Biological and Mechanistic Activities of Xanthorrizol and 4-(1′,5′-Dimethylhex-4′-enyl)-2-methylphenol Isolated from *Iostephane heterophylla*[1]

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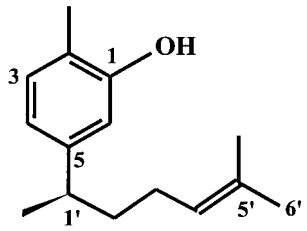
Received February 13, 2001

Xanthorrizol (**1**) and 4-(1′,5′-dimethylhex-4′-enyl)-2-methylphenol (**2**) were identified as the principal antimicrobial components of a CH_2Cl_2−MeOH (1:1) extract derived from *Iostephane heterophylla*. Compound **2** is a new natural product, but has been synthesized. Both compounds exhibited low level activity (MICs of 16−32 μg/mL) against methicillin-resistant staphylococci and vancomycin-resistant enterococci. They were either inactive or poorly active against Gram-negative bacteria and yeast. Mechanistic studies performed in *Escherichia coli imp* suggested nonspecific inhibition of DNA, RNA, and protein synthesis by both of these compounds. Compound **1** was tested in an in vivo model; it did not provide protection to mice infected with *Staphylococcus aureus*.

Iostephane Benth. (Asteraceae) is a genus of four species native to Mexico. While three species are restricted to Chiapas, Oaxaca, and Chihuahua, *Iostephane heterophylla* (Cav.) Hemsl. has the widest distribution (from Chihuahua in the north to Oaxaca in the south) and the greatest morphological variation. This yellow-flowered perennial herb, known as "raíz de indio", "hierba de manso", and "escorcionera", is widely used as a medicinal agent throughout México and is available in the markets. The root, when processed as a fresh cataplasm, a decoction, or a roasted powder, is commonly employed in treating skin abrasions, wounds, sores and inflammations. This decoction as well as an alcoholic tincture can be topically applied to reduce arthritis, rheumatism, back pain, and general body pain. In addition, the weak decoction is drunk to alleviate diabetes and gastrointestinal ailments such as dysentery. A stronger potion is an effective purgative. Minor reports include its use in treating liver ailments and sterility in women.[2,3] Previous chemical investigation of *I. heterophylla* led to the isolation and identification of a number of bioactive bisabolene-type sesquiterpenoids, including xanthorrizol (**1**), a few diterpenoids, scopoletin, and 8-hydroxy-6-acetyl-2,2,-dimethylchromene.[4−6] It was also demonstrated that xanthorrhizol (**1**) possessed antifungal activity against *Candida albicans* (MIC = 68.75 μg/mL), toxicity to *Artemia salina* (LC_{50} = 4.3 μg/mL), cytotoxicity against human nasopharyngeal carcinoma cell line (KB, EC_{50} = 4.90 μg/mL),[5] inhibitory activity of rat uterus contractions induced by KCl, $CaCl_2$, and BAY K 8644,[7] and induced endothelium-independent relaxation of rat thoracic aorta.[8]

During the course of our research program to discover bioactive agents from dryland plants of Latin America, a CH_2Cl_2−MeOH (1:1) extract from the roots of *I. heterophylla* was selected for bioassay-guided fractionation on the basis of its important antimicrobial activity against *Staphylococus aureus* and *Enterococcus faecium*. In the present investigation we describe the identification of a new naturally occurring bisabolene, namely, 4-(1′,5′-dimethylhex-4′-enyl)-2-methylphenol (**2**), as well as the mechanism of antibacterial activity of xanthorrhizol (**1**) and **2**.



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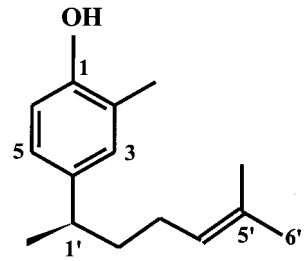

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Results and Discussion

At concentrations of 0.2 or 1 μg/mL, a CH_2Cl_2−MeOH (1:1) root extract of *I. heterophylla* exhibited antimicrobial activity against *S. aureus* and *E. faecium* when tested in the agar diffusion method. The active extract was fractionated on a silica gel column to yield 11 primary fractions (F001−F011). Fractions F002−F005 concentrated the antimicrobial activity. Extensive chromatography of the active fractions led to the isolation of xanthorrizol (**1**),[4,9,10] 16-α-hydroxy-*ent*-kaurane,[4] trachyloban-19-oic acid,[4] 16-α-hydroxy-*ent*-kaur-11-ene,[4] *ent*-kauren-16-en-19-oic acid,[4] *ent*-

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10.1021/np010076o CCC: $20.00
Published on Web 06/30/2001

Table 1. NMR Data for Compound **2**

position	δ_H[a] (m, *J* in Hz)	δ_C[b]	HMBC (H → C)	NOESY
1		151.7	3, 5, 2-Me	
2		122.1	6, 4	
3	6.92 (d, 2)	129.6	2-Me	2-Me, 1′, 2′, 1′-Me
4		139.9	6, 1′	
5	6.88 (dd, 8, 2.5)	125.3	3, Me-1′	6, 1′, 2′, 1′-Me
6	6.69 (d, 8)	114.7		1-OH, 5
1′	2.59 (qt 7, 7)	38.6	4, 6, 3′, Me-1′	3, 5, 2′, 1′-Me
2′	1.56 (m)	38.6	4′, Me-1′	3, 5, 3′, 1′-Me
3′	1.86 (p, 7.5)	26.1	1′	1′, 2-Me, 5′-Me, 6′-Me
4′	5.08 (tqq 7.25, 1.5, 1.5)	124.6	Me-5′, 6′, 2′	3′,2′,6′-Me
5′		131.3	3′	
6′	1.55 (d, 0.5)	17.60	Me-5′, 4′	5′- Me, 4′
2-Me	2.23 (bs)	15.8	3	3, 3′,2′, Me-5′
1′-Me	1.19 (d, 7)	22.5	4, 6, 3′	1′, 3, 5
5′-Me	1.67 (d, 1)	25.7	6′, 4′	6′,4′, Me-2
OH-1	4.5 (bs)			6

[a] 500 MHz in in $CDCl_3$. [b] 125 MHz in $CDCl_3$.

beyer-15-en-19-oic acid,[4] and 4-(1′,5′-dimethylhex-4′-enyl)-2-methylphenol (**2**). Compound **2** is a new naturally occurring bisabolene, previously synthesized as a racemic mixture to confirm the structure of 2-(1′,5′-dimethylhex-4′-enyl)-4-methylphenol, a phenolic sesquiterpene isolated from *Elvira biflora* DC. (Asteraceae).[11] In this report only partial ^{1}H NMR information on the racemate synthesized was reported. The remaining compounds were previously isolated from this species.[4]

Compound **2** was obtained as a viscous optically active oil which showed phenolic absorptions in its IR and UV spectra. HREIMS established its molecular formula as $C_{15}H_{22}O$. The one-dimensional NMR spectra (Table 1) were clearly analogous to those of xanthorrhizol, showing also an isopropylidene group, a secondary benzylic methyl, an aromatic methyl, and three aromatic protons, which appeared as an ABX system in the ^{1}H NMR. The most obvious difference between the ^{1}H NMR spectra of both compounds resulted in the chemical shift of the aromatic protons comprising the ABX system [6.92 (1H, d, J = 2 Hz, H-3), 6.88 (1H, dd, J = 8, 2.5 Hz, H-5), 6.69 (1H, d, J = 8 Hz, H-6) in **2**; 7.01 (1H, d, J = 8 Hz, H-3), 6.67 (1H, dd, J = 8, 1.5 Hz, H-4), 6.60 (1H, d, J = 2.1 Hz, H-6) in **1**], suggesting that the positions of the phenolic and methyl groups in **2** were the reverse of that in **1**. Detailed analysis of the NOESY and HMBC spectra confirmed this assumption. Thus, the HMBC correlations C-1/Me-2, H-3, H-5, C-3/Me-2, H-1′, H-5, and C-2/H-6 as well as the NOESY correlations H-3/Me-2, H-1′/H-3, H-5 were consistent with the placement of the phenolic and the aromatic methyl groups at C-1 and C-2, respectively. The optical rotation of compound **2** was negative, revealing that the absolute configuration at C-1′ was identical to that of **1** and (−)-curcuphenol.[4,9,10,12] Furthermore the CD spectra of **1** and **2** were identical, possessing a strong negative cotton effect at 205 nm and a weak negative Cotton effect at 277 nm.

Compound **2** is the second natural rearranged bisabolene reported in the literature, the first one being 2-(1′,5′-dimethylhex-4′-enyl)-4-methylphenol isolated from *Elvira biflora* DC. (Asteraceae).[11]

Xanthorrizol (**1**) and compound **2** were found to be equipotent against a panel of Gram-positive and Gram-negative bacteria and *C. albicans* (Table 2). The MIC values of both compounds were 16−32 μg/mL against a panel of Gram-positive bacteria, including methicillin-resistant *S. aureus* and vancomycin-resistant *E. faecium*. However, **1** and **2** were essentially inactive against *E. coli*, *P. aeruginosa*, and *C. albicans*, with MIC values equal to or greater than 128 μg/mL.

Table 2. Antimicrobial Activity[a] of Xanthorrizol (**1**) and Compound **2**

organism	MIC (μg/mL) xanthorrizol (**1**)	compound **2**	penicillin G[b]
Staphylococcus aureus 375	16	16	0.06
S. aureus 310 (MR)	32	32	64
S. aureus ATCC 25923	16	16	0.06
Enterococcus faecium 379 (VR)	16	16	32
Enterococcus faecium 436	16	16	32
Bacillus subtilis 327	16	16	32
Escherichia coli imp 389	32	32	2
E. coli 442	>128	>128	32
E. coli ATCC 25922	>128	>128	128
Klebsiella pneumoniae 425	32	32	128
Pseudomonas aeruginosa 339	>128	>128	>128
Candida albicans 54	128	128	>128

[a] Method: microbroth dilution method. [b] Standard drug.

Table 3. Mechanism of Action of Xanthorrizol (**1**) and Compound **2** in *E. coli imp*[a]

compound	conc (μg/mL)	Tdr	Udr	AA
xanthorrizol (**1**)	128	1.52	1.91	17.12
	64	7.03	8.72	23.39
	32	35.75	48.56	33.57
	16	99.98	91.13	96.92
	8	95.33	96.87	102.10
2	128	30.71	34.04	25.34
	64	37.82	39.08	39.06
	32	48.24	50.02	44.20
	16	63.06	60.35	52.28
	8	75.57	66.73	68.00
ciprofloxacin-HCl	0.25	4.42	87.10	96.16
rifampin	0.25	89.56	1.17	27.82
chloramphenicol	25	82.98	85.76	18.96

[a] Data presented as % of untreated control. Tdr = tritiated thymidine. Udr = tritiated uridine. AA = tritiated amino acids.

Inhibition of DNA, RNA and protein synthesis was determined by measuring the incorporation of ^{3}H-Tdr, ^{3}H-Udr, and ^{3}H-AA, respectively, into TCA-precipitable material of a logarithmic-phase culture of *E. coli imp*. Control drugs affected the anticipated macromolecular processes, whereas treatment with xanthorrizol (**1**) and compound **2** inhibited incorporation of all three radiolabled precursors, suggesting disruption of membrane integrity. Although xanthorrizol (**1**) at the higher concentrations (128 and 64 μg/mL) demonstrated a greater degree of inhibition of DNA and RNA synthesis than compound **2** at similar concentrations, both compounds inhibited all three macromolecular processes at their MIC or higher (Table 3). Thus, the mechanistic studies performed in *Escherichia coli imp*

Table 4. In Vivo Activity (Acute Lethal Infection with *S. aureus* in Mice) and Toxicity

	xanthorrizol (**1**)		vancomycin	
route	ED_{50}	LD_{50}/ED_{50}	ED_{50}	LD_{50}/ED_{50}[d]
SOD[a]	ND[c]	ND[c]	230	ND[c]
SSC[b]	>32	ND[c]	1	>1000

[a] Single oral dose. [b] Single subcutaneous dose. [c] Not determined. [d] LD_{50} of vancomycin were determined to be >1024 mg/kg by oral and subcutaneous.

suggested nonspecific inhibition of DNA, RNA, and protein synthesis by compounds **1** and **2**.

Xanthorrizol (**1**) was also tested in an in vivo model. The results summarized in Table 4 clearly reveal that it did not provide protection to mice infected with *Staphylococcus aureus*, even at the higher dose tested.

The remaining isolates were all tested, and only trachyloban-19-oic acid exhibited weak activity against susceptible *S. aureus* (MIC 128 μg/mL); therefore compounds **1** and **2** made up the bulk of the antibacterial activity within the extracts.

Experimental Section

General Experimental Procedures. Melting point determinations for the known diterpenes were performed on a Fisher-Johns apparatus and are uncorrected. IR spectra were obtained using KBr disks or films on a Perkin-Elmer FT 1605 spectrophotometer. UV spectra were obtained on a Lambda II UV spectrometer in MeOH solution. NMR spectra including COSY spectra, NOESY, HMBC, and HMQC experiments were recorded on a Bruker DMX500 at 500 MHz (^{1}H) or 125 MHz (^{13}C) NMR or on a Varian Unity INOVA at 300 MHz (^{1}H) or 75 MHz (^{13}C). CD spectra were measured at room temperature in MeOH using a CD6 spectropolarimeter. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. EIMS were obtained on a JEOL SX 102A mass spectrometer. Open column chromatography: Si gel 60 (70−230 mesh, Merck). TLC: silica gel 60 F_{254} (Merck).

Plant Material. The roots of *I. heterophyla* were collected in San Luis Potosí, San Luis Potosí, México, on October 25, 1995. A voucher specimen (R. Bye, E. Linares, G. Morales, M. Mendoza 20528) was deposited in the Ethnobotanical Collection of the National Herbarium (MEXU), Instituto de Biología, UNAM.

Antimicrobial Activity. In vitro antimicrobial activities against methicillin-sensitive (MSSA Sa 375) and -resistant (MRSA Sa 310) *Staphylococcus aureus*, vancomycin-resistant *Enterococci faecium* (VREF Ef 379), *E. coli*, *E. coli imp* Ec 389 (a mutant strain with increased permeability to large molecular weight compounds),[13] and *Candida albicans* Ca 54 were determined by the agar diffusion method.[14] Media used were Difco nutrient agar (pH 6.8) for *S. aureus*, LB (Luria-Bertani) agar for *E. faecium* and *E. coli*, and YM agar for *C. albicans*. Assay plates (12 in. × 12 in. Sumilon) were prepared by pouring 125 mL of agar medium (tempered at 50 °C) inoculated with an overnight broth culture of the test organisms (adjusted to approximately 10^6 cells per μL), as previously described.[14] Ten microliters of the antibiotic solution diluted in DMSO were spotted onto the agar surface, and the plates were incubated at 37 °C for 18 h. The zone of growth inhibition was measured using a hand-held digital caliper. DMSO control (20 mL/well or 2.5% v/v in liquid broth) did not inhibit bacterial growth.

Minimum inhibitory concentrations (MICs) were determined by the standard broth microdilution method as described earlier.[15] Briefly, 5 μL of an overnight broth bacterial culture (adjusted to a density of 1×10^7 to 5×10^7 CFU/mL) was added to 0.1 mL of broth medium in polystyrene plates containing the drug at 0.03−128 μg/mL concentration. The MIC was defined as the lowest concentration of antibiotic that prevented visual turbidity after 18−20 h of incubation at 37 °C.

Mechanistic Studies. Macromolecular synthesis in *E. coli imp* was studied by measuring the incorporation of appropriate radiolabeled precursors into trichloroacetic acid (TCA)-precipitable material.[15] An overnight culture was diluted 1:500 in fresh modified minimal medium (50 mL medium/250 mL Erlenmeyer flask) and incubated at 37 °C and 200 rpm to an A_{600} of 0.20. Aliquots of 100 μL were dispensed into microtiter wells containing 5 μL of antibiotic, and plates were incubated for 2−14 min at 37 °C with vigorous agitation. Cells were pulse-labeled for 5 min by adding one of the following radiolabeled precursors at the indicated final concentrations: [^{3}H]-Tdr (1 μCi/mL with 0.05 μg/mL of unlabeled thymidine/mL), [^{3}H]Udr (1 μCi with 0.12 μg unlabeled uridine/mL), or [^{3}H]AA (10 μCi/mL). To determine specific incorporation into DNA, RNA, and protein, 100 μL of chilled (4 °C) TCA (10%) supplemented with 0.5 mg of unlabeled precursors per mL was added to each well, and the plate was immediately refrigerated for 1 h. The precipitate was collected on a glass fiber filter (Wallac filtermat B, Wallac 1205−404) using a Skatron 96-well cell harvester (Model 11050) programmed for a 3 s prewet with chilled DI water, a 12 s wash with 5% chilled TCA, and a 5 s drying cycle. Filter mats were dried for 7 min at high power in a microwave oven (Quasar, 700 W), solid scintillant (Meltilex B, Pharmacia 1205-402) was applied, and the isotope that was retained on the filter was quantitated in an LKB Betaplate scintillation counter (Wallac 1205). The levels of incorporation of [^{3}H]Tdr, [^{3}H]Udr, and [^{3}H]AA are expressed as a percent of that of the untreated control.

In Vivo Activity. The in vivo activity was assessed in female mice, strain CD-1 (from Charles River Laboratories, NY), weighing 20 ± 2 g each, infected intraperitoneally with sufficient bacterial cells suspended in broth or about 5% mucin to kill 95−100% of the untreated mice within 48 h. Antibiotic was administered subcutaneously in single doses 30 min after infection. Seven-day survival ratios from 3 or 4 separate tests each with 5 dose levels and 5 animals per dose level were pooled for the determination of the median effective dose (ED_{50}) by probit analysis.[15]

Extraction and Bioassay-Directed Fractionation. The air-dried plant material (2.4 kg) was ground into powder and extracted by maceration with CH_2Cl_2−MeOH (1:1) at room temperature. After filtration, the extract was evaporated under reduced pressure to yield 263 g of residue, which was subjected to column chromatography over Si gel (2.5 kg) and eluted with a gradient of hexane−CH_2Cl_2 (10:0 → 0:10) and CH_2Cl_2−MeOH (9:1 → 3:7). Fractions of 500 mL each were collected and pooled based on TLC profiles to yield 11 major fractions (F001−F011). Fractions F002−F005 were active against Gram-positive bacteria. Extensive preparative TLC of fractions F002 (200 mg) and F003 (19 g) using hexane−CH_2Cl_2 (1:1) led to the isolation of **1** (100 mg and 12 g, respectively) as a yellowish oil. Further column chromatography of fraction F004 (123 g) on Si gel (1.2 kg), eluting with a concentration gradient of hexane−CH_2Cl_2−MeOH, starting with hexane afforded 15 secondary fractions (F004-I−F004-XV). Fractions F004-II to F004-IV exhibited very good activity and F004-1 and F004-V through F004-XV showed poor activity against the Gram-positive organisms. From active fraction F004-II [1 g, eluted with hexane−CH_2Cl_2 (9:1)] spontaneously crystallized 500 mg of 16-α-hydroxy-*ent*-kaurane. From fraction F004-X, eluted with CH_2Cl_2−MeOH (9:1), crystallized 500 mg of trachyloban-19-oic acid. Fractions F004-III−IX rendered an additional 41.28 g of **1**. Active fraction F005 (92 g) was further fractionated by column chromatography on silica gel (795 g) and eluted with a gradient of hexane−CH_2Cl_2 (10:0 → 0:10) and CH_2Cl_2−MeOH (9:1 → 0:10). This chromatographic process led to 12 secondary fractions (F005-I to F005-XII) of which F005-III and -IV were more active than the rest. From fractions F005III to F005VI, eluted with hexane−CH_2Cl_2 (7.5:2.5, 6:4, and 4.5:5.5, respectively) crystallized additional amounts (8 g) of trachyloban-19-oic acid. From fraction F005-X, eluted with CH_2Cl_2−MeOH (6:4), crystallized 406 mg of 16-α-hydroxy-*ent*-kaur-11-ene-19-oic. The mother liquors from fractions F005-VI and fraction F005-VII were combined (14 g) and rechromatographed on a silica gel column (185 g), eluting with a

concentration gradient of hexane−CH_2Cl_2 (10:0 → 0:10), to yield eight tertiary fractions (F005-XIII−F005-XX). Extensive preparative TLC [CH_2Cl_2, four runnings] of fraction F005-XX (740 mg), eluted with hexane−CH_2Cl_2 (8:2), yielded **2** (19.3 mg) and additional amounts of **1** (280 mg). From fraction F005-XVIII, eluted with hexane−CH_2Cl_2 (1:1), crystallized 1.6 g of a mixture of *ent*-kauren-16-en-19-oic and *ent*-beyer-15-en-19-oic acids.

4-(1′,5′-Dimethylhex-4′-enyl)-2-methylphenol (2): colorless oil; $[\alpha]_D$ −60 (*c* 1 mg/mL MeOH); UV (MeOH) λ_{max} (ϵ) 276 (2218), 257 (1709), 204 (22581) nm; CD (MeOH) $\Delta\epsilon$ (nm) -1.44×10^3 (277), -2.75×10^4 (205); IR ν_{max} (film) 3436, 2955, 2924, 2854, 1611, 1510, 1454, 1376, 1264, 1121 cm^{-1}; ^1H and ^{13}C NMR (Table 1); EIMS *m*/*z* 218 [M^+ (90)], 203 (10), 161 (68), 148 (90), 135 (100), 121 (45); HRMS *m*/*z* 218.164 (calcd for $C_{15}H_{22}O$, 218.162).

Acknowledgment. This work was supported by the "Bioactive Agents from Dryland Biodiversity of Latin America" grant U01 TW 00316 from the National Institute of Health (NIH) and National Science Foundation, and USAID. We thank Rosa I. Del Villar, Oscar S. Yañez-Muñoz, Graciela Chávez, Marisela Gutiérrez, and Georgina Duarte-Lisci, Facultad de Química, UNAM, for obtaining some NMR, IR, and HRFABMS spectra. Special thanks are due to Guadalupe Pérez Castorena, Laura Acevedo, Edelmira Linares, and Mirna Mendoza for technical assistance. E.M. acknowledges the fellowship awarded by CONACyT, México, to carry out graduate studies.

References and Notes

(1) This work represents part of the MS thesis submitted to the Graduate School of Chemistry, UNAM, México, by E.M.
(2) Hernández, F. Historia Natural de Nueva España. Obras Completas [II:37−38]; México DF: Universidad Nacional Autónoma de México, 1959.
(3) Bye, R. *Econ. Bot.* **1986**, *40*, 103−124, and references therein.
(4) Aguilar, M. I.; Delgado, G.; Bye, R.; Linares, E. *Phytochemistry* **1993**, *33*, 1161−1163.
(5) Aguilar M. I.; Delgado, G. *Nat. Prod. Lett.* **1995**, *7*, 155−162.
(6) Delgado, G.; Aguilar, M. I. In *Secondary Metabolites from Mexican Plants: Chemistry and Biological Properties*; Rodríguez, L., Ed.; Research Signpost: Trivandrum, India, 1996; pp 69−76.
(7) Ponce-Monter; H.; Campos, M. G.; Aguilar, I.; Delgado G. *Phytother. Res.* **1999**, *13*, 202−205.
(8) Campos, M. G.; Oropeza, M. V.; Villanueva, T.; Aguilar, M. I.; Delgado G.; Ponce, H. *Life Sci.* **2000**, *67*, 327−333.
(9) Rimpler, H.; Hansel, R.; Kochendoerfer, L. *Z. Naturforsch.* **1970**, *25b*, 995.
(10) John, T. K.; Krishna-Rao, G. S. *Indian J. Chem.* **1985**, *24B*, 35−37.
(11) Dennison, N. R.; Mirrington, R. N.; Stuart, A. D. *Aust. J. Chem.* **1975**, *28*, 1339−1343.
(12) McEnroe, F. J.; Fenical, W. *Tetrahedron* **1978**, *34*, 1661−1664.
(13) Sampson, B. A.; Misra, R.; Benson, S. A. *Genetics* **1989**, *122*, 491−501.
(14) Hewitt, W.; Vincent, S. *Theory and Application of Microbiological Assay*; Academic Press: London, 1988; pp 38−79.
(15) Singh, M. P.; Petersen, P. J.; Weiss, W. J.; Kong, F.; Greenstein, M. *Antimicrob. Agents Chemother.* **2000**, *44*, 2154−2159.

NP010076O