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Bisabolane Type Sesquiterpenes from a Marine *Didiscus* Sponge

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Two bisabolane type sesquiterpene phenols, (+)-curcuphenol (**1**) and (+)-curcudiol (**2**), were isolated from a Philippine marine sponge, *Didiscus* sp., in addition to β-sitosterol (**3**) and phenethylamine (**4**). The structures of the metabolites were established on the basis of spectral evidence (1D- and 2D NMR, $[\alpha]_D$, EIMS). (+)-Curcuphenol (**1**) showed cytotoxicity, which is indicative of a p53 independent mechanism.

Key Words: Marine sponge, *Didiscus*, (+)-curcuphenol, (+)-curcudiol, β-sitosterol, phenethylamine, HCT-116.

Introduction

Cancer is a multistep process characterized by hyperproliferation and genetic alterations that drive the progressive transformation of normal cells into malignant derivatives. It is now widely accepted that tumorogenesis results from the accumulation of mutations in oncogenes and in both tumor supressor and apoptosis regulatory genes. These acquired mutations result in the loss of regulatory function and the inability to control proliferation and homeostasis.

Eukaryotic cell division is driven by a regulated series of events collectively defined as the cell cycle. Although progression through the individual phases of the cell cycle is driven by cyclin dependent kinases (CDKs), superimposed checkpoint controls provide the orderly succession of cell cycle events. The best-studied checkpoint regulator, the p53 tumor suppressor gene, is the most frequently mutated gene in human cancers[1]. Although a number of diverse genes that contain wild type p53-binding sequences are known,

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it seems that p21 ($p21^{Waf1/Cip1}$) protein is the key mediator of the ability of p53 to regulate these cell cycle checkpoints. In response to DNA damage, p53 induces the expression of several genes, including p21. Increased levels of p21 results in cell cycle arrest by inhibiting CDKs[2]. Mutations in either p53 or p21 can lead to loss of checkpoint control and genetic instability. In addition, many anticancer agents show decreased activity against tumor cells with p53 mutations[3]. Anticancer agents that work via a p53 independent mechanism should have greater clinical efficacy. Compounds that show no dependence on p53 while showing increased cytotoxicity in p21 deficient cells would be advantageous for the treatment of many types of cancer.

As part of our continuing search for biologically active natural products from marine organisms, we screened marine invertebrate extracts in a set of isogenic colorectal cancer cells: wild type human colon tumor [HCT-116, $p53^{+/+}$ and $p21^{+/+}$], p53-deficient ($p53^{-/-}$) or p21-deficient ($p21^{-/-}$) HCT-116 cell lines in which the p53 and p21 genes were individually disrupted through homologous recombination[4,5]. The crude MeOH extract of a marine sponge, *Didiscus* sp., collected from the Philippines showed some differential between the $p53^{+/+}$ and $p53^{-/-}$ HCT cell lines. Bioactivity-guided isolation carried out on the hexane and the $CHCl_3$ extracts of this sponge yielded two bisabolane type sesquiterpenes, (+)-curcuphenol (**1**) and (+)-curcudiol (**2**), and β-sitosterol (**3**). Phenethylamine (**4**) was also isolated and characterized from the remaining aqueous MeOH extract. This paper describes isolation and identification of compounds **1**-**4**. The cytotoxicity of **1** and **2** toward a panel of isogenic HCT-116 colon carcinoma cells is also discussed.

Experimental

General Procedures. Optical rotations were measured on a Jasco DIP-370 Digital Polarimeter. UV spectra were recorded in MeOH on a Hewlett-Packard 8452A diode array spectrophotometer. IR spectra were recorded using a Jasco FTIR-420 spectrophotometer (NaCl disc). NMR spectra were obtained on a Varian instrument operating at 500 MHz for ^1H and 125 MHz for ^{13}C NMR spectra. All NMR spectra were recorded at 26°C using the residual signal of $CDCl_3$ and CD_3OD, as internal reference. Mass spectra were taken on a Finnigan MAT 95 mass spectrometer. The NIST library for EIMS was used to compare the fragmentation pattern of the known compound **4**. Prediction of EIMS fragmentation pattern was made by the High Chem Mass Frontier program (version 2.0). SiO_2 used for flash chromatography (FC) was Merck Kieselgel 60, particle size 0.040-0.063 mm (Merck 230-400 mesh ASTM). C-18 material (J.T. Baker, 40 μm, 275 Å) was utilized for reversed phase FC. Sephadex LH-20 gel (25-200 μm bead size) was purchased from Sigma.

Animal material. *Didiscus* sp. (order Halichondrida, sample # PBat99-5-93) was collected from Batanes, Northern Philippines, in April 1999 and kept frozen until workup. Vouchers were deposited at both the University of Utah and University of the Philippines. The identification of the animal material was done by one of us (M.K.H.).

Extraction and Isolation. Thawed sponge material was soaked in MeOH for 24 h, and the solution decanted. This procedure was repeated two more times. The combined MeOH extracts were dried *in vacuo* and dissolved in 10% H_2O in MeOH (200 ml) before partitioning against hexane (3 x 200 ml). The water content of the MeOH phase was then adjusted to 30% by adding 80 ml water. This solution was finally partitioned against $CHCl_3$ (3 x 200 ml). Hexane-soluble material (367 mg) was subjected to SiO_2 FC using

hexane with increasing proportions of EtOAc. (+)-Curcuphenol (**1,** 203 mg), β-sitosterol (**3**, 38 mg) and (+)-curcudiol (**2**, 39 mg) were eluted with Hexane:EtOAc mixtures of 8:2, 6:4, and 5:5, respectively. An aliquot of the $CHCl_3$ extract (203 mg) was chromatographed using the same procedure as above to yield additional amounts of **1** (70 mg) and **2** (77 mg). The remaining aqueous MeOH extract (727 mg) was triturated with MeOH to remove salts and fractionated by C-18 FC using a multistep MeOH gradient (0-100% MeOH) in water (0.05% TFA). The fractions eluted with 20 and 40% MeOH were found to be of interest. These fractions were combined and further purified on a Sephadex LH-20 column, eluting with MeOH (0.1% TFA), to yield phenethylamine (**4**, 5.3 mg).

(+)-Curcuphenol (**1**, 273 mg): orange oil; $[\alpha]_D$: + 27.0° (*c*: 0.54, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 232 (2.94), 266 (2.92), 286 (2.93) nm; IR (film) ν_{max} 3465 (broad), 2923, 1619, 1583, 1516, 1453, 1418, 1287, 1216, 809 cm^{-1}; EIMS m/z 218 $[M]^+$ (28), 148 (31), 135 $[M\text{-}CH_2CH_2CH{=}C(CH_3)_2]^+$ (100), 95 (9), 55 (5); HREIMS m/z 218.1662 (calcd for $C_{15}H_{22}O$, 218.1670); ^1H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1.

Table 1. NMR data of **1** and **2** (^1H NMR: 500 MHz; ^{13}C NMR: 125 MHz; $CDCl_3$).

$^1H/^{13}C$	**1**			**2**	
	^1H NMR	^{13}C NMR	HMBC corr. (H to C)	^1H NMR	^{13}C NMR
1	1.79 (s)	25.6 (q)	2, 3, 4, 15	1.20 (s)	29.6 (q)
2		131.8 (s)			71.7 (s)
3	5.28 (m)	124.6 (d)	1, 4, 5, 15	1.46 (m) 1.54 (m)	43.3 (t)
4	2.09 (m, J = 7.0 Hz)	26.0 (t)	2, 3, 5, 6	1.30 (m)	22.0 (t)
5	1.75 (m)	37.2 (t)	3, 7	1.50 (m) 1.64 (m)	37.5 (t)
6	3.15 (m, J = 7.0 Hz)	31.3 (d)	4, 5, 7, 8, 12, 14	3.10 (m, J = 7.0 Hz)	31.1 (d)
7		130.1 (s)			130.6 (s)
8		152.8 (s)			153.1 (s)
9	6.68 (d, J = 0.8 Hz)	116.2 (d)	7, 8, 10, 11, 13	6.58 (br s)	116.3 (d)
10		136.4 (s)			136.3 (s)
11	6.85 (br d, J = 7.8 Hz)	121.7 (d)	7, 8, 9, 10, 12, 13	6.71 (br d, J = 7.8 Hz)	121.5 (d)
12	7.18 (d, J = 7.8 Hz)	126.8 (d)	6, 8, 9, 10, 11	7.03 (d, J = 7.8 Hz)	126.8 (d)
13	2.38 (s)	20.7 (q)	8, 9, 10, 11	2.25 (s)	20.9 (q)
14	1.36 (d, J = 7.0 Hz)	21.0 (q)	5, 6, 7	1.22 (d, J = 7.0 Hz)	21.0 (q)
15	1.68 (s)	17.5 (q)	1, 2, 3, 4	1.17 (s)	28.6 (q)

(+)-Curcudiol (**2**, 116 mg): yellow oil; $[\alpha]_D$: + 10.0° (*c*: 0.6, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 222 (3.5), 278 (3.2) nm; IR (film) ν_{max} 3366 (broad), 2925, 1456, 1420, 1289, 1231, 808 cm^{-1}; EIMS m/z 236 $[M]^+$ (< 1), 148 (47), 135 (100); HREIMS m/z 236.1766 (calcd for $C_{15}H_{24}O_2$, 236.1776); ^1H NMR (500 MHz, $CDCl_3$) see Table 1; ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1.

Bioassay. The $p53^{-/-}$, $p53^{+/+}$, $p21^{-/-}$, and $p21^{+/+}$ isogenic cell lines were obtained from Dr. Bert Vogelstein at John Hopkins University (USA). All cell culture reagents were purchased from GIBCO with the exception of the antibiotic/antimycotic (Sigma). The HCT cells were cultured in McCoy's 5A medium containing 10% fetal bovine serum, 1.0 mM sodium pyruvate, 2.0 mM L-glutamine, 40 units/ml penicillin, 40 μg/ml streptomycin, and 0.1 μg/ml amphotericin B. Cultures were incubated at 37°C in a 5% CO_2 atmosphere. Cells were washed with 2 ml Versene (1:5000) and harvested in 3 ml of a 0.25% trypsin 0.03%

EDTA solution. The trypsin was quenched by the addition of 12 ml of culture medium, and the cells pelleted by centrifugation for 5 min at room temperature. The pellet was resuspended in 15 ml of fresh medium and the cell concentration was determined using a Coulter counter. Cells were seeded in 96-well plates (3000 cells/well) in 200 μL of medium and allowed to adhere for 24 h. The medium was aspirated and replaced with 180 μL of fresh medium. Cells were treated with a 20 μL solution of compound dissolved in 10% DMSO in PBS. Plates were subsequently incubated for 72 h, the media aspirated, and 100 μL of fresh medium added. 11 μL of 2.5 mg/ml MTT (3,[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was added to assess cell viability. Plates were incubated with MTT for 4 h, the media aspirated, and 100 ml of DMSO added to each well. Plates were shaken to dissolve the reduced MTT and the absorbance measured at 570 nm using a multiscan plus 96-well plate reader. The percent survival in each well was determined by dividing the absorbance of the test well by the average absorbance in wells treated only with DMSO. All samples were tested in quadruplicate.

Results and Discussion

(+)-Curcuphenol (**1**) was isolated as an orange oil. The molecular formula $C_{15}H_{22}O$ was deduced by HREIMS (m/z 218.1662, $\Delta = 0.8$ mmU). The UV spectrum exhibited absorption bands at λ_{max} 232, 266 and 286 nm. The IR spectrum displayed bands characteristic for a hydroxyl group (3465 cm^{-1}), an olefinic bond (1619 cm^{-1}) and an aromatic ring (1583, 1453, 1418, 809 cm^{-1}). The ^{1}H NMR spectrum of **1** (Table 1) revealed the presence of four methyl groups; one secondary (δ 1.36, d, $J = 7.0$ Hz), two tertiary (δ 1.68 s, 1.79 s) and one aromatic (δ 2.38 s); an aromatic ABX system [δ 6.85, (br d, $J = 7.8$ Hz), 7.18 (d, $J = 7.8$ Hz), 6.68 (d, $J = 0.8$ Hz)], an olefinic proton (δ 5.28 m) and a benzylic methine (δ 3.15 m, $J = 7.0$ Hz). The ^{13}C NMR spectrum of **1** (Table 1) revealed 15 carbon signals. These data, together with the results of a DEPT-135 experiment, showed that **1** was a sesquiterpene composed of a trisubstituted benzene ring, a trisubstituted double bond, one methine, two methylenes, and four methyl groups. Since the aromatic ring and the olefinic bond accounted for all five degrees of unsaturation, **1** was inferred to be monocyclic. The complete NMR assignments were established by the combined analysis of gHSQC, gCOSY and gHMBC (Table 1) data. The gCOSY spectrum of **1** contained only two spin systems. The first spin network included the benzylic methine proton (H-6, δ 3.15), which shared a 7.0 Hz coupling with the secondary methyl group (H_3-14, δ 1.36). Sequential couplings were detected between H-6/H-5, H_2-5/H_2-4 and finally H_2-4 and the olefinic proton, H-3. This left C-2 without two bonding partners. The appearance of two olefinic methyl groups (H_3-1 and H_3-15) as singlets in the ^{1}H NMR spectrum, as well as the HMBC cross peaks observed from both methyls to C-2 (δ 131.8), C-3 (δ 124.6), C-4 (δ 26.0) and also with each other, proved that they were bonded to C-2. This completed the first structural fragment **a**, as shown in Figure 1. In the aromatic region, H-12 (δ 7.18 d, $J = 7.8$ Hz) *ortho* coupled to H-11 (δ 6.85 br d, $J = 7.8$ Hz), which in turn showed a weak *meta* coupling with H-9 (δ 6.68, d, $J = 0.8$ Hz), thus completing the second spin system. The substitution pattern of the trisubstituted aromatic ring was revealed by a gHMBC experiment ($J = 8.0$ Hz). Long range ^{1}H-^{13}C correlations were observed from the aromatic methyl group (H_3-13) to C-8, C-9, C-10, C-11 and C-12, indicating that H_3-13 was attached to C-10. Also the residence of the aromatic hydroxy function at C-8 (δ 152.8) was evident from HMBC cross peaks observed between H-9/C-7, H-9/C-8, H-9/C-10, H-9/C-11 as well as H-6/C-8 and H-12/C-8, giving the second structural fragment, **b**. Fragments **a** and **b** could be

connected through the HMBC correlations between H-6/C-7, H-6/C-8, H-6/C-12 and H_3-14/C-6 and H_3-14/C-7. Thus, the gross structure of **1** was determined to be curcuphenol[6]. The unambigious assignment of the relative stereochemistry of the only chiral center (C-6) within **1** was not possible by coupling constant analysis or a NOESY experiment. However, the sign of the optical rotation value ($[\alpha]_D$: + 27.0°) indicated that **1** possessed *S* configuration ($[\alpha]_D$ in ref. 7: + 24.6°, in ref. 8: + 29.1°). All these data were in a good agreement with those reported for (+)-curcuphenol[7,8].

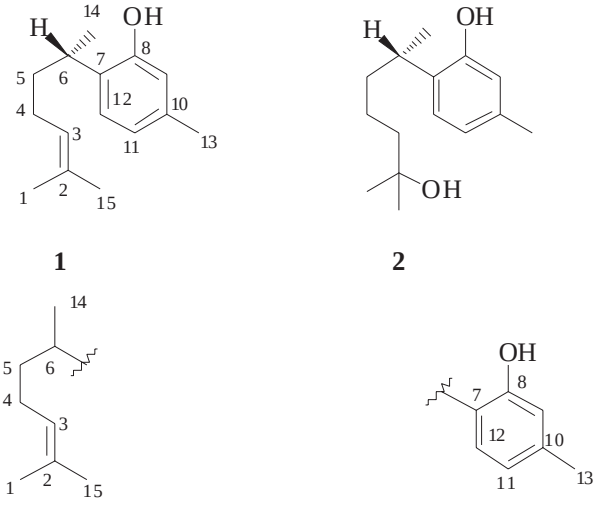

Figure 1. Structural fragments within compound **1**.

(+)-Curcudiol (**2**) was isolated as a yellow oil. High resolution EIMS of **2** gave the molecular formula of $C_{15}H_{24}O_2$ (m/z 236.1766, calcd 236.1778). The UV and IR data of **2** was very similar to those of **1** (see Experimental). The ^{1}H and ^{13}C NMR data indicated that **2** possessed the trisubstituted aromatic ring of (+)-curcuphenol (**1**). The only difference between these two compounds was confined to the end of the acyclic chain; the olefinic bond ($\Delta^{2(3)}$) was replaced by a methylene group (δ_H 1.46 and 1.54, δ_C 43.3 t) and a tertiary hydroxyl moiety (δ_C 71.7 s). As a result of this, two geminal methyl functions were shifted upfield (δ 1.17 s, H_3-15; 1.20 s, H_3-1) in comparison to **1**. The combination of these data with the cross peaks observed in the gCOSY (H_2-2/H_2-3) and gHMBC (H_3-1/C-2, H_3-15/C-2, H-3/C-2, H_3-1/H_3-15) spectra suggested **2** to be the hydration product of **1**. On the basis of the spectral data including the $[\alpha]_D$ value, and the comparison with the published data in the literature[8], **2** was identified as (+)-curcudiol.

Compound **3** was identified as β-sitosterol based on comparison of its HREIMS and NMR data with those given in the literature[9].

Compound **4** was obtained as a colorless glass. Its ^{1}H NMR spectrum contained a complex aromatic system (δ 7.25-7.36, 5H), and two triplets at δ 2.94 (2H) and δ 3.17 (2H). ^{13}C NMR data of **4** (δ_C 127.1 d, 128.6 (x2, d), 128.8 (x2, d), 136.7 s, 33.4 t, 40.8 t) clearly indicated the presence of a monosubstituted aromatic ring attached to an ethyl chain. The chemical shift of the latter CH_2 group (δ 40.8) was also indicative of a primary NH_2 function. The molecular weight (m/z 121 $[M]^+$, $C_8H_{11}N$) and the observance of the m/z 30 peak (CH_2NH_2) in the EI-mass spectrum suggested the residence of the NH_2 terminus on the

ethyl moiety. Thus, the structure of **4** was determined to be phenethylamine and confirmed by comparison of the EIMS fragmentation pattern with the NIST library of the known compound.

(+)-Curcuphenol (**1**) showed moderate activity against our panel of HCT-116 cells (Table 2). Interestingly, (+)-curcuphenol does not show a pattern indicative of a p53 dependant mechanism. Whereas, the etoposide control clearly shows a dependence on p53. The mechanism by which curcuphenol causes cell death is unknown and warrants further investigation.

Table 2. IC_{50} values (μg/ml) for **1** and **2** in isogenic HCT-116 cells.

Compound	$p53^{+/+}$	$p53^{-/-}$	$p21^{+/+}$	$p21^{-/-}$
(+)-Curcuphenol (**1**)	27	33	33	35
(+)-Curcudiol (**2**)	>50	>50	>50	>50
Etoposide	2	10	2	15

Conclusions

Curcuphenol and curcudiol are representatives of α-curcumene type monocyclic aromatic sesquiterpenes that are widely distributed in nature. (+)-Curcuphenol (**1**) and (+)-curcudiol (**2**) have thus far been isolated from three halichondrid sponge genera, *Didiscus*[8,10,11], *Myrmekioderma*[12], and *Epipolasis*[7]. However, the gorgonian *Pseudopterogorgia rigida*[6,13,14] and the terrestrial plant *Lasianthaeae podocephala*[15] contain (-)-curcuphenol and other similar sesquiterpenes with *R* stereochemistry at C-6. The volatile oil of the rhizomes of several *Curcuma* sp. have also been reported to contain curcuphenol[16−18], probably the *R* (-) enantiomer, as a minor constituent.

Several *Didiscus* species, such as *D. flavus*[8], *D. oxeata*[10,11] have yielded (+)-curcuphenol and (+)-curcudiol. We have observed that our specimens collected from different sites within the Philippines over the last decade are quite conservative, from a chemical point of view, and contain these two metabolites as major components. Therefore, (+)-curcuphenol and (+)-curcudiol may be considered as chemotaxonomic markers for the genus *Didiscus*, at least for those collected from the Philippines. We have confirmed the structures of these two compounds by advanced 2D NMR methods for the first time. Members of the genus *Didiscus* have also been reported to contain acyclic diterpenes[19] and 3,5-dibromo-2-methoxy-benzoic acid[20]. This is the first report of β-sitosterol (**3**) and phenethylamine (**4**) from this marine sponge genus.

Both (+)-curcuphenol (**1**) and (+)-curcudiol (**2**) have exhibited several biological activities such as the cytotoxic[8,10], antimicrobial[8,11], antifouling[12], ichtyotoxic[21], and stomachic[22]. (+)-Curcuphenol and its dehydro derivative inhibit the activity of gastric H,K-ATPase at subnanomolar concentrations[7]. For the first time we report the activity of (+)-curcuphenol on a panel of isogenic HCT-116 cells. It is worth noting that (+)-curcuphenol shows nearly identical activity in both $p53^{+/+}$ and $p53^{-/-}$ cell lines.

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