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INHIBITORS OF PROTEIN TYROSINE KINASE pp60$^{v\text{-}src}$: STEROL SULFATES FROM THE BRITTLE STAR *OPHIARACHNA INCRASSATA*

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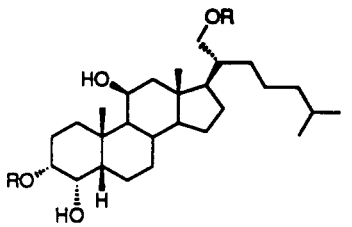
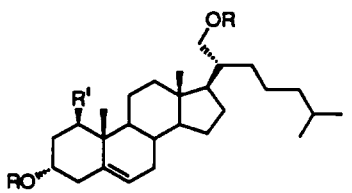
ABSTRACT.—Bioactivity-guided fractionation of the extracts of the green brittle star *Ophiarachna incrassata* using a protein tyrosine kinase pp 60$^{v\text{-}src}$ inhibition assay led to the isolation of a new sterol sulfate [**2**] together with four known ones (**1**, **3**–**5**). All five compounds were found to inhibit protein tyrosine kinase pp 60$^{v\text{-}src}$.

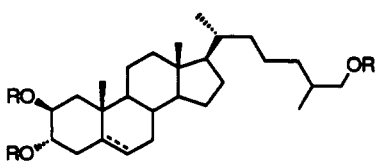
Protein tyrosine kinases (PTK) comprise a large family of enzymes that regulate cell growth and intracellular signalling pathways (1–4). The concept that inhibition of these enzymes might result in control of cancer and other hyperproliferative conditions (5) has stimulated a search for selective PTK antagonists as leads for new anticancer agents (6). A small number of marine natural products have been reported to inhibit PTK (7–10), including some sterol sulfates (11). In our ongoing enzyme inhibition assay-directed search for additional PTK inhibitors we have isolated one new sterol sulfate [**2**] and four known representatives [**1**, **3**–**5**] from the brittle star *Ophiarachna incrassata* (Lamarck, 1816), family Ophiadermatidae, collected in Palau. We report here the structure elucidation of **2** and the PTK inhibiting activity of **1**–**5**.



1 R=SO_3Na



2 R=SO_3Na; R′=OH
3 R=SO_3Na; R′=H



4 R=SO_3Na; 5α,6-Dihydro
5 R=SO_3Na; Δ^5

The MeOH and MeOH-CH_2Cl_2 (1:1) extracts of the frozen specimens were concentrated and subjected to solvent partitioning to furnish, after evaporation of solvent under reduced pressure, hexane, CH_2Cl_2, *n*-BuOH, and H_2O-soluble fractions. Only the residue from the *n*-BuOH extract (1.89 g) showed protein tyrosine kinase inhibition, and this was chromatographed over a Si gel open column using increasing amounts of MeOH in CH_2Cl_2 as eluent. The active fractions were further separated by gel filtration over Sephadex LH-20 using MeOH as eluent. 5β-Cholestane-3α,4α,11β-21-tetrol 3,21-disulfate [**1**], cholest-5-ene-1β,3α,21-triol 3,21-disulfate [**2**], and cholest-5-ene-3α,21-diyl sulfate [**3**] were finally purified by reversed-phase C-18 hplc using H_2O-MeOH (42:58), while

5α-cholestane-2β,3α,26-triyl sulfate [**4**] and cholest-5-ene-2β,3α,26-triyl sulfate [**5**] were obtained by C-18 hplc using H_2O-MeOH (37:63) containing 2 mM Na_2SO_4 as eluent. The isolates were desalted by chromatography over a small column of polystyrene beads (Diaion HP-20).

The known compounds **1**, **3**, **4**, and **5** were identified by comparison of their ^{1}H-nmr and ms data, and for **1** also ^{13}C-nmr data, with published data (12–14). Sterol sulfates **1**, **4**, and **5** have previously been reported from *O. incrassata* collected at Noumea, New Caledonia (12).

The new metabolite **2** was obtained as an amorphous solid, [α]D +16.7° [*c*=0.62, H_2O-MeOH (1:2)]. The molecular formula $C_{27}H_{44}O_9S_2Na_2$ was deduced from the negative-ion fabms, which showed a major peak at *m/z* 599 [M−Na^+] and a small ion at *m/z* 615, corresponding to the monosodium and monopotassium salts of the dianion, respectively. Fragmentation ion peaks at *m/z* 497 and 479 were also displayed in the mass spectrum which correspond to the loss of $NaSO_3$ (+H) and $NaHSO_4$, respectively, from *m/z* 599. The ^{13}C-nmr spectrum of **2** showed 27 resolved resonances, consistent with the above molecular formula.

The ir spectrum of **2** exhibited bands at 3400, 1650, 1230–1250 (strong) cm^{-1} indicating the presence of hydroxyl, double bond, and sulfate groups. The ^{1}H-nmr spectrum contained two methyl singlet peaks at δ 0.73 and 1.02 ppm, assigned to Me-18 and Me-19, respectively, and two overlapping methyl doublet signals at δ 0.88 (6H, *J*=6.3 Hz), assigned to the isopropyl methyl groups (C-26, C-27). From the ^{1}H-^{1}H COSY nmr spectrum it was determined that the doublet signals at δ 0.88 were coupled to a proton signal at δ 1.56 ppm which was assigned to H-25; this confirmed the presence of the conventional sterol side-chain isopropyl group. Other prominent features in the ^{1}H-nmr spectrum were one olefinic proton signal at δ 5.50 (1H, br d, *J*=4.9 Hz), and four lower field signals at δ 3.74 (1H, dd, *J*=11.7 and 4.2 Hz), 3.95 (1H, dd, *J*=9.5 and 6.5 Hz), 4.19 (1H, dd, *J*=9.5 and 3.5 Hz) and 4.63 (m, $W_{1/2}$=7.5 Hz) due to protons on oxygenated carbons. The ^{13}C-nmr spectrum verified the presence of three oxygenated carbons: an oxymethylene signal at δ 70.5 (DEPT) and two oxymethine resonances at δ 76.7 and 77.9.

Because only three oxygenated carbon signals were observed in the ^{13}C-nmr spectrum of **2**, eight of the oxygens were assigned to two sulfate groups, which were assigned to C-3 and C-21 on the basis of the following nmr data. In the ^{1}H-^{1}H COSY spectrum of **2**, doublets of doublet signals at δ 3.95 and 4.19 were correlated with each other and also with a proton signal at δ 1.60 consistent with expectations for a C-21 oxygenated steroid moiety. Since the chemical shifts for the oxygenated methylene protons and the associated ^{13}C-nmr signal (δ 70.5) were nearly identical to those of the C-21 sulfated group in **1** (δ 3.99, 4.22/69.8) and other C-21 sulfated steroids (12), a sulfate group could be assigned to the C-21 position in **2**. The chemical shift of the narrow multiplet at δ 4.63 matched well that of H-3β (δ 4.64, $W_{1/2}$ 8 Hz) of cholest-5-ene-3α,21-diol 3,21-disulfate (12) and hence **2** was assigned a 3α-OSO_3Na substituent. The signal for H-3β in the free sterol, cholest-5-ene-3α,21-diol, occurs at much higher field, δ 4.03.

The remaining oxygen could be accounted for by a hydroxyl group which was apparent from an ir absorption at 3400 cm^{-1} and a ^{13}C-nmr signal at δ 76.7. That this hydroxyl group was located at C-1 was evident from ^{1}H-^{1}H COSY data which showed that the double doublet at δ 3.74 (H-1α) was coupled to resonances at δ 2.08 (H-2β) and 2.20 (H-2α), which in turn were coupled to each other and also to the H-3β signal at δ 4.63. The latter signal was coupled further to two geminal proton signals at δ 2.33 (H-4α) and 2.50 (H-4β). A *W*-type

long-range coupling between H-2α (δ 2.20) and H-4α (δ 2.33) was also evident in the ^{1}H-^{1}H COSY spectrum. The olefinic proton signal at δ 5.50 showed a small allylic coupling to H-4β and thus the double bond was fixed at C-5,6. Hence, the structure of **2** was determined to be cholest-5-ene-1β,3α,21-triol 3,21-disulfate. The stereochemistry at C-1 and C-3 was defined by coupling constants for H-1 (dd, J=11.7 and 4.2 Hz) and H-3 (m, $W_{1/2}$=7.5 Hz). The position and relative configuration of the 1-OH were also supported by pyridine-induced solvent shifts of 19-Me (Δ=−0.22) (15).

Compounds **1–5** were found to show moderate inhibition of protein tyrosine kinase (PTK) pp60$^{v\text{-}src}$ with IC_{50}s of 62, 65, 31, 11, and 12 μM, respectively. These activities are in the same range as those of the cycloartanol disulfates we reported earlier (11), but the trisulfates **4** and **5** are clearly the most active of all these sterol sulfates. The sterol sulfate PTK inhibitors represent a chemotype different from the few other marine natural product PTK inhibitors which have been reported. These include the quinone/hydroquinol pair helenaquinone and helenaquinol (9,10), IC_{50} 1.5 and 0.6 μM, respectively, the quinone melemeleone (8), IC_{50} ca. 28 μM, all of which inhibited PTK pp60$^{v\text{-}src}$, and the substituted cyclohexadiendiol aeroplysinin-1 which strongly inhibited the kinase activity of the EGF-receptor-associated tyrosine kinase at 0.5 μM (7). A limited number of microbial and plant natural product inhibitors of various PTKs are known which are active in the nanogram to microgram/ml range (16,17). The chemotypes include flavonoids, quinols, quinones, indole alkaloids, and substituted styrenes.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All nmr spectra were obtained on a Varian XL-300 spectrometer at 300 MHz for ^{1}H and 75 MHz for ^{13}C; negative-ion fabms were measured with a VG ZAB mass spectrometer. Ir spectra were recorded on a Bio-Rad 3240-spec FT instrument. Prep. hplc was performed using a Spherex 5 C-18 column (300×10 mm) with ri detection. Diaion HP-20 is a macroporous styrene-based polymer bead resin providing an aromatic non-polar surface (Mitsubishi Kasei America, Inc., White Plains, New York).

ANIMAL MATERIAL.—The green brittle stars, *Ophiarachna incrassata* (Lamarck, 1816), family Ophiodermatidae, were collected in Palau in 1993 and frozen shortly after collection.

EXTRACTION AND ISOLATION OF STEROID SULFATES.—Freshly thawed specimens (340 g wet wt, 123 g dry wt after extraction) were extracted twice with MeOH (24 h for each extraction) and twice with CH_2Cl_2-MeOH (1:1). The extracts were concentrated *in vacuo* and combined to give a residue which was dissolved in 300 ml of 10% aqueous MeOH. The solution was partitioned against hexane (3×300 ml) and the resulting aqueous solution was diluted to 30% H_2O in MeOH and partitioned against $CHCl_3$ (3×120 ml). The aqueous MeOH phase was concentrated *in vacuo* and the aqueous concentrate was extracted with *n*-BuOH (3×120 ml). Only the residue from the *n*-BuOH extract (1.89 g) showed protein tyrosine kinase (PTK) activity (IC_{50} <50), and was therefore fractionated on an open column of SiO_2 using increasing amounts of MeOH in CH_2Cl_2 as eluent. Eight fractions were collected. The last two fractions were most active in the PTK assay and hence were pooled and further fractionated on a column of Sephadex LH-20 using MeOH as eluent to yield eight fractions. Analysis of fractions 6 and 7 by ^{1}H-nmr spectroscopy indicated that both contained polyoxygenated steroids and they were therefore pooled and further purified by reversed-phase hplc using H_2O-MeOH (42:58) as eluent to furnish 5β-cholestane-3α,4α,11β,21-tetrol 3,21-disulfate [**1**] (11) (21.5 mg), cholest-5-ene-1β,3α,21-triol 3,21-disulfate [**2**] (8.3 mg), and cholest-5-ene-3α,21-diyl sulfate [**3**] (12) (2.9 mg).

The last fraction of sterol sulfates could not be resolved using a C-18 hplc column and a variety of MeOH/H_2O ratios. However, this mixture was resolved by C-18 hplc using MeOH-H_2O (63:37) containing 2 mM Na_2SO_4. The Na_2SO_4 was removed from the eluates by passing them through a small column of Diaion HP20, eluting first with H_2O and then MeOH to give pure 5α-cholestane-2β,3α,26-triyl sulfate [**4**] (11) (4.0 mg) and cholest-5-ene-2β,3α,26-triyl sulfate [**5**] (11) (3.1 mg).

Cholestane-3α,4α,11β-21-tetrol 3,21-disulfate [**1**] was obtained as a white powder: negative-ion lrfabms m/z 633 [st $(OSO_3K)(OSO_3^-)$](st=steroid skeleton), 617 [st $(OSO_3Na)(OSO_3^-)$], 515, 497.

Cholest-5-ene-1β,3α,21-triol, 3,21-disulfate

[**2**] was obtained as a white powder: $[\alpha]_D$ +16.7° [c=0.62, MeOH-H_2O (2:1)]; ir (neat) ν max 3400 (OH), 1650 (C=C), 1230–1250 (strong, OSO_3^-) cm^{-1}; ^1H nmr (CD_3OD, 300 MHz) δ 0.73 (3H, s, Me-18), 0.88 (6H, d, J=6.3 Hz, Me-26, -27), 1.02 (3H, s, Me-19), 1.56 (1H, m, H-25), 1.60 (1H, m, H-20), 2.08 (1H, m, H-2β), 2.12 (1H, m, H-7), 2.20 (1H, m, H-2α), 2.33 (1H, dt, J=15 Hz, sm, H-4α), 2.50 (1H, br d, J=15 Hz, H-4β), 3.74 (1H, dd, J=11.7 and 4.2 Hz, H-1α), 3.95 (1H, dd, J=9.5 and 6.5 Hz, H-21), 4.19 (1H, dd, J=9.5 and 3.5 Hz, H-21), 4.63 (1H, m, $W_{1/2}$=7.5 Hz, H-3β), 5.50 (1H, br d, J=4.9 Hz, H-6); ^{13}C nmr (CD_3OD, 75 MHz) δ 139.0 (s, C-5), 127.1 (d, C-6), 77.9 (d, C-3), 76.7 (d, C-1), 70.5 (t, C-21), 57.5 (d, C-14), 51.8, 51.3 (d, C-17), 44.8, 42.9, 40.8 (C-20), 40.4 (C-24), 40.3, 38.2, 37.1, 34.2, 32.8, 30.9 (C-22), 28.9 (C-16), 28.6 (C-25), 25.7, 25.3 (C-15), 24.9 (C-23), 23.7 (C-26), 23.5 (C-27), 13.7 (C-18), 13.5 (C-19); negative hrfabms, found m/z 599.2355, calcd for $C_{27}H_{44}O_9S_2Na$ $(M-Na^+)^-$, 599.2324 (Δ 3.1 mmu); lr negative fabms m/z 615 [st $(OSO_3K)(OSO_3^-)$] (st=steroid skeleton), 599 (major peak) [st $(OSO_3Na)(OSO_3^-)$], 497 [st $(OSO_3Na)(OSO_3H)-NaSO_3$], 479 [st $(OSO_3Na)(OSO_3^-)-NaHSO_4$].

Cholest-5-ene-3α,21-diyl sulfate [**3**] was obtained as a white powder: negative-ion lrfabms m/z 583 $[M-Na^+]^-$.

5α-Cholestane-2β,3α,26-triyl sulfate [**4**] was obtained as a white powder: negative-ion lrfabms m/z 703 $[M-Na^+]^-$.

Cholest-5-ene-2β,3α,26-triyl sulfate [**5**] was obtained as a white powder: negative-ion lrfabms m/z 701 $[M-Na^+]^-$.

PROTEIN TYROSINE KINASE ASSAY.—PTK $pp60^{v\text{-}src}$ was purified from Rous sarcoma virus-transformed cells and used to screen extracts, fractions and pure compounds as described previously (10).

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