Ceanothane- and Lupane-Type Triterpenes with Antiplasmodial and Antimycobacterial Activities from *Ziziphus cambodiana*

Sunit SUKSAMRARN,*[,a] Panomwan PANSEETA,[a] Soykam KUNCHANAWATTA,[a] Thanomsin DISTAPORN,[a] Saovaluk RUKTASING,[b] and Apichart SUKSAMRARN[c]

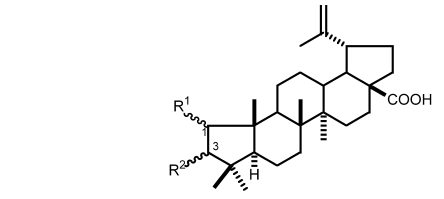
[a] *Department of Chemistry, Faculty of Science, Srinakharinwirot University; Sukhumvit 23, Bangkok 10110, Thailand:* [b] *National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency; Paholyothin Road, Pathumthani 12120, Thailand: and* [c] *Department of Chemistry, Faculty of Science, Ramkhamhaeng University; Bangkok 10240, Thailand.* Received September 1, 2005; accepted December 9, 2005

One new and eight known ceanothane- and lupane-type triterpenes were isolated from the root bark of *Ziziphus cambodiana* PIERRE (Rhamnaceae). Based on spectral analyses, the structure of the new compound was elucidated as 3-*O*-(4-hydroxy-3-methoxybenzoyl)ceanothic acid (3-*O*-vanillylceanothic acid) (1), while the known compounds were identified as lupeol (2), betulinaldehyde (3), betulinic acid (4), 2-*O*-*E*-*p*-coumaroyl alphitolic acid (5), alphitolic acid (6), zizyberanalic acid (7), zizyberenalic acid (8) and ceanothic acid (9). Compounds 1, 5 and 8 exhibited significant *in vitro* antiplasmodial activity against the parasite *Plasmodium falciparum*, with inhibitory concentration (IC_{50}) values of 3.7, 0.9 and 3.0 μg/ml, respectively. Compounds 1 and 3—8 showed antimycobacterial activity against *Mycobacterium tuberculosis* with respective MIC values of 25, 25, 25, 12.5, 50, 50 and 100 μg/ml.

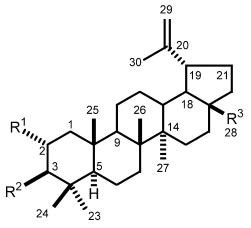
Key words *Ziziphus cambodiana*; Rhamnaceae; triterpene; antimycobacterial activity; antiplasmodial activity

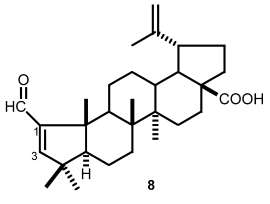
The Rhamnaceous *Ziziphus* is well-known for its content of triterpenes (betulinic and alphitolic acids),[1] saponins (jujubisides and joazeirosides)[2,3] and cyclopeptide alkaloids (lotosines).[4] Some *Ziziphus* plants have been found to possess biological activities, for example sedative,[5] hypoglycemic,[6] antibacterial and antifungal activities.[7] *Ziziphus cambodiana* Pierre is a thorny Rhamnaceous scandent widely distributed in the north-east of Thailand and used traditionally for its antiinfectious abilities.[8] No phytochemical study of this plant has been reported so far. In our search for biologically active substances of new structural types from Thai natural resources,[9—12] we investigated the chemical constituents and biological activities of this plant species and have found that the ethyl acetate extract of the root bark of *Z. cambodiana* showed pronounced *in vitro* antimalarial potential against *Plasmodium falciparum*. In this paper, we report the isolation and characterization of a new tritrpene ester (**1**), together with five known lupane constituents: lupeol (**2**),[13] betulinaldehyde (**3**),[14] the major metabolite betulinic acid (**4**),[13] 2-*O*-*E*-*p*-coumaroylalphitolic acid (**5**),[1] and alphitolic acid (**6**),[1] and three ceanothane triperpenes: zizyberanalic acid (**7**),[15] zizyberenalic acid (**8**)[15] and ceanothic acid (**9**),[16] as the antiplasmodial and antituberculosis constituents of the root bark of this plant species. This is the first report of *in vitro* antiplasmodial and antimycobacterial activities from the ceanothane-type triterpenes.

Compound **1** was obtained as a colorless solid, mp 183—185 °C. Negative high-resolution fast atom bombardment mass spectrometry (HR-FAB-MS) established a pseudomolecular ion at *m*/*z* 635.3592, compatible with a molecular formula of $C_{38}H_{52}O_8$. This compound exhibited IR absorption bands for hydroxyl (3430 cm^{-1}), conjugated carbonyl ester (1699 cm^{-1}) and aromatic (1602, 1512 cm^{-1}) functionalities. The ^{1}H-NMR spectrum (Table 1) revealed an isopropenyl group (δ_H 4.82, 4.62, 1.63), and five additional singlet methyls (δ_H 1.53, 1.23, 1.11, 1.10, 1.02), as well as two singlet methine protons at δ_H 5.94 and 3.15 which coupled to each other, as suggested by the correlated spectroscopy (COSY) spectrum. In the ^{13}C-NMR and distortionless enhancement by polarization transfer (DEPT) spectra, 38 carbon signals were observed, including six methyls, one methoxyl, nine methylenes, ten methines, and nine quaternary carbons, as well as one ester carbonyl and two carboxylic acids carbon signals (Table 1). The presence of a vanillyl moiety in the molecule was supported by aromatic protons at δ_H 7.86 (1H, br s), 7.28 (1H, d, J=8.2 Hz) and 7.90 (1H, br d, J=8.2 Hz) and one aromatic methoxyl group at δ_H 3.75 (3H, s). A nuclear Overhauser enhancement



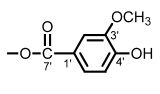
1 R^1=α COOH, R^2=β **A**
7 R^1=β CHO, R^2=α OH
9 R^1=α COOH, R^2=β OH



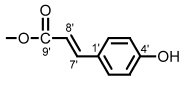


8

2 R^1=H, R^2=OH, R^3=CH_3
3 R^1=H, R^2=OH, R^3=CHO
4 R^1=H, R^2=OH, R^3=COOH
5 R^1=**B**, R^2=OH, R^3=COOH
6 R^1=R^2=OH, R^3=COOH



A



B

* To whom correspondence should be addressed. e-mail: sunit@swu.ac.th

© 2006 Pharmaceutical Society of Japan

Table 1. ^{1}H- and ^{13}C-NMR Data of Compound **1** (C_5D_5N)

Position	δ_C	δ_H
1	64.3	3.15 (1H, s)
2	176.8	
3	86.0	5.94 (1H, s)
4	43.8	
5	56.9	2.18 (1H, m)
6	18.8	1.43 (2H, m)
7	34.6	1.38 (2H, m)
8	43.5	
9	45.4	2.12 (1H, m)
10	49.6	
11	24.2[a)]	2.15 (1H, m), 1.58 (1H, m)[b)]
12	26.3[a)]	1.97 (1H, m), 1.23 (1H, m)[b)]
13	39.1	2.74 (1H, br t, J=10.1 Hz)
14	42.2	
15	30.5	1.86 (1H, m), 1.20 (1H, m)
16	32.9	2.57 (1H, br d, J=12.3 Hz), 1.47 (1H, m)
17	56.6	
18	49.6	1.66 (1H, m)
19	47.6	3.47 (1H, m)
20	151.1	
21	31.3	2.17 (1H, m), 1.20 (1H, m)
22	37.6	2.18 (1H, m), 1.51 (1H, m)
23	30.7	1.53 (3H, s)
24	20.3	1.11 (3H, s)
25	18.5	1.23 (3H, s)
26	17.1	1.10 (3H, s)
27	15.0	1.02 (3H, s)
28	179.0	
29	109.8	4.82 (1H, br s), 4.62 (1H, br s)
30	19.7	1.63 (3H, s)
1′	121.9	
2′	116.4	7.86 (1H, br s)
3′	148.5	
4′	153.4	
5′	113.5	7.28 (1H, br d, J=8.2 Hz)
6′	124.6	7.90 (1H, br d, J=8.2 Hz)
7′	166.3	
OMe	55.8	3.75 (3H, s)

a, *b*) Interchangeable within a column.

(NOE) displayed between H-2′ (δ_H 7.86) and the methoxyl group in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum, supporting the placement of the methoxyl group at the meta position in the vanillyl unit (Fig. 1). The NMR spectral data of **1** are very similar to those of ceanothic acid (**9**), except for the presence of a vanillyl ester moiety in **1**. The ester substituent was placed at C-3 as a result of downfield shifts observed for H-3 and C-3 in the ^{1}H- and ^{13}C-NMR spectra, respectively, as well as from correlations exhibited between H-3 (δ_H 5.94) and C-1 (δ_C 64.3), C-2 (δ_C 176.8) and C-7′ (δ_C 166.3) of the vanillyl group in the heteronuclear multiple bond correlation (HMBC) spectrum. The relative configuration of H-1 and H-3 for compound **1** was further suggested by a NOESY experiment (Fig. 1), wherein NOE enhancements were displayed between H-3 and H-23, H-1 and H-24 and H-25, confirming the H_β-1 and H_α-3 orientation of the ceanothic acid nucleus. ^{13}C-NMR assignments of **1** were made by one-dimensional (1D-) and 2D-NMR spectral data analysis, and by comparison with those of ceanothic acid[16] and 3-*O*-protocatechuoylceanothic acid.[17] Compound **1**, therefore, was elucidated as 3-*O*-(4-hydroxy-3-methoxybenzoyl)ceanothic acid or 3-*O*-vanillylceanothic acid. To our knowledge, the triterpene ester **1** is the third novel naturally occurring aromatic acid ester of the ceanothane-type triterpene, the previous ones being 2-*O*-*E*-*p*-coumaroylceanothanolic acid[15] and 3-*O*-protocatechuoylceanothic acid.[17]

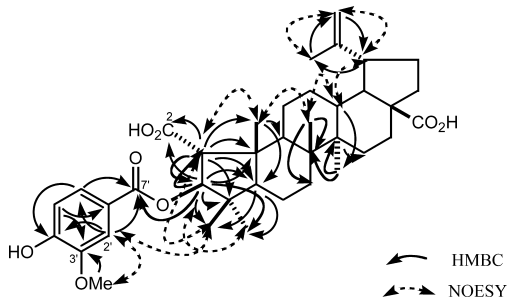


Fig. 1. HMBC and NOESY Correlations of Compound **1**

Table 2. IC_{50} Values for Antiplasmodial and MIC Values for Antimycobacterial Activities of Triterpenes **1**—**9**

Compound	IC_{50} (μg/ml)	MIC (μg/ml)
3-*O*-Vanillylceanothic acid (**1**)	3.7	25
Lupeol (**2**)	Inactive[a)]	Inactive[b)]
Betulinaldehyde (**3**)	6.5	25
Betulinic acid (**4**)	Inactive[a)]	25
2-*O*-*E*-*p*-Coumaroylalphitolic (**5**)	0.9	12.5
Alphitolic acid (**6**)	Inactive[a)]	50
Zizyberanalic acid (**7**)	Inactive[a)]	50
Zizyberenalic acid (**8**)	3.0	100
Ceanothic acid (**9**)	Inactive[a)]	Inactive[b)]

a) Inactive at 10 μg/ml. *b*) Inactive at >200 μg/ml.

The antiplasmodial and antituberculosis activities of the isolates **1**—**9** against *P. falciparum* and *M. tuberculosis* were tested, and the 50% inhibitory concentration (IC_{50}), and minimum inhibitory concentration (MIC) values were determined (Table 2). Weak antiplasmodial activities for compounds **2**, **4** and **6**[18,19] and antimycobacterial activities for compound **2**[20] were previously recorded, however they were in the inactive range of our test. The antituberculosis activity of **4** was similar to the reported value.[20] A comparison of compounds **5** with **6** and **1** with **9** indicated that the *p*-coumarate moiety in **5** and the vanillyl group of compound **1** were crucial for high antiplasmodial and antituberculosis potentials. Introduction of a double bond in ring A of the ceanothane-type triterpene **8** (IC_{50} 3.0 μg/ml) highly increased the inhibitory activity in the antiplasmodial assay as compared to compound **7**.

Experimental

General Experimental Procedures Melting points were determined using a Griffin melting point apparatus. Optical rotations were determined on a Jasco P1010 digital polarimeter. UV spectra were obtained on a Shimadzu UV-2401 PC spectrophotometer. IR spectra were measured on a Perkin Elmer FT-IR Spectrum BX spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AVANCE 300 FT-NMR spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C). For the spectra taken in pyridine-d_5, the residual nondeuterated solvent signals at δ_H 8.70 and the solvent signals at δ_C 149.9 were used as references for ^{1}H- and ^{13}C-NMR spectra, respectively. Electron impact (EI) and FAB mass spectra were run on Thermo Finnigan Polaris Q and Finnigan MAT 90 instruments. Unless indicated otherwise, column chromatography and TLC were carried out using Merck silica gel 60 (<0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Plates of silica gel PF_{254}, thickness 1.25 mm, were used for preparative TLC. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4, followed by heating.

Plant Material The dried root bark of *Z. cambodiana* was collected from Chamni District, Burirum Province, Thailand in March, 2001. A voucher specimen (Wicharn Wisetsri 001) is deposited at the CMU Herbarium, Faculty of Science, Chiang Mai University, Thailand.

Extraction and Separation Pulverized, dry root bark (4.85 kg) of *Z. cambodiana* was defatted with hexane and then extracted successively with ethyl acetate (EtOAc) and methanol (MeOH) at 50 °C for 50 h, and the solvents were evaporated to yield EtOAc (60.4 g) and MeOH (629.3 g) extracts, respectively. The EtOAc extract exhibited antiplasmodial and antimycobacterial activities, whereas the MeOH extract was found to be inactive. Thus, the EtOAc soluble fraction was investigated extensively through serial fractionations by quick column chromatography,[21] and eluted with a gradient of hexane, hexane–$CHCl_3$, $CHCl_3$, $CHCl_3$–EtOAc, EtOAc, EtOAc–MeOH, MeOH (5% increment of polar solvent, each 300 ml) to provide six major fractions (Fr. 1—6). Recrystallization of fraction 1 (0.8 g) with hexane and fraction 3 (9.9 g) with CH_2Cl_2, gave **2** (15 mg) and **4** (2.6 g), respectively. The filtrate from fraction 3 (6.9 g) was further subjected to silica gel column chromatography employing solvent gradient hexane–CH_2Cl_2, and ten fractions (fr. 3a to 3j) were collected. Fraction 3d (327 mg) was further separated with silica gel chromatography, and eluted with hexane–CH_2Cl_2 of increasing polarity to yield 11 fractions (fr. 3d-1 to 3d-11). Compounds **3** (5 mg), and **8** (5 mg) were isolated from repeated column chromatography of fraction 3d-3 (16 mg) and 3d-9 (16 mg), respectively. Repeated column chromatography of fraction 3d-8 (381 mg) with CH_2Cl_2–MeOH of increasing polarity, followed by a Sephadex LH20 column (in MeOH), yielded **1** (9.5 mg). A portion of fraction 4 (17.0 g) was fractionated by silica gel chromatography, and eluted with CH_2Cl_2–MeOH of increasing polarity to provide 8 fractions (fr. 4a to 4h), in which **7** (9 mg) and **5** (3.5 mg) were obtained from two individual repeated column chromatographies (silica gel, eluting with $CHCl_3$–MeOH of increasing polarity) of fractions 4d (0.5 g) and 4e (1.6 g), respectively. Fraction 5 (15.4 g) was chromatographed in a similar manner to afford 14 selected fractions (fr. 5a to 5n). **9** (14 mg) and **6** (16 mg) were furnished from two successive column chromatographies (silica gel, $CHCl_3$–MeOH of increasing polarity) of fractions 5e (270 mg) and 5f (279 mg), respectively. All known compounds were identified by comparison of their spectroscopic data (NMR and MS) with those reported in the literature. Complete ^{13}C-NMR data of **5** and **6**, which have not previously been published, are also reported in this paper.

3-*O*-(4-Hydroxy-3-methoxybenzoyl)ceanothic Acid (**1**): Colorless solid, mp 183—185 °C. $[\alpha]_D^{27}$ −22.0° (c=0.229, MeOH). UV λ_{max} (MeOH) nm (log ε): 216 (4.44), 263 (4.07), 294 (3.89). IR (KBr) cm^{-1}: 3430, 2943, 1699, 1602, 1512, 1458, 1378, 1281, 1216, 1104, 1032, 985, 884, 765. HR-FAB-MS *m*/*z*: 635.3592 $[M-H]^-$ (Calcd for $C_{38}H_{52}O_8$-H 635.3584). FAB-MS *m*/*z* 635 ($[M-H]^-$, 22), 423 (10), 219 (10), 205 (15), 167 (100). ^{1}H- and ^{13}C-NMR (C_5D_5N): Table 1.

2-*O*-*E*-*p*-Coumaroylalphitolic Acid (**5**): ^{13}C-NMR (C_5D_5N) δ: 178.8 (s, C-28), 167.5 (s, C-9′), 161.4 (s, C-4′), 151.3 (s, C-20), 144.7 (d, C-7′), 130.5 (d, C-2′), 130.5 (d, C-6′), 126.2 (s, C-1′), 116.8 (d, C-3′, C-5′), 116.0 (d, C-8′), 110.0 (t, C-29), 79.8 (d, C-3), 73.8 (d, C-2), 56.5 (s, C-17), 55.7 (d, C-5), 50.8 (d, C-9), 49.8 (d, C-18), 47.7 (d, C-19), 45.0 (t, C-1), 42.9 (s, C-14), 41.1 (s, C-8), 40.5 (s, C-4), 38.8 (s, C-10), 38.6 (d, C-13), 37.6 (t, C-22), 34.6 (t, C-7), 32.8 (t, C-16), 31.1 (t, C-21), 30.2 (t, C-15), 29.1 (q, C-23), 26.0 (t, C-12), 21.3 (t, C-11), 19.4 (q, C-30), 18.7 (t, C-6), 17.4 (q, C-24 and C-25), 16.3 (q, C-26), 14.8 (q, C-27).

Alphitolic Acid (**6**): ^{13}C-NMR (C_5D_5N) δ: 179.1 (s, C-28), 151.3 (s, C-20), 110.0 (t, C-29), 83.3 (d, C-3), 68.9 (d, C-2), 56.7 (s, C-17), 56.1 (d, C-5),[a] 51.0 (d, C-9), 49.8 (d, C-18), 48.2 (t, C-1), 47.8 (d, C-19), 42.9 (s, C-14), 41.2 (s, C-8), 39.9 (s, C-4), 38.7 (s, C-10),[a] 38.6 (d, C-13), 37.6 (t, C-22), 34.8 (t, C-7), 32.9 (t, C-16), 31.2 (t, C-21), 30.2 (t, C-15), 29.2 (q, C-23), 26.1 (t, C-12), 21.4 (t, C-11), 19.5 (q, C-30), 18.8 (t, C-6), 17.7 (q, C-25), 17.5 (q, C-24), 16.5 (q, C-26), 14.9 (q, C-27), ('a' indicates for the assignments may be reversed for signals with the same superscript).

Antimycobacterial Assay The antimycobacterial activity was assessed against *M. tuberculosis* H_{37}Ra using the Microplate Alamar Blue Assay.[22] Briefly, initial candidate compound dilutions were prepared in DMSO, and subsequent two-fold dilutions were performed in 0.1 ml of 7H9GC medium in the microculture plates. 100 μl of 5×10^4 CFU/ml of *M. tuberculosis* in 7H9GC-Tween was added to each well of 96-well microculture plates containing the test compound. Plates were incubated at 37 °C for 7 d. To three control wells which contained drug and medium, bacteria and medium, or medium only, Alamar Blue dye solution (20 μl of Alamar Blue solution and 12.5 μl of 20% Tween) was added daily until a color change from blue to pink occurred, at which time the dye was added to all remaining wells. Plates were incubated at 37 °C, and results were recorded at 24 h post-dye addition. Fluorescence was measured in a Cytofluoro Series 4000 Fluorescence Multi-Well Plate Reader (Per-Septive Biosystems, Framingham, MA, U.S.A.) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. Percent inhibition was defined as (1−test well FU/mean FU of triplicate control wells)×100. The lowest drug concentration effecting inhibition of ≥90% was considered the MIC. Experiments were usually completed within 10 d. Standard drugs rifampicin, isoniazid and kanamycin sulfate showed MIC of 0.004, 0.06 and 2.5 μg/ml, respectively.

Antiplasmodial Assay Antiplasmodial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug resistant strain) which was cultured continuously according to the method of Trager and Jensen.[23] Quantitative assessment of antiplasmodial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method described by Desjardins *et al.*[24] The inhibitory concentration is that which causes 50% reduction in parasite growth, as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 ng/ml was observed for the standard drug, artemisinin, in the same test system.

Acknowledgments This work was supported by a research team strengthening grant from the National Center for Genetic Engineering and Biotechnology (BIOTEC) and a senior research scholar grant from the Thailand Research Fund (TRF). We are grateful to Chulabhorn Research Institute for recording the mass spectra, to the Department of Chemistry, Silpakorn University for collecting optical rotation data and to James F. Maxwell, Department of Biology, Faculty of Science, Chiang Mai University, for identifying the plant species.

References

1) Yagi A., Okamura N., Haraguchi Y., Noda K., Nishioka I., *Chem. Pharm. Bull.*, **26**, 1798—1802 (1978).
2) Yoshikawa M., Murakami T., Ikebata A., Wakao S., Murakami N., Matsuda H., Yamahara J., *Chem. Pharm. Bull.*, **47**, 1186—1192 (1997).
3) Schuhly W., Heilmann J., Sticher O., *Helv. Chim. Acta*, **83**, 1509—1516 (2000).
4) Ghedira K., Chemli R., Caron C., Nuzillard J.-M., Zeches M., Le Men-Olivier L., *Phytochemistry*, **38**, 767—772 (1995).
5) Han B. H., Park M. H., *Arch. Pharm. Res.*, **10**, 208—211 (1987).
6) Anand K. K., Singh B., Chand D., Chandan B. K., Gupta V. N., *J. Ethnopharmacol.*, **27**, 121—127 (1989).
7) Pandy V. B., Devi S., *Planta Med.*, **56**, 649—650 (1990).
8) Chuakul W., Jaikaew C., *Thai J. Phytopharmacy*, **5**, 28—55 (1998).
9) Suksamrarn S., Suwannapoch N., Ratananukul P., Aroonrerk N., Suksamrarn A., *J. Nat. Prod.*, **65**, 761—763 (2002).
10) Suksamrarn S., Suwannapoch N., Phakodee W., Tanuhiranlert J., Chimnoi N., Ratananukul P., Suksamrarn A., *Chem. Pharm. Bull.*, **51**, 857—859 (2003).
11) Suksamrarn S., Wongkrajang K., Kirtikara K., Suksamrarn A., *Planta Med.*, **69**, 877—879 (2003).
12) Suksamrarn S., Suwannapoch N., Aunchai N., Kuno M., Ratananukul P., Haritakun R., Jansakul C., Ruchirawat S., *Tetrahedron*, **61**, 1175—1180 (2005).
13) Sholichin M., Yamasaki K., Kasai R., Tanaka O., *Chem. Pharm. Bull.*, **28**, 1006—1008 (1980).
14) Monaco P., Previtera L., *J. Nat. Prod.*, **47**, 673—676 (1984).
15) Lee S.-S., Shy S. N., Liu K. C. S., *Phytochemistry*, **46**, 549—554 (1997).
16) Lee S.-S., Su W.-C., Liu K. C., *J. Nat. Prod.*, **54**, 615—618 (1991).
17) Lee S.-S., Lin B.-F., Liu K. C., *Phytochemistry*, **43**, 847—851 (1996).
18) Ziegler H. L., Franzyk H., Sairafianpour M., Tabatabai M., Tehrani M. D., Bagherzadeh K., Hagerstrand H., Staerk D., Jaroszewski J. W., *Bioorg. Med. Chem.*, **12**, 119—127 (2004).
19) Ballin N. Z., Traore M., Tinto H., Sittie A., Molgaard P., Olsen C. E., Kharazmi A., Christensen S. B., *J. Nat. Prod.*, **65**, 1325—1327 (2002).
20) Cantrell C. L., Franzblau S. G., Fischer N. H., *Planta Med.*, **67**, 685—694 (2001).
21) Pederson D. S., Rosenbohm C., *Synthesis*, **16**, 2431—2434 (2001).
22) Collins L., Franzblau S. G., *Antimicrob. Agents Chemother.*, **41**, 1004—1009 (1997).
23) Trager W., Jensen J. B., *Science*, **193**, 673—675 (1976).
24) Desjardins R. E., Canfield C. J., Haynes J. D., Chulay J. D., *Antimicrob. Agents Chemother.*, **16**, 710—718 (1979).