PAPER

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Ancistrobreveines A–D and related dehydrogenated naphthylisoquinoline alkaloids with antiproliferative activities against leukemia cells, from the West African liana *Ancistrocladus abbreviatus*†

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A unique series of six biaryl natural products displaying four different coupling types (5,1′, 7,1′, 7,8′, and 5,8′) were isolated from the roots of the West African liana *Ancistrocladus abbreviatus* (Ancistrocladaceae). Although at first sight structurally diverse, these secondary metabolites all have in common that they belong to the rare group of naphthylisoquinoline alkaloids with a fully dehydrogenated isoquinoline portion. Among the African *Ancistrocladus* species, *A. abbreviatus* is so far only the second one that was found to produce compounds with such a molecular entity. Here, we report on four new representatives, named ancistrobreveines A–D (**12–14**, and **6**). They were identified along with the two known alkaloids 6-*O*-methylhamateine (**4**) and *ent*-dioncophylleine A (**10**). The two latter naphthylisoquinolines had so far only been detected in *Ancistrocladus* species from Southeast Asia. All of these fully dehydrogenated alkaloids have in common being optically active despite the absence of stereogenic centers, due to the presence of the rotationally hindered biaryl axis as the only element of chirality. Except for *ent*-dioncophylleine A (**10**), which lacks an oxygen function at C-6, the ancistrobreveines A–D (**12–14**, and **6**) and 6-*O*-methylhamateine (**4**) are 6-oxygenated alkaloids, and are, thus, typical 'Ancistrocladaceae-type' compounds. Ancistrobreveine C (**14**), is the first – and so far only – example of a 7,8′-linked fully dehydrogenated naphthylisoquinoline discovered in nature that is configurationally stable at the biaryl axis. The stereostructures of the new alkaloids were established by spectroscopic (in particular HRESIMS, 1D and 2D NMR) and chiroptical (electronic circular dichroism) methods. Ancistrobreveine C (**14**) and 6-*O*-methylhamateine (**4**) exhibited strong antiproliferative activities against drug-sensitive acute lymphoblastic CCRF-CEM leukemia cells and their multidrug-resistant subline, CEM/ADR5000.

Introduction

Ancistrocladus abbreviatus Airy Shaw (Ancistrocladaceae)[1–3] is a West African scandent shrub abundantly occurring in wet evergreen forests, along roadsides, river banks, and islands. In the juvenile phase, the plants start growing as erect, self-supporting saplings with leaves arranged in terminal rosettes. Reaching maturity, they form climbing stems from elongated shoots that bear vegetative, often robust leaves. Adult specimens can reach a height of more than 6 m, supported by means of recurved to spiraling woody hooks, as typical of the Ancistrocladaceae.[1,4]

Phytochemical studies on *A. abbreviatus* collected in the Parc National de Taï, Southwestern Côte d'Ivoire (Ivory Coast), resulted in the discovery of *ca.* 30 structurally most diverse naphthylisoquinoline alkaloids displaying four different coupling types (5,1′, 7,1′, 5,8′, and 7,8′).[5–11] The metabolic pattern of this West African plant is strongly dominated by the presence of 5,1′- and 7,1′-linked naphthylisoquinolines. All of the alkaloids with a 5,1′-biaryl linkage constitute typical Ancistrocladaceae-type compounds[5,12] like *e.g.*, ancistrobrevine E (**1**) (Fig. 1), *i.e.*, with *S*-configuration at C-3 and an oxygen function at C-6.[5,6,10,11] Most of the 7,1′-coupled alkaloids of *A.*

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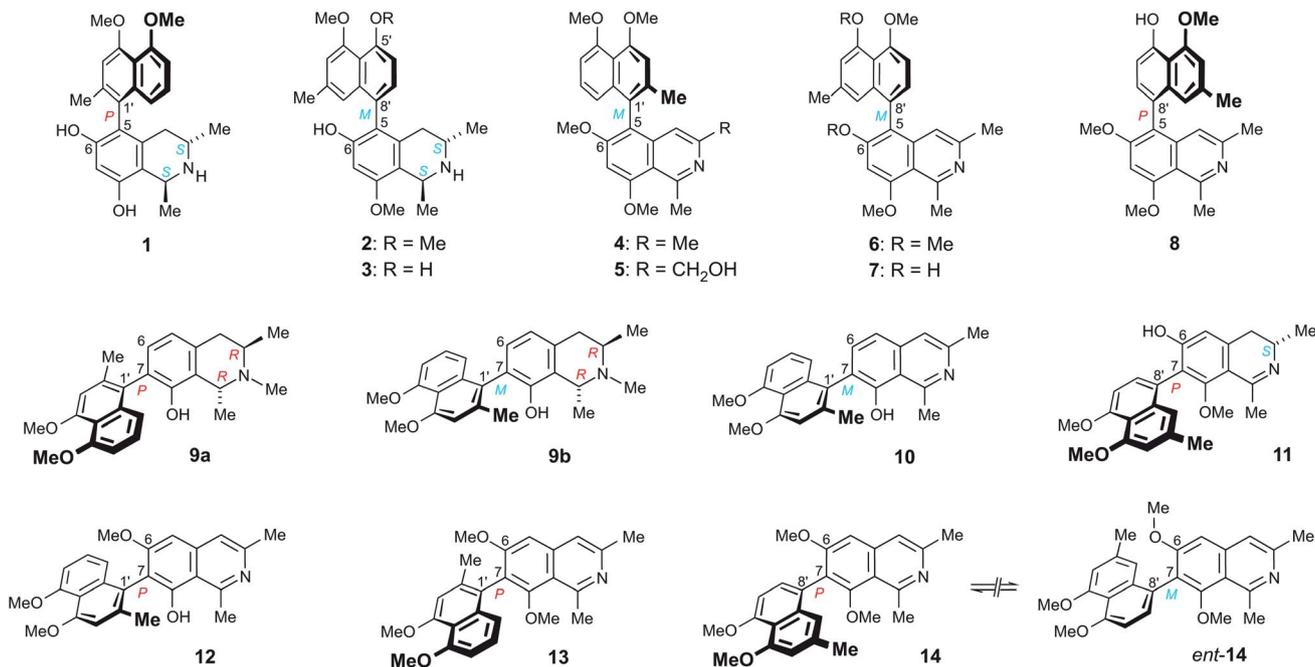
‡ Deceased on January 14, 2014.

Fig. 1 Naphthylisoquinolines from the roots of the West African shrub *Ancistrocladus abbreviatus* displaying four different coupling types, among them a unique series of six metabolites belonging to the small subgroup of alkaloids with a fully dehydrogenated isoquinoline portion, the four new ancistrobreveines A (**12**), B (**13**), C (**14**), and D (**6**), and two previously known[26,27] alkaloids, 6-*O*-methylhamateine (**4**) and *ent*-dioncophylleine A (**10**); ancistrobenomine B (**5**) and the ancistrolikokines G (**7**) and J$_2$ (**8**) are examples of fully dehydrogenated naphthylisoquinoline alkaloids displaying strong antiproliferative activities against leukemia cells.[21,30]

abbreviatus, by contrast, among them the two main constituents, *N*-methyldioncophylline A (**9a**) and its atropodiastereomer **9b** (Fig. 1), are 3*R*-configured and lack an oxygen substituent at C-6.[5,9,10] They are thus categorized as Dioncophyllaceae-type compounds,[5,12] because the only other plant family that likewise produces naphthylisoquinoline alkaloids, the Dioncophyllaceae,[13] exclusively forms alkaloids with these two structural features.[5,12,14] They are – similar to *A. abbreviatus* – endemic to West Africa.[1–3,13]

Remarkably, only two 5,8′-coupled alkaloids, ancistrobrevine B (**2**) and its 5′-*O*-demethyl analog **3** (Fig. 1), have so far been detected in *A. abbreviatus*,[6,11] whereas naphthylisoquinoline alkaloids of this coupling type are the most prevalent ones in Central African *Ancistrocladus* lianas.[5,12,15–25] *Ancistrocladus abbreviatus* furthermore contains a substantial number of representatives (six compounds) of the otherwise rare group of 7,8′-linked naphthylisoquinoline alkaloids, one of them is ancistrobrevine I (**11**) (Fig. 1).[10,11] All of the 5,8′- and 7,8′-coupled alkaloids are typical Ancistrocladaceae-type compounds.

The by far largest group of constituents of *A. abbreviatus* are naphthyltetrahydroisoquinolines,[5–7,9–11] whereas only four of the alkaloids had a dihydroisoquinoline subunit, among them compound **11**.[8,11]

Here, we describe the isolation and structural elucidation of an unprecedented series of minor metabolites possessing a fully dehydrogenated isoquinoline half. The series comprises four new compounds, named ancistrobreveines A–D (**12–14**, and **6**) and the two previously known[26,27] naphthylisoquinolines 6-*O*-methylhamateine (**4**) and *ent*-dioncophylleine A (**10**) (Fig. 1).

Since they were devoid of any stereogenic centers, the only element of chirality in these compounds was the rotationally hindered biaryl axis.

Fully dehydrogenated naphthylisoquinoline alkaloids have as yet been found quite rarely in nature. Prior to this work, only 14 such compounds (out of a total of more than 250 known naphthylisoquinoline alkaloids) had been identified in *Ancistrocladus* lianas, most of them from Asian taxa.[20,21,26–30] Only recently, phytochemical studies on the Congolese liana *A. likoko* has shown the presence of fully dehydrogenated naphthylisoquinolines for the first time in an African species, too.[20,21]

Each of the six alkaloids discovered in *A. abbreviatus* was obtained in an optically active form, initially leaving open whether they were enantiomerically pure or just scalemic. Analysis by HPLC chromatography on a chiral phase in combination with ECD spectroscopy revealed all of them to be enantiopure, except for one of the alkaloids, the new 7,8′-linked ancistrobreveine C (**14**). This compound was found to be produced as an atropo-enantiomeric mixture, with the *P*-enantiomer being the by far major one occurring in the plant. Likewise noteworthy, compounds **12–14** are the first 7,1′- and 7,8′-coupled Ancistrocladaceae-type alkaloids found in nature that possess a fully dehydrogenated isoquinoline subunit.

Some of the dehydrogenated alkaloids isolated from other *Ancistrocladus* species, such as ancistrobenomine B (**5**) and the ancistrolikokines G (**7**) and J$_2$ (**8**) (Fig. 1), have attracted attention due to their strong antiproliferative activities against human drug-sensitive CCRF-CEM leukemia cells and their multidrug-resistant subline, CEM/ADR5000, similar to the

effects induced by the established anticancer drug doxoru-bicin.[21,30] These findings prompted us to evaluate the cytotoxic potential of the six alkaloids of *A. abbreviatus* presented in this paper. The results provide further insight into structural prerequisites for the activities and into the impact of axial chirality on the antileukemic potential of fully dehydrogenated naphthylisoquinoline alkaloids, in particular.

Results and discussion

Isolation and structural elucidation of ancistrobreveines A–D and related naphthylisoquinoline alkaloids

An HPLC-UV-MS-guided screening of root bark extracts of *A. abbreviatus* gave hints at the presence of minor constituents, with UV and MS profiles typical of naphthylisoquinoline alkaloids with a non-hydrogenated isoquinoline subunit. For the isolation of these compounds, air-dried ground material of the roots was exhaustively extracted with MeOH–H₂O (9 : 1, v/v), followed by liquid–liquid partitioning with *n*-hexane and fractionation of the crude methanolic extract by column chromatography on silica gel. The alkaloid-containing subfractions were directly subjected to preparative reversed-phase HPLC, which permitted isolation of six fully dehydrogenated naphthylisoquinoline alkaloids. Two of them, 6-*O*-methylhamateine (**4**) and *ent*-dioncophylleine A (**10**), were well known from previous phytochemical studies[26,27] on related *Ancistrocladus* species from Southeast Asia.

The 5,1′-linked 6-*O*-methylhamateine (**4**) had so far been identified only in the leaves of the Vietnamese species *A. cochinchinensis*, but its enantiomeric purity was never analyzed.[26] HPLC analysis of the material of **4** isolated from the roots of *A. abbreviatus* on a chiral phase (Lux Cellulose-1) resulted only in one sharp peak (Fig. 2, left), thus revealing this fully dehydrogenated naphthylisoquinoline to occur in this West African species in an enantiomerically pure form. The ECD spectra recorded at different positions of the peak (*e.g.*, at the left or right slope) were all identical, and virtually opposite to the ECD curve of ancistrocladeine (**15**)[31] (Fig. 2, right), a known, likewise fully dehydrogenated and 5,1′-coupled, but *P*-configured (and 6-*O*-demethylated) naphthylisoquinoline, previously obtained by semi-synthesis from ancistrocladine.[31,32]
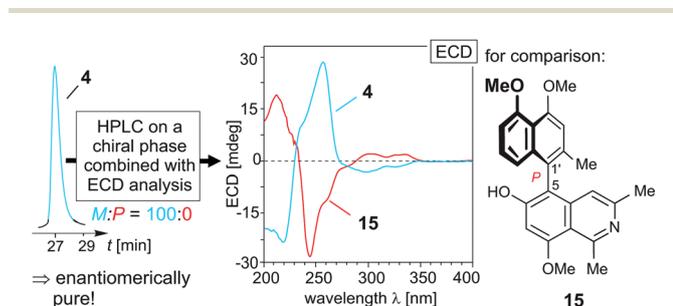

Fig. 2 HPLC analysis of 6-*O*-methylhamateine (**4**) on a chiral phase (Lux Cellulose-1) evidencing that **4** is produced by *A. abbreviatus* in an enantiomerically pure form, and comparison of the ECD spectrum of **4** with that of the known,[31,32] likewise 5,1′-coupled, but *P*-configured ancistrocladeine (**15**).

With its oxygen function at C-6, 6-*O*-methylhamateine (**4**) had the constitution of an Ancistrocladaceae-type naph-thylisoquinoline, whereas the constitution of the second known compound isolated from the roots of *A. abbreviatus*, was categorized as a Dioncophyllaceae-type alkaloid, since it lacked an oxygen group at C-6. Within this classification, the presence or absence of the 6-oxygen function is given priority over the – here not applicable – absolute configuration at C-3.

The second fully dehydrogenated naphthylisoquinoline was readily identified as the 7,1′-linked *ent*-dioncophylleine A (**10**), which had previously been discovered in the leaves of the Malaysian highland liana *A. benomensis*.[27] In that species, **10** had been found to be produced in a scalemic form, as a 93 : 7-mixture of **10** and its *P*-configured enantiomer dioncophylleine A.[5,27,32]

The material of **10** isolated from the roots of *A. abbreviatus*, by contrast, was determined to be enantiomerically pure, as obvious from HPLC analysis on a chiral Lux Cellulose-1 column, giving rise to only one peak (see ESI†), the ECD spectra measured in intervals at different sections of the peak were all identical.

The other four metabolites isolated from the root bark extracts of *A. abbreviatus* were as yet undescribed.

The first of these new alkaloids, compound **12**, obtained as a yellow amorphous solid, had a molecular formula of $C_{25}H_{25}NO_4$, as deduced from its monoprotonated molecular ion, $[M + H]^+$, at *m/z* 404.1861 by HRESIMS. This, together with the UV spectrum displaying a second maximum around 258 nm, suggested it to be yet another fully dehydrogenated naphthylisoquinoline alkaloid. This assumption was corroborated by the appearance of an aromatic singlet at δ_H 7.77 in the ¹H NMR spectrum (Table 1), instead of the signals of two diastereotopic geminal protons at C-4 usually observed for alkaloids with a tetra- or dihydroisoquinoline subunit. This finding was in accordance with the lack of resonances for protons at C-1 and C-3, *i.e.*, with the missing quartet of H-1 around δ_H 4.20 and the absence of the multiplet of H-3 in the region between δ_H 3.20 and 4.00. Likewise typical of fully dehydrogenated naph-thylisoquinolines was the significant downfield shift of the three-proton singlet of Me-1 (δ_H 3.20) in the ¹H NMR spectrum and the likewise down-field-shifted ¹³C NMR resonances of C-1 (δ_C 158.4), C-3 (δ_C 143.7), and C-4 (δ_C 121.3) (Table 1). The new alkaloid **12** possessed three methoxy functions, two of them (δ_H 3.92 and 3.98) located at C-4′ and C-5′ in the naphthalene part, as obvious from NOESY correlations with H-3′ (δ_H 6.94) and H-6′ (δ_H 6.87) (Fig. 3A). In the isoquinoline half, the position of the remaining methoxy group was established to be at C-6 as deduced from a NOESY interaction between OMe-6 (δ_H 3.81) and H-5 (δ_H 7.15), leaving the fourth oxygenated carbon atom, C-8 (δ_C 158.0), to bear a free hydroxy function. In the aromatic region of the ¹H NMR spectrum, the appearance of a spin system of three neighboring protons giving rise to two doublets (δ_H 6.87 and 6.73) and one pseudo-triplet (δ_H 7.18) indicated that the coupling site of the naphthalene moiety was either C-1′ or C-3′. The latter possibility was excluded by two NOESY correlation sequences, {Me-2′ ↔ H-3′ ↔ OMe-4′} and {OMe-5′ ↔ H-6′ ↔ H-7′ ↔ H-8′}, and by the high-field shift of the signal

Table 1 ¹H (600 MHz) and ¹³C (150 MHz) NMR data of ancistrobreveines A (12), B (13), C (14), and D (6) in methanol-d_4 (δ in ppm)

Position	12 δ_H (J in Hz)	δ_C	13 δ_H (J in Hz)	δ_C	14 δ_H (J in Hz)	δ_C	6 δ_H (J in Hz)	δ_C
1		158.4		157.7		157.5		158.6
3		143.7		144.0		143.8		141.8
4	7.77, s	121.3	7.90, s	122.0	7.89, s	122.0	6.86, s	119.3
5	7.15, s	98.9	7.40, s	102.5	7.37, s	102.3		116.9
6		166.7		166.9		167.0		166.3
7		117.9		126.5		128.1	7.20, s	97.8
8		158.0		160.4		160.7		164.1
9		114.5		118.0		118.0		114.2
10		142.2		142.8		142.8		142.4
1′		119.8		121.8	6.68, s	118.2	6.42, s	118.0
2′		138.8		137.4		138.7		138.2
3′	6.94, s	110.2	6.93, s	109.8	6.79, s	109.7	6.78, s	109.9
4′		159.0		158.8		158.7		158.9
5′		158.9		158.8		159.2		158.8
6′	6.87, d (7.4)	107.2	6.87, d (7.3)	107.0	6.96, d (8.1)	106.1	6.97, d (8.0)	106.5
7′	7.18, dd (7.8, 8.3)	128.0	7.21, dd (7.8, 8.4)	128.0	7.26, d (8.0)	130.7	7.14, d (7.9)	131.0
8′	6.73, d (7.5)	118.2	6.78, dd (0.9, 8.5)	118.9		122.7		124.2
9′		138.0		137.6		137.4		137.6
10′		118.0		117.5		117.2		117.5
1-Me	3.20, s	23.3	3.20, s	22.5	3.20, s	22.5	3.23, s	23.6
3-Me	2.69, s	18.5	2.74, s	18.5	2.74, s	18.4	2.40, s	18.5
2′-Me	2.11, s	20.4	2.16, s	20.8	2.28, s	22.0	2.19, s	21.9
6-OMe	3.81, s	57.0	3.85, s	57.2	3.31^a	57.2	3.92, s	56.9
8-OMe			3.30^a	61.1	3.85, s	61.6	4.25, s	57.2
4′-OMe	3.98, s	56.7	3.98, s	56.7	3.93, s	56.8	3.93, s	57.1
5′-OMe	3.92, s	56.9	3.93, s	56.8	3.97 s	56.6	3.98, s	56.7

^{a} Overlapped with the methanol peak.

of Me-2′ (δ_H 2.11). Consequently, the biaryl axis had to be located at C-1′ (δ_C 158.4). This was also confirmed by HMBC long-range interactions from H-8′ (δ_H 6.73) and H-3′ to C-1′ (Fig. 3A). In the isoquinoline part, the coupling position was deduced to be at C-7 (δ_C 117.9), based on the NOESY correlation sequence {OMe-6 ↔ H-5 ↔ H-4} and on an HMBC interaction from H-5 to C-7. In conclusion, the new compound was a 7,1′-coupled alkaloid with a constitution similar to that of ent-dioncophylleine A (10), except for the additional methoxy group at C-6.[27] The absolute configuration of this new Ancistrocladaceae-type naphthylisoquinoline 12 at the biaryl axis was attributed to be P, due to the fact that the new metabolite and 10 displayed nearly identical ECD spectra (Fig. 3B, left); for formal reasons, however, the two alkaloids have opposite descriptors, according to the Cahn–Ingold–Prelog denotion. The new fully dehydrogenated naphthylisoquinoline 12 thus possessed the stereostructure presented in Fig. 1. It was named ancistrobreveine A.

Like in the case of ent-dioncophylleine A (10) isolated from A. abbreviatus, ancistrobreveine A (12) was found to be enantiomerically pure by HPLC-ECD analysis on a chiral phase (Lux Cellulose-1), yielding only one peak (Fig. 3B, left). The ECD chromatogram monitored for one single wavelength (here at 258 nm, where 12 has a strong negative ECD signal) resulted in one single – and negative – peak. The online ECD spectra obtained from different peak positions were all identical.

HRESIMS analysis of the second new alkaloid, compound 13, gave a molecular formula of $C_{26}H_{27}NO_4$, which thus had 14 mass units more than ancistrobreveine A (12). Similar to the latter, the new compound 13 again displayed NMR signals (see Table 1) typical of a fully dehydrogenated 7,1′-coupled naphthylisoquinoline. This metabolite had nearly the same constitution as 12, but possessed a methoxy function (δ_H 3.92) at C-8 (instead of a hydroxy group as in 12) (Fig. 3A). Its nearly mirror-image-like ECD spectrum compared to that of ancistrobreveine A (12) (Fig. 3B, right) revealed the isolated metabolite to exhibit an opposite axial stereo-array. For formal reasons, however, the two compounds had the same P-descriptor according to the Cahn–Ingold–Prelog denotion. Consequently, the new alkaloid had the full stereostructure 13 as presented in Fig. 1. It was named ancistrobreveine B. Similar as in the case of 12, HPLC analysis on a chiral phase coupled to ECD spectroscopy revealed 13 to occur in the plant in an enantiomerically pure form.

According to HRESIMS and ¹³C NMR, the third new alkaloid, compound 14, likewise a minor metabolite of the root bark extract, corresponded to a molecular formula of $C_{26}H_{27}NO_4$, identical to that of ancistrobreveine B (13). NMR spectroscopic data (Table 1) again suggested the presence of a fully dehydrogenated naphthylisoquinoline alkaloid equipped with four methoxy functions, resonating at δ_H 3.30, 3.85, 3.93, and 3.97. Different from 13, the new compound 14 displayed a normal, not high-field shifted signal of Me-2′ (δ_H 2.28) and two NOESY
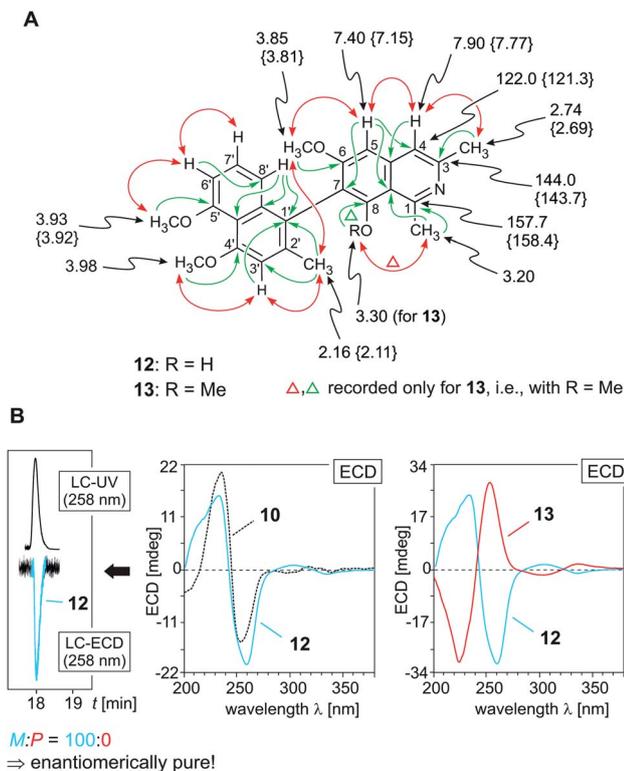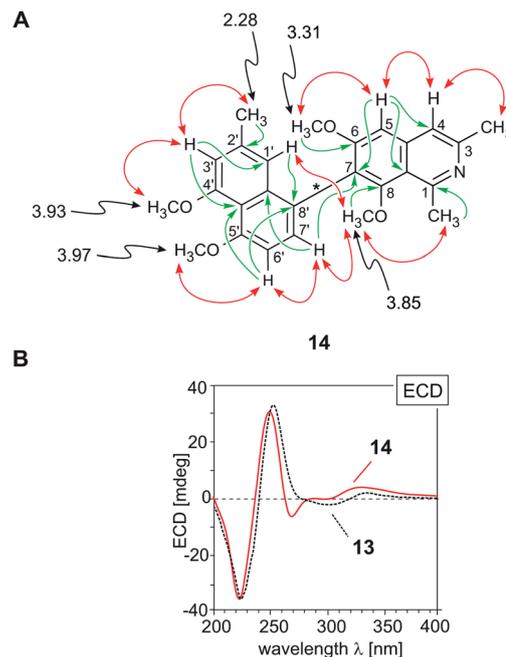
Fig. 3 (A) Selected ^{1}H and ^{13}C NMR data (in methanol-d_4, δ in ppm), HMBC (single green arrows), and NOE interactions (double red arrows) of ancistrobreveines A (**12**) and B (**13**); the values of **13** that are different from those of **12** are given in {}; (B) HPLC-ECD analysis on a chiral phase (Lux Cellulose-1) giving only one peak, revealing that in *A. abbreviatus* **12** occurs in an enantiopure form (left), and assignment of the absolute axial configuration of **12** (center) and **13** (right) by comparison of the ECD spectrum of **12** with that of the 7,1′-coupled known[27] *M*-configured *ent*-dioncophylleine A (**10**; for its structure, see Fig. 1), and by comparison of the ECD spectrum of **13** with that of **12**.

correlation sequences, {H-1′ ↔ Me-2′ ↔ H-3′ ↔ OMe-4′} and {OMe-5′ ↔ H-6′ ↔ H-7′}, which evidenced the naphthalene portion to be connected to the isoquinoline part *via* its methyl-free ring, establishing C-8 to be the coupling position. This was further confirmed by HMBC interactions from H-1′ (δ_H 6.68) and H-6′ (δ_H 6.96) to the quaternary carbon atom C-8′ (δ_C 160.7). In the isoquinoline moiety, C-7 was determined to be the axis-bearing carbon atom, as obvious from NOESY interactions in the series {H-4 ↔ H-5 ↔ OMe-6} and from HMBC cross peaks to C-7 (δ_C 128.1), observed for both, the signals of H-5 (δ_H 7.37) and H-7′ (δ_H 7.26). In conclusion, the new alkaloid **14** was a 7,8′-coupled naphthylisoquinoline with the constitution shown in Fig. 4A.

Its absolute axial configuration was deduced by comparison of its ECD spectrum with those of *ent*-dioncophylleine A (**10**)[27] (see ESI†) and ancistrobreveine B (**13**) (Fig. 4B), the latter differing from the new alkaloid **14** only by the position of the methyl group on the naphthalene moiety. The influence of that methyl group on the ECD behavior can be assumed as small compared with that of the large naphthalene chromophore. The ECD spectrum of the new metabolite **14** was nearly identical to

Fig. 4 (A) Selected ^{1}H NMR data (in methanol-d_4, δ in ppm), HMBC (single green arrows), and NOE interactions (double red arrows) of ancistrobreveine C (**14**); (B) assignment of the absolute axial configuration of **14**, by comparison of its ECD spectrum with that of the structurally related likewise *P*-configured, but 7,1′-coupled ancistrobreveine B (**13**, for its structure, see Fig. 1).

that of **13** and virtually opposite to that of **10**, which evidenced the biaryl axis of the isolated 7,8′-linked alkaloid to be *P*-configured. The new compound **14** thus had the full stereostructure presented in Fig. 1; it was named ancistrobreveine C.

HPLC-UV analysis of **14** on a chiral phase (Lux Cellulose-1) resulted in a nearly unresolved chromatographic peak (Fig. 5A, left), but the ECD trace of an HPLC run at 255 nm showed a small negative signal at the rising slope of the UV-detected peak and a large positive one on the descending side (see ESI†), thus suggesting that in the plant ancistrobreveine C (**14**) occurs in a not entirely enantiopure form. This was further confirmed by full LC-ECD spectra recorded online, in stopped-flow mode, directly taken at the left slope of the UV peak and at the right one, giving mirror-imaged ECD curves (Fig. 5A).

Consequently, one enantiomer indeed eluted faster than the other one, but the interactions of the two enantiomers with the adsorbent material were not different enough to achieve a baseline resolution of these two compounds. The more rapidly eluting minor peak (peak **A**, Fig. 5A, left) was easily identified to correspond to the *M*-configured enantiomer of ancistrobreveine C, *ent*-**14**, as obvious from its ECD curve, which was fully opposite to that of the prevalent enantiomer **14** (peak **B**, Fig. 5B, right). The ratio of the two enantiomers, **14** : *ent*-**14**, was determined to be *ca.* 93 : 7.

As in numerous earlier cases,[33–38] this finding again demonstrated the usefulness of the hyphenation of HPLC with ECD spectroscopy. This analytical device permitted to reliably distinguish between the two enantiomers of **14**, although they
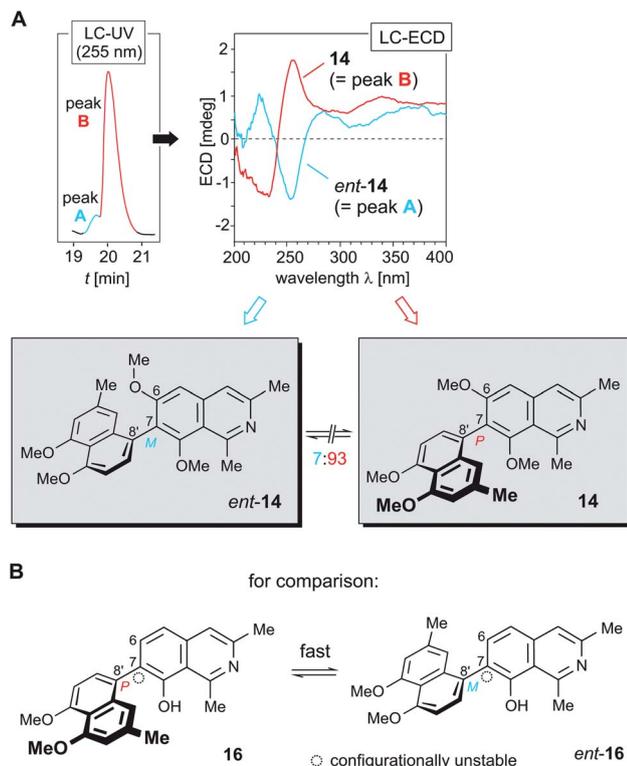
Fig. 5 (A) HPLC-ECD analysis on a chiral phase (Lux Cellulose-1), revealing that in *A. abbreviatus* 14 occurs as a mixture of its two enantiomers 14 and *ent*-14 in a ratio of *ca.* 93 : 7; (B) the structurally closely related, likewise 7,8′-coupled dioncophylleine D (16),[27] which lacks a methyl group *ortho* to its biaryl axis: this structural feature results in the occurrence of 16 as a racemic mixture of its rapidly interconverting enantiomers.

exhibited only slightly different retention times and were present in quite different concentrations.

Ancistrobreveine C (14) is the as yet only example of a 7,8′-linked fully dehydrogenated naphthylisoquinoline alkaloid that is optically active. The only other known alkaloid of this coupling type with a similar molecular framework, dioncophylleine D (16)[27] (Fig. 5B), a Dioncophyllaceae-type alkaloid isolated from the Malaysian liana *A. benomensis*, was found to occur as a racemic mixture of its two rapidly interconverting atropo-enantiomers, which are configuratively unstable due to the lack of an oxygen function at C-6.[27]

HRESIMS analysis of the fourth new metabolite (*m/z* 418.1925 [M + H]$^+$), compound 6, again gave a molecular formula of $C_{26}H_{27}NO_4$. Likewise similar to the ancistrobreveines B (13) and C (14), its ^{1}H NMR spectrum (Table 1) showed the chemical shifts of a fully dehydrogenated naphthylisoquinoline with four *O*-methyl groups (δ_H 3.92, 3.93, 3.98, and 4.25), but its proton pattern in the aromatic region was distinctly different from those of the 7,1′- and 7,8′-coupled alkaloids described above.

In the naphthalene part, two *meta*-coupled protons (δ_H 6.42 and 6.78) were observed, together with an AB spin system (δ_H 6.97 and 7.14), indicating that either C-8′ or C-6′ was the axis-bearing carbon atom. The latter possibility was excluded as

one of the protons of the AB spin system, the doublet signal at δ_H 6.97, was located at C-6′, as obvious from a COSY correlation between H-6′ and H-7′ (δ_H 7.14). NOESY cross-peaks in the series {H-1′ ↔ Me-2′ ↔ H-3′ ↔ OMe-4′} and joint HMBC interactions from H-1′ (δ_H 6.42) and H-6′ to C-8′ assigned the coupling position in the naphthalene part to be at C-8′ (δ_C 124.2) (Fig. 6A).

In the isoquinoline subunit, ^3J HMBC correlations from H-7 (δ_H 7.20) and H-7′ to C-5 (δ_C 116.9) established the biaryl axis to be located at C-5, which was confirmed by the NOESY correlation sequence {Me-1 ↔ OMe-8 ↔ H-7 ↔ OMe-6}. The new alkaloid 6 was hence 5,8′-coupled and possessed the constitution as presented in Fig. 6A. Its absolute axial configuration was deduced to be *M*, since it displayed a virtually mirror-imaged ECD spectrum (Fig. 6B, center) compared to that of the constitutionally identical ancistrolikokine J_3 (*ent*-6),[21] which is *P*-configured at the biaryl axis. This alkaloid has only recently been identified in the Congolese liana *A. likoko* and represents the enantiomer of this 5,8′-linked compound 6 isolated from *A. abbreviatus*. The new alkaloid 6 presented here thus had the full absolute stereostructure shown in Fig. 1. In continuation of the series of fully dehydrogenated metabolites discovered in the roots of *A. abbreviatus*, it was named ancistrobreveine D.

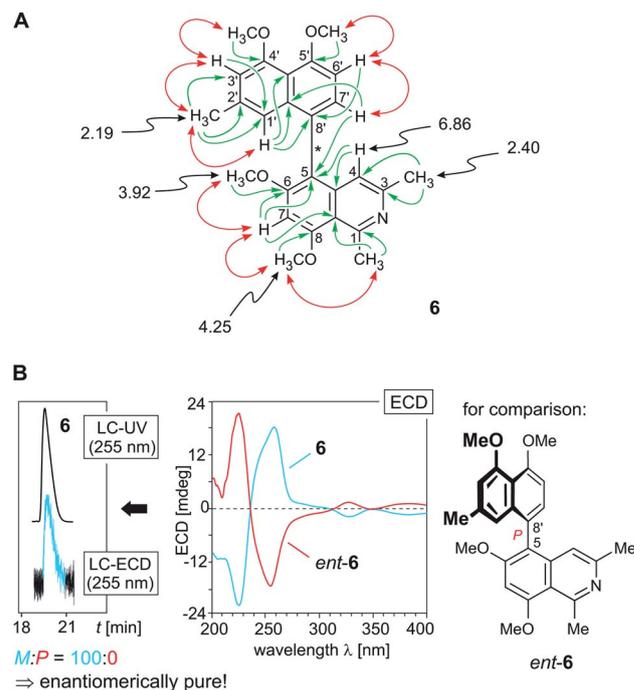Investigations on the enantiomeric purity of 6 by chromatography on a chiral phase coupled to ECD spectroscopy

Fig. 6 (A) Selected ^{1}H NMR data (in methanol-d_4, δ in ppm), HMBC (single green arrows), and NOE interactions (double red arrows) of ancistrobreveine D (6); (B, right) assignment of the absolute axial configuration of 6 by comparison of its ECD spectrum with that of the known,[21] likewise 5,8′-coupled, but *P*-configured ancistrolikokine J_3 (*ent*-6), earlier isolated from *A. likoko*, which is the enantiomer of ancistrobreveine D; (B, left) HPLC-ECD analysis on a chiral phase (Lux Cellulose-1) giving only one peak, revealing that 6 occurs in *A. abbreviatus* in an enantiopure form.

resulted in only one peak. The online ECD spectra, which were monitored in intervals at the left and the right slopes of the peak, were all identical. In the plant, ancistrobreveine D (**6**) was thus present in an enantiopure form, similar to its *P*-configured enantiomer ancistrolikokine J$_3$ (*ent*-**6**), which was likewise found to be produced stereochemically pure in *A. likoko*.[21]

Antileukemic activities of the ancistrobreveines A–D and of related fully dehydrogenated naphthylisoquinoline alkaloids

Multi-drug resistance (MDR) leading to recurrent and refractory leukemia is one of the major problems in the treatment of this severe malignancy.[39,40] Overexpression of P-glycoprotein (P-gp), an ATP-dependent drug pump, is considered to be the most important factor for the development of MDR, because it results in an increased drug efflux and, thus, in a dramatic reduction of intracellular drug concentrations of the antileukemic compounds in the cells.[41–43] Therefore, the search for novel therapeutic agents to overcome MDR[44,45] and to efficiently target leukemia is still an urgent task.

More recently, some representatives of naphthylisoquinolines possessing a fully dehydrogenated isoquinoline portion were found to strongly inhibit the viability of drug-sensitive CCRF-CEM leukemic cells and their multidrug-resistant P-gp-overexpressing subline, CEM/ADR5000.[21,30] In particular, the 5,1′-coupled ancistrobenomine B (**5**)[30] (Fig. 1) from the Chinese liana *A. tectorius*, additionally equipped with a hydroxymethylene function at C-3, and the 5,8′-linked ancistrolikokine G (**7**)[20,21] (Fig. 1), a minor metabolite of the Congolese liana *A. likoko*, attracted attention, since they inhibited cell proliferation at a low micromolar range, revealing only a small degree of cross-resistance, or, as in the case of ancistrolikokine J$_2$ (**8**)[21] (Fig. 1), likewise from *A. likoko*, even collateral sensitivity.[46] The alkaloid **8** displayed a more pronounced growth-retarding activity towards the CEM/ADR5000 cells as compared to that against the parental CCRF-CEM cell line (Table 2).[21] This finding indicated that these alkaloids may bear a substantial therapeutic potential.

Here, we report on the evaluation of the antileukemic effects of 6-*O*-methylhamateine (**4**), *ent*-dioncophylleine A (**10**), and the four new ancistrobreveines A–D (**12–14**, and **6**) (Table 2), especially focussing on the influence of the position of the biaryl linkage in these fully dehydrogenated naphthylisoquinoline alkaloids and on the impact of axial chirality on the cytotoxic activities of these compounds.

The lymphoblastic leukemia cells were treated with different concentrations of the respective naphthylisoquinolines in a range from 0.001 to 100 μM or with the reference drug doxorubicin. Cell viability was assessed by the resazurin assay.

Within this series of six naphthylisoquinolines exhibiting four different coupling types (5,1′, 5,8′, 7,1′, and 7,8′), the 5,1′-linked 6-*O*-methylhamateine (**4**) was by far the most potent compound (Table 2). Dose–response curves of **4** (Fig. 7) revealed excellent half-maximum inhibitory concentrations

Table 2 IC$_{50}$ values (μM) of drug-sensitive human lymphoblastic CCRF-CEM and multidrug-resistant CEM/ADR5000 leukemia cells treated with ancistrobreveines A (**12**), B (**13**), C (**14**), and D (**6**), 6-*O*-methylhamateine (**4**), and *ent*-dioncophylleine A (**10**), or with doxorubicin

| Compound | IC$_{50}$[a] [μM] | | |
	CCRF-CEM	CEM/ADR5000	Degree of resistance[b]
Doxorubicin	0.017 ± 0.002	30.07 ± 11.81	1769
4	3.948 ± 1.667	5.523 ± 1.756	1.40
5[c]	3.501 ± 0.069	21.38 ± 1.302	6.11
6	24.27 ± 1.398	29.51 ± 4.297	1.21
7[c]	4.731 ± 0.690	7.731 ± 0.992	1.63
8[c]	23.34 ± 1.630	20.06 ± 2.231	0.86
10	26.88 ± 0.285	31.41 ± 3.550	1.17
12	24.78 ± 3.910	>100	—
13	39.94 ± 6.864	50.64 ± 7.465	1.27
14	12.44 ± 1.661	12.64 ± 2.290	1.02

[a] The lymphoblastic leukemia cells were treated with different concentrations of **4**, **6**, **10**, and **12–14** or with doxorubicin. Cell viability was assessed by the resazurin assay. Mean values and standard deviation of three independent experiments with each six parallel measurements are shown. [b] The degrees of resistance were calculated by division of the IC$_{50}$ values of the compounds for CEM/ADR5000 cells by the corresponding IC$_{50}$ values for CCRF/CEM cells. [c] Values reported earlier, see ref. 21 and 30.

in the low micromolar range towards CCRF-CEM cells (IC$_{50}$ = 3.948 μM), and against the resistant CEM/ADR5000 subline (IC$_{50}$ = 5.523 μM). The MDR leukemia cell line CEM/ADR5000, which is known to be highly resistant to the standard drug doxorubicin (*ca.* 1.770-fold compared to CCRF-CEM), showed only a low degree of cross-resistance for 6-*O*-methylhamateine (**4**) (1.4-fold) (Table 2), thus indicating that **4** may be a strong and efficient inhibitor also for other drug-sensitive cancer cells.

Considerable antiproliferative activities against the drug-sensitive CCRF-CEM and the multidrug-resistant CEM/
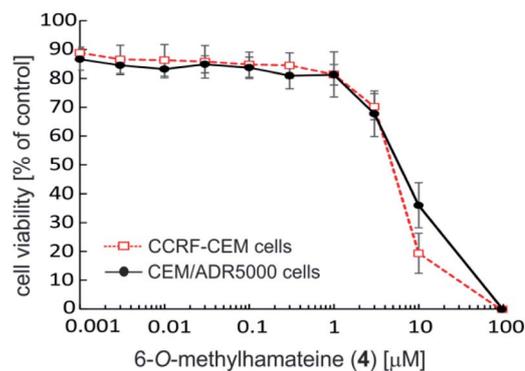

Fig. 7 Cytotoxic activity of 6-*O*-methylhamateine (**4**) against parental drug-sensitive CCRF-CEM leukemia cells and their multi-drug resistant subline, CEM/ADR5000. The compound was dissolved in DMSO (<1%) and cell culture medium at concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, or 100 μM. Cell viability was assessed by the resazurin assay. Mean values and standard deviation of three independent experiments with each six parallel measurements are shown.

ADR5000 leukemia cells were also observed for the 7,8′-coupled ancistrobreveine C (**14**) (Table 2). Its growth-inhibitory potential was distinctly lower than that of **4**, with an IC_{50} value of 12.44 μM determined in CCRF-CEM cells, showing that, in comparison to **4**, the cytotoxicity of **14** was reduced down to *ca.* one third. No cross-resistance was observed for ancistrobreveine C (**14**). Its IC_{50} values were nearly the same in both cell lines, thus inhibiting the cell viability of sensitive and multidrug-resistant leukemia cells with similar efficacies (Table 2).

The three 7,1′-coupled alkaloids ancistrobreveines A (**12**) and B (**13**), and *ent*-dionocophylleine A (**10**), structurally closely related to the 7,8′-linked ancistrobreveine C (**14**), by contrast, showed only moderate to weak antiproliferative activities against leukemia cancer cells. As outlined in Table 2, their IC_{50} values ranged from 24.8 to 50.6 μM. Ancistrobreveine A (**12**) even did not display any considerable cytotoxic effect at all against the multidrug-resistant CEM/ADR5000 subline. Similar to ancistrobreveine C (**14**), two of the 7,1′-coupled compounds, **10** and **13**, showed growth inhibitory activities that were nearly the same in both cell lines, thus they did not exhibit any cross-resistances.

The previously investigated 5,8′-coupled fully dehydrogenated ancistrolikokine G (**7**),[21] with its particular OH/OMe-substitution pattern in the naphthalene and isoquinoline portions, had displayed strong cytotoxic effects against CCRF-CEM and CEM/ADR5000 leukemia cells, with IC_{50} values in the low micromolar range (Table 2). The now isolated *O*-permethylated ancistrobreveine D (**6**), by contrast, exhibited only moderate growth-retarding activities: compared to the IC_{50} values of ancistrolikokine G (**7**),[21] the growth-inhibitory potential of compound **6** was reduced by a factor of *ca.* 5.1 for the drug-sensitive CCRF-CEM cells, and by a factor of *ca.* 3.8 for the multi-drug resistant subline CEM/ADR5000. These test results showed that the degree of *O*-methylation in the two molecular portions may have a distinct impact on the antileukemic activities of 5,8′-coupled naphthylisoquinoline alkaloids.

The position of the biaryl axis seemed to be of crucial importance, too, as seen from the fact that the 5,1′-linked 6-*O*-methylhamateine (**4**) strongly inhibited the cell viability of leukemia cells (Fig. 7), whereas the related 5,8′-coupled ancistrobreveine D (**6**), which, in other words, differed from **4** only by the position of its methyl group in the naphthalene subunit, was only moderately active.

In summary, from previous studies[21,30] and the results presented here, 5,1′- and 5,8′-coupled alkaloids were the most potent ones among the fully dehydrogenated naphthylisoquinolines tested. Most of these alkaloids were found to inhibit drug-sensitive CCRF-CEM and multidrug-resistant CEM/ADR5000 leukemia cells with nearly similar efficacies, revealing only minimal (*ca.* 1.2- to 6-fold) or even no cross-resistances. With respect to these findings, the compounds might also serve as promising agents for the treatment of cancer cells unresponsive towards chemotherapeutics routinely used in clinical treatment.

Experimental

General experimental procedures

All organic solvents were of analytical-grade quality. Ultra-pure water was obtained from an Elga Purelab Classic system. Optical rotations were measured on a Jasco P-1020 polarimeter operating with a sodium light source (λ = 589 nm). UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer. IR spectra were taken on a Jasco FT/IR-410 spectrometer. ECD spectra were acquired on a Jasco J-715 spectropolarimeter at room temperature, using a 0.1 cm standard cell and spectrophotometric-grade MeOH. The ECD data were processed using SpecDis.[47,48] 1D and 2D NMR measurements were performed on a Bruker DMX 600 instrument using methanol-d_4, with the ^1H or ^{13}C signals of methanol (^1H, δ 3.31 ppm, ^{13}C, δ 49.0 ppm) in the deuterated solvent as the internal reference. Chemical shifts (δ) are reported in parts per million (ppm), and coupling constants (J) are given in Hertz (Hz). NMR signal multiplicities are denoted as singlet (s), doublet (d), doublet of doublets (dd), quartet (q), or multiplet (m). LC-MS investigations were performed on an Agilent 1100 system equipped with a photodiode array (PDA) detector and an Esquire 3000 Plus ion-trap mass spectrometer using an electrospray ionization interface. HRESIMS measurements were acquired in positive mode on a Bruker Daltonics micrOTOF-focus mass instrument. Preparative HPLC was carried out on a Jasco System (PU-1580 Plus) in combination with UV/Vis detection at 200–680 nm (Jasco MD-2010 Plus diode array detector) at room temperature. For the isolation and purification of the constituents of the root bark extracts, a SymmetryPrep™ C18 column (19 × 300 mm, 7 μm, Waters) was used; mobile phases: (A) 90% H_2O with 10% MeCN (0.05% trifluoroacetic acid) and (B) 90% MeCN with 10% H_2O (0.05% trifluoroacetic acid). For further purification, a Waters XSelect HSS PFP column (10 × 250 mm, 5 μm) was applied; mobile phases: (A′) 90% H_2O with 10% MeOH (0.05% trifluoroacetic acid) and (B′) 90% MeOH with 10% H_2O (0.05% trifluoroacetic acid). The enantiomeric purities of the fully dehydrogenated compounds were analyzed by HPLC-ECD employing a chiral Lux Cellulose-1 column (250 × 4.6 mm, 5 μm, Phenomenex), with H_2O (0.05% trifluoroacetic acid) (A″) and MeCN (0.05% trifluoroacetic acid) (B″) as the eluents.

Plant material

Roots of *Ancistrocladus abbreviatus* Airy Shaw (Ancistrocladaceae) were collected by late Prof. L. Aké Assi (Centre National de Floristique, Université d'Abidjan) in May 1996, in the Parc National de Taï, in the South-Western region of the Ivory Coast. A voucher specimen (no. 3) has been deposited at the Herbarium Bringmann, Institute of Organic Chemistry, University of Würzburg.

Extraction and isolation

Air-dried, powdered root bark material (*ca.* 420 g) of *A. abbreviatus* was repeatedly extracted for 1 h with MeOH (2 × 3

L) at 40 °C, assisted by ultrasonication. The extract was filtered and then concentrated *in vacuo*. The solid residue was dissolved in MeOH/H$_2$O (9 : 1) and repeatedly washed with *n*-hexane to remove the non-polar impurities. The methanolic layer was dried under reduced pressure to yield *ca.* 17.5 g of a viscous alkaloid-rich solution. It was directly subjected to silica gel column chromatography (CC) using a linear solvent system of MeOH in CH$_2$Cl$_2$ (10 → 80%).

Resolution of fraction F1 (2.1 g) on a SymmetryPrep C18 column, using the solvent systems A and B, with a linear gradient (0 min 26% B, 27 min 65% B), at a flow rate of 10 mL min^{-1}, furnished some alkaloid-containing subfractions, which were further purified by semi-preparative HPLC on an XSelect HSS PFP column. Applying isocratic solvent systems of the mobile phases A′ and B′ (v/v), containing 57 or 59% of B′, at a flow rate of 4 mL min^{-1}, provided pure ancistrobreveine A (12) (1.4 mg) (retention time 15.4 min), ancistrobreveine B (13) (0.9 mg) (retention time 16.0 min), ancistrobreveine C (14) (2.3 mg) (retention time 22.5 min), 6-*O*-methylhamateine (4) (0.5 mg) (retention time 23.2 min), *ent*-dioncophylleine A (10) (0.6 mg) (retention time 23.4 min), and ancistrobreveine D (6) (1.1 mg) (retention time 26.2 min).

Ancistrobreveine A (12). Yellow amorphous solid; $[\alpha]_D^{20}$ −11.1 (*c* 0.1, MeOH); UV (MeOH) (log *ε*): λ_{max} 234 (2.6) and 258 (2.1) nm; ECD (*c* 0.1, MeOH) λ_{max} (Δ*ε*): 218 (+10.5), 223 (+12.7), 231 (+14.9), 258 (−19.1), 302 (+0.9), and 334 (−0.8) nm; IR (ATR) ν_{max}: 3363, 2925, 1668, 1612, 1199, and 1131 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS: *m/z* 404.1861 [M + H]$^+$ (calcd for C$_{25}$H$_{26}$NO$_4$, 404.1856).

Ancistrobreveine B (13). Yellow amorphous solid; $[\alpha]_D^{23}$ −180 (*c* 0.04, MeOH); UV (MeOH) (log *ε*): λ_{max} 228 (3.2), 257 (2.8), 305 (2.6), and 334 (2.5) nm; ECD (*c* 0.1, MeOH) λ_{max} (Δ*ε*): 223 (−5.0), 252 (+4.6), 299 (−0.3), and 335 (+0.2) nm; IR (ATR) ν_{max}: 3362, 2979, 1670, 1461, 1385, 1258, 1200, 1132, and 947 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS: *m/z* 418.2015 [M + H]$^+$ (calcd for C$_{26}$H$_{28}$NO$_4$, 418.2013).

Ancistrobreveine C (14). Yellow amorphous solid; $[\alpha]_D^{20}$ +5.4 (*c* 0.10, MeOH); UV (MeOH) (log *ε*): λ_{max} 236 (3.8) and 258 (3.4) nm; ECD (*c* 0.1, MeOH) λ_{max} (Δ*ε*): 223 (−36.2), 250 (+30.1), 269 (−6.9), 286 (−0.7), 303 (−0.6), and 331 (+3.3) nm; IR (ATR) ν_{max}: 3383, 2900, 1666, 1585, 1375, 1195, and 1112 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS: *m/z* 418.2013 [M + H]$^+$ (calcd for C$_{26}$H$_{28}$NO$_4$, 418.2013).

Ancistrobreveine D (6). Yellow amorphous solid; $[\alpha]_D^{20}$ −11.4 (*c* 0.10, MeOH); UV (MeOH) (log *ε*): λ_{max} 234 (2.8) and 258 (2.1) nm; ECD (*c* 0.1, MeOH) λ_{max} (Δ*ε*): 207 (−13.9), 223 (−24.6), 257 (+19.8), 274 (+2.1), 326 (−1.8), and 380 (−0.9) nm; IR (ATR) ν_{max}: 3349, 2945, 1673, 1349, 1198, and 1015 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS: *m/z* 418.1925 [M + H]$^+$ (calcd for C$_{26}$H$_{28}$NO$_4$, 418.2013).

Known alkaloids isolated

The two fully dehydrogenated naphthylisoquinoline alkaloids 6-*O*-methylhamateine (4) and *ent*-dioncophylleine A (10) (Fig. 1), well known from previous phytochemical studies on *A. benomensis* and *A. cochinchinensis*, now isolated for the first time

from root bark extracts of *A. abbreviatus*, were found to be identical in their spectroscopic, physical, and chromatographic behavior with the data reported previously.[26,27]

Antileukemic assay

The cytotoxic effects (Table 2) of ancistrobreveines A (12), B (13), C (14), and D (6), 6-*O*-methylhamateine (4), and *ent*-dioncophylleine A (10) on drug-sensitive leukemia CCRF-CEM and multidrug-resistant P-glycoprotein-overexpressing CEM/ADR5000 cells[49–51] were monitored by the resazurin assay as previously described.[52,53] Doxorubicin (Sigma Aldrich, Munich, Germany) was applied as the positive control, while DMSO, used to dissolve the compounds, was employed as the negative control. The highest concentration of DMSO was less than 1.0%. Fluorescence was measured on an Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany), using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. All experiments were performed in triplicate. The viability was evaluated based on the comparison with untreated cells. IC$_{50}$ values represent the concentrations of the compounds required to inhibit 50% of cell proliferation. They were calculated from a calibration curve by linear regression using Microsoft Excel.[54,55]

Conclusions

The alkaloid pattern of the West African liana *Ancistrocladus abbreviatus* is characterized by a remarkable structural diversity, comprising naphthylisoquinoline alkaloids of four different coupling types (5,1′, 5,8′, 7,1′, and 7,8′),[5–11] among them Ancistrocladaceae-type compounds,[5,6,10,11] which are 6-oxygenated and 3*S*-configured, but also Dioncophyllaceae-type alkaloids[5,9,10] were found, which are deoxygenated at C-6 and have the *R*-configuration at C-3. Most of them represent naphthyltetrahydroisoquinolines,[5–7,9–11] whereas compounds possessing a dihydroisoquinoline subunit have been isolated much less frequently.[8,11] The newly discovered series of six naphthylisoquinoline alkaloids, presented in this paper, are all equipped with a fully dehydrogenated isoquinoline portion, comprising compounds displaying the four coupling types mentioned above. Four of these metabolites, named ancistrobreveines A–D (12–14, and 6), were identified for the first time in nature, whereas 6-*O*-methylhamateine (4) and *ent*-dioncophylleine A (10) had been known from previous phytochemical studies on related *Ancistrocladus* species.[26,27] Ancistrobreveine C (14) is the first example of a 7,8′-linked fully dehydrogenated naphthylisoquinoline that is configurationally stable at the biaryl axis. It was found not to occur in an enantiopure form, but accompanied by 7% of its atropo-enantiomer.

The occurrence of such a large series of fully dehydrogenated naphthylisoquinoline alkaloids in a distinct species is remarkable since so far only a total of 14 representatives with such a structural entity had been identified in Ancistrocladaceae lianas,[20,21,26–30] with very few examples from African species in particular. Only the Malaysian highland liana *A. benomensis* was likewise found to produce such a high number of fully

dehydrogenated compounds.[27] Of all of the *Ancistrocladus* plants investigated so far,[5,12,56] among them 16 accepted[5,15–17,19–21,23,57–59] and six as yet botanically undescribed[18,22,24,25,60,61] species, only four taxa were found to contain naphthylisoquinolines with a fully dehydrogenated isoquinoline subunit.[20,21,26–30]

Furthermore, 6-*O*-methylhamateine (**4**) and ancistrobreveine C (**14**) have proven to be potent bioactive compounds displaying good to strong inhibitory effects against drug-sensitive (CCRF-CEM) and multidrug-resistant (CEM/ADR5000) leukemia cells. Since the efficiency of many recommended drugs routinely used for the treatment of malignant disorders is threatened by the increasing emergence of resistance,[39–45] the development of new potent agents preventing the overexpression of Pgp is an important goal. The promising antiproliferative activities of fully dehydrogenated naphthylisoquinoline alkaloids warrant more-in-depth studies regarding their potential as effective MDR suppressors. This work is in progress.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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