E/*Z*-Rubrolide O, an Anti-inflammatory Halogenated Furanone from the New Zealand Ascidian *Synoicum* n. sp.

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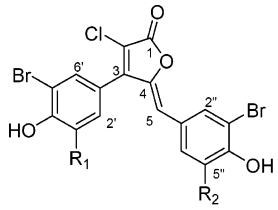
Bioassay-directed fractionation of extracts of a *Synoicum* n. sp. ascidian from New Zealand led to the isolation of the principal anti-inflammatory component, which was identified by spectroscopic methods as a new member of the rubrolide family, rubrolide O (**1**), existing as a mixture of *E*/*Z* isomers.

Worth an estimated U.S.$23 billion in 2004, the analgesic market is a major item of revenue for the pharmaceutical industry, with nonsteroidal anti-inflammatory drugs (NSAIDs) accounting for a large percentage of that income.[1] About one and a half billion people worldwide suffer from moderate to severe acute and chronic pain, ranging from sports injuries to headaches, back pain, and crippling diseases such as arthritis.[1] NSAIDs such as aspirin, rofecoxib (Vioxx), withdrawn from the market in 2004, and diclofenac (Voltaren), which target the cyclooxygenase enzymes, are effective but can have serious adverse effects.[2] In an effort to avoid these side effects, our program of discovery utilizes a preliminary step in the inflammatory cascade, namely, the activation of the respiratory burst involving the production of superoxide by human neutrophils. Quantifying neutrophil activation thereby provides a screen that can yield new classes of anti-inflammatory compounds.[3]

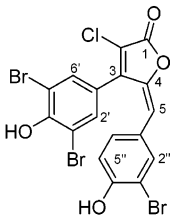
During our screening of New Zealand marine organisms for new anti-inflammatory lead compounds,[4] we investigated a specimen of a *Synoicum* n. sp. ascidian, an extract of which gave inhibition of superoxide production. A reversed-phase C18 analytical HPLC trace with diode array UV−vis detection indicated the presence of a large number of rubrolides or related compounds as the main component of the extract. Rubrolides A−H were first described in 1991 as antibacterial, phosphatase-inhibiting metabolites of the tunicate *Ritterella rubra*[5] followed in 2000 by the discovery of rubrolides I−N in a *Synoicum blochmanni* ascidian, with rubrolides I, K, L, and M exhibiting significant cytotoxicity.[6] Rubrolide A has also been detected in the Australian ascidian *Synoicum prunum*, from which were isolated the related prunolides,[7] and from an ascidian of the genus *Botryllus*, which also yielded the cadiolides, other related furanone metabolites. This latter isolation of rubrolide A reported the presence of the unusual *E*/*Z* isomeric mixture with the *Z* isomer at the $\Delta^{4,5}$ double bond forming the major component in a 3:1 mixture.[8] Hitherto all rubrolides had been reported solely as the *Z* isomer.

We now describe the identification of rubrolide O (**1**), a new rubrolide that not only exists as a mixture of *E*/*Z* isomers but is, moreover, the first anti-inflammatory rubrolide reported.

Freeze-dried specimens of the erect red *Synoicum* n. sp. were extracted with methanol followed by CH_2Cl_2. Anti-inflammatory bioassay-guided fractionation involving exhaustive reversed-phase C18 flash chromatography and Sephadex LH20 size exclusion chromatography was performed on fractions containing mixtures of ions isomeric with rubrolides A, B, C, D, I, and J, as identified by (−)-ESI FTICRMS, and other rubrolide-like compounds as detected by analytical C18 reversed-phase HPLC. Finally, reversed-phase C18 semipreparative HPLC led to the active fraction that contained two separable, but interconvertible compounds (*Z*/*E*-**1**) in a 9:1 ratio as measured by ^{1}H NMR analysis. It is worthwhile noting that of all the rubrolide-like metabolites present in this organism, only fractions containing **1** inhibited respiratory burst.



(4*Z*) - 1 R_1 = Br, R_2 = H
2 R_1 = H, R_2 = Br



(4*E*) - 1

A molecular formula of $C_{17}H_8O_4ClBr_3$ in the HRFABMS was obtained for the bright yellow, amorphous solid, isomeric with rubrolide I (**2**). Comparison with literature ^{1}H NMR data reported for **2**, however, revealed substantial differences between the chemical shift values observed for **2** and those for the major compound **1**. As observed previously for **2**, inspection of ^{1}H NMR data (Table 1) for **1** revealed the presence of one olefinic methine (δ 6.35 (s)), a symmetrical 1,3,4,5-tetrasubstituted benzene ring, and a 1,3,4-trisubstituted benzene ring. The observation of long-range COSY correlations between olefinic H-5 (δ 6.35), and H-2″ (δ 8.05) and H-6″ (δ 7.72) established connectivity between C-5 and C-1″. Further confirmation of this connectivity was secured by the observation of HMBC correlations between H-5 and C-2″ (δ 136.4) and C-6″ (δ 132.6). The attachment of the 1,3,4,5-tetrasubstituted benzene ring to furanone position C-3 (δ 148.4) was established by the observation of HMBC correlations from H-5 and H-2′/H-6′ (δ 7.82) to C-3, yielding the skeletal structure of **1**. The geometry at Δ^4 of **1** was investigated by means of a NOESY experiment. H-5 showed an NOE enhancement with both H-2′/H-6′ and H-2″/H-6″, appropriate for a *Z* configuration where H-5 is in close proximity to both phenyl rings.

Closer inspection of the ^{1}H, ^{13}C, HSQC, and HMBC NMR data acquired on the 9:1 mixture revealed the presence of the minor compound and indicated that it was structurally related to 4*Z*-**1**. Significant differences in ^{1}H NMR chemical shifts, comprised of considerable shielding ($\Delta\delta$ = 0.37−0.97 ppm) observed for the protons of both phenyl rings, with concomitant deshielding of H-5 ($\Delta\delta$ = 0.67 ppm), in conjunction with the interconvertible nature of the two compounds, suggested the presence of the 4*E*-geometrical

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Table 1. ^{1}H and ^{13}C NMR Data for 4*Z*-Rubrolide O (4*Z*-**1**) and 4*E*-Rubrolide O (4*E*-**1**)[a]

	4*Z*-rubrolide O (4*Z*-**1**)			4*E*-rubrolide O (4*E*-**1**)		
pos	δ_C	δ_H (mult, *J* in Hz)	NOESY	δ_C	δ_H (mult, *J* in Hz)	NOESY
1	164.4			not obs[b]		
2	118.7			not obs		
3	148.4			146.0		
4	145.7			not obs		
5	114.1	6.35 (s)	H-2′/H-6′, H-2″, H-6″	117.6	7.02 (s)	H-2″, H-6″
1′	123.0			123.7		
2′/6′	134.0	7.82 (s)	H-5	133.6	7.38 (s)	H-2″, H-6″
3′/5′	111.9			111.9		
4′	153.5			153.0		
1″	126.8			124.5		
2″	136.4	8.05 (d, 2.1)	H-5	135.3	7.08 (d, 2.1)	H-5, H-2′/H-6′
3″	110.9			110.0		
4″	156.8			156.0		
5″	117.7	7.20 (d, 8.6)	H-6″	116.6	6.83 (d, 8.5)	H-6″
6″	132.6	7.72 (dd, 8.6, 2.1)	H-5, H-5″	131.5	6.94 (dd, 8.5, 2.1)	H-5, H-2′/H-6′, H-5″

[a] ^{1}H NMR (observed at 600 MHz) and ^{13}C NMR data (150 MHz) recorded in $(CD_3)_2CO$. [b] Not observed.

isomer of **1**. Examination of NOESY data on the mixture determined that H-5 of the minor compound (4*E*-**1**) exhibited correlations only to H-2″/H-6″, and in addition, correlations were observed between H-2′/H-6′ and H-2″/H-6″, indicating that the phenyl rings were closer in space than in the *Z* isomer. These findings were consistent with those previously reported for both geometrical isomers of rubrolide A,[8] thus confirming the structure of the minor component as 4*E*-rubrolide O (4*E*-**1**).

Compound **1** as a 9:1 ratio of *Z*/*E* isomers inhibited superoxide production by human neutrophils in vitro with an IC_{50} of 35 μM.[3] Over the time course of the anti-inflammatory assay (30 min), cell viability was observed to be 87% in the most concentrated sample (500 μM), statistically insignificant in comparison to the DMSO control of 93%, indicating that there was no short-term toxicity of the compounds. It was also observed that the presence of **1** inhibited PMA-induced adhesion of neutrophils to the microtitre plate (data not shown). Antiproliferative activity (IC_{50} of 33 μM) was observed in the more classical 48 h exposure MTT-detected assay.[9] Although rubrolides have been reported to exhibit antibacterial activity, phosphatase inhibition, and cytotoxicity, this example with anti-inflammatory activity would appear to be unprecedented among this group of biologically active tunicate metabolites.

Experimental Section

General Experimental Procedures. Ultraviolet−visible spectra were run as MeOH solutions on a UV-2102PC Shimadzu UV−vis scanning spectrophotometer. Infrared spectra were recorded as dry films on a Spectrum One Fourier transform infrared spectrometer. NMR spectra were recorded on a Bruker Avance 600 spectrometer at 600 MHz for ^{1}H and 150 MHz for ^{13}C. The solvent signal (δ_H 2.04, δ_C 29.8) was used as reference. MS were recorded on a VG-7070 mass spectrometer. FTMS were recorded on a Thermo LTQ-FT spectrometer. Analytical reversed-phase HPLC was run on a Waters 600 HPLC photodiode array system using an Alltech Rocket C18 column (3 μm Econosphere, 33 × 7 mm) and eluting with a linear gradient of H_2O (0.05% TFA) through to MeCN. Semipreparative HPLC utilized an Alltech C18 column (10 μm Econosphere, 10 × 250 mm) with aqueous TFA (0.05%)/MeCN.

Taxonomy. *Synoicum* n. sp. (Terra Marine accession nos. MNP7213, MNP7346) collected from Tom Bowling Bay, Northland, New Zealand, inhabits subtidal reefs to 25 m depth. The zooids form circular systems on high brick-red, strawberry-shaped lobes of up to 50 mm high, raised above the sandy reef flat by a short stalk. The branchial sac has 15 rows of stigmata with 14 pairs of stigmata per mesh. The stomach is smooth; the intestine ends in a bilabate anus, which opens into the atrial cavity one-third along the length of the branchial sac. The branchial aperture has six low lobes; the atrial aperture, a long spatulate atrial lip. This species is distinguished from *S. apectetum* and *S. arenaceum* by colony structure, the atrial lip, and number of stigmata per row.

Isolation and Purification. A voucher specimen of *Synoicum* n. sp. is held at the National Institute for Water and Atmospheric Research (NIWA) Marine Biotechnology Collection, Greta Point, Wellington, as MNP7213 and MNP7346. The organism was frozen until used, then freeze-dried (59 g dry wt) and extracted with MeOH (3 × 100 mL) followed by CH_2Cl_2 (2 × 100 mL). The crude extract was subjected to reversed-phase C18 flash CC, which localized the anti-inflammatory activity in the 100% MeOH fraction. Repeated size exclusion Sephadex LH20 and C18 CC followed by reversed-phase C18 semipreparative HPLC eluting with MeCN/H_2O, 0.05% TFA (85%:15%, 5 mL/min), yielded the active component, rubrolide O (**1**), in a 9:1 ratio of *Z* to *E* isomers (7 mg, 0.012% dry wt).

Rubrolide O (*Z*/*E* 9:1) (1): amorphous, yellow solid; IR (film) 3442, 1746, 1681, 1595, 1471, 1206, 1136 cm^{-1}; UV (MeOH) λ_{max} 208 (ϵ 44 609), 261 (ϵ 16 001), 374 (ϵ 22 490) nm; ^{1}H and ^{13}C NMR data shown in Table 1; FABMS *m*/*z* (rel int) 555, 554, 553, 552, 551, 550, 549, 548 (48:50:94:100:81:73:31:28); HRFABMS *m*/*z* [M$^+$] 555.7588, $C_{17}H_8{}^{81}Br_3{}^{37}ClO_4$ requires 555.7570; *m*/*z* 553.7609, $C_{17}H_8{}^{79}Br^{81}Br_2{}^{37}ClO_4$ requires 553.7591; *m*/*z* 553.7609, $C_{17}H_8{}^{81}Br_3{}^{35}ClO_4$ requires 553.7600; *m*/*z* 551.7621, $C_{17}H_8{}^{79}Br_2{}^{81}Br^{37}ClO_4$ requires 551.7611; *m*/*z* 551.7621, $C_{17}H_8{}^{79}Br^{81}Br_2{}^{35}ClO_4$ requires 551.7620; *m*/*z* 549.7654, $C_{17}H_8{}^{79}Br_3{}^{37}ClO_4$ requires 549.7632; *m*/*z* 549.7654, $C_{17}H_8{}^{79}Br_2{}^{81}Br^{35}ClO_4$ requires 549.7641; *m*/*z* 547.7683, $C_{17}H_8{}^{79}Br_3{}^{35}ClO_4$ requires 547.7661.

Biological Assays. The anti-inflammatory superoxide assay was carried out using human neutrophils as described.[3] To measure cell viability, neutrophils were incubated with trypan blue dye (10% v/v) for 5 min. The cells were loaded onto a hemocytometer and counted differentially under a microscope. The cell viability was determined as the percentage of live cells (unstained) versus total cells (stained plus unstained) in the sample. HL60 cells were used for the antiproliferative assay as described.[9]

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