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Synthesis and Study of NOS-Inhibiting Activity of 2-*N*-Acylamino-5,6-dihydro-4*H*-1,3-thiazine

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Abstract—The synthesis and results of in vitro and in vivo testing of 2-*N*-acetylamino-, 2-*N*-benzoylamino-, 2-*N*-cyclohexanecarbonylamino-, and 2-*N*-(1-adamantanecarbonyl)amino-5,6-dihydro-4*H*-1,3-thiazines for NOS-inhibiting activity have been described.

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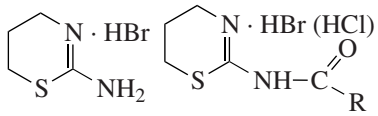
2-Amino-5,6-dihydro-4*H*-1,3-thiazine (**1**) is a well-known inhibitor of NO synthase (NOS) [1, 2] and shows antihypotensive activity in in vivo experiments [3]. With the aim to prolong the duration of antihypotensive action of this compound, we suggested to synthesize more lipophilic analogues that can behave as prodrugs.[1] In this work, we obtained and tested in vitro and in vivo four N-acyl derivatives of thiazine **1** with structurally variable substituents, namely: 2-*N*-acetylamino-, 2-*N*-benzoylamino-, 2-*N*-cyclohexanecarbonylamino-, and 2-*N*-(1-adamantanecarbonyl)amino-5,6-dihydro-4*H*-1,3-thiazines (**2a**–**2d**, Scheme 1). It is worth noting that the selection of substituents was determined not only by their lipophilic properties but also by their possible effect on the rate of hydrolysis of the amide bond in vivo.



1 **2a**–**2d**

2a R = –CH_3
2b R = –Ph
2c R = –cyclohexyl
2d R = –adamantyl

Scheme 1.

Compounds **2a**–**2d** were obtained by Scheme 1. At the first step, parent compound **1** was obtained from 3-bromopropylamine hydrobromide and thiourea through the cyclization of *S*-(aminopropyl)isothiourea dihydrobromide [4]. Then, acylation was carried out using acetic anhydride (for **2a**, Scheme 2) or corresponding acyl chlorides (for **2b**–**2d**).[2] The composition and structure of the obtained compounds were confirmed by elemental analysis, NMR spectroscopy, and mass spectrometry. At the next step, we carried out experiments to assess approximately the inhibiting activity of compounds **2a**–**2d** toward two isoforms of NO synthase, inducible NOS (*i*NOS) and neuronal NOS (*n*NOS) (Fig. 1). The testing was performed in vitro by radiometric method with the use of [^{3}H]-*L*-arginine (a natural substrate of NO synthase). The results indicate that compounds **2a**–**2d** show markedly lower inhibition of NO synthase as compared with parent compound **1** (Figs. 1 and 2).

[1] Prodrug is a compound undergoing biotransformation before it produces a pharmacological effect (this compound is usually inactive or shows low activity prior to biotransformation).

[2] To obtain compound **2d**, adamantanecarbonyl chloride was reacted with 2-amino-5,6-dihydro-4*H*-1,3-thiazine as a base.

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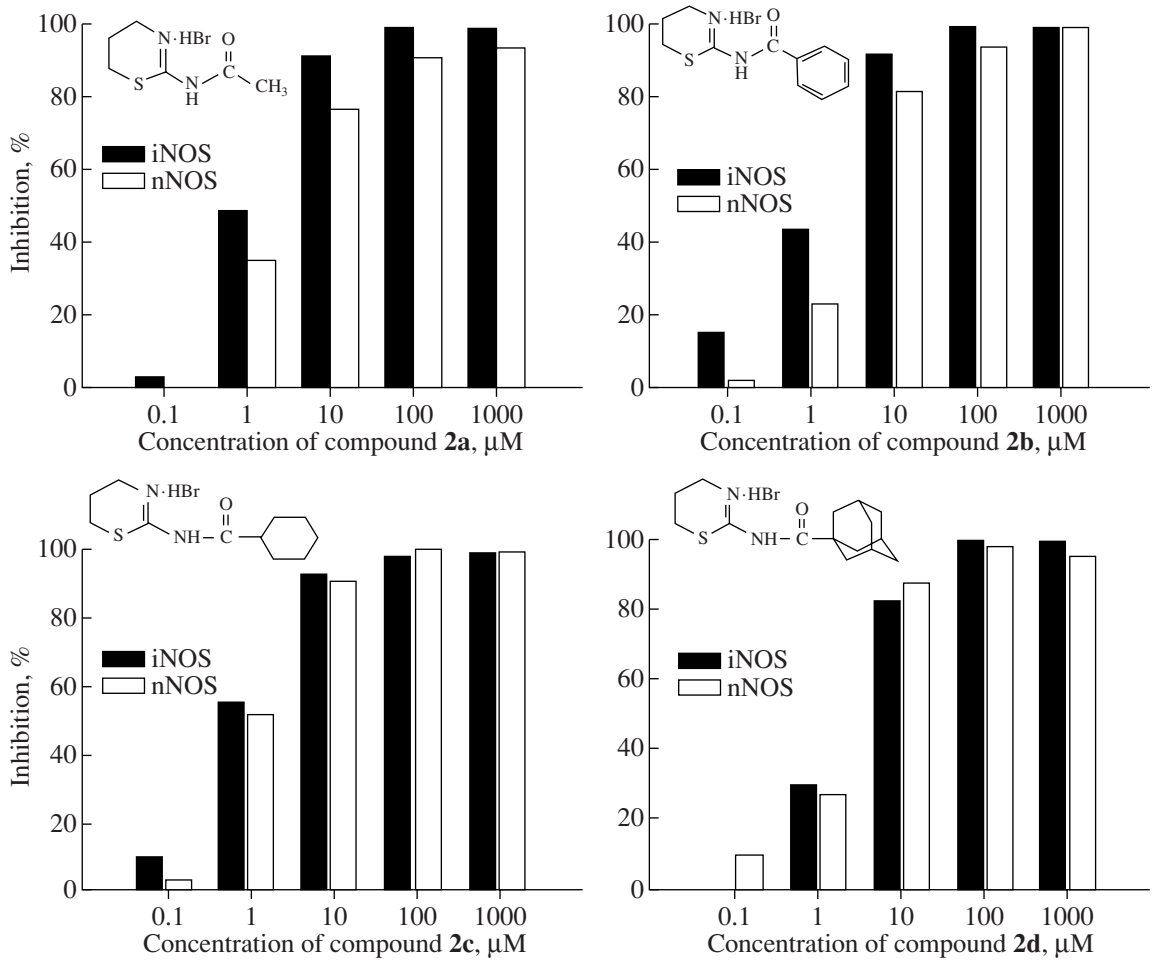
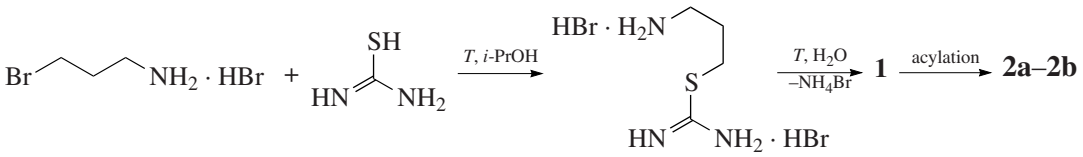


Fig. 1. Concentration dependence for the inhibition of NOS isoforms by compounds **2a**–**2d** (see Experimental for the procedure).



Scheme 2.

At the final step of the study, we tested the physiological activity of compounds **2a**–**2d** in laboratory animals. Originally, the compounds were assessed for nitrogen oxide (NO) production in mouse liver. Compounds **2a** and **2b** tested ex vivo showed almost the same inhibiting effect on NO production in mouse liver as parent thiazine **1** (the dose necessary to inhibit NO production by 50% (ID_{50}) is about 3 μmol/kg). The NO-inhibiting activity ex vivo of acyl derivatives **2c** and **2d** was found to be much lower (by one to two orders of magnitude) than that of the parent thiazine. The study of antihypotensive activity of compounds **2a** and **2b** in vivo carried out in rats, septic shock model including, showed that the antihypotensive effect of compound **2a** is approximately two times longer than that of thiazine **1**.

Thus, the best result in the series of the compounds obtained was achieved for compound **2a**, which proved to be an efficient prodrug of thiazine **1**. Taking into consideration that compound **2a** shows very low toxicity in vivo, we applied for patent for 2-acetylamino-5,6-dihydro-4*H*-1,3-thiazine **2a** as a potential antihypotensive remedy [5].

EXPERIMENTAL

^1H and ^{13}C NMR spectra were recorded on a Bruker WP-100SY and Bruker CXP-200 spectrometers operating at 100 and 200 MHz, respectively, using TMS as

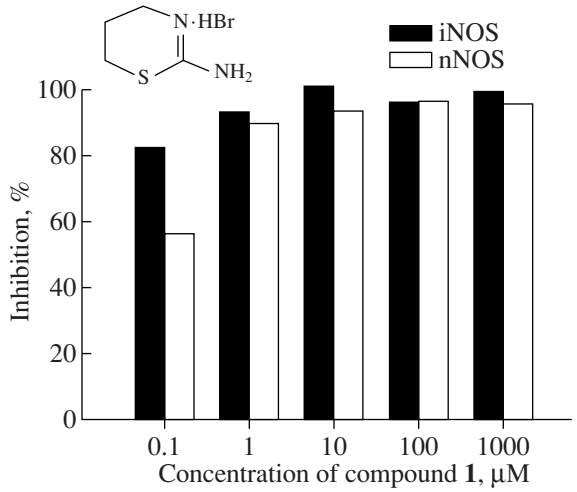


Fig. 2. Concentration dependence for the inhibition of NOS isoforms by parent thiazine **1**.

an internal reference. Reaction course was monitored by thin-layer chromatography (TLC) on Silufol UV-254 plates with the use of the butanol–acetone–formic acid (1 : 1 : 1) eluent system. Mass spectra were obtained on a Shimadzu LCMS-2010A quadrupole mass spectrometer with electrospray ionization.

2-*N*-Acetylamino-5,6-dihydro-4*H*-1,3-thiazine hydrobromide 2a. A 5-mL (0.053 mol) portion of acetic anhydride was added to 0.7 g (0.0036 mol) of 2-aminothiazine hydrobromide, and the mixture was heated at 90°C under magnetic stirring for 10.5 h. After cooling, the precipitate was filtered off and washed with ether to give 0.72 g of compound **2a**. Yield 84%. Mp 192–194°C (lit. [4]: mp 179–180°C). For $C_6H_{11}BrN_2OS$ anal. calcd. (%): C, 30.14; H, 4.64; N, 11.71. Found (%): C, 30.25, 30.32; H, 4.39, 4.52; N, 11.62, 11.86. MS (*m*/*z*): 117 ($C_4H_9N_2S^+$), 159 ($C_6H_{12}N_2SO^+$). ^{13}C NMR (DMSO-d_6–CCl_4 (1 : 4), δ, ppm): 172.39 (C-7), 166.42 (C-2), 41.97 (C-4), 26.80 (C-6), 24.30 (C-8), 19.22 (C-5). ^{1}H NMR (DMSO-d_6–CCl_4 (1 : 4), δ, ppm, *J*, Hz): 2.15 (m, 2H, CH_2), 2.32 (s, 3H, CH_3), 3.40 (t, 2H, *J* = 5.6, CH_2S), 3.75 (t, 2H, *J* = 5.6, CH_2N), 12.10 (br s, 2H, NH + N^+H).

2-*N*-Benzoylamino-5,6-dihydro-4*H*-1,3-thiazine hydrobromide 2b. A 2-mL (0.017 mol) portion of benzoyl chloride was added to 0.3 g (0.0015 mol) of 2-aminothiazine hydrobromide, and the mixture was heated at 140°C under magnetic stirring for 15–20 min. After cooling, the precipitate was filtered off and washed with ether to give 0.35 g of compound **2b**. Yield 76%. Mp 220–223°C (lit. [4]: mp 218–220°C). For $C_{11}H_{13}BrN_2OS$ anal. calcd. (%): C, 43.86; H, 4.35; N, 9.30. Found (%): C, 43.62, 43.98; H, 4.46, 4.28; N, 9.48, 9.32. ^{1}H NMR (DMSO-d_6–CCl_4 (1 : 4), δ, ppm, *J*, Hz): 2.15 (m, 2H, CH_2), 3.35 (t, 2H, *J* = 5.6, CH_2S), 3.80 (t, 2H, *J* = 5.6, CH_2N), 7.78–8.45 (m, 5H, H_{arom}), 12.10 (br s, 2H, NH + N^+H).

2-*N*-Cyclohexanecarbonylamino-5,6-dihydro-4*H*-1,3-thiazine hydrobromide 2c. A 4-mL (0.017 mol) portion of cyclohexanecarbonyl chloride was added to 0.5 g (0.0025 mol) of 2-aminothiazine hydrobromide, and the mixture was heated at 170°C under magnetic stirring for 20–25 min. After cooling, 10 mL of diethyl ether was added, the precipitate was filtered off and washed with ether to give 0.67 g of compound **2c**. Yield 86%. Mp 153–154°C. For $C_{11}H_{19}BrN_2OS$ anal. calcd. (%): C, 43.00; H, 6.23; N, 9.12. Found (%): C, 43.16, 43.32; H, 6.34, 6.28; N, 8.94, 9.18. ^{1}H NMR (DMSO-d_6–CCl_4 (1 : 4), δ, ppm, *J*, Hz): 1.40 (m, 5H, C_6H_{11}), 1.85 (m, 3H, C_6H_{11}), 1.95 (m, 2H, CH_2), 2.20 (m, 2H, CH_2), 2.65 (m, 1H, CH–CO), 3.35 (t, 2H, *J* = 6, CH_2S), 3.70 (t, 2H, *J* = 6, CH_2N), 12.10 (br s, 2H, NH + N^+H).

2-*N*-(1-Adamantanecarbonyl)amino-5,6-dihydro-4*H*-1,3-thiazine hydrochloride 2d. A 1-mL portion of a 10% NaOH solution was added to a solution of 0.5 g (0.0025 mol) of 2-aminothiazine hydrobromide in 5 mL of water with stirring. Five minutes later, 10 mL of $CHCl_3$ was added and the mixture was stirred for 1 h. The organic layer was separated and dried with $MgSO_4$, and the solvent was removed. Triethylamine (0.33 mL) and 1-adamantanecarbonyl chloride (0.48 g, 0.017 mol) were added to the residue. The mixture was stirred for 1 h, the precipitate of triethylamine hydrochloride was filtered off, and the filtrate was concentrated on a rotary evaporator. The residue was dissolved in 3 mL of isopropanol, and 5 mL of concentrated HCl was added dropwise with stirring. The precipitate was filtered off and dissolved in acetone, the solution was filtered, and the filtrate was concentrated to give 0.27 g of compound **2d** as white crystals. Yield 36%. Mp 243–245°C. For $C_{15}H_{23}ClN_2OS$ anal. calcd. (%): C, 57.22; H, 7.36; N, 8.90. Found (%): C, 57.38, 57.45; H, 9.07, 8.99; N, 8.94, 9.18. ^{1}H NMR (DMSO-d_6–CCl_4 (1 : 4), δ, ppm, *J*, Hz): 1.75–2.20 (m, 15H, cage), 2.40 (m, 2H, CH_2), 2.65 (m, H, CH–CO), 3.40 (t, 2H, *J* = 6.5, CH_2S), 3.85 (t, 2H, *J* = 6.5, CH_2N), 12.30 (br s, 2H, NH + N^+H).

Testing in vitro. The activity of NO synthase was measured for two samples: *i*NOS isolated from lipopolysaccharide-stimulated mouse macrophages produced by Cayman Chemical, United States, (22.43 units/mg, 4.97 mg/mL, 111.5 units/mL, catalog number 60862) and a soluble fraction of rat cerebellum homogenate (NO synthase activity detected in this sample is represented mainly by calcium-dependent *n*NOS). The activity of NOS was determined by the radiometric method (Wallac-1414, WinSpectral, Finland) from the rate of accumulation of [^{3}H]-L-citrulline in the NOS-catalyzed reaction of oxidation of [^{3}H]-L-arginine (0.37 Ci/mmol) [6] in an in-house experimental modification [7]. The results are represented (see Figs. 1 and 2) as an inhibition percent (i.e., the difference between the activities of the samples without inhibitors and those containing inhibitors expressed as a percent of the activity of samples without inhibitors).

Testing in vivo. Nitrogen oxide production in the liver of laboratory animals was carried out ex vivo in white non-inbred female mice 5 months old, weighing 27–30 g, derived from Swiss line. Four hours before euthanasia with ether, *E. coli* lipopolysaccharide (LPC) was injected into the animals (of tested and control groups) (1.5 mg/kg, 0.5 mL of physiological salt solution per mouse). Three hours after injection, compounds **2a**–**2d** were injected intraperitoneally into the tested animals (physiological salt solution was injected into the animals of the control group); 1 h later, liver samples were removed and frozen in liquid nitrogen. $NO^{\bullet}$ production was determined by EPR spectroscopy using the spin trap technique by A.F. Vanin [8, 9].

The antihypotensive activity in vivo was studied in rats (Wistar, male and female weighting 200–310 g). The animals were narcotized by intraperitoneal injection of Nembutal (55 mg/kg), and the background values of systolic and diastolic pressure from the left carotid artery, heart rate, and respiration rate were recorded. Then, compounds **2a** and **2b** in a 0.9% aqueous NaCl solution were injected and the above parameters were monitored (until stable and distinct decrease in arterial pressure was achieved). Compounds **2a** and **2b** were tested in a similar manner in experimental animals with permanent hypotonia, a model of septic shock induced by LPC (18 mg/kg of LPC solution was injected into an animal into jugular vein in 2–3 min immediately after recording the background values).

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