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New 1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrimidinium alkaloids (phloeodictynes) from the New Caledonian shallow-water haplosclerid sponge *Oceanapia fistulosa*. Structural elucidation from mainly LC-tandem-MS-soft-ionization techniques and discovery of antiplasmodial activity

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Received 24th October 2003, Accepted 23rd December 2003
First published as an Advance Article on the web 11th February 2004

We report here on new 6-hydroxy-1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrimidinium alkaloids, belonging to the phloeodictyne family, isolated from the haplosclerid sponge *Oceanapia* [= *Phloeodictyon*] *fistulosa* (Bowerbank, 1873) from New Caledonian shallow waters. Online LC-ESI-MS analysis, coupled to tandem fragmentation experiments on the crude alkaloid mixture, allowed us to clarify their flat structures, including structural isomers. At least 25 different components, of which 17 are new members with variable terminus and length chains, were characterised, besides less abundant analogues bearing a thioethylguanidine side chain. Crude mixtures and HPLC enriched fractions proved active against chloroquine-resistant *Plasmodium falciparum*, with IC_{50} values ranging from 0.6 to 6 μM, while cytotoxicity against human A-549 cell line was low. This makes these alkaloids a good prospect as leads for novel antimalarial agents.

Introduction

Novel 1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrimidinium alkaloids, called phloeodictynes, endowed with strong antibacterial and cytotoxic activity towards human cancer cells, have been isolated from the haplosclerid sponge, *Phloeodictyon* [= *Oceanapia*] sp. from deep New Caledonian waters.[1] The phloeodictyne framework is characterized by a fused alkaloidal skeleton, 1,2,3,4-tetrahydropyrrolo[1,2-*a*]pyrimidinium, bearing a C-6 variable-length alkyl (or alkenyl) side chain and an *N*-1 four/five methylene chain ending in a guanidine group, while a C-7 thioethylguanidine chain may be present or not (Chart 1).[1] Included are phloeodictyne **4**,**5a** (= phloeodictine A4),[2,1b] phloeodictyne **4**,**7a** (= phloeodictine A2),[1b] phloeodictyne **4**,**8i** (= phloeodictine A7),[1b] phloeodictyne **4**,**9a** (= phloeodictine A),[1a] phloeodictyne **5**,**7a** (= phloeodictine A1),[1b] phloeodictyne **5**,**4a** (= phloeodictine A5),[1b] phloeodictyne **5**,**5a** (= phloeodictine A3),[1b] phloeodictyne **5**,**8i** (= phloeodictine A6),[1b] phloeodictyne **B** (= phloeodictine B),[1a] phloeodictyne **C1** (= phloeodictine C1),[1b] and phloeodictyne **C2** (= phloeodictine C2)[1b] (Chart 1). The total synthesis of phloeodictyne **5**,**7a**[1b] in racemic form has recently been reported.[3]

While the indolizine nucleus is known in nature, for example in the octahydro form in alkaloids from marine sponges,[4] a rare example of pyrrolo[1,2-*a*]pyrimidines is represented by adenopeptin, an antitumor peptidic alkaloid isolated from the culture broth of a filamentus fungus, *Chrysosporium* sp.[5]

We report here that phloeodictynes are not confined to the deep-sea species of *Phloeodictyon* [= *Oceanapia*] sp., which are also found in a wide structural variety in a shallow-water New Caledonian sponge of the same genus, *Oceanapia fistulosa* (Bowerbank, 1873). We report also that, for some of these new phloeodictynes, *in vitro* antiplasmodial activity prevails over antitumour activity, making good prospects for novel antimalarial agents.

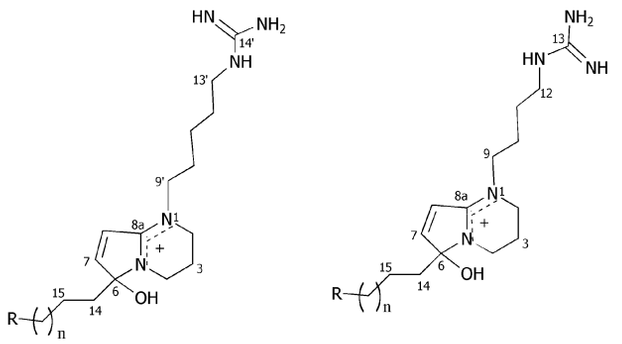
Results and discussion

Structural studies

O. fistulosa proved to be a rich source of both the known (see above and Chart 1)[1] and new phloeodictynes **4**,**5i**; **4**,**6a**; **4**,**6i**; **4**,**7i**; **4**,**8a**; **4**,**10a**; **4**,**10i**; **4**,**11a**; **5**,**4i**; **5**,**5i**; **5**,**6i**; **5**,**7i**; **5**,**8a**; **5**,**9a**; **5**,**9i**; **5**,**10a** and **5**,**10i**, besides a number of less well characterized minor analogues bearing a thioethylguanidine side chain at C-7 (Chart 1). The complexity of the mixtures, and the much similar behaviour of their components, prevented their isolation in pure form. However, enriched mixtures suitable for bioassays could be obtained, while modern soft-ionization, tandem mass spectrometry allowed adequate molecular characterization.

^{1}H NMR spectra in CD_3OD of the crude mixture of phloeodictynes obtained by reversed-phase chromatography of a methanolic extract of the sponge, in comparison with previous work, showed (i) characteristic deshielded doublets at δ_H = 7.29 and 7.06 ppm attributable to 7-H and 8-H, (ii) typical patterns at δ_H = 5.80, 5.01 and 4.92, attributable to a terminal alkenyl group, the single unsaturated point along the aliphatic chain,

DOI: 10.1039/b313348f



A5 5,4a n= 4, R= -$CH_2CH{=}CH_2$
5,4i n= 4, R= -$CH(CH_3)_2$
A3 5,5a n= 5, R= -$CH_2CH{=}CH_2$
5,5i n= 5, R= -$CH(CH_3)_2$
5,6i n= 6, R= -$CH(CH_3)_2$
A1 5,7a n= 7, R= -$CH_2CH{=}CH_2$
5,7i n= 7, R= -$CH(CH_3)_2$
5,8a n= 8, R= -$CH_2CH{=}CH_2$
A6 5,8i n= 8, R= -$CH(CH_3)_2$
5,9a n=9, R= -$CH_2CH{=}CH_2$
5,9i n=9, R= -$CH(CH_3)_2$
5,10a n=10, R= -$CH_2CH{=}CH_2$

A4 4,5a n= 5, R= -$CH_2CH{=}CH_2$
4,5i n= 5, R= -$CH(CH_3)_2$
4,6a n= 6, R= -$CH_2CH{=}CH_2$
4,6i n= 6, R= -$CH(CH_3)_2$
A2 4,7a n= 7, R= -$CH_2CH{=}CH_2$
4,7i n= 7, R= -$CH(CH_3)_2$
4,8a n= 8, R= -$CH_2CH{=}CH_2$
A7 4,8i n= 8, R= -$CH(CH_3)_2$
A 4,9a n= 9, R= -$CH_2CH{=}CH_2$
4,10a n=10, R= -$CH_2CH{=}CH_2$
4,10i n=10, R= -$CH(CH_3)_2$
4,11a n=11, R= -$CH_2CH{=}CH_2$

B m= 4, $\Delta^{7,8}$
C1 m= 5
C2 m= 4

Chart 1 Structure of phloeodictynes isolated from the sponge *Oceanapia fistulosa.*The numbering used for convenience and the unprotonated form for the guanidine unit are according to reference 1b.

and (iii) doublets at δ_H = 0.87 ppm for the isopropyl group (see Experimental section). Both ^{1}H and ^{13}C NMR spectra revealed also diversification in the length of the C-6 and N-1 chains. As to the C-6 chain, it could be established from the resonances for the methyl and an olefinic ring proton in the uncrowded spectral regions (δ_H = 0.87, d, J = 6.7 Hz, and 5.80 ppm, ddt, J = 17.0, 10.2, and 6.9 Hz, respectively), that isopropyl and alkenyl terminal groups occur in a *ca.* 1 : 1 ratio. As to the *N*-1 chain, the δ_H = 1.45 m, coupled to δ_C = 24.18 t, attributable to 11′-H_2, suggests preponderance in the mixture of $\Delta^{7,8}$ non-sulfurated, isopropyl-type, phloeodictynes. In contrast, preponderance of alkenyl-type phloeodictynes was observed for *Phloeodictyon* sp.[1b]

At this point, LC-tandem-MS soft-ionization techniques proved to be a great aid in assigning the molecular structure of these alkaloids. Soft, electrospray-ionization MS spectra, obtained in the positive mode by direct infusion of the crude methanolic sponge extract, showed a complex cluster of homologues in the range *m*/*z* 390–476, with *m*/*z* 434, 448 and 460 as major signals, together with a cluster for doubly charged ions. The intensity of the latter increased on acidifying the extract. In agreement, online LC-MS analysis for both the methanolic sponge extract and the crude mixture of phloeodictynes, under the optimized chromatographic conditions given in the Experimental section, also revealed the presence of numerous chromatographically-distinct, structurally-isomeric phloeodictynes. Selection, followed by MS^2 mass fragmentation, of the molecular ion corresponding to each component in the mixture, cleanly generated the $[M^+ - H_2O]^+$ signal, which was isolated and further fragmented, yielding both an intense $[M^+ - H_2O - NH_3]^+$ signal and a diagnostic MS^3 ion deriving from the specific loss of the guanidine chain at N-1 (Chart 2).

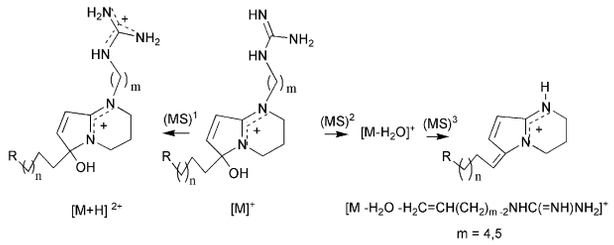


Chart 2 Tandem fragmentation patterns used for the structural characterisation of phloeodictynes **5**,**4a**–**5**,**10i** and **4**,**5a**–**4**,**11a** by ESI-MS^n experiments on the crude mixture from reversed-phase chromatography.

Performing similar tandem fragmentation experiments on the enriched mixtures of the minor compounds, all structural details, except the stereochemistry, were secured for each component. The composition of all new compounds (Table 1) was based on the close analogy of NMR and MS spectra with those for phloeodictynes **A** and **A1-A7**, for which HR-FAB-MS data were provided,[1] and was supported by a detailed tandem analysis, relying on the most recent MS techniques, as illustrated below. Data in Table 1 show also that the reversed-phase HPLC chromatographic profile correlates fairly well with the sum of chain lengths, the longer retention times pertaining to the more lipophilic, *i.e.*, the longest saturated, hydrocarbon chain.

MS experiments, carried out at low cone voltage on the crude desalted sponge extract, revealed the presence of three series of clusters with a pattern of complexity and relative abundance of the component peaks similar to the M^+ signals located in the region at *m*/*z* 390–476 (Experimental section). One of the series was constituted of doubly charged $[M^+ + H^+]^{2+}$ cluster ions of higher intensity than in spectra recorded under more energetic conditions. Another very weak cluster could be attributed to $[M^+ + HCl]^+$, on the basis of the observation of a 128 mass unit increase for the cluster at *m*/*z* 390–476 on the treatment of the crude mixture of phloeodictynes with aqueous KI. Another, very weak, cluster at *m*/*z* 546–630 attracted our attention: tandem MS fragmentation on the most intense portion revealed not only signals corresponding to the loss of a H_2O molecule, like for the major cluster at *m*/*z* 390–476, but also a more facile loss of a *m*/*z* 154 fragment (Experimental). This fragmentation resulted in the same ion peaks already observed on tandem fragmentation of the main components of the M^+ cluster for the most abundant phloeodictynes. The *m*/*z* 154 fragment, attributable to the unit $SCH_2CH_2NHC(=NH)NH_2{\cdot}HCl$, recalled the thioethylguanidine chain at C-7 of phloeodictyne B (**B**),[1a] and phloeodictynes C1 (**C1**) and C2 (**C2**).[1b] ^{1}H- and ^{13}C-NMR spectra showed a deshielded doublet for 8-H, suggesting that the sulfurated phloeodictynes in the mixture are of type **B**. The complexity of their NMR spectra indicates that the pattern of the N-1 and C-6 chains is like in the non-sulfurated analogues.

The bioactivity

The raw sponge extracts proved preliminary *in vitro* activity towards bacteria (*Staphilococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*), fungi (*Candida albicans*), and parasites (*Plasmodium falciparum*), while showing only moderate cytotoxicity towards KB and P388 tumor cell lines, suggesting possible antiplasmodial value. On this basis, an enriched mixture of phloeodictynes were positively assayed on a chloroquine-resistant FCB1 strain of the malaria parasite, *Plasmodium falciparum*. The activity level was similar to partially HPLC-purified fractions (Table 2), mirroring the high abundance of the active agents in the sponge. The activity of mixture 1, constituted of phloeodictynes **5**,**7i** and **4**,**7i** in a 7 : 3 ratio, nearly matched the level of a much used antimalarial drug, chloroquine sulfate (Table 2). This is of interest in view of

Table 1 Data from LC-ESI-MS experiments on the crude mixture of phloeodictynes

$[M]^+/m/z$	Molecular composition	Normalized abundance in the crude extract[a]	Structural characterization[b]				HPLC retention time[c]/min	Phloeodictyne
			$m+n$	m	n	R		
390	$C_{22}H_{40}N_5O$	5	9	4	5	Unsat.	3.0	**4**,**5a**
			9	5	4	Unsat.		**5**,**4a**
392	$C_{22}H_{42}N_5O$	5	9	4	5	^{i}Pr	3.0	**4**,**5i**
			9	4	5	^{i}Pr		**5**,**4i**
404	$C_{23}H_{42}N_5O$	10	10	4	6	Unsat.	3.0	**4**,**6a**
			10	5	5	Unsat.		**5**,**5a**
406	$C_{23}H_{44}N_5O$	20	10	5	5	^{i}Pr	3.0	**5**,**5i**
			10	4	6	^{i}Pr		**4**,**6i**
418	$C_{24}H_{44}N_5O$	20	11	4	7	Unsat.	4.0	**4**,**7a**
420	$C_{24}H_{46}N_5O$	50	11	5	6	^{i}Pr	4.0	**5**,**6i**
			11	4	7	^{i}Pr	5.6	**4**,**7i**
432	$C_{25}H_{46}N_5O$	70	12	5	7	Unsat.	4.0	**5**,**7a**
			12	4	8	Unsat.	4.7	**4**,**8a**
434	$C_{25}H_{48}N_5O$	100	12	5	7	^{i}Pr	5.6	**5**,**7i**
			12	4	8	^{i}Pr	6.7	**4**,**8i**
446	$C_{26}H_{48}N_5O$	56	13	5	8	Unsat.	4.7	**5**,**8a**
			13	4	9	Unsat.	6.7	**4**,**9a**
448	$C_{26}H_{50}N_5O$	95	13	5	8	^{i}Pr	6.7	**5**,**8i**
460	$C_{27}H_{50}N_5O$	90	14	5	9	Unsat.	6.7	**5**,**9a**
			14	4	10	Unsat.	7.7	**4**,**10a**
462	$C_{27}H_{52}N_5O$	30	14	5	9	^{i}Pr	7.7	**5**,**9i**
			14	4	10	^{i}Pr	11.7	**4**,**10i**
474	$C_{28}H_{52}N_5O$	44	15	5	10	Unsat.	11.7	**5**,**10a**
			15	4	11	Unsat.	7.7	**4**,**11a**
476	$C_{28}H_{54}N_5O$	26	14	5	10	^{i}Pr	11.7	**5**,**10i**

[a] From the intensities of MS signals by direct infusion both of the raw methanolic extract, or of the crude mixture of phloeodictynes from reversed-phase chromatography. [b] From MS^3 fragment experiments on the molecular ion M^+ indicated in the row. [c] From online LC-MS analysis by elution with MeOH–H_2O 85 : 15 +1.5% TFA on RP-18 stationary phase.

Table 2 Activities of phloeodictynes against chloroquine-resistant *Plasmodium falciparum* FCB1 strain and human tumour cell line A-549

Sample	FCB1 strain			A-549 cell-line
	Conc./μg cm^{-3}	Growth inhibition (%)	IC_{50}/μM	IC_{50}/μM
Crude mixture	10	100	0.98	
	1	100		
	0.1	60		
Mixture 1 (**5**,**7i**–**4**,**7i** = 7 : 3)	10	100	0.62	28
	1	73		
	0.1	11		
Mixture 2 (**4**,**10a**–**4**,**11a**–**5**,**9i** = 6 : 3 : 1)	10	97	1.09	9.7
	1	79		
	0.1	11		
Mixture 3 (**4**,**10i**–**5**,**10a**–**5**,**10i** = 6 : 3 : 1)	10	100	6.36	10.3
	1	14		
	0.1	26		
N-Methyl derivatives from the crude mixture			8.00	
Chloroquine sulfate			0.43	
Artemisin			0.025	
Vinblastine				$0.82 \cdot 10^{-3}$
Nocodazole				0.05

providing, from these phloeodictynes as leads, new drugs against such a devastating disease as malaria, which accounts for two million deaths annually and has no easy vaccine prevention.

The data in Table 2 for the enriched mixtures of phloeodictynes may serve to illustrate structure–activity relationships: it can be noticed that the length of the C-6 chain has greater influence on the bioactivity level than the nature of its terminal portion. We also noticed that on methylation of the guanidine moiety the activity was lowered. On the treatment of the crude mixture of phloeodictynes with MeI–K_2CO_3 in acetone, methylated products were formed, as evidenced by both ^{1}H NMR spectra (singlets at δ_H = 3.51 and 2.92 ppm in CD_3OD as solvent) and ESI-MS spectra (which revealed that a dimethyl derivatives were more abundant than trimethyl derivatives). The cluster suggested a mixture of complexity and relative intensities like before methylation. MS^3 tandem fragmentation experiments on the major components of the mixture showed typical ions from loss of one molecule of dimethylamine and one or two molecules of methylamine (Experimental section).

Experimental

General procedures

All evaporations were carried out at room temperature under reduced pressure. Flash chromatography (FC) was carried out on reversed-phased Merck LiChroprep RP-18 (40- 63 μm) and TLC on Merck Kieselgel 60 PF_{254} and Merck RP-18 F_{254}. HPLC was performed with a 25 × 1 cm column packed with Merck LiChrospher RP18 (7 μm), under UV monitoring at λ = 276 nm and solvent flux = 1 mL min^{-1}. ^{1}H and ^{13}C NMR spectra were taken on a Varian XL-300 spectrometer operating at 299.94 and 75.43 MHz, respectively; δ values are given in

ppm and J values in Hz with respect to the solvent (CD_3OD) residual signals (δ_H = 3.31 ppm and δ_C = 49.00 ppm). Multiplicity is from DEPT. Spectral assignments were aided by one-bond and long-range ^1H–^1H COSY and HMQC experiments. ESI-MS experiments were performed in positive ion mode on a Bruker Esquire-LC™ ion-trap mass spectrometer *via* an electrospray interface, as either direct infusion or LC-ESI-MS, with cone voltage 36~42 V, if not otherwise stated. In the latter case, the mass spectrometer was coupled with a HP Mod. 1100 liquid chromatograph equipped with a 250 × 4 mm column packed with 5 μm Merck LiChrospher RP-18, under UV-diode array monitoring at λ = 276 nm, with solvent flux = 1 mL min^{-1} 7 : 3 split for UV and ESI detectors.

Collection of the sponge and isolation of metabolites

The sponge of this study, *Oceanapia fistulosa* (Bowerbank, 1873) (Haploslerida, Oceanapiidae), was collected in 1993 by scuba diving at a depth of 45 m on the Touho bank off the eastern coast of New Caledonia Main Island. Freeze-dried sponge (575 g) was repeatedly extracted with fresh 95% EtOH, filtered and evaporated to leave a residue that was subjected to RP-18 FC, with water–MeOH gradient elution, collecting 22 fractions of 50 mL each. Fractions 8–20 (160 mg), combined on the basis of similar TLC, NMR and ESI-MS data, gave clear signals for the phloeodictynes of type A.

Positive-ion direct-infusion MS of both the desalted raw extract and crude RP-18 FC mixture gave superimposable spectra. The signals suggested a complex mixture of molecular ions in the range *m/z* 390–476, and a similar cluster for the $[M^+ + H^+]^{2+}$ ions in the region *m/z* 195.5–238.5, with similar relative intensities of the components in both the clusters (Table 1). Raw methanol extracts of the same sponge above, collected in the years 1992 and 1995, proved identical according to ESI-MS analysis. In spite of repeated efforts, optimized HPLC conditions for online LC-MS analysis (LiChrospher R-18, MeOH–H_2O 85 : 15 with 1.5% TFA added, λ = 276 nm) did not allow in most cases easy, neat separation the components.

Crude mixture of non-sulfurated phloeodictynes

UV absorptions: λ_{max} (EtOH)/nm 276 (ε, calculated with respect to the average value of the molecular weight of the components in the mixture) 3 000), 224 (8800). NMR assignments, based on previous values,[1] were confirmed by one-bond and long-range homo- and hetero- correlations: δ_H (CD_3 OD): 7.29 (d, J 6.4, 7-H), 7.06 (d, J 6.4, 8-H), 5.80 (ddt, J 17.0, 10.2, 6.9, CH_2CH=CH_2), 5.02 (dd, J 17.0, 2.0, CH_2CH=CH_aH_b), 4.90 (m, CH_2CH=CH_aH_b overlapping residual solvent signals), 3.70–3.50 (m, 2-H, 4-Ha, 9′-H), 3.35–3.20 (m, 4-H_b, 12-H, 13′-H), 2.20–2.00, 1.80–1.60, 1.40–1.10 (series of m), 0.87 (d, J 6.7, CHMe_2), with 5.80 : 0.87 ratio for a 45 : 55 mixture of allyl and isopropyl-type phloeodictynes, respectively; δ_C (CD_3 OD): 160.19 (s, C-8a), 156.54 (s, C-13 and C-14′), 154.31 (d, C-7), 140.04 (d, CH_2CH=CH_2), 120.32 (d, C-8), 114.73 (t, CH_2CH=CH_2), 99.09 (s, C-6), 53.3, 53.08 (t, C-9 and C-9′), 46.56 (t, C-2), 42.12, 42.01 (t, C-13′ and C-12), 36.86 (t, C-4), 35.91 (t, C-14), 34.84 (t, CH_2CH=CH_2), 30.63–30.16 (series of t), 29.38 (d, CHMe$_2$), 29.08–28.05 (series of t), 26.61, 26.30 (t, C-10 and C-11), 24.70, 24.35 (t), 23.06 (q, CHMe_2), 20.24 (t, C-3). MS: *m/z* 390–476 (Table 1) and *m/z* 195–238, as $[M^+ + H^+]^{2+}$ clusters of similar complexity and relative intensities of the components. LC-MS and LC-MS^n experiments: at t_R = 3.0 min, *m/z* 390, 392, 404, 406; 390 ⟶ 372 ⟶ 355, 259 and 245; 392 ⟶ 374 ⟶ 357, 261 and 247; 404 ⟶ 386 ⟶ 369, 273 and 259; 406 ⟶ 388 ⟶ 371, 261. At t_R = 4.0 min, *m/z* 406, 418, 420, 432; 406 ⟶ 388 ⟶ 371, 275 and 261; 418 ⟶ 400 ⟶ 383, 287 and 273; 420 ⟶ 402 ⟶ 385, 275; 432 ⟶ 414 ⟶ 397, 287. At t_R = 4.7 min, *m/z* 432, 446; 432 ⟶ 414 ⟶ 397, 301; 446 ⟶ 428 ⟶ 411, 301. At t_R = 5.6 min, *m/z* 420, 434; 420 ⟶ 402 ⟶ 385, 285; 434 ⟶ 416 ⟶ 399, 289. At t_R = 6.7 min, *m/z* 434, 446, 448, 460; 434 ⟶ 416 ⟶ 399, 303; 446 ⟶ 428 ⟶ 411, 315; 448 ⟶ 430 ⟶ 413, 303; 460 ⟶ 442 ⟶ 425, 315. At t_R = 7.7 min, *m/z* 460, 462, 474; 460 ⟶ 442 ⟶ 425, 329; 462 ⟶ 444 ⟶ 427, 317; 474 ⟶ 456 ⟶ 439, 329. At t_R = 11.7 min, *m/z* 462, 474, 476; 462 ⟶ 444 ⟶ 427, 331; 474 ⟶ 456 ⟶ 439, 329; 476 ⟶ 458 ⟶ 441, 331. These tandem fragmentations were later confirmed by the same experiments on the components of each HPLC fraction collected, under the same conditions, at identical indicated retention times.

Minor, sulfurated phloeodictynes

ESI-MS of the crude desalted methanolic extract, operating at a cone voltage of 19 V, showed a cluster at *m/z* 546–630, with main signals at *m/z* 574, 588, 600, 602, 614 in *ca.* 1 : 2 : 1 : 2 : 2 ratios. Their intensity was less than 8% with respect to major cluster at *m/z* 390–476. MS/MS (574): 556, 420; (588): 570, 434; (600): 582, 446; (602): 584, 448; (614): 596, 460.

Partially purified phloeodictynes for bioassay

Mixture 1 (Table 1). HPLC separation of a small portion of the crude mixture of phloeodictynes, and evaporation of the fraction collected at t_R = 5.6 min (2.6 mg), gave phloeodictynes **5**,**7i** and **4**,**7i** in a 7 : 3 ratio. The signals δ_H (CD_3 OD): 7.29 (d, J 6.4, 7-H), 7.06 (d, J 6.4, 8-H), 0.87 (d, J 6.7, CHMe_2) in *ca* 1 : 1 : 6 ratios, and only vanishingly small signals at δ_H = 5.80 ppm, indicated the presence of only isopropyl-type phloeodictynes. MS: *m/z* 434 and 420 in a 7 : 3 ratio, given with respect to the width of the signals. **Mixture 2** (Table 1). Evaporation of the fraction collected at t_R = 7.7. min (5.0 mg, from the same HPLC separation that gave mixture 1 above), gave phloeodictynes **5**,**9a**; **4**,**11a** and **5**,**9i** in 6 : 3 : 1 ratios. δ_H (CD_3 OD): the intensity ratio 9 : 1 between the signals 5.80 (ddt, J 17.0, 10.2, 6.9, CH_2CH=CH_2), 0.87 (d, J 6.7, CHMe_2) suggests a 9 : 1 mixture of allyl- and isopropyl-type phloeodictynes. MS: *m/z* 460, 474, 462, in 6 : 3 : 1 ratio, given with respect to the width of the signals. **Mixture 3** (Table 1). Evaporation of the fraction collected at t_R = 11.7 min (1.8 mg), under the same HPLC conditions used to get mixtures 1 and 2 above, gave phloeodictynes **4**,**10i**; **5**,**10a** and **5**,**10i** in 6 : 3 : 1 ratios. The intensity ratio 7 : 3 between the signals δ_H (CD_3 OD): 5.80 (ddt, J 17.0, 10.2, 6.9 Hz, CH_2CH=CH_2), 0.87 (d, J 6.7, CHMe_2) suggests a 7 : 3 mixture of isopropyl- and allyl-type phloeodictynes; MS: *m/z* 462, 474, 476, in a 6 : 3 : 1 ratio, given with respect to the width of the signals. For all three mixtures above, MS^3 fragmentation was observed in full agreement with the data obtained by LC-MS^n experiments on the components of the crude mixture eluted at the same retention time.

N-Methyl derivatives

To a solution of crude mixture of non sulfurated phloeodictynes (Chart 1) (13 mg) in acetone–MeOH 1 : 1 (1 mL) was added solid K_2CO_3 and MeI in excess (0.25 mL, neat). The mixture was stirred at rt overnight, then evaporated and the residue purified by FC on a Lichrolut RP-18 column with MeOH–H_2O 8 : 2, to give 11 mg of *N*-methyl derivatives. ESI-MS: *m/z* 418–504, as M^+ cluster ions for the dimethyl derivatives, and *m/z* 224–258, as doubly charged cluster ions for the trimethyl derivatives, in a 6 : 1 ratio, with similar complexity and relative intensities as for the components present in the crude mixture of phloeodictynes. Tandem MS fragmentation data are reported for only two of the most intense signals of the cluster at *m/z* 418–504: MS/MS (476): 458 ($M^+ - H_2O$), 445 ($M^+ - MeNH_2$), 431($M^+ - Me_2NH$); MS^3 experiments: 476–458–427 ($M^+ - H_2O - MeNH_2$); 476–445–414 ($M^+ - 2MeNH_2$), 427 ($M^+ - Me_2NH - H_2O$); 476–431–414 ($M^+ - Me_2NH - H_2O$). MS/MS (462): 444 ($M^+ - H_2O$), 431 ($M^+ - MeNH_2$), 417 ($M^+ - Me_2NH$); MS^3 experiments: 462–431–413, 399; 462–431–418, 400.

Bioassays

Antiplasmodial bioassays

In vitro activity against a chloroquine-resistant strain FCB1 of *Plasmodium falciparum* (kindly furnished by Dr A.Valentin from the Laboratory of Parasitology, Faculty of Pharmacy, Montpellier, France) was evaluated by a micromethod using the lactate dehydrogenase (LDH) enzyme of *Plasmodium falciparum*.[6] Erythrocytes infected with *P. falciparum*, from parasites cultures obtained using the method of Trager and Jensen,[7] were re-suspended in the complete culture medium at a haematocrit of 1.5%. The suspension was distributed in 96-well, microtitre plates (200 μdm^3 per well). Drug testing was performed in triplicate with cultures mostly at ring stage at 1% parasitemia. For each assay, a parasite culture was incubated with the drug for 48 h in 5% CO_2 at 95% relative humidity, and frozen until the biochemical assay could be run. A 20 μdm^3 sub-sample of the contents of each well was mixed with 100 μdm^3 of a substrate solution containing 20 mg of lithium L-lactate (Sigma), 5.5 mg of TRIS (Sigma), and 3.7 mg of 3-acetyl pyridine adenine dinucleotide (APAD; Sigma) per cm^3, in the well of another microtitre plate. After incubation for 30 min, 25 μdm^3 of a mixture of NBT (1.6 mg cm^{-3}; Sigma) and PES (0.1 mg mL^{-1}, Sigma) were added to each well. After a further 35 min of incubation, the reaction was stopped by the addition of 25% acetic acid (25 μL $well^{-1}$). Formation of the reduced form of APAD was measured at $\lambda = 650$ nm, using a spectrophotometer (microplate reader, Metertech). IC_{50} values were determined graphically in a concentration *versus* percent inhibition curve.

Cytotoxicity biossays

In vitro cytotoxicity against human lung carcinoma cell line A-549 was evaluated by the chemosensitivity method using 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[8] at four days. EC values were plotted as a function of eight values of concentrations, in the 10^{-4}–10^{-8} M range for phloeodictynes and 10^{-6}–10^{-10} M for vinblastine and nocodazole, taken as reference standard compounds.

Acknowledgements

We warmly thank Dr Jane Fromont, Western Australian Museum, Perth, for the sponge taxonomy and Mr A. Sterni for his technical contribution with mass spectra. This work has been financially supported in Italy by MIUR (Rome) and in France by the French Ministry for Research and New Technologies, through the program Pal+.

References and notes

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2 A capital letter followed by a Roman number refers to the original phloeodictine naming.[1] The latter does not match the genus name of the source sponge, while the former, based on chromatographic retention times,[1] fails to provide adequate mnemonical aid and does not match retention times on the different chromatographic substrates we used. To remedy this unsatisfactory situation, we have changed the name to phloeodictyne, while using a suffix that specifies the number of interposed methylene groups in the two side chains (the first number refers to the guanidine chain and the second number to the isopropyl (i)/alkenyl (a)).

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