Metabolites with Cytotoxic Activity from the Formosan Soft Coral *Cladiella australis*

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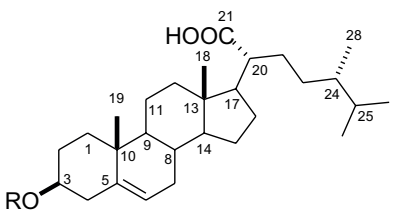
The metabolites, (24*S*)-3β-acetoxyergost-5-en-21-oic acid (**2**), 5′-*O*-acetylthymidine (**3**), 3′-*O*-acetylthymidine (**4**), and *p*-vinylbenzyl alcohol (**5**), along with a known steroid (**1**) were isolated from the EtOAc extract of the Formosan soft coral *Caldiella australis*. The structures of new metabolites were determined on the basis of spectroscopic (including 1D and 2D NMR) analyses and by comparison of their NMR spectral data with those of related compounds. Except for **3**, all compounds exhibited cytotoxic activity of various degrees of potency against a limited panel of human liver and breast cancer cell lines.

Keywords: *Cladiella australis*; Steroids; 5′-*O*-acetylthymidine; 3′-*O*-acetylthymidine; *p*-Vinylbenzyl alcohol; Cytotoxicity; Soft coral.

INTRODUCTION

In the course of our chemical study on the octocorals, several metabolites of versatile structures possessing cytotoxic activities have been isolated and identified.[1-6] Previous investigation on the EtOAc extract of *Cladiella australis* (Macfadyen, 1936) by our research group has resulted in the isolation of new eunicellin-based diterpenoids, australins A-D.[7] Further chromatographic purification for the rest of the fractions of the same extract has again led to the isolation of five metabolites including two steroids (**1** and **2**), two nucleosides (**3** and **4**), and *p*-hydroxymethylstyrene (**5**). Except for the known steroid **1**, the (24*S*)-3β-acetoxyergost-5-en-21-oic acid (**2**) was obtained as a new compound, while 5′-*O*-acetylthymidine (**3**), 3′-*O*-acetylthymidine (**4**), and *p*-vinylbenzyl alcohol (**5**) were found to be new natural products. The molecular structures of these metabolites were established on the basis of spectroscopic analyses and by comparison of their NMR spectral data with those of the related compounds. Metabolites **1**, **2**, **4**, and **5** also were found to exhibit cytotoxic activity of different degrees of potency against the cell lines of human hepatocellular carcinoma Hep G2 and Hep 3B, human breast carcinoma MCF-7 and MDA-MB-231.



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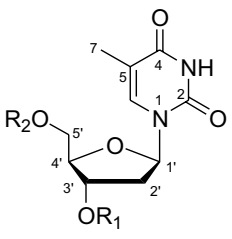
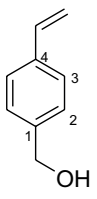


3: R_1 = H, R_2 = Ac
4: R_1 = Ac, R_2 = H



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RESULTS AND DISCUSSION

The organism was homogenized with EtOAc, filtered, and the organic layer was then concentrated under reduced pressure. The residue was fractionated using silica gel column chromatography and the selected fractions were further purified by open column chromatography on silica gel and/or normal phase HPLC to afford compounds **1**-**5** (see Experimental). The spectral data of **1** was found to be in full agreement with those reported previously.[8]

Metabolite **2** was isolated as a white solid and was found to possess a molecular formula $C_{30}H_{48}O_4$, as established by the HRFABMS *m/z* 473.3632 $(M + H)^+$ and NMR data (Table 1). The IR absorption bands at ν_{max} 3000-3800, 1699, and 1732 cm^{-1} revealed the presence of carboxyl and ester carbonyl functionalities, respectively. The ion peaks appearing in the FABMS at *m/z* 429 and 413 suggested the elimination of carbon dioxide and acetic acid from the molecule and suggested **2** as an acylated and a carboxylated compound. The ^{13}C NMR of **2** exhibited thirty carbon signals which were assigned, by the aid of DEPT spectra, into six methyls, ten methylene, eight sp^3 methines (including one oxygenated), one sp^2 methine, two sp^3 quaternary carbons, and three sp^2 quaternary carbons (including those of two carbonyls). The signals of two tertiary methyls (δ 0.74 and 1.00, each 3H, s) and the three secondary methyls (δ 0.85, 0.76, and 0.78, each 3H, d, J = 7.0 Hz) appeared in the ^1H NMR spectrum of **2** were assigned for H_3-18, H_3-19, H_3-26, H_3-27, and H_3-28 of a 24-methylsteroid,[8] respectively. A proton (1H, δ 2.20, ddd, J = 10.0, 10.0, 3.0 Hz) at-

Table 1. ^1H and ^{13}C NMR chemical shifts of **2** and **1**

	2[a]		**1**[b]	
Atom	δ_C	δ_H	δ_C	δ_H
1	37.0 (CH_2)[c]	1.82 m, 1.10 m	38.3 (CH_2)	1.69 m, 1.10 m
2	27.7 (CH_2)	1.84 m, 1.56 m	33.2 (CH_2)	2.07 m, 1.75 m
3	73.9 (CH)	4.60 m	71.7 (CH)	3.85 m
4	38.1 (CH_2)	2.32 m, 2.27 m	44.0 (CH_2)	2.64 2H, m
5	139.6 (C)		142.5 (C)	
6	122.5 (CH)	5.37 br d (5.0)[d]	121.7 (CH)	5.43 br d (4.0)
7	32.1 (CH_2)	1.98 dddd (17.5, 5.0, 5.0, 1.5), 1.54 m	32.7 (CH_2)	1.99 m, 1.61 m
8	31.8 (CH)	1.41 m	32.7 (CH)	1.51 dd (10.5, 5.5)
9	50.0 (CH)	0.95 dd (12.0, 5.0)	51.1 (CH)	0.99 m
10	36.5 (C)		37.4 (C)	
11	20.9 (CH_2)	1.43 m, 1.39 m	21.7 (CH_2)	1.44 m, 1.40 m
12	37.4 (CH_2)	1.70 d (13.0), 1.14 dd (13.0, 2.5)	38.5 (CH_2)	1.80 m, 1.13 m
13	41.9 (C)		42.9 (C)	
14	56.0 (CH)	1.03 dd (12.0, 4.0)	57.1 (CH)	1.07 m
15	23.6 (CH_2)	1.62 m, 1.11 m	24.5 (CH_2)	1.62 m, 1.12 m
16	27.1 (CH_2)	1.90 m, 1.30 m	28.1 (CH_2)	1.94 m, 1.39 m
17	52.5 (CH)	1.65 m	53.7 (CH)	1.94 m
18	12.0 (CH_3)	0.74 3H, s	12.7 (CH_3)	0.97 3H, s
19	19.2 (CH_3)	1.00 3H, s	20.1 (CH_3)	1.04 3H, s
20	47.4 (CH)	2.20 ddd (10.0, 10.0, 3.0)	49.4 (CH)	2.52 ddd (10.5, 10.5, 3.5)
21	181.7 (C)		179.9 (C)	
22	31.8 (CH_2)	1.55 m, 1.48 m	31.1 (CH_2)	1.77 m, 1.68 m
23	32.1 (CH_2)	1.58 m, 1.44 m	33.1 (CH_2)	1.67 m, 1.33 m
24	38.5 (CH)	1.28 m	39.4 (CH)	1.38 m
25	31.3 (CH)	1.53 m	32.0 (CH)	1.58 m
26	20.4 (CH_3)	0.85 3H, d (7.0)	21.1 (CH_3)	0.85 3H, d (7.0)
27	17.4 (CH_3)	0.76 3H, d (7.0)	17.8 (CH_3)	0.78 3H, d (7.0)
28	15.2 (CH_3)	0.78 3H, d (7.0)	15.8 (CH_3)	0.84 3H, d (7.0)
3-OAc	21.4 (CH_3)	2.04 3H, s		
	170.6 (C)			

Spectra recorded at 500 MHz for ^1H and 125 MHz for ^{13}C at 25 °C, [a] in $CDCl_3$ and [b] in C_5D_5N, [c] Attached protons were determined by DEPT experiments. [d] The J values are in Hz in parentheses.

tached to a methine carbon (δ 47.4, CH) showed ^{1}H-^{1}H COSY correlation with H-17 (1H, δ 1.65, m), and was assigned as H-20. The HMBC correlations (Fig. 1) observed from H-20 to the carboxy carbon (δ 181.7, C) and C-17 (δ 52.5, CH) suggested the location of carboxylic acid at C-20 of the side chain. Moreover, the ion peaks of the molecular fragments at *m/z* 341 [M - AcOH - C_5H_{11}]$^+$, 313 [M - AcOH - C_7H_{15}]$^+$, and 255 [M - AcOH - $C_9H_{17}O_2$]$^+$ also imply the presence of a carboxy group in a saturated side chain in the molecule of **2**. Comparison of the NMR spectral data of **2** with those of the known metabolite **1** (Table 1), which was also isolated from the same organism by us, suggested that **2** is the 3-*O*-acetyl derivative of **1**. This was further supported by the downfield shifts observed for H-3 (δ 4.60) and C-3 (δ 73.9) relative to those of **1**. The planar structure of **2**, including the positions of acetoxy group, carboxylate, and the olefinic double bond of this metabolite, could be further deduced from the detailed analyses of the ^{1}H-^{1}H COSY, HMQC, and HMBC spectral correlations (Fig. 1). Finally, the relative stereochemistry of **2** was established by the analysis of the NOE correlations in NOESY spectrum of **2**, as illustrated in Fig. 2. The *S*-configuration at C-24 was confirmed by the close chemical shifts of the side chain carbons in comparison with those of (24*S*)-3β-hydroxyergost-5-en-21-oic acid (**1**).[8] From the above findings the structure of **2** was thus determined as (24*S*)-3β-acetoxyergost-5-en-21-oic acid.

Metabolite **3** was obtained as a pale yellow gum. On the basis of its FABMS (*m/z* 285, [M+H]$^+$) and NMR spectral data (Table 2), the molecular formula of **3** was suggested as $C_{12}H_{16}O_6N_2$. The IR spectrum suggested the presence of hydroxy (ν_{max} 3445 cm^{-1}, broad) and carbonyl (ν_{max} 1710 and 1690 cm^{-1}) functionalities in **3**. The ion peaks appearing in the FABMS at *m/z* 267 ([M - H_2O + H]$^+$) and 207 ([M - H_2O - AcOH + H]$^+$) further confirmed the presence of hydroxy and acetoxy groups in **3**. The ^{1}H NMR spectrum of **3** exhibited three sharp singlets at δ 1.94 (3H), 7.28 (1H), and 8.55 (1H) which were found to be the signals of a methyl substituent at C-5, H-6, and the proton of the secondary amide of thymine moiety, respectively. Also, four sp^2 carbon signals appearing at δ 163.4 (C), 150.1 (C), 135.1 (CH), 111.1 (C), together with a carbon signal at δ 12.7 (CH_3), were thought to be attributable to a thymine base.[9,10] The other ten proton signals resonating at δ 2.17 and 2.44, 4.14, 4.30 and 4.39, 4.40, 6.28 (each 1H), and 2.13 (3H, s), were found by HMQC spectra to be correlated to the carbon signals at δ 40.4 (CH_2), 84.2 (CH), 63.7 (CH_2), 71.5 (CH), 85.2 (CH), and 20.9 (CH_3), respectively, of an acetylated deoxypentose. The above data, together with ^{1}H-^{1}H COSY, HMBC and NOESY correlations (see Figs. 3 and 4) further assigned **3** as an acetyl derivative of thymidine. The downfield shifts of the 5′-oxymethylene carbon and proton signals of **3** relative to those of normal thymidine[9,10] further confirmed the C-5′ location of the acetoxy group. Therefore, the structure of compound **3** was determined as 5′-*O*-acetylthymidine. It was found that although 5′-*O*-acetylthymidine (**3**) has been synthesized previously,[11] it was isolated for the first time from natural

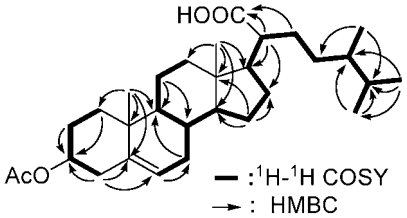

Fig. 1. ^{1}H-^{1}H COSY and HMBC correlations for **2**.

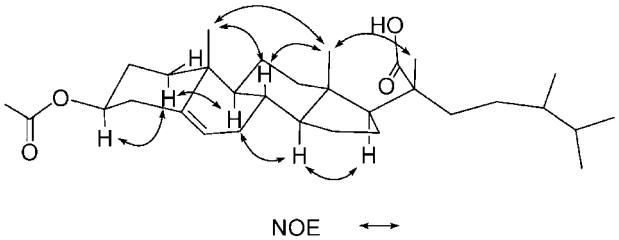

Fig. 2. Key NOESY correlations of **2**.

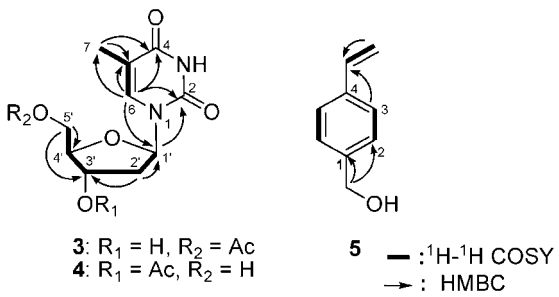

Fig. 3. ^{1}H-^{1}H COSY and HMBC correlations for **3**-**5**.

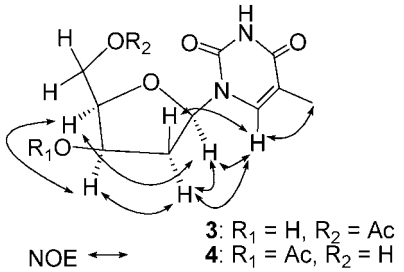

Fig. 4. Key NOESY correlations of **3** and **4**.

Table 2. ^{1}H and ^{13}C NMR chemical shifts of **3** and **4**

	3[a]		**4**[b]	
Atom	δ_C	δ_H	δ_C	δ_H
2	150.1 (C)[c]		150.2 (C)	
4	163.4 (C)		163.3 (C)	
5	111.1 (C)		111.4 (C)	
6	135.1 (CH)	7.28 s	136.2 (CH)	7.50 s
7	12.7 (CH_3)	1.94 3H, s	12.6 (CH_3)	1.93 3H, s
1′	85.2 (CH)	6.28 t (6.4)[d]	86.0 (CH)	6.25 t (6.0)
2′	40.4 (CH_2)	2.44 ddd (13.6, 6.4, 4.0), α	37.2 (CH_2)	2.42 m, α
		2.17 dd (13.6, 6.4), β		2.40 m, β
3′	71.5 (CH)	4.40 m	74.7 (CH)	5.35 m
4′	84.2 (CH)	4.14 dd (7.8, 4.2)	85.0 (CH)	4.10 m
5′	63.7 (CH_2)	4.39 dd (12.0, 4.2)	62.6 (CH_2)	3.94 dd (12.0, 2.5)
		4.30 dd (12.0, 4.2)		3.93 dd (12.0, 2.5)
3-OAc			21.0 (CH_3)	2.11 3H, s
			170.7 (C)	
5-OAc	20.9 (CH_3)	2.13 3H, s		
	170.7 (C)			
NH		8.55 s		8.23 s

Spectra recorded [a] at 400 MHz for ^{1}H and 100 MHz for ^{13}C and [b] at 500 MHz for ^{1}H and 125 MHz for ^{13}C, at 25 °C in $CDCl_3$, [c] Attached protons were determined by DEPT experiments. [d] The *J* values are in Hz in parentheses.

sources.

Metabolite **4** was also obtained as a pale yellow gum and showed similar FABMS and IR as those of **3**, suggesting that **4** is a geometric isomer of **3**. The NMR spectral data of **4** (Table 2) also exhibited the characteristic signals of thymine and acetylated 2-deoxyribose units as those found in **3**. The downfield shift observed for C-3′ (Δδ + 3.2 ppm) relative to that of **3**, which was long-range correlated with H_2-2′ and H_2-5′, clearly indicated the C-3′ location of the acetoxy group. The above observations combined with 2D-correlations revealed in the ^{1}H-^{1}H COSY, HMBC, and NOESY spectra (Figs. 3 and 4) established the structure of **4** as 3′-*O*-acetylthymidine. This is the first time that 3′-*O*-acetylthymidine has been obtained from a natural source, although it has been obtained previously by chemical synthesis.[12]

Metabolite **5** was obtained as a pale yellow oil. This compound was found to possess the molecular formula $C_9H_{10}O$ from the EIMS (*m/z* 134 $[M]^+$) and NMR spectral data (see experimental). The IR (ν_{max} 3443 cm^{-1}) indicated the existence of a hydroxy group. In the ^{1}H NMR spectrum, the signals appeared at δ 6.83 and 7.22 (each 2H, d, *J* = 8.8 Hz) assigned a *p*-substituted phenyl moiety. The *ABX* system shown by the olefinic protons at δ 6.49 (1H, dd, *J* =16.8, 9.6 Hz), 6.30 (1H, d, *J* =16.8 Hz), and 6.09 (1H, d, *J* = 9.6 Hz) together with a 2H singlet appearing at δ 4.17 indicated the presence of a vinyl and a hydroxymethyl, respectively, as the two *para*-substituents in the benzene ring. The above findings, together with ^{13}C NMR and 2D NMR analysis (Fig. 3), unambiguously established **5** as *p*-vinylbenzyl alcohol. Although **5** has been prepared previously by a chemical reaction,[13] our present study affords **5** for the first time from a natural source.

Evaluation of *in vitro* cytotoxic activity of metabolites **1**-**5** was carried out, and the results are represented in Table 3. Although **3** has been shown to be not cytotoxic, the other four metabolites were found to exhibit cytotoxic activities against the four cancer cell lines with different potencies. The known steroidal carboxylic acid **1** exhibited significant activity against the two liver cancer cell lines Hep G2 and Hep 3B (ED_{50}′s 2.2 and 2.8 μg/mL, respec-

Table 3. Cytotoxicity (IC_{50} μg/mL) of metabolites **1**-**5**

	Hep G2	Hep 3B	MCF-7	MDA-MB-231
1	2.2	2.8	14.5	13.3
2	8.6	3.9	29.0	26.4
3	-[a]	-	-	-
4	7.3	27.7	-	-
5	9.2	16.4	29.0	15.3

[a] IC_{50} > 30 μg/mL.

tively), while its acetate **2** also showed significant cytotoxicity against Hep 3B (ED_{50} 3.9 μg/mL) and moderate activity against Hep G2 (ED_{50} 8.6 μg/mL). The 3′-*O*-acetylatedthymidine (**4**) exhibited moderate activity against Hep G2 (ED_{50} 7.3 μg/mL) but was inactive against the breast cancer cells (MCF-7 and MDA-MB-231). Metabolite **5** also showed moderate activity against Hep G2 (ED_{50} 9.2 μg/mL). All of compounds **1**, **2**, and **5** showed only weak cytotoxicity against the breast cancer cell lines (ED_{50}′s 13.3-29.0 μg/mL).

EXPERIMENTAL SECTION

General Experimental Procedures

Melting points were determined using a Fisher-Johns melting point apparatus and were uncorrected. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. Ultraviolet spectra were recorded on a Hitachi U-3210 UV spectrophotometer, and IR spectra were recorded on a Jasco FT/IR-5300 infrared spectrophotometer. EIMS was obtained with a VG Quattro GC/MS spectrometer. FABMS and HRFABMS spectra were recorded on a Jeol JMS-700 mass spectrometer. The NMR spectra were recorded on a Bruker AMX-400 FT-NMR or on a Varian Unity INOVA 500 FT-NMR, in $CDCl_3$ using TMS as internal standard, unless otherwise indicated. Si gel (Merck, 230-400 mesh) was used for column chromatography. Precoated Si gel plates (Merck, Kieselgel 60 F-254, 0.2 mm) were used for analytical TLC.

Animal Material

The soft coral *C. australis* was collected at a depth of 15-20 m off the coast of the southernmost tip of Taiwan in 2001, Febrauary and freezed until use. A voucher specimen was deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

Extraction and Separation

The EtOAc extract of *C. australis* was prepared by exhaustive homogenization of the organism (1.6 Kg) with EOAc, which was filtered and concentrated under reduced pressure. The resulting residue (23.5 g) was fractionated as previously described,[7] using silica gel column chromatography and EtOAc-*n*-hexane (stepwise, 0-100% EtOAc) followed by MeOH-EtOAc (stepwise, 0-50% MeOH) as elution systems to yield 76 fractions. Fraction 42 eluted with EtOAc-*n*-hexane (1:3), was purified by normal phase HPLC, using EtOAc-*n*-hexane (1:4) to obtain **2** (3.5 mg). Fraction 54 eluted with EtOAc-*n*-hexane (2:3) was further purified by column chromatography over silica gel using EtOAc-*n*-hexane (1:3) to afford **1** (10 mg). Fraction 57 eluted with EtOAc-*n*-hexane (2:3) was purified by normal phase HPLC using EtOAc-*n*-hexane (1:3) to give **5** (2 mg). Fraction 71 eluted with EtOAc-*n*-hexane (7:3) was subjected to normal phase HPLC using acetone-*n*-hexane (gradient, 1:4 to 1:3) to give **4** (2.5 mg). The more polar fraction 75 eluted with pure EtOAc was further separated using normal phase HPLC using MeOH-EtOAc (1:99) to yield **3** (3 mg).

(24*S*)-3β-Hydroxyergost-5-en-21-oic acid (1)

White solid; mp 273-275 °C; $[\alpha]_D^{25}$ -27° (*c* 1.1, pyridine) (lit.,[9] -30°); IR (neat) ν_{max} 3399, 2955, 2935, 2870, 1699, 1684, 1541, 1458, 1385, 1232, 1196, 1044 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), see Table 1; FABMS *m/z* 453 (0.1, $[M + Na]^+$), 431 (0.4, $[M + H]^+$), 413 (0.7, $[M - H_2O + H]^+$), 366 (1.4, $[M - HCOOH - H_2O]^+$), 341 (2.6, $[M - H_2O - C_5O_{11}]^+$), 338 (2.1), 329 (0.7), 313 (1.6, $[M - H_2O - C_7O_{15}]^+$), 307 (5.7), 289 (3.4), 255 (8.5, $[M - H_2O - C_9H_{17}O_2]^+$, 242 (1.6). The above data were found to be in full agreement with those reported previously.[8]

(24*S*)-3β-Acetoxyergost-5-en-21-oic acid (2)

White solid; mp 213-215 °C; $[\alpha]_D^{25}$ -25° (*c* 0.6, pyridine); IR (neat) ν_{max} 3000-3800, 2951, 2934, 2844, 1732, 1699, 1647, 1539, 1456, 1385, 1283, 1037 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 1; FABMS *m/z* 495 (0.2, $[M + Na]^+$), 473 (0.3, $[M + H]^+$), 429 (0.4, $[M - CO_2 + H]^+$), 413 (9.2, $[M - AcOH + H]^+$), 399 (8.8), 381 (1.2), 367 (1.8, $[M - AcOH - HCOOH + H]^+$), 353 (1.7), 341 (1.8, $[M - AcOH - C_5O_{11}]^+$), 313 (1.8, $[M - AcOH - C_7O_{15}]^+$), 289 (2.3), 255 (8.5, $[M - AcOH - C_9H_{17}O_2]^+$ or/and $[M - CO_2 - AcOH - C_8H_{17}]^+$), 239 (2.4); HRFABMS *m/z* 473.3632 (calcd for $C_{30}H_{49}O_4$, 473.3633).

5′-*O*-Acetylthymidine (3)

Pale yellow gum; $[\alpha]_D^{25}$ -9° (*c* 0.6, $CHCl_3$); IR (neat) ν_{max} 3445, 2951, 2930, 2870, 1710, 1690, 1645, 1474, 1375, 1275, 1086, 1049 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 2; FABMS *m/z*

285 (3.5, $[M + H]^+$), 267 (4.3, $[M - H_2O + H]^+$), 207 (5.3, $[M - AcOH - H_2O + H]^+$), 154 (93.7), 136 (100), 107 (40.1).

3′-*O*-Acetylthymidine (4)

Pale yellow gum; $[\alpha]_D^{25}$ -21° (*c* 0.4, $CHCl_3$); IR (neat) ν_{max} 3443, 2951, 2930, 2870, 1710, 1690, 1647, 1474, 1375, 1244, 1096, 1049 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 2; FABMS *m/z* 307 (1.9, $[M + Na]^+$), 285 (2.0, $[M + H]^+$), 267 (2.6, $[M - H_2O + H]^+$), 207 (2.5, $[M - AcOH - H_2O + H]^+$), 154 (98.0), 136 (78.7), 107 (39.1).

***p*-Vinylbenzyl alcohol (5)**

Pale yellow oil; IR (neat) ν_{max} 3443 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), δ 7.22 (2H, d, *J* = 8.8 Hz, H-3,5), 6.83 (2H, d, *J* = 8.8 Hz, H-2,6), 6.49 (1H, dd, *J* = 16.8, 9.6 Hz, vinyl-H-2), 6.30 (1H, d, *J* = 16.8 Hz, vinyl-H-1_a), 6.09 (1H, d, *J* = 9.6 Hz, vinyl-H-1_b), 4.17 (2H, s) and ^{13}C NMR ($CDCl_3$, 100 MHz), δ 156.4 (C, C-4), 135.2 (CH, vinyl), 132.3 (2CH, C-2,6), 131.1 (CH_2, vinyl), 119.5 (C, C-1), 115.9 (2CH, C-3,5), 60.4 (CH_2, hydroxymethyl); EIMS *m/z* 134 (5.0, $[M]^+$), 107 (39.3, $[M - C_2H_3]^+$).

Cytotoxicity Testing

Cell lines were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assays of the test compounds **1**-**5** were performed using the MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[14,15]

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