Haloxylines A and B, Antifungal and Cholinesterase Inhibiting Piperidine Alkaloids from *Haloxylon salicornicum*

Sadia Ferheen,[b] Ejaz Ahmed,[a] Nighat Afza,[b] Abdul Malik,*[,a] Muhammad Raza Shah,[a] Sarfraz Ahmad Nawaz,[a] and Muhammad Iqbal Choudhary[a]

[a] *International Center for Chemical Sciences, H.E.J. Research Institute of Chemistry, University of Karachi; Karachi-75270, Pakistan: and* [b] *Pharmaceutical Research Center, PCSIR Laboratories Karachi Complex; Karachi–75280, Pakistan.*
Received November 2, 2004; accepted February 14, 2005

Haloxylines A (1) and B (2), new piperidine alkaloids, have been isolated from the chloroform soluble fraction of *Haloxylon salicornicum* and their structures elucidated by spectroscopic techniques including 2D-NMR. Both the compounds displayed antifungal and cholinesterase enzymes inhibitory potentials.

Key words *Haloxylon salicornicum*; piperidine alkaloid; haloxyline A; haloxyline B; antifungal activity; cholinesterase inhibition

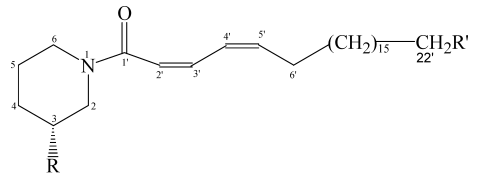
Haloxylon salicornicum (Moq.-Tand.) Boiss belongs to the family Chenopodiaceae which comprises 100 genera and 1200 species. Most of the members of this family are weedy and grow in waste and unfertile areas of soil. In Pakistan, this family is represented by 35 genera. Only five species of genus *Haloxylon* are found in Pakistan. *Haloxylon salicornicum* is a diffuse shrub, pale, much branched, almost leafless, 25—60 cm tall, with woody stem. It is widely distributed in Egypt, Palestine, Jordan, Iraq, Kuwait, Iran, and Pakistan.[1)] The plant is traditionally reported for its toxicity and applied externally on insect stings.[2)] The ash of the plant is used for internal ulcers.[3)] Three alkaloids and a pyranone derivative have so far been reported from this species.[4—6)] A methanolic extract of this plant showed strong cytotoxicity in brine shrimp lethality test.[7)] On further fractionation, the major cytotoxicity was detected in the chloroform-soluble fraction. Further pharmacological screening of this fraction revealed strong antifungal and cholinesterase inhibiting activities. This prompted us to carry out bioassay-guided isolation of bioactive compounds from the chloroform-soluble fraction. As a result of these studies we have isolated two new piperidine alkaloids named as haloxylines A (**1**) and B (**2**). Both **1** and **2** showed antifungal and cholinesterase inhibiting activities.

Results and Discussion

The methanolic extract of the whole plant of *H. salicornicum* was processed as described in Experimental to afford new piperidine alkaloids named as haloxylines A (**1**) and B (**2**). Haloxyline A (**1**) was isolated as colorless crystals (14 mg). The high-resolution (HR) EI-MS of (**1**) showed the molecular ion peak at *m*/*z* 419.3749 (Calcd for $C_{27}H_{49}NO_2$, 419.3763) possessing four degrees of unsaturation. The IR spectrum of **1** showed an amide carbonyl at 1640 cm^{-1} and conjugated double bonds at 1620 cm^{-1}. The UV spectrum displayed absorption at 262 nm, indicating the presence of a conjugated dienamide system. The presence of unsubstituted piperidine ring was indicated by EI-MS and HR-EI-MS at *m*/*z* 84 and 84.0809 $(C_5H_{10}N)^+$. The piperidine ring was further supported by the ^{1}H-NMR spectrum, which had three broad multiplets at δ 3.49 (4H, m, H_2-2, H_2-6), δ 1.60 (2H, m, H_2-4) and δ 1.58 (4H, m, H_2-3, H_2-5). The ^{1}H-NMR further showed olefinic protons at δ 5.32 (1H, d, $J_{2',3'}$=11.5 Hz, H-2′), δ 7.42 (1H, dd, $J_{3',2'}$=$J_{3',4'}$=11.5 Hz, H-3′), δ 6.80 (1H, dd, $J_{4',3'}$=$J_{4',5'}$=11.5 Hz, H-4′) and δ 5.82 (1H, dt, $J_{5',4'}$=11.5 Hz and $J_{5',6'}$=7.2 Hz, H-5′) indicative of a α, β, γ, δ-unsaturated amide. The values of coupling constant between the olefinic protons allowed us to assign *Z* configuration to both the double bonds.[8)] The assignments of these protons were confirmed by analysis of the ^{1}H–^{1}H COSY spectrum and by homodecoupling experiments. Irradiation of the signal of H-2′ (δ 5.32) collapsed the double doublet of H-3′ (δ 7.42) into a doublet (J=11.5 Hz). The irradiation at δ 7.42 caused the doublet at δ 5.32 to collapse into a singlet and that of H-4′ at δ 6.80 into a doublet (J=11.5 Hz). Similarly irradiation at δ 6.80 collapsed the doublet of triplet of H-5′ at δ 5.82 into a triplet (J=7.2 Hz). The presence of a terminal oxymethylene group was revealed by a triplet at δ 3.64 (2H, $J_{22',21'}$=6.5 Hz) while the methylene group adjacent to the olefinic carbon resonated at δ 2.19 (2H, dt, $J_{6',5'}$=7.2 Hz and $J_{6',7'}$=6.5 Hz, H_2-6′). A broad hump between δ 1.22—1.42 integrated for fifteen methylene groups (30-H, H-7′—H-21′). The broad band and distortionless enhancement by polarization transfer (DEPT) ^{13}C-NMR spectra showed the amide carbonyl at δ 167.0 and four olefinic carbons at δ 125.0, 140.9, 130.8 and 128.8, respectively. The hydroxymethylene carbon appeared at δ 63.1. The signals of the piperidine ring were observed at δ 46.7 (C-6), 46.5 (C-2), 25.5 (C-3), 25.3 (C-5) and 24.6 (C-4). All the assignments were confirmed by hetronuclear multiple quantum coherence (HMQC). The cumulative evidences allowed us to assign the structure of haloxyline A (**1**) as (*Z*,*Z*)-1-(22-hydroxy-1-oxo-2,4-docosadienyl) piperidine. The heteronuclear multiple-bond correlations (HMBC) were in exact agreement to the assigned structure.

Haloxyline B (**2**) was also obtained as colorless crystals (12 mg). The molecular formula was established by HR-EI-MS showing molecular ion peak at *m*/*z* 435.3701 (Calcd for $C_{27}H_{49}NO_3$, 435.3712) with four degrees of unsaturation. The UV and IR spectrum were almost superimposable to those of **1**. The presence of hydroxy substituted piperidine ring was revealed by peaks in EI-MS and HR-EI-MS at *m*/*z* 100 and 100.0058 respectively. Therefore, haloxyline B (**2**) differs from (**1**) in having additional alcoholic group in the piperidine ring. In ^{1}H-NMR spectrum the signals of piperidine differed from those of **1**. The hydroxymethine proton

* To whom correspondence should be addressed. e-mail: abdul.malik@iccs.edu



1 R = H, R`=OH
2 R = R`= OH
2a R = R'= (*R*) MTPA
2b R = R'= (*S*) MTPA

Fig. 1. Structures of Haloxyline A (**1**) and Haloxyline B (**2**)

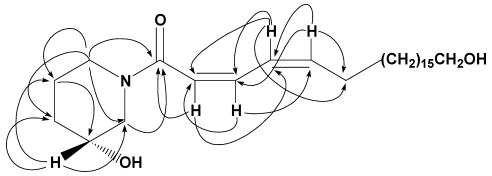


Fig. 2. Important HMBC Correlations of Haloxyline B (**2**)

was observed at δ 4.18 (dt, J=4.0, 8.8 Hz). In ^{1}H–^{1}H COSY spectrum this proton showed correlations to methylene protons at δ 3.51 and δ 1.50, respectively, thereby excluding positions 2 and 4. This allowed us to assign the hydroxyl group at C-3. Other signals of the piperidine ring were in complete agreement to the assigned structure; δ 3.51 (2H, d, $J_{2,3}$= 8.8 Hz, H_2-2), δ 3.40 (2H, t, $J_{6,5}$=6.8 Hz, H_2-6), δ 1.71 (2H, m, H_2-5) and δ 1.50 (2H, m, H_2-4). The larger coupling constant of hydroxymethine proton allowed us to assign β and pseudoaxial configuration. The α and pseudoequatorial configuration of the hydroxyl group was further substantiated by the non observance of hydrogen bonding between the hydroxyl group and piperidine nitrogen which is commonly observed for related compounds of opposite stereochemistry of hydroxyl group at C-3.[9] The (*S*) absolute configuration at C-3 was finally established by Mosher's method. Esterification of **2** with *R*-(+)MTPA-Cl and *S*-(−)MTPA-Cl afforded the corresponding diesters **2b** and **2c**, respectively.

The $\Delta(\delta_S-\delta_R)$ following the MTPA rules,[10,11] indicated (*S*)-configurations at hydroxylic center of **2**. On the basis of these cumulative evidences haloxyline B (**2**) was assigned the structure (*Z*,*Z*)-1-(22-hydroxy-1-oxo-2,4-docosadienyl)-3α-(3*S*) hydroxypiperidine. The ^{13}C-NMR spectrum was in agreement to the assigned structure. The HMBC correlations (Fig. 2) were similar to those observed for **1** except additional correlations due to hydroxyl moiety at C-3. The hydroxylmethine proton at δ 4.18 showed ^2J correlations with C-2 (δ 53.3), C-4 (δ 32.8), and ^3J correlation with C-5 (δ 25.7). The methylene protons at δ 3.51 showed ^2J correlation with C-2 and ^3J correlation with C-22 (δ 167.0). The methylene protons at δ 3.40 showed ^2J correlation with C-5 (δ 25.7) and ^3J correlations with C-2 (δ 53.3) and C-22 (δ 167.0), respectively.

The antifungal activity was determined by agar tube dilution method.[12—14] Haloxylines A (**1**) and B (**2**) showed significant to moderate activities against *Trichophyton longifusus*, *Candida albicans*, *Aspergillus flavus*, *Microsporum canis*, *Candida glabrata* and *Fusarium solani*. The Haloxyline B was slightly more potent probably due to the presence of additional alcoholic moiety at C-3 (Table 1).

Cholinesterases are a class of enzymes represented by

Table 1. Antifungal Activity of Haloxyline A (**1**), and Haloxyline B (**2**) in Agar Tube Dilution Method

Microorganisms	Compound		Standard antifungal drug	MIC (μg/ml)
	1	**2**		
Trichophyton longifusus	70	85	Miconazole	70
Candida albicans	65	80	Miconazole	110
Aspergillus flavus	45	48	Amphotericin B	20
Microsporum canis	70	75	Miconazole	98.4
Candida glabrata	80	85	Miconazole	110.8
Fusarium solani	54	60	Miconazole	73.25

Values are % inhibition of radial growth.

Table 2. *In Vitro* Quantitative Inhibition of AChE and BChE by Compounds **1** and **2**

Compounds	IC_{50}±S.E.M.[a] [μM]	
	AChE	BChE
1	25.3±0.02	19.0±0.03
2	20.2±0.01	14.7±0.02
Galanthamine	0.5±0.05	8.5±0.01

a) Standard mean error (SEH) of three experimental determinations. *c*) Positive control used in assays.

acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), respectively.[15] Their inhibition is an effective tool for the treatment of Alzheimer disease and related dementias.[16] The inhibitory activities of both **1** and **2** against AChE and BChE are illustrated in Table 2.

Experimental

General Experimental Procedure UV and IR spectra were recorded on Hitachi-UV-3200 and Jasco-320-A spectrometers, respectively. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AM-400 spectrometer with tetramethylsilane (TMS) as an external standard. The 2D-NMR spectra were recoded on a Bruker AMX 500 NMR spectrometer. Optical rotations were measured on a Jasco DIP-360 digital polarimeter using a 10 cm cell tube. Mass spectra (EI and HR-EI-MS) were measured in an electron impact mode on Finnigan MAT 12 or MAT 312 spectrometers and ions are given in *m*/*z* (%). TLC was performed with precoated silica gel G-25-UV_{254} plates and detection was done at 254 nm, and by spraying with ceric sulphate in 10% H_2SO_4. Silica gel (E. Merck, 230—400 mesh) was used for column chromatography. Melting points were determined on a Gallenkemp apparatus and are uncorrected.

For antifungal bioassay miconazole and amphotericin B were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.), Sabouraud dextrose agar was purchased from Oxoid Ltd. (Basingstoke, Hampshire, England) and DMSO was purchased from BDH Laboratory Supplies (Poole, England).

Plant Material The whole plant of *Haloxylon salicornicum* (Moq.-Tand.) Boiss was collected from Cholistan desert near district Bahawalpur, Pakistan, in April, 2003 and identified by Dr. Muhammad Arshad, Cholistan Institute of Desert Studies (CIDS), Islamia University Bahawalpur, where a voucher specimen (019/CIDS/IUB/PK) has been deposited.

Extraction and Purification The air dried whole plant (20 kg) was exhaustively extracted with methanol (50 l×3) at room temperature. The extract was evaporated to yield the residue (550 g), which was partitioned between *n*-hexane (60 g), chloroform (75 g), ethyl acetate (40.5 g), *n*-butanol (70 g) and water (38 g). The chloroform-soluble fraction was subjected to column chromatography over silica gel eluted with *n*-hexane–$CHCl_3$, $CHCl_3$, $CHCl_3$–MeOH, in increasing order of polarity. The fractions which were obtained from $CHCl_3$: *n*-hexane (7.5 : 2.5), were combined and rechromatographed over silica gel eluted with *n*-hexane–$CHCl_3$ in increasing order of polarity. The fractions obtained in $CHCl_3$: *n*-hexane (7.2 : 2.8) were subjected to preparative TLC ($CHCl_3$: hexane; 8 : 2) to afford the pure com-

pounds **1** (14 mg) and **2** (12 mg).

Haloxyline A (**1**): Colorless crystals; mp 161—162 °C. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: 7.42 (1H, dd, $J_{3',2'}=J_{3',4'}=11.5$ Hz, H-3′), 6.80 (1H, dd, $J_{4',3'}=J_{4',5'}=11.5$ Hz, H-4′), 5.82 (1H, dt, $J_{5',4'}=11.5$ Hz, $J_{5',6'}=7.2$ Hz, H-5′), 5.32 (1H, d, $J_{2',3'}=11.5$ Hz, H-2′), 3.64 (2H, t, $J_{22',21'}=6.5$ Hz, H-22′), 3.49 (4H, m, H-2, 6), 2.19 (2H, dt, $J_{6',5'}=11.5$ Hz, $J_{6',7'}=6.5$ Hz, H_2-6′), 1.60 (2H, m, H_2-4) and δ 1.58 (4H, m, H_2-3, H_2-5), 1.22—1.42 (30H, br m, H-7′—H-21′). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.0 (C-1′), 140.9 (C-3′), 130.8 (C-4′), 128.8 (C-5′), 125.0 (C-2′), 63.1 (C-22′), 46.7 (C-6), 46.5 (C-2), 25.5 (C-3), 25.3 (C-5), 24.6 (C-4), 32.5 (C-6′), 31.9—24.8 (C-7′—C-21′). IR (KBr) ν_{max} cm^{-1}: 2920, 1640, 1620, 1475, 1100, and 730. UV λ_{max} ($CHCl_3$) nm (log ε): 262 (3.92). EI-MS *m/z* (rel. int.) 419 (16), 335 (18), 241 (22), 234 (28), 206 (15), 178 (30), 112 (33), 84 (100). HR-EI-MS: Found *m/z* 419.3749 (Calcd for $C_{27}H_{49}NO_2$, 419.3763).

Haloxyline B (**2**): Colorless crystals; mp 142—143 °C. ^{1}H-NMR ($CDCl_3$; 500 MHz) δ: 7.42 (1H, dd, $J_{3',2'}=J_{3',4'}=11.5$ Hz, H-3′), 6.80 (1H, dd, $J_{4',3'}=J_{4',5'}=11.5$ Hz, H-4′), 5.82 (1H, dt, $J_{5,4'}=11.5$ Hz, $J_{5',6'}=7.2$ Hz, H-5′), 5.32 (1H, d, $J_{2',3'}=11.5$ Hz, H-2′), 4.18 (dt, $J=4.0$, 8.8 Hz, H-3), 3.51 (2H, d, $J_{2,3}=8.8$ Hz, H_2-2), 3.40 (2H, t, $J=6.8$ Hz, H_2-6), 2.18 (2H, dt, $J_{6',5'}=11.5$ Hz, $J_{6',7'}=6.5$ Hz, H_2-6′), 1.71 (2H, m, H_2-5), 1.50 (2H, m, H_2-4), 1.22—1.42 (30H, br m, H-7′—H-21′). ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 167.0 (C-1′), 139.9 (C-3′), 129.7 (C-4′), 128.8 (C-5′), 124.1 (C-2′), 63.1 (C-22′), 53.3 (C-2), 46.3 (C-6) 32.8 (C-4), 25.7 (C-5) 65.5 (C-3) 32.5 (C-6′), 31.9—24.8 (C-7′—C21′). IR (KBr) ν_{max} cm^{-1}: 3450, 2925, 1640, 1620, 1470, 1100 and 750. UV (MeOH) λ_{max} (log ε): 262 nm (4.0). EI-MS *m/z* (rel. int.) 435 (20), 335 (31), 307 (25), 278 (24), 241 (28), 194 (60), 128 (20), 100 (100). HR-EI-MS *m/z* 435.3701 (Calcd for $C_{27}H_{49}NO_3$, 435.3712). $[\alpha]_D^{20}$ −21.5° ($c=1.0$, $CHCl_3$).

Preparation and Purification of Mosher Ester (*R*)-MTPACl or (*S*)-MTPACl was added to a stirred solution of **2** in CH_2Cl_2 (0.2 ml/0.01 mmol of substrate) and trietylamine (12 eq) separately. The reaction mixture was stirred for 18 h at room temperature, then quenched by adding 1 N HCl and the products were extracted with CH_2Cl_2. The organic phase was washed with $NaHCO_3$ and brine, dried (Na_2SO_4), and concentrated under *vacuo*. Subsequent purification by preparative TLC (CH_2Cl_2 : ethyl acetate 8 : 2) afforded gummy products **2a** and **b**, respectively.

(*R*)-MTPA Ester (**2a**): Gummy product; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.43 (2H, m, ArH), 7.32 (4H, m, ArH), 7.22 (4H, m, ArH), 7.21 (1H, d, H-3′), 6.79 (1H, d, H-4′), 5.81 (1H, dt, H-5′), 5.32 (1H, br d, H-2′), 4.79 (1H, dd, H-3), 3.58 (6H, s, 2×OMe), 3.52 (2H, dd, H-2), 3.35 (2H, t, H-6), 2.18 (2H, dt, H-6′), 1.70 (2H, m, H-5), 1.44 (2H, m, H-4), 1.22—1.42 (30 H, br m, H-7′—H-21′). HR-EI-MS *m/z* 867.4546 (Calcd for $C_{47}H_{63}F_6NO_7$, 867.4531). IR (KBr) ν_{max} cm^{-1}: 2925, 1742, 1640, 1620, 1604, 1470, 1290, 1250, 1100 and 750. $[\alpha]_D^{20}$ +31.2° ($c=1.0$, $CHCl_3$).

(*S*)-MTPA Ester (**2b**): Gummy product; ^{1}H-NMR ($CDCl_3$, 400 MHz) δ: 7.42 (2H, m, ArH), 7.32 (4H, m, ArH), 7.21 (4H, m, ArH), 7.20 (1H, d, H-3′), 6.79 (1H, d, H-4′), 5.81 (1H, dt, H-5′), 5.32 (1H, br d, H-2′) 4.81 (1H, dd, H-3), 3.56 (6H, s, 2×OMe), 3.51 (2H, dd, H-2), 3.40 (2H, t, H-6), 2.18 (2H, dt, H-6′), 1.71 (2H, m, H-5), 1.50 (2H, m, H-4), 1.22—1.42 (30H, br m, H-7′—H-21′). HR-EI-MS *m/z* 867.4567 (Calcd for $C_{47}H_{63}F_6NO_7$, 867.4531). IR (KBr) ν_{max} cm^{-1}: 2925, 1742, 1642, 1619, 1604, 1472, 1289, 1250, 1100 and 750. $[\alpha]_D^{20}$ −29.6° ($c=1.0$, $CHCl_3$).

***In Vitro* Antifungal Bioassay** Antifungal activity was performed using agar tube dilution method.[10—12] Each compound (1.5 mg) dissolved in 1 ml of sterile dimethylsulphoxide (DMSO) served as stock solution. Sabouraud dextrose agar (SDA) (4 ml) was added into screwcaped tubes and autoclaved at 121 °C for 15 min and then cooled to 50 °C. The non-solidified SDA media was poisoned with 66.6 μl of the stock solution to give 200-μg compound/ml of SDA. Tubes were then allowed to solidify in slanting position at room temperature. Each tube was inoculated with 4 mm diameter piece of the inoculum removed from a 7 d old culture of fungi. For non-mycelial growth, an agar surface streak was employed. Inhibition of fungal growth was observed after 7 d of incubation at 28±1 °C. Negative and positive control experiments were also carried out with DMSO and reference antifungal drugs, respectively.

Cholinesterase Inhibition Assay The AChE and BChE inhibiting activities were measured by spectrophotometric method developed by Ellman *et al.*[15] Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (E.C 3.1.1.8), acetylthiocholine iodide, butyrylthiocholine chloride, 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), and galanthamine were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade. Assay conditions and protocol was the same as reported previously.[16] All the experiments were performed in 96-well microtitre-plates by using SpectraMax 340 (Molecular Devices, CA, U.S.A.).

The concentration of test-compounds that inhibited the hydrolysis of substrates (acetylthiocholine and butyrylthiocholine) by 50% (IC_{50}) was determined by monitoring the effect of various concentrations of the compound in the assays on the inhibition values. The IC_{50} (inhibitor conc. that inhibits 50% activity of AChE and BChE) values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, U.S.A.).

References and Notes

1) Ali S. I., Qaiser M., "Flora of Pakistan," Vol. 204, Department of Botany, University of Karachi, Karachi, 2001, p. 185.
2) Chopra R. N., Nayar S. L., Chopra I. C., "Glossary of Indian Medicinal Plants," Council of Scientific and Industrial Research, New Delhi, 1956, p. 130.
3) Sastri B. N., "The Wealth of India," Vol. 5, Council of Scientific and Industrial Research, New Dehli, 1959, p. 5.
4) Gibbons S., Denny B. J., Ali A. S., Mathew K. T., Skelton B. W., White A. H., Gray A. I., *J. Nat. Prod.*, **63**, 839—840 (2000).
5) Michel K. H., Sandberg F., *Acta Pharm. Suec.*, **4**, 97—116 (1967).
6) Michel K. H., Sandberg F., Haglid F., Norin T., *Acta Pharm. Suec.*, **4**, 97—116 (1967).
7) Meyer B. N., Ferrigni N. R., Putnam J. E., Jacobsen L. B., Nicholas P. E., McLaughlin J. L., *Planta Medica*, **45**, 31—34 (1982).
8) Alecio A. C., Bolzani V. S., Young M. C. M., Kato M. J., Furlan M., *J. Nat. Prod.*, **61**, 637—639 (1998).
9) Bolzani V. S., Gunatilaka A. A., Leslie Kigston G. I., *Tetrahedron*, **51**, 5929—5934 (1995).
10) Ohtani I., Kusumi T., Kashman Y., Kkisawa H., *J. Am. Chem. Soc.*, **113**, 4092 (1991).
11) Kusumi T., *Synth. Org. Chem.*, **51**, 462 (1993) and references therein.
12) Brass C., Shainhouse J. Z., Stevens D. A., *Antimicrob. Agents Chemother.*, **15**, 763—768 (1979).
13) Tougu V., *Curr. Med. Chem.*, **1**, 155—170 (2001).
14) Yu S. Q., Holloway H. W., Utsuki T., Brossi A., Greig N. H., *J. Med. Chem.*, **42**, 1855—1861 (1999).
15) Ellman G. L., Courtney K. D., Andres V., Featherstone R. M., **7**, 88—95 (1961).
16) Riaz N., Malik A., Rehman A. U., Muhammad P., Nawaz S. A., Choudhary M. I., *Chem. Biodiv.*, **1**, 1289—1295 (2004).