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Communication

Design, synthesis and biological evaluation of novel β-substituted indol-3-yl ethylamido melatoninergic analogues

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Abstract

A series of new melatonin analogues have been synthesized. Interestingly, two of the new compounds, **11c** and **11e**, which did not show any appreciable affinity for the melatonin receptor, were found to be potent inhibitors of lipid peroxidation in rat liver microsomes. Analogue **11c**, in particular, is a better antioxidant than melatonin.

Introduction

The pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine) is present in all mammalian species including humans. Melatonin has been shown to have a physiological role in regulating seasonal breeding in photoperiodic species (Bartness et al 1993) and can entrain circadian rhythms (Arendt 1995). The hormone also increases vascular tone in rat tail artery (Ting et al 1997) and cerebral vascular bed (Capsoni et al 1995) and inhibits [^{3}H]dopamine release from rabbit retina (Dubocovich 1983). In addition, a sleep-promoting action of melatonin has been repeatedly observed in animal and human studies (Holmes & Sugden 1982; Dollins et al 1994). Melatonin analogues are currently being examined as a therapy for treating circadian rhythm disturbances resulting from various causes (e.g. jet-lag, shift-work, blindness) and the use of melatonin as a hypnotic has been advocated (Garfinkel et al 1995). Molecular cloning data suggest that melatonin exerts all of these effects through a family of specific, high-affinity, G-protein-coupled cell-membrane receptors, MT_1, MT_2 and Mel_{1c}, (Reppert et al 1994, 1995), which are particularly abundant in tissues known to respond to melatonin (e.g. in the retina and the suprachiasmatic nuclei of the hypothalamus). Various other effects have been ascribed to melatonin including effects on the immune system (Maestroni 1993), an ability to scavenge hydroxyl and peroxy free radicals (Reiter et al 1999) and an oncostatic action (Molis et al 1995).

Notwithstanding the renewed interest in melatonin and its actions in recent years, the pharmacology of the hormone is still in its infancy as a limited number of structurally diverse melatonin agonists and antagonists, which can be used as pharmacological tools, is available. Our previous work has been directed at mapping the interaction of melatonin with its receptor by synthesizing and evaluating a variety of melatonin analogues (Garratt et al 1994a, 1994b, 1995, 1996; Davies et al 1998). We have shown, in a structure–activity relationship study of a

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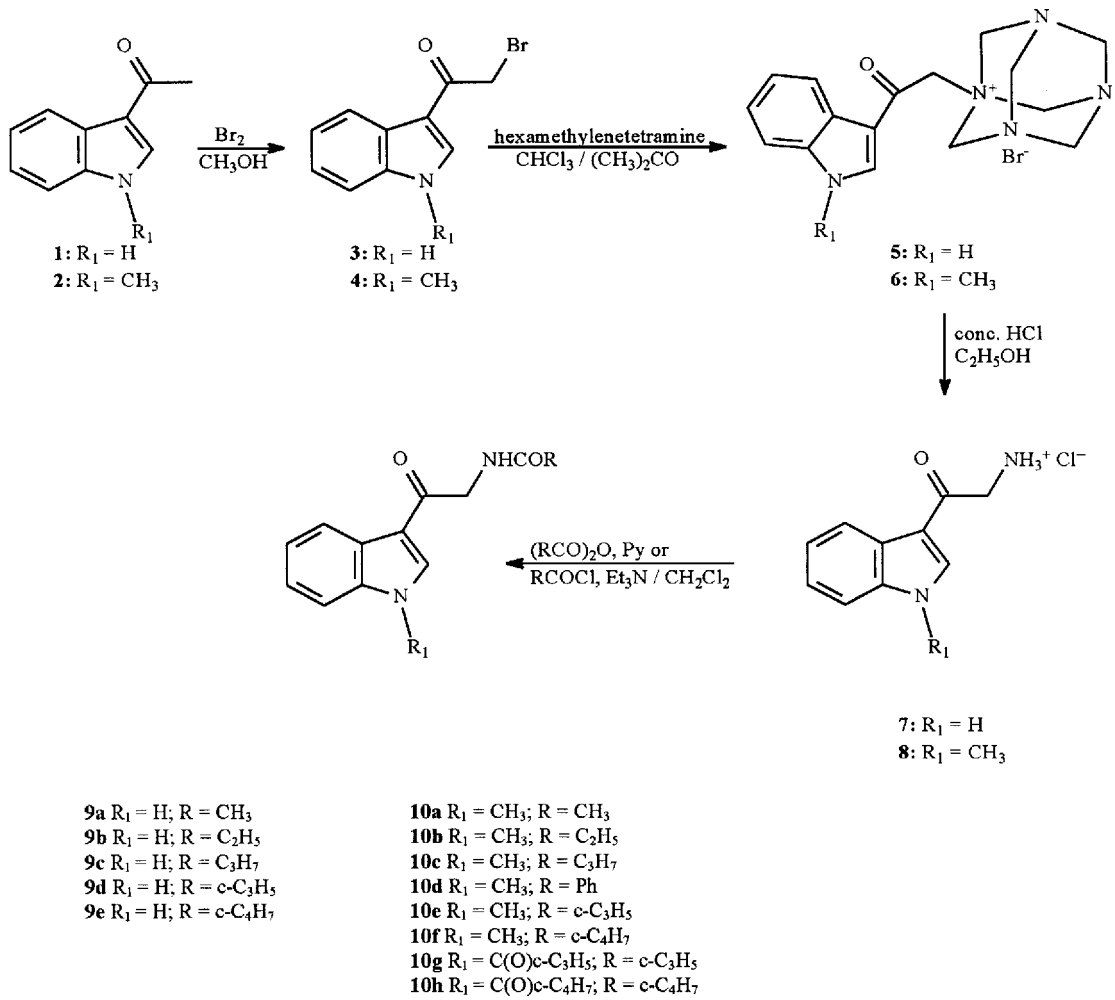


Figure 1 Structures and synthesis of series **9a–e** and **10a–h** analogues of melatonin.

series of simple aromatic molecules, that the minimal structural features for receptor recognition encompass an anisole or resorcinol dimethyl ether nucleus and an alkylamide moiety (Garratt et al 1996). Furthermore, we and others have demonstrated that the 5-methoxyl group of melatonin is an important site for binding to the receptor, but it is not an essential requirement for agonist activity (Garratt et al 1994a; Davies et al 1998). The active conformation of the 3-ethanamide side chain has also been established from studies with conformationally restricted indole (Spadoni et al 1993; Garratt et al 1994b; Davies et al 1998) and non-indole analogues (Copinga et al 1993; Sugden et al 1995; Grol & Jansen 1996; Jansen et al 1996; Leclerc et al 1996).

In our ongoing effort to probe the stereoelectronic requirements for optimal activity we have recently reported on the synthesis and biological activity of novel 2-phenyltryptamines annelated on the α-face of the pyrrole moiety by the introduction of 1, 2 or 3 methylene groups (Faust et al 2000). These molecules were prepared to probe the constraints at the receptor site with regard to the lower *N*1–C2 region of the indole nucleus.

In this study, we report the synthesis of analogues **9a–e**, **10a–h** and **11a–e** (Figures 1 and 2, respectively), which bear a keto and a hydroxy moiety β to the C3 acylamido chain. The constraint conferred on the side chain by the introduction of these groups functions as a vicarious probe for the investigation of the interaction of the upper part of the indole nucleus and especially the C3 side chain with the receptor. Furthermore, a number of reports in recent years have supported the claim that melatonin possesses antioxidant activity (for review see Reiter 1997). As there is just one report on the antioxidant action of analogues of melatonin (Gozzo et al

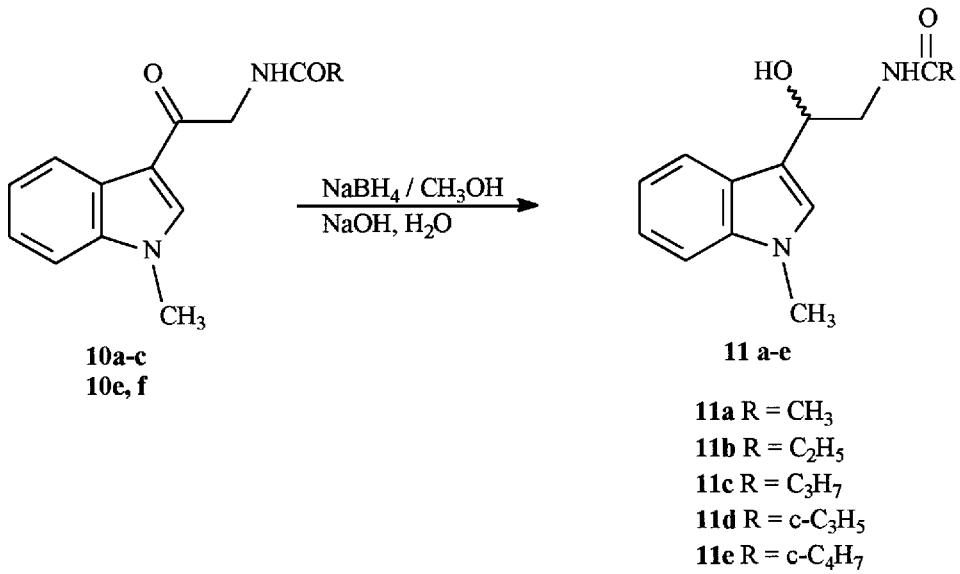


Figure 2 Synthesis of series **11a–e** analogues of melatonin.

1999), representative members of the new series were tested for their ability to inhibit in-vitro non-enzymatic peroxidation of microsomal membrane lipids.

Materials and Methods

Chemistry

Melting points were determined on a Büchi 530 apparatus and are uncorrected. ^{1}H NMR spectra were recorded either on a Bruker AC200 or a Bruker DRX400 MHz spectrometer, and the spectra are reported in δ. ^{13}C NMR spectra were taken at 50 MHz on a Bruker AC200 spectrometer. Tetramethylsilane was used as internal standard. All the experiments were carried out under an atmosphere of Argon. The solvents used were dried as follows: benzene using sodium wire; triethylamine over sodium hydroxide; dimethylformamide and dichloromethane over molecular sieves (4Å); diethyl ether and tetrahydrofuran over calcium hydride and acetone over molecular sieves (4Å). DC-Alufolien plates (Kieselgel 60 F_{254}, Schichtdicke 0.2 mm, Merck) were used for analytical TLC and were visualized with ultraviolet light or developed with iodine or phosphomolybdic acid. Microanalyses were carried out by the Microanalytical Section of the Institute of Organic and Pharmaceutical Chemistry, NHRF. Analyses indicated by the symbols of the elements were within $\pm$0.4% of the theoretical values.

*1-(1-Methyl-1*H*-indol-3-yl)ethanone (**2**)*

Methyl iodide (0.1 mL, 2.1 mmol) was added to a stirred mixture of 1-(1*H*-indol-3-yl)ethanone (**1**) (0.3 g, 1.9 mmol) and powdered potassium hydroxide (80%) (0.28 g, 4.4 mmol) in dimethylformamide (10 mL) at 0°C. The resulting suspension was then stirred at room temperature for 2 h before being poured into ice-water. Extraction with chloroform followed by washing of the organic phase with brine and drying over anhydrous sodium sulfate afforded a yellow solution, which was concentrated under reduced pressure to give 0.20 g, 90% of the title compound as a white solid pure enough to be used as such in the next step, mp 92–93°C (Kurihara et al 1987, 94–96°C). ^{1}H NMR (DMSO-d_6): 2.33 (s, 3H, $COCH_3$), 3.77 (s, 3H, *N*-CH_3), 7.35 (m, 3H, arom.), 7.77 (s, 1H, H2), 8.46 (m, 1H, arom).

General method for the preparation of the α*-bromo ketones (**3** and **4**)*

Bromine (0.1 mL, 1.9 mmol) was added drop-wise to a stirred suspension of 1-(1*H*-indol-3-yl)ethanone (**1**) (1.9 mmol) or 1-(1-methyl-1*H*-indol-3-yl)ethanone (**2**) (1.9 mmol) in methanol (3 mL) at 0°C. The resulting solution was then heated under reflux for 2 h, cooled to ambient temperature and the solvent was removed under reduced pressure. The residue formed was treated with water and made alkaline by adding sodium hydrogen carbonate. Extraction with ethyl acetate followed by drying of the organic phase over anhydrous sodium sulfate and concentration in-vacuo gave the desired

compounds **3** and **4** as mixtures with starting materials **1** and **2**, respectively. The mixtures were not separated but used as such for the synthesis of compounds **5** and **6**.

*General method for the preparation of the analogues (**7** and **8**)*

Hexamethylenetetramine (0.02 mol) was added at ambient temperature to a stirred solution of **3** (0.02 mol) or **4** (0.02 mol) in acetone–chloroform (50:50) (40 mL). The resulting solution was stirred at this temperature for 3 h. At the end of this period a precipitate was formed, which corresponds to analogues **5** and **6**, respectively. The precipitate was filtered, air-dried and treated with conc. hydrochloric acid (2.5 mL) and ethanol (15 mL). The mixture was stirred at room temperature for 19 h and then extracted with ethyl acetate. The organic phase was discarded and the aqueous phase was evaporated to dryness under reduced pressure to give mixtures of the corresponding analogues **7** or **8** and inorganic ammonium salts.

General method for the preparation of the N-*[2-(1*H-*indol-3-yl)-2-oxo-ethyl]alkanamides (**9a**–**e**) and* N-*[2-(1-methyl-1*H-*indol-3-yl)-2-oxo-ethyl]alkanamides (**10a**–**h**)*

For the synthesis of analogues **9a**–**c** and **10a**–**d** the appropriate acid anhydride was added to the hydrochloride **7** or **8** (3.1 mmol) at 0°C. The mixture was then treated with pyridine (0.8 mL), acetone (5 mL) and left stirring at room temperature for 45 min. The solvents were then removed in-vacuo and the residue formed was acidified with dilute hydrochloric acid and taken up in ethyl acetate. The aqueous phase was discarded and the organic layer was washed with H_2O and brine. In the case of compound **10d** the organic layer was washed with sodium hydroxide (10%) to remove benzoic acid, which was formed during the work up. Drying over anhydrous sodium sulfate and evaporation of the solvent under reduced pressure gave the desired compounds as off-white solids.

For the synthesis of analogues **9d**, **e** and **10 g**, **h** triethylamine (4.7 mmol, 0.7 mL) was added drop-wise to a chilled solution of the appropriate acid chloride (2.5 mmol) in dichloromethane (3 mL). For the synthesis of analogues **10e**, **f** the quantity of the triethylamine added to the above solution was 8.7 mmol, 1.2 mL. Then, the hydrochloric acid salt of the β-keto amine **7** or **8** (0.5 g) was added and the resulting mixture was allowed to thaw before stirring for 45 min. The suspension was then poured into ice-water and taken up in dichloromethane. The aqueous layer was decanted off and the organic phase was acidified with dilute hydrochloric acid, washed with water, dried over anhydrous sodium sulfate and concentrated in-vacuo to give **10e** and **10f** as sole products and amides **9d** and **10 g**, **9e** and **10h** as mixtures. Crystallization of the latter pairs of mixtures from benzene and tetrachloromethane/cyclohexane, respectively afforded the respective sole compounds as white solids.

N-*[2-(1*H-*Indol-3-yl)-2-oxo-ethyl]acetamide (**9a**)*
Yield (based on 1-(1*H*-indol-3-yl)ethanone **1**: 26%. Mp 220°C (dec.) (Guella et al 1994, 225°C). ^{1}H NMR ($CDCl_3$): 2.16 (s, 3H, $COCH_3$), 4.66 (s, 2H, CH_2), 6.71 (brs, 1H, NHCO), 7.34–7.44 (m, 3H, arom), 7.99 (s, 1H, H2), 8.32–8.36 (m, 1H, arom), 8.77 (s, 1H, NH). ^{13}C NMR ($CDCl_3$): 28.5, 45.2, 111.2, 113.5, 119.9, 121.4, 122.1, 124.6, 131.6, 135.8, 169.3, 188.8.

N-*[2-(1*H-*Indol-3-yl)-2-oxo-ethyl]propionamide (**9b**)*
Yield (based on 1-(1*H*-indol-3-yl)ethanone **1**: 22%. Mp 212°C (dec.). ^{1}H NMR ($CDCl_3$): 1.22 (t, 3H, J = 7.5 Hz, CH_2CH_3), 2.34 (q, 2H, J = 7.5 Hz, CH_2CH_3), 4.66 (s, 2H, $COCH_2NH$), 6.87 (s, 1H, NHCO), 7.28 (m, 2H, arom.), 7.32 (m, 1H, arom.), 7.98 (s, 1H, H2), 8.30 (m, 1H, arom.), 11.05 (s, 1H, NH). Anal. ($C_{13}H_{14}N_2O_2$) C, H, N.

N-*[2-(1*H-*Indol-3-yl)-2-oxo-ethyl]butyramide (**9c**)*
Yield (based on 1-(1*H*-indol-3-yl)ethanone **1**: 26%. Mp 215°C. ^{1}H NMR ($CDCl_3$): 0.99 (m, 3H, CH_3), 1.73 (m, 2H, CH_2CH_3), 2.31 (m, 2H, $NHCOCH_2$), 4.68 (s, 2H, $COCH_2NH$), 6.68 (s, 1H, NHCO), 7.27 (m, 2H, arom.), 7.35 (m, 1H, arom.), 7.98 (s, 1H, H2), 8.67 (s, 1H, NH). Anal. ($C_{14}H_{16}N_2O_2$) C, H, N.

*Cyclopropanecarboxylic acid [2-(1*H *-indol-3-yl)-2-oxo-ethyl]amide (**9d**)* Yield (based on 1-(1*H*-indol-3-yl)-ethanone **1**: 29%. Mp 238°C. ^{1}H NMR ($CDCl_3$): 0.68 (m, 4H), 1.72 (m, 1H), 4.47 (s, 2H, CH_2), 7.18 (m, 2H, arom.), 7.48 (m, 1H, arom.), 8.12 (m, 1H, arom.), 8.37 (brs, 2H, 1H arom. +1H NH indole). Anal. ($C_{14}H_{14}N_2O_2$) C, H, N.

*Cyclobutanecarboxylic acid [2-(1*H*-indol-3-yl)-2-oxo-ethyl]amide (**9e**)* Yield (based on 1-(1*H*-indol-yl)-ethanone **1**: 25%. Mp 198°C. ^{1}H NMR ($CDCl_3$): 1.9 (m, 2H), 2.3 (m, 4H), 3.1 (m, 1H), 4.63 (d, 2H, J = 4.4 Hz, CH_2), 6.60 (brs, 1H, NHCO), 7.38 (m, 3H, arom.), 7.95 (s, 1H, H2), 8.30 (m, 1H, arom.), 8.96 (brs, 1H, NH). Anal. ($C_{15}H_{16}N_2O_2$) C, H, N.

N-*[2-(1-Methyl-1*H*-indol-3-yl)-2-oxo-ethyl]acetamide* (***10a***) Yield (based on 1-(1-methyl-1*H*-indol-3-yl)-ethanone **2**: 20%. Mp 164°C. ^{1}H NMR ($CDCl_3$): 2.1(s, 3H, $COCH_3$), 3.86 (s, 3H, N-CH_3), 4.61 (d, 2H, J = 4.0 Hz, CH_2), 6.72 (s, 1H, NHCO), 7.30 (m, 3H, arom.), 7.83 (s, 1H, H2), 8.30 (m, 1H, arom.). Anal. ($C_{13}H_{14}N_2O_2$) C, H, N.

N-*[2-(1-Methyl-1*H*-indol-3-yl)-2-oxo-ethyl]propionamide* (***10b***) Yield (based on 1-(1-methyl-1*H*-indol-3-yl)ethanone **2**: 29%. Mp 135°C. ^{1}H NMR ($CDCl_3$): 1.23 (t, 3H, J = 7.7 Hz, CH_2CH_3), 2.34 (q, 2H, J = 7.7 Hz, CH_2CH_3), 3.88 (s, 3H, N-CH_3), 4.64 (d, 2H, J = 4.0 Hz, $COCH_2$), 6.75 (brs, 1H, NHCO), 7.36 (m, 3H, arom), 7.85 (s, 1H, H2), 8.32 (m, 1H, arom.). ^{13}C NMR ($CDCl_3$): 9.6, 29.3, 35.5, 46.1, 109.7, 113.6, 121.9, 122.8, 123.5, 125.8, 135.3, 173.7, 188.4. Anal. ($C_{14}H_{16}N_2O_2$) C, H, N.

N-*[2-(1-Methyl-1*H*-indol-3-yl)-2-oxo-ethyl]butyramide* (***10c***) Yield (based on 1-(1-methyl-1*H*-indol-3-yl)ethanone **2**: 28%. Mp 147°C. ^{1}H NMR ($CDCl_3$): 0.9 (t, 3H, J = 7.3 Hz, CH_2CH_3), 1.74 (m, 2H, CH_2CH_3), 2.27 (t, 2H, J = 7.5 Hz, $NHCOCH_2$), 3.87 (s, 3H, N-CH_3), 4.62 (d, 2H, J = 4.0 Hz, $COCH_2$), 6.72 (brs, 1H, NHCO), 7.38 (m, 3H, arom.), 7.86 (s, 1H, H2), 8.34 (m, 1H, arom.). Anal. ($C_{15}H_{18}N_2O_2$) C, H, N.

N-*[2-(1-Methyl-1*H*-indol-3-yl)-2-oxo-ethyl]benzamide* (***10d***) Yield (based on 1-(1-methyl-1*H*-indol-3-yl)-ethanone **2**: 24%. Mp 94°C. ^{1}H NMR ($CDCl_3$): 3.87 (s, 3H, N-CH_3), 4.84 (d, 2H, J = 4.0 Hz, CH_2), 7.26–8.17 (m, 10H arom. + 1H NHCO). Anal. ($C_{18}H_{16}N_2O_2$) C, H, N.

*Cyclopropanecarboxylic acid [2-(1-methyl-1*H*-indol-3-yl)-2-oxo-ethyl]amide* (***10e***) Yield (based on 1-(1-methyl-1*H*-indol-3-yl)ethanone **2**: 20%. Mp 171°C. ^{1}H NMR ($CDCl_3$): 0.82 (m, 2H), 1.02 (m, 2H), 1.56 (m, 1H), 3.88 (s, 3H, N-CH_3), 4.66 (d, 2H, J = 4.0 Hz, $COCH_2$), 6.90 (brs, 1H, NHCO), 7.37 (m, 3H, arom.), 7.85 (s, 1H, H2), 8.34 (m, 1H, arom.). Anal. ($C_{15}H_{16}N_2O_2$) C, H, N.

*Cyclobutanecarboxylic acid [2-(1-methyl-1*H*-indol-3-yl)-2-oxo-ethyl]amide* (***10f***) Yield (based on 1-(1-methyl-1*H*-indol-3-yl)ethanone **2**: 21%. Mp 150°C. ^{1}H NMR ($CDCl_3$): 1.90 (m, 2H), 2.22 (m, 4H), 3.16 (m, 1H, CH), 3.86 (s, 1H, N-CH_3), 4.62 (d, 2H, J = 4.0 Hz, CH_2), 6.64 (brs, 1H, NHCO), 7.31 (m, 1H, arom.), 7.34 (m, 2H, arom.), 7.85 (s, 1H, H2), 8.32 (m, 1H, arom.). Anal. ($C_{16}H_{18}N_2O_2$) C, H, N.

*Cyclopropanecarboxylic acid [2-(1-cyclopropanecarbonyl-1*H*-indol-3-yl)-2-oxo-ethyl]amide* (***10g***) Yield (based on 1-(1*H*-indol-3-yl)ethanone **1**: 26%. Mp 193°C. ^{1}H NMR ($CDCl_3$): 0.8 (m, 2H), 1.00 (m, 2H), 1.24 (m, 4H), 1.38 (m, 2H), 4.72 (d, 2H, J = 4.2 Hz, $COCH_2$), 6.74 (brs, 1H, NHCO), 7.40 (m, 2H, arom.), 8.32 (m, 2H, arom.), 8.48 (m, 1H, arom.). Anal. ($C_{18}H_{18}N_2O_3$) C, H, N.

*Cyclobutanecarboxylic acid [2-(1-cyclobutanecarbonyl-1*H*-indol-3-yl)-2-oxo-ethyl]amide* (***10h***) Yield (based on 1-(1*H*-indol-3-yl)ethanone **1**: 24%; Mp 108°C. ^{1}H NMR ($CDCl_3$): 1.84–2.61 (m, 12H), 3.01–3.23 (m, 1H, COCH), 3.79–3.96 (m, 1H, $COCH(CH_2)_3$), 4.64 (d, 2H, J = 4.4 Hz, $COCH_2$), 6.54 (brs, 1H, NHCO), 7.25–7.44 (m, 2H, arom.), 8.09 (s, 1H, H2), 8.23–8.28 (m, 1H, arom.), 8.44–8.48 (m, 1H, arom.). Anal. ($C_{20}H_{22}N_2O_3$) C, H, N.

General method for the preparation of the N-[2-*hydroxy*-2-(1-*methyl*-1*H*-*indol*-3-*yl*)*ethyl*]*alkanamides* (***11a–e***)

A suspension of sodium hydroxide (0.015 mmol) in water (0.1 mL) and methanol (0.6 mL) was added to a chilled mixture of the β-keto alkanamides **10a–c**, **10e**, **f** and sodium borohydride (0.1 mmol). The mixture was then allowed to reach ambient temperature and left stirring for 1 h. Upon completion of the reaction the solvent was removed under reduced pressure and the residue formed was acidified with dilute hydrochloric acid and extracted with ethyl acetate. The organic phase was dried over anhydrous sodium sulfate and concentrated in-vacuo to give the title compounds as off-white solids, which were recrystallized from cyclohexane.

N-*[2-Hydroxy-2-(1-methyl-1*H*-indol-3-yl)ethyl]acetamide* (***11a***) Yield: 82%. Mp 145°C. ^{1}H NMR ($CDCl_3$): 2.1 (s, 3H, $COCH_3$), 3.6–3.8 (m, 6H, N-CH_3, CH(OH) and CH_2NH), 6.75 (brs, 1H, NHCO), 6.90–7.35 (m, 4H, arom.), 7.62 (m, 1H, arom.). Anal. ($C_{13}H_{16}N_2O_2$) C, H, N.

N-*[2-Hydroxy-2-(1-methyl-1*H*-indol-3-yl)ethyl]propionamide* (***11b***) Yield: 79%. Mp 140°C. ^{1}H NMR ($CDCl_3$): 1.05 (m, 3H, $COCH_2CH_3$), 2.1 (q, 2H, J = 7.9 Hz, $COCH_2CH_3$), 3.55–3.80 (m, 6H, N-CH_3, CH(OH)

and CH_2NH), 6.70 (brs, 1H, NHCO), 6.80–7.30 (m, 4H, arom.), 7.61 (m, 1H, arom.). Anal. (CHN) C, H, N. Anal. ($C_{14}H_{18}N_2O_2$) C, H, N.

N-*[2-Hydroxy-2-(1-methyl-1*H*-indol-3-yl)ethyl]butyramide (**11c**)* Yield: 75%. Mp 115°C. ^{1}H NMR ($CDCl_3$): 0.64–0.90 (m, 2H, $COCH_2CH_2CH_3$), 2.07 (s, 2H, $COCH_2CH_2CH_3$), 3.55–3.80 (m, 6H, N-CH_3, C*H*(OH) and CH_2NH), 6.70 (brs, 1H, NHCO), 6.80–7.30 (m, 4H, arom.), 7.55 (m, 1H, arom.). Anal. ($C_{15}H_{20}N_2O_2$) C, H, N.

*Cyclopropanecarboxylic acid [2-hydroxy-2-(1-methyl-1*H*-indol-3-yl)ethyl]amide (**11d**)* Yield: 52%. Mp 121°C. ^{1}H NMR ($CDCl_3$): 0.53–1.00 (m, 4H), 1.26 (m, 1H), 3.59 (brs, 2H, CH_2NH), 3.76 (s, 3H, N-CH_3), 3.87 (brs, 1H, C*H*(OH)), 6.80 (brs, 1H, *NH*CO), 6.90–7.20 (m, 4H, arom.), 7.62 (m, 1H, arom.). Anal. ($C_{15}H_{18}N_2O_2$) C, H, N.

*Cyclobutanecarboxylic acid [2-hydroxy-2-(1-methyl-1*H*-indol-3-yl)ethyl]amide (**11e**)* Yield: 71%. Mp 112°C. ^{1}H NMR ($CDCl_3$): 1.78–2.14 (m, 6H), 2.97 (m, 1H), 3.44–3.69 (m, 6H, N-CH_3, C*H*(OH) and CH_2NH), 6.74–7.63 (m, 5H, arom.). Anal. ($C_{16}H_{20}N_2O_2$) C, H, N.

Pharmacology

Xenopus melanophore model for the evaluation of agonistic and antagonistic activity

Melanophore cells were grown in 96-well tissue culture plates, and growth medium (Daniolos et al 1990; Potenza & Lerner 1992) was replaced with 0.7×L-15 culture medium 18 h before analogues were tested. Initial absorbance of cells (*Ai*, 630 nm) was measured in each well using a Bio-Tek microtiter plate reader (model EL3115, Anachem, UK), then cells were treated with the concentrations of the analogues indicated. All experiments used triplicate wells at six concentrations (10^{-9}–10^{-4} M) of analogue (each well contains ~5000 cells and so is the average response of a large number of melanophores). S.e.m.s were always less than 5% of the mean. The final absorbance (*Af*) was measured after 60 min, and the fractional change in absorbance ($1-Af/Ai$) was calculated. Vehicle did not alter pigment granule distribution itself or inhibit responses to melatonin. The concentration of analogue producing 50% of the maximum agonist response (EC50) was determined from concentration–response curves. For evaluation of antagonist potency, cells were treated with vehicle (1% dimethyl sulfoxide (DMSO) or methanol) or varying concentrations (10^{-4}–10^{-9} M) of the analogues for 60 min before melatonin (10^{-9} M) was added. The concentration of analogue reducing melatonin-induced pigment aggregation by 50% (IC50) was determined.

In-vitro lipid peroxidation assay

The hepatic microsomal fraction from untreated female Fischer-344 rats (180–220 g) was prepared as described elsewhere (Rekka et al 1989). The incubation mixture contained heat inactivated (90°C for 90 s) hepatic microsomal fraction corresponding to 2.5 mg protein per mL (final concentration) or 4 mM fatty acid residues (Eichenberger et al 1982), ascorbic acid (0.2 nM) in Tris-HCl/KCl buffer (50 mM/150 mM, pH 7.4) and various concentrations (1.0–0.5 mM) of the test compounds dissolved in DMSO. The reaction was started by the addition of a freshly prepared $FeSO_4$ solution (10 μM) and the mixture was incubated at 37°C for 45 min. Samples (0.3 mL) from the incubation mixture were taken at various time intervals. Lipid peroxidation was assessed spectrophotometrically (535 against 600 nm) by the determination of the 2-thiobarbituric acid (TBA) reactive material (Kourounakis et al 1999). Under the above experimental conditions, all compounds, including DMSO, were tested and found not to interfere with the assay. Each experiment was performed at least in duplicate.

Hydrophobicity, expressed as clogP values, of compounds, was calculated with the programme CLOGP, v4.62, Daylight Chemical Information Systems Inc.

Results and Discussion

Chemistry

The synthetic route followed for the preparation of the new derivatives **9a**–**e** and **10a**–**h** is depicted in Figure 1. Thus, commercially available 1-(1*H*-indol-3-yl)ethanone (**1**) and 1-(1-methyl-1*H*-indol-3-yl)ethanone (**2**), prepared from **1** upon treatment with iodomethane in the presence of potassium hydroxide, were reacted with bromine in methanol to give the *a*-bromo ketones **3** (Guella et al 1994) and **4**, respectively. These were in turn treated with hexamethylenetetramine in acetone–chloroform to afford the tetra-aza analogues **5** and **6**. The latter were hydrolysed with concentrated hydrochloric acid in ethanol to the corresponding ammonium chloride salts **7** (Guella et al 1994) and **8**, which were converted to the target molecules **9a** (Guella et al 1994), **9b**–**e** and **10a**–**h** either by reaction with the appropriate acid anhydride, compounds **9a**–**c** and **10a**–**d**, or with the

analogous acid chloride, compounds **9d**, **e** and **10e–h** (Faust et al 2000).

The synthetic route followed for the preparation of the β-hydroxy amides **11a–e** is shown in Figure 2. It involved reduction of the ketones **10a–c** and **10e**, **f** with an alkaline solution of sodium borohydride in methanol.

Pharmacology

As mentioned above, the agonist and antagonist potency of the new analogues was assessed in the *Xenopus laevis* melanophore model. None of the tested compounds exhibited any agonist action at concentrations up to 10^{-4} M, while six of them (**9d**, **9e**, **10g**, **10h**, **11a** and **11b**) were melatonin antagonists at this maximum concentration (Table 1). The most potent antagonist was compound **11b** (pIC50 = 4.68), which inhibited pigment aggregation due to melatonin (10^{-9} M) by 87%. This compound, like its counterpart **11a**, bears a β-hydroxyl group in its side chain. The diminution in potency observed in the case of **11a** (57% inhibition) compared with that of **11b** might be due to the presence of the more lipophilic acylamido chain in the latter, which could enhance hydrophobic interactions with the receptor.

The introduction of a hydroxyl group in **11a** in place of the β-methyl moiety in the C-3 side chain of its congener alkanamide **I** (Figure 3) (Garratt et al 1995) seems to lead to antagonism. Interestingly, in the case of **11e** the replacement of the β-methyl substituent in **II** (Figure 3) (Garratt et al 1995) by a β-hydroxyl group does not enhance its antagonistic activity as for **11e** no antagonist effect was detected at concentrations up to 10^{-4} M.

In the β-keto series, **9a–e** and **10a–h**, the influence on antagonism of the constraint imposed on the side chain does not seem to be a critical factor. Thus, within these series compound **10h** inhibited the binding of the melatonin response by 65%. Next in potency was analogue **9e** (37% inhibition), which, like **10h**, bears an *N*-cyclobutanoyl group in its side chain, a moiety which is known to exert a detrimental effect on agonistic activity while it enhances antagonism (Leclerc et al 1998). The marked difference in potency observed between compounds **10h** and **9e** might be attributed to the presence of a second *N*-cyclobutanoyl group in the former at *N*-1. Presumably the extra *N*-cyclobutanoyl moiety at this position reduces further the ability of the receptor binding pocket to recognize and bind this molecule, thus leading to reduced antagonistic activity. However, an analogous trend was not observed in the case of the *bis N*-cyclopropyl analogue **10 g**, which was equipotent with its mono *N*-cyclopropanoyl counterpart, **9d**.

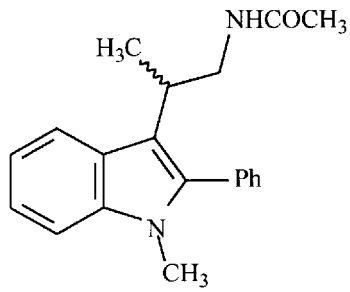
Besides investigating the agonist and antagonist profile of the new compounds, the free radical scavenging properties of selected analogues of both series were also studied. Many reports claim that melatonin exhibits antioxidant activity. For example, it has been reported that melatonin has the ability to detoxify the LOO• radical, which, apart from being highly reactive, re-initiates (propagates) the process of the oxidation of lipids (Pieri et al 1994). Lipid peroxidation is especially devastating in the CNS, which is particularly rich in lipids that can be readily oxidized. Besides melatonin (Pappolla et al 1997), it has been shown that analogues like indole-3-propionic acid (Chyan et al 1999) are capable of reducing lipid peroxidation caused by amyloid β-protein (A_β), thus protecting primary neurons and neuroblastoma cells against oxidative damage and consequent death.

To the best of our knowledge there is only one report on melatonin analogues as inhibitors of non-enzymatic lipid peroxidation (Gozzo et al 1999), so we examined the effect of seven representative compounds reported herein (**9c**, **10a**, **10c**, **10d**, **10f**, **11c** and **11e**) on the in-vitro peroxidation of rat hepatic microsomal membrane lipids. Most of these compounds exhibited a modest (**11e**, **9c**, **10d**, **10f**) or no (**10a**, **10c**) antioxidant activity, with the exception of **11c**, which significantly inhibited

Table 1 Antagonistic activity of compounds **9–11** in the *Xenopus laevis* melanophore assay; clogP values of selected analogues.

Compound	R_1	R	clogP	Antagonist inhibition (%) at 10^{-4} M
Melatonin				—
9a	H	CH_3		NA
9b	H	C_2H_5		NA
9c	H	C_3H_7	1.95	NA
9d	H	c-C_3H_5		35
9e	H	c-C_4H_7		37
10a	CH_3	CH_3	1.14	NA
10b	CH_3	C_2H_5		NA
10c	CH_3	C_3H_7	2.19	NA
10d	CH_3	C_6H_5	2.79	NA
10e	CH_3	c-C_3H_5		NA
10f	CH_3	c-C_4H_7	2.05	NA
10g	C(O)c-C_3H_5	c-C_3H_5		32
10h	C(O)c-C_4H_7	c-C_4H_7		65
11a	CH_3	CH_3		57
11b	CH_3	C_2H_5		87
11c	CH_3	C_3H_7	1.68	NA
11d	CH_3	c-C_3H_5		NA
11e	CH_3	c-C_4H_7	1.53	NA

NA, no antagonist effect detected at concentrations up to 10^{-4} M.



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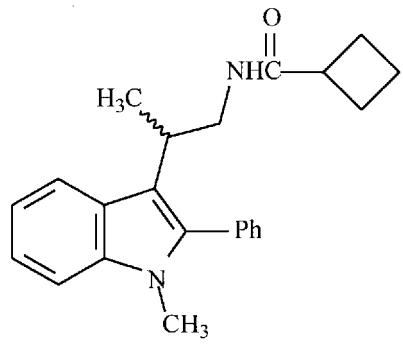


II

Figure 3 Structures of compounds **I** and **II** (Garratt et al 1995).

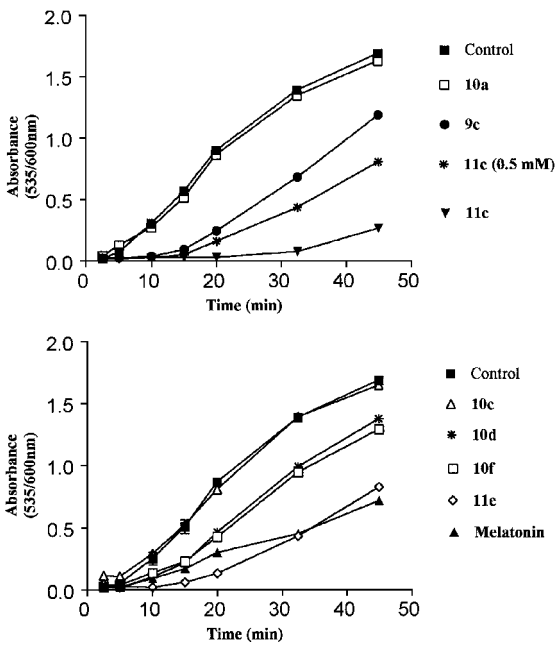
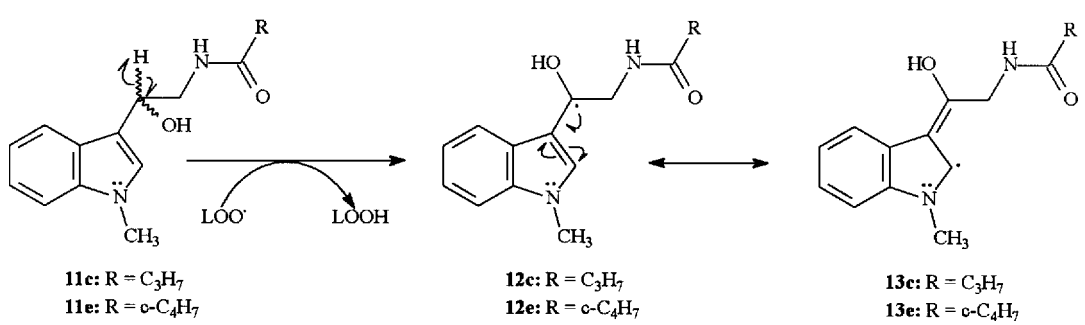


Figure 4 Time course of lipid peroxidation as affected by 1 mM (and 0.5 mM) of compounds **9c**, **10a**, **10c**, **10d**, **10f**, **11e** and **11c**.

lipid peroxidation with an $IC50_{(45\ min)}$ of approximately 0.5 mM, (Figure 4).

The enhanced potency of **11c** and, to a lesser extent, **11e** might be attributed to the presence of the benzylic-like β-hydrogen in their acylamido side chains. Abstraction of this hydrogen by the LOO• radical may lead to detoxification of the latter and to the formation of the indolyl radicals **12c**, **e** (Figure 5), which are stabilized by the presence of the hydroxyl group and their conjugation with the aromatic system (cf. resonance structures **12c**, **e**–**13c**, **e**). Conversely, the absence of benzylic-like hydrogens in the β-keto analogues, **9c**, **10a**, **10c**, **10d** and **10f**, leads to reduced or no potency. Also, the inactive compounds have the tendency to participate much less in free radical processes than the active compounds due to the greater stability of the keto-indole system through extended conjugation.

As hydrophobicity is known to affect antioxidant activity in many cases (Ohkawa et al 1991), clogP values were calculated for the tested compounds, Table 1. The results obtained indicate that the β-hydroxy analogues **11c** and **11e** are less lipophilic than their β-keto counter-



11c: R = C_3H_7
11e: R = c-C_4H_7

Figure 5 Proposed mechanism of detoxification of the LOO• radical by analogues **11c**, **e**.

parts, a fact that might be partially attributed to the enhanced capability of the former to form hydrogen bonds. Nevertheless, the difference in lipophilicity of **11c** and **11e**, compared with that of **9c**, **10a**, **10c**, **10d** and **10f**, appears to play a less significant role towards antioxidant activity than the presence of the benzylic-like hydrogen.

Conclusion

Under our experimental conditions, melatonin was equipotent with **11e**, less potent than **11c** and more potent than the other analogues. It is probable, however, that the antioxidant activity of **11c** and **11e** follows different pathways to that of melatonin, which does not possess a side chain hydroxyl group. Melatonin, upon donation of one of the two electrons of the *N*-1 lone pair, is transformed to an indolyl cation radical (Reiter 1998).

Based on these findings we are currently involved with the synthesis and screening of melatonin analogues of diverse structures.

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