A New Tyrosine Kinase Inhibitor from the Marine Brown Alga *Stypopodium zonale*

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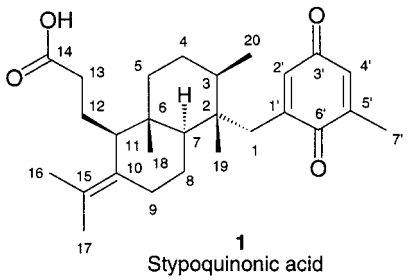
From the lipophilic extract of the marine brown alga *Stypopodium zonale* (Dictyotaceae) the new terpenoid compound stypoquinonic acid (**1**) together with the known compounds taondiol (**2**) and atomaric acid (**3**) were isolated. The structures of all isolates were determined from their spectroscopic data, including 1- and 2-dimensional NMR methods. The new compound, **1**, and atomaric acid (**3**), showed inhibition of tyrosine kinase (p56^{lck}).

In the course of our research on marine organisms for biologically active low molecular weight compounds, the brown alga *Stypopodium zonale* (Lamouroux) Papenfuss (Dictyotaceae, Dictyotales) was investigated. This alga is known to contain several polycyclic terpenoids with ichthyotoxic and cytotoxic activity.[1] The three pentacyclic sesterterpenoids stypoldione, stypotriol and epistypodiol were found to be mainly responsible for the observed biological effects, stypotriol having the strongest and epistypodiol the weakest activities.[1] Stypoldione, which is the product of aerial oxidation of stypotriol, was further investigated, and found to be a potent inhibitor of the first cleavage of fertilized sea urchin eggs, and an in vitro inhibitor of microtubule polymerization.[2] Crude extracts of *S. zonale* showed only weak antimicrobial activities.[3]

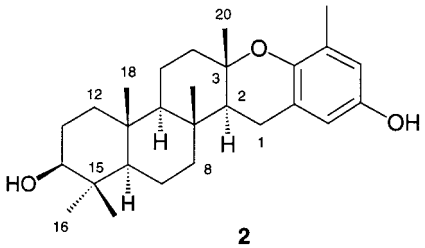
In this report the chemical and biological activity investigations of a specimen of *S. zonale* collected off the coast of Lanzarote (Canary Islands, Spain) in April 1991 are presented. Fractionation and purification were guided by TLC, ^{1}H NMR and bioassays. Separations were carried out by repeated column chromatography, including HPLC. Resultant extracts and pure compounds were tested for their antimicrobial activities against two bacteria (*Escherichia coli, Bacillus megaterium*), four fungi (*Fusarium oxysporum, Eurotium repens, Ustilago violacea, Mycotypha microspora*), the green alga *Chlorella fusca*, and inhibition of reverse transcriptase of the human immunodeficiency virus type 1 (HIV-1-RT) and p56^{lck} tyrosine kinase (TK).

The CH_2Cl_2 extract of the brown alga *S. zonale* showed an inhibitory effect in the TK assay (59% inhibition of the enzyme at 200 μg/mL), as well as having interesting signals in the low-field region of its ^{1}H NMR spectrum. Separation, guided by TLC, ^{1}H NMR, and the TK assay yielded the new compound **1** (stypoquinonic acid) and the known compounds **2** and **3**.

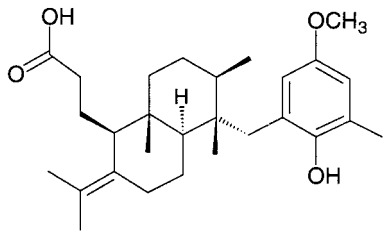
The ^{13}C NMR and mass spectral data of **1** indicated it to have the molecular formula $C_{27}H_{38}O_4$, and thus have nine elements of unsaturation. The ^{13}C NMR spectrum also showed the presence of three carbonyl groups, including two α,β-unsaturated carbonyls, one carboxyl group, and three carbon−carbon double bonds with two tertiary and four quaternary carbons as the only multiple bonds. The molecule is tri-cyclic. Further analysis of DEPT and ^{13}C NMR spectra revealed the presence of a further two quaternary carbons, three methine, seven methylene, and



1
Stypoquinonic acid



2
Taondiol



3
Atomaric acid

six methyl groups in **1**. The resonances for the methyl groups in the ^{1}H NMR spectrum of **1** were present as one doublet and five singlets, indicating that they were connected to one methine and a maximum of five quaternary carbons, respectively. Two further ^{1}H NMR signals, one at 6.63 (s) ppm and the other at 6.54 (s) ppm, for two olefinic protons, revealed that two of the three double bonds were trisubstituted. The third carbon−carbon double bond was thus fully substituted.

After all proton resonances were assigned to those of the directly bonded carbon atoms, aided by an HMQC spectrum, it was possible to deduce several molecular fragments. Thus, from the ^{1}H−^{1}H COSY spectrum of **1** three ^{1}H−^{1}H spin systems could be deduced. Coupling was observed between H_3-20 and H-3, H-3 further coupled to

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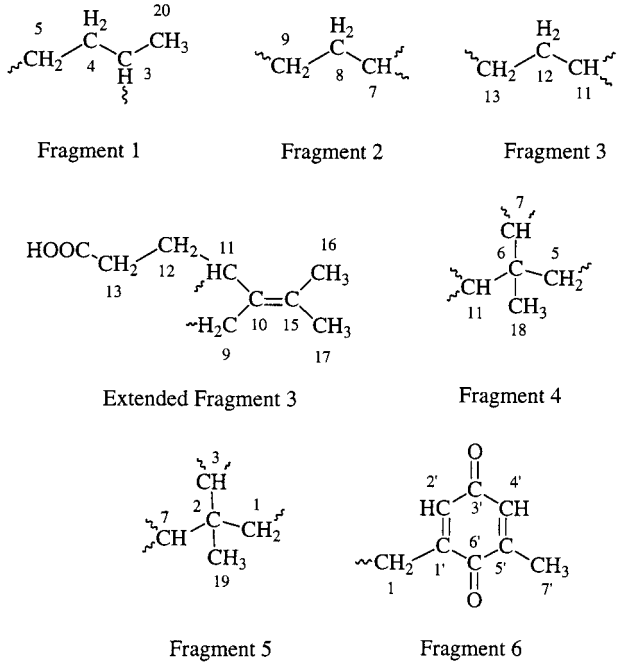
Table 1. Biological Activities of Stypoquinonic Acid (**1**), Taondiol (**2**), and Atomaric Acid (**3**)

test/compound	1	2	3
tyrosine kinase IC_{50}[a]	79.7 μg/mL	not active	92.0 μg/mL
bacteria[b] (50 μg/disk)	1 mm for B.m and E.c	not active	1 mm for B.m and E.c
fungi[c] (50 μg/disk)	not active	2 mm for E.r	not active
alga[d] (50 μg/disk)	not active	not active	5 mm

[a] Positive control piceatannol, $IC_{50} = 20$ μg/mL. [b] Tested against *B. megaterium* (B.m) and *E. coli* (E.c). Positive control, diethylphthalate: inhibition zones 10 mm (B.m) and 1 mm (E.c). Inhibition zones were measured in mm from the edge of the filter disks. [c] Tested against *E. repens* (E.r), *F. oxysporum* (F.o), *U. violacea* (U.v), and *M. microspora* (M.m), miconazol was positive control: inhibition zones 20 mm (E.r), 3 mm (F.o), 32 mm (U.v), and 5 mm (M.m). [d] Tested against *C. fusca*, miconazol (M) and diethylphthalate (D) were positive controls: inhibition zones 4 mm (M) and 13 mm (D).

Scheme 1. Molecular Fragments of **1** As Deduced from COSY and HMBC Spectra



H_2-4, which in turn coupled with H_2-5 (fragment 1, Scheme 1). Furthermore, couplings were observed between H-7 and H_2-8 and between H_2-8 and H_2-9 to give fragment 2. Couplings between H-11 and H_2-12 and between H_2-12 and H_2-13 afforded fragment 3. This information in conjunction with the interpretation of the HMBC spectrum finally led to the planar structure of **1** being deduced. Thus, starting with fragment 3, cross-peaks in the HMBC spectrum were seen between H_2-12, H_2-13, and the carbonyl C-14, positioning the carboxyl function. Further, HMBC cross-peaks between H-11 and the double-bond carbons C-15 and C-10 extended fragment 3. Both of these carbons coupled with the methyl protons H_3-16 and H_3-17, as well as H_2-9, extended fragment 3. Heteronuclear multiple bond couplings were also seen from H-11 to C-6, which in turn coupled with H_3-18, H_2-5, and H-7, fragment 4. Fragments 2 and 4 as deduced from ^{1}H−^{1}H COSY and HMBC could now be connected to extended fragment 3 between C-7 and C-9 to give the first ring. Further cross-peaks in the HMBC spectrum between H-7 and C-2, the latter showing coupling with H_2-1, H_3-19, and H-3 (fragment 5), enabled fragment 1 to be placed between C-2 and C-6 giving rise to the second ring. The last part of the molecule (fragment 6) was also elucidated through cross-peaks in the HMBC spectrum between H_2-1 and C-1′, C-2′, and C-6′, between H-2′ and C-3′, between H-4′ and C-2′, C-3′, and C-7′, and between H_3-7′ and C-4′, C-5′, and C-6′.

Comparison of the ^{13}C NMR data of the C-1−C-20 part of **1** with those of **3** (see Table 2) showed them to be in good agreement, differences being less than ±1.5 ppm. The ^{1}H NMR data of **1**, especially coupling constant values were also comparable, thus the identical relative stereochemistry at comparable centers is suggested for **1** and **3**.

Table 2. ^{13}C NMR Data (ppm) for Compounds **1** (75.5 MHz, $CDCl_3$), **2** (100.6 MHz, $CDCl_3$), and **3** (75.5 MHz, $CDCl_3$)[a]

carbon	1	2	3	CH long-range correlations
1	34.1 t[b]	22.6 t	35.3 t	
2	40.7 s	52.5 d	40.6 s	H-7, H_3-19
3	35.1 d	76.1 s	35.3 d	
4	25.2 t	41.0 t	25.3 t	H_3-20
5	36.2 t	18.7 t	36.5 t	
6	39.0 s	60.6 d	38.9 s	H_2-5, H-7, H-11, H_3-18
7	42.1 d	36.9 s	41.9 d	
8	22.4 t	41.1 t	22.4 t	
9	23.4 t	17.9 t	23.5 t	
10	123.7 s	55.4 d	123.2 s	H_2-9, H-11, H_3-16, H_3-17
11	53.0 d	37.2 s	53.2 d	
12	24.8 t	27.3 t	25.1 t	
13	32.9 t	38.4 t	33.7 t	
14	180.3 s	78.8 d	180.9 s	H_2-13, H_2-12
15	132.5 s	38.9 s	133.0 s	H_3-16, H_3-17
16	20.7 q	28.0 q	20.4 q	H_3-17
17	20.4 q	15.3 q	20.8 q	H_3-16
18	17.6 q	16.4 q	17.9 q	
19	21.7 q	15.8 q	20.3 q	
20	15.6 q	20.8 q	15.8 q	
1′	147.7 s	122.4 s	126.8 s	H_2-1
2′	134.0 d	145.4 s	114.4 d	H_2-1, H-4′
3′	187.6 s	127.1 s	152.5 s	H-2′, H-4′
4′	132.8 d	115.4 d	113.2 d	H-2′, H_3-7′
5′	146.2 s	147.8 s	124.0 s	H_3-7′
6′	188.3 s	112.9 d	146.8 s	H_2-1, H_3-7′
7′	16.4 q	16.1 q	16.7 q	
8′			55.5 q	

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HMQC, COSY). [b] Multiplicities determined by DEPT (*s* = C, d = CH, t = CH_2, q = CH_3).

An interesting phenomenon was encountered when compound **1** was isolated with a hydrophilic solvent such as aqueous methanol. When the resultant sample was submitted for ^{1}H and ^{13}C NMR measurement in $CDCl_3$ the signals for the C-12 to C-14 part of the molecule were not observed, all other spectroscopic data were, however, identical. A possible explanation for this observation is the formation of micelles involving the carboxyl function of **1**. Formation of micelles might result in either a significant increase or decrease in the relaxation times of the atoms associated with CH_2-12, CH_2-13, and C-14, and thus NMR signals are not observed under standard measuring conditions.

Together with **1** the known compounds taondiol (**2**) and atomaric acid (**3**) were isolated. As the ^{1}H and ^{13}C NMR data for these compounds were either incomplete or unassigned, they were recorded and assigned (see Tables 2 and 3).

Previous investigations of *S. zonale* revealed the presence of pentacyclic sesterterpenoids stypotriol, epistypodiol, stypoldione, **2**, its epimers, and **3** in the alga.[1,2] It has been shown that the chemical contents of *S. zonale* samples vary

Table 3. ^{1}H NMR Data of Compounds (ppm) **1** (300 MHz, $CDCl_3$), **2** (400 MHz, $CDCl_3$), and **3** (300 MHz, $CDCl_3$)[a]

proton	1	2	3
1	2.85 (1H, d, J = 13.9 Hz)	2.56 (1H, d, J = 5.1 Hz)	2.85 (1H, d, J = 14.3 Hz)
	2.04 (1H, d, J = 13.9 Hz)	2.54 (1H, s)	2.25 (1H, d, J = 14.3 Hz)
2		1.60 (1H, m)	
3	1.67 (1H, m)		1.73 (1H, m)
4	1.24 (1H, m)	1.65 (1H, m)	1.23 (1H, d, J = 15.4 Hz)
	1.86 (1H, m)	1.03 (1H, m)	1.88 (1H, m)
5	1.48 (2H, m)	1.70 (1H, m)	1.49 (2H, m)
		1.36 (1H, d, J = 15.3 Hz)	
6		0.96 (1H, d, J = 12.2 Hz)	
7	1.30 (1H, dd, J = 6.4, 11.7 Hz)		1.38 (1H, dd, J = 5.7, 11.7 Hz)
8	1.69 (1H, m)	2.03 (1H, d, J = 14.2 Hz)	1.75 (1H, m)
	1.52 (1H, m)	1.79 (1H, d, J = 14.2 Hz)	1.52 (1H, m)
9	2.38 (1H, q, J = 8.7, 12.4 Hz), 1.93 (1H, m)	1.47 (1H, m)	2.39 (1H, q, J = 8.7, 13.2 Hz), 1.96 (1H, m)
		1.59 (1H, m)	
10		0.79 (1H, d, J = 11.7 Hz)	
11	2.29 (1H, dd, J = 3.8, 9.0 Hz)		2.32 (1H, m)
12	1.79 (1H, m)	1.63 (2H, m)	1.81 (1H, m)
	1.59 (1H, m)		1.58 (1H, m)
13	2.30 (2H, m)	1.76 (1H, d, J = 14.8 Hz)	2.26 (2H, m)
		1.00 (1H, m)	
14		3.22 (1H, dd, J = 4.6, 11.2 Hz)	
16	1.64 (3H, s)	0.98 (3H, s)	1.68 (3H, s)
17	1.66 (3H, s)	0.79 (3H, s)	1.66 (3H, s)
18	1.00 (3H, s)	0.86 (3H, s)	1.02 (3H, s)
19	0.89 (3H, s)	0.88 (3H, s)	0.94 (3H, s)
20	0.99 (3H, d, J = 6.4 Hz)	1.13 (3H, s)	1.15 (3H, d, J = 6.8 Hz)
2′	6.63 (1H, s)		6.69 (1H, d, J = 3.0 Hz)
4′	6.54 (1H, s)	6.45 (1H, d, J = 2.5 Hz)	6.54 (1H, d, J = 2.6 Hz)
6′		6.38 (1H, d, J = 2.5 Hz)	
7′	2.04 (3H, s)	2.09 (3H, s)	2.22 (3H, s)
8′			3.73 (3H, s)

[a] All assignments are based on extensive 1D and 2D NMR measurements (HMBC, HMQC, COSY).

both qualitatively and quantitatively depending on where and when the alga was collected.[4] In the currently investigated specimen from Lanzarote, Spain, no stypotriol, epistypodiol, or stypoldione were detected. Instead, **1**−**3** were isolated. Clearly, the secondary metabolite chemistry of the sample from Lanzarote differs from the samples investigated by other researchers,[1,4] their samples coming from locations in the Caribbean Sea and off the coast of Palau. It is likely that environmental pressures and conditions in the vicinity of Lanzarote were quite different from those of the previously investigated samples and as a response the alga may produce a different array of secondary metabolites. **2** and **3** may be of ecological relevance for *S. zonale* since concentrations of 10 μg/mL produce a distinct lethargic behavior and narcosis in herbivorous fish.[1] The biological activity of **1** against herbivorous fish was not investigated. Even though the secondary metabolite chemistry varies in all of the samples investigated to date, all of the reported isolates belong to the same biosynthetic group.

1 and **3** both inhibit tyrosine kinase (p56^{lck}) with IC_{50} values of 79.7 μg/mL (187 μM; 3-fold determination), and 92.0 μg/mL (208 μM; 2-fold determination), respectively. The inhibition of the TK is relatively weak when compared to the positive control piceatannol (IC_{50} 20 μg/mL = 82 μM). Comparison of the basic structural features of **1** with those of some potent quinoid TK inhibitors, such as anthraquinones, and naphthoquinones may explain the observed activity.[6]

1 and **3** have weak antimicrobial activities toward *Bacillus megaterium* and *Escherichia coli*, and **2** has a weak activity toward the fungus *Eurotium repens* in agar diffusion assays. Inhibition of reverse transcriptase of the human immunodeficiency virus type 1 (HIV-1) was not observed for any of the compounds.

Experimental Section

General Experimental Procedures. The general experimental procedures were carried out as previously described.[8]

Collection and Isolation. *S. zonale* (Lamouroux) Papenfuss was collected in April 1991, by hand using SCUBA at a depth of 14 m off the coast of Lanzarote. Collected material was stored at −20 °C until used. A voucher specimen, number CT911K, is stored at the Institute for Pharmaceutical Biology, Technical University of Braunschweig, Mendelssohnstrasse 1, D-38106 Braunschweig, Germany.

Algal tissue was freeze-dried (dry weight 96.5 g) and exhaustively extracted with CH_2Cl_2 (2 L) and then with MeOH (2 L). Solvents were removed in vacuo, and the extracts filtered through a pad (5 mm thick) of silica gel. The MeOH extract was extracted with CH_2Cl_2, and the two lipophilic fractions were combined to yield 9.5 g (9.84%) of CH_2Cl_2-soluble and 12.6 g (13.06%) of MeOH-soluble material. Of the CH_2Cl_2-soluble fraction 5.5 g was applied to a vacuum liquid column (VLC, silica gel) and gradient-eluted from hexane to EtOAc, and thereafter with MeOH to yield 13 fractions, each of 90 mL. On the basis of their TLC and ^{1}H NMR data fractions 9−12 were combined and rechromatographed using VLC over silica gel employing a gradient elution from hexane/acetone 85/15 to 55/45 and finally MeOH to yield 16 sub-fractions, each of 40 mL.

Isolation of 1 and 2. VLC subfractions 5−9 were combined and eluted from a RP-18 open column with 90% methanol. The eluate was further separated into five fractions by HPLC (RP-18, 90% aqueous MeOH as eluent). Fraction 3 was further purified by HPLC (silica gel, hexane/acetone 75/25 as eluent) to yield **1** (73.6 mg, 0.13%), and **2** (10.5 mg, 0.02%).

Isolation of Atomaric Acid (3). VLC subfractions 10−13 were combined and separated by HPLC (RP-18, 90% aqueous methanol as eluent) to yield **3** (71.2 mg, 0.13%) and **1** (14.5 mg, 0.03%).

Stypoquinonic acid (1): an oil (88.1 mg, 0.16%); $[\alpha]^{25}_D$ +68.9° (c 0.27, MeOH); UV λ_{max} (MeOH) 255 nm (ϵ 1243); IR ν_{max} 3570−3240, 2930, 1655, 1440, 1385, 1030 cm^{-1}; ^{1}H NMR, see Table 3; ^{13}C NMR, see Table 2; EIMS m/z [M$^+$] 426 (22),

396 (15), 353 (16), 289 (100), 205 (24), 191 (33), 163 (63), 137 (63), 109 (47); HREIMS *m/z* 426.2770 (calcd for $C_{27}H_{38}O_4$, 426.2770).

Taondiol (2): an oil (10.5 mg, 0.02%); $[\alpha]^{25}_D$ −52.3° (*c* 0.34, $CHCl_3$), lit.[7] −76.0°; IR ν_{max} 3470−3250, 2933, 2857, 1609, 1471, 1385, 1228, 1029 cm^{-1}; ^1H NMR, see Table 3; ^{13}C NMR, see Table 2; remaining physical and spectral properties as published.[1,7]

Atomaric acid (3): 71.2 mg (0.13%); $[\alpha]^{25}_D$ +17.3° (*c* 0.32, MeOH), lit.[5] +49.0°; ^1H NMR ($CDCl_3$, 300 MHz) see Table 3; ^{13}C NMR ($CDCl_3$, 75.5 MHz) see Table 2; remaining physical and spectral properties as published.[1,5]

Biological Tests. Antimicrobial[9] and RT inhibition[10] assays were carried out as previously described.

Tyrosine Kinase (TK) Inhibition Assay. Tyrosine kinase ($p56^{lck}$) inhibitory activity was determined by ELISA using a commercial test kit (Boehringer Mannheim, catalog no. 1,534,-505). Experimental procedures followed, in principle, the descriptions of the supplier (Boehringer Mannheim), but were modified as stated below. Compounds and extracts were dissolved in half of their weight (μL) of dimethyl sulfoxide (DMSO), and diluted with water (1:1 v/v) to yield appropriate sample solutions. Pure compounds were tested in different concentrations. Assays were carried out using T cell tyrosine kinase $p56^{lck}$ (Upstate Biotechnology). Sample solutions were incubated with 1 μM tyrosine kinase substrate II, biotin-labeled (Boehringer Mannheim), 1 mM ATP, 10 mM $MgCl_2$, 1 unit of tyrosine kinase $p56^{lck}$, 20 μL of dilution buffer, and 20 μL of assay buffer for 1 h at 30 °C. The resultant concentration of the extract in the test mixture was 200 μg/mL. Dilution buffer (pH 7.0) contained 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10% glycerol, and 0.1% ethylphenolpoly(ethylene−glycol ether)$_n$ (NP-40). Assay buffer (pH 7.5) contained 250 mM Tris, 25 mM NaF, 2.5 mM EDTA-Na_2, 4.0 mM [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 5 mM DTT, and 150 μM Na_3VO_4. In the ELISA reader the measuring wavelength was 415 nm. In each test series 3 mM piceatannol (Boehringer Mannheim) was included as a positive control. Inhibitory activity was expressed as remaining enzyme activity (%) relative to a negative control.

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