Topsentins, new toxic bis-indole alkaloids from the marine sponge *Topsentia genitrix*[1]

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Three new bis-indole alkaloids, topsentin-A (**1**), -B1 (**2**), and -B2 (**3**) have been isolated from the Mediterranean sponge *Topsentia genitrix* and their structure determined by spectroscopic methods. These compounds are weakly toxic for fish and for dissociated cells of the freshwater sponge *Ephydatia fluviatilis* and thus might be partially responsible for the chemical defense of the sponge.

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Trois alcaloïdes bis-indoliques nouveaux, les topsentines-A (**1**), -B1 (**2**) et -B2 (**3**), ont été isolés de l'éponge méditerranéenne *Topsentia genitrix*, et leurs structures déterminées par des méthodes spectroscopiques. Ces composés, qui sont faiblement toxiques vis-à-vis des poissons et des cellules dissociées de l'éponge d'eau douce *Ephydatia fluviatilis*, sont donc probablement responsables de la défense chimique de cette éponge.

[Traduit par la revue]

Introduction

Among marine organisms, sponges appear to be one of the richest phylum in toxicogenic species. They are also remarkable for their ability to synthesize a wide variety of secondary metabolites (1, 2) that, in several cases, have been demonstrated to be responsible for the observed toxicity (3, 4). It is now generally admitted that these noxious compounds may act to minimize predation by mobile animals and (or) to reduce settlement and overgrowth of fouling organisms (5).

In the course of our search for compounds involved in the defense mechanism of sponges, we observed that the methanolic extract of the sponge *Topsentia genitrix* (Halichondriidae) is weakly toxic to the fish *Lebistes reticulatus* (LD: <50 mg/L) and to mice (LD_{50}: 10 mg/kg), kills dissociated cells of the freshwater sponge *Ephydatia fluviatilis* before early aggregation (<100 mg/L) (6), and shows antibacterial activities. We wish to report here on the isolation and structure determination of three new bis-indole alkaloids partially responsible for these activities.

Results and discussion

A large specimen of *Topsentia genitrix*, a rarely encountered Mediterranean sponge, was collected near Banyuls (France) by scuba diving. The three major compounds of the active fraction, named topsentin-A (**1**), topsentin-B1 (**2**), and topsentin-B2 (**3**), were separated by droplet counter current chromatography (DCCC: $CHCl_3$, CH_3OH, H_2O, 13:7:8) and further purified by flash chromatography on silica gel ($CHCl_3$, CH_3OH, NH_3 25%, 19:0.5:1 or 9:1:1).

Topsentin-A (1)

The pattern of the uv (CH_3OH, λ_{max} at 209 (43 000) sh 228, 253 (21 200) sh 270, 373 nm (17 930)) and ir spectra (KBr, no aliphatic ν C—H, aromatic out-of-plane bending band at 735 cm^{-1}), together with the absence in the ^1H nmr spectrum of topsentin-A of signals between 0 and 7 ppm, are indicative of an aromatic skeleton. The EI mass spectrum shows an intense molecular ion at m/z 326 and high resolution measurements provide the molecular formula $C_{20}H_{14}N_4O$ (*Exact Mass* calculated: 326.1168; measured: 326.1169). Although the behaviour of topsentin-A, both in tlc and hplc, suggests that it is a pure compound, its ^1H nmr spectrum, recorded in CD_3COCD_3, shows signals ($2 \times 14H$) attributable to a mixture of two closely related derivatives in a ratio 60:40. Four peaks amounting to a total of 6 protons (3H per derivative) disappear on treatment with D_2O and the addition of a few drops of CF_3COOD induces a dramatic simplification of the spectrum, which under these conditions shows signals integrating for 11 protons only. Such behaviour is reminiscent of the existence of two slowly interconverting compounds whose protonated forms are identical.

The ^{13}C nmr spectra of **1** (CD_3COCD_3–D_2O–CF_3COOD; BBD and DEPT) ascertain the presence of 20 carbon atoms (9 quaternary and 11 tertiary). The $^1H/^1H$ correlated 2D nmr spectrum of the protonated form of **1** shows that the four protons absorbing at 8.35, 7.65, 7.36, and 7.35 ppm and the four protons absorbing at 7.96, 7.60, 7.28, and 7.25 ppm form two separated 4-spin systems attributable to two 1,2-disubstituted benzene rings. The simulation of the two 4-spin systems using the PANIC program (7) confirms this attribution and allows accurate determination of the chemical shifts and coupling constants. The assignment of the carbon–proton connectivities of these protons was deduced from the $^1H/^{13}C$ correlated 2D nmr spectrum. SPI experiments (8) showed that the 1H singlet at δ 8.72 undergoes long range heteronuclear coupling

[1]This paper is dedicated to the memory of Dr. N. Defay.

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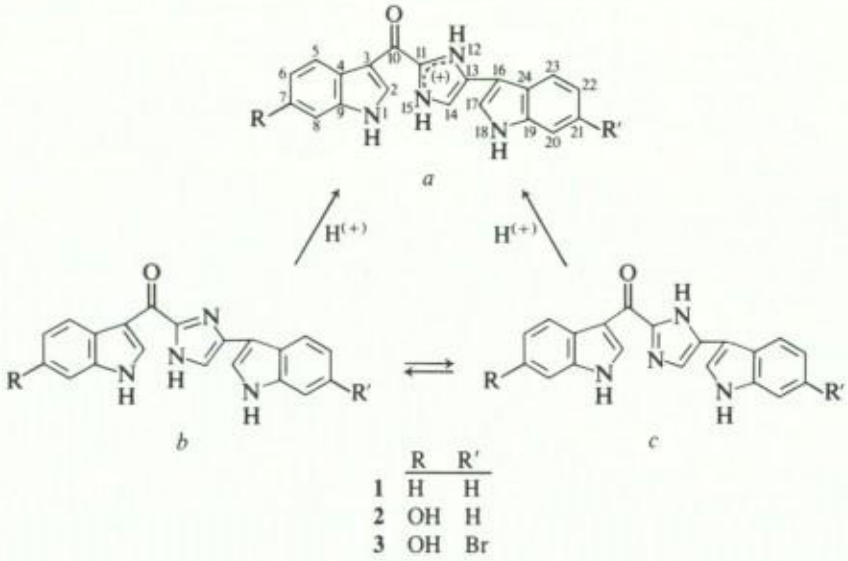


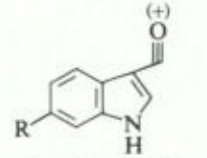
	R	R′
1	H	H
2	OH	H
3	OH	Br

with the ^{13}C signals at δ 114.8, 126.9, and 138.0, while the 1H singlet at δ 8.15 is long-range coupled with the ^{13}C signals at δ 125.4, 137.6, and 103.1. All these data, together with the presence of a deshielded carbon atom at δ 172.1, are consistent with the presence in topsentin-A of both a 3-ketoindole and a 3-substituted indole chromophore. The comparison of the ^{1}H and ^{13}C chemical shifts with those of model compounds (9–15) strongly supports this hypothesis. Thus, all signals in the ^{1}H and ^{13}C nmr spectra of **1** have been assigned, except for a 1H singlet at 8.08 ppm and 3 peaks corresponding to 1 tertiary (116.6) and two quaternary (141.5 and 132.5) carbon atoms in the ^{13}C nmr spectra. That these signals are attributable to a C-disubstituted imidazole ring follows from comparison with reported values (16) and from SPI experiments, which showed that the proton at δ 8.08 is long-range coupled with the ^{13}C signal at δ 141.5 and 132.5. The presence of an imidazole ring explains the modifications observed in the ^{1}H nmr spectrum of topsentin-A on addition of CF_3COOD. In neutral solution, asymmetrically C-disubstituted imidazoles exist as mixtures of slowly interconverting tautomers, and protonation yields a symmetrical imidazolium cation (17). It is known that, of the three carbon atoms of imidazole derivatives, the C-2 (imidazole numbering) is the most deshielded (δ ~ 140) (16). Since in topsentin-A this signal (δ 141.5) corresponds to a quaternary carbon atom, the imidazole ring is necessarily substituted at the carbon bearing the two nitrogen atoms. Moreover, further SPI experiments indicated that the signal at δ 8.15, attributable to the proton H-17 α to the nitrogen atom of the indole chromophore, is coupled (^{3}J) with the C-4 (or C-5) of the imidazole ring (δ 132.5). It follows from all these data that the protonated form of topsentin-A may be represented by structure **1***a*.

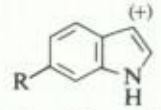
A careful analysis of the ^{1}H nmr spectrum of topsentin-A measured in CD_3COCD_3 permits us to assign the signals of both tautomers. Interestingly, the two signals at 7.62 and 7.70 ppm attributable to H-14 show different NH/CH coupling constants (2.2 and 1.5 Hz respectively). Since it is reasonable to assume that the larger *J* infers coupling between adjacent protons (17), the signal at 7.7 ppm may be assigned to tautomer **1***b*, which is thus the most abundant. The mass spectrum of topsentin-A entirely corroborates the proposed structure. It shows characteristic fragments corresponding to cleavages α to the carbonyl group. Cleavage of the C10—C11 bond gives rise to fragments *a* (*m/z* 144), *a* + H$^{\bullet}$ (*m/z* 145), and *d* + H$^{\bullet}$ (*m/z* 183), while cleavage of the C3—C10 bond gives rise to fragments *c* (*m/z* 210) and *c* − H$^{\bullet}$ (*m/z* 209). Subsequent loss of a molecule of carbon monoxide leads to fragments *b* (*m/z* 116; *a* − CO), *b* + H$^{\bullet}$ (*m/z* 117; *a* + H$^{\bullet}$ − CO), and *d* − H$^{\bullet}$ (*m/z* 181; *c* − H$^{\bullet}$ − CO) respectively. The composition of all these fragments was confirmed by high resolution measurements. The parent–daughter relationships $M^{+\bullet} \rightarrow c - H^{\bullet} \rightarrow d - H^{\bullet}$ and $a \rightarrow b$ are ascertained by the presence of metastable peaks at *m/z* 134, 157, and 94 respectively.

*Topsentin-B1 (**2**)*

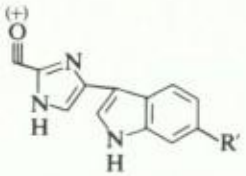
The spectroscopic properties of topsentin-B1 are closely related to those of topsentin-A. The EI mass spectrum shows an intense molecular ion at *m/z* 342 and high resolution measurements provide the molecular formula $C_{20}H_{14}N_4O_2$ (*Exact Mass* calculated: 342.1117; measured: 342.1129). Thus, topsentin-B1 possesses one more oxygen atom than topsentin-A. Moreover, in the ms of **2**, the peaks corresponding to fragments *a*, *a* + H$^{\bullet}$, *b*, and *b* + H$^{\bullet}$ in the ms of **1** are shifted to *m/z* 160 (*a′*), *m/z* 161 (*a′* + H$^{\bullet}$), *m/z* 132 (*b′*), and *m/z* 133 (*b′* + H$^{\bullet}$), respectively, while those corresponding to fragments *c*, *c* − H$^{\bullet}$, *d* − H$^{\bullet}$, and *d* + H$^{\bullet}$ are unchanged. This suggests that the extra oxygen atom is located on the 3-ketoindole moiety of the molecule. The presence in the ^{1}H nmr of 4 exchangeable protons implies that this oxygen atom is involved in an hydroxyl group. Further examination of the ^{1}H nmr data shows that the 4-spin system attributable to protons H-5 to H-8 in topsentin-A



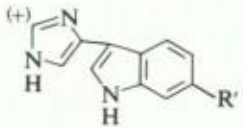
a R = H *m/z* 144
a′ R = OH *m/z* 160



b R = H *m/z* 116
b′ R = OH *m/z* 132



c R′ = H *m/z* 210
c′ R′ = Br *m/z* 288/290



d R′ = H *m/z* 182
d′ R′ = Br *m/z* 260/262

is replaced, in the spectrum of topsentin-B1, by a 3-spin system whose pattern indicates a 1,2,4-benzene substitution. The positioning of the hydroxyl group at C-6 follows from comparison of the chemical shifts of these three protons and their adjacent carbon atom in the ^1H and ^{13}C nmr spectra of topsentin-B1 with the chemical shifts calculated from the shielding effects[2] induced by the introduction of an hydroxyl group at C-6 or C-7 in topsentin-A, taken as reference compound. It follows that topsentin-B1 is 6-hydroxytopsentin-A. This structure is fully confirmed by the $^1H/^1H$ and $^1H/^{13}C$ correlated 2D nmr spectra.

In CD_3COCD_3, topsentin-B1 behaves as topsentin-A, existing as a mixture of two tautomeric forms (55:45) in slow interconversion. Again the *b* tautomer is the most abundant.

*Topsentin-B2 (**3**)*

Topsentin-B2 has the same R_f as topsentin-B1 in tlc and the two compounds can only be separated by DCCC. Their spectroscopic properties are closely related and suggest that topsentin-B2 is a monobromo derivative of topsentin-B1. The EI mass spectrum of **3** shows an intense molecular ion cluster at *m/z* 420–422 (50:50) which analyzes for $C_{20}H_{13}N_4O_2Br$ (measured: 420.0237/422.0215; calculated: 420.0222/422.0203) by high resolution mass spectrometry. Moreover, the peaks corresponding to fragments *c*, $c - H^\bullet$, and $d + H^\bullet$ in **1** and **2** are shifted 78–80 daltons to the high masses in the mass spectrum of **3** while those corresponding to fragments *a'* and *b'* are unchanged. These data demonstrate that the bromine atom is attached to the indole moiety of the molecule. Additional support for this hypothesis is provided by the ^1H nmr spectrum, which shows two 3-spin systems characteristic of the presence of two 1,2,4-trisubstituted benzene rings. The locations of the OH and Br substituents in **3** are based, as discussed above, on calculations of the ^1H and ^{13}C shielding effects taking **1** and **2** as reference compounds. Thus, topsentin-B2 is 21-bromotopsentin-B1. The $^1H/^1H$ and $^1H/^{13}C$ correlated 2D nmr spectra are in complete agreement with the proposed structure. As **1** and **2**, topsentin-B2 exists in neutral solution as a mixture of two tautomeric forms (67:33) in slow interconversion, with tautomer *b* being preponderant.

The LD of these three bis-indole alkaloids, for the fish *Lebistes reticulatus*, is between 15 and 20 mg/L, indicating that they are, at least in part, responsible for the toxicity of the methanolic extract of the sponge *Topsentia genitrix*. From a biogenetic point of view, the topsentins most probably derive from the combination of 2 tryptamine (or tryptophane) units.

Until now, only a few tryptamine-derived indole compounds have been isolated from marine invertebrates in general, and from sponges in particular (1, 2, 19, 20). Most of the marine indoles are rather simple compounds, presumably representing small deviations or sidelines in tryptophane metabolism. The topsentins fit nicely into this scheme and reinforce the idea that the complex indole alkaloid metabolic pathways that have appeared in higher plants probably have no counterpart in the marine environment.

Experimental

Thin-layer chromatographic analyses (tlc) were realized on SilG UV_{254} (Macherey Nagel) and visualized by using uv light at 254 nm. Silica gel column chromatographies were performed by the flash technique (21) on MN-Kieselgel 60 (0.04–0.063 mm) and droplet counter current chromatographies (DCCC) on a Büchi B670 (288 tubes, l = 40 cm, i.d. = 2.7 mm).

The ^1H and ^{13}C nmr spectra were recorded at 250 and 62.8 MHz respectively, using a Bruker WM 250 spectrometer. Chemical shifts are quoted in δ values downfield from TMS as internal standard. The 2D experiments were performed using the Bruker microprograms. EI hrms were recorded on a Micromass 7070 F mass spectrometer.

Extraction and purification procedures

The fresh animal, collected by scuba diving near Banyuls (France), was preserved in MeOH and transported to the laboratory in Brussels. The sponge was cut into small pieces and exhaustively extracted with MeOH. Evaporation of the organic solvent under reduced pressure led to an aqueous solution that was extracted with dichloromethane (fraction A: 1.5 g) and dichloromethane–ethanol 3:2 (fraction B: 8.1 g) respectively. The residual aqueous solution was evaporated to dryness and the resulting solid was treated with ethanol (fraction D: 18 g). This latter fraction was percolated on silica gel (eluent:hexane–acetone 6:4 to 0:10).

The less polar fraction (3.1 g) contained mainly topsentin-A, -B1, and -B2, which were separated by DCCC (descending mode; solvent: $CHCl_3$–CH_3OH–H_2O, 13:7:8; flow 7 mL/h; fractions of 10 mL). Fractions 4–11 yielded crude topsentin-A (~500 mg), fractions 35–52 crude topsentin-B1 (~350 mg), and fractions 65–85 crude topsentin-B2 (~100 mg). All three derivatives were purified by silica gel flash chromatography ($CHCl_3$–CH_3OH–NH_3 25%, 19:0.5:1). They were found to be homogenous by hplc (CH_3OH–H_2O, 1:1; RP-18 5μm).

Topsentin-A: mp 290–292°C; uv (CH_3OH) λ_{max}: 209 (43 000), 253 (21 200), 373 nm (17 930) shoulders at 228 and 270 nm; ir (film): characteristic bands at 3250 (br), 1700, 1230, 845, and 735 cm^{-1}; EI ms: 326 ($M^\ddagger$, 100), 210 (13), 209 (78), 183 (23), 181 (7), 163 (15), 154 (13), 144 (56), 117 (16), 116 (21), 89 (15). *Exact Mass* calcd. for $C_{20}H_{14}N_4O$: 326.1168; found (hrms): 326.1169.

Topsentin-B1: mp 270°C; uv (CH_3OH) λ_{max}: 209 (34 900), 237 (23 600), 278 (13 350), 375 nm (13 000) shoulders at 254 nm; ir (film): characteristic bands at 3300 (br), 1710, 1525, 870, and 745 cm^{-1}; EI ms: 342 ($M^\ddagger$, 100), 210 (11), 209 (37), 183 (30), 182 (5), 181 (5), 171 (17), 161 (6), 160 (44), 155 (11), 133 (57), 132 (14), 105 (14). *Exact Mass* calcd. for $C_{20}H_{14}N_4O_2$: 342.112; found (hrms): 342.113.

Topsentin-B2: mp 260°C; uv (CH_3OH) λ_{max}: 285 (17 270), 375 nm (15 870) shoulders at 236 and 256 nm; ir (film): characteristic bands at 3300 (br), 1705, 1590, 1520, 1160, 870 cm^{-1}; EI ms: 422/420 ($M^\ddagger$, 24), 290/288 (3), 289/287 (9), 263/261 (7), 161 (14), 160 (72), 134 (17), 133 (100), 132 (22). *Exact Mass* calcd. for $C_{20}H_{13}N_4O_2Br$: 420.022/420.020; found (hrms): 420.024/422.020.

^1H nuclear magnetic resonance spectra: *: Assignments may be reversed. [a]: Becomes singlet after D_2O treatment; [b]: Disappears after D_2O treatment; s = singlet; bs = broad singlet; d = doublet; dd = double doublet; m = multiplet.

1*a* (CD_3COCD_3, D_2O, CF_3COOD): 8.72 (s, H-2), 8.35 (m, H-5), 8.15 (s, H-17), 8.08 (s, H-14), 7.96 (m, H-23), 7.65 (m, H-8), 7.60 (m, H-20), 7.36 (m, H-7), 7.35 (m, H-6), 7.28 (m, H-21), 7.25 (m, H-22).

1*b* (CD_3COCD_3): 12.05*[b] (bs, H-1), 11.12*[b] (bs, H-18), 10.34[b] (bs, H-15), 9.63 (d,[a] H-2, J = 3 Hz), 8.52 (m, H-5), 8.22 (m, H-23), 7.84 (d,[a] H-17, J = 3 Hz), 7.70 (d,[a] H-14, J = 2.2 Hz), 7.57 (m, H-8), 7.47 (m, H-20), 7.24 (m, H-6 + H-7), 7.15 (m, H-21 + H-22).

1*c* (CD_3COCD_3): 12.05*[b] (bs, H-1), 11.12*[b] (bs, H-18), 10.60[b] (bs, H-12), 9.41 (d,[a] H-2, J = 3 Hz), 8.52 (m, H-5), 8.07 (d,[a] H-17, J = 3 Hz), 7.96 (m, H-23), 7.62 (d,[a] H-14, J = 1.5 Hz), 7.57 (m, H-8), 7.47 (m, H-20), 7.24 (m, H-6 + H-7), 7.15 (m, H-21 + H-22).

2*a* (CD_3COCD_3, D_2O, CF_3COOD): 8.42 (s, H-2), 8.13 (s, H-17), 8.08 (d, H-5, J = 8.6 Hz), 8.04 (s, H-14), 7.90 (m, H-23), 7.56 (m, H-20), 7.25 (m, H-21 + H-22), 7.07 (d, H-8, J = 2 Hz), 6.91 (dd, H-6, J = 8.6 and 2 Hz).

2*b* (CD_3COCD_3): 11.93*[b] (bs, H-1), 10.82*[b] (bs, H-18), 10.33*[b] (bs, H-15), 9.44 (d,[a] H-2, J = 3 Hz), 8.29 (d, H-5, J = 8.6 Hz), 8.21 (m, H-23), 8.14[b] (s, OH), 7.81 (d,[a] H-17, J = 2.6 Hz), 7.67

[2]The shielding effects utilized are those reported for monosubstituted benzene (18).

(d,[a] H-14, J = 2.2 Hz), 7.46 (m, H-20), 7.16 (m, H-21 + H-22), 7.00 (d, H-8, J = 2.2 Hz), 6.82 (dd, H-6, J = 8.6 and 2.2 Hz).

2*c* (CD_3COCD_3): 11.93*[b] (bs, H-1), 10.82*[b] (bs, H-18), 10.77*[b] (bs, H-12), 9.22 (d,[a] H-2, J = 3 Hz), 8.27 (d, H-5, J = 8.6 Hz), 8.11[b] (s, OH), 8.05 (d,[a] H-17, J = 2.6 Hz), 7.94 (m, H-23), 7.55 (d,[a] H-14, J = 1.6 Hz), 7.51 (m, H-20), 7.21 (m, H-21 + H-22), 6.99 (d, H-8, J = 2.2 Hz), 6.80 (dd, H-6, J = 8.6 and 2.2 Hz).

3*a* (CD_3COCD_3, D_2O, CF_3COOD): 8.43 (s, H-2), 8.14 (s, H-17), 8.09 (s, H-14), 8.08 (d, H-5, J = 8.6 Hz), 7.84 (d, H-23, J = 8.5 Hz), 7.78 (d, H-20, J = 1.7 Hz), 7.33 (dd, H-22, J = 8.5 and 1.7 Hz), 7.08 (d, H-8, J = 2.2 Hz), 6.93 (dd, H-6, J = 8.6 and 2.2 Hz).

3*b* (CD_3COCD_3): 12.03*[b] (bs, H-1), 10.8*[b] (bs, H-15), 10.5*[b] (bs, H-18), 9.41 (d,[a] H-2, J = 3 Hz), 8.28 (d, H-5, J = 8.6 Hz), 8.19 (d, H-23, J = 8.5 Hz), 8.11[b] (s, OH), 7.83 (d,[a] H-17, J = 2.5 Hz), 7.69 (d,[a] H-14, J = 2.1 Hz), 7.67 (d, H-20, J = 1.7 Hz), 7.23 (dd, H-22, J = 8.5 and 1.7 Hz), 7.00 (d, H-8, J = 2.2 Hz), 6.83 (dd, H-6, J = 8.6 and 2.2 Hz).

3*c* (CD_3COCD_3): 12.03*[b] (bs, H-1), 10.8*[b] (bs, H-12 and H-18), 9.22 (d,[a] H-2, J = 3 Hz), 8.27 (d, H-5, J = 8.6 Hz), 8.10[b] (s, OH), 8.07 (d,[a] H-17, J = 2.6 Hz), 7.88 (d, H-23, J = 8.5 Hz), 7.72 (d,[a] H-14, J = 1.6 Hz), 7.57 (d, H-20, J = 1.7 Hz), 7.30 (dd, H-22, J = 8.5 and 1.7 Hz), 6.99 (d, H-8, J = 2.2 Hz), 6.82 (dd, H-6, J = 8.6 and 2.2 Hz).

^{13}C nuclear magnetic resonance spectra: *♣: Assignments may be reversed. The multiplicity of the carbon atom signals have been assigned by using the DEPT pulse sequence. d = CH and s = C.

1*a* (CD_3COCD_3, D_2O, CF_3COOD): 172.1 (C-10, s), 141.5 (C-11, s), 138.5 (C-2, d), 138.0 (C-9, s), 137.6 (C-19, s), 132.5 (C-13, s), 126.9 (C-4, s), 126.4 (C-17, d), 125.4 (C-24, s), 125.3 (C-6*, d), 124.0 (C-7*, d), 123.5 (C-21♣, d), 122.5 (C-5, d), 121.7 (C-22♣, d), 119.7 (C-23, d), 116.6 (C-14, d), 114.8 (C-3, s), 113.7 (C-8, d), 113.2 (C-20, d), 103.1 (C-16, s).

2*a* (CD_3COCD_3, D_2O, CF_3COOD): 171.4 (C-10, s), 156.2 (C-7, s), 141.3 (C-11, s), 139.5 (C-9, s), 137.9 (C-2, d), 137.7 (C-19, s), 132.2 (C-13, s), 126.6 (C-17, d), 125.4 (C-24, s), 123.7* (C-21, d), 123.2 (C-5, d), 121.7* (C-22, d), 120.0 (C-4, s), 119.7 (C-23, d), 116.1 (C-14, d), 115.2 (C-3, s), 114.2 (C-6, d), 113.3 (C-20, d), 102.7 (C-16, s), 99.2 (C-8, d).

3*a* (CD_3COCD_3, D_2O, CF_3COOD): 171.3 (C-10, s), 156.3 (C-7, s), 141.6 (C-11, s), 139.5 (C-9, s), 138.5 (C-19, s), 137.8 (C-2, d), 131.5 (C-13, s), 127.5 (C-17, d), 124.8 (C-24, s), 124.5 (C-22, d), 123.3 (C-5, d), 121.4 (C-23, d), 120.0 (C-4, s), 116.7 (C-21, s), 116.5 (C-14, d), 116.1 (C-20, d), 115.2 (C-3, s), 114.1 (C-6, d), 103.2 (C-16, s), 99.1 (C-8, d).

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