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p-Sulfooxyphenylpyruvic acid from the red macro alga *Ceratodictyon spongiosum* and its sponge symbiont *Haliclona cymaeformis*

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Abstract

Investigation of a Philippine specimen of the red alga *Ceratodictyon spongiosum* and its sponge symbiont *Haliclona cymaeformis* led to the isolation of *p*-sulfooxyphenylpyruvic acid, whose structure was elucidated using spectroscopic methods, with the *Z*-enol geometry determined through analysis of ^3J(C,H) coupling constants. The metabolite was tested for tyrosine kinase inhibition using a ^{3}H-thymidine incorporation assay, but was found inactive.

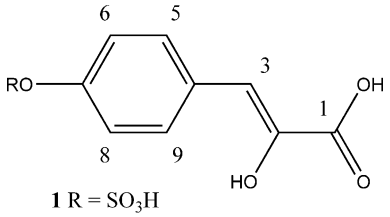
Keywords: *Ceratodictyon spongiosum*; Rhodymeniales; *Haliclona cymaeformis*; Haploslerida; gHSQMBC; ^3J(C,H) coupling constants

1. Introduction

In our continuing study of marine natural products, we investigated the red macro alga, *Ceratodictyon spongiosum* Zanardini (Rhodymeniales) and its sponge symbiont *Haliclona cymaeformis* (Esper) (Haplosclerida). To date, there have been only two reports (Tan et al., 2000; Lo et al., 2001) on the chemistry and associated bioactivity of *Ceratodictyon*/*Haliclona* metabolites, although these reports have indicated that metabolites from these organisms may be of pharmacological interest.

2. Results and discussion

The *Ceratodictyon*/*Haliclona* investigated was collected in the Philippines in 1999 from Mahatao, Batanes. The sample was extracted with MeOH and 1:1 MeOH/$CHCl_3$ followed by rotary evaporation and subsequent solvent partitioning between aqueous MeOH/hexanes and aqueous MeOH/$CHCl_3$. Column chromatography of the aqueous/MeOH soluble material on Sephadex LH-20 (70% MeOH, 30% H_2O) yielded *p*-sulfooxyphenylpyruvic acid (**1**), as well as a mixture of **1** and *p*-hydroxyphenylpyruvic acid (**2**).



1 R = SO_3H
2 R = H

Compound **1** was isolated as a white amorphous solid. Negative ion HRFABMS gave a molecular formula of $C_9H_7SO_7$ and negative ion ESIMS showed two peaks at *m*/*z* 259 $[M]^-$ and *m*/*z* 179 $[M-SO_3]^-$. The ^{1}H NMR spectrum showed three signals [δ 6.80 *dd* (2H), δ 7.09 *s* (1H), δ 7.78 *dd* (2H)] representing five protons. Integration and coupling constants supported a *para* substituted benzene ring with *ortho* coupling constants of 8.8 Hz. The ^{13}C NMR spectrum showed seven signals, two of which were double intensity (δ 134.2, δ 116.5), assigned to the four aromatic methines C-5, C-9 (δ 134.2) and C-6, C-8 (δ 116.5). The signal at δ 7.78

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(H-5) was shifted downfield relative to tyrosine (Pretsch et al., 1989) supporting a sulfated phenol. HMBC data showed correlations from the methine at δ 7.09 (H-3) to C-4, C-5, and C-9 which suggested that the methine was directly attached to the aromatic ring, leaving two carbons unassigned: one was a conjugated carboxylic acid (^{13}C NMR signal at δ 169.0, Pretsch et al., 1989) and the other (δ 137.9), C-2 must have an attached hydroxyl based on the molecular formula, hence the structure of *p*-sulfooxyphenylpyruvic acid (**1**). Interestingly, only NMR signals of the enol tautomers of **1** and **2** were observed, which is consistent with published studies on enol-keto tautomerism in phenylpyruvate derivatives (Hanai et al., 1989).

The geometry of the enol was determined by analysis of long-range coupling constants using gHSQMBC (Williamson et al., 2000). The ^3J(C,H) coupling constants between C-1 and H-3 of **1** and **2** (4.9 Hz and 7.9 Hz, respectively) are consistent with a *Z* olefin geometry (Breitmaier and Voelter, 1990). ^3J(C,H) values greater than 10 Hz would be expected if the compounds had *E* geometry (Pretsch et al., 1989; Chan et al., 1993).

A substructure query performed in the Chemical Abstracts database using STN online led to the report of cephamycin A and B (Albers-Schonberg et al., 1972). Cephamycin A has a *p*-sulfooxyphenylpyruvate incorporated into its structure while cephamycin B incorporates a *p*-hydroxyphenylpyruvate. The ^{1}H NMR data reported for the phenylpyruvate moiety of the cephamycins helped confirm an aromatic sulfate in **1**, as opposed to the enol sulfate. The ^{1}H chemical shifts of **1** were nearly identical to those reported for the corresponding methylenol ether obtained from hydrolysis of cephamycin A (Albers-Schonberg et al., 1972). Furthermore, the difference in ^{13}C and ^{1}H NMR shifts between **1** and **2** were mainly localized to the aromatic ring. The sulfate containing metabolite has also been identified in mammalian urine as a result of deamination of L-tyrosine *O*-sulphate (Hext et al., 1973).

The structure of **2** was elucidated from a mixture of **1** and **2** by comparing the NMR data to that reported for the methylenol ether derived from cephamycin B. Further purification of **2** was not pursued after failed attempts using reversed-phase HPLC.

Based on its structural similarity to tyrosine, we investigated **1** for tyrosine kinase inhibitory activity. A431 epidermoid carcinoma cells were chosen for the study because these cells express high levels of the epidermal growth factor receptor (EGFR) (Osherov and Levitzki, 1994), which renders them highly sensitive to EGF induced mitogenesis (Lipson et al., 1998). Tyrosine kinase inhibitors, such as tyrphostin AG1478 (Lipson et al., 1998), have been shown to block DNA synthesis in these cells after treatment with EGF. Since A431 cells are highly sensitive to EGF induced mitogenesis, the difference in ^{3}H-thymidine incorporation between cells treated with a tyrosine kinase inhibitor versus a negative control is profound and makes the assay highly sensitive to tyrosine kinase inhibitors. When tested for cytotoxic/cytostatic activity in the A431 cell line using an MTT assay (Carmichael et al., 1987), both **1** and the mixture of **1** and **2** exhibited minimal activity with an extrapolated IC_{50} greater than 1 mM (data not shown). To investigate possible tyrosine kinase inhibition, ^{3}H-thymidine incorporation was measured after treating A431 cells with **1** using DMSO as a negative control and tyrphostin AG1478 as a positive control. **1** showed only a minor decrease in ^{3}H-thymidine incorporation, whereas tyrphostin AG1478 showed 93% inhibition of ^{3}H-thymidine incorporation at 10 μM, relative to the DMSO control.

The isolation of the two pyruvic acid derivatives provides more insight about the alga and sponge symbiosis. The specimen studied and presented here showed different chemistry than previously described. No significant biological activity was associated with the pyruvic acid derivatives.

3. Experimental

3.1. General

^{1}H and ^{13}C experiments were performed with a Varian Mercury 400 MHz spectrometer in the indicated solvent at 25 °C. Spectra were referenced to residual undeuterated MeOD (3.30 ppm) or to the MeOD ^{13}C signal (49.0 ppm). UV spectra were obtained in MeOH on a Hewlett Packard 8452A diode array spectrophotometer. High- and low-resolution FAB MS measurements were recorded on a Finnegan MAT 95 high-resolution spectrometer. ESIMS were recorded in the negative ion mode on a Finnigan LCQDECA ion trap mass spectrometer.

3.2. Algal material

Ceratodictyon spongiosum/*Haliclona cymaeformis* (20 g) was collected by hand from Mahatao, Batanes, Philippines at a depth of 5–10 m. The specimen was identified by one of us (M.K.H.), and a voucher specimen (PBAT-99–1-11) is held at the University of Utah.

3.3. Extraction and isolation

The specimen was extracted three times with 100 ml methanol and subsequently extracted twice with 100 ml 1:1 $CHCl_3$/ MeOH. The combined extracts were evaporated and dried in vacuo to yield 690 mg of crude material. The crude material was dissolved in 50 ml 10% H_2O/MeOH and extracted with hexanes prior to dilution with water to 30% H_2O and subsequent extraction with $CHCl_3$. The aqueous methanol soluble

Table 1
^1H and ^{13}C NMR data (δ in ppm, J in Hz, 5% D_2O/CD_3OD) for **1** and **2**

Position	Compound **1**			Compound **2**		
	δ_C	δ_H	HMBC[a]	δ_C	δ_H	HMBC
1	169.0			168.0		
2	137.9			138.7		
3	128.8	7.09 (*s*)	1, 2, 4, 5	127.2	6.79 (*s*)	1, 2, 4, 5
4	125.8			124.8		
5, 9	134.2[b]	7.78 (*dd*, 8.8)	3	131.0[b]	7.27 (*d*, 8.6)	3
6, 8	116.5[b]	6.80 (*dd*, 8.8)	7, 4	115.1[b]	6.77 (*d*, 8.6)	4
7	159.8			156.2		

[a] The numbers are the carbons to which the proton had a correlation.
[b] These signals were doubled and represent two carbons each.

material (610 mg) was fractionated on Sephadex LH-20 (4 cm×101 cm) using 70:30 MeOH/H_2O as eluent. **2** was collected as a mixture of **1** and **2** (5.0 mg), pure **1** (2.0 mg) was collected following elution of the mixture.

3.4. *p*-Sulfooxyphenylpyruvic acid (*1*)

UV (MeOH) λ_{max} (log ε) 208 (3.6), 220 (3.6), 298 (3.8); ^1H (5% D_2O/CD_3OD, 400 MHz), ^{13}C (5% D_2O/CD_3OD, 100 MHz), and HMBC data: Table 1; negative ion HRFABMS m/z 258.9888 (calcd for $C_9H_7SO_7$, 258.9912).

3.5. *p*-Hydroxyphenylpyruvic acid (*2*)

^1H (5% D_2O/CD_3OD, 400 MHz), ^{13}C (5% D_2O/CD_3OD, 100 MHz), and HMBC data: Table 1.

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