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Isolation and structure of vanitaracin A, a novel anti-hepatitis B virus compound from *Talaromyces* sp.

Hiroki Matsunaga [a,†], Shinji Kamisuki [a,†], Manabu Kaneko [a,b], Yasuhiro Yamaguchi [a], Toshifumi Takeuchi [a], Koichi Watashi [a,b], Fumio Sugawara [a,*]

[a] Department of Applied Biological Science, Tokyo University of Sciences, Noda, Chiba 278-8510, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT

Two new tricyclic polyketides, vanitaracin A (**1**) and B (**2**), together with three novel compounds **3**, **4** and **5**, were isolated from a culture broth of a fungus, *Talaromyces* sp. The chemical structures of these compounds were determined from spectroscopic data (1D/2D NMR, MS and IR). The five isolated compounds were then tested for anti-hepatitis B virus (HBV) activity and vanitaracin A was found to exhibit an IC_{50} value of 10.5 μM using a HBV-susceptible cell line. By contrast, the derivative **2** displayed weak anti-HBV action, which suggested that the substituents at C-9 in **1** are likely to be important for its antiviral activity. We believe the two vanitaracin derivatives constitute a new class of anti-HBV agents.

Chronic infection of hepatitis B virus (HBV) is a common cause of liver diseases such as liver cirrhosis and hepatocellular carcinoma.[1] It is estimated that over 240 million people worldwide are chronically infected with HBV, of whom 780,000 people will eventually die of either liver disease or hepatocellular carcinoma each year.[2] Current therapies to combat chronic HBV infection include interferon treatment (interferon-α and pegylated-interferon-α), which primarily modulates immune response, and nucleos(t)ide analogs (Lamivudine, Adefovir, Entecavir, Telbivudine and Tenofovir), which inhibit the reverse transcriptase of HBV.[3,4] Although these drugs can deliver significant clinical improvement, there are serious problems associated with this treatment including the emergence of drug-resistant viruses and severe side effects.[5,6] Consequently, there is an urgent need to develop a novel class of anti-HBV agents with a different molecular target.

Bioactive products obtained from fungal metabolites have yielded some of the most important natural products for the pharmaceutical industry.[7] In the past, we have focused on fungi isolated from sand, seaweed, mosses and plants to obtain natural products to construct a library of compounds. Subsequent screening of our library has resulted in the discovery of several valuable bioactive compounds, including anti-hepatitis C virus agents.[8–11] Here, we describe the isolation, structural elucidation and anti-HBV activity of two new tricyclic polyketides; vanitaracin A (**1**) and B (**2**). During the course of this investigation we also identified three novel compounds **3**–**5**.

Repeated separation of a culture extract from a fungus, *Talaromyces* sp.,[12] using silica gel chromatography yielded compounds **1**–**5** (Fig. 1).[13] The molecular formula of $C_{25}H_{34}O_7$ for compound **1**[14] was determined by HRESIMS. The IR spectrum showed the presence of a hydroxy group (3409 cm^{-1}) and carbonyl groups (1735 cm^{-1} and 1693 cm^{-1}). The ^{13}C NMR spectrum revealed all twenty-five carbon atoms, comprising; three carbonyl carbons, seven quaternary carbons, five methine carbons, four methylene carbons and six methyl carbons (Table 1). ^{1}H NMR signals at δ 10.51 (1H, br s), δ 6.27 (1H, s) and δ 4.61 (1H, s) and HMQC spectrum indicated the presence of three hydroxy groups. The ^{13}C NMR spectrum and HMBC correlations of H-7 (δ 7.12) with C-5 (δ 128.9), C-6 (δ 160.3), C-8 (δ 146.4) and C-13 (δ 119.1) showed the presence of a tetrasubstituted phenol (Fig. 2). The phenol ring was found to be connected to a partial structure of $-CH_2-CH(OH)-CH_2-CH(CH_3)-$ established on the basis of ^{1}H–^{1}H COSY correlations from H-1 to H-4 and HMBC correlations from H_3-15 to C-3, C-4 and C-5. Furthermore, HMBC correlations from H_3-17 to two ketone carbons, C-10 (δ 204.5) and C-12 (δ 191.3), and C-11, and correlations from H_3-16 to C-8, C-9 and C-10 revealed the tricyclic core structure of **1** as shown in Figure 1. ^{1}H–^{1}H COSY correlations, and HMBC correlations from H_3-7′ to C-1′ (δ 174.4), C-2′ and C-3′ revealed that **1** possessed a 2,4-dimethylhexanoate unit. All

* Corresponding author. Tel.: +81 4 7124 1501; fax: 81 4 7123 9767.
E-mail address: sugawara@rs.noda.tus.ac.jp (F. Sugawara).
† These authors contributed equally to this work.

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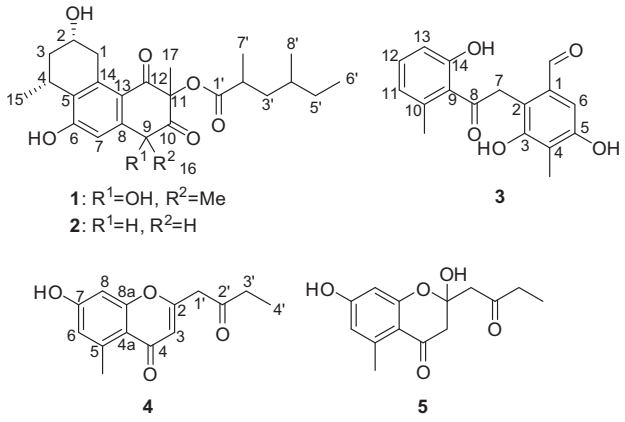

Figure 1. Structures of compounds **1–5**.

hydroxy proton signals could be assigned to 2-OH, 6-OH and 9-OH on the basis of HMBC correlations of these protons, which suggested that the 2,4-dimethylhexanoic acid unit was attached to the core structure of **1** at C-11. The *syn* relationship of OH-2/H_3-15 was deduced from the NOESY correlation between H-2 and H-4 (Fig. 2). Thus the structure of compound **1** was determined as shown in Figure 1, and named vanitaracin A. Efforts to determine the configurations at C-9, C-11, C-2′ and C-4′ are currently underway.

The molecular formula of $C_{24}H_{32}O_6$ for compound **2**[15] was determined by HRESIMS. The ^{1}H NMR spectrum suggested that the structure of compound **2** was similar to compound **1**, except

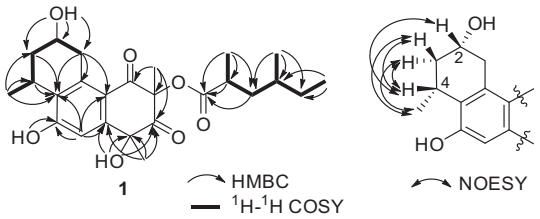

Figure 2. Key HMBC, ^{1}H–^{1}H COSY and NOESY correlations of compound **1**.

for and the presence of a methylene proton signal (δ 3.88) in **2** and the absence of methyl and hydroxy proton signals at C-9 in **1** (Table 1). The HMBC spectrum of **2** allowed us to assign the methylene proton signal to be H_2-9 on the basis of correlations from these methylene protons to C-8, C-10, C-11 and C-13. Thus, the structure of **2** (vanitaracin B) was elucidated (Fig. 1), and was further confirmed by ^{1}H–^{1}H COSY, HMQC and HMBC experiments. The *syn* relationship of OH-2/H_3-15 was determined from the NOESY correlation between H-2 and H-4.

The molecular formula of $C_{17}H_{16}O_5$ for compound **3**[16] was determined by HRESIMS. The IR spectrum showed the presence of a hydroxy group (3369 cm^{-1}) and carbonyl groups (1727 cm^{-1} and 1675 cm^{-1}). In the ^{1}H NMR, a methine proton signal at δ 10.07 (1H, s) indicated the presence of an aldehyde group (Table 2). The ^{13}C NMR spectrum revealed seventeen carbon signals ascribable to two carbonyl, twelve aromatic, one methylene and two methyl carbons. The ^{13}C NMR spectrum and HMBC correlations suggested that **3** had *m*-cresol and 3,5-dihydroxy-4-

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data for compounds **1** and **2**

Position	**1**[a]		**2**[b]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	36.6, CH_2	3.46, m	37.7, CH_2	3.61, dd (17.6, 3.5)
		2.57, m		2.94, dd (17.6, 7.1)
2	64.6, CH	3.88, m	66.8, CH	4.04, m
2-OH		4.61, s		
3	37.7, CH_2	1.99, m	38.3, CH_2	2.17, m
		1.51, ddd (13.1, 6.8, 6.8)		1.60, m
4	27.4, CH	3.10, ddq (6.8, 6.8, 6.8)	27.9, CH	3.16, m
5	128.9, C		129.1, C	
6	160.3, C		159.9, C	
6-OH		10.51, brs		
7	110.3, CH	7.12, s	112.4, CH	6.41, s
8	146.4, C		136.3, C	
9	74.5, C		42.8, CH_2	3.88, s
9-OH		6.27, s		
10	204.5, C		200.8, C	
11	84.1, C		85.1, C	
12	191.3, C		192.6, C	
13	119.1, C		121.0, C	
14	138.9, C		140.8, C	
15	22.2, CH_3	1.31, d (6.8)	22.2, CH_3	1.35, d (6.8)
16	32.4, CH_3	1.47, s		
17	22.9, CH_3	1.42, s	21.7, CH_3	1.55, s
1′	174.4, C		176.1, C	
2′	36.2, CH	2.61, m	36.4, CH	2.69, m
3′	40.5, CH_2	1.62, ddd (13.5, 9.4, 5.2)	40.7, CH_2	1.77, m
		1.10, m		1.13, m
4′	31.5, CH	1.44, m	31.8, CH	1.50, m
5′	29.2, CH_2	1.27, m	29.4, CH_2	1.32, m
		1.13, m		1.13, m
6′	11.2, CH_3	0.84, t (7.4)	11.1, CH_3	0.88, t (7.3)
7′	18.1, CH_3	1.15, d (6.9)	17.8, CH_3	1.22, d (7.0)
8′	19.1, CH_3	0.87, d (6.6)	19.1, CH_3	0.91, d (6.7)

[a] Recorded in DMSO-d_6.
[b] Recorded in $CDCl_3$.

Table 2
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data for compound **3** in acetone-d_6

Position	3	
	δ_C, type	δ_H (J in Hz)
1	138.2, C	
1-CHO	194.2, CH	10.07, s
2	112.3, C	
3	163.6, C	
4	118.0, C	
4-Me	6.4, CH_3	2.05, s
5	162.6, C	
6	110.9, CH	6.44, s
7	46.8, CH_2	4.51, s
8	203.2, C	
9	128.3, C	
10	136.4, C	
10-Me	18.6, CH_3	2.12, s
11	121.9, CH	6.83, d (7.8)
12	130.5, CH	7.16, dd (7.8, 7.8)
13	113.4, CH	6.73, d (7.8)
14	154.5, C	

Table 3
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectroscopic data for compounds **4** and **5**

Position	4[a]		5[b]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
2	164.2, C		100.3, C	
3	118.6, CH	6.01, s	48.2, CH_2	2.63, d (16.4)
				3.18, d (16.4)
4	181.8, C		190.1, C	
4a	115.4, C		114.2, C	
5	143.7, C		144.5, C	
5-Me	23.1, CH_3	2.61, s	23.0, CH_3	2.56, s
6	113.6, CH	6.58, s	113.1, CH	6.27, s
7	161.6, C		160.9, C	
8	101.2, CH	6.55, s	102.2, CH	6.12, s
8a	162.9, C		161.1, C	
1′	49.6, CH_2	3.82, s	48.8, CH_2	2.76, s
2′	206.8, C		212.0, C	
3′	36.7, CH_2	2.58, q (7.3)	38.3, CH_2	2.55, q (7.2)
4′	7.8, CH_3	1.10, t (7.3)	7.2, CH_3	1.10, t (7.2)

[a] Recorded in CD_3OD.
[b] Recorded in $CDCl_3$.

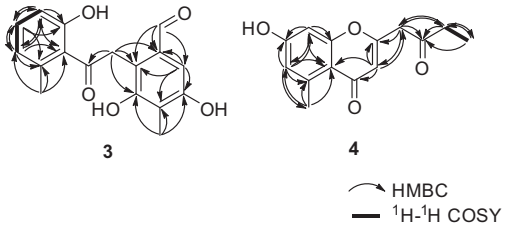

Figure 3. Key HMBC correlations of compounds **3** and **4**.

Table 4
Anti-HBV activity of compounds **1**–**5**

Compounds	IC_{50} (μM)[a]
1	10.5 ± 0.6
2	91.2 ± 34.5
3	51.4 ± 15.4
4	72.4 ± 70.5
5	52.1 ± 25.8
PreS1 peptide[b]	0.00083 ± 0.00048

[a] All values are the mean ± SD (n = 3 or 6).
[b] PreS1 peptide was used as a positive control.

methylbenzaldehyde moieties (Fig. 3). On the basis of HMBC correlations from H_2-7 to C-1, C-2, C-3 and a ketone carbon (C-8, δ 203.2), the structure of compound **3** was determined to be 3,5-dihydroxy-2-(2-(2-hydroxy-6-methylphenyl)-2-oxoethyl)-4-methylbenzaldehyde (Fig. 1).

The molecular formula of $C_{14}H_{14}O_4$ for compound **4**[17] was determined by HRESIMS. The IR spectrum showed the presence of a hydroxy group (3401 cm^{-1}) and carbonyl groups (1720 cm^{-1} and 1650 cm^{-1}). The ^{13}C NMR spectrum revealed all fourteen carbon atoms, comprising two ketone carbons, eight aromatic or olefinic carbons, two sp^3 methylene and two methyl carbons (Table 3). The HMBC correlations from H_3-5 to C-4a, C-5, C-6, from H-3 to C-2 (δ 164.2), C-4 (δ 181.8) and C-4a, and from H-8 to C-4a, C-6, C-7 and C-8a, indicated that 7-hydroxy-5-methyl-4*H*-chromen-4-one was the partial structure (Fig. 3). The ^{1}H–^{1}H COSY and HMBC correlations established the structure of a 2-butanone unit, which was connected at C-2 of the 7-hydroxy-5-methyl-4*H*-chromen-4-one moiety on the basis of HMBC correlations from H-1′ to C-2 and C-3. Thus the structure of compound **4** was determined to be 7-hydroxy-5-methyl-2-(2-oxobutyl)-4*H*-chromen-4-one (Fig. 1).

The molecular formula of $C_{14}H_{16}O_5$ for compound **5**[18] was determined by HRESIMS. Compound **5** was found to possess an additional oxygen atom and two additional hydrogen atoms compared with that of **4**. The ^{1}H NMR spectrum of compound **5** was similar to that of **4**, except for the presence of two aliphatic protons at H_2-3 (δ 2.63 and δ 3.18) in **5** and the absence of the olefinic proton in **4** (Table 3). The location of the aliphatic protons (H_2-3) was determined by HMBC correlations from H_2-3 to C-1′, C-2, C-4 and C-4a. In the ^{13}C NMR spectrum, a quaternary carbon at δ 100.3 indicated the presence of a hemiketal moiety. Therefore, the structure of compound **5** was determined to be 2,7-dihydroxy-5-methyl-2-(2-oxobutyl)chroman-4-one (Fig. 1).

Compounds **1**–**5** were evaluated for their anti-HBV activity using HBV-susceptible HepG2-hNTCP-C4 cells according to a procedure described previously.[19,20] The corresponding IC_{50} values for all five compounds are shown in Table 4. All compounds showed >90% cell viabilities at all concentrations tested. Of these compounds, **1** exhibited the strongest anti-HBV activity with an IC_{50} value of 10.5 μM. Intriguingly, the anti-HBV activity of compound **2**, which lacks methyl and hydroxy groups at C-9, was 10-fold weaker than that of **1**. These observations suggest the methyl and hydroxy substituents at C-9 of **1** are likely to be important for its anti-HBV activity. Weak anti-HBV activity was also displayed by compounds **3** and **5**.

In conclusion, two new tricyclic polyketides, vanitaracin A (**1**) and B (**2**), together with novel compounds, 3,5-dihydroxy-2-(2-(2-hydroxy-6-methylphenyl)-2-oxoethyl)-4-methylbenzaldehyde (**3**), 7-hydroxy-5-methyl-2-(2-oxobutyl)-4*H*-chromen-4-one (**4**), and 2,7-dihydroxy-5-methyl-2-(2-oxobutyl)chroman-4-one (**5**) were isolated from a fungal culture broth of *Talaromyces* sp. The anti-HBV activities of the isolated metabolites were evaluated, and vanitaracin A was found to be the most potent of the tested compounds. Dothideomycetide A that has a similar tricyclic structure to vanitaracins exhibited cytotoxicity against cancer cell lines and antibacterial activity.[21] To our knowledge, there are no reports on anti-HBV activity of the related compounds to vanitaracins. We believe the vanitaracin derivatives constitute a new class of anti-HBV agents.

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12. *Isolation of fungi:* Sand was collected in Kasai, Tokyo, Japan and suspended in sterilized H_2O. The suspension was spread on potato dextrose agar (PDA) plates, and the plates were incubated for 2–3 days at 37 °C. Fungi growing on this plate were transferred onto individual PDA plates and cultured under the same conditions. Cultures were repeated 2–5 times to obtain pure mycelium strains. The fungus producing the new compounds reported in this paper was identified as *Talaromyce* sp. based on its 5.8S rDNA sequence (Techno Suruga Laboratory Co., Ltd, Shizuoka, Japan).
13. *Purification of compounds:* The isolated fungal strain was cultured by transferring a small piece of agar from the cultured plate into four 2-L Erlenmeyer flasks containing potato dextrose broth (24 g) (Difco, Franklin Lakes, NJ) in H_2O (1 L). The culture (24 L) was grown under static conditions at room temperature in the dark for 39 days. The culture was then filtered through sterile cheesecloth to remove fungal mycelia and the resultant filtrate extracted using CH_2Cl_2. The organic layer was evaporated in vacuo to obtain a crude extract (387.9 mg). This crude extract was separated by silica gel column chromatography with $CHCl_3$–MeOH (10:0–10:1) to give fractions 1–7. Fraction 6 was subjected to silica gel column chromatography with toluene–EtOAc (9:1–0:1) to give compound **1** (19.2 mg) as a pale yellow oil. Fraction 4 was subjected to silica gel column chromatography with toluene–EtOAc (20:1–9:1) to give fractions 4-1–4-4. Fraction 4-2 was subjected to silica gel column chromatography with toluene–EtOAc (9:1–4:1) to give compound **3** (2.8 mg) as a white powder and compound **5** (2.4 mg) as a brown oil. Fraction 4-3 was subjected to silica gel column chromatography with hexane–EtOAc (9:1–0:1) to give compound **2** (18.6 mg) as a pale yellow oil. Fraction 4-4 was subjected to silica gel column chromatography with hexane–EtOAc (9:1–3:1) to give compound **4** (3.0 mg) as a pale brown powder.
14. *Vanitaracin A* (**1**) pale yellow oil; $[\alpha]_D^{23}$ −96.1 (c 0.96, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 290 (4.95) nm; IR (film) ν_{max} 3409, 2960, 2931, 1735, 1693, 1583, 1461, 1294, 1099, 1041 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HRESIMS *m*/*z* 469.2188 $[M+Na]^+$ (calcd for $C_{25}H_{34}O_7Na$, 469.2196).
15. *Vanitaracin B* (**2**) pale yellow oil; $[\alpha]_D^{23}$ +40.7 (*c* 0.46, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 288 (4.78) nm; IR (film) ν_{max} 3419, 2962, 2933, 2875, 1729, 1676, 1583, 1461, 1295, 1086 cm^{-1}; ^1H and ^{13}C NMR see Table 1; HRESIMS *m*/*z* 439.2101 $[M+Na]^+$ (calcd for $C_{24}H_{32}O_6Na$, 439.2091).
16. *3,5-Dihydroxy-2-(2-(2-hydroxy-6-methylphenyl)-2-oxoethyl)-4-methylbenzaldehyde* (**3**) white powder; UV (MeOH) λ_{max} (log ε) 294 (4.96) nm; IR (film) ν_{max} 3369, 2958, 2927, 1727, 1675, 1619, 1581, 1461, 1295, 1251, 1160, 1122, 1093 cm^{-1}; ^1H and ^{13}C NMR see Table 2; HRESIMS *m*/*z* 323.0888 $[M+Na]^+$ (calcd for $C_{17}H_{16}O_5Na$, 323.0889).
17. *7-Hydroxy-5-methyl-2-(2-oxobutyl)-4H-chromen-4-one* (**4**) pale brown powder; UV (MeOH) λ_{max} (log ε) 293 (4.76) nm; IR (film) ν_{max} 3401, 2977, 2931, 1720, 1650, 1604, 1581, 1457, 1388, 1357, 1272, 1157 cm^{-1}; ^1H and ^{13}C NMR see Table 3; HRESIMS *m*/*z* 269.0782 $[M+Na]^+$ (calcd for $C_{14}H_{14}O_4Na$, 269.0784).
18. *2,7-Dihydroxy-5-methyl-2-(2-oxobutyl)chroman-4-one* (**5**) brown oil; UV (MeOH) λ_{max} (log ε) 278 (4.63) nm; IR (film) ν_{max} 3519, 2971, 2929, 2337, 1702, 1658, 1608, 1581, 1461, 1313, 1286, 1157 cm^{-1}; ^1H and ^{13}C NMR see Table 3; HRESIMS *m*/*z* 287.0883 $[M+Na]^+$ (calcd for $C_{14}H_{16}O_5Na$, 287.0889).
19. Anti-HBV activity was measured as described previously.[20] In brief, HepG2-hNTCP-C4 cells were infected with HBV at 12,000 genome equivalent (GEq)/cell in the presence of 4% PEG8000 and the tested compounds for 16 h. After washing, cells were cultured in normal growth medium without compounds for 12 days. The viral envelope surface protein (HBs) secreted into the medium was quantified by ELISA, and cell viability was simultaneously measured by MTT assay. Normalized infectivity was calculated as HBV infectivity divided by cell viability. PreS1 peptide[22] was used as a positive control.
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