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THE MARINE NATURAL PRODUCT, HALISTANOL TRISULFATE, INHIBITS pp60$^{v\text{-}src}$ PROTEIN TYROSINE KINASE ACTIVITY

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Summary: Halistanol trisulfate, a sulfated steroid derivative, was isolated from the extracts of two different marine sponges (genus Topsentia) by bioassay-guided fractionation. It exhibited an IC50 of approximately 4 μM against pp60$^{v\text{-}src}$, the oncogenic protein tyrosine kinase encoded by Rous sarcoma virus. Removing the sulfate groups by acid hydrolysis produced the inactive tris-alcohol, halistanol. The kinetics of inhibition were examined and revealed that halistanol trisulfate is a competitive inhibitor with respect to the peptide substrate, [val^5]-angiotensin II, and a mixed inhibitor with respect to ATP. A number of monosulfated steroids were studied for protein tyrosine kinase inhibitory activity, but were found to be inactive.

Several natural product inhibitors of protein tyrosine kinases have been described (1, 2), including erbstatin (3), herbimycin A (4), genistein (5), lavendustin A (6), staurosporine (7), desmal (8), melemeleone (9), and halenaquinone (10). Erbstatin has been reported to compete for the peptide substrate binding site on the epidermal growth factor receptor kinase (11), while herbimycin A covalently modifies a cysteine residue in pp60$^{v\text{-}src}$ (12). Michael addition is probably involved in the irreversible inhibition of pp60$^{v\text{-}src}$ by halenaquinone and related analogs (10, 13).

In a systematic screen for marine organisms producing protein tyrosine kinase inhibitors, a crude extract from a Topsentia sponge showed potent activity against pp60$^{v\text{-}src}$. The active component was isolated by bioassay-guided fractionation, and identified as halistanol trisulfate (compound 1 in Figure 1), a sulfated steroid first identified by Fusetani *et al.* in 1981 (14, 15). This compound had an IC50 of approximately 4 μM (2.7 μg/ml); acid hydrolysis to remove the sulfate groups produced the inactive triol, halistanol (compound 2). Seven synthetic monosulfated steroids

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were also tested, but did not inhibit pp60$^{v\text{-}src}$ at concentrations up to 200 μM. Kinetic analysis revealed that halistanol trisulfate competed for the peptide binding site on the enzyme.

Materials and Methods

Compounds: Using standard reversed phase HPLC methods, halistanol trisulfate (compound 1 in Figure 1) was isolated from two marine sponges (Topsentia species, order Halichondrida, family Halichondriidae, collection numbers 90007 and 90028, collected from Vanuatu). Its spectroscopic properties were consistent with those published previously (14). Acid hydrolysis of compound 1 yielded halistanol (compound 2), with properties in agreement with those reported by Fusetani *et al.* (14). The seven synthetic steroid sulfates shown in Figure 1 (compounds 3-9) were prepared and their structures confirmed at Syntex Discovery Research.

Enzyme Assay: pp60$^{v\text{-}src}$ was purified from Rous sarcoma virus-transformed rat cells and its protein tyrosine kinase activity assayed essentially as decribed by Wong and Goldberg (16). Test compounds were solubilized in DMSO and incubated with the enzyme for 15 min prior to the addition of [val^5]-angiotensin II (4 mg/ml final concentration, Sigma Chemical Company, St. Louis, MO) and ATP (30 μM final concentration, Sigma) to start the reaction. Approximately 1-2 μCi of γ[^{32}P]-ATP (New England Nuclear, Boston, MA) was used per reaction. The IC50 is defined as the concentration of compound which inhibits 50% of enzyme activity compared to solvent control. Kinetic analysis was performed by varying the concentrations of halistanol trisulfate, [val^5]-angiotensin II, and ATP. Lineweaver-Burke plots were analyzed by Dr. Seth Michelson, Syntex Discovery Research Biomathematics.

Results and Discussion

Halistanol trisulfate (1), halistanol (2), and seven synthetic sulfated steroids (3-9), whose structures are shown in Figure 1, were tested for their ability to inhibit pp60$^{v\text{-}src}$ at concentrations up to 200 μM. Only halistanol trisulfate was active with an IC50 of approximately 4 μM. The inhibition was shown to be competitive with respect to the peptide substrate, and mixed with respect to ATP (Figure 2).

The finding that halistanol trisulfate competes for [val^5]-angiotensin II binding may be related to the preference for a negatively charged amino acid at position -3 in peptide substrates for protein tyrosine kinases (17) and the predicted locations of positively charged amino acids in tyrosine kinase active sites based on modeling studies (18). We have recently identified other sulfated natural products as inhibitors of pp60$^{v\text{-}src}$ protein tyrosine kinase, including cycloartanol sulfates (19), malhamensilipin A (20), and halenaquinol sulfate (10). Not all sulfated lipids are inhibitors of the enzyme, however, as can be seen from the results with synthetic compounds tested in this study. Under our assay conditions, the well-known detergent, sodium dodecyl sulfate, was found to inhibit pp60$^{v\text{-}src}$ with an IC50 of approximately 43 μM (unpublished data).

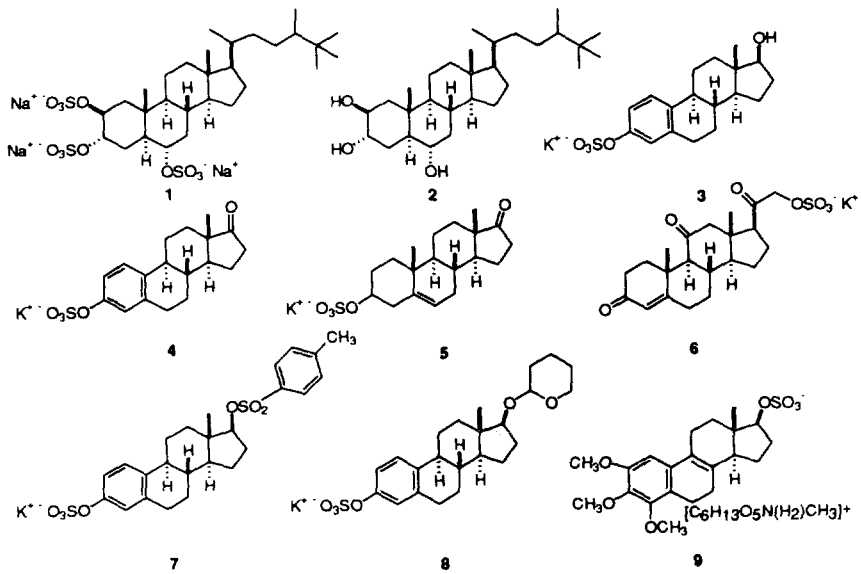
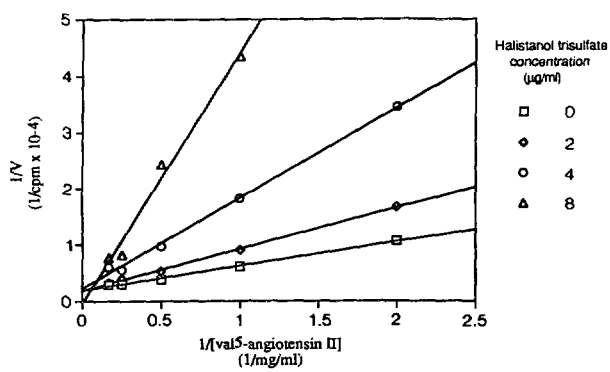


Figure 1. Structures of natural (1), semisynthetic (2), and synthetic (3-9) steroid derivatives tested for protein tyrosine kinase inhibitory activity.



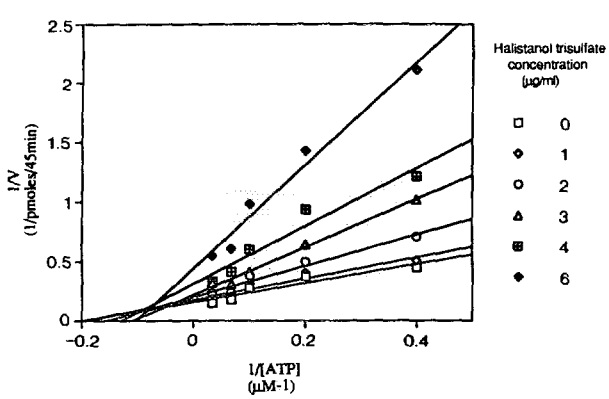


Figure 2. Inhibition kinetics for halistanol trisulfate.

Halistanol trisulfate was first isolated from a Halichondrid sponge (*Halichondria* cf. *moorei*) as a novel antimicrobial sulfated steroid (14). It was subsequently isolated from a Choristid sponge, Epipolasis (15) and another Halichondrid sponge, *Pseudoaxinissa digiitata* (21). This work expands the sources of this compound to two additional Halichondrid sponges.

A wide variety of biological effects have been observed with halistanol trisulfate. It has been suggested that it functioned as a detergent in depressing melanin synthesis in melanoma cells (22), and Moni *et al.* also reported that it exhibited hemolytic activity around its critical micelle concentration, 14.5 μM (23). Other related compounds (halistanol sulfates A-E) were described by Kanazawa *et al.* with antifungal and anti-thrombin activities (15), and Bifulco *et al.* concluded that halistanol sulfates F and G were cytoprotective against HIV (21). This report adds protein tyrosine kinase inhibition to the biological activities of this interesting class of sulfated natural products.

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