NF-κB-Inhibiting Naphthopyrones from the Fijian Echinoderm *Comanthus parvicirrus*

Florence Folmer,[†] William T. A. Harrison,[†] Jioji N. Tabudravu,[†] Marcel Jaspars,*[,†] William Aalbersberg,[‡] Klaus Feussner,[‡] Anthony D. Wright,[§] Mario Dicato,[⊥] and Marc Diederich[⊥]

Department of Chemistry, University of Aberdeen, Old Aberdeen, AB24 3UE, U.K., Institute of Applied Sciences, Faculty of Science and Technology, University of the South Pacific, P.O. Box 1168, Suva, Fiji Islands, Australian Institute of Marine Science, Townsville 4810, Queensland, Australia, and Laboratoire de Biologie Moléculaire et Cellulaire du Cancer, Hôpital Kirchberg, 9, Rue Edward Steichen, L-2540 Luxembourg, Luxembourg

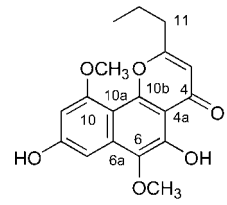
Received June 18, 2007

The two naphthopyrones 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**) were isolated from a bioactive methanol-soluble extract of the Fijian echinoderm *Comanthus parvicirrus*. Their structures were assigned on the basis of spectroscopic methods. X-ray diffraction analysis was used to confirm the structure of **1**. Both compounds were tested for their potential to inhibit the activation of the transcription factor NF-κB, which plays an important role in cancer development and inflammation, and the mechanism of action of the two compounds was investigated. Both naphthopyrones **1** and **2** completely inhibit TNF-α-induced NF-κB activation at a concentration of 300 μM by inhibiting the enzymatic activity of the kinase IKKβ.

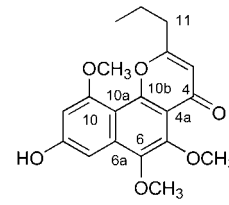
Nuclear factor κB (NF-κB) is an inducible transcription factor that plays a key role in cancer development and inflammation.[1–3] Because of its strong implications in cancer development, in inflammation, and in a wide range of other diseases including AIDS, atherosclerosis, septic shock, and Alzheimer's disease, NF-κB has become a major target in drug discovery.[1,2,4–7] A large number of natural and synthetic compounds are currently being investigated for NF-κB inhibitory activity, and natural products with MIC values as low as 60 nM have been identified as promising NF-κB inhibitors. The majority of the compounds known to date as potent NF-κB inhibitors are plant-derived isoprenoids and polyphenolics.[8,9]

The transcription factor NF-κB is a dimer of proteins belonging to the Rel family. The most common NF-κB dimer is composed of the two proteins p50 and p65. NF-κB is present in the cytoplasm of virtually every mammalian cell, in an inactive form as it is bound to its inhibitor IκB (inhibitory protein of the nuclear factor-κB). Several factors are known to activate NF-κB, including the exposure to cytokines, to proliferation agents, to various carcinogens, or to physical stress factors.[10] All NF-κB activation signals converge on the IκB kinase complex IKK (IκB kinase).[4] IKK's subunit IKKβ phosphorylates IκB.[4] Upon phosphorylation and consequent ubiquitinilation, IκB is rapidly degraded by the 26S proteasome, and NF-κB is freed to translocate to the nucleus, where it binds to its target DNA sequences to initiate gene transcription. The majority of the genes transcribed by NF-κB are genes implicated in cancer or in inflammation.[1,2,11–13]

In our search for new NF-κB inhibitors, we screened over two hundred extracts from Fijian marine algae and invertebrates using NF-κB reporter gene assays. Fourteen percent of the tested extracts were shown to have a strong NF-κB inhibitory potential, as they inhibited 65% or more of the NF-κB activity induced by the NF-κB-activating cytokine TNF-α (tumor necrosis factor α) at the test concentration of 100 μg/mL. An extract from the echinoderm *Comanthus parvicirrus* M. (Comasteridae), which was among the 10 most active samples in the large-scale screening assay, was selected for further chemical and biological studies. In general, echinoderms belonging to the genus *Comanthus* exhibit a low structural diversity, consisting mainly of aromatic polyketides.[14] Only a few natural products, including quinones, anthraquinones, and naphthopyrones, have been isolated from representatives of the genus *Comanthus* so far.[14–19] The extract of *C. parvicirrus* yielded only two major compounds, namely, 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**). Both **1** and **2** were shown to inhibit TNF-α-induced NF-κB activation by ≥74% at a concentration of 100 μg/mL. In the current paper, we present the 2D NMR assignment of 6-methoxycomaparvin (**1**) and of 6-methoxycomaparvin 5-methyl ether (**2**), the X-ray crystal structure of 6-methoxycomaparvin (**1**), and the mechanism of action of **1** and **2** with respect to NF-κB inhibition.



1



2

Results and Discussion

A MeOH extract (1.1 g, dry weight) of *C. parvicirrus* collected in Fiji was partitioned by liquid–liquid fractionation into H_2O-, MeOH-, CH_2Cl_2-, and C_6H_{14}-soluble fractions. Bioassay-guided fractionation of the CH_2Cl_2-soluble fraction by reversed-phase HPLC yielded **1** (61 mg) and **2** (34 mg).

The HSQC NMR spectrum for **1** indicated the presence of 10 quaternary C, three CH, two CH_2, and three CH_3 units. The LRESIMS data (m/z = 331 $[M + H]^+$) indicated the probable presence of a further six oxygen atoms, leading to a molecular formula of $C_{18}H_{18}O_6$. This deduction was confirmed by HRESIMS (ion peak at m/z = 331.1176 $[M + H]^+$, 1.21 ppm from calc for $C_{18}H_{19}O_6$). Dereplication based on the taxonomic information and on the molecular formula pointed toward **1** being naphthopyrone 6-methoxycomaparvin, which was first isolated by Smith and Sutherland in 1971,[15] and for which no ^{13}C NMR data have been reported. To assign the ^{13}C data for **1**, we used HSQC and HMBC experiments, as well as information derived from a $^1H-^1H$ COSY experiment (see Table 1).

Additionally, the structure of **1** as 6-methoxycomaparvin was confirmed by single-crystal X-ray diffraction analysis. Pale yellow slabs and rods of **1** were recrystallized from an aqueous solution. The single-crystal analysis revealed that two molecules of **1** and

* To whom correspondence should be addressed. Tel: +44 1224272895. Fax: +44 1224 272921. E-mail: m.jaspars@abdn.ac.uk.
[†] University of Aberdeen.
[‡] University of the South Pacific.
[§] Australian Institute of Marine Science.
[⊥] Laboratoire de Biologie Moléculaire et Cellulaire du Cancer.

10.1021/np070290y CCC: $40.75
Published on Web 12/19/2007

Table 1. NMR Spectroscopic Data (400 MHz, $CDCl_3$) for 6-Methoxycomaparvin (**1**) and 6-Methoxycomaparvin 5-Methyl Ether (**2**)

	6-methoxycomaparvin (**1**)				6-methoxycomaparvin 5-methyl ether (**2**)	
position	δC	δH (*J* in Hz)	HMBC[a]	COSY	δC	δH (*J* in Hz)
2	170.1, C				167.0, C	
3	109.5, CH	6.23, s	2, 4[b], 4a[b], 5, 11		111.8, CH	6.21, s
4	183.4, C				178.3, C	
4a	147.1, C				146.9, C	
5	109.3, C				114.6, C	
6	134.7, C				135.2, C	
6a	158.3, C				158.0, C	
7	96.3, CH	7.01, d (1.6)	6[b], 6a[b], 8, 9, 10[b], 10a, 10b[b]	9	97.4, CH	7.12, d (1.6)
8	104.9, C				108.4, C	
9	96.7, CH	6.43, d (1.6)	7, 8, 10, 10b	7	99.0, CH	6.61, d (1.6)
10	160.2, C				160.1, C	
10a	136.1, C				143.6, C	
10b	152.4, C				153.0, C	
11	36.5, CH_2	2.68, t (7.7)	2, 3, 12, 13	12, 13	36.0, CH_2	2.61, t (7.4)
12	20.0, CH_2	1.84, m	2, 11, 13	11, 13	19.9, CH_2	1.83, m
13	13.7, CH_3	1.01, t (7.7)	11, 12	11, 12	13.8, CH_3	1.00, t (7.4)
14 (in **2**)			4a, 6		62.0, CH_3	3.93, s
15 (14 in **1**)	60.4, CH_3	3.95, s			61.4, CH_3	3.94, s
16 (15 in **1**)	56.2, CH_3	3.94, s	9[b], 10[b], 10a		56.3, CH_3	3.95, s
OH-5 (in **1**)		12.90, s	4a[b], 5, 6[b]			

[a] HMBC correlations are from proton(s) stated to the indicated carbon. [b] Correlation only seen in **1**, on a 600 MHz spectrometer, using a cryoprobe.

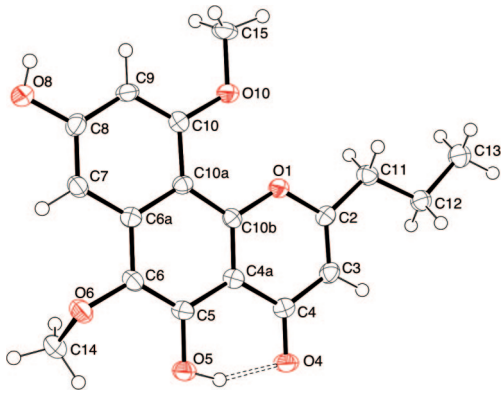


Figure 1. View of the C2 molecule of 6-methoxycomaparvin (**1**)·3/2 H_2O, showing 50% displacement ellipsoids. H atoms are represented by arbitrary spheres. The intramolecular H bond is indicated by a dashed line.

three of water make up the asymmetric unit of the centrosymmetric, monoclinic unit cell. One of the two molecules of **1** that form this unit cell is shown in Figure 1, with the atom numbers assigned according to IUPAC conventions. The detailed crystallographic data for **1** is provided in the Supporting Information.

Compound **2** was dereplicated in the same way as **1** and differed from **1** only by 14 amu (LRESIMS ion peak at $m/z = 345$). In terms of the NMR data this difference corresponded to the lack of the ^1H signal at 12.9 ppm characteristic of an −OH group, and the presence of a signal for an a $-OCH_3$ unit in the HSQC in its place, and indicated a molecular formula of $C_{19}H_{20}O_6$. This deduction was confirmed by HRESIMS (accurate $m/z = 345.1337$ $[M + H]^+$, 1.16 ppm from calcd for $C_{19}H_{21}O_6$). Compound **2** was identified as naphthopyrone 6-methoxycomaparvin 5-methyl ether, which was first isolated by Smith and Sutherland in 1971, together with 6-methoxycomaparvin (**1**),[15] and which, like **1**, was still lacking ^{13}C assignments. The replacement of the −OH group at C-5 in **1** by an $-OCH_3$ group in **2** was established using the HMBC correlations between C-4a and H-14. The HMBC correlations between C-10 and H-16 and between C-10a and H-16 in **2** confirmed the ^{13}C assignment of C-10a in both **1** and **2**.

The effects of **1** and **2** on TNFα-induced transcriptional activity of NF-κB were examined using a luciferase reporter gene assay. The results showed that neither compound had any effect on basal NF-κB transcription (Figure 2). Both **1** and **2** reduced TNF-α-induced NF-κB activation in a concentration-dependent manner. At 50 μg/mL (150 μM), 6-methoxycomaparvin (**1**) reduced TNF-α-induced NF-κB activation by 53% compared to the control,

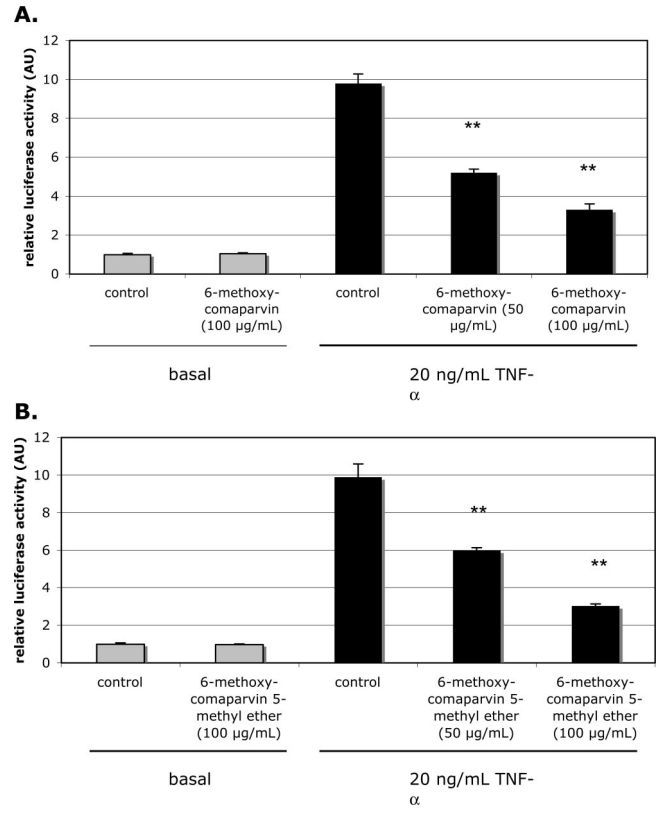


Figure 2. Luciferase activity of pNF-κBLuc K562 cells pretreated for 2 h with different concentrations (in μg/mL) of 6-methoxycomaparvin (**1**) (A) or 6-methoxycomaparvin 5-methyl ether (**2**) (B), and treated for 2 h with 20 ng/mL TNF-α. The results of the luciferase NF-κB reporter gene assay are given as a ratio of the luminescence measured for the firefly luciferase divided by the luminescence measured for the *Renilla* luciferase. The negative control refers to the basal transcription activity of NF-κB in the absence of any test substance. Results shown as mean ± standard deviation (SD) of eight individual measurements. ** represents $p < 0.01$ compared to the negative control.

and 6-methoxycomaparvin 5-methyl ether (**2**) reduced TNF-α-induced NF-κB activation by 43%. At 100 μg/mL (300 μM), the reduction in NF-κB activation was increased to 75% and 77%, respectively, compared to the control.

In order to discard false positive results caused by cytotoxicity of the compounds to K562 test cells or by interference of the compounds with the enzymatic activity of luciferase during the luciferase reporter gene assay, a K562 cell viability assay was performed, and the enzymatic activity of recombinant luciferase exposed to 6-methoxycomaparvin (**1**) or to 6-methoxycomaparvin 5-methyl ether (**2**) was measured. The results showed that **1** and **2** had no significant effect on the viability of K562 cells or on the enzymatic activity of luciferase over the luciferase reporter gene assay test period (data presented in the Supporting Information).

The potential of 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**) to inhibit TNF-α-induced NF-κB−DNA binding was assessed by electrophoretic mobility shift assays (EMSA). Jurkat cells were pretreated for 2 h with various concentrations of 6-methoxycomaparvin (**1**) or 6-methoxycomaparvin 5-methyl ether (**2**) before being treated for 2 h with 20 ng/mL TNF-α. EMSA experiments were performed by incubating 10 μg of nuclear extract for 20 min, with an oligonucleotide probe containing the consensus NF-κB binding site C-κB. The results confirmed that both **1** and **2** completely inhibit NF-κB activation and NF-κB−DNA binding at a MIC of 300 μM (Figure 3).

In order to determine whether **1** and **2** inhibit TNF-α-induced NF-κB activation by targeting IκBα degradation and the consequent nuclear translocation of NF-κB, Western blot assays were performed. The results show that both **1** and **2** completely inhibit TNF-α-induced IκBα degradation and the consequent translocation of NF-κB into the nucleus (Figure 4). We further investigated whether the observed inhibition of TNF-α-induced IκBα degradation could result from an inhibition of the kinase activity of IKKβ by the two active compounds. The results showed that **1** and **2** inhibit the kinase activity of IKKβ in a concentration-dependent manner (Figure 5). Both **1** and **2** significantly ($p < 0.05$) inhibit the kinase activity of IKKβ at a MIC of 100 μg/mL (300 μM). The IKKβ inhibitory activity of **1** and **2** remains, however, low compared to the IKKβ inhibitor CalbioIV ([5-(*p*-fluorophenyl)-2-ureido]thiophene-3-carboxamide) provided as a positive control by Calbiochem, as the latter achieved the same level of IKKβ inhibition at a concentration as low as 1 μM. [5-(*p*-Fluorophenyl)-2-ureido]thiophene-3-carboxamide has been reported to have an IC_{50} of 18 nM for its IKKβ inhibition.[20]

Additionally, we investigated the effects of **1** and **2** on the proteolytic activity of the 26S proteasome using a Proteasome-Glo assay kit (Promega). 6-Methoxycomaparvin 5-methyl ether (**2**), but not 6-methoxycomaparvin (**1**), significantly ($p < 0.01$) inhibits the proteolytic activity of the 26S proteasome at a concentration of 300 μM. Nevertheless, the bioactivity of **2** at the latter concentration remains low compared to the bioactivity of the known 26S proteasome inhibitor MG-132 (*Z*-Leu-Leu-Leu-CHO), which was active at concentrations as low as 10 μM (Figure 6).[21]

We conclude that the naphthopyrones 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**) isolated from the Fijian echinoderm *C. parvicirrus*, and for which we describe the complete 2D NMR assignment as well as the single-crystal X-ray diffraction structure (for **1**), completely inhibit TNF-α-induced NF-κB activation and NF-κB−DNA binding at a MIC of 300 μM. The results show the kinase IKKβ to be the major target of **1** and **2** along the NF-κB activation pathway. IKKβ is known to be the subunit of IKK responsible for the phosphorylation of the IκB leading to the degradation of IκB by the 26S proteasome and to the consequent release of free, biologically active NF-κB from the IκB-NF−κB complex. To date, naphthopyrones and anthraquinones have been mainly isolated from terrestrial plants. Only few naphthopyrones or anthraquinones have been reported to target NF-κB so far. Among the rare reports of NF-κB-inhibiting anthraquinones or naphthopyrones are the two anthraquinones emodin and aloe-emodin isolated from the rhubarb plant (*Rheum palmatum*) and from the roots of Japanese knotweed (*Polygonum cuspidatum*).[22–25] Emodin, which inhibits NF-κB by preventing IκBα degradation,[25] has been shown to block cancer progression in human cervical cancer[26] and lung carcinoma.[24,27] Naphthopyrones with anti-inflammatory or anticancer activity have been reported from the roots of the terrestrial plant *Pleuropterus ciliinervis*[28] and from the lichen *Flavoparmelia euplecta*.[29]

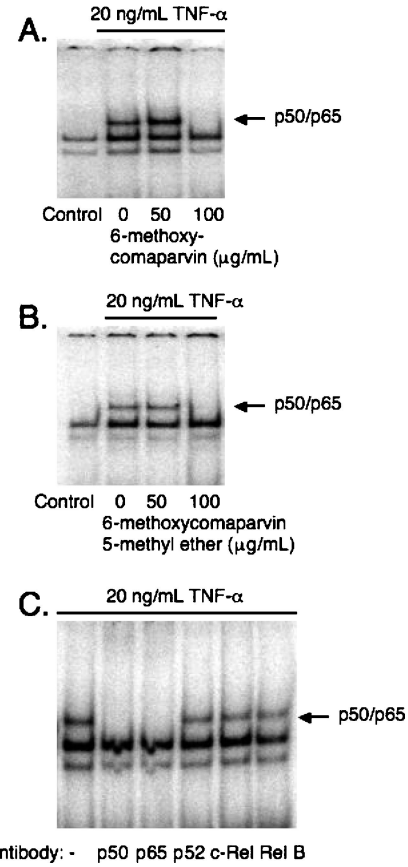


Figure 3. Inhibition of TNF-α-induced NF-κB−DNA binding activity by 6-methoxycomaparvin (**1**) (A) and 6-methoxycomaparvin 5-methyl ether (**2**) (B). Binding activity of NF-κB to the κB binding site. Jurkat cells were pretreated for 2 h with 50–100 μg/mL 6-methoxycomaparvin (**1**) or 6-methoxycomaparvin 5-methyl ether (**2**), before being treated for 2 h with 20 ng/mL TNF-α. EMSA experiments were performed by incubating 10 μg of nuclear extract for 20 min with an oligonucleotide probe containing the consensus NF-κB binding site C-κB. For supershift/immunodepletion experiments, the nuclear extracts and labeled probes were incubated in the reaction mixture for 30 min on ice prior to a 30 min incubation with 2 μg of anti-p50, anti-p52, anti-p65, anti c-Rel, and anti-RelB antibodies (C).

Experimental Section

General Experimental Procedures. UV spectra were measured in MeOH on a Perkin-Elmer Lambda 15 UV/vis spectrometer. IR spectra were recorded on an ATI Mattson Genesis FTIR, using KBr discs. ^1H, ^{13}C, and all other NMR 2D experiments were recorded in $CDCl_3$, either on a Varian Unity INOVA 400 MHz spectrometer or on a Bruker Avance 600 MHz spectrometer with a cryoprobe. TMS was used as an internal reference for the ^1H NMR spectra, and the $CDCl_3$ signal at 77.0 ppm was used as a reference for ^{13}C NMR spectra. ESIMS was carried out on a Perseptive Biosystem Mariner LC-MS. HRESIMS analysis was carried out at the EPSRC National Mass Spectrometry Service Centre in Swansea (UK). HPLC separations were carried out using a Phenomenex reversed-phase (C_{18}, 10 × 250 mm, 5 μm) HPLC

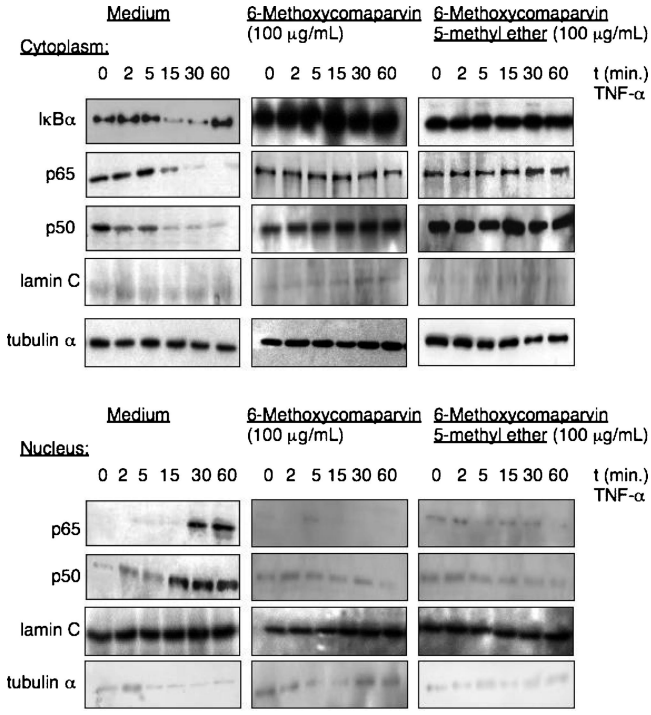


Figure 4. Western blot analysis showing that 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**) inhibit TNF-α-induced degradation of IκBα and the consequent translocation of p50 and p65 into the nucleus. Jurkat cells (2×10^6 cells/mL) were incubated with 100 μg/mL 6-methoxycomaparvin (**1**) or 6-methoxycomaparvin 5-methyl ether (**2**) for 2 h at 37 °C, treated with 20 ng/mL TNF-α for the indicated times at 37 °C, and then tested for IκBα, p50, and p65 in cytosolic fractions and for p50 and p65 in nuclear fractions by Western blot analysis. Equal protein loading and purity of the cytosolic/nuclear extracts were evaluated by tubulin α (cytosolic) and lamin C (nuclear) Western blots.

column and an HP1100 HPLC system (Agilent Technologies) with a diode array detector (DAD).

Biological Material. A sample (140 g wet weight) of the echinoderm *C. parvicirrus* was collected in Thanggalai, Caqalai Island (Fiji) (18°47′5″ S, 178°44′7″ E) by researchers of the University of the South Pacific (USP), Fiji, in 2001. The green, brittle organism was identified by one of us (K.F.). The collected material was stored at −20 °C until used. A voucher specimen (FJ01-199) is stored at the South Pacific Herbarium, University of the South Pacific, Fiji Islands.

Extraction and Isolation. The sample of *C. parvicirrus* was extracted with MeOH to yield 2.11 g of crude extract. A sample of this extract (1.1 g) was shipped from USP to the University of Aberdeen and partitioned between H_2O and CH_2Cl_2. The CH_2Cl_2 part was dried and the residue partitioned between hexanes and 10% aqueous MeOH. The MeOH layer was then phase adjusted to 50% aqueous MeOH and extracted with CH_2Cl_2. The resultant CH_2Cl_2 solubles were separated by semipreparative reversed-phase HPLC using a C_{18} column and a 50 to 85% (v/v) CH_3CN/H_2O gradient solvent system to yield 61 mg of **1** and 34 mg of **2**.

6-Methoxycomaparvin (1): brown needle-shaped crystals; UV (MeOH) λ_{max} (log ϵ) 245 (4.57), 290 (4.37), 380 (3.77) nm; IR (KBr) ν_{max} 3341, 2950, 1667, 1615, 1571, 1537, 1448 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 331.1176 $[M + H]^+$ (1.21 ppm from 331.1172 calcd for $C_{18}H_{19}O_6$).

6-Methoxycomaparvin 5-methyl ether (2): brown needle-shaped crystals; UV (MeOH) λ_{max} (log ϵ) 240 (4.58), 280 (4.51), 365 (4.05) nm; IR (KBr) ν_{max} 3229, 2960, 1642, 1584, 1415 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; HRESIMS *m/z* 345.1337 $[M + H]^+$ (1.16 ppm from 345.1333 calcd for $C_{19}H_{22}O_6$).

X-ray Structure Determination of 6-Methoxycomaparvin (1). Intensity data for a yellow slab (0.28 × 0.12 × 0.06 mm) of **1**·3/2 H_2O were collected using a Nonius Kappa CCD diffractometer

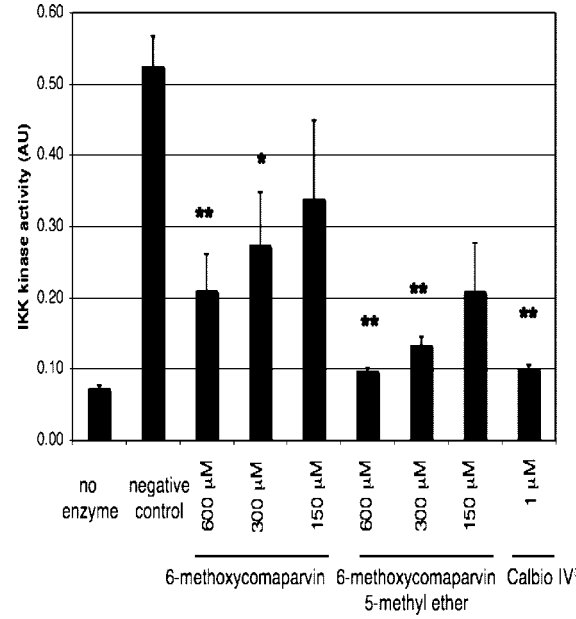


Figure 5. Effects of 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**) on the kinase activity of IKKβ, over an incubation period of 30 min at 30 °C, and at various concentrations. "No enzyme" refers to a control in the absence of IKKβ. The negative control was performed in the presence of IKKβ, but in the absence of any test compound. Calbio IV ([5-(*p*-fluorophenyl)-2-ureido]thiophene-3-carboxamide (Calbiochem)), which is an IKKβ inhibitor provided with the IKKβ K-ELISA test kit as a positive control, was used at the test concentration recommended by Calbiochem (1 μM). Results shown as mean ± SD of three individual measurements. * and ** represent $p < 0.05$ and $p < 0.01$, respectively, compared to the negative control.

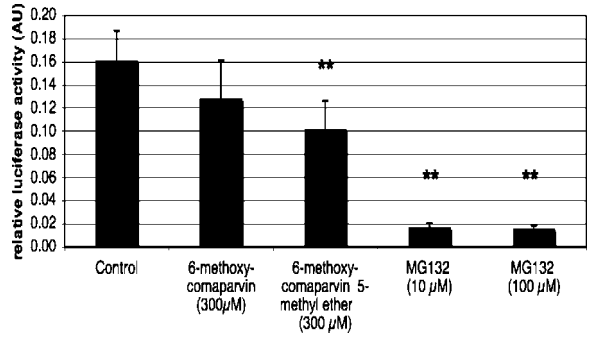


Figure 6. Effects of 6-methoxycomaparvin (**1**) and 6-methoxycomaparvin 5-methyl ether (**2**) on the proteolytic activity of the 26S proteasome in K562 cells, at a concentration of 100 μg/mL (300 μM). Results are shown as a ratio of the firefly luciferase activity measured in the proteasome assay (using a Proteasome-Glo kit (Promega)) over the firefly luciferase activity measured in a viability assay (using a Cell-Titer Glo kit (Promega)) run in parallel with the proteasome assay to standardize the results of the proteasome assay to the number of live cells in each sample at the end of the assay. "Control" refers to a negative control without any test compound. Different concentrations of the known proteasome inhibitor MG132 (*Z*-Leu-Leu-Leu-CHO) were used as positive controls. SD shown for four individual measurements. ** refers to $p < 0.01$ compared to the negative control.

at 120(2) K with graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). A monoclinic unit cell with a = 22.1769(10) Å, b = 7.2797(2) Å, c = 21.9466(9) Å and β = 109.8017(17)° [V = 3333.6(2) $Å^3$] was established and refined based on 7974 reflection positions. A multiscan absorption correction was applied with SADABS (min., max. transmission factors = 0.969, 0.994). A total

of 37 765 scanned data were merged ($R_{Int} = 0.086$) to 7663 unique data [4458 with $I > 2\sigma(I)$] with $2\theta < 55.3°$. The systematic absences indicated space group $P2_1/c$ (No. 14), and all the non-hydrogen atoms in the structure were located by direct methods with SHELXS-97. The structural model was refined against $|F|^2$ with SHELXL-97. The C-bound H atoms were placed geometrically (C−H = 0.95–0.98 Å) and refined as riding with $U_{iso}(H) = 1.2U_{eq}$(carrier) or $1.5U_{eq}$ (methyl carrier). All the O-bound H atoms were located in difference maps. Their positions were freely refined, and the constraint $U_{iso}(H) = 1.2U_{eq}$(carrier) was applied. Final residuals of $R(F) = 0.064$ [$I > 2\sigma(I)$] and $wR(F^2) = 0.146$ (all data) were obtained. Crystallographic data for **1**·3/2 H_2O including atomic positional and displacement parameters, all geometrical data, and full software details have been deposited in CIF format with the Cambridge Crystallographic Data Centre (deposition number CCDC 604780). 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@ccds.cam.ac.uk).

Biological Assays. TNF-α was purchased from Sigma and dissolved at 10 μg/mL in 1× phosphate-buffered saline (PBS) supplemented with 0.5% (w/v) bovine serum albumin (BSA) according to the manufacturer's instructions. K562 (human chronic myelogenous leukemia) and Jurkat (T-cell leukemia) cells (DSMZ) were cultured in RPMI medium (Bio-Whittaker) supplemented with 10% (v/v) fetal calf serum (FCS) (Perbio) and 1% (v/v) antibiotic-antimycotic (Bio-Whittaker) at 37 °C in air containing 5% CO_2. Compounds **1** and **2** were dissolved in DMSO (20 mg/mL) and tested at various concentrations.

Transient transfections of K562 cells were performed as described previously.[24] Five micrograms of luciferase reporter gene construct containing 5 repeats of a consensus NF-κB site (Stratagene) and 5 μg of *Renilla* luciferase plasmid (Promega) were used for each pulse. Following electroporation, the cells were resuspended in growth medium (RPMI/FCS 10%) and incubated at 37 °C in air containing 5% CO_2. Twenty hours after transfection, the cells were harvested and resuspended in growth medium (RPMI/FCS 10%) to a final concentration of 10^8 cells/mL and treated for 2 h with or without the test compounds. The cells were then challenged with 20 ng/mL TNF-α for 2 h. Dual-Glo luciferase reagent (Promega) (75 μL) was added to the cells for a 10 min incubation at 22 °C before luciferase activity was measured. Then, 75 μL of Dual-Glo Stop & Glo reagent (Promega) was added for 10 min at 22 °C in order to assay *Renilla* activity. Luciferase and *Renilla* activities were measured using an Orion microplate luminometer (Berthold) by integrating light emission for 10 s. The results are expressed as a ratio of arbitrary units of firefly luciferase activity normalized to *Renilla* luciferase activity.

The effects of **1** and **2** on the viability of K562 test cells during the reporter gene assay were assessed using a CellTiterGlo kit (Promega) as described in the manufacturer's protocol.

The effects of **1** and **2** on the enzymatic activity of luciferase were assessed by incubating 100 μL of QuantiLum recombinant firefly luciferase (Promega), diluted 10^5 times in a 10% BSA solution, with 100 μg/mL **1** or **2** for 1 h at room temperature. The enzymatic activity of luciferase at the end of the treatment was measured on a luminometer after an addition of 100 μL of luciferin substrate (SteadyGlo reagent (Promega)).

For the electrophoretic mobilitiy shift assay (EMSA), Jurkat cells were resuspended in growth medium (RPMI/FCS 10%) to a final concentration of 10^6 cells/mL and treated for 2 h with or without the test compounds. The cells were then challenged with 20 ng/mL TNFα for 2 h. Nuclear extracts were prepared using the method described by Müller et al.[27] and stored at −80 °C. The oligonucleotide NF-κB c (consensus NF-κB site) (Eurogentec) (5′-AGTTGAGGGGACTTTCCCAGGC-3′) and its complementary sequence were used as probe. The probe was hybridized and labeled with [γ-^{32}P]ATP (MP-Biomedicals), and the EMSA was performed as published previously.[26] Briefly, 10 μg of nuclear extract was incubated in binding buffer with the ^{32}P ATP-labeled probe for 20 min The DNA–protein complexes were analyzed by electrophoresis on a 4% native polyacrylamide gel using Tris-glycine buffer and visualized by autoradiography.

The effects of **1** and **2** on TNF-α-dependent IκBα degradation and on TNF-α-dependent translocation of p65 and p50 into the nucleus were investigated by Western blot analysis using Jurkat cells. Cytosolic and nucleic protein extracts were prepared as previously described.[24] Briefly, 2 × 10 cells/mL were pretreated with **1** or **2** (100 μg/mL) for 2 h and then exposed to 20 ng/mL TNF-α for various amounts of time. Ten micrograms of protein extract were resolved on a 10% SDS-PAGE gel, transferred onto a membrane, blocked with 5% nonfat milk, and probed with specific antibodies against IκBα, p65, and p50 (Santa Cruz). The blots were washed, exposed to horse radish peroxidase-conjugated secondary antibodies for 1 h, and finally detected by ECL reagent (GE Healthcare).

The kinase assay was performed using a K-LISA IKKβ inhibition assay kit (Calbiochem) as described in the manufacturer's protocol. Calbio IV ([5-(*p*-fluorophenyl)-2-ureido]thiophene-3-carboxamide (Calbiochem)) was used as a positive control.

The proteasome inhibiton assay was performed using a Proteasome-Glo assay kit (Promega) as described in the manufacturer's protocol.

Acknowledgment. We wish to thank the "Qoliqoli" owners of Caqalai Island, Fiji Islands, for sample collection and V. Momoivalu of the University of the South Pacific for the sample extraction. We thank the EPSRC National Mass Spectroscopy Centre (University of Wales, Swansea) for mass spectrometric analysis, the Luxembourg government for a Ph.D. research fellowship to F.F. (BFR03/059), and the "Recherche Cancer et Sang" Foundation, the "Recherches Scientifiques Luxembourg" Association, "Een Häerz fir kriibskrank Kanner" a.sbl (Luxembourg), and the Scottish Association for Marine Science for funding. M.J. is the recipient of a BBSRC Research Development Fellowship.

Supporting Information Available: NMR spectra, including ^{1}H, ^{13}C, HSQC, ^{1}H−^{1}H COSY, and HMBC in $CDCl_3$ of **1** and **2**; UV, IR, and MS spectra of **1** and **2**; detailed crystal data for **1**; cytotoxicity data for **1** and **2**; results of the testing of the effects of **1** and **2** on the enzymatic activity of luciferase. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Aggarwal, B. B.; Takada, Y.; Shishodia, S.; Gutierrez, A. M.; Oommen, O. V.; Ichikawa, H.; Baba, Y.; Kumar, A. *Indian J. Exp. Biol.* **2004**, *42*, 341–353.
(2) Karin, M.; Yamamoto, Y.; Wang, Q. M. *Nat. Rev. Drug Discovery* **2004**, *3*, 17–26.
(3) Karin, M. *Nature* **2006**, *441*, 431–436.
(4) Garcia-Pineres, A. J.; Lindenmeyer, M. T.; Merfort, I. *Life Sci.* **2004**, *75*, 841–856.
(5) Mayo, M. W.; Baldwin, A. S. *Biochim. Biophys. Acta: Rev. Cancer* **2000**, *1470*, M55–M62.
(6) Haefner, B. *Drug Discovery Today* **2002**, *7*, 653–663.
(7) Muller, S.; Murillo, R.; Castro, V.; Brecht, V.; Merfort, I. *J. Nat. Prod.* **2004**, *67*, 622–630.
(8) Aggarwal, B. B.; Shishodia, S. *Biochem. Pharmacol.* **2006**, *71*, 1397–1421.
(9) Bremner, P.; Heinrich, M. *J. Pharm. Pharmacol.* **2002**, *54*, 53–472.
(10) Garg, A.; Aggarwal, B. B. *Leukemia* **2002**, *16*, 1053–1068.
(11) Sun, Z. W.; Andersson, R. *Shock* **2002**, *18*, 99–106.
(12) Pande, V.; Sharma, R. K.; Inoue, J. I.; Otsuka, M.; Ramos, M. J. *J. Comput.-Aided Mol. Design* **2003**, *17*, 825–836.
(13) Bharti, A. C.; Aggarwal, B. B. *Biochem. Pharmacol.* **2002**, *64*, 883–888.
(14) Kent, R.; Smith, I. R.; Sutherland, M. D. *Aust. J. Chem.* **1970**, *23*, 2325–2335.
(15) Smith, I. R.; Sutherland, M. D. *Aust. J. Chem.* **1971**, *24*, 1487–1489.
(16) Powell, V. H.; Sutherland, M. D.; Wells, J. W. *Aust. J. Chem.* **1967**, *20*, 535–540.
(17) Sutherland, M. D.; Wells, J. W. *Aust. J. Chem.* **1967**, *20*, 515–533.
(18) Sakuma, Y.; Tanaka, J. I.; Higa, T. *Aust. J. Chem.* **1987**, *40*, 1613–1616.
(19) Francesconi, K. A. *Aust. J. Chem.* **1980**, *33*, 2781–2784.
(20) Podolin, P.; Callahan, J. F.; Bolognese, B.; Li, Y.; Carlson, K.; Davis, T.; Mellor, G.; Evans, C.; Roshak, A. *J. Pharmacol. Exp. Ther.* **2005**, *312*, 373–381.
(21) Banerjee, D.; Liefshitz, A. *Anticancer Res.* **2001**, *21*, 3941–3947.
(22) Huang, Q.; Lu, G.; Shen, H. M.; Chung, M.; Ong, C. N. *Med. Res. Rev.* **2006**.
(23) Aggarwal, B. B.; Ichikawa, A.; Garodia, P.; Weerasinghe, P.; Sethi, G.; Bhatt, I.; Pandey, M.; Shishodia, S.; Nair, M. G. *Expert Opin. Ther. Targets* **2006**, *10*, 87–118.

(24) Duvoix, A.; Delhalle, S.; Blasius, R.; Schnekenburger, M.; Morceau, F.; Fougere, M.; Henry, E.; Galteau, M. M.; Dicato, M.; Diederich, M. *Biochem. Pharmacol.* **2004**, *68*, 1101–1111.
(25) Kumar, A.; Dhawan, S.; Aggarwal, B. B. *Oncogene* **1998**, *17*, 913–918.
(26) Srinivas, G.; Anto, R. J.; Srinivas, P.; Vidhyalakshmi, S.; Senan, V. P.; Karunagaran, D. *Eur. J. Pharmacol.* **2003**, *473*, 117–125.
(27) Lee, H. Z. *Br. J. Pharmacol.* **2001**, *134*, 11–20.
(28) Min, B.; Lee, J.; Na, M.; An, R.; Lee, S.; Lee, H.; Bae, K.; Kang, S. *Chem. Pharm. Bull.* **2003**, *51*, 1322–1324.
(29) Ernst-Russell, M.; Chai, C.; Wardlaw, J.; Elix, J. *J. Nat. Prod.* **2000**, *63*, 129–131.

NP070290Y