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Chloroscabrolides, chlorinated norcembranoids from the Indonesian soft coral *Sinularia* sp.

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ABSTRACT

Chemical analysis of the Indonesian soft coral *Sinularia* sp. (order Alcyonacea, family Alcyoniidae) afforded two known and three new C-4 norcembranoids, named chloroscabrolides A (**3**) and B (**4**) and prescabrolide (**5**). Chloroscabrolide A is a pentacyclic norcembranoid including an unprecedented THF-type ring to connect C-13 and C-15; furthermore, it is only the second chlorinated cembranoid derivative to be reported in the literature. The relative configuration of chloroscabrolide A has been established on the basis of a comparison between experimental ^{13}C NMR data and DFT-calculated ^{13}C NMR chemical shifts. All the isolated norcembranoids have been evaluated for iNOS protein inhibition.

1. Introduction

Cembranoids constitute a large group of natural products isolated from both marine and terrestrial sources. These macrocyclic diterpenoids have been largely found in gorgonians and alcyonarians (soft corals) of the genera *Lobophytum*, *Sinularia*, *Sarcophyton*, and *Clavularia*[1–3] and they are believed to play an important role within the chemical defense arsenal against other reef organisms.[4] Anti-inflammatory,[2] antimicrobial,[5] cytotoxic,[6] and several other bioactivities have been disclosed for members of the cembranoid class, which have also stimulated a number of elegant synthetic strategies.[7,8]

As part of our continuing research program aimed at the discovery of bioactive metabolites from marine organisms, we have been recently studying the chemical composition of marine invertebrates from the Indonesian coasts,[3,9,10] held as one of the richest biodiversity hot spot of the oceans. In this context, we had the opportunity to analyze a specimen of the soft coral *Sinularia* sp. (order Alcyonacea, family Alcyoniidae), which probably belongs to the largest zooxanthellate shallow-water soft coral genus of the Indo-Pacific reefs. This organism was collected along the island of Siladen, in the Bunaken Marine Park of Manado (North Sulawesi, Indonesia), and from its organic extract we have isolated two known (**1**–**2**) and three new C-4 norcembranoids, named chloroscabrolides A (**3**) and B (**4**) and prescabrolide (**5**). Herein we discuss the details of the stereostructural characterization of the three new norcembranoids and the results of pharmacological evaluation of the isolated compounds for iNOS protein inhibition.

2. Isolation and stereostructure elucidation of the new norcembranoids

Colonies of *Sinularia* sp. (580 g wet weight) was homogenized and repeatedly extracted with MeOH and $CHCl_3$ at room temperature. The obtained organic extract (6.8 g) was chromatographed by MPLC over silica gel eluting with a gradient system of increasing polarity from *n*-hexane to EtOAc to MeOH, and the obtained fractions were further purified by analytical HPLC (*n*-hexane/EtOAc mixtures) to afford leptocladolide B (**1**),[11] scabrolide D (**2**),[12] chloroscabrolide A (**3**), chloroscabrolide B (**4**), and prescabrolide (**5**). The structures of the known metabolites leptocladolide B (**1**),[11] and scabrolide D (**2**)[12] were assigned through the comparison of their spectral data with those reported in the literature.

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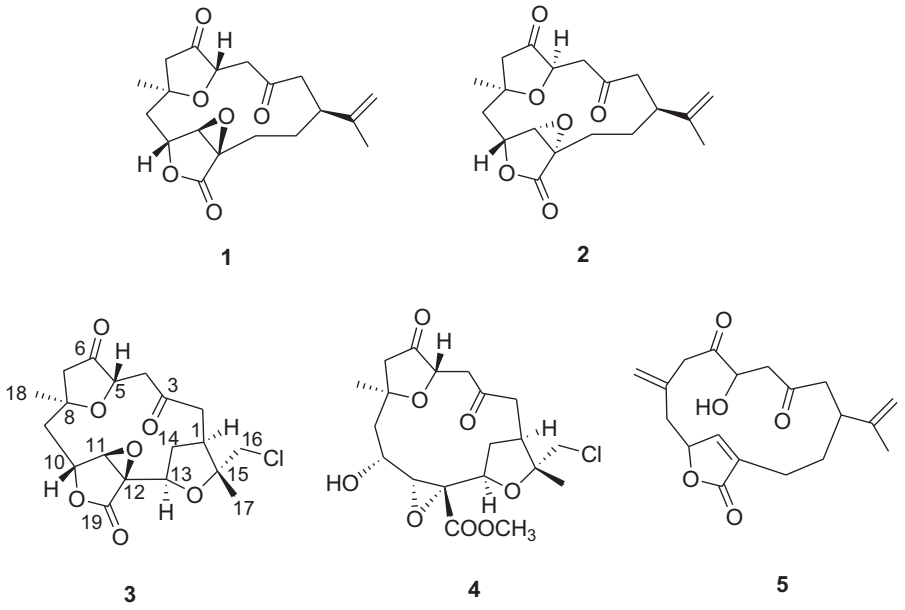

Chloroscabrolide A (**3**) was obtained as a colorless viscous oil with $[\alpha]_D^{25}$ +18.2. ESI-MS spectrum (positive ions) of **3** showed two pseudomolecular ion peaks at *m*/*z* 421 and 423 $[M+Na]^+$ in about 3:1 ratio. High-resolution measurement on the peak at lower mass indicated the molecular formula $C_{19}H_{23}ClO_7$ (*m*/*z* 421.1022 calcd for $C_{19}H_{23}{}^{35}ClNaO_7$ *m*/*z* 421.1030), implying eight degrees of unsaturation. ^{1}H NMR of **3** ($CDCl_3$, Table 1) showed three multiplets between δ_H 4.70 and 4.50, a broad singlet at δ_H 4.27, an AB system at δ_H 3.74/3.78, and a series of multiplets between δ_H 2.00 and 3.20, while the higher field region (between δ_H 1.00 and 2.00) was particularly poor of signals, showing only a multiplet at δ_H 1.78 and two methyl singlets at δ_H 1.24 and 1.49. The ^{13}C NMR of **3** ($CDCl_3$, Table 2) showed resonances of two ketone carbonyls (δ_C 207.7 and 211.2) and one ester carbonyl (δ_C 168.4), in addition to seven carbon resonances located between δ_C 62.0 and 84.0 and attributable to oxygenated carbons. The remaining nine resonances were located between δ_C 28.0 and 52.0. The 1D NMR spectra were analyzed in more detail with the help of a 2D HSQC NMR experiment, which allowed the association of all the proton signals with those of the directly attached carbon atoms. Thus, chloroscabrolide A (**3**) must include five methines (four of which are oxygenated), six diastereotopic methylenes, and two methyls. The remaining six carbon atoms are unprotonated and they include, in addition to the above mentioned carbonyls, three oxygenated carbon atoms at δ_C 62.3, 80.6 and 83.6. The three C–O double bonds resulting from the above analysis left unassigned five of the eight unsaturation degrees implied by the molecular formula, suggesting a pentacyclic structure for **3**.

The ^{1}H–^{1}H COSY NMR spectrum of **3** was instrumental to sort the proton multiplets into the three spin systems *A*–*C* highlighted in bold in Fig. 1. These small subunits were then connected with the help of the key $^{2,3}J_{H,C}$ correlations evidenced by means of a 2D g-HMBC experiment (Fig. 1). Correlations of H-1, H_2-2, H_2-4, and H-5 with the signal at δ_C 207.7 identified the ketone carbonyl (C-3) as

Table 1
^{1}H (500 MHz) NMR data of chloroscabrolides A (**3**) and B (**4**) and of prescabrolide (**5**) in $CDCl_3$

Pos.	**3**	**4**	**5**
	δ_H, mult., *J* in Hz	δ_H, mult., *J* in Hz	δ_H, mult., *J* in Hz
1	2.84–2.87, m	2.93–2.96, m	2.07–2.09, m
2a	3.13, dd, 11.3, 1.8	2.57[a]	2.50, dd, 13.0, 4.0
b	2.16[a]	2.40, dd, 13.0, 7.5	2.27, dd, 13.0, 7.5
4a	2.84, t, 11.5	2.64, dd, 13.0, 10.5	3.00, dd, 15.7, 8.0
b	2.69, dd, 11.5, 1.0	2.41[a]	2.57, dd, 15.7, 4.5
5	4.56, dd, 11.5, 1.0	4.61, dd, 10.5, 1.5	4.13–4.15, m
7a	2.60, d, 15.0	2.62, d, 15.0	2.48, d, 14.0
b	2.53, d, 15.0	2.43[a]	2.36, d, 14.0
9a	2.51[a]	2.53[a]	2.52[a]
b	2.12[a]	2.16[a]	2.21[a]
10	4.67, dd, 4.0, 3.0	4.37–4.39, m	5.13–5.17, m
11	4.27, s	3.51, d, 2.3	7.21, bs
13a	4.53, dd, 7.4, 5.7	4.37, dd, 5.5, 2.5	2.63[a]
b			2.17[a]
14a	2.05, dd, 13.0, 7.4	2.12[a]	1.82–1.85, m
b	1.78, dd, 13.0, 5.7	1.80, dd, 13.4, 6.2	1.77–1.79, m
16a	3.78, d, 11.3	3.52, d, 12.3	4.88, s
b	3.74, d, 11.3	3.46, d, 12.3	4.74, bs
17	1.24, s	1.38, s	1.66, bs
18a	1.49, s	1.40, s	4.80, bs
b			4.68, bs
10-OH		3.82, bs	
19-OMe		3.80, s	

[a] Overlapped with other signals.

Table 2
^{13}C (125 MHz) NMR data of chloroscabrolides A (**3**) and B (**4**) and of prescabrolide (**5**) in $CDCl_3$

Pos.	**3**	**4**	**5**
	δ_C, mult.	δ_C, mult.	δ_C, mult.
1	39.3, CH	35.7, CH	38.5, CH
2	45.5, CH_2	44.8, CH_2	45.4, CH_2
3	207.7, C	205.5, C	207.5, C
4	44.2, CH_2	44.2, CH_2	45.5, CH_2
5	79.2, CH	79.9, CH	74.7, CH
6	211.2, C	212.7, C	212.6, C
7	51.1, CH_2	50.0, CH_2	50.5, CH_2
8	80.6, C	79.0, C	150.8, C
9	42.3, CH_2	41.3, CH_2	42.9, CH_2
10	76.7, CH	64.7, CH	78.6, CH
11	65.1, CH	64.4, CH	149.3, CH
12	62.3, C	65.3, C	133.0, C
13	73.0, CH	73.3, CH	20.3, CH_2
14	36.5, CH_2	37.5, CH_2	30.5, CH_2
15	83.6, C	84.0, C	145.6, C
16	51.8, CH_2	49.1, CH_2	113.4, CH_2
17	29.7, CH_3	29.3, CH_3	19.3, CH_3
18	28.8, CH_3	27.4, CH_3	112.9, CH_2
19	168.4, C	167.7, C	174.4, C
19-OMe		52.0, CH_3	

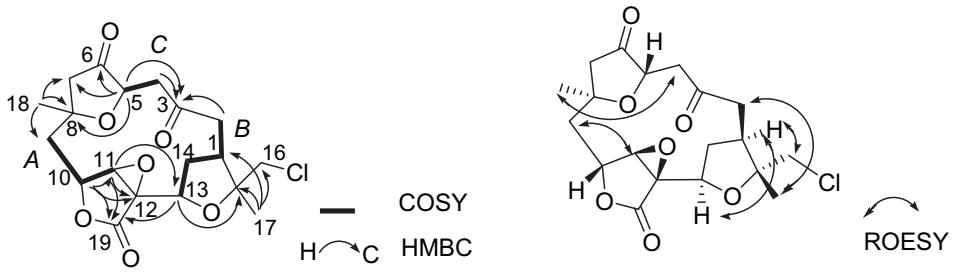

Fig. 1. COSY, $^{2,3}J_{H\rightarrow C}$ HMBC (left) and ROESY (right) correlations for chloroscabrolide A (**3**).

the connection point between moieties *B* and *C*. Analogously, the unprotonated carbon at δ_C 62.3 (C-12) must link moieties *A* and *B*, as indicated by the cross-peaks of H-10, H-11, and H-13 with C-12 and by the cross-peak H-11/C-13. Correlations of H-5 with C-7 and with the ketone signal at δ_C 211.2 (C-6) and correlations of the methyl singlet at δ_H 1.49 (Me-18) with C-7, the oxygenated unprotonated C-8, and with C-9 established the connection between subunits *A* and *C*, also indicating the presence of a 14-membered ring, typical of the cembranoid framework. Then, the ester carbonyl resonating at δ_C 168.4 (C-19) was attached at C-12 on the basis of the g-HMBC cross-peaks of both H-11 and H-13 with C-19, while the cross-peak H-10/C-19 indicated that this carbonyl is actually part of a γ-lactone ring. Similarly, the g-HMBC cross-peak H-5/C-8 was indicative of the presence of an oxygen bridge between C-5 and C-8 to build a THF-type ring. Both the above five-membered rings are typical features of norcembranoids of the scabrolide/leptocladolide family.

At this stage, in order to define the planar structure of chloroscabrolide A (**3**), a C_3H_5Cl moiety and two further rings should be located on its structure. The g-HMBC cross-peaks of the unprotonated carbon at δ_C 83.6 (C-15) with H-1, H_2-2, Me-17, H_2-16, and H-13 were only compatible with the presence of a further five-membered oxygenated ring and with the attachment of methyl and chloromethylene (δ_H 3.74 and 3.78, δ_C 51.8) groups at C-15. The g-HMBC cross-peaks H-17/C-16, H-17/C-1, and H_2-16/C-1 further supported this conclusion. Finally, an epoxide ring connecting C-11 and C-12, suggested by the relatively low-field resonances of the involved carbons (C-11: δ_C 65.1; C-12: δ_C 62.3) and by comparison with data of other scabrolide cembranoids, accounted for the last unsaturation degree implied by the molecular formula, and completely defined the planar structure of chloroscabrolide A (**3**).

Chloroscabrolide A is the first member of a new class of pentacyclic norcembranoids, whose structural framework, in addition to the 14-membered macrocycle, includes an epoxide ring and three five-membered rings. Among them, the THF-type ring originating from the C-13/C-15 ether linkage is unprecedented. Furthermore, in spite of the hundreds cembranoids isolated to date, chloroscabrolide A is only the second chlorinated cembranoid to be reported in the literature.[13]

Assignment of the relative configuration at the eight stereogenic carbon atoms of chloroscabrolide A (**3**) was next attempted. In this regard, a comparison between the structures of leptocladolide B (**1**) and scabrolide D (**2**), both related to **3**, well highlights the extreme variability in the configurational arrangements found within this class of compounds. A careful inspection of the 2D NMR ROESY spectrum of **3** (Fig. 1) provided unambiguous evidence to deduce the geometry around the three five-membered rings. In particular, an intense cross-peak between Me-18 and H_2-4 indicated the cis orientation of these groups, while correlations H-13/H_2-16, H-1/H-13, Me-17/H_2-2, and H_2-16/H-1 unambiguously defined the relative geometry around the second THF ring. Finally, the spatial proximity between H-11 and H_2-9 indicated that the epoxide ring and H-10 should be cis-oriented on the lactone ring. This latter assignment was further supported by the NMR resonance of the epoxide proton H-11 (δ_H 4.27); indeed, it can be observed that in those scabrolides where the epoxide ring and H-10 are trans-oriented, H-11 resonates at relatively higher fields (e.g., δ_H 3.95 in **2**)[12] compared to those with cis orientation of the epoxide ring and H-10 (e.g., δ_H 4.15 in **1**).[11]

Unfortunately, the ROESY spectrum of **3** showed no significant transannular couplings, which could be utilized to connect to each other the relative configurations deduced for the three five-membered rings. Therefore, at this stage, the four stereoisomers A–D shown in Fig. 2 could be possible candidates for the relative configuration of chloroscabrolide A. We reasoned that the recent methodology based on the quantum-mechanical prediction of ^{13}C NMR chemical shifts[14,15] could be particularly suitable to solve this stereochemical problem. Among the different computational possibilities, we decided to utilize the Gauge Including Atomic Orbitals (GIAO) calculation of ^{13}C NMR chemical shifts by ab initio DFT method and using 6-31G(d,p) basis set,[16] since it proved to perform very well in other cases of medium-small terpenes.[15]

Each of the possible candidates A–D was then subjected to conformational search using the Simulated Annealing procedure (InsightII Software Package), obtaining a set of conformations, which was optimized using the CVFF force field and a quasi-Newton–Raphson minimization method (VA09A). The resulting conformers were then pooled into families and ranked on the basis of their conformational energy values. As a result of this analysis, all the structures A–D proved to possess significantly low flexibility, since in each case a single conformational family accounted for more

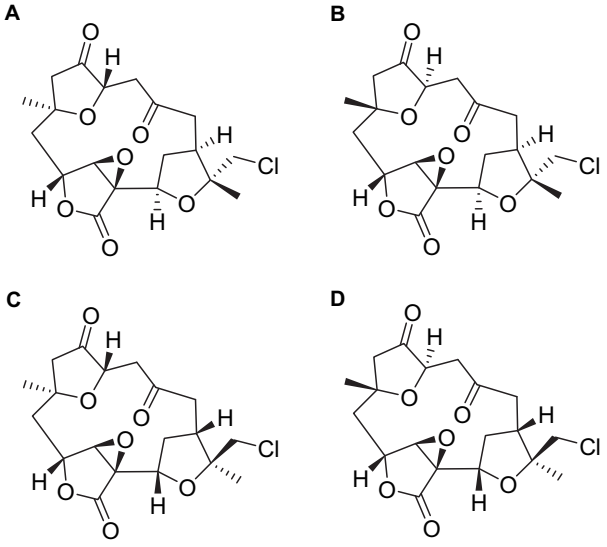

Fig. 2. The four candidate relative configurations for chloroscabrolide A (**3**).

than 98% of the reasonably populated conformers (Fig. 3). Finally, for each conformation, the geometries were fully optimized and the NMR chemical shifts were calculated at the same level with the GIAO option using the MPW1PW91/6-31G(d,p) DFT method. All these calculations were carried out using the Gaussian 03 program.[17]

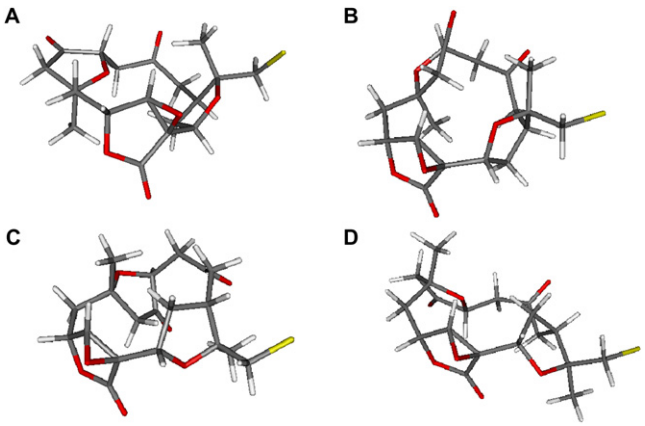

Fig. 3. Minimum energy conformations calculated for structures A–D.

In order to evaluate which of the four stereoisomers A–D best fits with the experimental data, the experimental shifts were plotted against the calculated shifts and the least-squares fit values of slope, intercept and correlation factor (r^2) were determined. Difference plots were determined by subtracting the so corrected chemical shifts from the experimental chemical shifts. In Fig. 4 we report the mean absolute error (MAE=$\sum[|(\delta_{exp}-\delta_{calcd})|]/n$) for each stereoisomer, expressed in $\Delta\delta$ units. The lowest value of 3.09 ppm obtained for A clearly evidences the better agreement between the chemical shifts calculated for this stereoisomer and the experimental values, compared to all the other stereoisomers. Therefore, quantum-mechanical calculations of ^{13}C NMR chemical shifts indicated that structure A (or its enantiomer) should be assigned to chloroscabrolide A (**3**).

Chloroscabrolide B (**4**) was isolated as a colorless amorphous solid with the molecular formula $C_{20}H_{27}ClO_8$, differing from that of chloroscabrolide A (**3**) for the presence of an additional CH_4O fragment. This difference was explained with the opening of the lactone ring and the formation of a methyl ester, on the basis of the following spectral evidences: (i) the ^{1}H NMR spectrum of **4** was very similar to that of **3** but showed an additional methoxy singlet at δ_H 3.80 and a broad exchangeable signal at δ_H 3.82 (OH-10); (ii) chloroscabrolides A (**3**) and B (**4**) featured exactly parallel spin systems (Fig. 5), including also very similar proton resonances (Table 1), with the exception of the shift at higher fields for H-10 (δ_H 4.39 in **4** instead of 4.67 in **3**) and H-11 (δ_H 3.51 in **4** instead of 4.27 in **3**); (iii) the ^{13}C NMR spectra of the two molecules were practically superimposable (Table 2), apart from the presence of the methoxy carbon at δ_C 52.0 and the marked high-field shift of C-10 (δ_C 64.7 in **4** instead of 76.7 in **3**); (iv) the g-HMBC spectrum of **4** (Fig. 5) allowed the attachment of all the partial moieties and confirmed a planar structure very similar to that of chloroscabrolide A, with the single difference of the opening of the lactone ring and the formation of a methyl ester. This was unambiguously indicated by the HMBC cross-peak between the methoxy singlet at δ_H 3.80 and the ester carbon (C-19, δ_C 167.7), and by the absence of a cross-peak between H-10 and C-19.

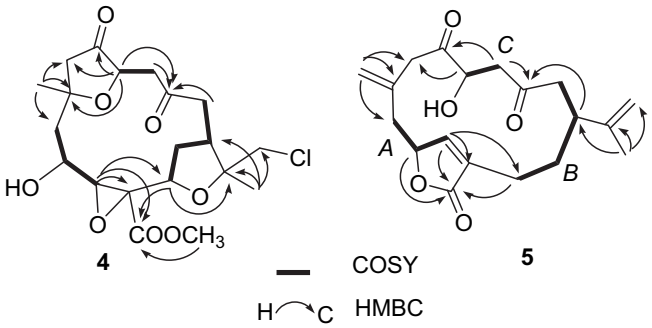

Fig. 5. COSY and $^{2,3}J_{H\rightarrow C}$ HMBC correlations for chloroscabrolide B (**4**) (left) and prescabrolide (**5**) (right).

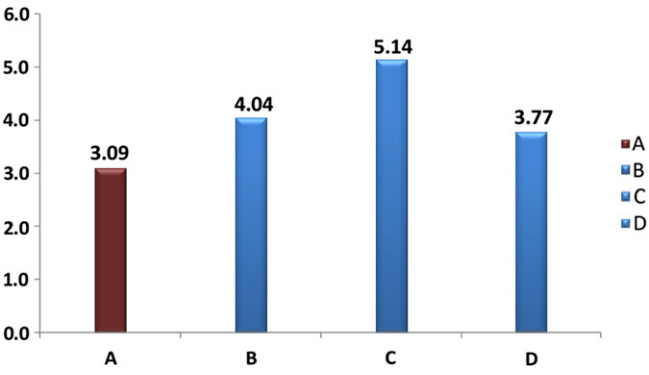

Fig. 4. Mean absolute error ($\sum[|(\delta_{exp}-\delta_{calcd})|]/n$) relative to stereoisomers A–D, in $\Delta\delta$ (ppm) units.

The relative configuration around the two five-membered rings of **4** was established on the basis of the ROESY cross-peaks Me-18/H_2-4, H-1/H-13, and H-13/H_2-16, while the relative configuration at C-10, C-11, and C-12 was assigned through the ROESY cross-peaks H-11/19-OMe and H-10/H-13. All these relative configurations exactly parallel those previously established for **3**.

The structural relationship between chloroscabrolides A (**3**) and B (**4**) raised the question whether compound **4** is actually an artifact produced from **3** during the extraction process with methanol. To shed light on this issue, 1.5 mg of pure chloroscabrolide A (**3**) was dissolved in MeOH (5.0 mL) and the solution was left at room temperature for 4 days (extraction conditions). The ^{1}H NMR spectrum revealed the absence of any conversion of chloroscabrolide A (**3**) in chloroscabrolide B (**4**).

HR-ESIMS assigned the molecular formula $C_{19}H_{24}O_5$, implying eight degrees of unsaturation, to prescabrolide (**5**), isolated in minute amounts after repeated purifications of the same MPLC fraction containing chloroscabrolide A (**3**). The ^{1}H NMR spectrum of **5** ($CDCl_3$, Table 1) included a broad singlet at δ_H 7.21, two multiplets at δ_H 5.16 and 4.14 and four broad singlets between δ_H 4.65 and 4.90. The ^{1}H NMR spectrum was completed by a series of multiplets between δ_H 1.75 and 3.00 and by a single methyl singlet at δ_H 1.66. All these proton signals were associated to those of the directly linked carbon atoms through the 2D NMR HSQC spectrum, which allowed the identification of one sp^2 methine, two sp^2 methylenes (δ_H 4.68 and 4.80, δ_C 112.9; δ_H 4.74 and 4.88, δ_C 113.4), three sp^3 methines (two of which are oxymethines), six sp^3 methylenes and one methyl group. Inspection of the ^{13}C NMR spectrum allowed us to identify the remaining unprotonated carbons as two ketone carbonyls (δ_C 207.5 and 212.6), one ester carbonyl (δ_C 174.4), and three additional sp^2 carbons involved in carbon–carbon double

bonds. These data clearly accounted for six out of the eight unsaturations, indicating that prescabrolide (**5**) is a bicyclic compounds.

The spin systems deduced from inspection of the 2D NMR COSY spectrum of **5** (Fig. 5) closely paralleled those defined for **3** (Fig. 1) and **4** (Fig. 5), but with two significant exceptions: (i) spin system A terminates with the deshielded sp^2 methine at δ_H 7.21 (H-11) and not with the epoxide signal; (ii) spin system B starts with a methylene (H_2-13) and not with an oxymethine.

Also in the case of prescabrolide the g-HMBC spectrum provided information of pivotal importance to join the fragments and deduce the planar structure. Similarly to chloroscabrolides, fragments B and C were joined through a ketone carbonyl, while correlations of the sp^2 methylene H_2-18 with C-7, C-8, and C-9, and those of the oxymethine H-5 with the ketone carbonyl C-6 and with C-7 defined the connection between fragments A and C. The g-HMBC cross-peaks of both H-10 and H-11 with the ester carbonyl (C-19) and with C-12, as well as the cross-peaks H-11/C-13 and H_2-13/C-19 completely defined the junction between moieties A and B identifying the presence of a butenolide (α,β-unsaturated γ-lactone) ring. Finally, the attachment of an isopropenyl group at C-1 was deduced by the g-HMBC cross-peaks of Me-17 with C-15, C-16 (the second sp^2 methylene), and C-1 and completely defined the planar structure of prescabrolide (**5**). Given their location on the 14-membered ring, the relative configurations at the three stereogenic centers of compound **5** (C-1, C-5, C-10) could not be deduced from inspection of the ROESY spectrum. Unfortunately, the very small amounts obtained for prescabrolide prevented the application of strategies for relative (or absolute) configuration assignments, which are mainly based on the derivatization of the starting material.

The isolation of prescabrolide (**5**) is particularly interesting since it can be conceived as a likely precursor of the scabrolide/leptocladolide family of cembranoids. These compounds should originate from epoxidation of the C-11/C-12 double bond and linkage of the OH-5 at the sp^2 carbon C-8, which would give rise to the THF ring. In the case of chloroscabrolides a further oxygen bridge connects C-13 and C-15.

Since previous studies have reported that some (nor)cembrane-type diterpenoids possess inducible NO-synthase (iNOS) protein inhibitory activity,[2,18] all the norcembranoids isolated during this study where evaluated for this activity. iNOS is regulated by inflammatory mediators (LPS, cytokines),[19] and the excessive production of NO by iNOS has been implicated in the pathogenesis of the inflammatory response.[20] In this assay, the production of NO_2^- (stable metabolite of NO) was evaluated as a parameter of macrophage activation and iNOS induction. Unstimulated J774 cells generated undetectable (<5 nmol/mL) amounts of NO_2^-, while stimulation of the cells with LPS (1 μg/mL) for 24 h produced a dose-dependent release of NO_2^- (15.6±0.1 nmol/mL). When the cells were incubated with scabrolide D (**2**) at concentration of 10 μM, a 15% inhibition of NO_2^- production was observed. All the other compounds tested resulted inactive at the same concentration. The structural features required to cembranoids for iNOS inhibition have not been investigated; however, it appears clearly that the presence of a further five-membered ring in chloroscabrolides (and/or of a chlorine atom) has deleterious effects on the pharmacological activity.

3. Conclusions

Chemical investigation of the organic extract obtained from *Sinularia* sp. yielded three new norcembranoids, two of which, named chloroscabrolides A and B, possess an unprecedented oxygen bridge connecting C-13 and C-15 and bear a chlorine atom at C-16. Chlorinated compounds are extremely rare among soft coral metabolites and, to the best of our knowledge, this is only the second example within the class of cembranoids. The isolation of chloroscabrolides well exemplifies the incredible chemical diversity associated to the cembrane diterpenoid family, which continue to yield structurally intriguing and biologically interesting secondary metabolites.

4. Experimental

4.1. General

Optical rotations ($CHCl_3$) were measured at 589 nm on a P2000 Jasco polarimeter using a 10 cm microcell. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were measured on a Varian INOVA spectrometer. Chemical shifts were referenced to the residual solvent signal ($CDCl_3$: δ_H 7.26, δ_C 77.0). Homonuclear ^1H connectivities were determined by the COSY experiment; one-bond heteronuclear $^1H-^{13}C$ connectivities by the HSQC experiment; two- and three-bond $^1H-^{13}C$ connectivities by gradient-HMBC experiments optimized for a $^{2,3}J$ of 9 Hz. Through-space ^1H connectivities were evidenced by using a ROESY experiment with a mixing time of 500 ms. Low- and high-resolution ESIMS spectra were obtained on an LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Medium pressure liquid chromatography was performed on a Büchi apparatus using a silica gel (230–400 mesh) column; HPLC were achieved on a Knauer apparatus equipped with a refractive index detector and analytical LUNA (Phenomenex) SI60 (250×4 mm) columns.

4.2. Animal material, extraction, and isolation

Colonies of *Sinularia* sp. (580 g wet weight) was collected in January 2010 in the Bunaken Marine Park of Manado along the coasts of the small island of Siladen (North Sulawesi, Indonesia) at a depth of 2–5 m. A small voucher sample is deposited at the Dipartimento per lo Studio del Territorio e delle sue Risorse, Università di Genova. The colonies have been repeatedly extracted with MeOH and $CHCl_3$ at room temperature and the obtained material (6.8 g) has been chromatographed by MPLC over silica gel eluting with a gradient system of increasing polarity from *n*-hexane to EtOAc to MeOH. Fractions eluted with *n*-hexane/EtOAc 7:3 were further purified by HPLC (*n*-hexane/EtOAc 1:1) to afford leptocladolide B (1, 3.5 mg); fractions eluted with *n*-hexane/EtOAc 6:4 were further purified by HPLC (*n*-hexane/EtOAc 6:4) to afford scabrolide D (**2**, 19.3 mg); fractions eluted with *n*-hexane/EtOAc 4:6 were further purified by HPLC (*n*-hexane/EtOAc 1:1) to afford chloroscabrolide A (**3**, 6.2 mg) and prescabrolide (**5**, 1.4 mg), while fractions eluted with *n*-hexane/EtOAc 2:8, purified by HPLC (*n*-hexane/EtOAc 3:7), afforded chloroscabrolide B (**4**, 4.6 mg).

*4.2.1. Chloroscabrolide A (**3**).* Colorless viscous oil; $[\alpha]_D^{25}$ +18.2 (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 2930, 1755, 1358, 1088 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) Table 2; (+) ESI-MS *m*/*z* 421, 423 (3:1) $[M+Na]^+$; HREI-MS found *m*/*z* 421.1022 calcd for $C_{19}H_{23}{}^{35}ClNaO_7$ *m*/*z* 421.1030.

*4.2.2. Chloroscabrolide B (**4**).* Colorless amorphous solid; $[\alpha]_D^{25}$ −8.5 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3479, 2942, 1737, 1709, 1374, 1096 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) Table 2; (+) ESI-MS *m*/*z* 453, 455 (3:1) $[M+Na]^+$; HREI-MS found *m*/*z* 453.1300 calcd for $C_{20}H_{27}{}^{35}ClNaO_8$ *m*/*z* 453.1292.

*4.2.3. Prescabrolide (**5**).* Colorless amorphous solid; $[\alpha]_D^{25}$ −5.5 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3429, 2924, 1740, 1699, 1407, 1215, 1080 cm^{-1}. H NMR ($CDCl_3$, 500 MHz) Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz) Table 2;

(+) ESI-MS m/z 355 $[M+Na]^+$; HREI-MS found *m/z* 355.1529 calcd for $C_{19}H_{24}NaO_5$ *m/z* 355.1521.

4.2.4. Computational calculations. A conformational search on each stereoisomer A–D was performed by Simulated Annealing in the INSIGHT II package. Using the steepest descent followed by quasi-Newton–Raphson method (VA09A) the conformational energy was minimized. Restrained simulations were carried out for 500 ps using the CVFF force field as implemented in Discover software (Accelrys, San Diego, USA). The simulation started at 1000 K, and then the temperature was decreased stepwise to 300 K. The final step was again the energy minimization, performed in order to refine the structures obtained, using the steepest descent and the quasi-Newton–Raphson (VA09A) algorithms successively. Both dynamic and mechanic calculations were carried out by using 1 Kcal/(mol $Å^2$) 2 flat well distance restraints. One hundred structures were generated. To simulate the solvent chosen for NMR analysis, a distance-dependent dielectric constant set to the value of $CHCl_3$ (ε 4.8) was used during the calculations. All optimizations were performed with the software package Gaussian 03, by using the DFT functional DFT MPW1PW91 and the basis set 6-31G(d). GIAO ^{13}C NMR chemical shifts were performed using the MPW1PW91 functional, the 6-31G(d, p) basis set, using as the input the geometry previously optimized at MPW1PW91/6-31G(d) level of theory.

4.2.5. iNOS inhibitory activity. The murine monocyte/macrophages J774 (ECACC) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Biowhittaker) and cultured at 37 °C in humidified 5% CO_2/95% air. The cells were plated in 24 well culture plates (Falcon) at a density of 2.5×10^6 cells/mL/well and allowed to adhere for 2 h. Thereafter, the medium was replaced with fresh medium and cells were activated by lipopolysaccharide (LPS 1 μg/mL) from *Escherichia coli* (Fluka) for 24 h in presence or absence of different concentrations of the test compounds **1**–**5**. Thereafter, culture medium was removed, centrifuged and the supernatant was used for the determination of nitrite (NO_2^-) production. Cell viability (>95%) was determined with the MTT assay (Denizot and Lang, 1986). NO_2^- levels in culture media from J774 macrophages were measured 24 h after LPS with the Griess reaction as previously described.[21] Results are expressed as nmol/mL of NO_2^- and represent the mean±S.E.M. of three experiments run in triplicates.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2011.08.024.

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