NO-Donor Melatonin Derivatives: Synthesis And In Vitro Pharmacological Characterization

NO-donor melatonin derivatives: synthesis and *in vitro* pharmacological characterization.

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Running title: NO-donor melatonin derivatives

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Abstract: several studies indicate that melatonin, the hormone principally produced by the pineal gland, possesses a broad-spectrum antioxidant activity. It traps a number of reactive oxygen species (ROS) such as hydroxy ($OH^{\bullet}$) and peroxy ($ROO^{\bullet}$) radicals, singlet oxygen ($^1O_2$) and hypochlorous acid (HClO). It also inhibits peroxynitrite-induced reactions. It is known that atherosclerosis progression involves ROS-induced oxidation of low density lipoproteins (LDL) in sub-endothelial space and the depletion of nitric oxide (NO) in blood vessels, as well as a decreased sensitivity of the vessels to the actions of NO. On these bases a series of new NO-donor antioxidants were designed and synthesised by joining melatonin with NO-donor nitrooxy and furoxan moieties as polyvalent agents potentially useful for the treatment of cardiovascular diseases involving atherosclerotic vascular changes. The *in vitro* antioxidant properties of the resulting products were assessed in the TBARS, the $ABTS^{+\bullet}$ as well as in the alkaline phosphatase (ALP) assays. The antioxidant capacities of NO-donor melatonins to inhibit lipoperoxidation (TBARS-IC_{50}) was predominantly dependent on their lipophilicity, and therefore on their partitioning process into membranes. On the other hand, their comparable capacity to inhibit protein oxidation (ALP-IC_{50}) was independent of their lipophilicity and is consistent with their similar ability to participate in electron transfer reactions. All the NO-donor melatonins were also evaluated for their ability to relax rat aorta strips pre-contracted with 1 μM phenylephrine. Finally, binding affinities and intrinsic activity studies, carried out at MT_1 and MT_2 receptor subtypes, showed a rather complex picture in need of further investigation.

Introduction.

Melatonin (**1**, Chart 1) is a hormone principally produced and released by the pineal gland. It exerts several biological effects; in particular, it plays a pivotal role in circadian

rhythms of vertebrates [1,2]. Many of these effects are exerted through the activation of two G-protein-coupled receptors named MT_1 and MT_2 [3]. Several studies indicate that melatonin possesses a broad-spectrum antioxidant activity. It traps a number of reactive oxygen species (ROS) such as hydroxy ($OH^{\bullet}$) and peroxy ($ROO^{\bullet}$) radicals, singlet oxygen ($^1O_2$) and hypochlorous acid (HClO) [4-7]. In addition to this, melatonin inhibits peroxynitrite-induced reactions [8]. ROS play relevant roles in the oxidative modifications of low density lipoproteins (LDL) in sub-endothelial space. This is one of the earliest phases in the development of atherosclerosis [9]. The modified LDL are phagocytosed by macrophages with a resulting production of ROS and an accumulation of cholesterol within these cells. This leads to their transformation in foam cells, then to the formation of the fatty streak and ultimately to the atherosclerotic plaque. Atherosclerosis progression induces, through a number of mechanisms, the depletion of nitric oxide (NO) in blood vessels and decreased sensitivity of the vessels to the actions of NO.

On these bases, in previous works [10,11] we proposed a new class of potential anti-atherosclerosis drugs, the NO-donor antioxidants. Generally speaking, these products could be useful not only in the treatment of atherosclerosis, but in all pathologies in which a deficiency of nitric oxide and an abnormal increase in the concentration of ROS occur [12]. As a development of this research, we now describe the synthesis, the physicochemical profiles and an *in vitro* pharmacological characterisation of products **16-19**, **31-35**, **40**, **42**, **45**, **46**. In particular, we evaluated their lipophilicity, some of their antioxidant properties, their NO-dependent vasodilator properties. Furthermore we give an accout of their ability to interact with melatonin MT_1 and MT_2 receptors.

This series of products was obtained by joining directly, or through appropriate spacers, melatonin with NO-donor nitrooxy and 1,2,5-oxadiazole 2-oxide (furoxan) moieties. In the first case we chose the nitrooxyalkyl moieties present in simple nitric esters **3** and **4**, which

are characterised by different vasodilator capacities. As furoxan moieties we selected the 3-(phenylsulfonyl)furoxan-4-yloxy and the 3-carbamoylfuroxan-4-yl-methyl substructures present in 4-ethoxy-3-(phenylsulfonyl)furoxan **5** (CHF 2363) and in 4-(hydroxymethyl)furoxan-3-carboxamide **6** (CAS 1609). Products **5** and **6** are potent, orally active vasodilators developed by the pharmaceutical companies Chiesi and Cassella-Hoechst respectively [13,14]. We also addressed our attention to the 3-phenylfuroxan-4-yloxy and 4-phenylfuroxan-3-sulfonylethyl moieties present in furoxans **7** and **8**. The NO-donor substructures were joined to the melatonin scaffold at different anchoring points, namely the methoxy group in position 5 of the indole ring, the *N*-acetyl group and the amide nitrogen on the side chain. Melatonin and its more lipophilic analogues **36-39** were also considered as reference compounds.

Materials and methods

Chemistry

The synthesis (Scheme 1) of NO-donor melatonins **16-19** obtained by modification of the 5-methoxy group of melatonin required the preliminary preparation of the intermediates **10**, **13** and the use of the previously described furoxans **14** [15], **15** [16]. The nitric ester **10** was easily obtained by the action of *p*-toluensulfonyl chloride (TsCl) in pyridine on 3-hydroxypropyl nitrate (**9**). The (bis)nitrooxypropyl substituted benzyl bromide **13** was synthesised starting from 4-allylbenzaldehyde (**11**). This latter product was treated with I_2 and $AgNO_3$ in acetonitrile solution to give the (bis)nitrooxy substituted aldehyde **12** that was transformed into **13** by action of $NaBH_4$ in methanol solution, followed by treatment with triphenylphosphine and *N*-bromosuccinimide (NBS) in CH_2Cl_2 solution. The reaction of products **10**, **13-15** with **2** in the presence of a base afforded the expected final nitrooxy substituted compounds **16**, **17** and the furoxan derivatives **18**, **19**.

The synthesis (Scheme 2) of NO-donor melatonins **31**-**35** modified at the acetyl group required the use of carboxylic acids **23**, **24**, **28**, **29** and of the already known furoxan ester **25** [17]. Furoxanyloxy acetic acids **23**, **24** were prepared by the action of glycolic acid ethyl ester **20** on furoxans **14**, **15** in THF in the presence of NaH. The intermediate esters **21**, **22** thus obtained were hydrolysed into the corresponding acids by the action of HCl in dioxan/water mixture. Bis(nitrooxy)propoxy substituted benzoic acid **28** was synthesised by $KMnO_4$ oxidation of the related aldehyde **27** in acetone solution. This latter intermediate was prepared from **26** following the same procedure used to prepare **12** from **11**. Oxidation of aldehyde **12** under the same conditions gave the corresponding carboxylic acid **29**. Coupling of the appropriate carboxylic acids with 5-methoxytryptamine **30** to yield the final models **31**, **32**, **34**, **35** was run in CH_2Cl_2 solution in the presence of *N*,*N'*-carbonyldiimidazole (CDI). The same procedure was used to prepare the lipophilic analogues of melatonine **36**-**39**. By contrast, compound **33** was obtained by coupling the ester **25** with **30** in the presence of a catalytic amount of 4-dimethylaminopyridine (DMAP).

The synthesis (Scheme 3) of model **40** containing a bis(nitrooxy) substituted moiety at the amide nitrogen atom of melatonin was produced by reductive amination in methanol using aldehyde **27**, the amine **30** and $NaBH_4$. The resulting secondary amine was not characterised, but it was immediately acetylated with acetic anhydride (Ac_2O) to give the final product, **40**. The melatonin derivative **42**, bearing a substituted furoxan at the amide nitrogen, was obtained in CH_2Cl_2 solution treating **30** with the Michael system **41** and then with Ac_2O and Et_3N. Finally, the target furoxan compounds **45**, **46** were prepared by action of **14** and **15** respectively on **44**, obtained by reduction of **43** with $LiAlH_4$ in dry THF, followed by acylation of the secondary amine by action of Ac_2O.

Melting points were determined with a capillary apparatus (Büchi 540). ^1H and ^{13}C-NMR spectra were recorded on a Bruker Avance 300 operating at 300 and 75 MHz respectively,

using $Si(CH_3)_4$ as an internal standard. The following abbreviations were used to indicate the peak multiplicity: s = singlet; d = doublet; t = triplet, q = quadruplet, m = multiplet. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 70-230 mesh ASTM) using the indicated eluents. Petroleum ether 40-70°C (PE) was used as a co-eluent. The progress of the reactions was followed by thin layer chromatography (TLC) on 5×20 cm plates with a layer thickness of 0.2 mm. Anhydrous magnesium sulfate was used as a drying agent for the organic phases. Organic solvents were removed under reduced pressure at 35-40°C. Analysis (C, H, N) of the target compounds was performed by REDOX (Monza) and the results were within ± 0.4 % of the theoretical unless otherwise stated. Products **5** [16], **7** [18], **9** [19], **11** [20], **14** [15], **15** [16], **25** [17], **41** [21] were synthesized as described elsewhere; product **6** was a gift from Sanofi-Aventis Deutschland GmbH. **43** was synthesized by the reaction of ethyl glycolate and **30** at reflux and its mp was in accordance with literature (mp 142-143°C (EtOAc), lit.[22] mp 142-143°C (Hex/$CHCl_3$)). Products **3** and **4** were synthesized from *n*-propanol and 1,2-propandiol respectively according to the procedure described in literature [23]; product **8** was synthesized from 4-phenyl-3-phenylsulfonylfuroxan according to the procedure described in literature [24].

3-(Nitrooxy)propyl 4-methylbenzenesulfonate (10)

9 (1.10 g, 9.1 mmol) was dissolved in dry pyridine (10 mL) at 0°C and *p*-toluensulfonyl chloride (2.50 g, 13.1 mmol) was added in one portion. The reaction mixture was kept at 0°C for 5 h and then it was poured into 1N HCl solution and extracted with EtOAc. The organic layer was washed with 1N HCl solution, brine, dried and evaporated under reduced pressure. The obtained oil was purified by flash chromatography (eluent: PE/AcOEt 95/5) to give the title compound as colourless oil which solidified upon standing (yield 55%). MS (EI) 275 (M^+). ^{1}H-NMR ($CDCl_3$): δ 2.08 (m, 2H, $CH_2CH_2CH_2$), 2.45 (s, 3H, CH_3), 4.12 (t, 2H), 4.46

(t, 2H) (C*H*$_2$CH$_2$C*H*$_2$ONO$_2$), 7.36 (d, 2H), 7.78 (d, 2H) (C$_6$*H*$_4$); ^{13}C-NMR ($CDCl_3$): δ 21.7, 26.6, 65.8, 68.6, 127.9, 130.0, 132.5, 145.3. The compound due to its instability was immediately reacted in the following step.

4-[2,3-Bis(nitrooxy)propyl]benzaldehyde (12)

To a vigorously stirred solution of **11** (1.15 g, 7.9 mmol) and $AgNO_3$ (3.50 g, 21 mmol) in CH_3CN, iodine (2.00 g, 7.9 mmol) was added in one portion at room temperature (RT). The reaction mixture was stirred until the iodine completely dissolved and then refluxed for 10 h. After that time the reaction was concentrated and the precipitate was filtered and washed with EtOAc. The organic phase was washed with H_2O, brine, dried and evaporated. The obtained oil was purified by flash chromatography (eluent: PE/EtOAc 8/2) and the obtained product was recrystallized from EtOH to give a pale-yellow solid (yield 52%), mp 60-61°C (EtOH). MS (EI) 270 (M^+). ^{1}H-NMR ($CDCl_3$): δ 3.08-3.23 (m, 2H, C$_6$H$_4$C*H*$_2$), 4.47 (dd, 1H, CH*H*ONO$_2$), 4.76 (dd, 1H, C*H*HONO$_2$), 5.46-5.54 (m, 1H, C*H*(ONO$_2$)), 7.44 (d, 2H), 7.88 (d, 2H) (C$_6$*H*$_4$), 10.01 (s, 1H, C*H*O); ^{13}C-NMR ($CDCl_3$): δ 35.7, 70.1, 78.8, 130.0, 130.4, 135.9, 141.1, 191.6.

1-[2,3-Bis(nitrooxy)propyl]-4-bromomethylbenzene (13)

To a suspension of **12** (2.26 g, 4.2 mmol) in CH_3OH, $NaBH_4$ (0.16 g, 4.2 mmol) was added in one portion at 0°C. The reaction was stirred at this temperature for 30 min and was then poured into 1N HCl and extracted with EtOAc. The organic phase was washed with H_2O, $NaHCO_3$ sat. sol., brine, dried and evaporated. The obtained colourless oil was used without further purification. To the solution of alcohol (0.20 g, 0.73 mmol) and PPh_3 (0.24 g, 0.91 mmol) in dry CH_2Cl_2, NBS (0.17 g, 0.92 mmol) was added in small portions at 0°C. The ice bath was removed and the mixture was stirred for 1 h. The solvent was evaporated and the obtained crude product was separated by flash chromatography (eluent: PE/EtOAc 97/3) to

give the title product as a colourless oil, which solidified on standing (yield 90%), mp 55.5-56.5°C. MS (EI) 334/336 (M^+). ^{1}H-NMR ($CDCl_3$): δ 2.97-3.13 (m, 2H, $C_6H_4CH_2$), 4.40-4.48 (m, 3H, CH_2Br, $CHHONO_2$), 4.72 (dd, 1H, $CHHONO_2$), 5.40-5.48 (m, 1H, $CHONO_2$), 7.22 (d, 2H), 7.38 (d, 2H) (C_6H_4); ^{13}C-NMR ($CDCl_3$): δ 32.8, 35.3, 70.0, 79.2, 129.7, 129.8, 133.4, 137.4.

***N*-{2-[5-(3-Nitrooxypropoxy)-1*H*-indol-3-yl]ethyl}acetamide (16)**

To a stirred solution of **2** (0.30 g, 1.4 mmol) in dry THF under positive N_2 pressure *t*-BuO^-K^+ (0.16 g, 1.4 mmol) was added and the mixture was stirred for 15 min. Then, **10** (0.40 g, 1.5 mmol) was added and the reaction mixture was heated at 50°C for 12 h. The reaction mixture was poured into EtOAc and the organic phase was washed with H_2O, brine, dried and evaporated. The obtained oil was separated by flash chromatography (eluent: CH_2Cl_2/MeOH 98/2) to give the title product as a pale yellow oil, which solidified by treating with Et_2O (yield 33%). An analytically pure sample was obtained by recrystallization from benzene, mp 101.5-102°C (benzene). MS (EI) 321 (M^+). ^{1}H-NMR (DMSO-d_6): δ 1.80 (s, 3H, CH_3CO), 2.15 (m, 2H, $CH_2CH_2CH_2$), 2.77 (t, 2H, IndCH_2), 3.29 (q, 2H CH_2CH_2NH), 4.06, 4.72 (2t, $CH_2CH_2CH_2$), 6.72 (m, 1H), 7.04 (d, 1H), 7.11 (s, 1H), 7.23 (d, 2H) (4 C*H*, Ind), 7.92 (t, 1H, $NHCOCH_3$), 10.65 (s, 1H, N*H* Ind); ^{13}C-NMR (DMSO-d_6): δ 23.6, 26.1, 27.4, 40.3, 65.3, 72.1, 102.3, 112.3, 112.6, 112.8, 124.2, 128.4, 132.4, 152.7, 169.9. Anal. ($C_{15}H_{19}N_3O_5$) C, H, N.

***N*-(2-{5-[4-(2,3-Bis(nitrooxy)propyl)benzyloxy]-1*H*-indol-3-yl}ethyl)acetamide (17)**

To a stirred solution of **2** (0.37 g, 1.7 mmol) in CH_3CN, K_2CO_3 (0.55 g, 4.0 mmol) was added and the mixture was stirred for 15 min at RT. Then **13** (0.57 g, 1.7 mmol) was added and the reaction was heated at 70°C for 12 h. The reaction mixture was poured into EtOAc and the organic phase was washed with H_2O, 0.1N NaOH, brine, dried and evaporated. The

obtained oil was separated by flash chromatography (eluent: CH_2Cl_2/MeOH 99/1) to give the title product as a yellow oil (yield 41%). MS (EI) 472(M^+). ^{1}H-NMR ($CDCl_3$): δ 1.94 (s, 3H, C*H*$_3$CO), 2.93 (t, 2H, C*H*$_2$CH$_2$NH), 2.98-3.15 (m, 2H, O_2NOCHC*H*$_2$C$_6$H$_4$), 3.58 (q, 2H, CH$_2$C*H*$_2$NH), 4.43 (dd, 1H, CH*H*ONO$_2$), 4.73 (dd, 1H, C*H*HONO$_2$), 5.10 (s, 2H, C$_6$H$_4$C*H*$_2$O), 5.45 (m, 1H, C*H*ONO$_2$), 5.61 (m, 1H, -N*H*CO-), 6.95 (dd, 1H), 7.03 (d, 1H); 7.13 (d, 1H); 7.25–7.46 (m, 3H) 7.47 (d, 2H) (C$_6$*H*$_4$, 4C*H* Ind), 8.20 (s, 1H, N*H* Ind); ^{13}C-NMR ($CDCl_3$): δ 23.4, 25.3, 35.3, 39.7, 70.1, 70.5, 79.4, 102.2, 112.1, 112.7, 113.0, 122.9, 127.7, 128.3, 129.4, 130.9, 133.6, 137.2, 153.1, 170.1. Anal. ($C_{22}H_{24}N_4O_8$) C, H, N.

***N*-{2-[5-(3-phenylfuroxan-4-yloxy)-1*H*-indol-3-yl]ethyl}acetamide (18)**

The product was synthesized starting from **14** using a procedure similar to that used for the preparation of **16** from **10**, but in this case the reaction was conducted at RT for 24 h using NaH as a base. The title compound was recrystallized from EtOH to give a white solid (yield 70%), mp 183-184°C (EtOH). MS (EI) 378 (M^+). ^{1}H-NMR (DMSO-d_6): δ 1.79 (s, 3H, C*H*$_3$CO), 2.80 (t, 2H, C*H*$_2$CH$_2$NH), 3.30 (m, 2H, CH$_2$C*H*$_2$NH), 7.20 (d, 1H), 7.28 (s, 1H), 7.42 (d, 1H), 7.60-7.68 (m, 4H), 8.14 (d, 2H) (C$_6$*H*$_5$, 4 C*H* Ind), 7.92 (m, 1H, N*H*CO), 11.05 (s, 1H, N*H* Ind); ^{13}C-NMR (DMSO-d_6): δ 22.8, 27.1, 39.7, 107.8, 109.4, 112.2, 112.8, 113.7, 122.1, 124.5, 126.3, 127.6, 128.8, 130.6, 134.5, 145.6, 162.8, 169.7. Anal. ($C_{20}H_{18}N_4O_4$) C, H, N.

***N*-{2-[5-(3-Benzenesulfonylfuroxan-4-yloxy)-1*H*-indol-3-yl]ethyl}acetamide (19)**

The product was synthesized starting from **15** using a procedure similar to the one used for preparation of **16** from **10**, but in this case the reaction was conducted at RT for 1 h using NaH as a base. The title compound was purified by flash chromatography (eluent: CH_2Cl_2/MeOH 98/2) to give an oil which becomes a white foam in desiccator (yield 84%). MS (EI) 442 (M^+). ^{1}H-NMR ($CDCl_3$): δ 1.92 (s, 3H, COC*H*$_3$) 2.90 (t, 2H, C*H*$_2$CH$_2$NH), 3.52

(q, 2H, CH_2CH_2NH), 5.79 (t, 1H, CONH), 7.03-7.07 (m, 2H), 7.37 (d, 1H), 7.46 (d, 1H) (4 CH Ind), 7.63 (t, 2H), 7.78 (t, 1H), 8.12 (d, 2H) (C_6H_5), 8.82 (s, 1H, NH Ind); ^{13}C-NMR ($CDCl_3$): δ 23.3, 25.2, 39.9, 109.6, 110.9, 112.3, 113.4, 114.4, 122.1, 124.3, 127.7, 128.6, 129.8, 134.6, 137.9, 146.1, 159.8, 170.4. Anal. ($C_{20}H_{18}N_4O_6S$) C, H, N.

3-Phenylfuroxan-4-yloxy acetic acid ethyl ester (21)

To a stirred solution of **20** (0.20 mL, 2.2 mmol) in dry THF under positive N_2 pressure NaH (0.10 g, 2.5 mmol) was added in one portion. The reaction was stirred for 15 min and then placed into an ice bath, subsequently **14** (0.60 g, 2.0 mmol) was added in one portion. The ice bath was removed and stirring was continued for 10 h. The reaction mixture was poured into H_2O and extracted with EtOAc. The organic phase was washed with brine, dried and evaporated. The obtained oil was purified by flash chromatography (eluent: PE/CH_2Cl_2 8/2) to give the title compound as a white solid (yield 72%), mp 66-67°C. MS (EI) 264 (M^+). ^{1}H-NMR ($CDCl_3$): δ 1.33 (t, 3H, CH_2CH_3), 4.93 (q, 2H, CH_2CH_3), 5.04 (s, 2H, $COCH_2O$), 7.44-7.54 (m, 3H), 8.14-8.17 (m, 2H) (C_6H_5); ^{13}C-NMR ($CDCl_3$): δ 14.1, 62.0, 65.9, 107.6, 122.1, 126.4, 128.9, 130.6, 161.6, 166.4.

3-Benzenesulfonylfuroxan-4-yloxy acetic acid ethyl ester (22)

The product was synthesized starting from **15** using a procedure similar to the one used for the preparation of **21** from **14**. The title compound was recrystallized from EtOH to give a white solid (yield 72%), mp 123.5-124°C (EtOH). MS (EI) 328 (M^+). ^{1}H-NMR ($CDCl_3$): δ 1.29 (t, 3H, CH_2CH_3), 4.27 (q, 2H, CH_2CH_3), 5.00 (s, 2H, $COCH_2O$), 7.63 (t, 2H), 7.77 (t, 1H), 8.10 (d, 2H) (C_6H_5); ^{13}C-NMR ($CDCl_3$) δ: 14.1, 62.1, 66.0, 110.9, 128.7, 129.7, 135.7, 138.3, 158.7, 166.2.

3-Phenylfuroxan-4-yloxy acetic acid (23)

A solution of **21** (0.33 g, 1.3 mmol) in dioxan (3 mL) and HCl 20% (3 mL) mixture was heated at 60°C for 12 h. Subsequently, the reaction mixture was poured into H_2O and extracted with EtOAc. The organic phase was washed with brine, dried and evaporated. The obtained solid was recrystallized from EtOH/H_2O mixture to give the title compound as a white solid (yield 68%), mp 143-144.5°C (EtOH/H_2O). MS (EI) 236 (M^+). ^{1}H-NMR (DMSO-d_6): δ 5.10 (s, 2H, COCH_2O), 7.55-7.66 (m, 3H), 8.00-8.10 (m, 2H) (C_6H_5); ^{13}C-NMR (DMSO-d_6): δ 65.5, 107.5, 121.8, 126.3, 129.2, 131.0, 161.7, 168.2.

3-Benzenesulfonylfuroxan-4-yloxy acetic acid (24)

The product was synthesized starting from **22** using a procedure similar to the one used for the preparation of **23** from **21**. The title compound was precipitated by pouring the reaction mixture into H_2O (yield 90%), mp 185-190°C (dec). MS (EI) 300 (M^+). ^{1}H-NMR (DMSO-d_6): δ 5.00 (s, 2H, COCH_2O), 7.68 (t, 2H), 7.83 (t, 1H), 8.07 (d, 2H) (C_6H_5); ^{13}C-NMR (DMSO-d_6): δ 66.2, 108.6, 128.3, 129.7, 135.8, 137.5, 158.3, 167.4.

4-[2,3-Bis(nitrooxy)propoxy]benzaldehyde (27)

The product was synthesized starting from **26** using a procedure similar to the one used for the preparation of **12** from **11**. The title compound was recrystallized from EtOH to give a pale yellow solid (yield 40%), mp 74-74.5°C (EtOH). MS (EI) 286 (M^+). ^{1}H-NMR ($CDCl_3$): δ 4.35 (d, 2H, OCH_2), 4.81 (dd, 1H, C*H*HONO$_2$), 4.95 (dd, 1H, CH*H*ONO$_2$), 5.62-5.68 (m, 1H, C*H*ONO$_2$), 7.02 (d, 2H), 7.87 (d, 2H) (C_6H_4), 9.91 (s, 1H, CO*H*); ^{13}C-NMR ($CDCl_3$): δ 64.8, 68.6, 76.4, 115.1, 131.6, 132.4, 162.4, 190.7.

4-[2,3-Bis(nitrooxy)propoxy]benzoic acid (28)

To a solution of **27** (3.56 g, 12 mmol) in acetone $KMnO_4$ (2.95 g, 19 mmol) was added at RT in one portion. After 30 min the $KMnO_4$ excess was destroyed by the addition of oxalic acid. The reaction mixture was filtered; the filtrate was diluted with CH_2Cl_2 and the organic

phase was washed with H_2O, brine, dried and evaporated. The title product was obtained as a white solid (yield 67%). An analytically pure sample was obtained by recrystallization from *i*-Pr_2O, mp 107.5-108.5°C (*i*-Pr_2O). MS (EI) 302 (M^+). ^{1}H-NMR (DMSO-d_6): δ 4.41 (dd, 1H, OCH*H*), 4.51 (dd, 1H, OC*H*H), 4.95 (dd, 1H, C*H*HONO$_2$), 5.07 (dd, 1H, CH*H*ONO$_2$), 5.81-5.86 (m, 1H, C*H*ONO$_2$), 7.06 (d, 2H), 7.91 (d, 2H) (C_6H_4), 12.72 (br. s, 1H, COO*H*); ^{13}C-NMR ($CDCl_3$): δ 66.1, 70.8, 78.7, 115.3, 124.7, 132.3, 162.0, 167.7.

4-[2,3-Bis(nitrooxy)propyl]benzoic acid (29)

The product was synthesized starting from **12** using a procedure similar to the one used for the preparation of **28** from **27** (yield 83%). An analytically pure sample was obtained by crystallization from *i*-Pr_2O, mp 133-134.5°C (*i*-Pr_2O). MS (EI) 286 (M^+). ^{1}H-NMR (DMSO-d_6): δ 3.18 (m, 2H, $C_6H_4CH_2$), 4.71 (dd, 1H, C*H*HONO$_2$), 4.96 (dd, 1H, CH*H*ONO$_2$), 5.72 (m, 1H, C*H*ONO$_2$), 7.45 (d, 2H), 7.92 (d, 2H) (C_6H_4), 12.96 (br. s, 1H, COO*H*); ^{13}C-NMR ($CDCl_3$): δ 34.3, 71.4, 80.1, 129.4, 129.5, 129.6, 140.5, 167.0.

General procedure for the synthesis of *N*-[2-(5-methoxy-1*H*-indol-3-yl)-ethyl]amides

The following procedure for the synthesis of 4-[2,3-bis(nitrooxy)propoxy]-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]benzamide (**34**) is representative of the synthesis of **31**, **32**, **34-39**.

4-[2,3-Bis(nitrooxy)propoxy]-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]benzamide (34)

To a solution of **28** (0.25 g, 0.8 mmol) in dry CH_2Cl_2 CDI (0.14 g, 0.9 mmol) was added in one portion. The reaction was allowed to stir at RT for 15 min, then **30** (0.15 g, 0.8 mmol) was added. After 2 h the reaction was poured into H_2O and extracted with CH_2Cl_2. The organic phase was washed with 1N HCl, H_2O, 0.1N NaOH, brine, dried and evaporated. The obtained solid was recrystallized from MeOH to give the title compound as a white solid (yield 87%), mp 133-134°C (MeOH). MS (EI) *m/z* 474 (M^+). ^{1}H-NMR (DMSO-d_6): δ 2.93 (t,

2H, C*H*$_2$CH$_2$NH), 3.54 (q, 2H, CH$_2$C*H*$_2$NH), 3.73 (s, 3H, C*H*$_3$O), 4.40 (dd, 1H, C*H*HO), 4.50 (dd, 1H, CH*H*O), 4.96 (dd, 1H, C*H*HONO$_2$), 5.08 (dd,1H, CH*H*ONO$_2$), 5.81-5.84 (m, 1H, C*H*ONO$_2$), 6.72 (dd, 1H), 7.03-7.07 (m, 3H), 7.14 (d, 1H), 7.24 (d, 1H), 7.87 (d, 2H), (4 C*H* Ind, C$_6$*H*$_4$), 8.50 (t, 1H, CON*H*); 10.65 (s, 1H, N*H*Ind); ^{13}C-NMR (DMSO-*d*$_6$): δ 26.1, 41.1, 56.1, 66.0, 70.8, 78.8, 101.0, 111.9, 112.7, 112.9, 114.9, 124.1, 128.5, 128.7, 129.9, 132.3, 153.9, 160.6, 166.3. Anal. (C$_{21}$H$_{22}$N$_4$O$_9$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]-2-(3-phenylfuroxan-4-yloxy)acetamide (31)**

White solid (yield 58%), mp 136-137°C (EtOH). MS (EI) 408 (M$^+$). ^{1}H-NMR (DMSO-*d*$_6$): δ 2.83 (t, 2H, C*H*$_2$CH$_2$NH), 3.40 (m, 2H, CH$_2$C*H*$_2$NH), 3.75 (s, 3H, OC*H*$_3$), 4.97 (s, 2H, OC*H*$_2$CO), 6.72 (d, 1H), 7.03 (s, 1H), 7.13 (s, 1H), 7.23 (d, 1H) (4 C*H* Ind), 7.58-7.62 (m, 2H), 8.06-8.11 (m, 3H) (C$_6$*H*$_5$), 8.39 (t, 1H, N*H*CO), 10.67 (s, 1H, N*H*Ind); ^{13}C-NMR (DMSO-*d*$_6$): δ 25.0, 39.3, 55.3, 68.2, 100.0, 107.6, 111.0, 111.2, 112.0, 121.8, 123.3, 126.3, 127.4, 128.9, 130.7, 131.3, 152.9, 161.9, 165.3. Anal. (C$_{21}$H$_{20}$N$_4$O$_5$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]-2-(3-benzenesulfonylfuroxan-4-yloxy)acetamide (32)**

White solid (yield 62%), mp 173-174°C (EtOH). MS (EI) 472 (M$^+$). ^{1}H-NMR (DMSO-*d*$_6$): δ 2.93 (t, 2H, C*H*$_2$CH$_2$NH), 3.54 (m, 2H, CH$_2$C*H*$_2$NH), 3.82 (s, 3H, OC*H*$_3$), 4.90 (s, 2H, OC*H*$_2$CO), 6.72-6.76 (m, 1H), 7.00 (d, 1H), 7.06 (d, 1H), 7.24 (d, 1H) (4 C*H* Ind), 7.65 (t, 2H), 7.78-8.01 (m, 2H), 8.03 (d, 2H) (C$_6$*H*$_5$, N*H*CO), 10.34 (s, 1H, N*H*Ind); ^{13}C-NMR (DMSO-*d*$_6$): δ 25.0, 39.5, 55.5, 68.3, 100.0, 107.8, 111.3, 111.5, 112.1, 123.3, 127.5, 128.4, 129.7, 131.6, 135.8, 137.4, 153.3, 158.4, 164.9. Anal. (C$_{21}$H$_{20}$N$_4$O$_7$S) C, H, N.

4-[2,3-Bis(nitrooxy)propyl]-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]benzamide (35)

White solid (yield 79%), mp 129.5-133°C (EtOH). MS (EI) *m/z* 458 (M$^+$). ^{1}H-NMR (DMSO-*d*$_6$): δ 2.92 (t, 2H, C*H*$_2$CH$_2$NH), 3.15 (m, 2H, C$_6$H$_4$C*H*$_2$), 3.54 (q, 2H, CH$_2$C*H*$_2$NH),

3.71 (s, 3H, C*H*$_3$O), 4.70 (dd), 4.96 (dd) (2H, C*H*$_2$ONO$_2$), 5.72 (m, 1H, C*H*ONO$_2$), 6.71 (dd, 1H), 7.05-7.24 (m, 3H) (4 C*H* Ind), 7.40 (d, 2H), 7.82 (d, 2H) (C$_6$*H*$_4$), 8.59 (t, 1H, CON*H*), 10.65 (s, 1H, N*H*Ind); ^{13}C-NMR (DMSO-*d*$_6$): δ 25.1, 34.1, 55.1, 61.4, 80.2, 100.0, 111.0, 111.6, 111.9, 123.2, 127.4, 127.5, 128.2, 129.0, 131.2, 133.4, 138.4, 152.8, 165.7. Anal. ($C_{21}H_{22}N_4O_8$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]propanamide (36)**

White solid (yield 86%), mp 104.5-105°C (*i*-Pr$_2$O). MS (CI) *m/z* 247 $(M+1)^+$. ^{1}H-NMR (CD$_3$OD): δ 1.09 (t, 3H, C*H*$_3$CH$_2$), 2.16 (q, 2H, CH$_3$C*H*$_2$), 2.90 (t, 2H, C*H*$_2$CH$_2$NH), 3.45 (t, 2H, CH$_2$C*H*$_2$NH), 3.82 (s, 3H, C*H*$_3$O), 6.74 (dd, 1H), 7.02-7.22 (m, 3H) (4 C*H* Ind); ^{13}C-NMR (CD$_3$OD): δ 10.5, 26.3, 30.3, 41.4, 56.3, 101.3, 112.6, 113.1, 119.2, 124.2, 129.1, 133.4, 155.0, 177.1. Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]pentanamide (37)**

The crude product was purified by flash chromatography (eluent: CH$_2$Cl$_2$/MeOH 95/5). Yellow oil (yield 86%). MS (CI) *m/z* 275 $(M+1)^+$. ^{1}H-NMR (CD$_3$OD): δ 0.90 (t, 3H, C*H*$_3$CH$_2$), 1.29 (m, 2H), 1.54 (m, 2H), 2.15 (t, 2H) ((C*H*$_2$)$_3$CH$_3$), 2.89 (t, 2H, C*H*$_2$CH$_2$NH), 3.45 (t, 2H, CH$_2$C*H*$_2$NH), 3.82 (s, 3H, C*H*$_3$O), 6.74 (dd, 1H), 7.02-7.22 (m, 3H) (4 C*H* Ind); ^{13}C-NMR (CD$_3$OD): δ 14.1, 23.3, 26.3, 29.2, 37.0, 41.3, 56.3, 101.3, 112.5, 112.8, 113.1, 124.2, 129.1, 133.4, 154.9, 176.3. Anal. ($C_{16}H_{22}N_2O_2 \cdot 0.25\ H_2O$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]decanamide (38)**

White solid (yield 68%), mp 79.5-80°C (MeOH). MS (CI) *m/z* 345 $(M+1)^+$. ^{1}H-NMR (CDCl$_3$): δ 0.87 (t, 3H, C*H*$_3$CH$_2$), 1.24 (m, 12H), 1.58 (m, 2H), 2.10 (t, 2H) ((C*H*$_2$)$_8$CH$_3$), 2.93 (t, 2H, C*H*$_2$CH$_2$NH), 3.59 (q, 2H, CH$_2$C*H*$_2$NH), 3.85 (s, 3H, C*H*$_3$O), 5.57 (m, 1H, N*H*), 6.86 (dd, 1H), 6.99-7.27 (m, 3H) (4 C*H* Ind), 8.20 (br. s, 1H, N*H* Ind); ^{13}C-NMR (CDCl$_3$): δ

14.1, 19.4, 25.4, 25.8, 29.3, 29.3, 29.4, 29.4, 31.9, 37.0, 39.5, 55.9, 100.5, 112.0, 112.5, 112.8, 122.8, 127.8, 131.5, 154.1, 173.2. Anal. ($C_{21}H_{32}N_2O_2$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]dodecanamide (39)**

White solid (yield 85%), mp 86.5-93°C (EtOH). MS (CI) *m/z* 373 $(M+1)^+$. ^{1}H-NMR (CD_3OD): δ 0.89 (t, 3H, C*H*$_3$CH$_2$), 1.27 (m, 16H), 1.55 (m, 2H), 2.14 (t, 2H) ((C*H*$_2$)$_{10}$CH$_3$), 2.89 (t, 2H, C*H*$_2$CH$_2$NH), 3.46 (t, 2H, CH$_2$C*H*$_2$NH), 3.81 (s, 3H, C*H*$_3$O), 6.74 (dd, 1H), 7.02-7.22 (m, 3H) (4 C*H* Ind); ^{13}C-NMR (CD_3OD): δ 14.7, 24.0, 26.6, 27.4, 30.6, 30.7, 30.8, 30.9, 31.0, 33.4, 37.6, 41.6, 56.6, 101.6, 112.8, 113.2, 113.3, 124.4, 129.4, 133.7, 155.2, 176.6. Anal. ($C_{23}H_{36}N_2O_2$) C, H, N.

3-Hydroxymethylfuroxan-4-carboxylic acid [2-(5-methoxy-1*H*-indol-3-yl)ethyl]amide (33)

To a solution of **30** (0.44 g, 2.3 mmol) and **25** (0.40 g, 2.3 mmol) in EtOH, DMAP (0.03 g, 0.25 mmol) was added in one portion. The reaction was stirred for 1 h at RT, then it was concentrated and the obtained suspension was cooled in an ice-salt bath. The precipitate was filtered, washed with a small quantity of cold ethanol and recrystallized from EtOH to give the title compound as a white solid (yield 91%), mp 170-170.5°C (EtOH). MS (EI) 332 (M^+). ^{1}H-NMR (DMSO-*d*$_6$): δ 2.89 (t, 2H, C*H*$_2$CH$_2$NH), 3.53 (m, 2H, CH$_2$C*H*$_2$NH), 3.75 (s, 3H, OC*H*$_3$), 4.71 (s, 2H, HOC*H*$_2$), 5.74 (br. s, 1H, O*H*), 6.69-6.72 (m, 1H), 7.06 (d, 1H), 7.14 (s, 1H), 7.21 (d, 1H) (4 C*H* Ind), 8.53 (s, 1H, N*H*CO), 10.67 (s, 1H, N*H*Ind); ^{13}C-NMR (DMSO-*d*$_6$): δ 25.6, 40.6, 56.2, 56.3, 101.1, 111.4, 111.7, 112.0, 112.9, 124.4, 128.3, 132.3, 153.3, 155.1, 159.8. Anal. ($C_{15}H_{16}N_4O_5$) C, H, N.

***N*-{4-[2,3-Bis(nitrooxy)propoxy]benzyl}-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide (40)**

To a suspension of **27** (0.37 g, 1.3 mmol) in MeOH **30** (0.20 g, 1.1 mmol) was added at 0°C. The reaction was stirred for 30 min; then $NaBH_4$ (0.05 g, 1.3 mmol) was added in one portion. After 30 min Ac_2O (0.11 mL, 1.13 mmol) was added and the reaction was stirred at 0°C for 1 h. The reaction mixture was diluted with H_2O and extracted with EtOAc. The organic phase was washed with brine, dried and the solvent removed under reduced pressure. The product was purified by flash chromatography (eluent: CH_2Cl_2/EtOAc 9/1) to give a colourless oil which solidified by treating with Et_2O (yield 64%). An analytically pure sample was obtained by recrystallization from *i*-PrOH/*i*-Pr_2O mixture, mp 102°C (*i*-PrOH/*i*-Pr_2O). MS (CI) *m/z* 503 $(M+1)^+$. ^{1}H-NMR ($CDCl_3$): δ 2.02, 2.15 (2s, 3H, COC*H*$_3$), 2.92-3.00 (m, 2H), 3.50, 3.64 (2t, 2H) (IndC*H*$_2$C*H*$_2$), 3.84, 3.85 (2s, 3H, OC*H*$_3$), 4.21 (d, 2H, C_6H_4OC*H*$_2$) 4.34, 4.57 (2s, 2H, C_6H_4C*H*$_2$N), 4.77 (dd, 1H, C*H*HONO$_2$), 4.90 (dd, 1H, CH*H*ONO$_2$), 5.56-5.60 (m, 1H, C*H*ONO$_2$), 6.81-7.27 (m, 8H, C_6H_4, 4 C*H* Ind), 8.07, 8.16 (2s, 1H, N*H*Ind). Due to amide resonance, the ^{13}C-NMR spectrum displays a complex pattern of signals which is poorly informative. Therefore, ^{13}C-NMR chemical shifts are not reported. Anal. ($C_{23}H_{26}N_4O_9$) C, H, N.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]-*N*-[2-(4-phenylfuroxan-3-sulfonyl)ethyl]acetamide (42)**

To a solution of **41** (0.30 g, 1.2 mmol) in CH_2Cl_2 **30** (0.22 g, 1.2 mmol) was added and the reaction mixture was stirred for 45 min. Then, Ac_2O (0.13 mL, 1.4 mmol) and Et_3N (0.19 mL, 1.4 mmol) were added and stirring was continued for 30 min. The reaction mixture was diluted with CH_2Cl_2 and the organic phase was washed with a phosphate buffer (pH 5.5), $NaHCO_3$ sat. sol., brine, dried and evaporated. The obtained oil was purified by flash chromatography (eluent: CH_2Cl_2/EtOAc 95/5) to give a colourless oil which solidified by treating with *i*-Pr_2O at –10°C (yield 82%). An analytically pure sample was obtained by recrystallization from EtOH, mp 121.5-122°C (EtOH). MS (EI) *m/z* 484 (M^+). ^{1}H-NMR

(DMSO-d_6): δ 1.80, 2.01 (2s, 3H, COCH_3), 2.84-2.89 (m, 2H), 3.44-3.51 (m, 2H), 3.60-3.65 (m, 2H) (IndCH_2CH_2, CH_2CH_2SO_2), 3.74 (s, 3H, OCH_3), 3.79-3.85, 3.96-3.99 (2m, 2H, CH_2CH_2SO_2), 6.71 (dd, 1H), 7.00 (d, 1H), 7.09 (d, 1H), 7.23 (d, 1H) (4 C*H* Ind), 7.53-7.71 (m, 5H, C_6H_5), 10.69, 10.71 (2s, 1H, N*H*Ind). Due to amide resonance, the ^{13}C-NMR spectrum displays a complex pattern of signals which is poorly informative. Therefore, ^{13}C-NMR chemical shifts are not reported. Anal. ($C_{23}H_{24}N_4O_6S \cdot 0.5\ H_2O$) C, H, N.

***N*-(2-Hydroxyethyl)-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide (44)**

To a suspension of **43** (2.10 g, 8.5 mmol) in THF dry under positive N_2 pressure $LiAlH_4$ (1.00 g, 26 mmol) was added in small portions at 0°C. After complete addition the ice bath was removed and the reaction was refluxed for 48 h. Then, the reaction mixture was cooled again and MeOH was added in small portions in order to destroy the excess of $LiAlH_4$. To the obtained mixture Ac_2O (4 mL, 42 mmol) was added at 0°C. After 10 min the reaction was poured into 1N HCl and extracted with EtOAc. The organic phase was washed with H_2O, $NaHCO_3$ sat. sol., brine, dried and evaporated. The obtained oil was purified by flash chromatography (eluent: EtOAc) to give a colourless oil (yield 65%). MS (EI) *m/z* 276 (M^+). ^{1}H-NMR (DMSO-d_6): δ 1.86, 2.04 (2s, 3H, COCH_3), 2.80-2.93 (m, 2H), 3.30-3.57 (m, 6H) (CH_2CH_2NCH_2CH_2OH), 3.76 (s, 3H, OCH_3), 4.70, 4.84 (2t, 1H, O*H*), 6.71 (dd, 1H), 7.02-7.23 (d, 3H) (4 C*H* Ind), 10.63, 10.70 (2s, 1H, N*H*Ind). Due to amide resonance, the ^{13}C-NMR spectrum displays a complex pattern of signals which is poorly informative. Therefore, ^{13}C-NMR chemical shifts are not reported.

***N*-[2-(5-Methoxy-1*H*-indol-3-yl)ethyl]-*N*-[2-(3-phenylfuroxan-4-yloxy)ethyl]acetamide (45)**

To a suspension of NaH (0.07 g, 1.8 mmol) in dry THF under positive N_2 pressure a solution of **44** (0.39 g, 1.4 mmol) in dry THF was added dropwise at RT. The reaction

mixture was cooled in an ice-salt bath and a solution of **14** (0.47 g, 1.52 mmol) in dry THF was added dropwise. The reaction mixture was stirred overnight, poured into brine and extracted with EtOAc. The organic phase was washed with H_2O, brine, dried and evaporated. The obtained product was crystallized twice from EtOH to give a beige crystalline powder (yield 67%), mp 135-136°C (EtOH). MS (EI) 436 (M^+). ^{1}H-NMR (DMSO-*d*$_6$): δ 1.90, 2.08 (2s, 3H, COC*H*$_3$), 2.85-2.98 (m, 2H), 3.36-3.85 (m, 7H), 4.56 (m, 2H) (C*H*$_2$C*H*$_2$NC*H*$_2$C*H*$_2$O, OC*H*$_3$), 6.71-6.74 (m, 1H), 7.03-7.26 (m, 3H) (4 C*H* Ind), 7.50 (m, 3H), 7.99-8.03 (m, 2H) (C$_6$*H*$_5$), 10.65, 10.72 (2s, 1H, N*H*Ind). Due to amide resonance, the ^{13}C-NMR spectrum displays a complex pattern of signals which is poorly informative. Therefore, ^{13}C-NMR chemical shifts are not reported. Anal. ($C_{23}H_{24}N_4O_5 \cdot 0.25\ H_2O$) C, H, N.

***N*-[2-(3-Benzenesulfonylfuroxan-4-yloxy)ethyl]-*N*-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide (46)**

To a suspension of NaH (0.22 g, 5.5 mmol) in dry THF under positive N_2 pressure a solution of **44** (1.30 g, 4.5 mmol) in dry THF was added dropwise at RT. The reaction mixture was cooled in a dry-ice/acetonitrile bath, and a solution of **15** (1.70 g, 4.6 mmol) in dry THF was added dropwise. The reaction mixture was allowed to reach RT overnight, poured into brine and extracted with EtOAc. The organic phase was washed with H_2O, brine, dried and evaporated. The obtained oil solidified when treated with cold EtOH. The solid product was collected, dissolved in acetone and precipitated at –15°C by adding Et_2O, to give pale pink crystals (yield 65%), mp 99-102°C (acetone/Et_2O). MS (EI) 500 (M^+). ^{1}H-NMR (DMSO-*d*$_6$): δ 1.90, 2.17 (2s, 3H, COC*H*$_3$), 2.84-3.00 (m, 2H), 3.57-3.76 (m, 7H), 4.50-4.53 (m, 2H) (C*H*$_2$C*H*$_2$NC*H*$_2$C*H*$_2$O, OC*H*$_3$), 6.71-6.74 (m, 1H), 7.04-7.26 (m, 3H) (4 C*H* Ind), 7.67 (m, 2H), 7.84-7.99 (m, 3H) (C$_6$*H*$_5$), 10.65, 10.73 (2s, 1H, N*H*Ind). Due to amide resonance, the ^{13}C-NMR spectrum displays a complex pattern of signals which is poorly informative.

Therefore, ^{13}C-NMR chemical shifts are not reported. Anal. ($C_{23}H_{24}N_4O_7S \cdot 0.3\ Et_2O$) C, H, N.

Lipophilicity measurements

Log P_{oct} of all the compounds was obtained by RP-HPLC analyses performed with a liquid chromatograph (LC-10AS, Shimadzu) equipped with a UV-Vis diode-array detector (SPD-M10A, Shimadzu) operating at 226 and 254 nm. Retention time measurements were performed on a Purospher® RP-18 endcapped column (250 × 4 mm, 5 μm, Merck Darmstadt, Germany) thermostated at 40°C. The mobile phase consisted of mixtures of 0.02 M pH 7.4 phosphate buffer and methanol in proportions varying from 40 to 70% (v/v). The phosphate buffer was filtered under vacuum through a 0.45 μm HA Millipore filter (Millipore, Milford, MA, USA). The flow rates ranged from 0.8 mL min^{-1} to 1.0 mL min^{-1}. Stock solutions (10^{-2} M) of compounds were prepared in methanol and diluted ($10^{-3} \div 10^{-4}$ M) in mobile phase for injection (50 μL). Thiourea was used as the unretained compound. The logarithm of the retention factors (log *k*) was determined for each compound working with a minimum of four different methanol-buffer ratios; all samples were injected at least three times for each mobile phase. A linear relationship between log *k* and the volume fraction of methanol in the eluent was found for all compounds (in all cases r^2 was higher than 0.99). Log k_w, namely the logarithm of the retention factor corresponding to 0% methanol modifier, was obtained by linear extrapolation. Log P_{oct} values for melatonin derivatives were calculated from their log k_w values using Eq.1 [25]; the 95% confidence intervals of regression coefficients are given within parentheses.

$$\log P = 1.131\ (\pm 0.040) \log k_w - 0.408\ (\pm 0.102) \qquad (1)$$

$n = 52$, $r^2 = 0.985$, $s = 0.110$, $F = 3206$

Antioxidant properties

TBARS assay

Hepatic microsomal membranes from male Wistar rats (200 g) were prepared by differential centrifugation (8000 × *g*, 20 min; 120000 × *g*, 1 h) in a HEPES/Sucrose buffer (10 mM, 250 mM, pH = 7.4) and stored at -80°C. Incubation was performed at 37°C in a Tris-HCl/KCl buffer (100 mM, 150 mM, pH = 7.4) containing microsomal membranes (2 mg/mL), sodium ascorbate (100 μM) and either DMSO solutions of the tested compounds or DMSO alone. Addition of DMSO alone (maximal amount 5%) did not significantly change the extent of peroxidation in the control experiments. Lipid peroxidation was initiated by the addition of 2.5 mM $FeSO_4$. Aliquots were taken from the incubation mixture at 5, 15 and 30 min and treated with 10% w/v trichloroacetic acid (TCA). Lipid peroxidation was assessed by spectrophotometric (543 nm) determination of the 2-thiobarbituric acid reactive substances (TBARS) consisting mainly of malondialdehyde (MDA); TBARS concentrations (expressed in nmol mg^{-1} protein) were obtained by interpolation with a MDA standard curve. The antioxidant activity of tested compounds was evaluated as % inhibition of TBARS production with respect to control samples using the values obtained at 30 min of incubation. TBARS-IC_{50} values were calculated by non-linear regression analysis (Table 1).

$ABTS^{+\bullet}$ assay

Acetonitrile solutions of melatonin antioxidants (12.5 μM) described in the present work were rapidly mixed with a pH 7.4 buffered solution of 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical cation ($ABTS^{+\bullet}$) (potassium phosphate buffer 50 mM, temperature controlled at 37°C, final concentration of the $ABTS^{+\bullet}$ about 31.5 μM). The percent of $ABTS^{+\bullet}$ reduction after 1 min and 10 min respectively (Table 1) was determined on the basis of the decrease of the $ABTS^{+\bullet}$ absorbance at 415 nm (Varian Cary

50BIO UV/Vis spectrophotometer) according to the procedure previously described [26]. Acetonitrile did not interfere in the detection. The progress of the reaction during the first 15 seconds in the presence of a fixed amount of $ABTS^{+\bullet}$ and variable of an amount of each melatonin derivative was also monitored by the decrease of $ABTS^{+\bullet}$ absorbance followed spectrophotometrically (Figure 1A). Plots of $1/[ABTS^{+\bullet}]$ *vs* time afforded straight lines (Figure 1B). The slopes of these lines were plotted against the ratio $[antioxidant]/[ABTS^{+\bullet}]$ (Figure 1C). Linear regression afforded parameter Z (slope of the line in mol L^{-1} s^{-1}); this procedure was the same as the one previously used to evaluate the capacity of diphenylpicrylhydrazyl radical ($DPPH^{\bullet}$) to react with phenols [27]. Log Z was taken as a quantification of the capacity of studied antioxidants to interact with the $ABTS^{+\bullet}$ (Table 1) in the initial phase of reaction ($t \rightarrow 0$).

ALP assay

The antioxidant capacity of NO-donor melatonin derivatives to protect proteins from peroxyl radical-induced activity loss was measured by their ability to preserve the catalytic effectiveness of alkaline phosphatase (ALP) despite peroxyl radical attack, as described previously [28].

Briefly, ALP solutions (2 mU mL^{-1} in 0.1 M glycine/NaOH-buffer pH 8.3, containing $MgCl_2$ and $ZnCl_2$ at 1 mM concentrations) were preincubated for 15 min at 40°C with DMSO solutions of melatonin antioxidants (final concentrations of 0.16 to 316 µM, final DMSO concentration 2%). Oxidation was initiated by an addition of 5 mM α,α'-azodiisobutyramidine dihydrochloride (AAPH) and continued for 90 min at 40°C. The catalytic activity of ALP was assessed by measuring the enzymatic dephosphorylation of 4-methylumbelliferyl phosphate (4-MUP) to fluorescent 4-methylumbelliferone (4-MU). After an addition of 20 µL of 5 µM 4-MUP, the fluorescence (λ_{Ex} 360 ± 20 nm, λ_{Em} 460 ± 20 nm) was monitored at room

temperature for 15 min using a Bio-Tek FL_X 800 microplate fluorescence reader and KC4 v3.3 software (Bio-Tec Instruments Inc., Winooski, USA). The remaining hydrolytic activity of oxidized ALP was calculated as the percentage of ALP activity obtained in the absence of oxidation but in the presence of AAPH during the activity determination, which was assumed to be 100%. The antioxidant potencies of test compounds were expressed as ALP-IC_{50} values and calculated by curve fitting according to the classical sigmoidal dose-response equation. The ALP-IC_{50} values of the antioxidants were determined in triplicate from at least ten test compound concentrations (Table 1).

Vasodilator activity

Thoracic aortas were isolated from male Wistar rats weighing 180-200 g. The purposes and the protocols of our studies have been approved by the Ministero della Salute, Rome, Italy. The endothelium was removed and the vessels were helically cut: three strips were obtained from each aorta. The tissues were mounted under 1.0 g tension in organ baths containing 30 mL of Krebs-bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12.0, glucose 11.1, maintained at 37°C and gassed with 95% O_2, 5% CO_2 (pH = 7.4). The aortic strips were allowed to equilibrate for 120 min and then contracted with 1 mM *L*-phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Results, expressed as EC_{50} ± SE (μM), are collected in Table 1; values are the mean of at least five experiments. The effects of 1 μM ODQ on relaxation were evaluated in a separate series of experiments in which it was added 5 minutes before the contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab®. An addition of the drug vehicle, DMSO, had no appreciable effect on contraction level.

MT_1 and MT_2 membrane receptor affinities

Binding affinities of all NO-donor melatonin derivatives at these two receptors were determined using 2-$I^{[125]}$ iodomelatonin as labelled ligand in competition experiments on cloned human MT_1 and MT_2 subtypes. Non-specific binding was assessed with 10 μM melatonin. To define the functional activity of the new compounds at each receptor subtype, [^{35}S]GTPγS binding assay was performed. The amount of [^{35}S]GTPγS is proportional to the level of the analogue-induced G-protein activation and related to the intrinsic activity. Binding affinities, expressed as pK_i values, and intrinsic activities are collected in Table 2.

Results and Discussion

All target compounds were assayed as inhibitors of ferrous salt/ascorbate induced peroxidation of microsomial membrane lipids of rat hepatocytes. Melatonin (**1**), the simple nitric esters **3**, **4**, and furoxans **5-7** were also considered for a comparison. Microsomial membranes were treated in the presence of increasing concentrations of the products, as well as in the absence of products. The progress of the peroxidation was followed by visible spectroscopy detection of 2-thiobarbituric acid reactive substances (TBARS) which are the final metabolites of the autooxidation. This is a widely used method even though the reaction is not very specific and reaction conditions have a significant effect on colour development [29]. All the products were able to inhibit autooxidation in a concentration-dependent manner. An example of this behaviour is shown in Figure 2 and the antioxidant potencies, expressed as TBARS-IC_{50}, are reported in Table 1. All the simple NO-donors taken as references inhibited lipid peroxidation at concentrations > 500 μM or were inactive. The only exception was furoxan **5**, possessing moderate antioxidant activity (TBARS-IC_{50} of 110 μM), as already previously reported [10,11]. All the NO-donor melatonins displayed higher antioxidant properties than that of melatonin. Among the products obtained by the modification of the 5-

substituent the most active compound was the highly lipophilic bis(nitrooxy)propylbenzyl structure **17** followed by the more polar furoxan derivative **19** and the nitrooxypropyl melatonin analogue **16** respectively. In the case of **18**, it was impossible to obtain a complete concentration-response curve owing to problems of solubility. The modulation of the acetyl substructure gave rise to the most active compounds **31**, **32**, **34**, **35**. The only exception is structure **33**, in which the hydrophilic 4-hydroxymethyl-3-furoxancarbonyl moiety was substituted for the acetyl group of melatonin. Finally, the introduction on the NH group of furoxan substructures afforded products (**40**, **42**, **45**, **46**) with quite good activities. The strict analogues of melatonin **36**-**39**, studied as reference compounds, displayed a wide range of antioxidant potencies which parallels their lipophilicity. From this qualitative picture, a role of the molecular lipophilicity in determining the antioxidant behaviour of the whole class of products seems to emerge. Indeed, a QSAR analysis showed that the antioxidant potencies (TBARS-pIC_{50}) are linearly correlated to log P by Eq.2; the 95% confidence intervals of regression coefficients are given within parentheses.

$$\text{TBARS-}pIC_{50} = 0.37\ (\pm 0.10)\ \log P + 3.10\ (\pm 0.39) \qquad (2)$$

$n = 17$, $r^2 = 0.805$, $s = 0.243$, $F = 61.78$ ($F_{1,15}$ (99%) = 8.68), $q^2 = 0.720$

A significant improvement of the correlation was obtained when the parabolic model was used (Eq.3, Figure 3A)

$$\text{TBARS-}pIC_{50} = -0.096\ (\pm 0.043)\ (\log P)^2 + 1.06\ (\pm 0.31)\ \log P + 2.03\ (\pm 0.54) \qquad (3)$$

$n = 17$, $r^2 = 0.926$, $s = 0.154$, $F = 88.07$ ($F_{2,14}$ (99%) = 6.51), $q^2 = 0.903$

By contrast, no significant improvement of the correlation occurred when the bi-linear model was applied. Equation 3 explains about 90% of the data variance and has a relevant predictive capacity ($q^2 = 0.903$). Its limit is a high co-linearity degree between log P and $(\log P)^2$ ($r^2 = 0.957$). It would be necessary to take into account additional members of the series endowed with very high lipophilicity in order to see if this co-linearity is broken down.

However, the probable very low solubility of such products discouraged this approach. Consequently, the question of whether the dependence of the antioxidant potencies of this series of products on the lipophilicity is linear, parabolic or bi-linear remains unanswered. In a recent work a linear dependence of the antioxidant potencies (TBARS assay) with log P_{oct} was found in a series of indole-based analogues of melatonin [30]. However, the lipophilicity range examined by the authors (log P 0.23 ÷ 3.50) was restricted with respect to the range studied in the presence work (log P 1.34 ÷ 6.10). This range includes the optimal lipophilicity value derived from equation 3 (log P_o = 5.50). These results indicate that NO-donor melatonin analogues with an appropriate lipophilicity can penetrate and diffuse into lipid membranes and act as scavengers of free radicals. The parabolic behaviour supported by Eq.3 suggests that the reduced antioxidant capacity can be attributed to lower diffusion rates into the membrane of compounds with low or very high lipophilicity. Therefore, the antioxidant potencies of these products in TBARS assay seems to be predominantly dependent on their partition process into membranes rather than on their intrinsic redox potential.

To confirm this hypothesis, the redox properties of melatonin derivatives were examined in more detail. Indeed, although the molecular mechanism of the antioxidant action of melatonin has not been fully established, an accredited hypothesis suggests that melatonin acts as an electron donor to afford a stable indolyl radical cation which could be further oxidized to N^1-acetyl-N^2-formyl-5-methoxykynurenamine [31]. The capability of undergoing electron transfer reactions of melatonin is frequently evaluated on the basis of its capacity of scavenging 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+\bullet}$). While the classical antioxidants scavenge one or less $ABTS^{+\bullet}$, each melatonin molecule can scavenge more than one $ABTS^{+\bullet}$, with a maximum of four [32]. Analysis of Table 1 shows that the NO-donor melatonin derivatives described in the present work are able to interact

with $ABTS^{+\bullet}$. After 1 min the % of reduction of $ABTS^{+\bullet}$ ranges from 31.3 to 92.2%, while after 10 min the reduction is almost complete for all members of the series. LogZ values are also distributed over a rather narrow range (2.63-4.11). These two reactivity parameters are very well linearly correlated (r^2 = 0.936). The introduction in the Eq.2 of logZ as a new independent variable does not improve the correlation and its coefficient is not significant. This indicates that the variation in the scavenging properties consequent to the structural modifications we performed on **1** plays a negligible role in comparison with lipophilicity, as far as their ability to inhibit lipid peroxidation is concerned.

The capacity of compounds **16-19**, **31-35**, **40**, **45** and **46** to protect proteins from peroxyl radical-induced damage was investigated by assessing their ability to preserve the catalytic effectiveness of alkaline phosphatase (ALP) despite oxidative stress. Protein oxidation was induced by the peroxyl radical generator α,α'-azodiisobutyramidine dihydrochloride (AAPH) and the decrease in catalytic activity of ALP to hydrolyze 4-methylumbelliferyl phosphate to fluorescent 4-methylumbelliferone was monitored as a marker of protein degradation. Melatonin **1**, the simple nitric esters **3**, **4** and furoxans **5-7** were tested as reference compounds. After pre-incubation of ALP with increasing antioxidant concentrations, or 2% of co-solvent for the control, protein oxidation by AAPH was accomplished during 90 min at 40°C. The antioxidant capacity of NO-donor melatonin derivatives to protect ALP from oxidation was expressed as ALP-IC_{50} (μM) and summarized in Table 1. All the tested NO-donor melatonin derivatives protected ALP against peroxyl radical-induced activity loss to about the same extent as the parent compound melatonin. Indeed, their ALP-IC_{50} values (5.5 to 22.3 μM) were very close to the ALP-IC_{50} of melatonin (10.3 μM). On the other hand, as expected, NO-donors **3-7** have shown no protection of ALP against oxidation at concentrations up to100 μM.

Moreover, no relationship between the capacity of NO-donor antioxidants to prevent protein oxidation and their capacity to inhibit lipid peroxidation appears. It can also be observed that the range of ALP-IC_{50} values obtained is narrower than the range of TBARS-IC_{50} values (Table 1). With the exception of inactive compounds, ALP-IC_{50} values vary from 5.5 to 22.3 μM whereas TBARS-IC_{50} values range from 11 to 372 μM. Hence, the potencies of the NO-donor antioxidants to inhibit ferrous salt/ascorbate-induced lipid peroxidation are more modulated than their potencies to prevent ALP from peroxyl radical-induced activity loss. This can be explained by considering the contribution of molecular lipophilicity and redox properties of the melatonin derivatives to their capacity to protect ALP from peroxyl radical-induced activity loss. Fig 3B shows that no correlation between the lipophilicity of NO-donor melatonin derivatives and their capacity to prevent ALP oxidation can be observed. In contrast to what has been shown for lipoperoxidation, lipophilicity has no influence on the capacity of these compounds to prevent protein oxidation. In fact, the protein oxidation assay is performed in an aqueous medium and no partitioning processes into membranes are implicated. Furthermore, despite a lack of correlation between the protection of protein oxidation and $ABTS^{+\bullet}$ scavenging activity, the similar ALP-IC_{50} values obtained for the NO-donor melatonins concur well with their comparable ability to participate in electron transfer reactions, as evidenced by the small distribution range of the log Z values found. The comparable capacity of these melatonin derivatives to inhibit peroxyl radical-induced protein oxidation is independent from their lipophilicity and in good agreement with their similar scavenging activity of $ABTS^{+\bullet}$.

The *in vitro* vasodilator activity of all NO-donor melatonin analogues was evaluated by assessing their ability to relax rat aorta strips pre-contracted with 1 μM phenylephrine. All the products were able to relax the contracted tissues in a concentration-dependent manner. An example of this behaviour is reported in Figure 4. Analysis of the data reported in Table 1

shows that the vasodilator potencies of the NO-donor melatonins parallels the potency sequence of the corresponding references quite well (Figure 5). When the experiments were repeated in the presence of 1 μM ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC), a decrease in the potencies was observed. This suggests the involvement of NO in the vasodilation.

Many actions displayed by melatonin can be explained as a direct consequence of its binding to MT_1 (formerly known as $Me1_a$) and MT_2 (formerly known as $Me1_b$) membrane receptors. These receptors belong to the family of G-protein coupled receptors linked to the inhibition of adenylyl cyclase. The affinity of melatonin for these targets is in the low picomolar and low nanomolar range respectively [33]. In particular, melatonin mediates two distinct responses in vascular smooth muscle, relaxation and constriction. Relaxation is mediated through MT_2 receptors while constriction is possibly induced via MT_1 receptors [34]. Analysis of the data reported in Table 2 shows that all the products are less effective ligands than melatonin. Most of them behave as partial agonists but some models display full agonist properties and product **19** is an antagonist at both receptor subtypes. The full agonist **16** was the structure with the greatest affinity towards both MT_1 and MT_2 receptors, about 13-fold lower with respect to melatonin. The lowest affinity was shown by the structures obtained by introducing the NO-donor moiety at the side-chain amide nitrogen. Interestingly, the partial agonist **18** exhibits a good selectivity, showing a MT_2/MT_1-K_i ratio of 103. This quite complex picture shows that joining NO-donor nitroxy and furoxan moieties to melatonin considerably affects its membrane receptor affinities, and brings to light the prospect of including MT_1 and MT_2 receptor binding in drug design considerations. However, in the present work, the goal in designing these products was to obtain NO-donor melatonin

derivatives with promising vasodilator and antioxidant properties rather than specific ligands to either subtype of melatonin receptors.

Conclusions

In conclusion, in this paper we describe a new class of melatonin derivatives bearing NO-donor moieties in different positions. All NO-donor melatonin derivatives were able to inhibit in a concentration-dependent manner ferrous salt/ascorbate induced peroxidation of microsomial membrane lipids and peroxyl radical induced oxidation of alkaline phosphatase, although their antioxidant reactivity differs between the two tests. The antioxidant capacities of NO-donor melatonin derivatives to inhibit lipoperoxidation was predominantly dependent on their lipophilicity, and therefore, on their partitioning process into membranes. On the other hand, the comparable capacity of these melatonin derivatives to inhibit protein oxidation was independent of their lipophilicity and in good agreement with their similar ability to participate in electron transfer reactions. All the products showed *in vitro* vasodilator activities which parallel the potency of the corresponding reference NO-donors quite well. Due to their interesting vasodilator and antioxidant properties, these products are promising leads for further *in vivo* studies.

Acknowledgments

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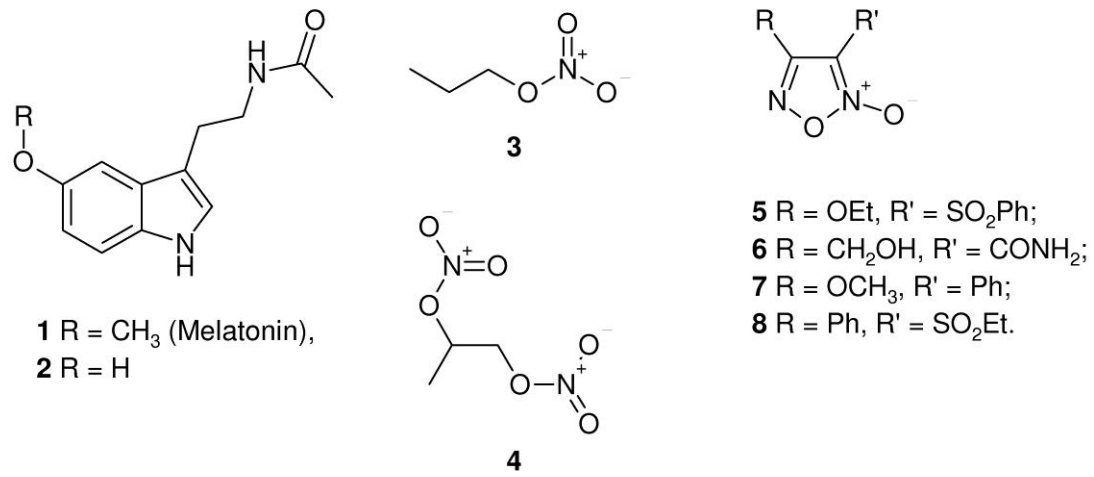
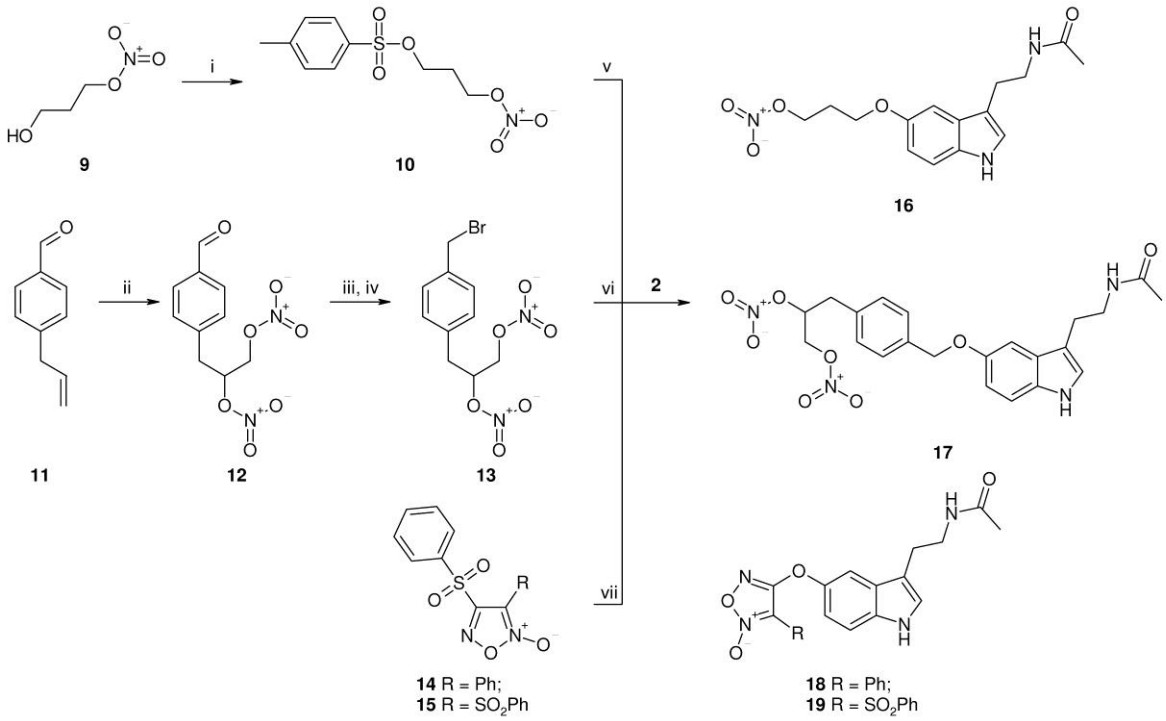
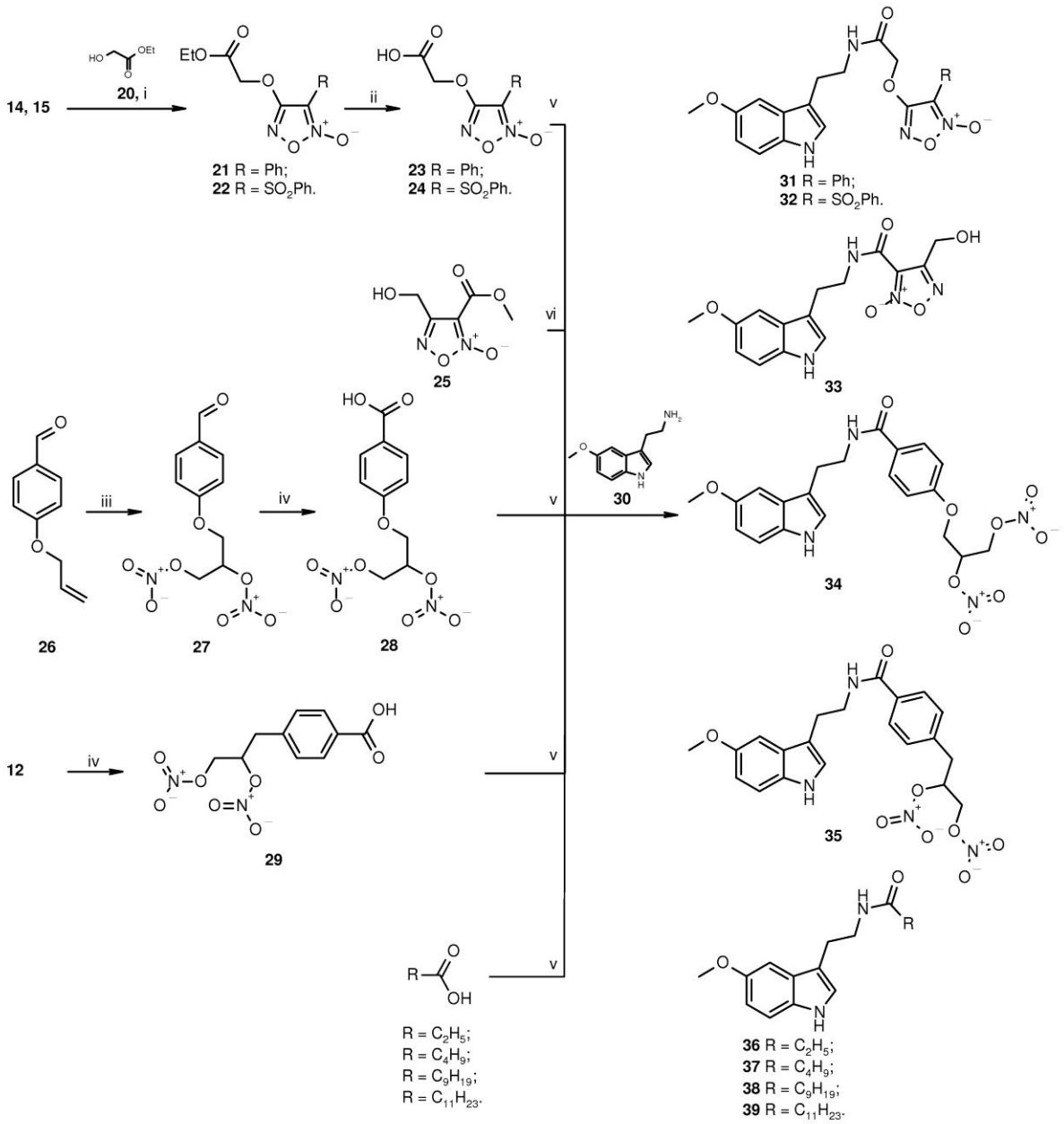


Chart 1.



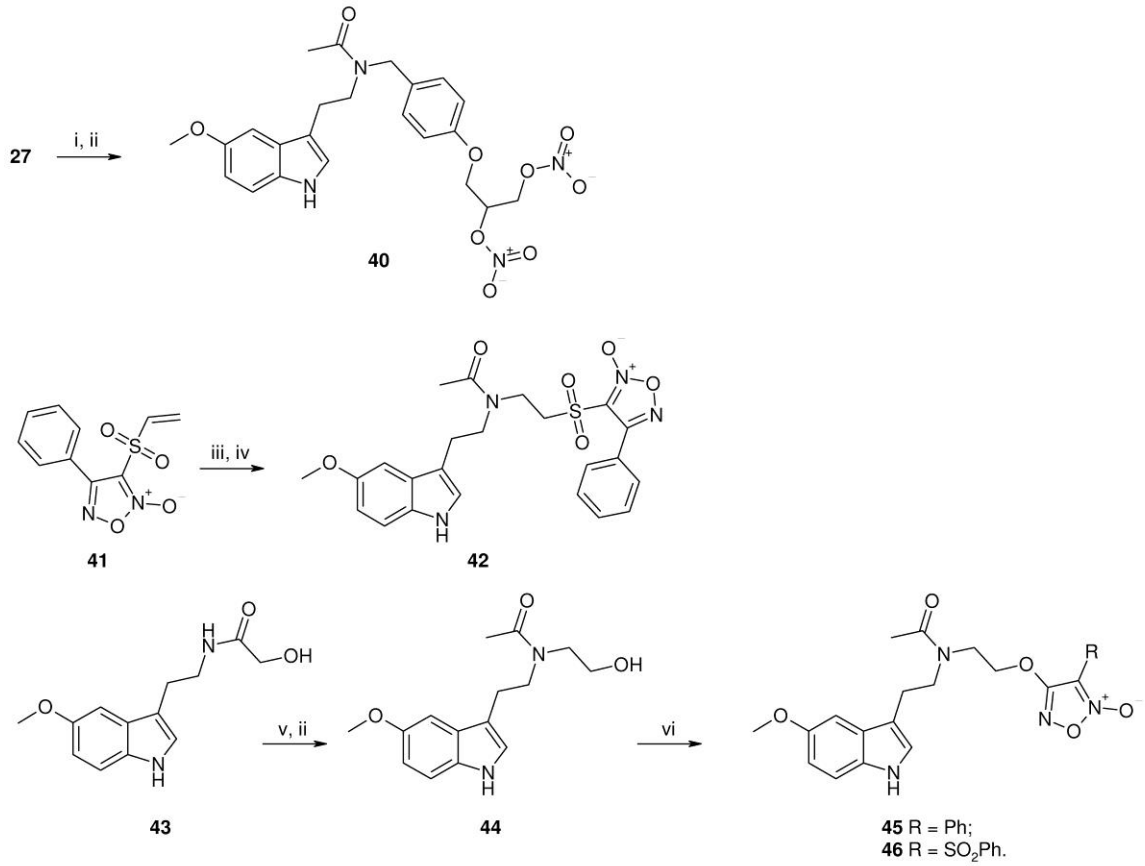
Scheme 1.

i) TsCl, Py, 0°C; ii) $AgNO_3$, I_2, CH_3CN, RT, then Δ; iii) $NaBH_4$, MeOH, 0°C; iv) NBS, PPh_3, CH_2Cl_2, 0°C; v) *t*BuO^-K^+, THF dry, 60°C; vi) K_2CO_3, CH_3CN, 60°C; vii) NaH, THF dry, RT.



Scheme 2.

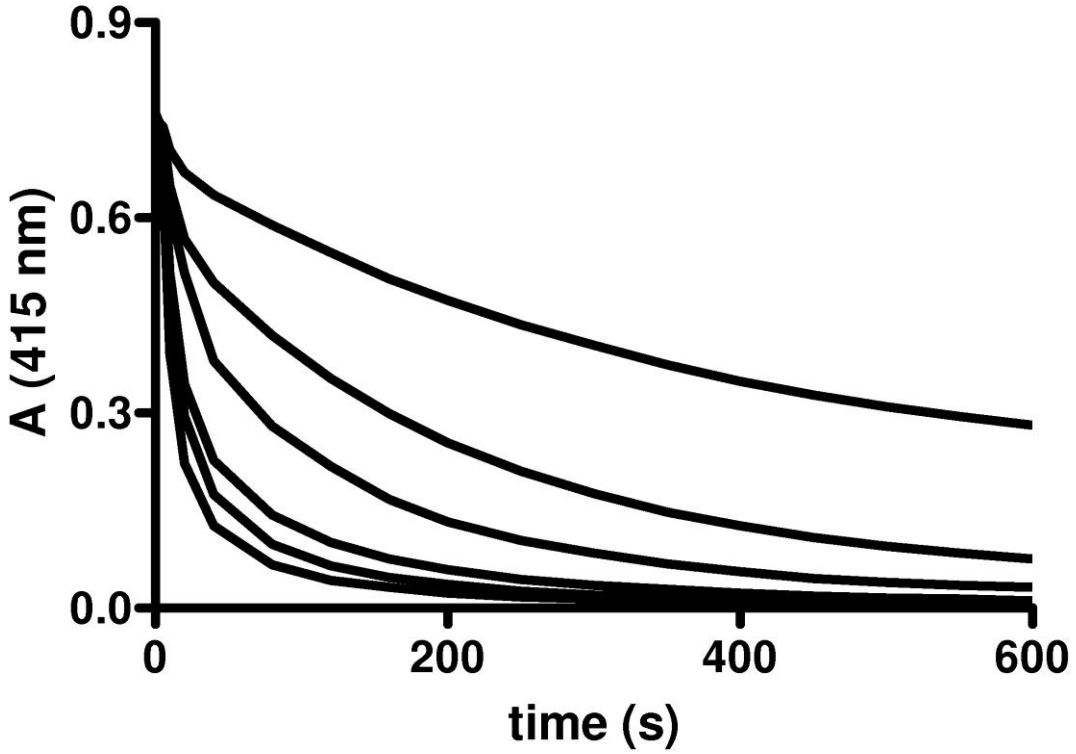
i) NaH, THF dry, 60°C; ii) Dioxane, HCl, Δ; iii) $AgNO_3$, I_2, CH_3CN, RT, then Δ; iv) $KMnO_4$, acetone, RT; v) CDI, CH_2Cl_2, RT; vi) DMAP, EtOH.



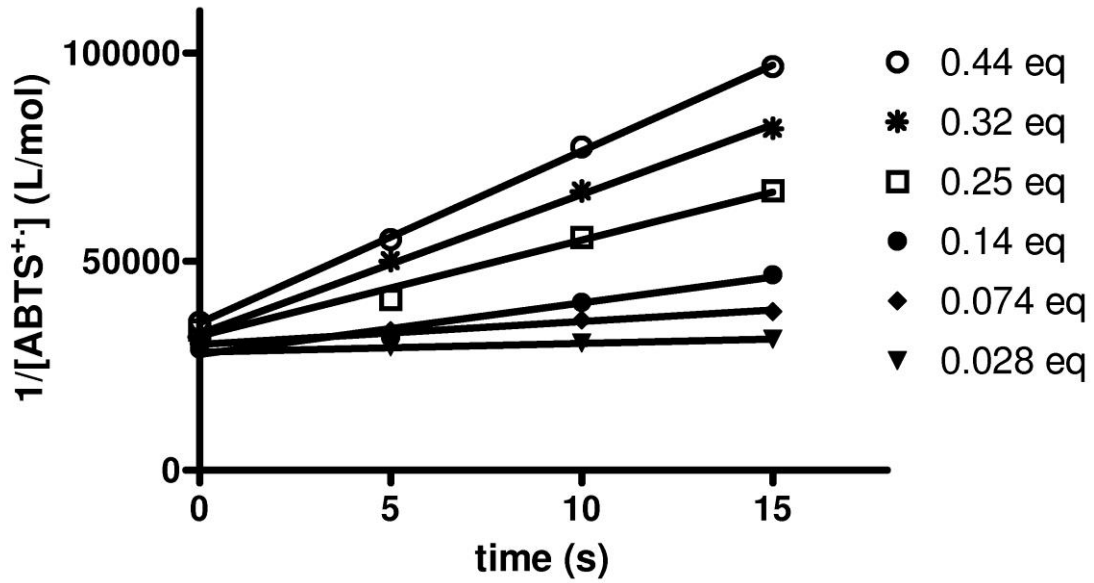
Scheme 3.

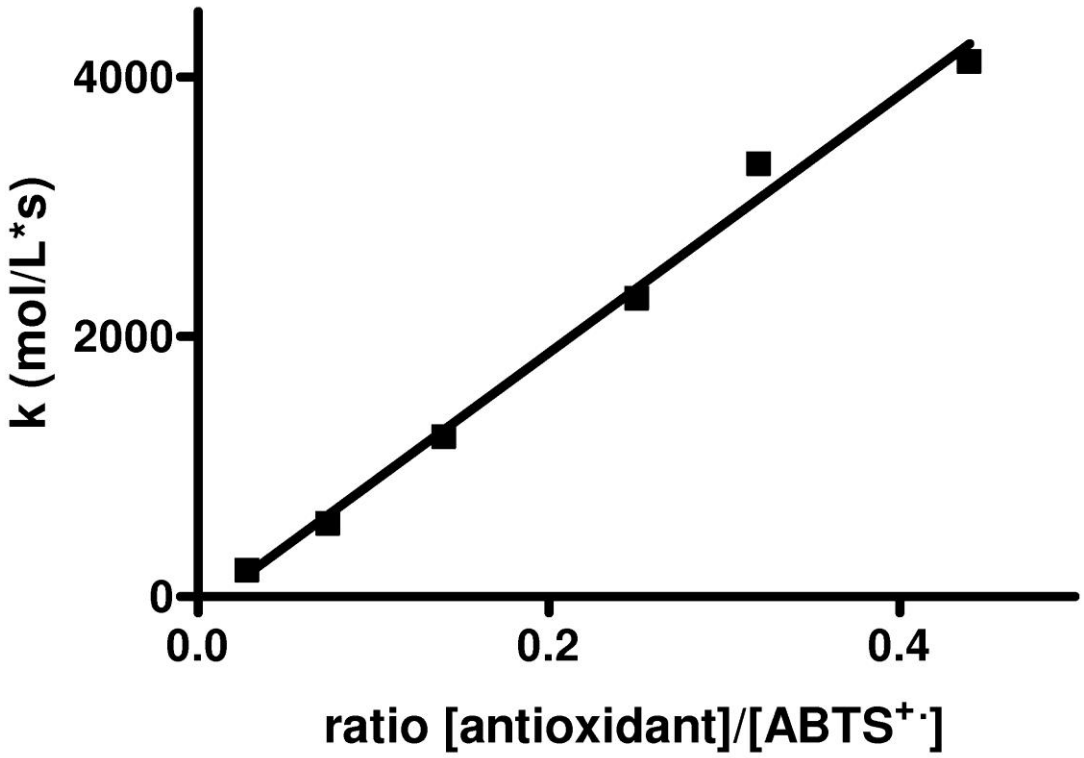
i) **30**, $NaBH_4$, MeOH, 0°C; ii) Ac_2O, 0°C; iii) **30**, CH_2Cl_2, 0°C; iv) Ac_2O, Et_3N, 0°C; v) $LiAlH_4$, THF dry; vi) **14**, NaH, THF dry, -15°C up to RT for **45**, **15**, -40°C up to RT for **46**.

(A)


0.9
0.6
0.3
0.0
A (415 nm)
0
200
400
600
time (s)

(B)


100000
50000
0
1/[ABTS+·] (L/mol)
0
5
10
15
time (s)
0.44 eq
0.32 eq
0.25 eq
0.14 eq
0.074 eq
0.028 eq



(C)

Figure 1. **(A)** Reaction of melatonin **1** with $ABTS^{+\bullet}$, monitored at 415 nm (0.028 eq, 0.074 eq, 0.14 eq, 0.25 eq, 0.32 eq, 0.44 eq). **(B)** Kinetics of the reaction of **1** with $ABTS^{+\bullet}$. **(C)** Calculation of Z for **1** (regression parameters: slope (Z) = 9923, r^2 = 0.992).

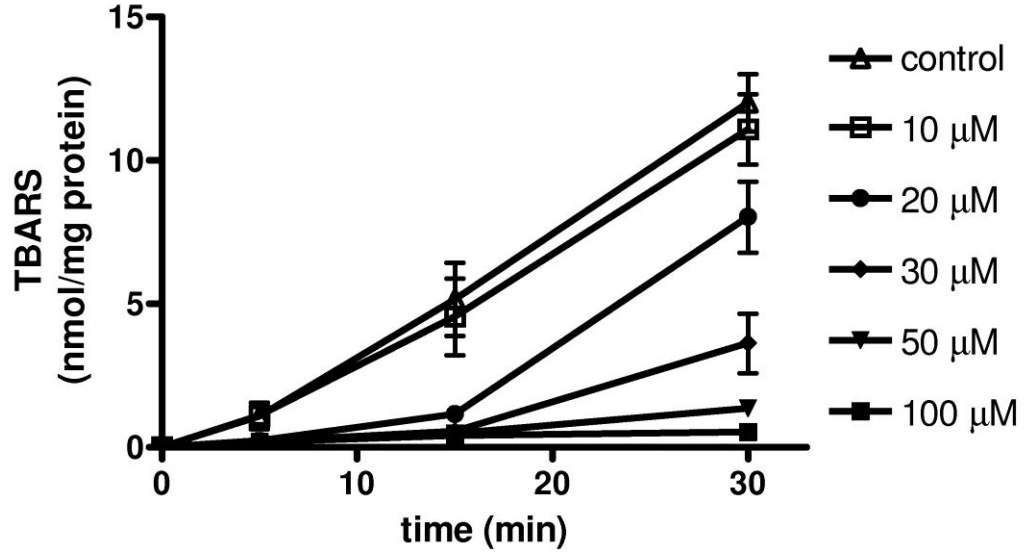
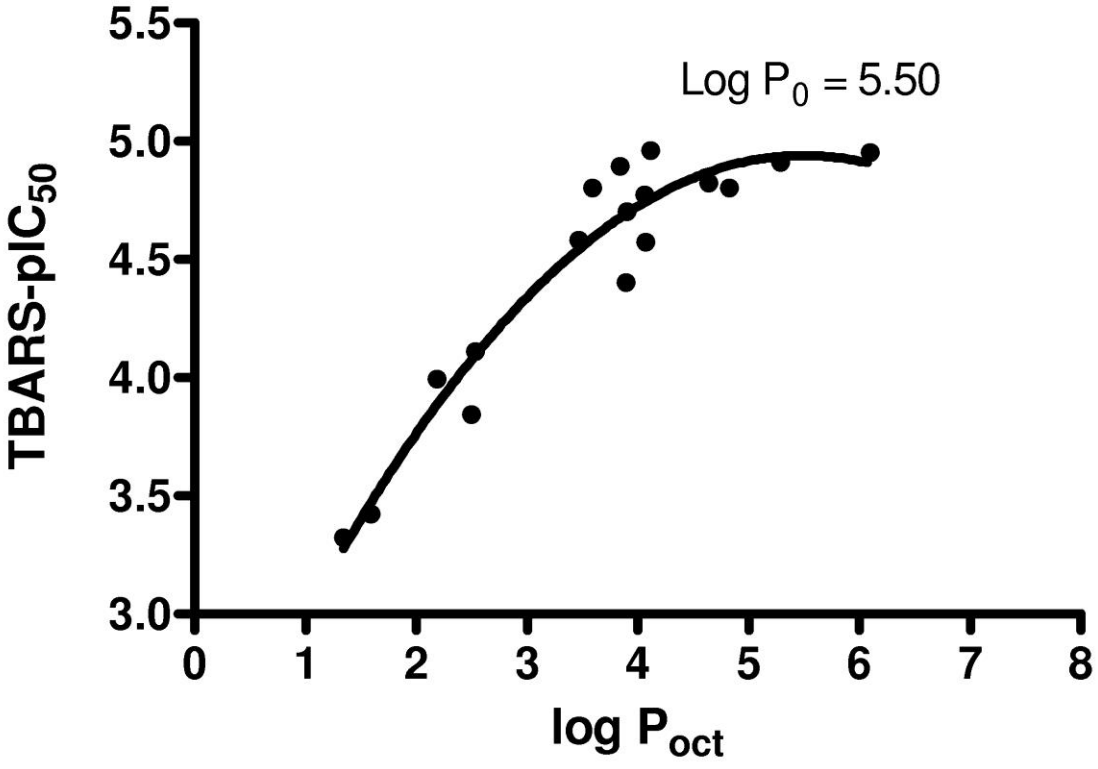


Figure 2. Effect of compound **19** on time course of lipid peroxidation.

(**A**)



(**B**)

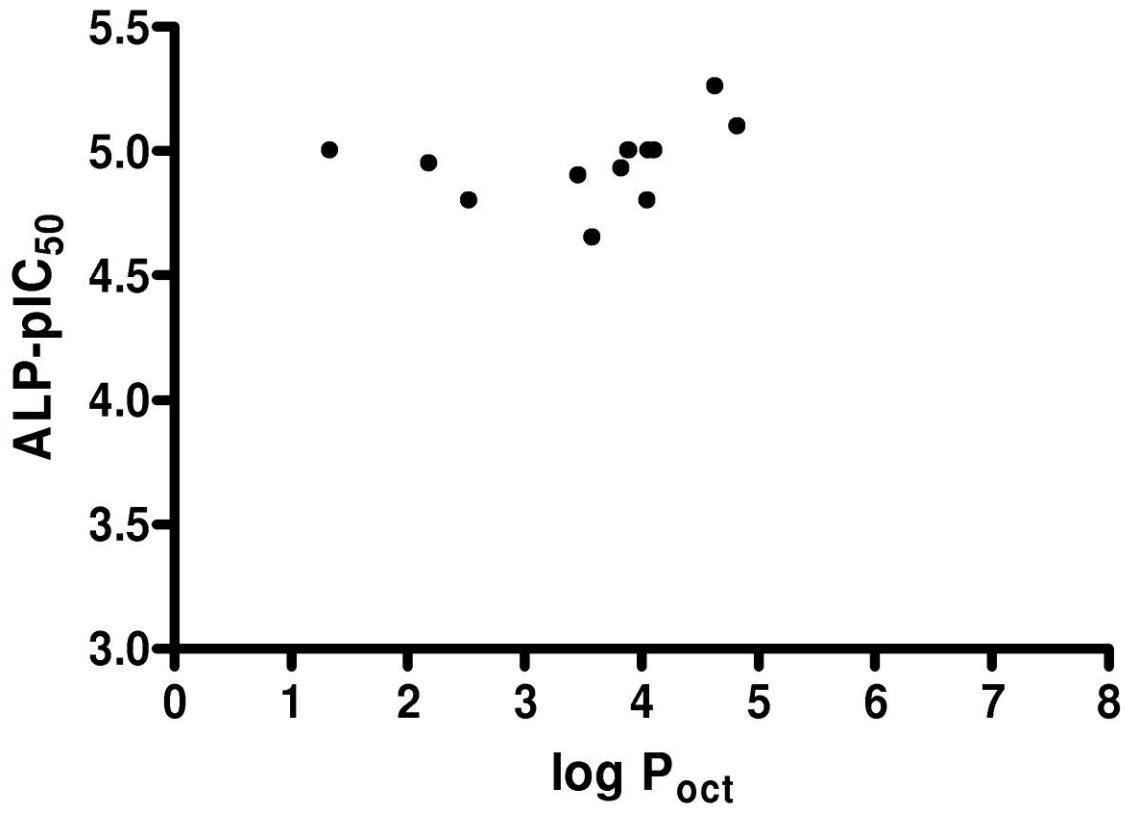


Figure 3. Correlation between antioxidant potencies and log P_{oct}: (**A**) TBARS-pIC_{50} values, (**B**) ALP-pIC_{50} values.

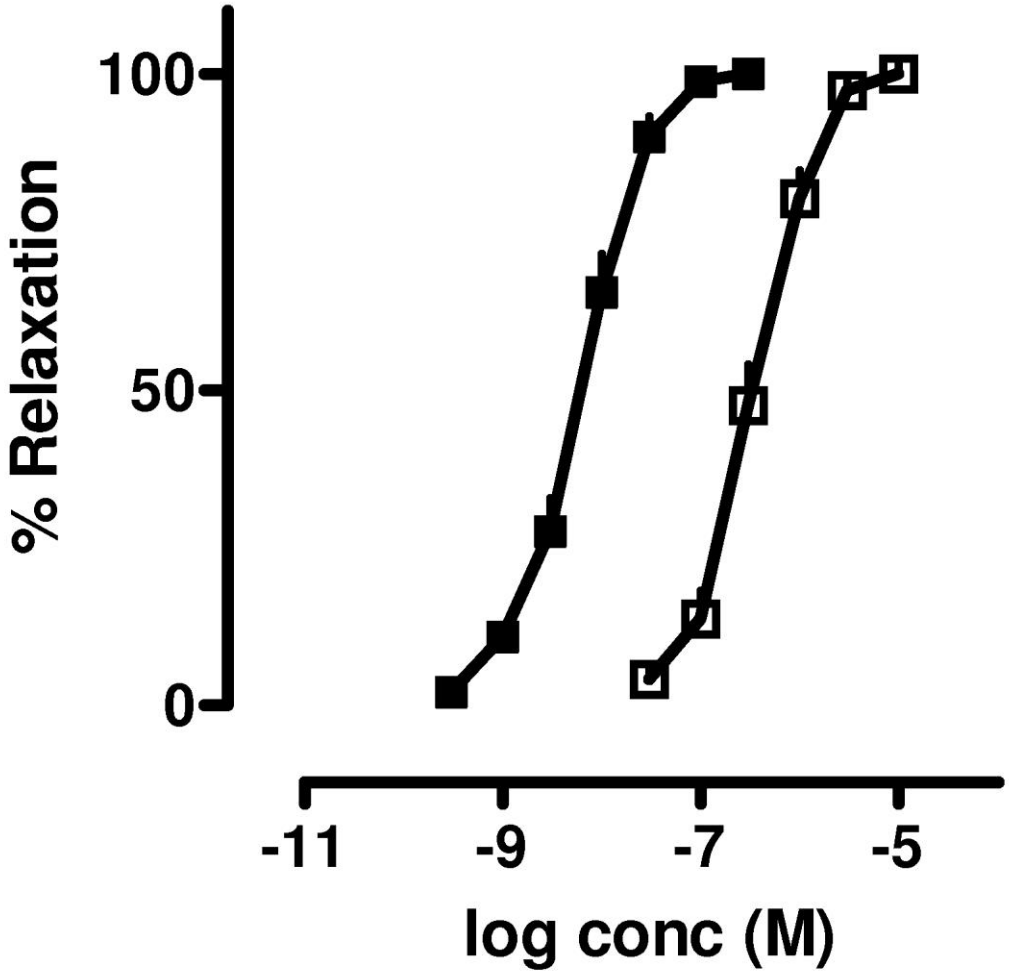


Figure 4. Concentration-response curves for vasodilating activity of compound **19** in the absence (solid square) and in the presence (open square) of ODQ.

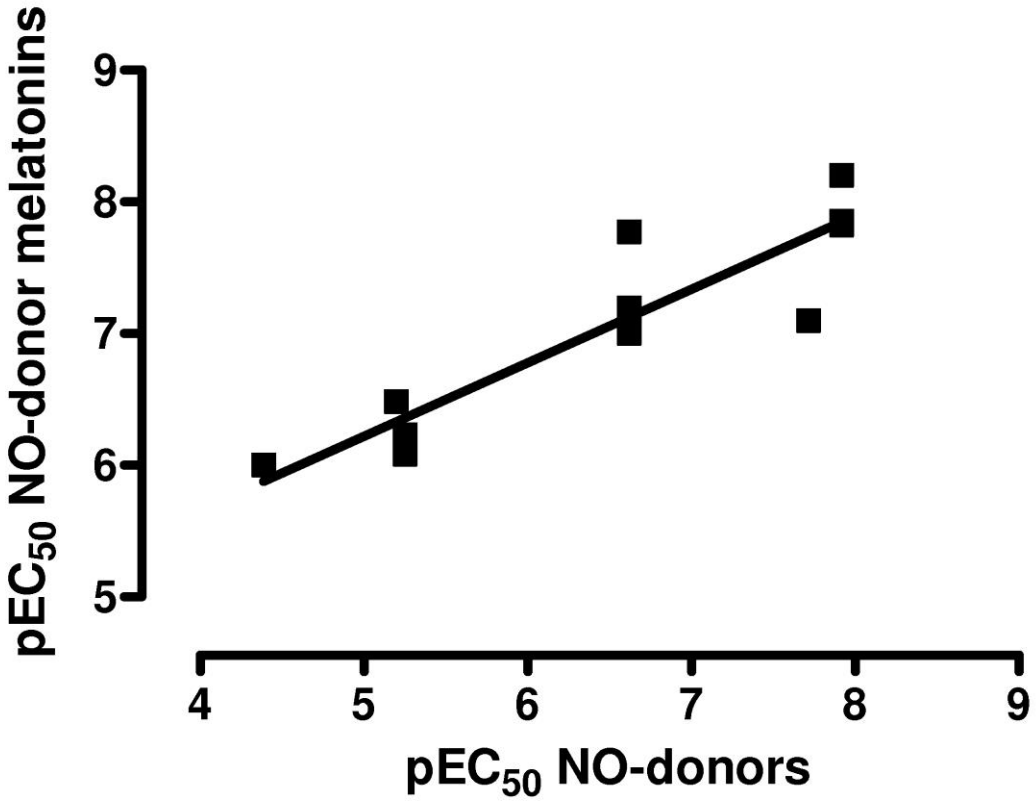


Figure 5. Relationship between the vasodilator potency of the NO-donor melatonins and the potency of the corresponding references.