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Novel Compounds Designed as Antistress Agents

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Agents against biologic stress were designed, containing GABA esterified with lorazepam and amidated with (*R*)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (**5**) or 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (**6**). Compounds **5** and **6** inhibited lipid peroxidation, IC_{50} 1.1 and 24 μM. Oxidative damage accompanied stress, **5** and **6** reduced radical attack, uropepsinogen, and morphological changes. Stress increased drug metabolism. Treatment with **5** reduced cytochrome P450 and *N*-demethylation of erythromycin, 35 and 40%. Compounds **5** and **6** decreased lipidemic indices of hyperlipidemic rats 40−63%.

1. Introduction

Biologic stress (BS[a]), the homeostatic response of the organism to various challenges, is a defensive phenomenon regulated by the central nervous, immune, and endocrine systems.[1−3] There is a hypothalamic−pituitary−adrenal axis activation, indicating the decisive role of the brain in the defense against stressors.[4] BS causes tissue injury, however, the underlying molecular mechanism only recently started being clarified. Thus, there is a close relationship between biologic and oxidative stress (OS).[5] The extensive overlap between the pathologic conditions where BS or OS are implicated further confirms their interconnection.

In this work, we designed antistress and antioxidant structures as agents against the pathologic consequences of stress. The central part of these compounds contains a residue of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). The carboxylic group of GABA is esterified with the anxiolytic benzodiazepine lorazepam, and the amino group of GABA is amidated by two known potent antioxidants, (*R*)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) and 3,5-di-*tert*-butyl-4-hydroxybenzoic acid (BHBA). Thus, they are expected to maintain the properties of the antioxidant carboxylic acids, the anxiolytic effect of the benzodiazepine, and to act as GABA-mimetics or GABA liberating agents in the brain. Their in vitro antioxidant activity is assessed and their effect on stressed rats is examined, measuring morphologic and biochemical indices of BS and oxidative insult.

2. Results and Discussion

2.1. Synthesis. The synthetic pathway is shown in Scheme 1. **5** and **6** are the target structures; however, **3** and **4** are also novel. Almost in all steps of these reactions, yields were about 60%. Compounds were isolated and identified spectroscopically and by combustion analysis. Compound **4**, highly hygroscopic with very deliquescent crystals, could not be handled for elemental analysis, however, spectroscopic evaluation agreed with its structure and was successfully used for the preparation of **5** and **6**.

2.2. Biological Evaluation. 2.2.1. In Vitro Effect on Lipid Peroxidation. The time course of lipid peroxidation, as affected by **5**, **6**, trolox, and BHBA, as well as their IC_{50} values after 45 min of incubation, were determined. The IC_{50} values, 1.1 and 24 μM for **5** and **6**, are by far better than the well-known antioxidants trolox and BHBA (IC_{50} values 25 and 257 μM, respectively). To the best of our knowledge, only a few antioxidant compounds are reported with IC_{50} lower than 1.1 μM.

2.2.2. In Vivo Studies. 2.2.2.1. Biologic Stress. Stress was induced by applying interrupted immobilization and food and water deprivation. For the evaluation of stress, morphologic changes were measured. These changes have been noticed and used by H. Selye ever since he described scientifically the phenomenon of stress.[6] Uropepsinogen (UPG) increase is a characteristic, easy to measure, and reliable biochemical change in stress. We have found that uropepsinogen is greatly increased in BS and runs in parallel with corticosterone concentration.[1,5] The effect of **5** and **6** on these indices of BS is shown in Table 1. **5** and **6** can efficiently reduce biologic stress. We attribute this not only to the presence of the GABA and benzodiazepine residues but also to their strong antioxidant ability, which can play substantial role in antistress properties, as we have shown using vitamin E.[5]

2.2.2.2. Oxidative Stress Caused by Biologic Stress. The applied BS caused oxidative damage, as this is assessed by lipid peroxidation of the liver. Treatment of the stressed animals with **5** and **6** significantly reduced malondialdehyde (MDA) formation (Table 1), thus confirming our previous results with vitamin E.[5]

2.2.2.3. Biologic Stress and Drug Metabolism. It has been known for many years that stress causes induction of the

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[a] Abbreviations: BHBA, 3,5-di-*tert*-butyl-4-hydroxybenzoic acid; BS, biologic stress; DCC, *N*,*N*-dicyclohexylcarbodiimide; DMAP, 4-dimethylamino-pyridine; MDA, malondialdehyde; OS, oxidative stress; *t*-BocON, (2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile; UPG, uropepsinogen.

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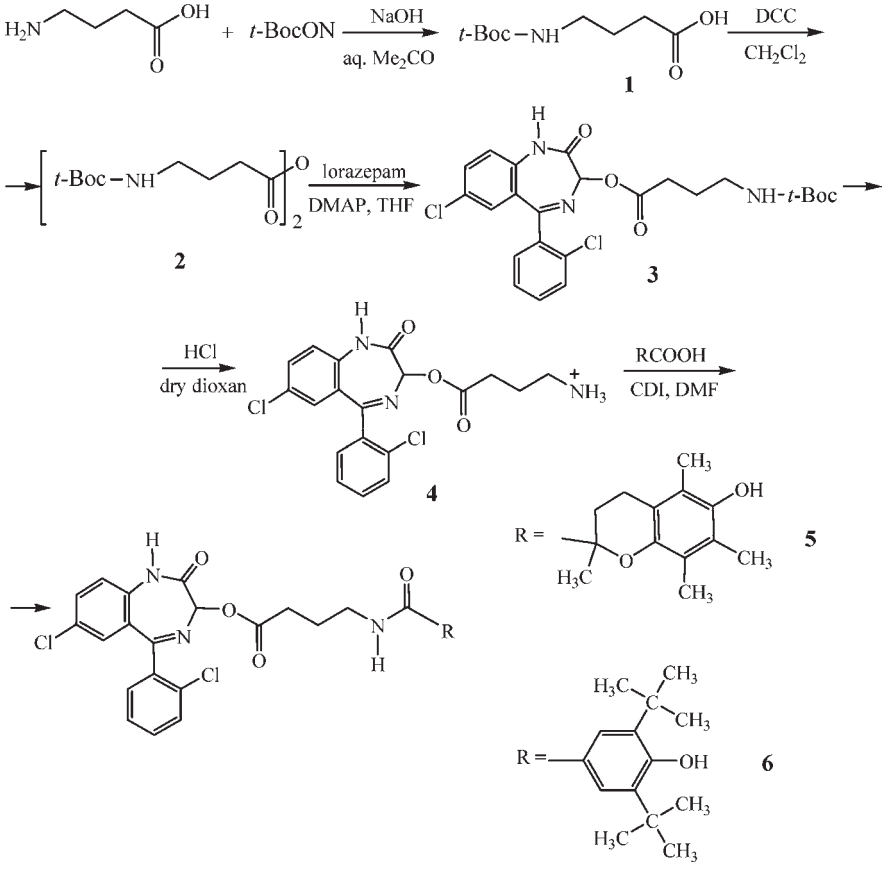
Table 1. Effect of Compounds **5** and **6** on Some Morphologic and Biochemical Indices of Biologic and Oxidative Stress

animal groups	thymus[a]	adrenals[a]	liver[a]	BW change %	UPG kU/h	MDA nmol/mg protein
stress +**5** (group 1)	$117 \pm 22^{d,e}$	34 ± 2^{f}	$4.20 \pm 0.01^{b,g}$	$-10.0 \pm 1.1^{d,f}$	$34 \pm 8^{d,g}$	$37.5 \pm 0.4^{d,g}$
stress +**6** (group 2)	$151 \pm 7^{d,g}$	$40 \pm 3^{d,f}$	4.50 ± 0.31^{e}	$-3.8 \pm 1.6^{d,g}$	$30 \pm 13^{c,g}$	$41.2 \pm 0.3^{d,g}$
stress (group 3)	87 ± 9	46 ± 1^{d}	3.40 ± 0.01^{d}	-16.8 ± 1.4^{d}	127 ± 18^{d}	52.5 ± 0.6^{d}
control (group 4)	214 ± 9	32 ± 1	4.60 ± 0.15	$+4.1 \pm 0.1$	15 ± 1	22.5 ± 0.3

[a] In mg/100 g body weight (BW). [b] $P < 0.05$. [c] $P < 0.01$. [d] $P < 0.001$, compared to control animals. [e] $P < 0.05$. [f] $P < 0.01$. [g] $P < 0.001$, compared to stressed animals (Student's *t*-test).

Scheme 1. Synthesis of Compounds: Protection on the GABA Amino Group with Boc-ON (**1**) Was Followed by Esterification of Lorazepam with GABA, Via the Symmetric Anhydride (**2**), Using DCC and DMAP (**3**); Deprotection of the Amino Group of **3** Gave Compound **4**, and Reaction with the Antioxidant Carboxylic Acids Using CDI Gave the Final Products **5** and **6**



drug metabolic activity of the liver[7] and increases resistance to drugs.[8] The higher resistance of the organism to xenobiotics is attributed to the induction of cytochrome P450 enzymes due to the increased corticoids during stress.[9] Glucocorticoids such as cortisol and corticosterone are mainly metabolized by CYP3A, the most abundantly expressed CYP in the liver, and they also induce this subfamily. The mechanism of this induction is complex, however it is believed that glucocorticoid receptor and possibly some other members of the nuclear hormone receptor superfamily are responsible for this phenomenon.[10] The effect of the most potent antioxidant, **5**, on P450 (nmol/g protein) and erythromycin *N*-demethylation (nmol/min/nmol P450) for control, stress and stress + **5** is (Student's *t*-test): 2.50 ± 0.02, 1.0 ± 0.1, 5.40 ± 0.04***, 2.5 ± 0.3***, 3.50 ± 0.03*** $^{+2}$, $1.5 \pm 0.2^{+}$, ***$P < 0.001$ compared to control; $^{+2}P < 0.01$, $^{+}P < 0.05$, compared to stress. These results show that stress increased significantly both total hepatic cytochrome P450 content as well as the metabolism of erythromycin, a CYP3A substrate.[11] Treatment of the stressed rats with **5** reduced cytochrome P450 and the oxidative *N*-demethylation of erythromycin by 35 and 40%, respectively. This decrease caused by **5** toward normal values could be attributed to the limited increase of corticoids due to the reduction of biologic stress as well as to its antioxidant properties.

2.2.2.4. Effect of Compounds 5 and 6 on Dyslipidemia. Compounds **5**, **6**, trolox, and BHBA were administered to hyperlipidemic rats at equimolar doses, and their effect on total cholesterol, triglyceride, and LDL-cholesterol plasma levels (mg/dL plasma) was as follows (Student's *t*-test): **5**: 101.6 ± 20*, 507.2 ± 83.0**, 33.4 ± 1.0**; trolox: 157.5 ± 21.7, 1033.6 ± 113.0, 43.1 ± 3.3; **6**: 75.6 ± 14.0**, 336.9 ± 119.0**, 30.8 ± 6.0**; BHBA: 146.1 ± 9.0, 630.5 ± 50.0, 49.2 ± 2.0; control: 206.3 ± 41.7, 846.4 ± 135.0, 60.0 ± 11.0; *$P < 0.05$, **$P < 0.01$, compared to dyslipidemic control rats. Compounds **5** and **6** had a remarkable effect, greater than the parent antioxidants, on all lipidemic indices, offering a decrease of 40−63%.

BS affects the cardiovascular system. During stress, free fatty acids are released under the influence of hormones. Stress has been found to develop atherosclerosis in cynomologous monkeys on atherogenic diet[12] and in the Watanabe

heritable hyperlipidemic rabbit.[13] In this respect, the antidyslipidemic activity of **5** and **6** is expected to widen the spectrum of their antistress effects.

In conclusion, BS induced by stressors such as immobilization and food and water deprivation and assessed by established morphological and biochemical markers can produce oxidative damage. Common antistress agents like anxiolytics alone cannot withdraw biologic stress completely, while they are not effective antioxidants.[1] Glucocorticoids, produced during stress, attenuate the inhibitory GABA neuronal pathway, and gabapentine, a synthetic GABA analogue, has been found to reduce oxidative damage, as well as anxiety, through GABA-A receptors.[14] The designed compounds, although quite lipophilic (calculated logP are 5.51 and 6.35, respectively) are able to reduce BS as well as the ensuing oxidative damage. Using lipoic acid or cysteine as the antioxidant acids, they may be useful in the development of less lipophilic, and thus possibly more active, molecules against biologic stress and its complications.

3. Experimental Section

3.1. General. Starting materials, reagents, and solvents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. ^{1}H NMR spectra were recorded using a BRUKER AC-400 MHz spectrometer. Melting points (mp) were determined with a MEL-TEMPII (Laboratory Devices, USA) apparatus and are uncorrected.

Purity of all compounds was found to be equal to or greater than 95% by combustion analysis.

Fischer-344 male rats were kept in a controlled temperature room (22 ± 2 °C), with free access to laboratory chow and tap water, under a 12 h light/dark cycle, and treated according to the European Communities Council Directive of 24 November 1986 (86/609/EEC).

3.2. Synthesis. 4-(*tert*-Butoxycarbonylamino)butanoic acid (**1**) was prepared according to the literature[15] with some modifications: GABA (10 mmol) was dissolved in 1N aq NaOH (10 mL), *t*-BocON (2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile, 11 mmol) in acetone (140 mL), the two solutions were mixed, the mixture was left at room temperature for 24 h, the solvents were evaporated, the residue was washed with ether, ethyl acetate was added (35 mL), and the pH was brought to 2−3 with the addition of $KHSO_4$ (15 mM, 50 mL). The aqueous layer was extracted with ethyl acetate, dried (Na_2SO_4), the solvent evaporated, and the residue kept at −20 °C to give **1** (89%) as a crystalline product, mp 52−54 °C.

4-(*tert*-Butoxycarbonylamino)butanoic acid anhydride (**2**) was prepared according to the literature[15] from **1** (4.4 mmol) and DCC (*N*,*N*-dicyclohexylcarbodiimide, 2.4 mmol) in dry CH_2Cl_2. **2** was recrystallized from ethyl acetate/petroleum ether 1/4, mp 98 °C, yield 93%.

***tert*-Butyl 3-((*E*)-7-chloro-5-(2-chlorophenyl)-1,3-dihydro-2-oxo-2*H*-1,4-benzodiazepin-3-yloxy)carbonyl)propylcarbamate (3).** Lorazepam ((*E*)-7-chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2*H*-1,4-benzodiazepin-2-one, 0.8 mmol) and **2** (0.95 mmol) were dissolved in peroxide-free, dry, freshly distilled tetrahydrofuran (15 mL), DMAP (4-dimethylamino-pyridine, 0.8 mmol) was added, and the mixture was left in a nitrogen atmosphere for 48 h. The solvent was evaporated, and ethyl acetate (30 mL) added and washed with water. The organic phase was dried (Na_2SO_4), the solvent evaporated, and the residue was purified by flash chromatography (ethyl acetate/petroleum ether 1/3), to give 1.2 g of **3** (68%) as crystals, mp 74 °C.

^{1}H NMR ($CDCl_3$ + DMSO-d_6) δ (ppm): 1.40 (s, 9H, $C(CH_3)_3$), 1.70−1.90 (m, 2H, C-3 GABA), 2,50 (t, J = 14,40 Hz, 2H, C-2 GABA), 3.20 (q, J = 16,00 Hz, 2H, C-4 GABA), 4.92 (s, 1H, −NHCO−), 6.05 (s, 1H, C-3 lorazepam), 6.90−7.60 (m, 7H arom), 9.50 (s, 1H, NH lorazepam).

(*E*)-7-Chloro-5-(2-chlorophenyl)-1,3-dihydro-2-oxo-2*H*-1,4-benzodiazepin-3-yl 4-aminobutanoate hydrochloride (4). To a solution of **3** (1.2 mmol) in dry dioxan (10 mL), a solution of HCl in dioxan (4 M, 3.6 mL) was added, the mixture was stirred (70 min), most of the solvent evaporated, ethyl acetate (40 mL) was added, the precipitate was collected, washed with dry ether, dried in vacuo over P_2O_5, and recrystallized (ethyl acetate/ether) to give 1.55 g (89%) of **4**, mp 152−160 °C, a very hygroscopic product.

^{1}H NMR ($CDCl_3$ + DMSO-d_6) δ (ppm): 1.70−2.30 (m, 2H, C-3 GABA), 2.60 (t, J = 16,00 Hz, 2H, C-2 GABA), 2.80−3.10 (m, 2H, C-4 GABA), 7.00 (s, 1H, C-3 lorazepam), 7.20−7.80 (m, 7Harom.), 8.20 (brs, 4H, NH lorazepam, NH_3^+).

(*E*,*R*)-7-Chloro-5-(2-chlorophenyl)-1,3-dihydro-2-oxo-2*H*-1,4-benzodiazepin-3-yl 4-(6-Hydroxy-2,5,7,8-tetramethylchroman-2-carbonylamino)butanoate (5). Trolox (0.5 mmol) was dissolved in dimethylformamide (DMF, 15 mL), cooled, and CDI (1,1-carbonyldiimidazole, 0.7 mmol) was added. **4** (0.55 mmol) in ethyl acetate (15 mL) was added and the mixture left at room temperature under nitrogen for 24 h. Then a small volume of water was added, the precipitate was collected, dissolved in CH_2Cl_2, washed with $NaHCO_3$ (5%), H_2O, dried ($CaCl_2$), the solvent evaporated, and the residue was purified by flash chromatography (petroleum ether/ethyl acetate 5/1), to give **5**, yield 70%, mp 144−145 °C.

^{1}H NMR ($CDCl_3$) δ (ppm): 1.10−1.40 (m, 3H, C-3 GABA C-3 chroman), 1.81 (s, 3H, 2-CH_3), 1.90 (m, 2H, C-2 GABA), 1,95 (s, 3H, 5-CH_3), 2.00 (s, 3H, 7-CH_3), 2.05 (s, 3H, 8−CH_3), 2.40−2.70 (m, 3H, C-3 and C-4 chroman), 4.10 (t, J = 8,00 Hz, 2H, C-4 GABA), 5.30 (bs, 1H, OH), 5.90 (s, 1H, C-3 lorazepam), 6.40 (bs, 1H, NHCO), 7.00−7.80 (m, 7H arom), 9.50 (s, 1H, NH lorazepam).

(*E*)-7-Chloro-5-(2-chlorophenyl)-1,3-dihydro-2-oxo-2*H*-1,4-benzodiazepin-3-yl 4-(3,5-Di-*tert*-butyl-4-hydroxy-benzoylamino)butanoate (6). The procedure described for **5** was applied. The final product was purified by flash chromatography (petroleum ether/ethyl acetate 4/1), to give **6**, yield 52%, mp 112−113 °C.

^{1}H NMR ($CDCl_3$) δ (ppm): 1.20 (s, 18H, 2 × $C(CH_3)_3$), 2.40−2.80 (m, 2H, C-3 GABA), 3.50 (t, J = 8.0 Hz, 2H, C-2 GABA), 4.00−4.30 (m, 2H, C-4 GABA), 5.50 (s, 1H, OH), 6.10 (s, 1H, C-3 lorazepam), 6.90−7.80 (m, 10H, 9H arom+NHCO), 9.50 (s, 1H, NH lorazepam).

3.3. In Vitro Effect on Lipid Peroxidation. It was assessed spectrophotometrically (535 vs 600 nm) according to the literature.[16] All compounds and solvents were found not to interfere with the assay.

3.4. Biologic Stress. 3.4.1. Protocol. Four groups of 6−10 rats (180 ± 10 g) were used.

Group 1: Rats were administered **5** (0.055 mmol/kg, ip, as an aq suspension with a few drops of Tween 80) and then kept immobilized without food and water for 10 h and left free in metabolic cages individually, with access to food and water for 2 h. The whole process was repeated three times, thus the treatment was given at 0, 12, 24, and 36 h. Group 2: Rats were treated as in group 1, compound **6** was given instead of **5**. Group 3: Rats were treated as in group 1, and the liquid vehicle was given instead of compounds. Group 4: Rats were kept in metabolic cages, and the liquid vehicle was given instead of compounds.

After the last application of stressors, rats were left in metabolic cages for 3 h, weighed, and thymus, adrenals, and liver were excised and weighed separately. Urine was also collected.

3.4.2. Tissue and Fluid Preparation. Hepatic microsomal fraction was prepared. Hepatic microsomes and urine were kept at −80 °C until use.

3.4.3. Determination of Uropepsinogen. Following conversion of uropepsinogen to uropepsin by an initial incubation of urine

speciments at acidic pH, urine was incubated with buffered homogenized milk, thus casein was converted to insoluble paracasein. The time required for visible appearance of aggregates of paracasein particles on the test tube wall was taken as the end point of the reaction. Results of uropepsinogen concentration are expressed in arbitrary units of uropepsinogen excreted per hour.[5]

3.4.4. Hepatic Lipid Peroxidation. Lipid peroxidation of hepatic microsomal fractions from treated rats was determined fluorometrically as thiobarbituric acid reactive substance.[17]

3.4.5. Hepatic Drug Metabolism. 3.4.5.1. Determination of Total Hepatic Cytochrome P450. Total hepatic P450 content was determined spectroscopically.[18]

3.4.5.2. Cytochrome P450-Mediated Metabolism of Erythromycin. The experimental procedure was performed according to the literature.[19]

3.4.5.3. Protein Determination. Total protein in liver microsomal fractions was estimated using bovine serum albumin as a standard.[20]

3.4.6. Development of Dyslipidemia. An aq solution of Triton WR 1339 was administered ip (200 mg/kg) to rats (230−280 g), and 1 h later the examined compounds (56 μmol/kg) dissolved in saline or saline only were given ip.[21] After 24 h, plasma total cholesterol, LDL-cholesterol and triglyceride concentrations were determined.

References

(1) Kourounakis, P. N.; Rekka, E.; Retsas, S. The pharmacochemistry of stress: effect of chlordiazepoxide and diazepam on some manifestations of stress. *Sci. Pharm.* **1990**, *58*, 389–393.

(2) Schneiderman, N.; Ironson, G.; Siegel, S. D. Stress and health: psychological, behavioural and biological determinants. *Annu. Rev. Clin. Psychol.* **2005**, *1*, 607–628.

(3) Hu, Y.; Cardounel, A.; Gursoy, E.; Anderson, P.; Kalimi, M. Anti-stress effects of dehydroepiandrosterone: protection of rats against repeated immobilization stress-induced weight loss, glucocorticoid receptor production, and lipid peroxidation. *Biochem. Pharmacol.* **2000**, *59*, 753–762.

(4) Kourounakis, P.; Selye, H.; Taché Y. Catatoxic steroids. In *Advances in Steroidal Biochemistry and Pharmacology*; Briggs, B. M., Christie, G. A., Eds.; Academic Press: London, 1977; pp 35−57.

(5) Tsiakitzis, K.; Kourounakis, A. P.; Tani, E.; Rekka, E. A.; Kourounakis, P. N. Stress and active oxygen species-effect of alpha-tocopherol on stress response. *Arch. Pharm.* **2005**, *338*, 315–321.

(6) Selye, H. *Stress in Health and Disease*; Butterworths: Boston, 1976.

(7) Bousquer, W. F.; Rupe, B. D.; Miya, T. S. Endocrine modification of drug responses in the rat. *J. Pharmacol. Exp. Ther.* **1966**, *147*, 376–379.

(8) Kourounakis, P.; Selye, H. Influence of steroids and stress on toxicity and disposition of tetraethylammonium bromide. *J. Pharm. Sci.* **1976**, *65*, 1838–1840.

(9) Kourounakis, P. N.; Rekka, E. Induction of drug metabolism can be a homeostatic response. *Arch. Pharm.* **1991**, *324*, 161–164.

(10) Cantiello, M.; Giantin, M.; Carletti, M.; Lopparelli, R. M.; Capolongo, F.; Lasserre, M.; Bollo, E.; Nebbia, C.; Martin, P. G. P.; Pineau, T.; Dacasto, M. Effects of dexamethasone, administered for growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. *Biochem. Pharmacol.* **2009**, *77*, 451–463.

(11) Rekka, E.; Ayalogu, E. O.; Lewis, D. F.; Gibson, G. G.; Ioannides, C. Induction of hepatic microsomal CYP4A activity and of peroxisomal beta-oxidation by two nonsteroidal anti-inflammatory drugs. *Arch. Toxicol.* **1994**, *68*, 73–78.

(12) Kaplan, J. R.; Manuck, S. B.; Clarkson, T. B.; Lusso, F. M.; Taub, D. M. Social status, environment and atherosclerosis in cynomolgus monkeys. *Arteriosclerosis* **1982**, *2*, 359–368.

(13) McCabe, P. M.; Gonzalez, J. A.; Zaias, J.; Szeto, A.; Kumar, M.; Herron, A. J.; Schneiderman, N. Social environment influences the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Circulation* **2002**, *105*, 354–359.

(14) Kumar, A.; Goyal, R. Possible involvement of GABAergic modulation in the protective effect of gabapentin against immobilization stress induced behaviour alterations and oxidative damage in mice. *Fundam. Clin. Pharmacol.* **2007**, *21*, 575–581.

(15) Shashoua, V. E.; Jacob, J. N.; Ridge, R.; Cambell, A.; Baldessarini, R. J. γ-Aminobutyric esters. 1. Synthesis, brain uptake and pharmacological studies of aliphatic and steroid esters of γ-aminobutyric acid. *J. Med. Chem.* **1984**, *27*, 659–664.

(16) Siskou, I. C.; Rekka, E. A.; Kourounakis, A. P.; Chrysselis, M. C.; Tsiakitzis, K.; Kourounakis, P. N. Design and study of some novel ibuprofen derivatives with potential nootropic and neuroprotective properties. *Bioorg. Med. Chem.* **2007**, *15*, 951–961.

(17) Kourounakis, A. P.; Tsiakitzis, K.; Paramithiotis, D.; Kotzampassi, K.; Kourounakis, P. N. Effect of a novel NSAID derivative with antioxidant moiety on oxidative damage caused by liver and cerebral ischeamia-reperfusion in rats. *J. Pharm. Pharmacol.* **2002**, *54*, 1091–1096.

(18) Omura, T.; Sato, R. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein-nature. *J. Biol. Chem.* **1964**, *239*, 2370–2378.

(19) Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **1953**, *55*, 416–421.

(20) Lowry, O. H.; Rosenbrough, N. J.; Farr, Y. L.; Randall, A. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.

(21) Chrysselis, M. C.; Rekka, E. A.; Kourounakis, P. N. Hypocholesterolemic and hypolipidemic activity of some novel morpholine derivatives with antioxidant activity. *J. Med. Chem.* **2000**, *43*, 609–612.