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NOTE

New steroidal compounds from an actinomycete strain, *Lechevalieria aerocolonigenes* K10-0216

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Secondary metabolites of actinomycetes represent important and major sources of new natural products. Since streptomycin was discovered by Selman Waksman, a large number of biological compounds, in many research areas, such as medicine, organic chemistry and cell biology, have been isolated from cultured broths of actinomycetes.[1–4] More than 22 000 bioactive compounds have been discovered from secondary metabolites of microorganisms, such as actinomycetes, fungi and bacteria. Of those, ~40% arose from secondary metabolites of actinomycetes.[2–4] The main strategy in discovering new compounds involves biological screening, in which guided assays are used for the isolation of bioactive compounds. However, the discovery of new compounds from microbial cultured broths suffers from duplication of isolated compounds. As the rate of discovery of completely novel natural products has slowed, new approaches are continually being sought.

The physicochemical (PC) properties of compounds, such as UV–visible absorption spectrum, MW and molecular formula, can be detected by LC/UV and LC/MS analyses of microbial cultured broths. PC screening of cultured broths uses advanced analytical technology to swiftly avoid chemical duplicates. Our ongoing PC screening program has recently identified trehangelins[5] and mangromicins[6,7] as new compounds, isolated from broths of rare actinomycetes, *Polymorphospora rubra* K07-0510 and *Lechevalieria aerocolonigenes* K10-0216, respectively. Moreover, PC screening identified two new compounds, K10-0216 KA (**1**) and KB (**2**), from same broth of *L. aerocolonigenes* K10-0216 that produced mangromicins. This paper describes the fermentation, isolation, structure determination and brief biological activity of **1** and **2**.

A loop of spores of strain K10-0216 was inoculated into 100 ml of seed medium consisting of 2.4% starch, 0.1% glucose, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract and 0.4% $CaCO_3$ (adjusted to pH 7.0 before sterilization) in a 500-ml Erlenmeyer flask. The flask was incubated on a rotary shaker (210 r.p.m.) at 27 °C for 3 days. A 1-ml portion of the seed culture was transferred to 500-ml Erlenmeyer flasks (total 16 l), each containing 100 ml of production medium consisting of 2.0% soluble starch, 0.5% glycerol, 1.0% defatted wheat germ, 0.3% Ehlrich meat extract from *Katsuwonus pelamis* (Kyokuto Pharmaceutical Inc., Tokyo, Japan) and 0.3% dry yeast, 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization) and fermentation was carried out on a rotary shaker (210 r.p.m.) at 27 °C for 7 days. The whole culture broth (16 l) was centrifuged to separate mycelium and supernatant. The supernatant was passed through a column of Diaion HP-20 (75 × 200 mm, Nihon Rensui Co, Tokyo, Japan) previously equilibrated with water. After washing with water, the fraction containing **1** was eluted with 40% MeOH. The fraction containing **2** was then eluted with 100% MeOH. The 40% MeOH fraction and 100% MeOH fraction were concentrated *in vacuo* to dryness to yield 150 and 611 mg of dry extract, respectively. The 40% fraction (150 mg) was applied on an ODS column (20 × 150 mm, Senshu Scientific Co, Tokyo, Japan) previously equilibrated with water. After washing with water and 40% MeOH, the fraction containing **1** was eluted with 60% MeOH. The 60% MeOH fraction was concentrated to yield 46.2 mg. The fraction was dissolved in a small amount of MeOH and purified by HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm, GL sciences Inc., Tokyo, Japan) with 18% acetonitrile at 6 ml min^{-1} detected at UV 254 nm. The yield of **1** was 3.0 mg. (Supplementary Scheme S1).

The 100% fraction (611 mg) was applied on an ODS column (20 × 150 mm, Senshu Scientific Co.) previously equilibrated with water. After washing with 80% MeOH, the fraction containing **2** was eluted with 100% MeOH. The 100% MeOH fraction was concentrated to yield 44.2 mg. The fraction was dissolved in a small amount of MeOH and purified with HPLC on an Inertsil ODS-4 column (10 i.d. × 250 mm, GL sciences Inc.) with 20% acetonitrile. The yield of **2** was 4.3 mg (Supplementary Scheme S1).

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Compounds **1** and **2** are both readily soluble in MeOH and showed absorption maxima at 237 and 240 nm in UV spectra, respectively. The IR absorption at ~3400 and 1680 cm^{-1} in both compounds suggested the presence of hydroxyl and carbonyl groups. The physicochemical properties are similar in both compounds (Supplementary Table S1).

Compound **1** was obtained as a pale-yellow powder determined to have the molecular formula of $C_{19}H_{26}O_4$ by HRESIMS $[M+H]^+$ *m/z* 319.1903 (calcd. for $C_{19}H_{27}O_4$, 319.1909), requiring seven unsaturation degrees. The 1D and 2D NMR spectra of **1** were measured in CD_3OD. The ^1H NMR data of **1** indicated the presence of three oxygenated sp^3 methines, one olefinic proton, six methylenes and two tertiary methyls. The ^{13}C NMR spectrum showed the resonances of 19 carbons, which were classified into four olefinic carbons at δ_c 121.4, 130.9, 132.8 and 169.4, one carbonyl carbon at δ_c 199.9, three oxygenated sp^3 methine carbons at δ_c 74.6, 78.4 and 81.3, one sp^3 methine carbon, two sp^3 quaternary carbons, six sp^3 methylene carbons and two methyl carbons with HSQC spectra (Supplementary Table S2). One carbonyl group and two olefin groups accounted for three of the seven unsaturation degrees, indicating the existence of four rings in **1**. The ^1H-^1H COSY indicated the presence of four partial structures: a, C-1/C-2; b, C-4/C-7; c, C-11/C-12; and d, C-14/C-17 (Figure 1a).

The HMBC correlations from H-1 to C-2, C-3, C-5, C-10 and C-10-Me; from H-2 to C-3; from H-4 to C-2, C-6 and C-10 revealed the presence of α,β-unsaturated cyclohexanone moiety. The HMBC correlations from H_2-6 to C-4, C-5, C-7, C-8 and C-10; from H_2-7 to C-5, C-6, C-8 and C-9; from H_3-10-Me to C-1, C-5, C-9 and C-10 indicated the presence of a decaline moiety, including partial structures a and b, in **1**. Finally, the planar structure of **1** was elucidated as shown in Figure 1a by the HMBC correlations from H_2-11 to C-10 and C-13; from H_2-12 to C-9, C-11, C-13, C-14 and C-13-Me, including partial structures c, from H-14 to C-8; from H_2-15 to C-8, C-13, C-14, C-16 and C-17; from H_2-16 to C-13, C-15 and C-17; from H-17 to C-12, C-16 and C-13-Me, including partial structures d, from H_3-13-Me to C-12, C-13, C-14 and C-17.

The relative configuration of **1** was estimated using ROESY experimentation (Figure 1b). The ROESY correlations were observed between H-1/H-2, H-1/H-11a, H-1/H_3-10-Me, H-2/H_3-10-Me, H-4/H6b, H-6a/H_3-10-Me, H-7a/H-15a, H-11a/H_3-10-Me, H-11a/H_3-13-Me, H-12b/H-14, H-12b/H-17, H-14/H-17 and H-15a/H_3-13-Me. These results indicate that H-1, H-2, H_3-10-Me and H_3-13-Me are located on the identical face. Therefore, the relative configuration of **1** was proposed as *1R**, *2R**, *10S**, *13S**, *14S** and *17S**, and a 14αH steroid skeletal compound (Figure 2).

Compound **2** was obtained as a pale-yellow powder determined to have the molecular formula of $C_{19}H_{24}O_4$ by HRESIMS $[M+H]^+$ *m/z* 317.1708 (calcd. for $C_{19}H_{25}O_4$, 317.1735). The 1D and 2D NMR spectra of **2** were measured in CD_3OD. From a comparison of chemical shifts in the ^1H and ^{13}C NMR of **2** with those of **1**, the signals of a carbonyl group (17-C; δ_C 222.7) were observed in **2** instead of those of the hydroxy group (17-CH; δ_H 3.68/δ_C 81.3) in **1** (Supplementary Table S2). Analysis of HMBC data confirmed the presence of a cyclopentanone, based on correlations from H_2-15 to C-17; from H_2-16 to C-14 and C-17; and from H_3-13-Me to C-14.

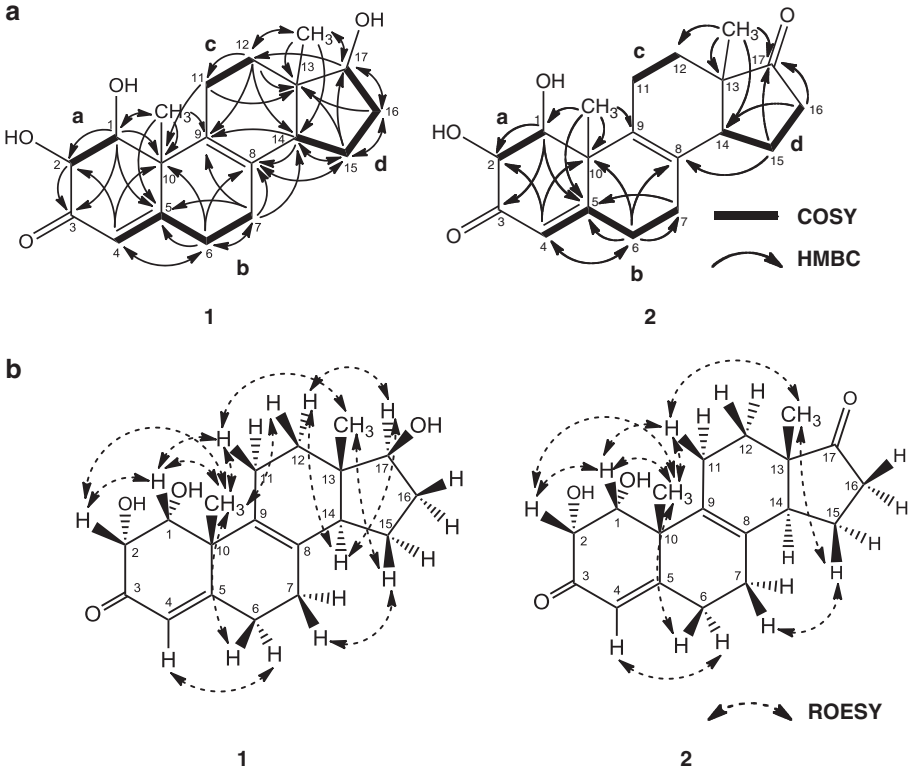
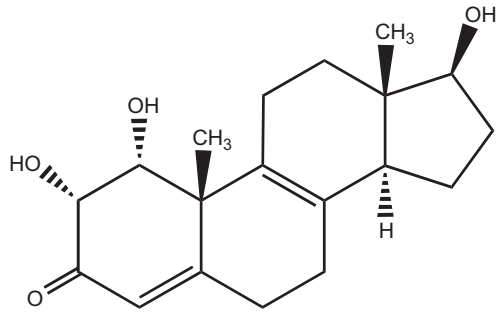


Figure 1 (**a**) ^1H-^1H COSY (bold) and selected HMBC (arrow) correlations of K10-0216 KA (**1**) and KB (**2**). (**b**) Key ROESY correlations (arrow dot) of K10-0216 KA (**1**) and KB (**2**).



1

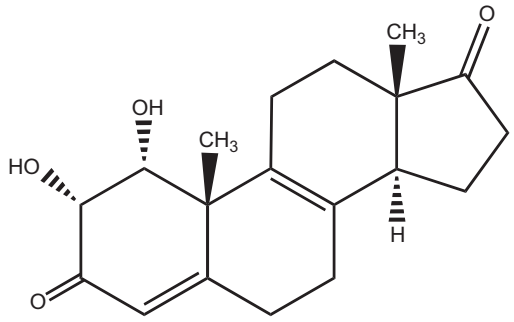


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Figure 2 Relative configurations of K10-0216 KA (**1**) and KB (**2**).

Therefore, the planar structure of **2** was elucidated as a 17-dehydroxyl-17-oxo analog of **1** (Figure 1). The ROESY correlations of **2** were almost similar to those of **1** (Figure 1). These results suggested that **2** had the relative configuration of *1R**, *2R**, *10S**, *13S** and *14S** (Figure 2).

Compound **2** showed slightly stronger inhibitory effect on the lipid accumulation in 3T3-L1 adipocytes than testosterone. However, no effect of **1** on the lipid accumulation was observed, even at 100 μM (Supplementary Figure S1). Moreover, lipid accumulation was inhibited by treatment with **2** in a concentration-dependent manner (Supplementary Figure S1). The structural difference between **1** and **2** was a hydroxyl group and carbonyl group, respectively, bound at the C-17 position. In comparision between **1** and **2**, it was suggested that the carbonyl group at the C-17 position is important in the inhibition of lipid accumulation in 3T3-L1 adipocytes.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)