New Anti-inflammatory Norcembranoids from the Soft Coral Sinularia numerosa

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Two new norcembranoids, sinumerolide A (1) and its epimer, 7α-sinumerolide A (2), were isolated from the ethyl acetate extract of the soft coral Sinularia numerosa. The structures of compounds 1 and 2 were established using spectroscopic methods. In the in vitro anti-inflammatory effect test, norcembranoids 1 and 2 were found to inhibit the accumulation of the pro-inflammatory inducible nitric oxide synthase protein of lipopolysaccharide-stimulated RAW264.7 macrophage cells significantly.

Key words norcembranoid; Sinularia numerosa; sinumerolide; anti-inflammatory activity; inducible nitric oxide synthase

Results and Discussion

Sinumerolide A (1) was isolated as colorless oil and the molecular formula for this compound was determined to be C₂₀H₂₆O₆ (8° of unsaturation) from a sodiated molecule at m/z 385 in the electrospray ionization (ESI)-MS spectrum, which was further supported by high-resolution (HR)-ESI-MS (m/z 385.16201, Calcd for 385.16216, [C₂₀H₂₆O₆Na]+). The ¹³C-NMR and distortion enhancement by polarization transfer (DEPT) spectra of 1 showed that 1 had 20 carbons (Table 1), including three methyls (including a methoxy group), four sp³ methylenes, an sp² methylene, four sp³ methines (including three oxymethines), two sp² methines, and six sp² quaternary carbons (including three carbonyls). Two trisubstituted and an 1,1-disubstituted carbon–carbon double bonds were identified from the NMR signals at δC 154.6 (C-8), 124.6 (CH-7), and δH 6.44 (1H, brs, H-7); δC 130.6 (C-12), 146.3 (CH-13), and δH 6.51 (1H, dd, J=11.6, 4.0Hz, H-13); and δC 146.0 (C-15), 111.2 (CH₂-16), and δH 4.94 (1H, brs, H-16), 4.75 (1H, brs, H-16), respectively.

From the ¹H–¹H correlation spectroscopy (COSY) spectrum of 1 (Table 1), the spin systems of H₂-2/H-1/H₂-14/H-13, H₅-4/H-5, and H₂-9/H-10/H-11 were differentiated. These data, together with the heteronuclear multiple-bond coherence (HMBC) correlations between H-1/C-3; H₂-2/C-1, -3, -14; H₅-4/C-3, -5, -6; H-7/C-6, -9; H₂-9/C-7, -8, -10, -11; H-10/C-11; H-11/C-10; and H-13/C-11, established the main carbon skeleton of 1. The vinyl methyls at C-8 and C-15 were confirmed by the HMBC correlations between H-7/C-18; H₂-18/C-7, -8, -9; and H₂-16/C-1, -17; and supported by the allylic couplings between H-7/H₂-18 and H₂-16/H₂-17 in the ¹H–¹H COSY spectrum of 1. The methoxy group at C-5 was confirmed by the HMBC correlations between the oxygen-bearing methyl protons at δH 3.44 and the C-5 oxymethine at δC 83.7; and the C-5 oxymethine proton at δH 4.08 and the methoxy carbon at δC 58.2. These data, together with the HMBC correlations between the C-10 and C-11 oxymethine protons at δH 4.70 and 4.52 with the ester carbonyl at δC 168.2, were used to establish the molecular framework of 1.

The relative configuration of compound 1 observed in the nuclear Overhauser effect spectroscopy (NOE) experiment corroborated the MM2 force field calculations which suggested the most stable conformation as shown in Fig. 1.¹⁵ The Z-geometry of the 7,8-double bond was established by the interaction between H-7 and H-18. One proton attaching at C-14 and resonating at δH 3.72 was found to show an interaction with H-1 and was assigned arbitrary as H-14α. Thus, the isopropenyl group at C-1 should be β-oriented. The other proton attaching at C-14, H-14β, showed an interaction with the olefinic proton H-13, confirming the upward orientation of H-13. The significant interaction shown between oxymethine proton H-11 and olefinic proton H-13 revealed the parallel orientation of C11-H and C13-H, and hence, the S* configuration at C-11 and the cis orientation.
of the 12,13-double bond, according to a molecular model presented in Fig. 1. One proton of C-9 methylene protons at \( \delta_H 3.52 \) showed correlation with H-11 and was assigned as H-9\( \alpha \). Therefore, the interaction observed between the other H-9 resonating at \( \delta_H 2.49 \) and H-10 depicted the \( \beta \)-orientation of H-10, and hence the \( R^\ast \) configuration at C-10. The interactions disclosed between both H-10 and H-9\( \beta \) and H-3-18, H3-18/H-7, and H-7/H-5, revealed that H-5 is positioned on the upward face of the 14-membered ring as shown in Fig. 1 and is syn oriented relative to H-10. On the basis of the above findings, the structure of sinumerolide A (1), was established as shown in formula 1. It was found that the structure of 1 was similar with that of a known leptocladolide A (3), isolated from a Taiwanese soft coral \textit{Sinularia leptoclados}, and compound 1 was found to be the 5-deethoxy-5-methoxy derivative of leptocladolide A.

The new metabolite, 7\( E \)-sinumerolide A (2), has the same molecular formula \( C_{20}H_{26}O_6 \) and was considered to be an isomer of 1 on the basis of HR-ESI-MS and NMR spectroscopic data (Table 2). Also, spectroscopic data revealed the presence of a hydroxy group and a methoxy group in 2. In general, the \( ^1H \)- and \( ^13C \)-NMR data of 2 were found to be similar to those of 1. Nevertheless, the chemical shifts of C-1 (\( \delta_C 39.3 \)) and C-14 (\( \delta_C 27.5 \)) of 2 were close to those of 1 (\( \delta_C 38.3 \) and 27.9), suggesting that the relative configuration at C-1 is possibly the same as that of 1. Moreover, the upfield shift for C-18, and the downfield shift of C-9 in comparison with those of 1 (Tables 1, 2), suggesting that 2 could possess different geometry for the 7,8-carbon, carbon double bond, in contrast to that of 1. According to the NOESY spectrum of 2 (Fig. 2), H-7 did not show response with H-3-18, indicating the \( E \)-geometry of 7,8-double bond. Furthermore, comparison of the \( ^1H \)-NMR chemical shifts, coupling pattern, and coupling constants of protons attaching at the chiral carbons C-1 (\( \delta_H 2.98, \text{m}, H-1 \)), C-5 (\( \delta_H 3.96, \text{dd}, J=6.8, 2.4\text{Hz}, H-5 \)), C-10 (\( \delta_H 4.73, \text{dd}, J=4.8, 2.4\text{Hz}, H-10 \)), and C-11 (\( \delta_H 4.63, \text{d}, J=2.4\text{Hz}, H-11 \)) in 1 with those of a known cembrane analogue, 7\( E \)-leptocladolide A (4) (\( \delta_H 2.97, \text{dd}, J=9.0, 4.5\text{Hz}, H-1; 4.05, \text{dd}, J=7.0, 2.5\text{Hz}, H-5; 4.73, \text{brdd}, J=4.5, 3.0\text{Hz}, H-10; 4.63, s, H-11 \)) indicated that the chiral carbons C-1, C-5, C-10, and C-11 in 2 possessed the same relative configurations as \( S^* \), \( S^* \), \( R^* \), and \( S^* \)-forms as those of 4, respectively. By consideration of the above findings, the structure of compound 2 was established as 7\( E \)-sinumerolide A and this compound

![Chart 1](image.png)

**Table 1. \( ^1H \)- and \( ^13C \)-NMR Data, \( ^1H \)-\( ^1H \) COSY, and HMBC Correlations for 1**

| C/H | \( \delta_H \) \( ^a \) | \( \delta_C \) \( ^b \) | \( ^1H \)-\( ^1H \) COSY | HMBC \( (H\rightarrow C) \) |
|-----|----------------|----------------|----------------------------|---------------------|
| 1   | 2.85 m         | 38.3 (CH\( ^0 \)) | H-2, H-14                  | C-3                 |
| 2a/\( \beta \) | 2.52 m; 2.82 m | 45.1 (CH\( _3 \)) | H-1                        | C-1, -3, -14, -15   |
| 3   | 205.8 (qC)     | 42.4 (CH\( _3 \)) | H-5                        | C-3, -5, -6         |
| 5   | 4.08 dd (9.6, 2.8) | 83.7 (CH) | H-4 | OCH\( _3 \) |
| 6   | 198.8 (qC)     | 124.6 (CH)      | H-18                       | C-6, -9, -18        |
| 7   | 6.44 brs       | 154.6 (qC)      | H-18                       | C-6, -9, -18        |
| 8   | 6.51 dd (13.2, 8.8); 2.49 dd (13.2, 7.6) | 35.5 (CH\( _3 \)) | H-10                       | C-7, -8, -10, -11, -18 |
| 10  | 4.70 dd (8.8, 7.6) | 81.0 (CH) | H-9, H-11                  | C-11, -19           |
| 11  | 4.52 s         | 75.7 (CH)       | H-10                       | C-10, -19           |
| 12  | 130.6 (qC)     | 146.3 (CH)      | H-14                       | C-11                |
| 13  | 6.51 dd (11.6, 4.0) | 111.2 (CH\( _3 \)) | H-17                       | C-1, -17           |
| 14a/\( \beta \) | 3.72 ddd (15.6, 11.6, 4.0); 2.25 ddd (15.6, 4.0, 4.0) | 27.9 (CH\( _3 \)) | H-1, H-13               | n.o.\(^c\) |
| 15  | 146.0 (qC)     | 11.2 (CH\( _3 \)) | H-17                       | C-1, -17           |
| 16  | 4.94 brs; 4.75 brs | 22.4 (CH\( _3 \)) | H-16                       | C-1, -15, -16      |
| 17  | 1.83 s         | 25.5 (CH\( _3 \)) | H-7                        | C-7, -8, -9        |
| 18  | 2.01 s         | 168.2 (qC)      |                            |                     |
| 19  | 5-OCH\( _3 \)  | 58.2 (CH\( _3 \)) |                            | C-5                |

\( ^a \) Spectra measured at 400 MHz in CDCl\( _3 \) at 25°C. \( ^b \) Spectra measured at 100 MHz in CDCl\( _3 \) at 25°C. \( ^c \) J value (in Hz) in parentheses. \( ^d \) Multiplicity was deduced by \( ^13C \), DEPT, and HMBC experiments. \( ^e \) n.o. = not observed.
was found to be the 5-deethoxy-5-methoxy derivative of 7E-leptocladolide A isolated from *S. parva*.12)

In the *in vitro* anti-inflammatory activity test, the upregulation of the pro-inflammatory inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression of lipopolysaccharide (LPS)-stimulated RAW264.7 macrophage cells was evaluated using immunoblot analysis. From anti-inflammatory assay results, both compounds 1 and 2 at doses of 10 \( \mu \)M showed significant inhibition of iNOS but not COX-2 protein expression compared to LPS-alone group (Fig. 3). To evaluate the cytotoxic effects of compounds 1 and 2 on the viability of RAW264.7 macrophage cells, we used
the alamar blue assay. The viability of macrophage cells at 16h after treatment with compounds 1 and 2 (10 μM). Both compounds (10 μM) did not significantly affect the viability of macrophage cells 16h after treatment. Thus, compounds 1 and 2 might be promising as anti-inflammatory agents, as they do not exhibit cytotoxicity to RAW264.7 macrophage cells.

**Experimental**

**General** Optical rotations were measured at a HORIBA SEPA-300 digital polarimeter. Infrared spectra were recorded on a JASCO 4100 FT-IR spectrometer; peaks are reported in cm⁻¹. NMR spectra were recorded on a VARIAN MERCURY PLUS 400 NMR spectrometer using the residual CHCl₃ signal as internal standard for ¹H-NMR (δH, 7.26 ppm) and CDCl₃ for ¹³C-NMR (δC, 77.0 ppm). Coupling constants (J) are given in Hz. ESI-MS and HR-ESI-MS data were recorded using a BRUKER 7 TESLA solariX FT-mass system. Column chromatography was performed on silica gel (230–400 mesh, Merck, Darmstadt, Germany). TLC was carried out on precoated Kieselgel 60 F 254 (0.25 mm, Merck); spots were visualized by spraying with 10% H₂O₂ solution followed by heating. The normal phase HPLC (NP-HPLC) was performed using a system comprised of a HITACHI L-7110 pump and a RHEODYNE 7725 injection port. A normal phase column (Supelco Ascentis® Si Cat #: 581514-U, 25 cm × 10 mm, 5 μm) was used for NP-HPLC.

**Animal Material** Specimens of the octocoral *Sinularia numerosa* were collected by hand in a 0.6 t tank with a flow-through sea water system located in the National Museum of Marine Biology and Aquarium (NMMBA), Taiwan, in 30 July 2014, and stored at −20°C until extraction. A voucher specimen (NMMBA-TWSC-14009) was deposited in the NMMBA, Taiwan. This organism was identified with previous descriptions.¹

**Extraction and Isolation** Specimens of the soft coral *Sinularia numerosa* (wet weight 487 g, dry weight 69 g) were minced and extracted with ethyl acetate (EtOAc). The EtOAc extract left after removal of the solvent (5.0 g) was chromatographed on silica gel by column chromatography and eluted with acetone in dichloromethane (DCM) (0−100%, gradient) to yield 22 fractions 1−22. Fraction 8 was separated by NP-HPLC, using a mixture of n-hexane and acetone (2:1) to yield 18 subfractions 8A−8R. Fraction 8J was repurified by NP-HPLC, using a mixture of DCM and EtOAc (4:1, flow rate: 2.0 mL/min) to yield 18 fractions 8A−8R. Fraction 8K was separated by NP-HPLC, using a mixture of n-hexane and EtOAc (1:1, flow rate: 2.0 mL/min) to yield 2 (1.3 mg, tR = 46 min).

**In Vitro Anti-inflammatory Assay** According to our previous and other studies for the in vitro anti-inflammatory activity assay, we used LPS induced RAW murine macrophage cell line which was purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) ¹⁴−¹⁷ The in vitro anti-inflammatory activity of compounds 1 and 2 was measured by examining the inhibition of lipopolysaccharide (LPS)-induced upregulation of pro-inflammatory iNOS and COX-2 protein expression in macrophage cells using Western blotting analysis.¹⁷−¹⁹ Briefly, inflammation in macrophages was induced by incubating them for 16h in a medium containing only LPS (10 ng/mL) without compounds. For the anti-inflammatory activity assay, compounds 1, 2, and dexamethasone (10 μM) were added to the cells 10min before the LPS challenge. The cells were then
for Western blot analysis. The immunoreactivity data were calculated with respect to the average optical density of the corresponding LPS-stimulated group. RAW264.7 macrophage cells viability was determined after treatment with alamar blue (Invitrogen, Carlsbad, CA, U.S.A.), the tetrazolium dye that is reduced by living cells to fluorescent products. This assay is similar in principle to the cell viability assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and has been validated as an accurate measure of the survival of RAW264.7 macrophage cells.\textsuperscript{20,21} For statistical analysis, the data were analyzed by a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls post hoc test for multiple comparisons. A significant difference was defined as a $p$-value of $<0.05$.

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Conflict of Interest The authors declare no conflict of interest.

References

1) Chen W.-T., Li Y., Guo Y.-W., \textit{Acta Pharm. Sin. B}, 2, 227–237 (2012).
2) Yang B., Zhou X.-F., Lin X.-P., Liu J., Peng Y., Yang X.-W., Liu Y., \textit{Curr. Org. Chem.}, 16, 1512–1539 (2012).
3) Rocha J., Peixe L., Gomes N. C. M., Calado R., \textit{Mar. Drugs}, 9, 1860–1886 (2011).
4) Su J., Yu X., Zeng L., Mak T. C. W., \textit{J. Nat. Prod.}, 52, 934–940 (1989).
5) Tillekeratne L. M. V., Liyanage G. K., Ratnasooriya W. D., Ksebati M. B., Schmitz F. J., \textit{J. Nat. Prod.}, 52, 1143–1145 (1989).
6) Tseng Y.-J., Yang Y.-C., Wang S.-K., Duh C.-Y., \textit{Mar. Drugs}, 12, 3371–3380 (2014).
7) Chen W.-F., Yin C.-T., Cheng C.-H., Lu M.-C., Fang L.-S., Wang W.-H., Wen Z.-H., Chen J.-J., Wu Y.-C., Sung P.-J., \textit{Int. J. Mol. Sci.}, 16, 3298–3306 (2015).
8) Sato A., Fenical W., Zheng Q.-T., Clardy J., \textit{Tetrahedron}, 41, 4303–4308 (1985).
9) Qin M.-L., Li X.-M., Wang B.-G., \textit{Oceanol. Limnol. Sin.}, 40, 540–544 (2000).
10) Yamashita T., Nakao Y., Matsunaga S., Oikawa T., Imahara Y., Fusetani N., \textit{Bioorg. Med. Chem.}, 17, 2181–2184 (2009).
11) Dai C.-F., \textit{Atoll Res. Bull.}, 354, 1–28 (1991).
12) Allinger N. L., \textit{J. Am. Chem. Soc.}, 99, 8127–8134 (1977).
13) Ahmed A. F., Shiuie R.-T., Wang G.-H., Dai C.-F., Kuo Y.-H., Sheu J.-H., \textit{Tetrahedron}, 59, 7337–7344 (2003).
14) Ho F.-M., Lai C.-C., Huang L.-J., Kuo T.-C., Chao C.-M., Lin W.-W., \textit{Br. J. Pharmacol.}, 141, 1037–1047 (2004).
15) Park J. S., Woo M. S., Kim S. Y., Kim W. K., Kim H. S., \textit{J. Neuroimmunol.}, 168, 56–64 (2005).
16) Lin Y.-F., Kuo C.-Y., Wen Z.-H., Lin Y.-Y., Wang W.-H., Su J.-H., Sheu J.-H., Sung P.-J., \textit{Molecules}, 18, 8160–8167 (2013).
17) Huang S.-Y., Chen N.-F., Chen W.-F., Hung H.-C., Lee H.-P., Lin Y.-Y., Wang H.-M., Sung P.-J., Sheu J.-H., Wen Z.-H., \textit{Mar. Drugs}, 10, 1899–1919 (2012).
18) Jean Y.-H., Chen W.-F., Sung C.-S., Duh C.-Y., Huang S.-Y., Lin C.-S., Tai M.-H., Tseng S.-F., Wen Z.-H., \textit{Br. J. Pharmacol.}, 158, 713–725 (2009).
19) Jean Y.-H., Chen W.-F., Duh C.-Y., Huang S.-Y., Hsu C.-H., Lin C.-S., Sung C.-S., Chen I.-M., Wen Z.-H., \textit{Eur. J. Pharmacol.}, 578, 323–331 (2008).
20) Chen L.-C., Lin Y.-Y., Jean Y.-H., Lu Y., Chen W.-F., Yang N.-S., Wang H.-M. D., Jang I.-Y., Chen I.-M., Su J.-H., Sung P.-J., Sheu J.-H., Wen Z.-H., \textit{Molecules}, 19, 14667–14686 (2014).
21) Oliveira T., Figueiredo C. A., Brito C., Stavroulakis A., Prakki A., Da Silva Velozo E., Nogueira-Filho G., \textit{Int. J. Cell Biol.}, 535789 (2014).