.0 (C-9), 150.0 (C-6), 150.3 (C-5), 155.0 (C-3), 183.3 (C-1), 184.4 (C-4); EIMS *m/z* (rel. int.): 220.1 $[M-Glc]^+$ (100), 192.1 $[M-Glc-H_2O]^+$ (24), 163.1 $[Glc]^+$ (19), 146.1 (25); HRESIMS *m/z*: 381.0827 $[M-H]^-$ (calcd. for $C_{17}H_{17}O_{10}^-$ 381.0827).

3.6. Dioncoquinone B (*5*)

Red needles; m.p. 218 °C; UV (MeOH) λ_{max} ($\log\varepsilon$) nm: 416 (0.33), 317 (0.67), 269 (1.12), 215 (1.57); IR (KBr) ν_{max} cm^{-1}: 3408 (m), 2922 (w), 2853 (w), 1618 (s), 1459 (m), 1297 (m), 1209 (m), 1105 (m), 430 (w); ^{1}H NMR (600 MHz, CD_3OD): δ 2.10 (3H, *s*, CH_3-2), 6.04 (1H, *s*, OH-6), 7.04 (1H, *s*, OH-3), 7.20 (1H, *d*, *J* = 8.3 Hz, H-7), 7.65 (1H, *d*, *J* = 8.3 Hz, H-8), 11.22 (1H, *s*, OH-5); ^{13}C NMR (150 MHz, CD_3OD): δ 8.9 (CH_3-2), 113.4 (C-10), 120.4 (C-7), 121.3 (C-8), 122.7 (C-2), 124.3 (C-9), 147.9 (C-5), 149.6 (C-6), 152.3 (C-3), 183.4 (C-1), 185.0 (C-4); EIMS *m/z* (rel. int.): 220.1 $[M]^+$ (46), 192.1 $[M-H_2O]^+$ (15), 146.1 (12); HRESIMS *m/z*: 219.0302 $[M-H]^-$ (calcd. for $C_{11}H_7O_5^-$ 219.0299).

3.7. Ancistroquinone B (*6*)

Orange needles; m.p. 218–220 °C; lit. 215–217 °C (Govindachari et al., 1971); lit. 168–170 °C (Brockmann and Zeeck, 1970); UV (CH_2Cl_2) λ_{max} ($\log\varepsilon$) nm: 417 (0.48), 299 (0.94), 257 (1.48), 203 (1.66); IR (KBr) ν_{max} cm^{-1}: 3365 (m), 2924 (w), 1632 (s), 1457 (m), 1372 (m), 1307 (m), 1264 (s), 1066 (m), 1018 (w), 746 (w), 713 (w); ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.01 (3H, *s*, CH_3-2), 3.91 (3H, *s*, OCH_3-6), 7.00 (1H, *d*, *J* = 8.3 Hz, H-7), 7.10 (1H, *s*, 3-OH), 7.59 (1H, *d*, *J* = 8.3 Hz, H-8), 11.38 (1H, *s*, OH-5); ^{13}C NMR (150 MHz, $CDCl_3$): δ 8.8 (CH_3-2), 56.3 (OCH_3-6), 113.0 (C-10), 115.9 (C-7), 120.9 (C-8), 122.5 (C-2), 124.3 (C-9), 151.7 (C-6), 152.7 (C-3), 153.0 (C-5), 183.4 (C-1), 184.9 (C-4); EIMS *m/z* (rel. int.): 234.1 $[M]^+$ (100), 188.1 (26), 149.1 (21); HRESIMS *m/z*: 233.0455 $[M-H]^-$ (calcd. for $C_{12}H_9O_5^-$ 233.0455).

3.8. Ancistroquinone C (*7*)

Yellow needles; m.p. 231 °C; lit. 235–238 °C (Govindachari et al., 1971); UV (CH_2Cl_2) λ_{max} ($\log\varepsilon$) nm: 365 (0.33), 291 (0.78), 263 (1.57), 199 (1.81); IR (KBr) ν_{max} cm^{-1}: 3854 (m), 2923 (w), 2852 (w), 1655 (s), 1637 (m), 1573 (m), 1383 (m), 1359 (m), 1277 (m), 1204 (w), 1069 (m), 1026 (w), 740 (w); ^{1}H NMR (600 MHz, $CDCl_3$): δ 1.99 (3H, *s*, CH_3-2), 3.87 (3H, *s*, OCH_3-6), 3.89 (3H, *s*, OCH_3-5), 7.11 (1H, *d*, *J* = 8.6 Hz, H-7), 7.46 (1H, *s*, OH-3), 7.87 (1H, *d*, *J* = 8.6 Hz, H-8); ^{13}C NMR (150 MHz, $CDCl_3$): δ 8.5 (CH_3-2), 56.2 (OCH_3-6), 61.2 (OCH_3-5), 116.5 (C-7), 118.9 (C-2), 122.4 (C-10), 124.4 (C-8), 126.4 (C-9), 149.7 (C-5), 152.5 (C-3), 157.8 (C-6), 180.1 (C-4), 184.2 (C-1); EIMS *m/z* (rel. int.): 248.1 $[M]^+$ (78), 234.1 $[M-CH_2]^+$ (100), 209.1 (94), 149.1 (61); HRESIMS *m/z*: 247.0612 $[M-H]^-$ (calcd. for $C_{13}H_{11}O_5^-$ 247.0612).

3.9. Ancistroquinone D (*9*)

Red needles; m.p. 218–220 °C; UV (CH_2Cl_2) λ_{max} ($\log\varepsilon$) nm: 411 (0.23), 305 (0.41), 261 (0.58), 215 (1.02); IR (KBr) ν_{max} cm^{-1}: 3349 (s), 2925 (w), 2854 (w), 1650 (s), 1609 (s), 1478 (m), 1426 (m), 1392 (m), 1334 (m), 1291 (m), 1260 (m), 1149 (s), 1022 (m), 861 (w), 461 (w); ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.07 (3H, *s*, CH_3-2), 3.89 (3H, *s*, OCH_3-5), 3.94 (3H, *s*, OCH_3-6), 6.70 (1H, *s*, H-7), 7.64 (1H, *s*, OH-3), 13.44 (1H, *s*, OH-8); ^{13}C NMR (150 MHz, $CDCl_3$): δ 8.1 (CH_3-2), 56.4 (OCH_3-6), 61.2 (OCH_3-5), 106.6 (C-10), 107.2 (C-7), 118.7 (C-2), 120.6 (C-9), 145.9 (C-5), 153.7 (C-3), 160.0 (C-6), 160.9 (C-8), 179.1 (C-4), 189.5 (C-1); CIMS *m/z* (rel. int.): 265.1 $[M+H]^+$ (100); HRESIMS *m/z*: 265.0709 $[M+H]^+$ (calcd. for $C_{13}H_{13}O_6^+$ 265.0706).

3.10. Ancistroquinone E (*10*)

Red solid; m.p. 242 °C (subl.); UV (CH_2Cl_2) λ_{max} ($\log\varepsilon$) nm: 460 (0.32), 343 (0.39), 263 (0.82), 233 (0.89); IR (KBr) ν_{max} cm^{-1}: 3384 (s), 2961 (m), 2923 (m), 2853 (w), 1654 (s), 1560 (m), 1459 (w), 1262 (m), 1098 (s), 1027 (m), 801 (m); ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.07 (3H, *s*, CH_3-2), 6.25 (2H, *s*, OCH_2O), 11.48 (1H, *s*, OH-5), 12.94 (1H, *s*, OH-8); ^{13}C NMR (150 MHz, $CDCl_3$): δ 8.1 (CH_3-2), 104.5 (OCH_2O), 107.8 (C-10), 109.0 (C-9), 120.8 (C-2), 140.8 (C-7), 144.4 (C-5), 144.6 (C-6), 144.9 (C-8), 153.9 (C-3), 180.9 (C-4), 188.7 (C-1); EIMS *m/z* (rel. int.): 264.0 $[M]^+$ (100), 236.0 (44), 149 (33); HREIMS *m/z*: 264.0266 $[M]^+$ (calcd. for $C_{12}H_8O_7^+$ 264.0273).

3.11. Ancistroquinone F (*12*)

Yellow solid; m.p. 220 °C (dec.); UV (CH_2Cl_2) λ_{max} ($\log\varepsilon$) nm: 377 (0.17), 319 (0.53), 271 (1.06), 220 (1.13); IR (KBr) ν_{max} cm^{-1}: 3412 (m), 2912 (s), 2924 (m), 2853 (w), 1734 (m), 1638 (s), 1584 (m), 1347 (m), 1245 (m), 1096 (m), 1033 (m), 747 (w); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.02 (3H, *s*, CH_3-2), 4.14 (3H, *s*, OCH_3-5), 6.11 (2H, *s*, OCH_2O), 7.39 (1H, *s*, H-8), 7.67 (1H, *s*, OH-3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 8.4 (CH_3-2), 60.6 (OCH_3-5), 102.5 (OCH_2O), 103.1 (C-8), 116.2 (C-10), 117.1 (C-2), 131.8 (C-9), 140.6 (C-6), 144.4 (C-5), 153.4 (C-3), 154.0 (C-7), 178.9 (C-4),

183.6 (C-1); EIMS *m/z* (rel. int.): 262.0 $[M]^+$ (20), 149.1 (100); HRESIMS *m/z*: 261.0402 $[M-H]^-$ (calcd. for $C_{13}H_9O_6^-$ 261.0404).

3.12. Preparation of dioncoquinone B (**5**) from dioncoquinone A (**4**)

Dioncoquinone A (4.0 mg, 10.4 μmol, isolated from cell cultures of *T. peltatum*) was dissolved in DMSO:H_2O (2 ml, 1:1) and incubated with β-glucosidase (from almonds) (0.5 mg) for 3 h. After extraction of the reaction mixture with CH_2Cl_2, the combined organic layers were dried over $MgSO_4$, and the solvents were removed *in vacuo* to give **5** as an orange-colored solid (1.2 mg, 5.5 μmol, 53%), identical with the natural product by m.p., TLC, HPLC coelution, and ^{1}H NMR.

3.13. Hydrolysis of glucoside **4** by treatment with HCl

A sample of **4** (3.0 mg, 7.84 μmol) was stirred in HCl:MeOH (4 ml, 1:1) at 60 °C for 2 h and cooled to room temperature. CH_2Cl_2 (15 ml) was added and the phases were separated. Evaporation of the aqueous phase gave D-glucose. Identity to an authentic sample (Aldrich) was confirmed by ^{1}H NMR, optical rotation, $[\alpha]_D^{20}$ +50.9 (H_2O; *c* 0.1), lit. +52.0 (H_2O; *c* 5) (Myhre and Smith, 1960), melting point, 146 °C (H_2O), lit. 146 °C (H_2O) (Myhre and Smith, 1960), and TLC comparison (silica gel, $CHCl_3$:MeOH:H_2O (9:6:1), detection by spraying with 10% vanillin in conc. H_2SO_4, followed by heating). Evaporation of the organic phase afforded the aglycon **5** (1.9 mg, 7.20 μmol, 91%). Its chromatographic (TLC; HPLC) and spectroscopic (^{1}H NMR) properties were identical to those of the sample isolated from *T. peltatum*.

3.14. Preparation of ancistroquinone C (**7**) by O-methylation of **5**

Diazomethane was prepared by adding dropwise a solution of KOH (0.5 g) in EtOH (25 ml) and H_2O (0.8 ml) to Diazald (2.15 g) in Et_2O (20 ml) at 60 °C (De Boer and Backer, 1963) and was directly distilled into a cooled solution (0 °C) of dioncoquinone B (5.9 mg, 26.9 μmol, isolated from cell cultures of *T. peltatum*) in Et_2O (15 ml). The reaction mixture was stirred at room temperature for 2 h. After removal of the solvent under reduced pressure, the resulting residue was submitted to column chromatography on silica gel, using an increasing gradient of CH_2Cl_2:MeOH (100:0 up to 100:2), to yield **7** as a yellow solid (4.2 mg, 16.6 μmol, 62%), identical with the natural product by m.p., TLC, HPLC coelution, and ^{1}H NMR.

3.15. Computational details

All calculations were done with the Gaussian 03 Software package (Frisch et al., 1971). The conformational analysis of dioncoquinone B (**5**) by using the DFT hybrid functional B3LYP (Becke, 1993; Lee et al., 1988) together with 6-31G* (Francl et al., 1982; Hariharan and Pople, 1973) as a basis set yielded two possible conformers. The energetic minimum thus obtained was used for the gauge invariant atomic orbitals ^{13}C NMR calculations, again with the B3LYP functional, but with the much larger, augmented, and polarized correlation-consistent basis set aug-ccpVDZ (Dunning, 1989; Kendall et al., 1992). ^{1}H NMR shielding tensors were calculated with B3LYP/6-31++G** (Clark et al., 1983; Francl et al., 1982; Hariharan and Pople, 1973) for an MP2/TZVP (Møller and Plesset, 1934; Head-Gordon et al., 1988; Schäfer et al., 1992, 1994) optimized minimum structure of **7**. The obtained shielding tensors were referenced against tetramethylsilane to get relative chemical shifts. The corresponding ^{13}C NMR and ^{1}H NMR shielding tensors of tetramethylsilane were calculated with B3LYP/aug-ccpVDZ on a B3LYP/6-31G* optimized structure and with B3LYP/6-31++G** on a MP2/TZVP optimized structure, respectively.

3.16. Cell viability assay

For the cell viability assays, cells of two different human B cell tumor models, B cell lymphoma and multiple myeloma, and human primary normal mononuclear blood cells were used. The B cell lymphoma cell lines DOHH-2 and SU-DHL-4, as well as the multiple myeloma cell line RPMI 8226, were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ) (ACC 47, ACC 495 and ACC 402, Braunschweig, Germany). The multiple myeloma cell line INA-6 had previously been described (Burger et al., 2001). Mononuclear cells from the peripheral blood of two healthy donors were separated by Ficoll-Hypaque density gradient centrifugation. To assess the percentage of apoptotic and viable cell fractions, a human annexin V-FITC/PI staining kit (Bender MedSystems, Vienna, Austria) was used. Cells were washed in PBS, incubated for 10 min in 100 ml binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 2.5 ml annexin V-FITC mix and 1 mg/ml propidium iodide (PI), subsequently diluted with 300 ml binding buffer and analyzed by flow cytometry (FACSCalibur/CELLQuest; Becton Dickinson, Heidelberg, Germany). Early apoptosis is characterized by a positive annexin V-FITC staining. Cells in a late apoptotic stage lose their membrane integrity and additionally incorporate PI. Viable cells are negative for both, annexin V-FITC and PI.

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