Oximidines I and II: Novel Antitumor Macrolides from *Pseudomonas* sp.

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Our screening for cell-cycle inhibitors in transformed cells resulted in the isolation of novel active compounds, oximidines I (**1**) and II (**2**), from *Pseudomonas* sp. Q52002. The molecular formulas of **1** and **2** were established as $C_{23}H_{24}N_2O_7$ and $C_{23}H_{24}N_2O_6$, respectively, by high-resolution FABMS. The structures of the oximidines were elucidated by NMR spectral analysis, including a variety of two-dimensional techniques. The absolute stereochemistry of **1** was determined by using the modified Mosher method. The oximidines are novel 12-membered macrolides containing an *O*-methyloxime moiety. Oximidines I and II selectively inhibited the growth of rat 3Y1 cells transformed with E1A, *ras*, or *src* oncogenes.

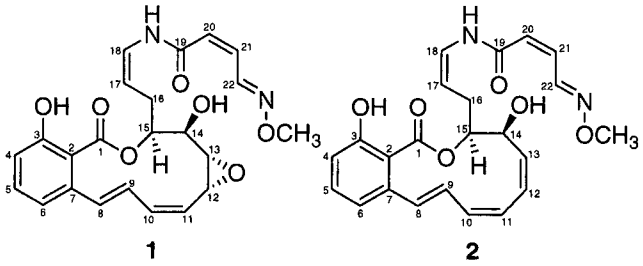
Many types of oncogenes are implicated in cell-cycle control.[1−3] Thus, cell-cycle inhibitors in oncogene-transformed cells may act as selective antitumor drugs. Our screening for such antitumor substances resulted in the isolation of novel active metabolites, oximidines I (**1**) and II (**2**), from *Pseudomonas* sp. Q52002. We report herein the structures and biological activities of **1** and **2**.

The molecular formulas of **1** and **2** were established as $C_{23}H_{24}N_2O_7$ and $C_{23}H_{24}N_2O_6$, respectively, by high-resolution FABMS. ^{13}C and ^1H NMR data for **1** and **2** are summarized in Table 1.

A series of NMR experiments including COSY and HMQC were used to construct three partial structures of **1**, depicted in Figure 2 by bold lines. The partial structure A was extended to be a 2,3-disubstituted phenol based on an HMBC experiment, which revealed long-range couplings from H-4 to C-2, H-5 to C-3 and C-7, H-6 to C-2, and a phenolic hydroxyl proton to C-2, C-3, and C-4. In the partial structure B, the presence of an epoxide between C-12 and C-13 was required by their characteristic ^{13}C and ^1H chemical shifts (Table 1). The geometrical configurations were determined to be 8*E*, 10*Z*, 17*Z*, and 20*Z* from their relevant proton coupling constants (J_{8-9} = 16.5 Hz, J_{10-11} = 11.5 Hz, J_{17-18} = 9.0 Hz, and J_{20-21} = 11.5 Hz).

Long-range correlations, from H-8 to C-2, C-6, and C-7 and from H-9 to C-7, established the connection of the partial structures A and B (Figure 2). Two aromatic protons (H-4 and H-6) displayed four-bond correlations to a carbonyl carbon (C-1), indicating its substitution at C-2. A long-range coupling from H-15 to C-1 identified an ester linkage between C-1 and C-15 which leads to a 12-membered lactone ring. The partial structures B and C were joined via an amide carbonyl (C-19) as determined from its long-range couplings to H-18, 18-NH, H-20, and H-21 (Figure 2).

The terminal sp^2 methine (C-22) in the partial structure C needed to be connected to the remaining nitrogen



1 **2**

Figure 1. Structures of oximidines I (**1**) and II (**2**).

Table 1. ^{13}C (125 MHz) and ^1H (500 MHz) NMR Data for Oximidines I (1) and II (2) in $CDCl_3$

	1		**2**	
no.	δ_C	δ_H (multiplicity, Hz)	δ_C	δ_H (multiplicity, Hz)
1	169.8		171.0	
2	114.2		109.9	
3	159.5		162.7	
4	116.5	6.88 (d, 8.0)	117.5	6.89 (d, 8.0)
5	133.4	7.29 (dd, 8.0, 7.5)	134.1	7.31 (dd, 8.0, 7.5)
6	120.1	6.70 (d, 7.5)	121.7	6.73 (d, 7.5)
7	140.8		141.0	
8	134.8	6.96 (d, 16.5)	130.9	6.84 (d, 16.5)
9	128.0	5.96 (dd, 16.5, 7.0)	132.9	6.70 (dd, 16.5, 9.5)
10	129.1	6.10 (ddd, 11.5, 7.0, 1.5)	130.1	6.39 (dd, 10.5, 9.5)
11	124.4	5.69 (d, 11.5)	130.7	6.28 (d, 10.5)
12	57.6	3.90 (m)	127.1	6.08 (d, 12.5)
13	57.7	3.49 (dd, 8.5, 3.5)	133.3	5.71 (dd, 12.5, 3.0)
14	67.5	3.69 (dd, 8.5, 5.5)	72.9	4.32 (br d, 11.0)
15	74.9	5.05 (dt, 5.5, 5.0)	77.6	5.27 (dd, 10.5, 4.5)
16	25.4	2.85 (m)	27.8	2.59 (m)
		2.47 (m)		2.26 (m)
17	108.5	5.11 (dt, 8.5, 8.0)	104.5	4.80 (dt, 9.5, 8.0)
18	123.4	6.81 (dd, 11.0, 8.5)	126.1	6.91 (dd, 10.0, 9.5)
19	162.4		162.1	
20	124.7	5.92 (d, 11.5)	124.5	5.90 (d, 11.5)
21	135.2	6.44 (dd, 11.5, 10.5)	135.5	6.49 (dd, 11.5, 10.5)
22	147.7	8.97 (d, 10.5)	147.7	9.01 (d, 10.5)
OMe	62.3	3.87 (s)	62.3	3.92 (s)
3-OH		9.77 (s)		10.73 (s)
14-OH		3.37 (br)		3.17 (d, 11.0)
18-NH		8.65 (d, 11.0)		8.36 (d, 10.0)

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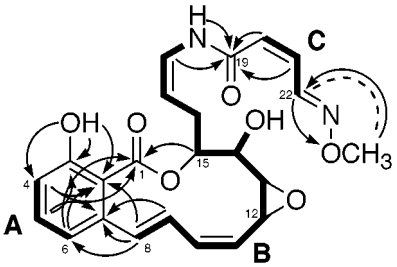

Figure 2. Connectivities among the partial structures of **1** by HMBC. Bold lines show proton spin systems, arrows indicate $^1H-^{13}C$ long-range correlations, and a dashed line indicates an NOE correlation.

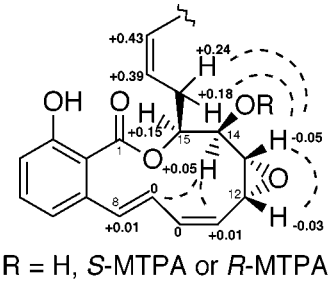

Figure 3. Absolute stereochemistry of **1**. $\Delta\delta$ ($\delta_{S-MTPA} - \delta_{R-MTPA}$) values are expressed in ppm. Dashed lines indicate NOEs.

atom in an imine-type bond. Four-bond $^1H-^{13}C$ correlations from a methoxyl group to the imine carbon confirmed the presence of an *O*-methyloxime moiety. A NOESY experiment revealed a weak NOE between H-22 and the methoxyl group, indicating *E* geometry for the oxime double bond. The planar structure of **1** thus obtained is shown in Figure 2.

A cis configuration for the epoxide was established by a significant NOE and a 3.5-Hz coupling between H-12 and H-13. In addition, NOEs between H-13 and H_2-16 indicated that H-12, H-13, and C-16 reside in the same orientation on the macrolide ring. A proton on C-14 displayed an NOE to H-11 but not to H-12 and was required to be in the orientation opposite that of H-12, thereby establishing the relative stereochemistry of **1** as shown in Figure 3.

The absolute stereochemistry of oximidine I (**1**) was elucidated by the modified Mosher method.[4] Both the (*R*)- and (*S*)-2-methoxy-2-(trifluoromethyl)phenyl acetate (MTPA) esters of **1** were prepared and subjected to ^1H NMR analysis. Only the two epoxide protons (H-12 and H-13) appeared downfield in the (*R*)-derivative spectrum relative to the (*S*)-derivative one, while most of the other protons including H-15, H_2-16, H-17, and H-18 exhibited upfield shifts in the (*R*)-derivative spectrum. These data allowed the absolute configuration at C-14 to be assigned as *R* and completed the total structure of **1** as shown in Figure 1.

The molecular formula of **2**, $C_{23}H_{24}N_2O_6$, showed a loss of one oxygen in comparison with that of **1**. In the ^1H NMR spectrum of **2**, H-12 and H-13 appeared downfield (H-12, δ 3.90 → 6.80; H-13, δ 3.49 → 5.71), and the corresponding carbon signals were observed at δ 127.1 and δ 133.3, respectively, indicating that the expoxide

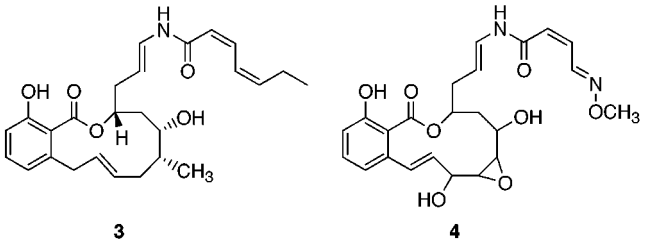

Figure 4. Structures of salicylihalamide A (**3**) and CJ-12,-950 (**4**).

Table 2. IC_{50} Values (ng/mL) of 1 and 2 against Normal and Transformed Rat 3Y1 Fibroblasts

cell line	oncogene	**1**	**2**
3Y1		510	270
SV-3Y1	SV40 large T	740	350
E1A-3Y1	E1A	30	17
Ad12-3Y1	E1A, E1B	62	36
HR-3Y1	v-H-*ras*	16	9.0
SR-3Y1	v-*src*	27	14

group in **1** is replaced by an olefinic double bond in **2**. The geometrical configuration of the new double bond was identified as *Z* on the basis of a significant NOE between H-12 and H-13, although their coupling constant exhibited a value (12.5 Hz) intermediate between those for *E* and *Z* configurations. The remaining spectral features were essentially identical with those of **1**. The structure of **2** (Figure 1), including the relative stereochemistry, was confirmed by COSY, HMQC, HMBC, and NOESY experiments (data not shown).

Recently, a series of 12-membered benzolactones have been isolated from natural sources, e.g., salicylihalamide A (**3**) and its geometrical isomer B from the marine sponge *Haliclona* sp.[5] and CJ-12,950 (**4**) and its geometrical isomer CJ-13,357 from the fungus *Mortierella verticillata* (Figure 4).[6] Oximidines I (**1**) and II (**2**) represent two new members of this family and are the first examples produced by a bacterium.

The antitumor effects of **1** and **2** were investigated using normal and transformed 3Y1 rat fibroblasts (Table 2).[7–9] Oximidines I and II inhibited the growth of 3Y1 cells transformed with the adenovirus E1A, v-H-*ras*, and v-*src* oncogenes at 15- to 30-fold lower concentrations than that of the parent 3Y1 cells, whereas SV40-transformed cells showed almost the same sensitivity as normal cells. Flow cytometric analysis revealed that **1** arrested the cell cycle of *ras*- and *src*-transformed 3Y1 cells at the G1 phase (data not shown). Further biological studies on the oximidines are in progress.

Experimental Section

Production and Isolation. The producing organism was inoculated into 500-mL Erlenmeyer flasks containing 100 mL of a production medium consisting of soluble starch 2%, glycerol 0.5%, soybean meal 1.5%, corn steep liquor 0.5%, NaCl

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0.3%, and $CaCO_3$ 0.3% (pH 7.0, before sterilization). The fermentation was carried out at 28 °C for 72 h on a rotary shaker at 200 rpm.

The culture supernatant (2 L) and the mycelial acetone extract were combined and extracted with EtOAc. The extract was chromatographed on a silica-gel column with $CHCl_3$−MeOH (80:1) and then subjected to HPLC using a YMC-Pack D-ODS-7 column. Development of the column with 60% MeOH gave active fractions I and II. The fraction I was further purified by HPLC using a PEGASIL ODS column with 55% MeOH. The active fraction was evaporated to dryness, to yield a colorless powder of **1** (108 mg). The fraction II was subjected to silica-gel TLC with hexane−EtOAc (1:1). The active fraction was purified by Sephadex LH-20 column chromatography with $CHCl_3$-MeOH (1:1), following which it was concentrated to dryness to give a colorless power of **2** (15 mg).

Oximidine I (1): mp 104−106 °C; $[\alpha]^{22}_D$ −286° (*c* 1.0, MeOH); UV λ_{max} 272 nm (ϵ 30500) in MeOH; 203 nm (ϵ 112000) and 270 nm (30500) in 0.01 M NaOH−MeOH; IR (KBr) ν_{max} 3350, 1710, and 1650 cm^{-1}; FABMS *m*/*z* 441.1673 MH^+ (calcd for $C_{23}H_{25}N_2O_7$, 441.1662).

Oximidine II (2): mp 113−115 °C; $[\alpha]^{26}_D$ −141° (*c* 0.75, MeOH); UV λ_{max} 282 nm (ϵ 30300) in MeOH; 203 nm (ϵ 138000) and 279 nm (28200) in 0.01 M NaOH−MeOH; IR (KBr) ν_{max} 3400, 1700, and 1650 cm^{-1}; FABMS *m*/*z* 425.1702 MH^+ (calcd for $C_{23}H_{25}N_2O_6$, 425.1713).

Preparation of the MTPA Esters of 1. To a solution of **1** (12 mg) in $CHCl_3$ (500 μL) were added 500 μL of pyridine, 0.1 mg of 4-(dimethylamino)pyridine, and 56 mg of (*S*)−(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride. The reaction mixture was allowed to stand at room temperature for 30 min. After 10 mL, each, of H_2O and $CHCl_3$ were added, the $CHCl_3$ layer was washed with aqueous HCl and aqueous NaOH, dried over Na_2SO_4, and evaporated to dryness. The dried material was purified by HPLC using a YMC-Pack D-ODS-7 column with 60% MeOH. The major UV peak was collected and evaporated to dryness to give a colorless powder of the (*R*)-MTPA ester of **1** (3.2 mg). Similarly, the (*S*)-MTPA ester of **1** (4.3 mg) was obtained using (*R*)-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride.

***(R)*-MTPA ester of 1:** ^{1}H NMR ($CDCl_3$) δ 9.81 (s, OH-3), 8.99 (d, *J* = 10.0 Hz, H-22), 7.88 (d, *J* = 11.0 Hz, 18-NH), 7.54 (d, *J* = 7.5 Hz, MTPA), 7.44 (t, *J* = 7.5 Hz, MTPA), 7.40 (dd, *J* = 8.0, 7.5 Hz, H-5), 7.40 (t, *J* = 7.5 Hz, MPTA), 7.07 (d, *J* = 16.0 Hz, H-8), 6.94 (d, *J* = 8.0 Hz, H-4), 6.75 (d, *J* = 7.5 Hz, H-6), 6.50 (dd, *J* = 11.0, 10.0 Hz, H-21), 6.38 (dd, *J* = 11.0, 8.5 Hz, H-18), 6.20 (ddd, *J* = 11.5, 6.0, 1.5 Hz, H-10), 5.86 (dd, *J* = 16.0, 6.0 Hz, H-9), 5.84 (d, *J* = 11.0 Hz, H-20), 5.81 (d, *J* = 11.5 Hz, H-11), 5.17 (dd, *J* = 9.0, 6.0 Hz, H-14), 4.90 (m, H-15), 4.41 (dt, *J* = 8.5, 8.0 Hz, H-17), 3.96 (m, H-12), 3.93 (s, OMe), 3.70 (s, MTPA−OMe), 3.62 (dd, *J* = 9.0, 3.5 Hz, H-13), 2.56 (m, H-16_a), 2.40 (m, H-16_b).

***(S)*-MTPA ester of 1:** ^{1}H NMR ($CDCl_3$) δ 9.86 (s, OH-3), 9.01 (d, *J* = 10.5 Hz, H-22), 8.20 (d, *J* = 11.0 Hz, 18-NH), 7.55 (d, *J* = 8.0 Hz, MTPA), 7.43 (t, *J* = 8.0 Hz, MTPA), 7.40 (dd, *J* = 8.0, 7.5 Hz, H-5), 7.40 (t, *J* = 8.0 Hz, MTPA), 7.08 (d, *J* = 16.5 Hz, H-8), 6.95 (d, *J* = 8.0 Hz, H-4), 6.81 (dd, *J* = 11.0, 8.5 Hz, H-18), 6.76 (d, *J* = 7.5 Hz, H-6), 6.52 (dd, *J* = 11.5, 10.5 Hz, H-21), 6.20 (ddd, *J* = 11.5, 6.0, 1.5 Hz, H-10), 5.91 (d, *J* = 11.5 Hz, H-20), 5.86 (dd, *J* = 16.5, 6.0 Hz, H-9), 5.82 (d, *J* = 11.5 Hz, H-11), 5.22 (dd, *J* = 9.0, 6.0 Hz, H-14), 5.05 (m, H-15), 4.80 (dt, *J* = 8.5, 8.0 Hz, H-17), 3.93 (s, OMe), 3.93 (m, H-12), 3.57 (dd, *J* = 9.0, 3.5 Hz, H-13), 3.48 (s, MTPA−OMe), 2.74 (m, H-16_a), 2.64 (m, H-16_b).

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Supporting Information Available: Spectral data (^{1}H NMR, ^{13}C NMR, COSY, HMQC, HMBC, and NOESY) are available for oximidines I and II in $CDCl_3$ (12 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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