Synthesis and Cytotoxic Profile of 3,4-Methylenedioxymethamphetamine ("Ecstasy") and Its Metabolites on Undifferentiated PC12 Cells: A Putative Structure−Toxicity Relationship

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The toxicological and redox profiles of MDMA and its major metabolites (MDA, α-methyldopamine, *N*-methyl-α-methyldopamine, 6-hydroxy-α-methyldopamine, 3-methoxy-α-methyldopamine) were studied to establish a structure-toxicity relationship and determine their individual contribution to cell death induction by apoptosis and/or necrosis. The results of the comparative toxicity study, using undifferentiated PC12 cells, strongly suggest that the metabolites possessing a catecholic group are more toxic to the cells than MDMA and metabolites with at least one protected phenolic group. Redox studies reveal that an oxidative mechanism seems to play an important role in metabolite cytotoxicity. Nuclear features of apoptosis and/or necrosis show that most of the metabolites, particularly *N*-methyl-α-methyldopamine, induce cell death by apoptosis, largely accompanied by necrotic features. No significant differences were found between MDMA and the metabolites, concerning overall characteristics of cell death. These results may be useful to ascertain the contribution of metabolism in MDMA neurotoxicity molecular mechanisms.

Introduction

3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy", **16**) is a ring-substituted phenethylamine derivative widely used as a recreational drug. The abuse of this group of amphetamine-like designer drugs is increasing among young adults, being at present a serious public health problem (*1*, *2*). The toxicity related to the misuse of this drug is extensively reported in recent literature, but the molecular mechanisms involved remain largely unknown. Among several biological targets, the neurotoxic (*3*), nephrotoxic (*4*), hepatotoxic (*5*), and cardiovascular (*6*) effects of MDMA were extensively studied *in vitro* and *in vivo*.

The research on the biotransformation reactions of xenobiotics, which play a major role in influencing the nature, intensity, and duration of wanted and unwanted effects, is an important task in the medicinal chemistry field (*7*). A majority of the investigation has been based on the overall effects induced by the use of the drug, but little attention has been given to the putative activities of the metabolites themselves. Several recent studies suggest that part of the toxicity ascribed to MDMA can be related to the formation of metabolites with higher activity (*8*−*10*). Several MDMA metabolic pathways have been proposed and reported (*11*−*12*), with the *N*-demethylation to 3,4-methylenedioxyamphetamine (MDA, **11**), also as a recreational drug, and the simultaneous or subsequent *O*-demethylenation to 3,4-dihydroxyamphetamine (α-methyldopamine, α-MeDA, **19**) and 3,4-dihydroxymethamphetamine (*N*-methyl-α-methyldopamine, *N*-Me-α-MeDA, **21**) being the most significant ones. It has also been assumed that the catecholic compounds **19** and **21** can undergo subsequent *O*-methylation mediated by catechol *O*-methyltranferase (COMT) to 4-hydroxy-3-methoxyamphetamine (3-methoxy-α-methyldopamine, 3-*O*-Me-α-MeDA, **13**) and 4-hydroxy-3-methoxymethamphetamine (Figure 1).

Aromatic hydroxylation has also been established as an important biotransformation step in *in vivo* metabolism of MDMA in rats. This reaction can occur at the remaining positions of the aromatic ring, but the one occurring at C_6 is the most reactive one. The resulting compound 2,4,5-trihydroxyamphetamine (6-hydroxy-α-methyldopamine, 6-OH-α-MeDA, **20**) is structurally similar to 6-hydroxydopamine (a potent neurotoxin obtained by *in vivo* aromatic hydroxylation of dopamine) (*13*, *14*). All compounds possessing a catecholic group can undergo further conjugation with other molecules like sulfate, glucuronide and thiol-containing endogenous substances (glutathione, cysteine, and *N*-acetylcysteine).

Animals exposed to MDMA show acute and long-term neurotoxic effects, causing irreversible damage to the central nervous system. Characterization of the *in vivo* and *in vitro* MDMA-induced neurotoxicity was widely accomplished (*15*−*17*), although the molecular mechanisms involved remain to be fully elucidated. Previously published studies suggested that bioactivation occurs after the administration of amphetamines, mediating a series of neurotoxic effects (*18*−*20*).

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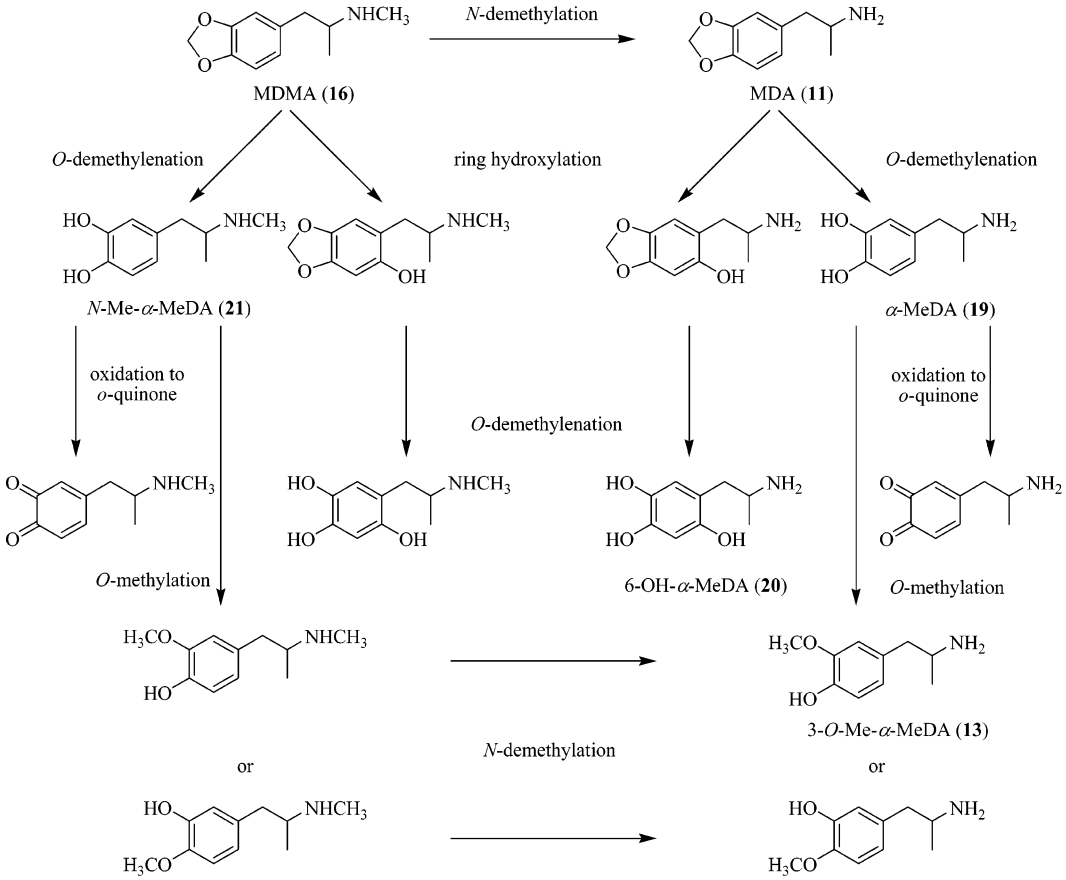

Figure 1. Proposed major metabolic pathways of MDMA; the numbered compounds correspond to the metabolites synthesized and used in the toxicological assays.

MDMA is a monoaminergic neurotoxin in both serotonin (5HT) and dopamine nerve terminals, depending on the animal species and conditions of administration. The neurotoxicity has been largely associated to a modification of the serotonergic function, namely, through an acute release of serotonin from central neurons, followed by prolonged depletion and structural neuronal damage (*21*−*23*). There is also considerable evidence that MDMA shows marked effects on dopamine release and can damage dopamine axon terminals, which could play a major role in some behavioral, mental, and thermal changes in animals (*24*, *25*).

Cell death by apoptosis or necrosis has been proposed to be involved in amphetamine-induced neurodegeneration (*26*, *27*). The activation of these pathways seems to be closely related to the occurrence of oxidative stress in the cellular medium, through redox cycles, which is intimately linked to the metabolism of MDMA and catecholamine (auto)oxidation (*10*, *28*).

Nonconjugated metabolites of MDMA are present in blood, brain, liver, feces, and urine for a 24-hour period following drug administration (*11*). It is generally accepted that one or more metabolites of MDMA or MDA may be responsible for neurotoxic effects because direct intracerebral injection of the drugs failed to reproduce the neurotoxicity revealed after peripheral administration (*29*). As MDMA does not seem to be neurotoxic, the evaluation of the metabolite's toxic effects is essential for the elucidation of the molecular mechanisms involved. However, there is controversy regarding the specific role of the metabolites. Intracerebroventricular administration of α-MeDA, *N*-Me-α-MeDA, and 3-*O*-Me-α-MeDA failed to produce the same neurotoxic features on central serotoninergic and dopaminergic systems induced by peripherally administered MDMA (*18*, *30*); nonetheless, other neurochemical effects were determined, and alterations were detected for the same metabolites, specially for 6-OH-α-MeDA (*18*, *31*, *32*).

In order to examine the hypothesis that the metabolites can contribute to the neurotoxicity of the parent compound, the cytotoxic potential of MDMA and five of its metabolites was investigated. The research project includes the synthesis of the main described metabolites of MDMA through standard synthetic methods and the evaluation of their cytotoxic profiles by using undifferentiated PC12 cells in culture, a well-established *in vitro* model of catecholaminergic neurons (*33*−*35*). Cell viability was assessed after incubation for 24 h with increasing concentrations of the compounds in order to determine the IC_{50} values. Because oxidative pathways have been proposed to play an important role in the toxicity profile of this type of compounds, the relationship between the redox profile and cytotoxicity was also studied. Accordingly, to get new insights on the molecular mechanisms underlying the biological activities, the redox properties of the drug and metabolites were determined using different electrochemical techniques.

Because MDMA-induced cell damage is related to programmed cell death in serotoninergic and neocortical neurons (*36*, *37*), assays of chromatin fragmentation/condensation were performed in PC12 cells in order to define drug-induced cell death as apoptotic and/or necrotic. To the best of our knowledge, there are no reports of apoptosis-like events induced by the MDMA metabolites in dopaminergic-like neurons.

Experimental Procedures

Materials and Solutions. PC12 cells were obtained from ATCC (Manassas, VA). 3,4-Dihydroxybenzaldehyde, 3,4-dimethoxybenzaldehyde, 4-hydroxy-3-methoxybenzaldehyde, 2,4,5- trimethoxy-

benzaldehyde, bromochloromethane, potassium carbonate, benzyl chloride, cesium carbonate, ammonium acetate, nitroethane, anhydrous magnesium sulfate, lithium aluminum hydride (1 M solution in tetrahydrofuran), ethyl chloroformate, boron tribromide (1 M solution in dichloromethane), 10% platinum in carbon, RPMI 1640 medium (modified with L-glutamine and 25 mM HEPES), streptomycin, penicillin, MTT, poly-L-lysine and propidium iodide, and all dry solvents were purchased from Sigma-Aldrich Química S.A. (Sintra, Portugal). Syto-13 was obtained from Molecular Probes (Eugene, OR). Horse serum and fetal bovine serum were purchased from BioChrom KG (Berlin, Germany). All other reagents and solvents were *pro analysis* grade and were acquired from Merck (Lisbon, Portugal). Deionized water (conductivity $<0.1\ \mu S\ cm^{-1}$) was used in all experiments. The gases, hydrogen, argon and nitrogen, were of the highest grade available. All reagents were used without further purification.

To perform electrochemical measurements, 10 mM stock solutions of the compounds were made by dissolving an appropriate amount in water. The voltammetric working solutions were prepared, in an electrochemical cell, by diluting 100 μL of the stock solution in 10 mL of supporting electrolyte to get a final concentration of 0.1 mM. The pH 7.3 supporting electrolyte, used for voltammetric determinations, was prepared by diluting 6.2 mL of 0.2 M dipotassium hydrogen phosphate and 43.8 mL of 0.2 M potassium dihydrogen phosphate to 100 mL.

Apparatus and Other Analytical Conditions. ^{1}H and ^{13}C NMR data were acquired, at room temperature, on a Brüker AMX 300 spectrometer operating at 300.13 and 75.47 MHz, respectively. Chemical shifts are expressed in δ (ppm) values, relative to tetramethylsilane (TMS) (as internal reference); coupling constants (J) are given in Hz. Dimethyl sulfoxide-d_6 was used as solvent unless specified in spectroscopic data. Assignments were also made from DEPT (distortionless enhancement by polarization transfer) experiments (values in italic). Electron impact mass spectra (EI-MS) were obtained on a VG AutoSpec instrument; data are reported as *m*/*z* (% of relative intensity for the most important fragments). Melting points were measured on a Köfler microscope (Reichert Thermovar) and were uncorrected. Analytical thin-layer chromatography (TLC) was carried out on 0.2 mm silica gel 60 F254 plates.

The purity of the final products (>95% purity) was verified using two different high-performance liquid chromatography (HPLC) systems, one equipped with a UV detector (DAD) and the other with an electrochemical detector. First, chromatograms were obtained in an HPLC/DAD system, a Jasco instrument (pumps model 880-PU and solvent mixing model 880-30, Tokyo, Japan), equipped with a commercially prepacked Nucleosil RP-18 analytical column (250 mm × 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany), and UV detection (Jasco model 875-UV) at the maximum wavelength determined by the analysis of the UV spectrum of each compound. The isocratic mobile phase consisted of 10 mM aqueous ammonium acetate (adjusting the final pH to 3 with hydrochloric acid) with 10% acetonitrile, at a flow rate of 1 mL/min at room temperature. The chromatographic data was processed in a Compaq computer, fitted with CSW 1.7 software (DataApex, Czech Republic).

A Waters 2690 Alliance system equipped with a CONCORDE Electrochemical Detector (Waters Corporation, Milford, USA) was also used. The electrochemical cell was a VT-03 flow cell (Antec Leyden, Zoeterwoude, Netherlands) with a confined wall-jet design in a three-electrode configuration: a 2 mm diameter glassy carbon working electrode, an *in-situ* Ag/AgCl reference electrode, and a stainless steel auxiliary electrode. The electrochemical detector was operated in oxidative amperometric mode with the working potentials between −100 mV and +1200 mV. The HPLC separation was carried out in a reverse phase LC-18-S Supelcosil analytical column (150 mm × 4.6 mm, 5 μm, Supelco, Bellefonte, USA); 100 mM sodium acetate (pH 4.3)/methanol (10:3) was used as mobile phase, in isocratic mode at 1 mL/min flow rate. Chromatograms were acquired in a Millenium 32 Chromatography Manager (Waters Corporation, Milford, USA).

Electrochemical data were obtained using an Autolab PGSTAT 12 potentiostat/galvanostat (Eco-Chemie, Netherlands) and a one-compartment glass electrochemical cell. Voltammetric curves were recorded at room temperature using a three-electrode system. A glassy carbon working electrode (GCE) (d = 2 mm), a platinum wire counter electrode, and an Ag/AgCl saturated KCl reference electrode were used. A Metrohm E520 pH-meter with glass electrode was used for pH measurements (Metrohm, Switzerland).

Synthesis of Metabolites. Synthesis of Aldehydes 1 and 5. 3,4-Methylenedioxybenzaldehyde (1). Because piperonal (**1**) is not commercially available in Portugal, it was synthesized by an adaptation of a previously described procedure (*38*). The dioxole ring was closed after the reaction of the starting material 3,4-benzaldehyde with bromochloromethane and using cesium carbonate as a catalyst.

3,4-Dibenzyloxybenzaldehyde (5). Briefly, 3,4-benzaldehyde or protocatechualdehyde (5.0 g, 36.2 mmol) was dissolved in *N*,*N*-dimethylformamide (DMF) (80 mL). Potassium carbonate (20.0 g, 144.9 mmol) and benzyl chloride (9.6 mL, 83.5 mmol) were then added to the solution, and the resulting suspension was stirred and refluxed for 5 h. The remaining residue was filtered and the solution poured on cold water/ice and acidified with HCl (pH 4). The yellow precipitate produced was filtered, washed with cold water, and identified as compound **5** (yield: 96%). The spectroscopic data is in accordance with an article published earlier (*39*).

General Synthetic Procedure for Obtaining β-Methyl-β-nitrostyrenes (6−10). The synthetic method used to obtain these compounds was an adaptation of references published earlier (*38*, *40*, *41*) with only minor modifications. The corresponding benzaldehyde (**1**−**5**), with the aromatic substitution pattern of the target compound, (30.0 mmol) and ammonium acetate (7.5 mmol) were dissolved in nitroethane (50 mL) and refluxed for 6 h. After cooling the reaction mixtures to room temperature, the solvent was partially evaporated, diluted with diethyl ether, and washed twice with 100 mL of water. The organic layers were dried over anhydrous magnesium sulfate, filtered, and concentrated. The remaining residues were recrystallized from diethylether/petroleum ether (40−60 °C) or from methanol/water to yield yellow to orange solids.

The reactions were all followed by thin layer chromatography (TLC). The synthesized β-methyl-β-nitrostyrene derivatives (**6**−**10**) were identified by both NMR and EI-MS. The compounds were systematically characterized in this work, even though some of them had been previously reported (*11*, *42*, *43*). The data of the remaining compound used in this study (**6**) is described elsewhere (*44*).

3,4-Dimethoxy-β-methyl-β-nitrostyrene (7). Yield 95%; ^{1}H NMR δ: 2.44 (3H, *s*, C*H*$_3$), 3.80 (3H, *s*, 3-OC*H*$_3$), 3.82 (3H, *s*, 4-OC*H*$_3$), 7.08 (1H, *d*, J = 8.5, H (5)), 7.22 (1H, *s*, H (2)), 7.23 (1H, *d*, J = 8.5, H (6)), 8.08 (1H, *s*, H(α)); ^{13}C NMR δ: *14.0* *C*H$_3$, *55.6* (4-OCH$_3$), *55.7* (3-OCH$_3$), *111.8*, *113.9* C (2) and C (5), *124.3* C (6), 124.4 C (1), *133.8* C(α), 145.6 C(β), 148.7 C (4), 150.7 C (3); EI-MS *m*/*z* (%): 223 (M$^{+\bullet}$, 100), 176 (78), 165 (22), 161 (37), 146 (47), 131 (46), 119 (36), 103 (37), 91 (47), 77 (38), 65 (34); mp 71−72 °C (lit (*43*). mp 72−74 °C).

4-Hydroxy-3-methoxy-β-methyl-β-nitrostyrene (8). Yield 98%; ^{1}H NMR δ: 2.44 (3H, *s*, C*H*$_3$), 3.81 (3H, *s*, OC*H*$_3$), 6.89 (1H, *d*, J = 8.2, H (5)), 7.12 (1H, *dd*, J = 8.2; 1.9, H (6)), 7.20 (1H, *d*, J = 1.9, H (2)), 8.05 (1H, *s*, H(α)), 9.85 (1H, bs, O*H*); ^{13}C NMR δ: *14.0* *C*H$_3$, *55.7* (OCH$_3$), *114.8*, *115.9* C (2) and C (5), 123.0 C (1), *124.8* C (6), *134.2* C(α), 144.7 C(β), 147.7 C (4), 149.2 C (3); EI-MS *m*/*z* (%): 209 (M$^{+\bullet}$, 100), 162 (81), 147 (53), 131 (33), 119 (30), 103 (84), 91 (56), 77 (36), 65 (32); mp 97−99 °C (lit (*42*). mp 100−101 °C).

2,4,5-Trimethoxy-β-methyl-β-nitrostyrene (9). Yield 92%; ^{1}H NMR δ: 2.39 (3H, *s*, C*H*$_3$), 3.74 (3H, *s*, 2-OC*H*$_3$), 3.88 (6H, *s*, 4- and 5-OC*H*$_3$), 6.79 (1H, *s*, H (3)), 7.01 (1H, *s*, H (6)), 8.20 (1H, *s*, H(α)); ^{13}C NMR δ: 14.1 CH$_3$, 56.0, 56.2, 56.4 (3×OCH$_3$), 97.5 C (3), 111.1, 113.3 C (1) and C (6), 128.8 C(α), 142.5 C (2), 145.1 C(β), 152.5 C (4), 153.9 C (5); EI-MS *m*/*z* (%): 253 (M$^{+\bullet}$, 100), 206 (64), 191 (40), 177 (36), 163 (19), 149 (15), 121 (17), 77 (16), 69 (16); mp 93−95 °C.

3,4-Dibenzyloxy-*β*-Methyl-*β*-nitrostyrene (10). Yield 79%; ^{1}H NMR *δ*: 2.32 (3H, *s*, C*H*$_3$), 5.20 (2H, *s*, 3-OC*H*$_2$), 5.22 (2H, *s*, 4-OC*H*$_2$), 7.20 (2H, *m*, H (2) and H (5)), 7.33−7.45 (11H, *m*, phenyl and H (6)), 8.03 (1H, *s*, H(α)); ^{13}C NMR *δ*: *13.9* CH_3, *69.8*, *70.0* (2×CH_2), *113.9*, *116.2* C (2) and C (5), 124.6 C (1), *124.9* C (6), *127.6-128.5* (10×*C*HAr), *133.5 C*(α), 136.9 (2×*C*Ar), 145.7 C(*β*), 147.8 C (3), 150.1 C (4); EI-MS *m*/*z* (%): 375 (M$^{+\bullet}$, 20), 284 (8), 181 (10), 91 (100), 65 (13); mp 107−109 °C (lit (*45*). mp 103−105 °C).

General Synthetic Procedure for the Reduction of Nitrostyrene Intermediates. The method applied for the synthesis of compounds **11**−**15** was a modification of those described earlier (*40*, *45*). Therefore, a solution of the appropriate *β*-methyl-*β*-nitrostyrene derivative (**6**−**10**) (8 mmol) in anhydrous tetrahydrofuran (THF) (30 mL) was added dropwise to a stirred suspension of lithium aluminum hydride (20 mmol) in anhydrous THF (50 mL) under nitrogen (N_2). The reaction was stirred, heated under reflux for 4 h, and then allowed to cool to room temperature. The excess $LiAlH_4$ was decomposed and the reaction quenched by the smooth addition of cold water/ice with vigorous stirring. The inorganic residue was removed by vacuum filtration; the solvent was dried under anhydrous magnesium sulfate, evaporated, and the residue diluted with diethyl ether and extracted with aqueous HCl (3 × 100 mL of a 2 M solution). The acidic extract was alkalinized with a NaOH solution and extracted with diethyl ether (3 × 100 mL). The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated to yield light brown oils.

When necessary for the systematic characterization of the compounds, the phenethylamine derivatives were isolated as salts (due mainly to their stability and simple of handling). Hence, the hydrochloride salts were prepared by acidifying the remaining residues with methanolic or ethanolic HCl and diluting with diethyl ether to obtain a white or almost white solid.

The identification of several of the amphetamine derivatives (**11**−**15**) had been previously reported (*10*, *11*, *43*, *45*−*48*). Nevertheless, some structural data was insufficiently accomplished, and the spectroscopic characterization was thoroughly performed in this work. For the accurate ^{1}H NMR assignment of the (CH_2C*H*)CH_3 group in all of the amphetamine derivatives, the spectra were also acquired in $CDCl_3$ and D_2O.

(*R*,*S*)-3,4-Dimethoxyamphetamine Hydrochloride (12). Yield 56%; ^{1}H NMR *δ*: 1.11 (3H, *d*, C*H*$_3$), 2.61 (1H, *dd*, *J* = 13.4; 8.7, C*H*$_2$), 2.93 (1H, *dd*, *J* = 13.4; 5.4, C*H*$_2$), 3.36 (1H, *m*, C*H*), 3.72 (3H, *s*, 3-OC*H*$_3$), 3.75 (3H, *s*, 4-OC*H*$_3$), 6.73 (1H, *dd*, *J* = 8.1; 1.8, H (6)), 6.85 (1H, *d*, *J* = 1.8, H (2)), 6.89 (1H, *d*, *J* = 8.2, H (5)), 8.11 (3H, bs, N*H*$_3^+$); ^{13}C NMR *δ*: *17.6* CH_3, *39.6* CH_2, *48.0* C*H*, *55.4*, *55.5* (2×OCH$_3$), *111.8*, *112.9* C (2) and C (5), *121.2* C (6), 129.0 C (1), 147.6 C (3), 148.7 C (4); EI-MS *m*/*z* (%): 195 (M$^{+\bullet}$, 5), 162 (7), 152 (95), 137 (17), 121 (6), 107 (14), 91 (12), 83 (100), 65 (15); mp 146−148 °C (lit (*43*). mp 145−148 °C).

(*R*,*S*)-4-Hydroxy-3-methoxyamphetamine Hydrochloride (13). Yield 35%; ^{1}H NMR *δ*: 1.10 (3H, *d*, C*H*$_3$), 2.56 (1H, *dd*, *J* = 13.5; 8.6, C*H*$_2$), 2.86 (1H, *dd*, *J* = 13.4; 5.5, C*H*$_2$), 3.38 (1H, *m*, C*H*), 3.75 (3H, *s*, 3-OC*H*$_3$), 6.60 (1H, *dd*, *J* = 8.0; 1.8, H (6)), 6.72 (1H, *d*, *J* = 8.0, H (5)), 6.79 (1H, *d*, *J* = 1.8, H (2)), 7.99 (3H, bs, N*H*$_3^+$), 8.91 (1H, bs, 4-O*H*); ^{13}C NMR *δ*: *17.7* CH_3, *39.5* CH_2, *48.2* C*H*, *55.5* (OCH_3), *113.2*, *115.5* C (2) and C (5), *121.5* C (6), 127.3 C (1), 145.4 C (4), 147.6 C (3); EI-MS *m*/*z* (%): 181 (M$^{+\bullet}$, 7), 138 (100), 122 (19), 94 (20), 89 (12), 77 (13), 65 (12); mp 245 °C (dec) (lit (*48*). mp 244−246 °C).

(*R*,*S*)-3,4,5-Trimethoxyamphetamine Hydrochloride (14). Yield 70%; ^{1}H NMR *δ*: 1.09 (3H, *d*, C*H*$_3$), 2.64 (1H, *dd*, *J* = 13.3; 8.4, C*H*$_2$), 2.86 (1H, *dd*, *J* = 13.3; 5.7, C*H*$_2$), 3.35 (1H, *m*, C*H*), 3.69 (3H, *s*, 6-OC*H*$_3$), 3.77 (3H, *s*, 3-OC*H*$_3$), 3.78 (3H, *s*, 4-OC*H*$_3$), 6.68 (1H, *s*, H (2)), 6.79 (1H, *s*, H (5)), 7.94 (3H, bs, N*H*$_3^+$); ^{13}C NMR *δ*: 17.8 CH_3, 34.3 CH_2, 47.0 C*H*, 55.8, 56.1, 56.3 (3×OCH_3), 98.2 C (5), 115.3, 115.7 C (1) and C (2), 142.4 C (6), 148.6 C (3), 151.6 C (4); EI-MS *m*/*z* (%): 225 (M$^{+\bullet}$, 11), 182 (100), 167 (32), 151 (15), 139 (8); mp 193−195 °C.

(*R*,*S*)-3,4-Dibenzyloxyamphetamine Hydrochloride (15). Yield 56%; ^{1}H NMR *δ*: 1.08 (3H, *d*, C*H*$_3$), 2.59 (1H, *dd*, *J* = 13.4; 8.8, C*H*$_2$), 2.93 (1H, *dd*, *J* = 13.4; 5.8, C*H*$_2$), 3.39 (1H, *m*, C*H*), 5.13 (2H, *s*, 3-OC*H*$_2$), 5.15 (2H, *s*, 4-OC*H*$_2$), 6.77 (1H, *dd*, *J* = 8.2; 1.8, H (6)), 7.00 (1H, *d*, *J* = 1.8, H (2)), 7.20 (1H, *d*, *J* = 8.2, H (5)), 7.33−7.50 (10H, *m*, phenyl), 8.07 (3H, bs, N*H*$_3^+$); ^{13}C NMR : 17.6 CH_3, 39.6 CH_2, 48.0 C*H*, 70.0 (2×OCH_2), 114.5, 115.6 C (2) and C (5), 121.9 C (6), 127.6−128.4 (10×*C*HAr), 129.6 C (1), 137.3, 137.4 (2×*C*Ar), 147.1 C (3), 148.1 C (4); EI-MS *m*/*z* (%): 348 (M$^{+\bullet}$, 5), 181 (12), 91 (100), 65 (14); mp 130−132 °C (lit (*45*). mp 130−133 °C).

General Synthetic Procedure for the N-Methylation of the Amphetamine Derivatives. Compounds **16**, **17**, and **18** were synthesized according to a synthetic route published earlier (*47*). The preparation of these secondary amines, starting from the respective primary amines, was preformed by a two-step reaction pathway, in which the intermediate was a carbamate derivative. The amine hydrochlorides (**11**, **12**, and **15**) (4.5 mmol) were dissolved in water (30 mL) at 0 °C. A solution of NaOH (0.3 g) in water (2.5 mL) was then added to the reaction followed by the addition of ethylchloroformate (0.5 mL, 5.3 mmol). The suspension was stirred at 0 °C for 3 h, and the reaction was then stopped by extraction with 3 × 100 mL of dichloromethane. The organic layer was dried, and the solvent was evaporated to obtain the corresponding urethane (R-NHCOOEt) (yields around 85%). These compounds were then reduced to the methylated amine by reaction with $LiAlH_4$. Briefly, the carbamates (4.1 mmol) were dissolved in THF (15 mL), and this solution was slowly added to lithium aluminum hydride (12 mmol) suspended in THF (25 mL), under N_2. The reaction was stirred and heated under reflux for 10 h. The quenching of the reaction, purification procedure, and the salification were performed by the same technique used in the previous reaction step. The purified compounds were fully characterized with the following structural features.

(*R*,*S*)-3,4-Dimethoxymethamphetamine Hydrochloride (17). Yield 55%; ^{1}H NMR *δ*: 1.09 (3H, *d*, C*H*$_3$), 2.54 (3H, *s*, NC*H*$_3$), 2.56 (1H, *dd*, *J* = 13.2; 8.2, C*H*$_2$), 3.07 (1H, *dd*, *J* = 13.2; 4.5, C*H*$_2$), 3.35 (1H, *m*, C*H*), 3.72 (3H, *s*, 3-OC*H*$_3$), 3.75 (3H, *s*, 4-OC*H*$_3$), 6.74 (1H, *dd*, *J* = 8.2; 1.8, H (6)), 6.86 (1H, *d*, *J* = 1.8, H (2)), 6.90 (1H, *d*, *J* = 8.2, H (5)), 8.90 (2H, bs, N*H*$_2^+$); ^{13}C NMR *δ*: *15.0* CH_3, *29.6* NCH_3, *37.9* CH_2, *55.3* C*H*, *55.4* (2×OCH_3), *111.8*, *112.9* C (2) and C (5), *121.3* C (6), 128.8 C (1), 147.7 C (3), 148.7 C (4); EI-MS *m*/*z* (%): 210 (M$^{+\bullet}$, 6), 152 (16), 107 (5), 72 (7), 58 (100); mp 118−120 °C (lit (*43*). mp 117−119 °C).

(*R*,*S*)-3,4-Dibenzyloxymethamphetamine Hydrochloride (18). Yield 42%; ^{1}H NMR *δ*: 1.04 (3H, *d*, C*H*$_3$), 2.53 (3H, *s*, NC*H*$_3$), 2.55 (1H, *dd*, *J* = 13.2; 8.4, C*H*$_2$), 3.03 (1H, *dd*, *J* = 13.2; 4.3, C*H*$_2$), 3.35 (1H, *m*, C*H*), 5.10 (2H, *s*, 3-OC*H*$_2$), 5.12 (2H, *s*, 4-OC*H*$_2$), 6.75 (1H, *dd*, *J* = 8.2; 1.9, H (6)), 6.99 (1H, *d*, *J* = 2.0, H (2)), 7.01 (1H, *d*, *J* = 8.2, H (5)), 7.29−7.46 (10H, *m*, phenyl), 8.74 (2H, bs, N*H*$_2^+$); ^{13}C NMR *δ*: *15.0* CH_3, *29.7* NCH_3, *37.9* CH_2, *55.3* C*H*, *70.1* (2×OCH_2), *114.5*, *115.7* C (2) and C (5), *121.9* C (6), *127.5-128.4* (10×*C*HAr), 129.4 C (1), 137.2, 137.4 (2×*C*Ar), 147.2 C (3), 148.1 C (4); EI-MS *m*/*z* (%): 361 (M$^{+\bullet}$, 5), 304 (22), 181 (4), 91 (46), 65 (7), 58 (100); mp 152−155 °C.

General Synthetic Procedure for the O-Demethylation. The process of *O*-demethylation to obtain the phenolic derivatives (**19**−**21**) was based on a method described earlier (*47*, *49*). The first step was the transformation of the hydrochloride salts in the respective free base amines, by dissolving them in water, making them alkaline with NH_3 solution, and extracting with dichloromethane. The di or trihydroxilated compounds **12**, **14**, and **17** (4.5 mmol) were then dissolved in anhydrous dichloromethane (50 mL) in an inert atmosphere (N_2) and at −60 °C. To this solution, boron tribromide (13 mL of 1 M solution in dichloromethane, 13 mmol) was added, and the reaction was kept at − 60 °C for 10 min and then allowed to reach room temperature for 4 h. The reaction was quenched by cautious addition of methanol (10 mL), and the solvent evaporated yielding a residue of the hydrobromide salt. Compound **19** was obtained as an almost white solid after recrystallization with acetonitrile. Compound **20** was recrystallized from dichloromethane as a white solid that quickly turns into a

dark oil at room temperature and when exposed to air. The data obtained was in accordance with that described in the literature (*10*−*13*).

(*R*,*S*)-3,4-Dihydroxyamphetamine Hydrobromide (19). Yield 57%; ^{1}H NMR δ: 1.08 (3H, *d*, CH_3), 2.46 (1H, *dd*, J = 13.4; 8.8, CH_2), 2.77 (1H, *dd*, J = 13.4; 5.4, CH_2), 3.30 (1H, *m*, C*H*), 6.46 (1H, *dd*, J = 8.0; 1.9, H (6)), 6.59 (1H, *d*, J = 1.9, H (2)), 6.68 (1H, *d*, J = 8.0, H (5)), 7.78 (3H, bs, NH_3^+), 8.85 (2H, bs, 2×O*H*); ^{13}C NMR δ: *17.6* CH_3, *39.6* CH_2, *48.3* C*H*, *115.7*, *116.5* C (2) and C (5), *119.9* C (6), 127.2 C (1), 144.2 C (3), 145.2 C (4); EI-MS *m*/*z* (%):168 ($M^{+\bullet}$, 8), 151 (12), 124 (42), 123 (70), 107 (8), 84 (62), 80 (62), 79 (21), 77 (97), 66 (100); mp 189−192 °C (lit (*10*). mp 193−195 °C).

(*R*,*S*)-2,4,5-Trihydroxyamphetamine Hydrobromide (20). Yield 65%; ^{1}H NMR δ: 1.06 (3H, *d*, CH_3), 2.50 (1H, *dd*, J = 13.3; 8.6, CH_2), 2.70 (1H, *dd*, J = 13.3; 5.5, CH_2), 3.38 (1H, *m*, C*H*), 6.34 (1H, *s*, H (2)), 6.44 (1H, *s*, H (5)), 7.74 (3H, bs, NH_3^+), 8.80 (3H, bs, 3×O*H*); ^{13}C NMR δ: *17.8* CH_3, *34.7* CH_2, *47.3* C*H*, *103.7* C (5), 112.3 C (1), *117.9* C (2), 137.6 C (6), 144.6 C (3), 147.9 C (4); EI-MS *m*/*z* (%): 183 ($M^{+\bullet}$, 4), 163 (14), 140 (5), 84 (77), 66 (100).

General Synthetic Procedure for the O-Debenzylation. The synthesis was carried out by catalytic hydrogenolysis of compounds **15** and **18** as described earlier (*46*). In a flask containing Argon and 10% Pd/C (300 mg) previously wetted with dry methanol, a solution of **15** and **18** (2.5 mmol) in methanol (30 mL) was added. The mixture was then hydrogenated catalytically in a PARR pump, at room temperature and at 40 psi until no more hydrogen was consumed, and the reaction ended (approximately 2 h). The suspension was filtered by suction to eliminate Pd/C, and methanol was partially evaporated, HCl was added to form the hydrochloride salt of the catecholic compounds. Acetonitrile was used as the recrystallization solvent and compound **21** was obtained as a light brownish oil and used directly.

(*R*,*S*)-3,4-Dihydroxymethamphetamine Hydrochloride (21). Yield 72%; ^{1}H NMR δ: 1.06 (3H, *d*, CH_3), 2.49 (3H, *s*, NCH_3), 2.53 (1H, *dd*, J = 13.2; 8.3, CH_2), 2.98 (1H, *dd*, J = 13.2; 4.2, CH_2), 3.33 (1H, *m*, C*H*), 6.45 (1H, *dd*, J = 8.0; 1.9, H (6)), 6.60 (1H, *d*, J = 1.9, H (2)), 6.67 (1H, *d*, J = 8.0, H (5)), 8.03 (2H, bs, 2×O*H*), 8.92 (2H, bs, NH_2^+); ^{13}C NMR δ: *14.9* CH_3, *29.6* NCH_3, *37.8* CH_2, *55.6* C*H*, *115.8*, *116.7* C (2) and C (5), *120.0* C (6), 127.3 C (1), 144.1 C (3), 145.3 C (4); EI-MS *m*/*z* (%): 180 ($M^{+\bullet}$, 8), 166 (13), 151 (6), 123 (57), 105 (12), 91 (11), 86 (100), 77 (53), 66 (24).

Biological Methods. Culture of Undifferentiated PC12 Cells. PC12 cells, derived from a pheochromocytoma of the rat adrenal medulla, were cultured in RPMI 1640 medium (with l-glutamine and HEPES) supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum (FBS), 50 U/mL penicillin, and 50 mg/mL streptomycin (final pH at about 7.4), as described before (*50*).

Cultures were preserved in 75 cm^2 flasks at 37 °C in a humidified incubator containing 95% air and 5% CO_2, and passed at least twice a week. The cells were plated on poly-L-lysine-coated multiwells at a density of 100 000 cells/cm^2 for MTT assays. In what is related with the study of chromatin condensation, the cells were plated in coverslips pre-coated with poly-L-lysine at a density of 180 000 cells/cm^2. The cells were additionally incubated with MDMA and its metabolites (placed as aqueous solutions) for more 24 h.

Analysis of Cell Viability. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (*51*). Following the incubation of the cells for 24 h with the tested compounds, they were washed with sodium solution (in mM:140 NaCl, 5 KCl, 1 $MgCl_2$, 1 NaH_2PO_4, 1.5 $CaCl_2$, 5.6 glucose, and 20 HEPES (pH 7.4)) and incubated further with 0.2 mL of MTT solution (0.5 mg/mL dissolved in sodium solution containing 1.0 mM of $CaCl_2$) for 2.5 h. The blue formazan precipitate, produced after the reduction of MTT by cellular dehydrogenases, was resuspended and dissolved after addition of 0.2 mL of acidic isopropanol (0.04 M of HCl). The capacity of treated cells in reducing the tetrazolium salt was expressed as a percentage of absorbance in control cells (ELISA microplate reader at 570 nm).

Analysis of Chromatin Condensation. The fluorescent probes SYTO-13 and PI (propidium iodide) were used to evaluate the morphological evidence of cell death by necrosis and/or apoptosis, after preincubation of the cells with the compounds in a concentration close to IC_{50}.

Necrosis and apoptosis were evaluated concomitantly on cultured PC12 cells after double fluorescence staining using vital fluorescence dyes SYTO-13 (a permeant intercalating probe that labels RNA and DNA in living cells with an UV-excited green-colored emission) and propidium iodide (a nonpermeant intercalating probe, which is excluded from viable cells with an UV-excited red emission). Normal nuclei exhibited loose chromatin colored green by SYTO-13; apoptotic nuclei exhibited condensed green-colored chromatin (postapoptotic necrosis or secondary apoptosis (*52*) was characterized by nuclei exhibiting the same apoptotic morphological features, but with red colored chromatin); necrotic cells exhibited red-colored nuclei with loose chromatin.

The cells were loaded for 3 min with sodium solution containing SYTO-13 (4 μM) and PI (4 μg/mL). The cells were then visualized and counted by fluorescence microscopy in a Nikon diaphot microscope equipped with a Xenon lamp and a FX 64 Omega Optical triple filter. Apoptotic and necrotic cells were scored by counting at least 200 nuclei in four or more random different fields in triplicates from three different experiments.

Statistical Analysis. Data presented are the mean ±SEM from the number of experiments indicated in the Figure legends, performed in triplicate. For the comparison of MTT reduction between treated cells and controls, we performed one-way statistical analysis of variance (ANOVA) followed by the Tukey−Kramer post-hoc test. For the analysis of chromatin condensation, statistical analysis was performed by the Kruskal−Wallis test followed by the Mann−Whitney multiple comparison with Bonferroni correction. In both cases, significance was accepted at $p < 0.05$.

Results and Discussion

Chemistry. MDMA and its major metabolites are ring-substituted phenethylamine derivatives structurally related to psychomotor stimulant amphetamines and the hallucinogen mescaline.

The total synthesis of the drug and its main metabolites was based on the general pathway presented in Figure 2.

Briefly, the synthesis of the target compounds was performed in a straightforward manner, starting from the appropriate substituted benzaldehydes (**1**−**5**), which were converted into the corresponding phenylisopropylamines (**11**−**15**) *via* β-methyl-β-nitrostyrenes (**6**−**10**) intermediates. First, a Knoevenagel condensation between the aldehyde, with the corresponding aromatic substitution pattern, and nitroethane was carried out, yielding the nitrostyrene derivatives (*53*). These compounds were then reduced to the unsaturated primary amines by lithium aluminum hydride ($LiAlH_4$). When necessary, the subsequent *N*-methylation of the amphetamine derivatives **11**, **12**, and **15** was accomplished by a two-step reaction path, beginning with the treatment with ethylchloroformate to provide the *N*-formyl derivatives followed by a reduction with $LiAlH_4$ to yield the designed *N*-methylated products (**16**−**18**). Finally and in order to obtain the phenolic amphetamine analogous (**19**−**21**), compounds **12**, **14**, and **17** were *O*-demethylated after reaction with boron tribromide (BBr_3).

Prior to the characterization procedures and biological assays, all of the compounds containing an amine group were converted to their hydrochloride or hydrobromide salts, according to the employed synthetic route and/or stability of the final compound.

Derivative **13** (4-hydroxy-3-methoxyamphetamine), as a hydrochloride, was also prepared in fairly good yield by

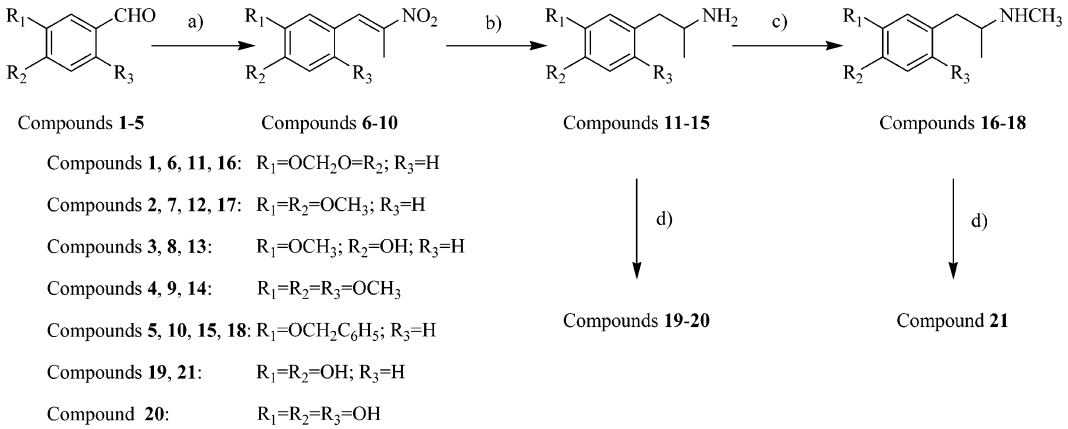

Figure 2. General synthetic procedure for MDMA and its metabolites. Chemical conditions: (a) $EtNO_2$, NH_4CH_3COO, reflux; (b) $LiAlH_4$, THF, reflux; (c) 1. $ClCOOC_2H_5$, H_2O, NaOH, 0 °C; 2. $LiAlH_4$, THF, reflux; (d) for the methylated compounds (**12**, **14**, **17**): BBr_3, CH_2Cl_2, −60 °C; for the benzylated compounds (**15**, **18**): H_2, Pd/C, MeOH. See Experimental Procedures.

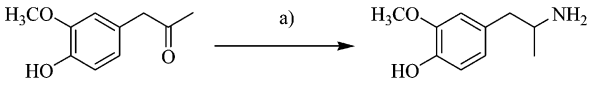

Figure 3. Reductive amination route for the synthesis of 3-*O*-Me-α-MeDA (**13**). Chemical conditions: (a) ammonium acetate (NH_4CH_3COO) and sodium cyanoborohydride ($NaCNBH_3$) in methanol (24 h/rt/N_2/pH 6).

reductive amination of the ketone 4-hydroxy-3-methoxyphenylacetone (Figure 3) (*10*).

The synthetic approach leading to the phenolic compounds **19**−**21** requires protection/deprotection steps to prevent alleged side reactions. Because the process of deprotection and following purification seems to be a limitative key step of the designed synthetic procedure, it was interesting to study different strategies to synthesize compounds **19** and **21** with the main goal of improving the overall yield. For the protection of the catechol of the starting material (3,4-dihydroxybenzaldehyde), the following protecting groups were used: benzyl (Bn or -$CH_2C_6H_5$), methoxymethyl (MOM), tetrahydropyranyl (THP), and di-*tert*-butyldichlorosilyl (DTBS) ethers (*54*). The yields of these protection reactions were not significantly different, except for benzylic ether, which seems to be a good alternative because the benzylated benzaldehyde was obtained in good yield, and the deprotected final amines (α-MeDA and *N*-Me-α-MeDA) can be easily achieved by hydrogenolysis (a selective, apparently smoother and cleaner reaction compared to that of boron tribromide demethylation; see Experimental Procedures) (Figure 4).

All of the synthesized compounds were identified by both NMR and electron impact mass spectroscopy (EI-MS), and their purity was confirmed using the HPLC systems reported in Experimental Procedures.

Biological Assays: Analysis of Cell Viability. The putative neurotoxic effects of this series of compounds were evaluated by two different types of assays: (i) analysis of PC12 cell viability after incubation with increasing concentrations of the tested compounds and (ii) analysis of chromatin fragmentation/condensation after incubation of PC12 cells with a concentration close to the respective IC_{50} values (concentration of the drug that decreases cell viability by 50%). The cell line selected for the cytotoxicity studies is derived from a catecholamine-secreting tumor (pheochromocytoma) of rat adrenal medulla, which upon exposure to nerve growth factor (NGF) differentiates into a neuronal phenotype similar to that of the postganglionic sympathetic (catecholaminergic) nerve-like cell (*34*). Undifferentiated PC12 cells are commonly used for the screening of cytotoxicity and to study the disruption by neurotoxicants related to various aspects of neuronal physiology and biochemistry (*55*, *56*). It is worthwhile to mention that undifferentiated PC12 cells release more dopamine and less acetylcholine than differentiated cells (*34*).

Studies of cell toxicity were performed by analyzing cell-reducing capacity through the examination of the ability of these cells to reduce MTT (3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium). The MTT measures the reducing capacity of the cells, a parameter dependent upon the maintenance of cellular metabolic function, thus reflecting cell viability and/or proliferation. This tetrazolium salt (yellow and soluble) is reduced to formazan (blue and insoluble) by active cellular dehydrogenases. The number of metabolically viable cells in the culture was directly correlated with the amount of formazan dye formed.

Changes in cell viability caused by exposure to MDMA or its five metabolites were evaluated and compared as shown in Figure 5. All of the compounds induced a dose-dependent decrease in cell viability after 24 h of incubation; however, the catechol-containing metabolites (**19**−**21**) were considerably more toxic to PC12 cells because their IC_{50} values are significantly lower than the ones of MDA and MDMA, as determined by the analysis of all of the IC_{50} values: IC_{50} ∼158 μM (*N*-Me-α-MeDA) < IC_{50} ∼247 μM (6-OH-α-MeDA) < IC_{50} ∼416 μM (α-MeDA) ≪ IC_{50} ∼3365 μM (MDA) < IC_{50} ∼4464 μM (MDMA) < IC_{50} ∼7526 μM (3-*O*-Me-α-MeDA), decreasing order of toxicity. The range of concentrations achieved is believed to occur *in vivo* after repeated drug usage (*57*).

Evaluation of Necrosis and Apoptosis. Apoptosis has been shown to play a key role in the cytotoxic effects of many drugs, including amphetamine derivatives (*36*, *37*). However, the precise role of apoptosis in the toxic effects of MDMA and its metabolites in dopaminergic cells has not been fully accomplished. Thus, upon analysis of cell viability, we attempted to characterize cell death as apoptotic and/or necrotic, by studying the morphological changes of the nuclei.

We analyzed the fragmentation/condensation of chromatin, a characteristic feature of apoptosis, by using the SYTO-13/PI assay. Apoptotic cells resulted in shrunken, brightly green fluorescent nuclei due to SYTO-13 labeling, showing condensed chromatin with high fluorescence compared with nonapoptotic cells. Simultaneous labeling with propidium iodide (PI), a non-permeable fluorescent dye, excluded from the living cells, allowed the detection of nucleic acids in intact nuclei of membrane-damaged cells (red fluorescence), characteristic of necrotic cell death. Late or secondary apoptosis was characterized by SYTO-13 plus PI labeling of fragmented/condensed nuclei.

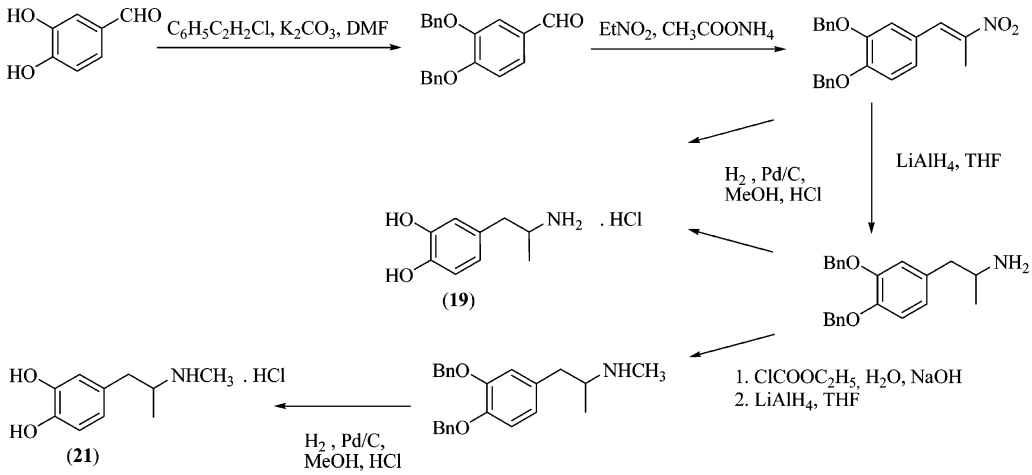

Figure 4. Proposed synthetic strategies for the total synthesis of α-MeDA (**19**) and *N*-Me-α-MeDA (**21**) using a benzyl group to protect the catechol.

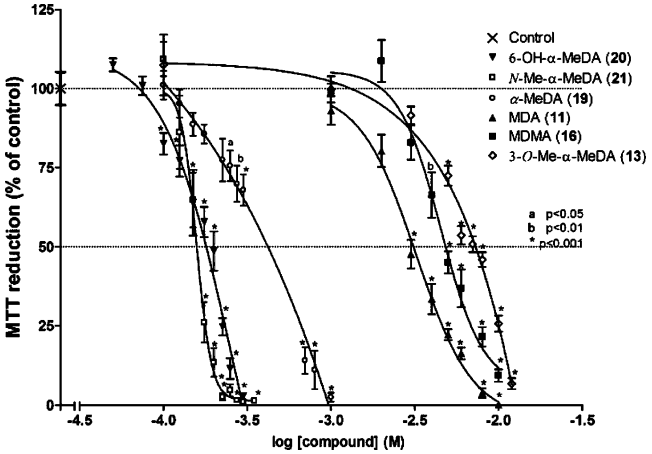

Figure 5. Dose−response curves of MDMA and metabolite-induced cytotoxicity. PC12 cells were incubated with increasing concentrations of the compounds for 24 h, and the toxic effects were evaluated by the MTT reduction assay (percentage of MTT reduction relative to the control). Data are the mean ± SEM of at least three experiments performed in triplicate.

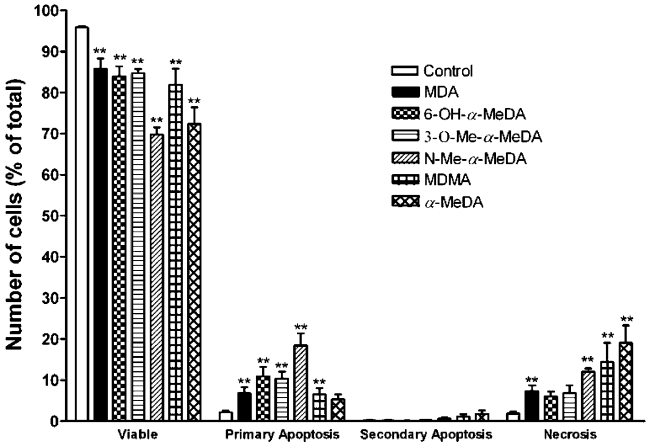

Figure 6. Analysis of chromatin condensation in PC12 cells exposed to the six compounds: *N*-Me-α-MeDA, 6-OH-α-MeDA, α-MeDA (300 μM each), MDA, MDMA, and 3-*O*-Me-α-MeDA (3 mM each) incubated for 24 h. SYTO-13 and propidium iodide (PI) were used to evaluate apoptotic or necrotic cell death. Data are the mean ± SEM of three experiments performed in triplicate. Statistical significance compared to the control (in the absence of the drug): $^{**}p < 0.01$ with Bonferroni correction is considered very significant.

To determine the fraction of fragmented/condensed nuclei, PC12 cells were incubated with the compounds for 24 h in concentrations in the vicinity of the IC_{50} values as determined in Figure 5. Under these conditions, there was a decrease by 15 to 30% in the number of viable cells (Figure 6). It should be noted that the loss of cell-reducing capacity (ascertained by the MTT assay) may not correspond immediately to cell death, as evidenced by the SYTO13/PI assay. Cells may have lost metabolic activity before showing evidence of a loss of membrane integrity or nuclear fragmentation or condensation.

All of the compounds tested, with the exception of α-MeDA, induced fragmentation and condensation of chromatin and, therefore, apoptosis when compared to the control, as observed by the labeling with SYTO-13 (Figure 6). MDMA, MDA, *N*-Me-α-MeDA, and α-MeDA revealed an increase of necrotic cell death compared to the control.

By comparing the relative proportion of the cells showing features of necrosis and apoptosis for each compound, it is important to highlight that although in a relatively small percentage *N*-Me-α-MeDA causes cell death mainly by apoptosis (by 6%), MDMA (by 8%) and α-MeDA (by 13.7%) cause cell death mostly by necrosis, whereas the other compounds did not induce a predominant type of cell death.

Comparison of features of apoptosis and necrosis observed for MDMA and its metabolites revealed no significant differences between the groups and, therefore, no major changes in the profile of cell death evoked by all of the compounds tested.

Structure−Oxidation−Toxicity Relationships. Because cytotoxic effects are frequently ascribed to be related to the occurrence of redox reactions, standard redox potentials of MDMA and its metabolites were determined in order to establish the relationship between the observed cytotoxicity and the oxidative profile. The electrochemical behavior of the compounds was studied at physiological pH 7.3, at a glassy carbon working electrode (GCE) using differential pulse, cyclic, and square wave voltammetry.

From the analysis of the electrochemical data of the drug and metabolites, it was possible to clearly distinguish two sets of compounds (Figure 7): one containing the metabolites with a catecholic group (α-MeDA (**19**), 6-OH-α-MeDA (**20**), *N*-Me-α-MeDA (**21**)) and the other including the compounds with a substituted catechol function (MDA (**11**), 3-*O*-Me-α-MeDA (**13**), MDMA (**16**)).

For 6-OH-α-MeDA (**20**), *N*-Me-α-MeDA (**21**), and α-MeDA (**19**), two well-defined anodic peaks were observed at physiological pH using differential pulse voltammetry (Figure 7a). The first peak is related to the oxidation of the catechol group to *o*-quinone and occurs at $E_p = -0.11$ V for compound **20**

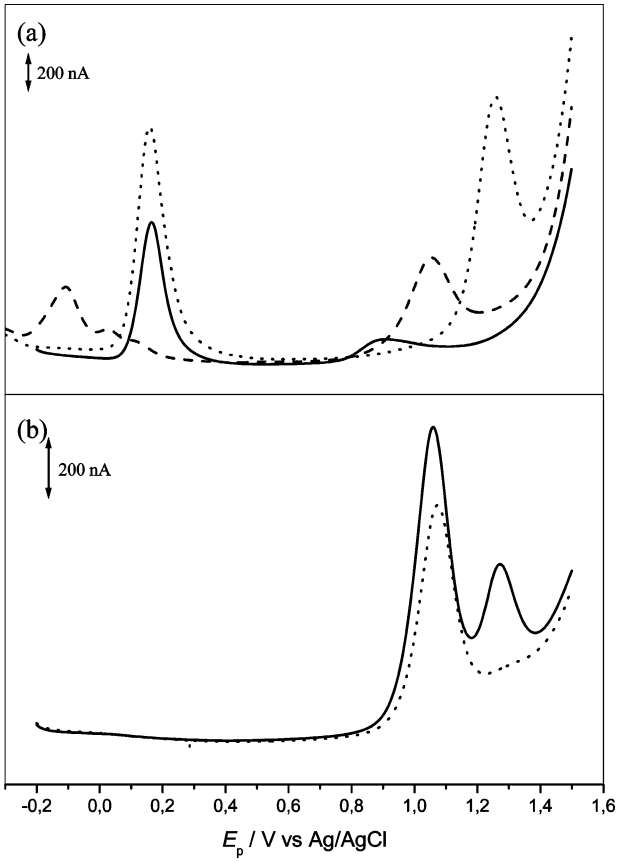

Figure 7. Differential pulse voltammograms in pH 7.3 phosphate supporting electrolyte of 0.1 mM solutions of (a) (---) 6-OH-α-MeDA, (·····) *N*-Me-α-MeDA and (—) α-MeDA; (b) (—) MDMA and (·····) MDA. Scan rate 5 mVs^{-1}.

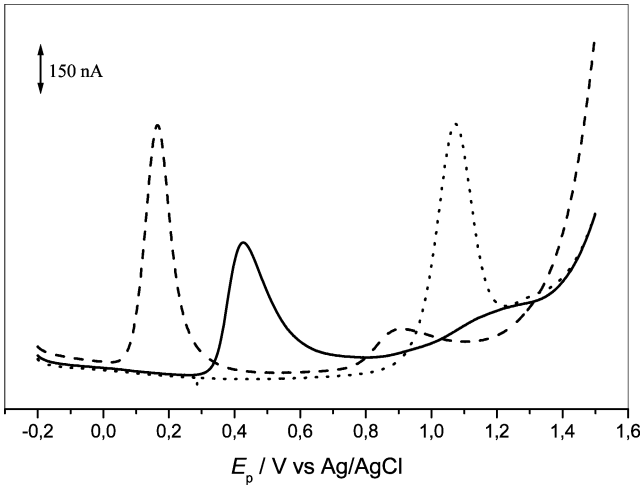

Figure 8. Differential pulse voltammograms in pH 7.3 phosphate supporting electrolyte of 0.1 mM solutions of (---) α-MeDA, (·····) MDA and (—) 3-*O*-Me-α-MeDA. Scan rate 5 mVs^{-1}.

Table 1. Redox Potential Values of MDMA and Its Metabolites

compound	peak	E_p/V vs Ag/AgCl
α-MeDA (**19**)	I	0.16
	II	0.89
6-OH-α-MeDA (**20**)	I	−0.11
	II	1.05
N-Me-α-MeDA (**21**)	I	0.16
	II	1.25
3-*O*-Me-α-MeDA (**13**)	I	0.43
	II	1.15
MDMA (**16**)	I	1.06
	II	1.27
MDA (**11**)	I	1.07

and +0.16 V for compounds **19** and **21**. Cyclic and square wave voltammograms recorded for these compounds clearly show the reversible character of this first electron-transfer oxidation reaction, which is consistent with the formation of quinonoid structures. The second anodic wave occurring at more positive potentials (Figure 7a) could be due to the oxidation of the quinone side-chain. MDMA (**16**) presents two well-defined anodic peaks at physiological pH using differential pulse voltammetry (Figure 7b). The first, $E_p = +1.06$ V, is due to the removal of one electron from the aromatic ring with the formation of a radical cation stabilized by the substituents, and the second peak, $E_p = +1.27$ V, could be attributed to the oxidation of the secondary amine present in the structure. MDA (**11**) showed only a single and well-defined anodic wave at physiological pH, $E_p = +1.07$ V (Figure 7b) attributed also to the removal of one electron from the aromatic ring, the same oxidation process as in MDMA. For 3-*O*-Me-α-MeDA (**13**), a well-defined anodic peak is observed at physiological pH using differential pulse voltammetry (Figure 8). This peak, $E_p = +0.43$ V, is due to the oxidation of the phenolic group. An indistinct second wave also appears at *ca.* +1.15 V (Figure 8), which might correspond to the formation of the radical aromatic cation.

Regarding the redox potentials obtained for the compounds, which are summarized in Table 1, it is possible to depict a clear correlation: all of the catechol-containing metabolites are more easily oxidized and remarkably more cytotoxic (Figure 5), a feature that could be intrinsically related with the *in situ* formation of electrophilic quinones. In fact, the obtained electrochemical data allow us to show that the catecholic metabolites share a common oxidative profile with the formation of quinones as primary oxidation products, which are highly reactive with respect to nucleophiles. The quinones can undergo an intracyclization process through the amine group of the side-chain, as an available nucleophile, leading to the formation of leucoaminochromes. Subsequently, these heterocyclic compounds can be easily oxidized to aminochromes. Moreover, the high predisposition to oxidation of these catecholic compounds, traduced by its redox potential, can also result in its autoxidation, leading to the formation of extracellular hydrogen peroxide and consequently to an increase in cytotoxicity.

Conclusion

The obtained data clearly indicate that some of the metabolites of MDMA cause stronger effects on PC12 cells than the parent compound, as shown by the analysis of their cytotoxic profiles. Given the large ammount of data implicating MDMA in neurodegeneration, the present results support the hypothesis that the mechanism of neurotoxicity can be related with biotransformation after the administration of the drug.

Although *N*-Me-α-MeDA, 6-OH-α-MeDA, and α-MeDA have not been undoubtedly established as *in vivo* neurotoxic metabolites of MDMA, it was demonstrated that these metabolites can account for the toxic properties of this drug. Note that *N*-Me-α-MeDA, a metabolite that attains human plasma concentrations similar to those of MDMA (*58*), was the most toxic metabolite, a result that is in agreement with toxicological data obtained in cardiomyocytes and hepatocytes (*9*, *59*). It is possible that adducts of these catecholic-containing compounds and major oxidative metabolites of MDMA could be generated and participate in its toxic effects (*60*−*62*).

Comparing the cytotoxicity of 6-OH-α-MeDA and α-MeDA, we conclude that the introduction of an hydroxyl group at the

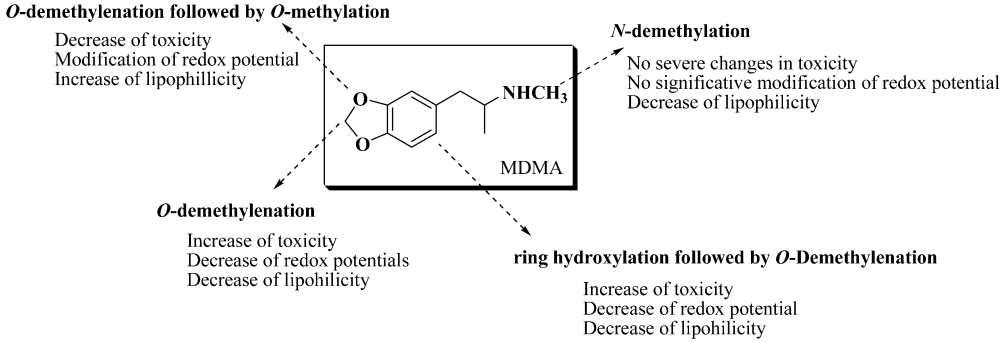

Figure 9. Consequences of structural modifications on toxicity and physicochemical properties of "ecstasy" (MDMA) and its metabolites.

ortho position to the amine side chain leads to an increase in cytotoxic properties. 6-OH-α-MeDA was previously described as neurotoxic because it has structural similarities with 6-hydroxydopamine, a potent neurotoxin that causes long-term alterations in central monoaminergic systems (*13*, *19*).

All compounds that possess a substituent in the catechol group seem to be less toxic than those with free aromatic hydroxyl groups. The ability of these latter compounds to form reactive *o*-quinones, leading to the formation of reactive species, is thought to represent a critical step in their toxicity, as suggested by previous studies (*63*, *64*). Furthermore, the oxidation of the metabolites to their respective aminochromes, which are toxic, must also be taken in consideration (*9*). Electrochemical results fully support and explain the differences observed in terms of the cytotoxicity of the compounds.

N-Methylation enhances cytotoxicity on PC12 cells. In contrast, O-methylation of the catecholamine-type compounds seems to be a detoxification step in the metabolism of MDMA because the resulting compound (3-*O*-Me-α-MeDA) is significantly less toxic than the parent compound and the catecholic precursors that will undergo O-methylation by the action of COMT.

It is important to notice that the experimental IC_{50} values obtained in the present work (ranging from 150 to 7000 μM) are in agreement with other similar published *in vitro* studies (*59*, *61*, *62*), particularly for the more toxic metabolites.

It was undoubtedly established that metabolism leads to changes of the redox potential of the parent compound. However, the authors believe that another physicochemical parameter, namely, lipophilicity, also contributes to its toxicity because a correlation was found between the partition coefficients of the metabolites and their toxicity as depicted in Figure 9.

The data on chromatin fragmentation/condensation reveal that the majority of MDMA metabolites significantly increase cell death by apoptosis. Programmed cell death was previously proposed to be involved in the neurotoxic effects of MDMA because the drug induces apoptosis on serotonergic and neocortical neurons (*36*, *37*). If one compares the metabolites and the drugs used recreationally (MDMA and MDA), the overall effects of the compounds as apoptosis inducers are quite similar, and their toxic effects in undifferentiated PC12 cells seem to be associated with the induction of apoptotic cell death. Nevertheless, it is important to note that some metabolites also led to significant death by necrosis. Accordingly, further experimental approaches are needed to clarify the precise cytotoxic mechanisms.

Systemic metabolism of MDMA seems to be needed for the occurrence of neurotoxicity, but the nature of any putative toxic metabolite is still unknown. Because drug metabolites are usually more hydrophilic than their parent drug, specific transporters are presumably needed to take up the neurotoxic metabolite into the brain (*10*, *23*). These results provide evidence that the metabolism of MDMA is a key factor for its toxicity and may prove to be useful in elucidating the amphetamine-like drug's putative activities.

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