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# Oxasetin, a New Antibacterial Polyketide Produced by Fungus *Vaginatispora aquatica*, HK1821

HAIYIN HE,[†,*] JEFFREY E. JANSO,[††] HUI Y. YANG,[†]
MAYA P. SINGH,[††] VALERIE S. BERNAN,[††]
MICHAEL GREENSTEIN[††] and GUY T. CARTER[†]

[†] Department of Natural Products Chemistry, Chemical Sciences and [††] Department of Microbiology, Infectious Disease Research, Wyeth Research,
401 N. Middletown Road,
Pearl River, NY 10965, U.S.A.

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The rapid increase in bacterial resistance to antibiotics in clinical practice has prompted renewed efforts to discover new types of antibacterial agents.[1] In our continuing search for new antibiotics from microorganisms, we examined a number of fungal cultures that were either recently acquired by our laboratory or fermented by unconventional methods.[2] The fungus HK1821 was isolated from a decaying piece of wood submerged in the Lam Tsuen River in Tai Po, Hong Kong. Through comparison of the ITS in GenBank, it was found to be highly related to *Vaginatispora aquatica*.[3] In antimicrobial screening, methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis* were shown to be susceptible to the culture whole broth of HK1821. A new 2-oxo-succinimide polyketide, designated oxasetin (**1**), was isolated from the fermentation broth and was found to be responsible for the antibacterial activity. In this paper the production, structure, and biological activity of **1** are reported.

## Identification of HK1821 as *Vaginatispora aquatica*

Genomic DNA was isolated from mycelia of an agar grown culture of HK1821 by a phenol/chloroform extraction. The internally transcribed spacer region 1, 5.8S rDNA, and the internally transcribed spacer region 2 (ITS1-5.8S-ITS2) were PCR-amplified. The purified PCR product was sequenced directly on an Applied Biosystems ABI 3700 sequencer. After editing in Sequencher™ version 4.0.5, the ITS of HK1821 was found to be 484 bp in length. A nucleotide Blast search (NCBI 2.2) was performed to compare this sequence to other sequences in the GenBank database. The analysis revealed that the ITS of HK1821 is most similar to the ITS of *Vaginatispora aquatica* (GenBank accession AF383968)[4] followed by several *Massarina* sp. and *Lophiostoma* sp., which are all members of the *Lophiostomaceae*. An alignment of the ITS of HK1821 with *Vaginatispora aquatica* in BestFit indicated that they are 99.6% identical with only three mismatched base pairs; there is a C/T transition at position 55 in the ITS 1, and there are two single base deletions in the ITS of HK1821; one at position 45 in the ITS 1 and the other at position 442 in the ITS 2.

Apparently, these two organisms have only recently diverged. A difference in the ITS of about 0.5% is well within the bounds of intraspecific variation found amongst other genera of fungi.[5] However, it is not enough variation to state that HK1821 and *Vaginatispora aquatica* (AF383968) are distinct species. Therefore, we propose that HK1821 is a *Vaginatispora aquatica* isolate.

HK1821 was plated onto potato dextrose agar (PDA) (Difco) and cornmeal agar (CMA) (Oxoid) to observe morphology. The plates were incubated at room temperature (about 22°C) with a natural light cycle, and morphology was noted every seven days. On PDA, HK1821 grew about nine millimeters per week. The colonies were velvety and mounded in the first two weeks and became more fleece-like with age. The young colonies were light gray to buff in color and darkened to a dark taupe after one month. A yellow soluble pigment was visible in the agar after two weeks incubation. Fruiting bodies were visible after one month and formed at the base of the petri dish as described by HYDE.[3] Although the fruiting structures were about 0.3 to 0.5 millimeters in diameter, spores were not produced within the fruiting bodies even after two months incubation.

On CMA, HK1821 grew at about eight millimeters per week. In contrast to the dense, mounded growth of HK1821 on PDA, the growth on CMA was much thinner. After two weeks of growth, the mycelia were furry or fuzzy and pigmented olivaceous; they became more gray and fleece-like with age. No fruiting bodies or soluble pigment were observed after two months incubation.

## Production, Structure, and Antibacterial Activity of Oxasetin (**1**)

The fungal culture HK1821 was plated onto Bennett's

* Corresponding author: heh@wyeth.com

medium from frozen stock and was incubated at 22°C until there was sufficient mycelial growth to inoculate into the first stage seed. The culture was transferred into a 25×150 mm tube, containing 11 ml of Difco potato-dextrose broth. The tube was shaken at 160 rpm at 22°C for 4 days. The first stage seed was transferred to a 250 ml Erlenmeyer flask containing 45 ml of potato-dextrose broth at pH 7.0. The second stage seed was incubated at 22°C with shaking at 200 rpm for an additional 4 days and was then inoculated into a 2.8-liter Fernbach flask containing 1 liter of potato-dextrose broth at pH 7.0. The fermentation was carried out under the same conditions as the second stage seed. The antibacterial activity was monitored by the agar diffusion method[6] and peaked after 10 days.

The whole broth of the 10-day fermentation (1 liter) was centrifuged, and the cells and supernatant were respectively extracted with methanol and ethyl acetate. The combined organic extract was concentrated under reduced pressure to give a reddish residue. This residue was chromatographed by reverse phase HPLC on a C18 column using a linear gradient of 90~100% acetonitrile in water containing 0.02% trifluoroacetic acid (TFA) to obtain oxasetin (**1**) (85.5 mg, physico-chemical data see Table 1).

The molecular formula of **1** was determined to be $C_{21}H_{29}NO_4$ by high-resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. The $^{13}C$ NMR spectrum (Figure 2) displayed the signal of a keto group at $\delta$ 200.2, in addition to 6 $sp^2$ carbon signals in the range between 106.3 and 171.7. The $^{13}C$ signals in the aliphatic region were assigned by DEPT experiments to 4 methyls, 5 methylenes, 4 methines, and 1 quaternary carbon. In the $^1H$ NMR spectrum (Figure 3), resonances of 2 methyl singlets, 1 methyl doublet, and 1 methyl triplet were observed. The signals at $\delta$ 5.07 and 10.75 ($D_2O$ exchangeable) were respectively assigned to an olefinic and an amide proton.

The analysis of 2-D COSY and TOCSY data revealed the homonuclear spin system of H-14 at $\delta$ 2.58, $H_2$-15 at 1.36 and 1.04, $H_2$-16 at 1.14 and 1.03, and $H_3$-17 at 0.70. In an HMBC spectrum, the strong 2- or 3-bond correlations between 5-Me at $\delta$ 1.13 and C-4 at 200.2, C-5 at 51.7, C-6 at 40.6, and C-14 at 45.1, between 13-Me at $\delta$ 1.63, and C-12 at 124.8, C-13 at 134.1, and C-14, between 9-Me at $\delta$ 0.85 and C-8 at 35.6, C-9 at 32.9, and C-10 at 42.0, and between H-12 at $\delta$ 5.07 and C-6 at 40.6, C-8, C-11 at 38.6, C-13, and C-14 signals were observed. These correlations,

Fig. 1. Structure of oxasetin (**1**).

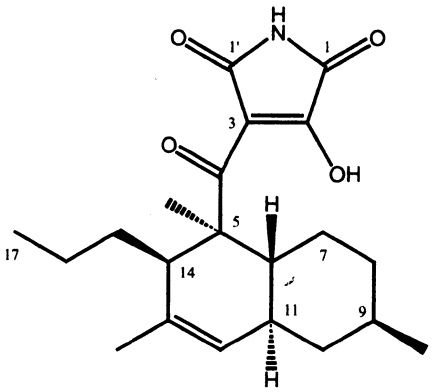



Fig. 2. $^{13}C$ NMR spectrum of **1** (100 MHz, DMSO-$d_6$).

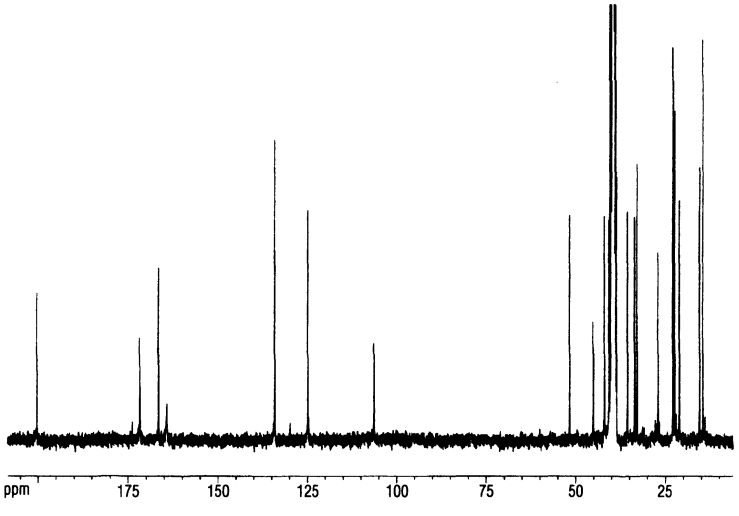
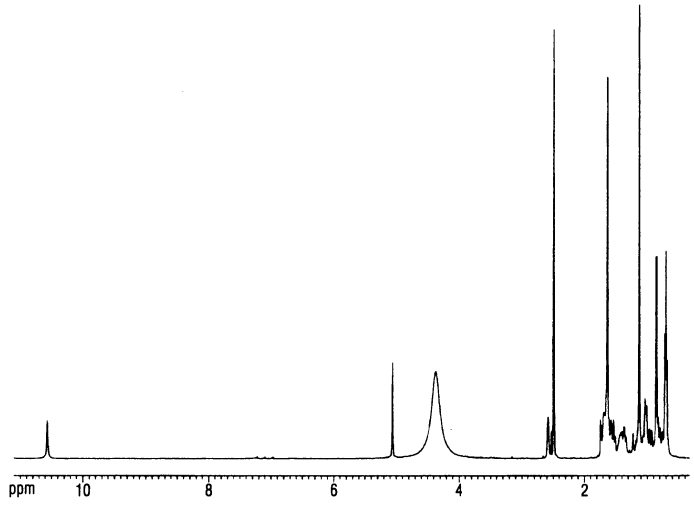


Fig. 3. $^1$H NMR spectrum of **1** (400 MHz, DMSO-$d_6$).




together with weaker 2-bond cross-peaks between C-7 at $\delta$ 27.1 and H-6 at 1.55 and $H_2$-8 at 1.67 and 0.95, led to the unambiguous assignment for the octahydronaphthalene moiety.

The remaining 4 carbon signals at $\delta$ 171.7, 166.5, 164.2, and 106.3 were all correlated to the amide proton at 10.75 in the HMBC spectrum, which was attributed to the presence of a 2-oxo-succinimide moiety connecting to the C-4 keto group. The assignments of $^1$H and $^{13}$C NMR spectral data and HMBC cross-peaks are listed in Table 2.

The relative stereochemistry of oxasetin (**1**) was determined by analysis of nuclear Overhauser effects (nOes). In a ROESY spectrum, the strong cross-peaks between H-9 at $\delta$ 1.44 and H-11 at 1.64, between H-11 and 5-Me at 1.13 and between 5-Me and H-14 at 2.58 were observed. These data suggested that the ring junction of the octahydronaphthlene system was *trans*, the 5-methyl was down, and the 9-methyl and 14-propyl were up (Figure 4).

Oxasetin (**1**) exhibited moderate *in vitro* activity against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*. Oxasetin showed no activity against the Gram-negative bacterium *Escherichia coli* and the yeast *Candida albicans*. MIC data obtained by the broth dilution method are listed in Table 3.

Oxasetin is structurally related to other fungal metabolites, the glycoside BU-4514N[7] and the enantiomeric homologs, equisetin[8] and phomasetin,[9] which are all 3-oxo-$\gamma$-lactam polyketides. Equisetin and phomasetin exhibit inhibitory activity to HIV-1 integrase.[9] Although the 3-oxo-succinimide is contained in many synthetic compounds as a pharmacophore, oxasetin is the first example of a natural product to bear this moiety. Biosynthetically, oxasetin is likely to derive from polyketide and amino acid origins. The labeling experiments designed to study its biosynthesis pathway are currently underway.

Table 1. Physico-chemical data of **1**.

| | | |
|---|---|---|
| Appearance | | Colorless oil |
| Molecular formula | | $C_{21}H_{29}NO_4$ |
| Molecular weight | | 359 |
| HRFTICRMS (neg, *m/z*) | found | 358.20179 $(M-H)^-$ |
| | Calcd | 358.20183 |
| UV $\lambda_{max}$ (MeOH, nm) | | 345, 261, 225 |
| IR $\nu_{max}$ ($CHCl_3$, $cm^{-1}$) | | 2952, 2923, 2868, 1782, 1726, 1625, 1562, 1454, 1408, 1341, 1162, 1025 |
| $[\alpha]_D$ (MeOH) | | −147.5 (c 0.367) |

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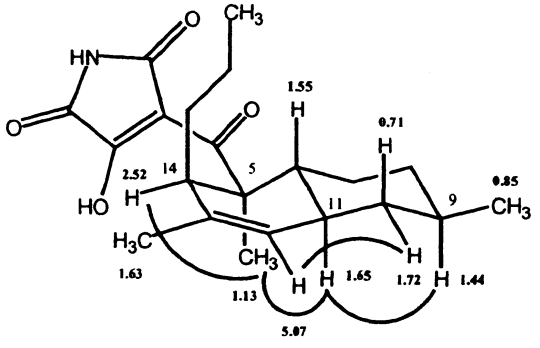
Table 2. $^{1}H$ and $^{13}C$ NMR spectral data of **1** in DMSO-$d_6$.

| Atom | $^{1}H$ (400 MHz, mult, $J$ in Hz) | $^{13}C$ (100 MHz) | HMBC ($J$ = 8 Hz) |
|---|---|---|---|
| 1 | | 171.7 (C) | |
| 2 | | 164.2[a] (C) | |
| 3 | | 106.3 (C) | |
| 4 | | 200.2 (C) | |
| 5 | | 51.7 (C) | |
| 5-Me | 1.13 (3H, s) | 15.5 ($CH_3$) | C-4, C-5, C-6, C-14 |
| 6 | 1.55 (m) | 40.6 (CH) | C-5, 5-Me, C-7, C-8, C-10, C-11 |
| 7 | 0.82 (m) | 27.1 ($CH_2$) | C-8, C-11 |
| | 1.58 (m) | | C-9 |
| 8 | 0.95(m) | 35.6 ($CH_2$) | C-7, C-9 |
| | 1.67 (m) | | C-6, C-7, C-9 |
| 9 | 1.44 (m) | 32.9 (CH) | |
| 9-Me | 0.85 (3H, d, 6.4) | 22.4 ($CH_3$) | C-8, C-9, C-10 |
| 10 | 0.71 (m) | 42.0 ($CH_2$) | C-9, C-11 |
| | 1.72 (m) | | C-6, C-8, C-9, C-11 |
| 11 | 1.64 (m) | 38.6 (CH) | C-7 |
| 12 | 5.07 (br s) | 124.8 (CH) | C-6, C-10, C-11, 13-Me, C-14 |
| 13 | | 134.1 (C) | |
| 13-Me | 1.63 (3H, s) | 22.8 ($CH_3$) | C-12, C-13, C-14 |
| 14 | 2.58 (m) | 45.1 (CH) | C-5, 5-Me, C-12, C-13, 13-Me |
| 15 | 1.04 (m) | 33.6 ($CH_2$) | C-13, C-16 |
| | 1.36 (m) | | C-5, C-13, C-14, C-16, C-17 |
| 16 | 1.03 (m) | 21.1 ($CH_2$) | C-14, C-15, C-17 |
| | 1.14 (m) | | C-15, C-17 |
| 17 | 0.70 (3H, t, 7.0) | 14.5 ($CH_3$) | C-15, C-16 |
| 1′ | | 166.5[a] (C) | |
| NH | 10.75[b] (s) | | C-1′, C-1, C-2, C-3 |

[a]— Assignments may be reversed. [b]— Exchanged when $D_2O$ was added.

Fig. 4. Relative stereochemistry of **1**.




Selected ROESY cross-peaks are indicated.

Table 3. Antimicrobial activity of **1**.

| Test organism | MIC (μg/ml)[a] |
|---|---|
| *Staphylococcus aureus* (3 strains, including a methicillin-resistant strain) | 16 |
| *Enterococcus faecalis* (2 strains, including a vancomycin-resistant strain) | 16 |
| *Streptococcus pneumoniae* (2 strains) | 16–32 |
| *Escherichia coli* | >128 |
| *Candida albicans* | >128 |

[a] Microbroth dilution method in Mueller-Hinton II, incubated at 35 °C for 18 hours.

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