Anti-inflammatory Thiazine Alkaloids Isolated from the New Zealand Ascidian *Aplidium* sp.: Inhibitors of the Neutrophil Respiratory Burst in a Model of Gouty Arthritis

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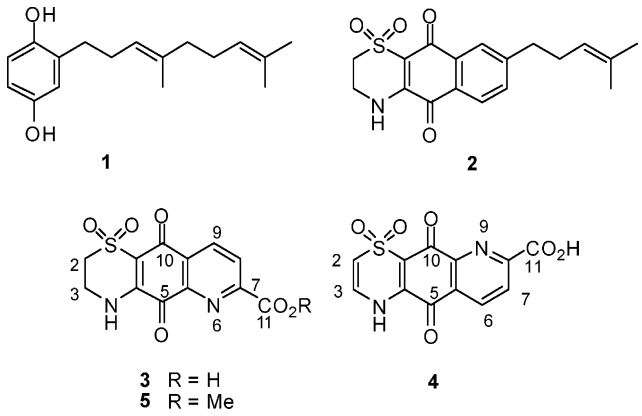
Ascidiathiazones A (**3**) and B (**4**), two new tricyclic thiazine-containing quinolinequinone alkaloids, were isolated from the New Zealand ascidian *Aplidium* species. Both compounds inhibited the *in vitro* production of superoxide by PMA-stimulated human neutrophils in a dose-dependent manner with IC_{50} 1.55 ± 0.32 and 0.44 ± 0.09 μM, respectively. *In vivo* inhibition of superoxide production by peritoneal neutrophils in a murine model of gout was observed for both compounds with oral doses of 25.6 μmol/kg. Ascidiathiazone A (**3**) was synthesized in four steps from 8-hydroxyquinoline-2-carboxylic acid.

Inflammation is a common characteristic of many debilitating human diseases including gout, rheumatoid arthritis, asthma, and chronic obstructive pulmonary disease. Many of the current treatments involve the use of nonsteroidal anti-inflammatory drugs (NSAIDs) that target the cyclooxygenase and phospholipase enzymes in the inflammatory cascade. Although these therapies can be effective, some individuals fail to respond to treatment or experience cardiovascular and gastrointestinal side effects.[1−3] Refractory disease and poor tolerance to side effects highlight a need for alternative anti-inflammatory treatments. Many inflammatory diseases, including gouty arthritis and non-atopic asthma, are associated with the infiltration of neutrophils and the subsequent production of damage-causing superoxide. Therefore inhibition of superoxide production by human neutrophils represents an alternate target of the acute inflammatory response.[4]

During screening for anti-inflammatory natural products from New Zealand biota, an extract of the ascidian *Aplidium* sp. exhibited strong inhibition of superoxide production by human neutrophils stimulated with PMA.[5] The genus *Aplidium* (family Polyclinidae) is one of the largest genera of ascidians and is found worldwide.[6] A wide variety of biologically active compounds have been reported from *Aplidium* species including simple prenylated quinones and hydroquinones (e.g., **1**), which exhibit antiproliferative activities.[7] Recently, several prenylated quinones bearing a fused 1,1-dioxo-1,4-thiazine ring, e.g., conicaquinone A (**2**), have been reported from the Mediterranean ascidian *Aplidium conicum*.[8−10] We now report that bioassay-guided fractionation of the extract of the New Zealand ascidian *Aplidium* sp. resulted in the discovery of two thiazine-containing 2-quinolinequinone carboxylic acid derivatives, which we have named ascidiathiazones A (**3**) and B (**4**). The structures of **3** and **4** were determined by spectroscopic methods, with confirmation of **3** also being achieved by X-ray crystallography. The major natural product **3** was synthesized, and *in vitro* and *in vivo* anti-inflammatory activities are reported.



Results and Discussion

Bioassay-guided fractionation of a MeOH−CH_2Cl_2 extract of *Aplidium* sp. using reversed-phase C18 flash CC (H_2O/MeOH/TFA) gave the activity concentrated in the water fraction. Repeated C18 chromatography followed by size exclusion chromatography on Sephadex LH-20 yielded two related biologically active compounds, **3**, a yellow powder (0.02% dry wt), and **4**, a pink powder (0.005% dry wt), which were characterized as the free acids.

The major compound, ascidiathiazone A (**3**), gave bright yellow solutions in neutral and acidic aqueous media, which turned pink upon the addition of base. The UV−visible spectrum revealed absorption maxima at 205, 237, 274, 290, and 420 nm, which exhibited a bathochromic shift under basic conditions to 211, 236, 329, and 467 nm, suggesting the presence of an extended aromatic chromophore and acidic/basic groups. The IR spectrum showed strong broad absorptions at 1660 and 1651 cm^{-1}, indicating the presence of a carboxylic acid and/or a 1,4-quinone. A molecular formula of $C_{12}H_8N_2O_6S$ for **3** was established by negative ion ESIFTMS.

The ^1H NMR spectrum of **3** (DMSO-d_6) was simple, comprised of two exchangeable singlets (δ 13.80 and 9.43), two ortho-coupled aromatic doublets at δ 8.51 and 8.38, and two coupled methylene resonances (δ 3.89 and 3.41, Table 1). The ^{13}C NMR spectrum of **3** (Table 1) contained 12 signals, consistent with the molecular formula, and was comprised of two methylene carbons (δ 48.1 and 39.0) and 10 sp^2 carbons (δ 110.7−176.2).

Extensive interpretation of COSY, $^1H-^{13}C$ gHSQC, and $^1H-^{13}C$ and $^1H-^{15}N$ gHMBC NMR data (Table 1) allowed construction of two fragments, comprised of a 6,7-disubstituted quinoline-quinone-2-carboxylic acid ring system and a second fragment of a 1,2-disubstituted ethane. The presence of a quinolinequinone

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Table 1. ^{1}H and ^{13}C NMR Data for Ascidiathiazones A (**3**) and B (**4**)

		ascidiathiazone A (**3**)		ascidiathiazone B (**4**)		
pos	δ_C/δ_N[a]	δ_H (mult, *J* in Hz)[b]	HMBC[c]	δ_C[d]	δ_H (mult, *J*, Hz)[f]	HMBC[c]
2	48.1	3.41 (t, 6)	N-4, 10*, 10a*	114.1	6.29 (d, 7.9)	10*, 10a
3	39.0	3.89 (m)	4a	141.8	7.34 (d, 7.9)	4a
4	103.0	9.43 (br s)		nd[e]		
4a	147.7			150.8		
5	176.2			181.1		
5a	146.2			127.8		
6	300.0			137.0	8.45 (d, 7.9)	5, 8, 9a, 10*
7	151.3			126.0	8.18 (d, 7.9)	5a, 11
8	128.9	8.38 (d, 8.1)	9a, 11	158.2		
9	135.6	8.51 (d, 8.1)	5*, 5a, 7, 10	nd		
9a	131.5			147.3		
10	173.2			176.5		
10a	110.7			113.9		
11	165.1			165.8		
COOH		13.80 (br s)		no[g]		

[a] ^{13}C NMR spectrum recorded in DMSO-d_6 at 100 MHz. ^{15}N chemical shifts deduced indirectly from ^{1}H−^{15}N HMBC NMR experiment (optimized for 6 Hz and referenced to liquid NH_3 using urea as an external standard). [b]Spectrum recorded in DMSO-d_6 at 400 MHz. [c] ^{1}H−^{13}C HMBC NMR experiment optimized for $^xJ_{CH}$ = 8.3 Hz. Correlations observed for experiment optimized for $^xJ_{CH}$ = 2 Hz are indicated by asterisks. [d] ^{13}C NMR spectrum recorded in DMSO-d_6 at 75 MHz. [e]nd: not determined. [f]Spectrum recorded in DMSO-d_6 at 300 MHz. [g]no: not observed.

fragment was suggested by the observation of ^{1}H−^{13}C HMBC correlations (experiment optimized for $^xJ_{CH}$ of 8.3 Hz) from H-9 (δ 8.51) to δ 151.3 (C-7) and 146.2 (C-5a) and a quinonoid resonance at δ 173.2 (C-10), while a carboxylic acid group could be placed at C-7 by virtue of an observed correlation from H-8 (δ 8.38) to a resonance at δ 165.1 (C-11). The presence of the carboxylic acid was supported by conversion of **3** to the methyl ester **5** by treatment with thionyl chloride in methanol (see Experimental Section). Placement of a nitrogen atom was achieved by detection of a strong ^{1}H−^{15}N HMBC correlation from H-8 (δ 8.38) to a pyridine-type ^{15}N resonance at 300.0 ppm.[11] Observation of a long-range correlation from H-9 to a second quinonoid carbon resonance at δ 176.2 (C-5) in a ^{1}H−^{13}C HMBC NMR experiment optimized for 2 Hz completed the quinolinequinone fragment.

The assignment of the two remaining quinolinequinone carbon resonances (δ 147.7 (C-4a) and 110.7 (C-10a)) and a 1,1-dioxo-1,4-thiazine ring was achieved in the following manner. Inspection of the ^{1}H NMR spectrum, combined with COSY, HSQC (^{1}H−^{13}C and ^{1}H−^{15}N), and HMBC (^{1}H−^{13}C and ^{1}H−^{15}N) NMR data analysis (Table 1), indicated the presence of a $-NHCH_2CH_2-$ spin system, while the chemical shifts of the two associated methylene ^{13}C resonances (δ 48.1 and 39.0) were similar to those previously reported for a 1,1-dioxo-1,4-thiazine moiety ($-NHCH_2CH_2SO_2-$).[8−10,12] Fusion of this moiety to positions 4a and 10a was deduced by the observation of an ^{1}H−^{13}C HMBC correlation from H_2-3 (δ 3.89) to C-4a (δ 147.7) in an experiment optimized for 8.3 Hz, while weaker correlations were observed from H_2-2 (δ 3.41) in a 2 Hz optimized ^{1}H−^{13}C HMBC experiment to carbons at δ 110.7 (C-10a) and crucially to the signal at δ 173.2, already assigned to C-10. This completed the structural assignment of compound **3**, including the proposed assignment of the thiazine ring regiochemistry. Confirmation of the proposed structure **3** was achieved with the aid of a single-crystal X-ray diffraction study (see Supporting Information).[13]

The minor compound, ascidiathiazone B (**4**), was characterized as an oxidized derivative of **3** by (−)-ESIFTMS, showing two fewer hydrogens than **3**, and NMR data, which indicated replacement of the H_2-2−H_2-3 spin system with two ortho-coupled vinylic protons at δ 7.34 and 6.29 (Table 1). As observed for **3**, long-range ^{1}H−^{13}C HMBC NMR correlations established the presence of a quinolinequinone−carboxylic acid fragment (Table 1). This left the possible structure of the minor natural product as being either **4** or the regioisomer **6**.

Compelling proof of the identity of **4** was achieved by direct comparison of NMR data observed for **4** and **6**, the latter of which

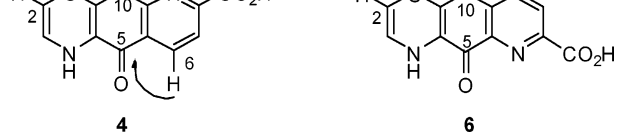


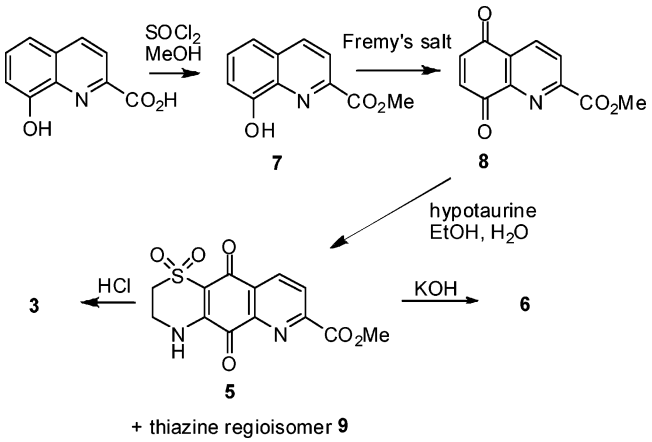
Figure 1. Selected ^{1}H−^{13}C HMBC correlations observed for **4** and **6**.

was prepared by total synthesis (*vide infra*). Significant chemical shift differences were observed for H-2/H-3/H-6/9 and H-7/8 ($\Delta\delta$ 0.12−0.33) and C-3/C-4a/C-5/C-5a and C-9a ($\Delta\delta$ 5.6−18.7), indicating that compound **6** was not the structure of the isolated minor metabolite. Further evidence for the regiochemistry of the thiazine ring was contained in correlations observed in ^{1}H−^{13}C HMBC NMR experiments on **4** and **6**. In the case of **6**, correlations were observed between both the relatively shielded β-enamine proton H-2 (δ 6.62) and pyridine ring proton H-9 (δ 8.57) and the *same* quinonoid resonance C-10 (δ 177.6), while for **4** a correlation from the β-enamine proton H-2 (δ 6.29) was observed to one quinonoid resonance C-10 (δ 176.5) and from the pyridine ring proton H-6 (δ 8.45) to the *other* quinonoid resonance C-5 (δ 181.1) (Figure 1). Thus the structure of the minor natural product was proposed to be **4**. The 4*H*-1,4-benzothiazine-1,1-dioxide ring system contained in **4** is rare in nature, with the only prior report being the euthyroidenone family of alkaloids isolated from a New Zealand bryozoan.[14]

Ascidiathiazone A (**3**) was synthesized via the route shown in Scheme 1. Commercially available 8-hydroxyquinoline-2-carboxylic acid was treated with $SOCl_2$ in MeOH at reflux to yield methyl ester **7**, which was oxidized to quinone **8** with Fremy's salt. Stirring quinone **8** in EtOH with cerium chloride heptahydrate and hypotaurine for 3 days yielded the chromatographically separable 1,1-dioxothiazine regioisomers **5** and **9** in a 10:1 ratio. Spectroscopic data observed for synthetic **5** were identical to those observed for semisynthetically prepared **5**. Hydrolysis of the ester group in **5** by heating in concentrated HCl yielded **3**, which was identical with the natural product in all respects. In contrast, saponification of **5** yielded oxidized acid **6**.

Ascidiathiazones A (**3**) and B (**4**) and compounds **5** and **8** strongly inhibited *in vitro* superoxide production by PMA-stimulated human neutrophils in a dose-dependent manner (Table 2).[4] Whereas the anti-inflammatory effect of synthetic intermediate **8** was associated with cell stasis or toxicity (AP_{50}), natural products

Scheme 1. Synthesis of Ascidiathiazone A (**3**) and the Regioisomer of Ascidiathiazone B (**6**)



3 and **4** selectively suppressed superoxide production. To rule out nonspecific antioxidant effects, **3**−**5** were also tested in a xanthine/xanthine oxidase enzyme-based superoxide scavenging assay. As shown in Table 2, all three compounds were considerably less effective as superoxide scavengers than as suppressors of superoxide production by neutrophils.

The *in vitro* results observed for **3**−**5**, with their indications of potent and selective inhibition of neutrophil superoxide production and low antiproliferative activity, prompted us to evaluate the compounds in an animal model for gout. In this model, monosodium urate crystals were injected intraperitoneally (ip) into mice, and the recruitment of neutrophils and their ability to produce superoxide were measured.[15]

Peritoneal neutrophils collected from mice treated orally with **5** (25.6 μmol/kg) exhibited marked inhibition of superoxide production (84 ± 3% inhibition) followed by **4** (59 ± 9% inhibition) and **3** (23 ± 11% inhibition) (Figure 2). Interestingly **5** showed the greatest inhibitory effect *in vivo*, whereas it was the weakest inhibitor *in vitro* (Table 2). Compound **5** also inhibited urate-induced neutrophil recruitment into the peritoneal cavity by 60% at 25 μmol/kg, whereas **3** and **4** were inactive (data not shown). Oral bioavailability and pharmacokinetics of the ester compared with the acid may play a role in these contrasting patterns of activity.

Renal and heptatic toxicity is a common problem with many existing NSAIDs.[16] Colchicine is one such drug that is still used therapeutically to treat gout despite the known hepatic side effects.[17] None of the test compounds caused significant elevation of liver and renal toxicity markers in sera from treated mice compared to the naïve and disease controls *in vivo* (Supporting Information Figure S3), and the serum markers were consistently lower than colchicine. These results provided evidence that the liver and renal side effects of these new anti-inflammatory compounds may be low or nonexistent in the context of the acute inflammation model.

In summary, the screening of New Zealand biota for bioactive molecules capable of inhibiting the respiratory burst of human neutrophils has led to the discovery of two new natural products, **3** and **4**, containing rare 1,1-dioxo-1,4-thiazine rings. We presume that these compounds are biosynthesized by a similar route to our synthesis, i.e., by addition of hypotaurine, which has been reported in another ascidian,[18] to quinoline-5,8-quinone-2-carboxylic acid. While this compound has not been reported as a natural product, a possible precursor, 5-hydroxyquinoline-2-carboxylic acid, is known.[19] The combination of *in vivo* anti-inflammatory activity by oral dosage, specificity for inhibition of superoxide production by neutrophils, lack of toxicity markers, and ease of synthesis makes this new compound class highly attractive for further study as potential anti-inflammatory pharmaceuticals.[20]

Experimental Section

General Experimental Procedures. UV−vis spectra were run as methanol solutions on a UV-2102 PC Shimadzu UV−vis scanning spectrophotometer. IR spectra were acquired as either dry films or Nujol mulls on a Spectrum One FTIR spectrometer with the 1603 cm^{-1} absorption band of polystyrene being used as reference. NMR spectra were recorded on either a Bruker Avance DRX-600 spectrometer at 600 MHz for ^1H and 150 MHz for ^{13}C, a Bruker Avance DRX-400 spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C, or a Bruker Avance 300 spectrometer at 300 MHz for ^1H and 75 MHz for ^{13}C. Residual solvent signals were used as reference (DMSO-d_6: δ_H, 2.50; δ_C, 39.4; CD_3OD: δ_H, 3.30; δ_C, 49.0; $CDCl_3$: δ_H, 7.25; δ_C, 77.0 ppm). MS were recorded on either a VG-7070 or a Thermo LTQ-FT mass spectrometer. Compound purity was determined by reversed-phase HPLC (Waters 600 HPLC photodiode array system, Alltech Econosil C18, 5 μm, 4.6 × 125 mm, H_2O (0.05% TFA) to MeCN over 25 min at 1.0 mL/min and monitoring at 254 nm).

Biological Material. The ascidian was collected (950 g) by scuba at a depth of 28 m from Tom Bowling Bay, North Cape, North Island, New Zealand, and kept frozen until used. A voucher specimen of the orange-brown colonial ascidian *Aplidium* sp. is held at the National Institute for Water and Atmospheric Research, Private Bag 14-901, Kilbirnie, Wellington, New Zealand, as MNP7215 and MNP7347. A color *in situ* photograph is included in the Supporting Information. *Aplidium* sp. (TerraMarine accession codes: MNP7215, MNP7347) forms erect lamellate colonies to 10 cm in maximum height and 1 cm thick. Colonies are occasionally attached to the substratum by a short, wide stalk. Common clocal apertures of 1−2 mm in diameter are evenly distributed throughout the test. Zooids are arranged in regular circular systems around slightly raised common clocal apertures. Living specimens have an opaque brown-colored test, but zooids are nonpigmented. *Aplidium* sp. most closely resembles *Aplidium gilvum*.[21] However, the morphology of the colony (sandy stalk), transparent test, pointed branchial lobes, and structure of the atrial lappet set the two species apart. *Apidium* sp. has not been described before in New Zealand and does not resemble any Australian *Aplidium* species described by Kott;[6] it is therefore considered to be a new species endemic to New Zealand waters.

Isolation and Purification. The frozen animals were freeze-dried (25.4 g) and extracted with MeOH (6 × 100 mL) followed by CH_2Cl_2 (2 × 100 mL). The combined extracts were filtered and dried to produce 9.96 g of crude extract, which was subjected to C18 reversed-phase flash CC (water through to MeOH/TFA). The anti-inflammatory assay showed the activity to be concentrated in the water fraction. Repeated C18 CC (water through to 100% MeOH) followed by size exclusion CC on Sephadex LH-20 yielded **3** (5.1 mg, 0.02% dry wt) and the later eluting compound **4** (1.3 mg, 0.005% dry wt). The compounds were characterized as the free acids.

Ascidiathiazone A (3): yellow powder; mp 155 °C (dec); UV (MeOH) λ_{max} (log ϵ) 416 (3.40), 268 (4.21), 236 (4.46), 216 (4.37); (MeOH/TFA) 421 (3.56), 274 (4.18), 237 (4.55), 205 (4.43); (MeOH/KOH) 467 (3.59), 329 (3.97), 236 (4.52), 211 (5.07) nm; fluorescence (MeOH/TFA), Ex 250 nm, Em 508 nm; (MeOH/KOH) Ex 250 nm, Em 507 nm; IR (film) ν_{max} 3034, 1660, 1651, 1585, 1417, 1193, 1129 cm^{-1}; NMR data are reported in Table 1; HRESI(−)FTMS *m*/*z* 307.0030 $[M - H]^-$ (calcd for $C_{12}H_7N_2O_6S$, 307.0025); purity 96%; t_R = 15.41 min.

Ascidiathiazone B (4): pink powder; mp 280 °C (dec); UV (MeOH/TFA) λ_{max} (log ϵ) 417 (3.11), 269 (3.91), 237 (4.02), 214 (4.50); (MeOH/KOH) 472 (3.29), 311 (3.62), 279 (3.81), 237 (4.04), 207 (4.95) nm; fluorescence (MeOH/TFA) Ex 250 nm, Em 505 nm; (MeOH/KOH) Ex 250 nm, Em 506 nm; IR (film) ν_{max} 3414, 1681, 1638, 1524, 1279, 1127 cm^{-1}; NMR data are reported in Table 1; HRESI(−)FTMS *m*/*z* 304.9874 $[M - H]^-$ (calcd for $C_{12}H_5N_2O_6S$, 304.9868); purity 99%; t_R = 15.54 min.

Semisynthesis of Ascidiathiazone A Methyl Ester (5). A sample of **3** (8 mg, 0.026 mmol) was stirred in dry MeOH (1.5 mL) under nitrogen and cooled to −5 °C in a salted ice-bath. Excess $SOCl_2$ (40 μL) was added, and the mixture was stirred 15 min before being allowed to come to room temperature. It was then stirred at 40 °C for 2.5 h and then dried under vacuum. The crude mixture was separated on a C18 Sep-pak, the product eluting with 10% MeOH/water, yielding **5** (3 mg, 36% yield) as a yellow gum: IR (film) ν_{max} 1728 cm^{-1}; ^1H NMR

Table 2. *In Vitro* Activities of Compounds **3**, **4**, **5**, **7**, and **8**[a]

compound	AI_{50}[b]	AP_{50}[c]	X/XO[d]
3	1.55 ± 0.32 (n = 7)	73.08 ± 7.33 (n = 5)	55.90 ± 7.90 (n = 2)
4	0.44 ± 0.09 (n = 4)	16.73 ± 2.02 (n = 2)	17.89 ± 1.14 (n = 2)
5	2.26 ± 0.31 (n = 4)	11.78 ± 2.28 (n = 2)	18.23 ± 5.66 (n = 2)
7	276.7 ± 56.95 (n = 2)	96.93 ± 96.52 (n = 2)	ND[e]
8	1.75 ± 0.15 (n = 2)	4.11 ± 0.27 (n = 2)	ND
SOD	0.65 ± 0.06		
methotrexate		6.92 nM ± 0.84 nM	
allopurinol			42.24 ± 5.86

[a] Values are IC_{50} values (units of μM unless stated otherwise) representing the mean of at least two determinations ± standard error. [b]AI_{50}: anti-inflammatory activity. Concentration of compound required to inhibit *in vitro* superoxide production by PMA-stimulated human neutrophils by 50%. [c]AP_{50}: antiproliferative activity. Concentration of compound required to inhibit HL60 cell proliferation by 50%. [d]X/XO: xanthine/xanthine oxidase inhibitory activity. Concentration of compound required to inhibit superoxide production in xanthine/xanthine oxidase assay by 50%. [e]ND: not determined.

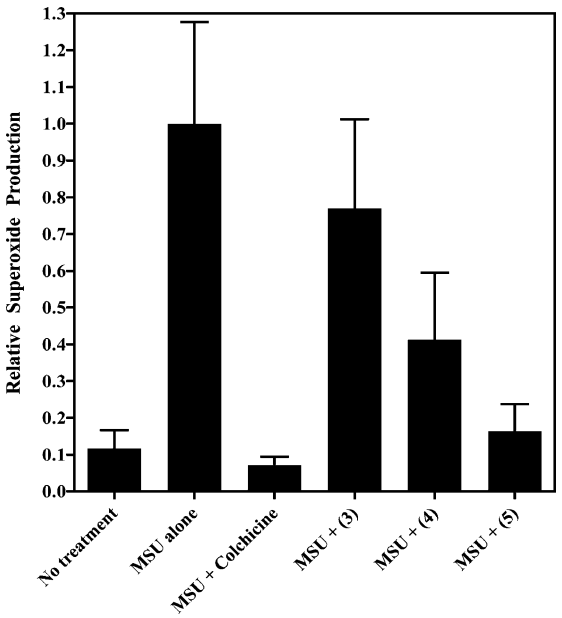


Figure 2. Inhibition of superoxide production by peritoneal neutrophils from gouty mice treated with **3**−**5** relative to monosodium urate (MSU) alone treated mice. Mice were injected intraperitoneally with MSU crystals (3 mg) in the presence or absence of compounds **3**−**5** (oral gavage, 25.6 μmol/kg) or colchicine (sc, 5 μmol/kg, control). The neutrophils were isolated from the peritoneum at 4 h and tested for superoxide production as described in the Experimental Section.

(DMSO-d_6, 400 MHz) δ 8.54 (1H, d, J = 8.1 Hz), 8.40 (1H, d, J = 8.1 Hz), 3.95 (3H, s), 3.89 (2H, t, J = 5.8 Hz), 3.40 (2H, t, J = 5.8 Hz); HMBC correlation from methyl protons δ 3.95 to a carbonyl carbon at δ 163.0; FABMS m/z 323 $[M + H]^+$; HRFABMS m/z 323.0336 (calcd for $C_{13}H_{11}N_2O_6S$, 323.0338).

Compound 6. Methyl ester **5** (87 mg, 0.27 mmol) was stirred in 2 N KOH (15 mL) at room temperature for 2 h. Concentrated HCl was added dropwise until the reaction mixture turned acidic, after which the solvents were removed under reduced pressure. The residue was taken up in water and subjected to C8 reversed-phase flash CC, yielding **6** (13 mg (16%)) as a bright yellow solid: mp 240 °C (dec); IR (Nujol mull) ν_{max} 3399, 1642, 1519, 1299, 1270, 1161, 1118 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 414 (3.55), 330 (3.77), 266 (4.26), 240 (4.35), 212 (4.42); (MeOH/TFA) 424 (3.55), 268 (4.23), 213 (4.73); (MeOH/KOH) 483 (3.78), 316 (3.99), 278 (4.19), 239 (4.45), 210 (5.04) nm; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 13.90 (1H, br s, CO*OH*), 11.51 (1H, d, J = 5.6 Hz, NH), 8.57 (1H, d, J = 8.1 Hz, H-9), 8.42 (1H, d, J = 8.1 Hz, H-8), 7.17 (1H, dd, J = 8.9, 5.6 Hz, H-3), 6.62 (1H, d, J = 8.9 Hz, H-2); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 177.6 (C-10), 175.5 (C-5), 165.1 (C-11), 151.8 (C-7), 146.5 (C-5a), 141.5 (C-4a), 135.8 (C-9), 130.8 (C-9a), 130.4 (C-3), 128.8 (C-8), 115.2 (C-10a), 112.0 (C-2); HRESI(−)FTMS m/z 304.9872 $[M - H]^-$ (calcd for $C_{12}H_5N_2O_6S$, 304.9868).

Synthesis of Ascidiathiazone A. Methyl 8-Hydroxyquinoline-2-carboxylate (7). 8-Hydroxyquinoline-2-carboxylic acid (100 mg, 0.53 mmol) was stirred in dry MeOH (2 mL) at 0 °C. $SOCl_2$ (60 μL, 0.82 mmol) was added dropwise, and the suspension was stirred at 0 °C for 10 min under N_2 and then at 65 °C for 2 h, during which time the bright yellow suspension dissolved into solution and became light brown in color. Water (10 drops) was added to quench the reaction mixture, which was evaporated to dryness under vacuum to yield **7** (103 mg (96%)) as a bright orange solid: mp 93−94 °C; ^{1}H NMR (CD_3OD, 400 MHz) δ 8.83 (1H, d, J = 8.6 Hz), 8.30 (1H, d, J = 8.6 Hz), 7.73 (1H, dd, J = 7.9, 7.9 Hz), 7.62 (1H, d, J = 8.0 Hz), 7.35 (1H, d, J = 7.8 Hz), 4.13 (3H, s); ^{13}C NMR (CD_3OD, 100 MHz) δ 164.1, 152.6, 143.8, 143.5, 134.9, 132.5, 131.9, 122.1, 119.3, 115.7, 54.6; EIMS m/z 203 $[M]^+$ (60), 171 925), 143 (100); HREIMS m/z 203.0581 (calcd for $C_{11}H_9NO_3$, 203.0582); purity 100%; t_R = 21.62 min.

Methyl Quinoline-5,8-dione-2-carboxylate (8). To a solution of Fremy's salt (370 mg, 1.38 mmol) in water (10 mL) and aqueous KH_2PO_4 (0.167 M, 3 mL) at 0 °C was added **7** (60 mg, 0.30 mmol) in MeOH (4 mL). The purple suspension was stirred for 10 min at 0 °C, after which time it was allowed to come to room temperature and stirred for 1 h. A second portion of Fremy's salt, water, and aqueous KH_2PO_4 (0.167 M) were added. The reaction mixture was stirred for a further 2 h and extracted with 3 × 20 mL portions of CH_2Cl_2. The organic extracts were dried under reduced pressure, affording crude **8** as an orange solid (58 mg (90%)). This crude material was used in the subsequent reaction without further purification. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.59 (1H, d, J = 8.1 Hz), 8.48 (1H, d, J = 8.1 Hz), 7.25 (1H, d, J = 10.5 Hz), 7.14 (1H, d, J = 10.5 Hz), 4.07 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 183.6, 182.0, 164.5, 152.3, 147.0, 139.6, 138.1, 136.2, 130.6, 128.5, 53.5.

Ascidiathiazone A Methyl Ester 5 and Regioisomer 9. To a solution of quinone **8** (58 mg, 0.27 mmol) and cerium chloride heptahydrate (101 mg, 0.27 mmol) in absolute EtOH (25 mL) was added hypotaurine (29 mg, 0.27 mmol) in water (2 mL). The reaction mixture was stirred at room temperature for 3 days, then dried under reduced pressure. The residue was triturated with MeOH, affording a MeOH-insoluble red-brown solid (**5**) (47 mg (55%)). The filtrate was subjected to C18 reversed-phase flash CC, yielding the thiazine isomer **9** (6.0 mg (7% yield)) as a bright yellow solid. Alternatively, to quinone **8** (220 mg, 1 mmol) in MeCN (7.5 mL) and EtOH (7.5 mL) was added hypotaurine (120 mg, 1.1 mmol) in water (3 mL). The reaction mixture was stirred at room temperature overnight and then solvents were removed *in vacuo*. MeOH was added and an insoluble orange product filtered off and washed with MeOH to yield pure **5** (122 mg, 37%).

Methyl ester 5: mp 254 °C (dec); IR (film) ν_{max} 1721 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.46 (1H, s, NH), 8.54 (1H, d, J = 8.1 Hz, H-9), 8.40 (1H, d, J = 8.1 Hz, H-8), 3.95 (3H, s, H_3-12), 3.89 (2H, br m, H_2-3), 3.38 (H_2-2, obscured by water peak); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 175.7 (C-5), 172.7 (C-10), 163.7 (C-11), 149.4 (C-7), 147.3 (C-4a), 145.9 (C-5a), 135.4 (C-9), 131.4 (C-9a), 128.7 (C-8), 110.3 (C-10a), 52.5 (C-12), 47.7 (C-2), 39.0 (C-3); FABMS m/z 323 $[M + H]^+$; HRFABMS m/z 323.0336 (calcd for $C_{13}H_{11}N_2O_6S$, 323.0338); purity 98%; t_R = 15.46 min.

Methyl ester 9: IR (film) ν_{max} 1727 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 9.67 (1H, s, NH), 8.51 (1H, d, J = 8.0 Hz, H-6), 8.35 (1H, d, J = 8.0 Hz, H-7), 4.09 (2H, m, H_2-3), 3.98 (3H, s, H_3-12), 3.49 (2H, t, J = 5.8 Hz, H_2-2); ^{13}C NMR (DMSO-d_6, 75 MHz) δ 177.7 (C-5), 168.2 (C-10), 163.7 (C-11), 150.8 (C-4a), 149.8 (C-8), 145.2 (C-9a), 136.9 (C-6), 129.8 (C-5a), 127.8 (C-7), 111.9 (C-10a), 53.1

(C-12), 48.4 (C-2), 40.3 (C-3); HRESI(−)FTMS *m/z* 321.0186 [M − H]$^-$ (calcd for $C_{13}H_9N_2O_6S$, 321.0176).

Ascidiathiazone A. Methyl ester **5** (17 mg, 0.05 mmol) was dissolved in concentrated HCl (2 mL) and stirred 5 h at room temperature, then at 100 °C for 1 h. The solution was dried under reduced pressure and subjected to C18 reversed-phase flash CC, yielding **3** as a bright yellow solid (11 mg, 68%) that was identical to the natural product in all respects. HRESI(−)FTMS *m/z* 307.0030 [M − H]$^-$ (calcd for $C_{12}H_7N_2O_6S$, 307.0025).

Neutrophil Superoxide Assay.[4] Human neutrophils were isolated from anticoagulated whole human blood using Polymorphprep (density 1.113 g/mL, centrifuge 500*g*, 35 min). Neutrophils, resuspended in Hank's balanced salt solution (HBSS), were plated out in 96-well flat-bottomed plates at 1×10^6 cells/well. Neutrophils were treated with different concentrations of the test compound (dissolved in HBSS, 1% v/v DMSO) for 30 min prior to addition of the detection dye WST-1 (final concentration of 0.35 mM). At this concentration, no effect of DMSO on the ability of neutrophils to generate a respiratory burst in response to PMA was observed. The respiratory burst was initiated by addition of phorbol 12-myristate 13-acetate (PMA, final concentration of 0.295 μM), and dye reduction was monitored in real time (450 nm) for 20 min at 37 °C. Respiratory burst activity was calculated as the rate of dye reduction over time compared to buffer-treated cells.

Cell Culture Conditions. HL60 cells were cultured in RPMI-1640 medium supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS), 2 mM GlutaMAX-1, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate, at 37 °C in a humidified atmosphere maintained at 5% CO_2. Cell densities were maintained at less than 0.5×10^6 cells/mL. Cell viability was confirmed by trypan blue exclusion.

Antiproliferative Assay.[22] HL60 cells (suspended in RPMI1640, 5% v/v FBS) were plated out in 96-well flat-bottomed plates at 2×10^4 cells/well. The cells were treated with different concentrations of the test compound (dissolved in RPMI1640, 5% v/v FBS, 1% v/v DMSO) and incubated for 48 h (37 °C in a humidified atmosphere maintained at 5% CO_2). 3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was then added to each well (final concentration of 1.10 mM), and the cells were incubated for 2 h. An equal volume of lysing buffer (10% w/v SDS, 45% v/v DMF in H_2O, pH 4) was added to each well, and the plates were incubated overnight to ensure complete lysis. Proliferative activity was determined by comparing the absorbance of the samples at 570 nm to the buffer-treated cells.

Xanthine Oxidase Assay.[4] Varying dilutions of test compound (dissolved in 0.1M Tris (pH 7,4), 0.2 mM EDTA, 1% v/v DMSO) were plated out in 96-well flat-bottomed plates. WST-1 and xanthine were added to a final concentration of 0.35 and 2.38 mM, respectively. Xanthine oxidase was then added to a final concentration of 15 mU/mL. The dye reduction was monitored in real time (450 nm) for 20 min at 37 °C. Enzyme activity was calculated as the rate of dye reduction over time compared to buffer-treated controls.

***In Vivo* Murine Gout Model (MSU-Induced Inflammation) Assay.** C57BL/6 male mice were bred and housed in a conventional animal facility at the Malaghan Institute of Medical Research. All animals used for the experiments were between 8 and 12 weeks old. Experimental procedures were approved by the animal ethics committee in accordance with Victoria University of Wellington (New Zealand) guidelines for the care of animals. Mice were injected intraperitoneally (ip) with monosodium urate (MSU) crystals (3 mg) suspended in 500 μL of phosphate-buffered saline (PBS, pH 7.4). Test compounds were administered by oral gavage (25.6 μmol/kg) at the same time as administration of MSU. The positive control, colchicine (5 μmol/kg), was administered subcutaneously (sc), immediately after ip injection of MSU crystals. Colchicine was dissolved in PBS (pH 7.4). Control mice received an equivalent volume of PBS (pH 7.4) by oral gavage.

Detection of Superoxide Production by Peritoneal Neutrophils. Four hours after MSU administration, mice were euthanized by CO_2 exposure. The peritoneal cavity was washed with PBS (3 mL, pH 7.4) containing 25 U/mL heparin. Total cell numbers were counted and the sample volume was adjusted to 1×10^6 neutrophils/mL. The cells were then tested for superoxide production as described above. To determine the number of neutrophils in each sample, a small aliquot (100 μL) of each cell sample was fixed onto slides using a cytocentrifuge and stained with the Diff-Quick staining kit (Dade Behring, Newark, NJ). The percentage of neutrophils in the peritoneal wash was determined microscopically using standard histological criteria. Superoxide production is represented as superoxide produced per million neutrophils.

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Supporting Information Available: Color photograph of *Aplidium* sp., X-ray crystallographic data for **3**, Figure S3 showing toxicity markers, and ^1H and ^{13}C NMR spectroscopic data for compounds **3**−**9**. This material is available free of charge via the Internet at http//pubs.acs.org.

References and Notes

(1) Levesque, L. E.; Brophy, J. M.; Zhang, B. *Ann. Intern. Med.* **2005**, *142*, 481−489.
(2) Davies, N. M.; Jamali, F. *J. Pharm. Pharm. Sci.* **2004**, *7*, 332−336.
(3) Breuil, V.; Drici, M. D. *Lett. Pharmacol.* **2003**, *17*, 91−96.
(4) Tan, A. S.; Berridge, M. V. *J. Immunol. Methods* **2000**, *238*, 59−68.
(5) (a) McNamara, C. E.; Larsen, L.; Perry, N. B.; Harper, J. L.; Berridge, M. V.; Chia, E. W.; Kelly, M.; Webb, V. L. *J. Nat. Prod.* **2005**, *68*, 1431−1433. (b) Pearce, A. N.; Chia, E. W.; Berridge, M. V.; Maas, E. W.; Page, M. J.; Webb, V. L.; Harper, J. L.; Copp, B. R. *J. Nat. Prod.* **2007**, *70*, 111−113.
(6) Kott, P. *Mem. Queensland Mus.* **1992**, *32*, 375−620.
(7) Zubia, E.; Ortega, M. J.; Salva, J. *Mini-Rev. Org. Chem.* **2005**, *2*, 389−399.
(8) Aiello, A.; Fattorusso, E.; Luciano, P.; Menna, M.; Esposito, G.; Iuvone, T.; Pala, D. *Eur. J. Org. Chem.* **2003**, 898−900.
(9) Aiello, A.; Fattorusso, E.; Luciano, P.; Mangoni, A.; Menna, M. *Eur. J. Org. Chem.* **2005**, 5024−5030.
(10) Aiello, A.; Fattorusso, E.; Luciano, P.; Macho, A.; Menna, M.; Muñoz, E. *J. Med. Chem.* **2005**, *48*, 3410−3416.
(11) Martin, G. E.; Hadden, C. E. *J. Nat. Prod.* **2000**, *63*, 543−585.
(12) Schmitz, F. J.; Bloor, S. J. *J. Org. Chem.* **1988**, *53*, 3922−3925.
(13) Crystallographic data for the structure reported in this paper have been deposited with the Cambridge Crystallographic Data Centre as CCDC 626466. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44-(0)1223−336033 or e-mail: deposit@ccdc.cam.ac.uk).
(14) Morris, B. D.; Prinsep, M. R. *J. Org. Chem.* **1998**, *63*, 9545−9547.
(15) Getting, S. J.; Flower, R. J.; Parente, L.; de Medicis, R.; Lussier, A.; Woliztky, B. A.; Martins, M. A.; Perretti, M. *J Pharmacol. Exp. Ther.* **1997**, *283*, 123−130.
(16) Conaghan, P. G.; Day, R. O. *Drug Saf.* **1994**, *11*, 252−258.
(17) Roberts, W. N.; Liang, M. H.; Stern, S. H. *JAMA, J. Am. Med. Assoc.* **1987**, *257*, 1920−1922.
(18) Nontratip, A.; Wada, S.; Yamanaka, H. *Nippon Suisan Gakkaishi* **1992**, *58*, 315−322.
(19) Noertemann, B.; Glaesser, A.; Machinek, R.; Remberg, G.; Knackmuss, H. J. *Appl. Environ. Microbiol.* **1993**, *59*, 1898−1903.
(20) Denny, W. A.; Copp, B. R.; Pearce, A. N.; Berridge, M. V.; Harper, J. L.; Perry, N. B.; Larsen, L.; Godfrey, C. A. PCT Int. Appl. WO 2006031134, 2006, 45 pp.
(21) Millar, R. H. *N. Z. Oceanogr. Inst. Mem.* **1982**, *85*, 5−116.
(22) Berridge, M. V.; Tan, A. S. *Arch. Biochem. Biophys.* **1993**, *303*, 474−482.

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