Synthesis and Neurotoxicity Profile of 2,4,5-Trihydroxymethamphetamine and Its 6-(N-Acetylcystein-5-yl) Conjugate

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#INTRODUCTION

3,4-Methylenedioxyamphetamine (MDMA, "ecstasy") is a drug of abuse that produces lasting decreases in various presynaptic serotonergic neuronal markers including 5-HT, its major metabolite, 5-hydroxyindoleacetic acid (5-HIAA), its biosynthetic enzyme, tryptophan hydroxylase (TPH), and its membrane reuptake site, the 5-HT transporter (SERT).1,2 These decrements in 5-HT axonal markers are accompanied by anatomical signs of 5-HT axonal damage3,4 and a lasting decrease in anterograde [3H]proline transport from the dorsal raphe nucleus to the forebrain.5 Collectively, these lasting chemical and anatomical alterations after MDMA exposure have been widely interpreted as being indicative of a toxic effect of MDMA on brain 5-HT axon terminals.1,2,6 Such neurotoxic effects of MDMA have been documented in a variety of species, including nonhuman primates. In most species (including primates), MDMA selectively damages brain 5-HT neurons.1,2,7 For uncertain reasons, in mice, MDMA selectively damages brain dopamine (DA) neurons.8 Despite knowledge of MDMA’s neurotoxic properties for more than two decades,9 the mechanisms by which MDMA damages brain axons and axon terminals are not known.5,10

Research demonstrating that central administration of MDMA fails to produce 5-HT neurotoxicity11–14 has led to the suspicion that systemic metabolism of peripherally administered MDMA is required for the expression of neurotoxicity. This suspicion has generally been taken to suggest that a toxic metabolite is responsible for the lasting effects of MDMA on brain 5-HT neurons. In this regard, thioether conjugates of MDMA metabolites have drawn particular attention.15–18

Of the various potentially toxic metabolites of MDMA (Scheme 1), recent evidence indicates that several metabolites do not appear to be directly involved. These include 3,4-dihydroxymethamphetamine (HHMA)14,19,20 and 4-hydroxy-3-methoxymethamphetamine (HMMA),20 both of which...
are phase I and phase II metabolites generated through O-demethylation, and 3,4-dihydroxyamphetamine (HHA), which is produced via N-demethylation followed by O-demethylation. In particular, exposure to these metabolites does not result in lasting 5-HT deficits. More recently, the thioether conjugate of HHMA, 5-(N-acetylcystein-S-yl) HHMA (5-NAC-HHMA) has been implicated in MDMA neurotoxicity, but efforts to replicate these findings have been unsuccessful.

One metabolite of MDMA that has not yet been excluded as the mediator of MDMA neurotoxicity is 2,4,5-trihydroxymethamphetamine (THMA). This metabolite, arising from the ring hydroxylation biotransformation step (Scheme 1), is a particularly intriguing candidate because it has structural similarity to the well-known DA neurotoxin, 6-hydroxydopamine (6-OHDA), has been reported to be formed endogenously in the liver, and has already been shown to produce lasting 5-HT deficits when administered centrally. Of note, however, THMA has never been detected in the brains of animals treated with MDMA.

Although extensive work has been done with thioether conjugates of HHMA, there are no published reports on thioether conjugates of THMA. Notably, thioether conjugates of 6-OHDA have been detected in the brains of rats and mice given 6-OHDA centrally, suggesting that similar processes may occur with THMA. To our knowledge, synthesis of the thioether conjugate of THMA has yet to be reported.

The goal of the present investigation was to accomplish high-yield syntheses of THMA and its thioether conjugate, 6-(N-acetylcystein-S-yl)-2,4,5-trihydroxymethamphetamine (6-NAC-THMA) (Scheme 1), and to determine if either of these MDMA metabolites plays a role in MDMA neurotoxicity. In particular, after confirming earlier findings that centrally administered THMA produces lasting effects on brain 5-HT neurons, we sought to (1) determine if the lasting effect of THMA on 5-HT can be blocked with a 5-HT transporter blocker, fluoxetine, which is known to protect against MDMA neurotoxicity; employ newly developed liquid chromatographic-mass spectrometric (LC-MS) methods to ascertain if THMA can be detected in the brains of rats treated with peripherally administered neurotoxic dose of MDMA; (3) test if the inherent instability of the THMA molecule impacts its detectability in brain tissue; and (4) determine if the thioether conjugate of THMA, 6-NAC-THMA, produces lasting effects on brain 5-HT neuronal markers. The present results cast doubt on the role of THMA and its thioether conjugate, 6-NAC-THMA, in MDMA neurotoxicity.

EXPERIMENTAL PROCEDURES

Chemicals. All reagents and solvents (HPLC grade) were commercial products of the highest available purity and were used as supplied. Analytical thin-layer chromatography was carried out on silica gel Macherey-Nagel Polygram SIL G/UV 254 (0.25 mm). Column
chemical research was performed on Macherey-Nagel Si 60 M silica gel (40–63 μm). Melting points were measured on a Köfler apparatus.

HPLC was carried out using a Waters system consisting of a 600E dual-column delivery system, a Rhodyne-type loop injector, and a 2487 dual-channels UV-visible detector set at 254 and 278 nm. A mixture of two solvents A and B constituted the mobile phase. Solvent A was prepared by adding 1% concentrated trifluoroacetic acid (TFA) to deionized water. Solvent B was prepared by adding 0.5% TFA to a 1:1 (v/v) mixture of methanol and deionized water. Spectroscopic data were acquired in a Bruker AC-300 spectrometer operating at 300 and 75 MHz, respectively. Chemical shifts are expressed as δ units (parts per million) downfield from TMS (tetramethylsilane). The measurements were carried out using the standard pulse sequences. The carbon type (methyl, methylene, methine, or quaternary) was determined by DEPT experiments. 

3,4-Dihydroxymethamphetamine hydrobromide (HHMA, HBr) was synthesized in four steps (Scheme 2) from commercially available 2,4,5-trimethoxybenzaldehyde and nitroethane, through a procedure close to those previously reported for the synthesis of (±)-2,4,5-trihydroxymethamphetamine hydrobromide (HHMA, HBr) with some modifications.

Scheme 2. Four-Step Synthesis of THMA 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Reaction Conditions</th>
<th>mp (°C)</th>
<th>Yield (%)</th>
<th>Spectral Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.52 g, 6.0 mmol</td>
<td>50 °C</td>
<td>95.5%</td>
<td>3.77 g, 14.90 mmol</td>
<td>94–96 °C</td>
</tr>
<tr>
<td>2.94 g, 15.0 mmol</td>
<td>60 mL of nitroethane was heated to reflux for 45 min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.43 g, 6.75 mmol, 1.5 equiv</td>
<td>240.1600</td>
<td>240.1594</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.07 g, 4.5 mmol</td>
<td>10 mL of methylamine in THF</td>
<td>55–57 °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>λ_max = 292 nm, ε = 4300 mol L cm⁻¹</td>
<td>1H NMR (CDCl₃) δ 1.14 (d, J = 6.5 Hz, 3H), 2.57 (s, 3H), 2.69 (m, 2H), 3.37 (m, 1H), 6.38 (s, 1H), 6.60 (s, 1H)</td>
<td>14.8, 30.1, 32.9, 55.8, 104.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2,4,5-Trimethoxymethamphetamine (3). To a stirred solution of compound 2 (1.0 g, 4.5 mmol) in dry dichloromethane (10 mL) cooled at −5 °C was added a 2 M solution of methylamine in THF (10 mL, 20.25 mmol, 4.5 equiv). After the reaction mixture was stirred at room temperature for 1 h, 1.15 mL of glacial acetic acid (4.5 equiv) was added, followed by sodium triacetoxylborohydride (Na(AsO₃)BH) (1.43 g, 6.75 mmol, 1.5 equiv) in small portions. Then, the reaction mixture was stirred at room temperature for 3 h and quenched with 2.5 M sodium hydrogen aqueous solution (5 mL). The aqueous layer was extracted with diethyl ether (3 × 100 mL), and the combined ether extracts were washed with 2.5 M sodium hydrogen aqueous solution (5 mL) and then washed over anhydrous MgSO₄ and evaporated to dryness to give compound 3 as a white solid (1.07 g, 4.5 mmol) in quantitative yield; mp 74–76 °C. 1H NMR (CDCl₃) δ 1.01 (d, J = 6.0 Hz, 3H), 1.55 (broad s, 1H), 2.36 (s, 3H), 2.52 (m, J = 13.0 Hz, 1H), 2.64 (m, J = 13.0 Hz, 1H), 7.33 (m, 3H), 3.78 (s, 3H), 3.80 (s, 3H), 3.85 (s, 3H), 6.49 (s, 1H), 6.66 (s, 1H). 13C NMR (CDCl₃) δ 19.8, 34.0, 37.5, 55.3, 56.1, 56.3, 56.6, 97.7, 115.1, 119.4, 142.7, 147.9, 151.9. HRMS (ESI) m/z calculated for [M + Na]⁺ 247.0946; found, 247.0942.

2,4,5-Trimethoxymethamphetamine hydrobromide (THMA, HBr) (4). THMA was previously described as the hydrochloric salt. A 48% aqueous solution of HBr (5.2 mL (46 mmol) was added to compound 3 (954 mg, 4.0 mmol). The resulting solution was heated to reflux for 2.0 h, under inert atmosphere. After evaporation under reduced pressure, the brown solid was recrystallized successively in dichloromethane-MeOH 90:10 v/v, and chloroform—isopropanol 85:15 v/v mixture, affording THMA, HBr 4 as a white solid (845 mg, 3.04 mmol) in 76% yield. The degree of purity for compound 4 (99%) was determined by analytical HPLC (eluents, solvent A/solvent B 93/7; flow rate, 0.6 mL min⁻¹); mp 172–174 °C. UV absorption (0.2 M aqueous HCl) λ_max = 292 nm, ε = 4300 mol L cm⁻¹. 1H NMR (D₂O) δ 1.14 (d, J = 6.5 Hz, 3H), 2.57 (s, 3H), 2.69 (m, 2H), 3.37 (m, 1H), 6.38 (s, 1H), 6.60 (s, 1H). 13C NMR (D₂O) δ 14.8, 30.1, 32.9, 55.8, 104.2, 113.9, 118.9, 137.1, 143.9, 147.7. Note, when the 1H NMR spectra of THMA was performed in DMSO D6, three additional signals were recorded at 8.09 (1H, OH, D₂O exchanged), 8.38 (2H, OH, D₂O exchanged), 7.60 (1H, NH, D₂O exchanged)
exchanged, and 8.74 (2H, MeNH₂, D₂O exchanged) that confirmed the presence of the three hydroxyl groups at C2, C4, and C5 and that of the protonated secondary amino group on the side chain. As reported earlier, HRMS did not allow us to obtain the exact mass of THMA but that of the 5,6-dihydroxyindole species. Only derivatization with methyl chloroformate provided a means of converting the highly reactive THMA to a stable derivative, then avoiding the cyclization into indole.²² HRMS (ESI) m/z calculated for [M + Na]⁺ 200.06822; found, 200.06823 (5,6-dihydroxyindole species). However, it was possible to detect THMA by LC-MS (see Figure 5 below).

**Electrochemistry.** Controlled-potential electrolyses were carried out in a cylindrical three-electrode divided cell (9 cm diameter), using a Voltalab 32 electrochemical analyzer (Radiometer, Copenhagen). In the main compartment, a cylindrical platinum grid (area = 60 cm²) served as the anode (working electrode). A platinum sheet was placed in the concentric cathodic compartment (counter-electrode), which was separated from the main compartment with a glass frit. The reference electrode was an Ag/AgCl electrode, to which all potentials quoted are referred.

6-(N-Acetylcysteinyl-5)-2,4,5-trihydroxymethamphetamine (5). Procedure A. A solution of THMA, HBr (69.5 mg, 0.25 mmol), in 0.2 M HCl (250 mL) was oxidized under nitrogen at 10 °C at a platinum grid whose potential was fixed at +1.0 V versus Ag/AgCl. After the consumption of 2 electrons per molecule, 2 equiv of N-acetylcysteine (83 mg, 0.50 mmol) was added to the pale orange solution, which slowly turned yellow. After 50 min, the reaction mixture was frozen at −80 °C and then freeze-dried. The residue was subdivided into fractions of about 25 mg. Each fraction was dissolved in 2 mL of NaOH solution, immediately frozen at −80 °C, and then freeze-dried. Compound 5 (diastereomeric mixture) was isolated as a pale pink solid (64 mg, 0.18 mmol) in 71% yield. Its degree of purity (96.5%) was determined by analytical HPLC (elucent, solvent A/B 90/10; flow rate, 0.65 mL min⁻¹; mp >110 °C (dec.). UV absorption (0.2 M aqueous HCl) λmax = 257 nm (ε = 2420 mol⁻¹L cm⁻¹) and 307 nm (ε = 3118 mol⁻¹L cm⁻¹).

1H NMR (D₂O) δ 1.30 (d, J = 6.5 Hz, 3H), 1.76 (s, 3H), 2.56 (s, 3H), 3.03 (m, 3H), 3.27 (m, 2H), 3.29 (m, 1H), 4.23 (m, 1H), 6.44 (s, 1H). 13C (CDCl₃) Diastereoisomers A: δ 14.8, 21.4, 30.2, 31.2, 34.5, 53.2, 56.4, 105.2, 117.6, 120.3, 139.4, 139.9, 147.9, 173.8. Diastereoisomer B: δ 14.8, 21.4, 30.2, 31.2, 34.5, 53.2, 56.4, 105.2, 117.7, 120.4, 139.9, 143.9, 147.9, 173.8. Note, when the 1H NMR spectra of 6-NAC-THMA was performed, four additional signals were recorded at 8.26 (1H, NHCOMe, D₂O exchanged), 8.38 (2H, OH, D₂O exchanged), 9.04 (1H, OH, D₂O exchanged), and 9.42 (1H, CO₂H, D₂O exchanged) that confirmed the presence of the three hydroxyl groups at C2, C4, and C5, that of the acetylated amino group, and that of the carboxylic acid function. As mentioned in the case of THMA, HRMS did not allow us to obtain the exact mass of 6-NAC-THMA but that of its corresponding 5,6-dihydroxyindole species. HRMS (ESI) m/z calculated for [M + Na]⁺ 361.08282; found, 361.08286.

For biological evaluation, a second purification was performed by semipreparative reversed-phase HPLC, using a mixture of solvent A/ solvent B 92/8 as the eluent (flow rate: 10.5 mL min⁻¹). Then, compound 5 could be isolated with 99.5% purity grade (see the Supporting Information).

Procedure B. A solution of THMA, HBr (4) (69.5 mg, 0.25 mmol) in 10⁻² M sodium phosphate buffer (250 mL, pH 7.4) containing 5 equiv of N-acetylcysteine (208 mg, 1.25 mmol) was oxidized under nitrogen at 10 °C at a platinum grid whose potential was fixed at +0.4 V versus Ag/ AgCl. After the consumption of 1.75 electron per molecule, the resulting red solution was acidified to pH 2.0 with 0.2 M HCl. The resulting solution was frozen at −80 °C and then freeze-dried. Semipreparative reversed-phase HPLC was performed using the following mobile phase gradient (flow rate: 5.0 mL min⁻¹): 0–10 min, mixture of solvent A/solvent B 80/20; 10–70 min, mixture of solvent A/solvent B from 80/20 to 100% solvent B. Fractions containing 6-(N-acetylcysteine-S-yl)-2,4,5-trihydroxymethamphetamine (5) were collected, immediately frozen at −80 °C, and then freeze-dried. Compound 5 was isolated in 27% yield (24.0 mg, 0.07 mmol). Twenty-five percent of the starting THMA (10.5 mg, 0.05 mmol) were also recovered because the electrolysis was stopped before the end of the oxidation (after the consumption of 1.75 electron per molecule) since, in pH 7.4 buffer, compound 5 underwent oxidative cyclization into an indole species which rapidly polymerized as do other indoles of this nature.

**Enzymatic Oxidation.** Mushroom tyrosinase (12500 units) was added to a solution of THMA, HBr (4) (69.5 mg, 0.25 mmol) containing 5 equiv of N-acetylcysteine (208 mg, 1.25 mmol) in 10⁻² M sodium phosphate buffer (250 mL, pH 7.4) at 37 °C. The solution became immediately red. After 10 min of reaction at 37 °C, the solution was acidified to pH 2.0 with concentrated HCl, frozen at −80 °C, and then freeze-dried. Semipreparative reversed-phase HPLC was performed using the following mobile phase gradient (flow rate: 5.0 mL min⁻¹): 0–10 min, mixture of solvent A/solvent B 80/20; 10–80 min, mixture of solvent A/solvent B from 80/20 to 100% solvent B. Fractions containing 6-(N-acetylcysteine-S-yl)-2,4,5-trihydroxymethamphetamine (5) were collected, immediately frozen at −80 °C, and then freeze-dried. Compound 5 was isolated in 21.5% yield (19.2 mg, 0.05 mmol). Note 14.5% of THMA (7.5 mg, 0.04 mmol) was also recovered.

**Biological Studies.** Male albino Sprague–Dawley rats weighing approximately 300 g were used. Animals were housed in a colony room maintained at 22 ± 2 °C with free access to food and water. Lighting in the room was automatically regulated on a 12:12 h light/dark cycle. Animals were single housed throughout. The facilities used are accredited by the American Association for the Assessment and Accreditation of Laboratory Animal Care. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University School of Medicine and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals Housing.

To evaluate of the lasting effects of THMA and 6-NAC-THMA on brain 5-HT axonal markers, five experiments were performed. The purpose and design of each of these experiments were as follows.

Experiment 1 assessed regional brain 5-HT and 5-HIAA levels one week after the administration of newly synthesized THMA. THMA, as the hydrobromide salt, was administered into the right lateral ventricle of rats at two different doses (250 and 1000 nmol). Doses refer to the base form. These doses were selected on the basis of previous studies.³²,³³ Rats received either 10 µL of artificial cerebrospinal fluid (aCSF) (control group, N = 5), 250 nmol of THMA (N = 6), or 1000 nmol of THMA (N = 7).

Experiment 2 determined if the lasting effect of THMA on central 5-HT neuronal markers could be blocked with a 5-HT transporter blocker, fluoxetine. There were four treatment groups (n = 5–7 per group): (1) SAL/aCSF; (2) Fluox/aCSF; (3) SAL/THMA; and (4) Fluox/THMA. Rats received saline or fluoxetine (10 mg/kg, i.p.) 65 and 5 min prior to injection of THMA (150 µg) into the lateral ventricle. THMA was dissolved in aCSF immediately prior to intraventricular injection. One week after treatment, rats were sacrificed for the determination of brain 5-HT and 5-HIAA levels.

Experiment 3 tested for the presence of THMA in the brain of animals treated with a high neurotoxic dose of MDMA (80 mg/kg; s.c.). Six rats were treated with MDMA: 3 were examined 1.5 h after MDMA administration; the other 3 were examined 3 h after MDMA administration. Cortical and hippocampal tissues from these animals were analyzed for THMA, MDMA, and various MDMA metabolites, as described below. These times for analysis were selected on the basis of prior pharmacokinetic studies of MDMA in the rat indicating that these times correspond to
peak concentrations of MDMA [i.e., times when maximum concentrations of the precursor would be available for potential conversion into THMA20]. Also, after intracerebroventricular administration, the highest levels of THMA were evident 30 to 120 min later (see Figure 6).

Experiment 4 determined if THMA, an inherently unstable molecule, could be reliably measured in brain tissue after central doses that produce a lasting effect on brain 5-HT and 5-HIAA, namely, 250 and 1000 nmol (see Figure 2). THMA (250 or 1000 nmol) was infused into the right lateral ventricle of rats (N = 12 for each dose). Animals were then divided into subgroups (N = 3 at each time point) and sacrificed at various time points (0.5, 1, 2, and 4 h) after treatment. Regional brain levels of THMA were measured, as described below.

Experiment 5 assessed regional brain 5-HT and 5-HIAA levels two weeks after the administration of 6-NAC-THMA, the thioether conjugate of THMA. 6-NAC-THMA was administered directly into the right frontal cortex. Rats received either 1 μL of aCSF (control group, N = 6) or 42 nmol of 6-NAC-THMA (N = 8), or 52 nmol of 5,7-dihydroxytryptamine (5,7-DHT) (positive control group, N = 6, single dose). Animals treated with aCSF and 6-NAC-THMA received four consecutive doses, with each dose administered 12 h apart. The neurotoxin 5,7-DHT was administered in a single dose. The dose and frequency chosen are the same as those that were used in previous studies to test for the neurotoxic potential of various thioether conjugates derived from HHMA and/or HHA.15,16,18,20

**Intracerebroventricular Administration.** Animals were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), and the head was shaved and placed into a stereotaxic apparatus. Using a surgical scalpel, a midsagittal incision was made to expose the skull. A small burr hole was made with a hand drill: (x0) 0.92 mm from bregma and (x0) 1.4 mm lateral to the midline. A 10 μL Hamilton 7000 series glass syringe (Hamilton Co., Reno, NV) containing the various injection solutions was inserted (x0) 3.5 mm dorsoventrally. Artificial CSF serving as a vehicle control was prepared as described previously by Miller et al.15 Ten microliters of the drug solution was injected manually into the right ventricle at a rate of 10 μL over 2 min. After the injection was completed, the needle was left in the ventricle for an additional 2 min.

**Intracortical Administration.** Animals were anesthetized with xylazine (25 mg/kg, i.p.) and ketamine (35 mg/kg i.p.). Guide cannulae (20 gauge; Plastic One, Roanoke, VA) were surgically implanted into the right frontal cortex [anteroanterior, 3.0 mm; mediolateral, 2.0 mm; dorsoventral, 2.0 mm35]. Cannulae were fixed to the skull with dental acrylic (Ortho-Jet, Lang Dental, Wheeling, IL) and two stainless steel screws. Dummy cannulae were placed in the guide cannulae, and animals were individually housed and allowed a 7-day recovery period. The dummy cannulae were replaced with internal cannulae (24 gauge; Plastic One) connected to PE 20 tubing that in turn was connected to PE 20 tubing that in turn was connected to a 1 μL Hamilton 7000 series glass syringe (Hamilton Co., Reno, NV) containing the various injection solutions. Artificial CSF serving as a vehicle control was prepared as described previously by Miller et al.15 Ten microliters of the drug solution was injected manually into the right frontal cortex at a rate of 10 μL over 2 min. After the injection was completed, the needle was left in the frontal cortex for an additional 2 min.

**Figure 1.** Spectrophotometric changes accompanying the electrosynthesis of 6-NAC-THMA 5. (Spectra a–e): electrochemical oxidation of THMA(1 mM), at a platinum anode (E = +1.0 V versus Ag/AgCl), in deaerated 0.2 M HCl aqueous solution; (a) 0 (before electrolysis), (b) 0.5, (c) 1.0, (d) 1.5, and (e) 2.0 mol of electrons. (Spectra f–i): evolution after the addition of 2 equiv of N-acetylcysteine to the two electron-oxidized solution; (f) 10 min after addition, (g) 20 min, (h), 40 min, and (i) 50 min (end of the process). Cell thickness is 0.1 cm.
Brain Dissection. Rats were euthanized by decapitation, and the striatum, hippocampus, and cerebral cortex were dissected free as previously described. Immediately after dissection, tissue parts were wrapped in aluminum foil and stored in liquid nitrogen until assayed.

Regional Brain 5-HT and 5-HIAA Determination. Tissue samples were analyzed for their content of 5-HT and 5-HIAA 1 or 2 weeks after drug treatment by means of high performance liquid chromatography coupled with electrochemical detection (HPLC-EC) as previously described.

Determination of Brain THMA and MDMA Metabolite Concentrations. For the determination of brain concentrations of THMA, samples were prepared and analyzed using LC-MS methods. In particular, aliquots of rat cortex (approximately 100 mg) and hippocampus (approximately 50 mg) were weighed and for each microgram of tissue, and 10 μL of internal standard solution (pholedrine) was added. After homogenization in 0.01 M HCl (preserved with 6% of each 250 mmol SMBS and 250 mmol EDTA) with a Polytron homogenization unit (model PT 10-35, 15 s, setting 6; Kinematica Inc., Bohemia, NY), the samples were centrifuged (16,000 g for 10 min), and the supernatant was transferred to autosampler vials. Aliquots (5 μL) were injected into the LC-MS system. All samples were analyzed using an Agilent Technologies (AT, Waldbronn, Germany) AT Series 1100 LC/MSD, VL version, using electrospray ionization (ESI) in positive ionization mode, including an AT 1100 Series HPLC system which consisted of a degasser, a quaternary pump, a column thermostat, and an autosampler. Isocratic elution was performed on a Zorbax 300-SCX column (NarrowBore 2.1 × 150 mm, 5 μm) and a Zorbax SCX guard column (4.6 × 12.5 mm, 5 μm). The mobile phase consisted of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid (elucent A) and acetonitrile (elucent B). Samples were analyzed in positive (selected ion monitoring) SIM mode with the following ions: m/z 198 (target ion), 167 for THMA, m/z 196 (t), 165 for the cyclic indoline form of THMA, and m/z 166 (t), 135 for pholedrine (IS). Fragmentor voltage was 100 V. The linear range was 5 to 100 μg/g for THMA as well as for the indoline species. The lowest point of the calibration curve was defined as the limits of quantitation of the method (5 μg/g). For the determination of MDMA and its metabolites HHMA, HMMA, and MDA, samples were prepared and analyzed using previously described LC-MS methods.

Statistics. The significance of differences between means was determined using one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. Statistical analyses were performed using Prism, version 3.02 (GraphPad Software, Inc. San Diego, CA, USA). Differences were considered significant if p < 0.05.

RESULTS AND DISCUSSION

Synthesis of THMA. THMA was synthesized in a straightforward manner (Scheme 2), starting from commercially available 2,4,5-trimethoxybenzaldehyde. Briefly, the previously reported Knoevenagel condensation between 2,4,5-trimethoxybenzaldehyde
and nitroethane quantitatively led to \(\beta\)-methyl-\(\beta\)-nitrostyrene (1), which was converted, after the reduction of the nitro group and hydrolysis of the imine function, into the corresponding 2,4,5-trimethoxyphenylacetone (2) in 80.5% yield. Then, acid-catalyzed reductive amination by sodium triacetoxyborohydride quantitatively yielded 2,4,5-trimethoxymethamphetamine (3). Finally, complete demethylation of 3 using hydrobromic acid heated at reflux, afforded THMA in 76% yield. Interestingly, this four-step reaction sequence produced THMA in a markedly improved overall yield (60%) when compared with that of the previously described procedure, starting from commercially available 3,4-(methyleneoxy)phenol, for which the overall yield did not exceed 5% after seven steps. Although it was previously reported that THMA could be synthesized from 2,4,5-trimethoxybenzaldehyde by modification of the procedure for 4-hydroxy-3-methoxymethamphetamine, no details of the experiments were provided. Therefore, the experiments and the spectroscopic characterizations are thoroughly detailed in this work.

**Electrosynthesis of 6-NAC-THMA 5.** As previously described in the case of 6-hydroxydopamine, 2,4,5-trihydroxymethamphetamine was poorly stable in potassium phosphate buffer (pH 7.4) and rapidly air oxidized to a putative \(p\)-quinone species. At pH 7.4, the ionization of the phenol group at the 4 position \([pK_a = 4 – 5, as reported for 6-OHDA]\) facilitated the deprotonation of the secondary amine function at the origin of the intramolecular cyclization, leading to an indoline intermediate. This was converted into a redox active 5,6-dihydroxyindole species which, after subsequent oxidation reaction, afforded insoluble melanin-like polymers (Scheme 3, path a). To overcome this problem, we decided to adapt our electrochemical procedure, previously described for the preparation of thioether conjugates of HHMA, to the synthesis of 6-NAC-THMA 5. Therefore, the anodic oxidation of THMA 4 was conducted under acidic conditions (Scheme 3, path b), in the absence of N-acetylcysteine (NAC) because 6-NAC-THMA 5 was also electroactive at the applied potential, giving no desired additional oxidation products.

Controlled potential electrolysis was used as a preparative method for the isolation of 6-NAC-THMA 5. When the controlled potential of the platinum anode was fixed at +1.0 V versus Ag/AgCl, which is at a potential at which THMA could be oxidized to the \(p\)-quinone species, a coulometric value of 2.0 ± 0.1 was found for the number of electrons involved in the oxidation of one molecule of THMA into the transient \(p\)-quinone species. The latter was rather stable in aqueous 0.2 M HCl as shown by monitoring the UV absorption spectrum in the course of the electrolysis (Figure 1). After the application of the
potential, a decrease in the UV absorption band shown by THMA at 292 nm ($\varepsilon = 4300$ mol$^{-1}$ L cm$^{-1}$) was observed, while two new bands at 264 and 389 nm developed. Spectral changes showed three isosbestic points at 230, 283, and 309 nm, indicating that a simple equilibrium between two species was shifted (Figure 1, spectra a–e). Subsequent addition of 2 equiv of N-acetylcysteine resulted in the slow discoloration of the pale orange solution due to the formation of the catechol–thioether conjugate 5, which was identified by the change in the UV absorption spectrum (Figure 1, spectra e–i), showing new absorption maxima at 261 and 298 nm. Treatment of the electrolysis solution afforded, after semipreparative reversed-phase HPLC (see the Experimental Procedures section), 6-NAC-THMA 5 in 71% yield.

In a second experiment aimed at comparing the results of anodic oxidation with that of the enzymatic procedure reported below, electrolysis was performed in potassium phosphate buffer (pH 7.4), in the presence of NAC, in other words, under the experimental conditions required for enzymatic oxidation. After the application of the potential, the electrolysis solution rapidly became red, in agreement with the formation of quinonoid species. This was substantiated by monitoring the UV absorption spectrum in the course of the electrolysis. A decrease in the UV absorption band at 292 nm was recorded while new bands at 278 and 489 nm developed. Contrary to what has been observed under acidic conditions, spectral changes did not show isosbestic points. Consequently, to limit the decomposition of the electro-generated catechol–thioether conjugate 5 which was

Figure 5. Mass chromatograms of the target ions of THMA (a), HHMA (b), and HMMA (c). Top chromatograms of each part (a–c) show given ions in rat brain tissue spiked with THMA, HHMA, and HMMA, respectively. Final tissue concentration of each spiked analyte was 5 μg/g. The bottom chromatograms of each part (a–c) show the given ions in authentic brain tissue samples taken from rats (N = 3) treated with a high neurotoxic dose of MDMA (80 mg/kg; SC) 3 h previously. Similar results were obtained in rats treated identically but sacrificed 1.5 (instead of 3) h after drug administration.
electroactive at the applied potential, the electrolysis was stopped after the consumption of 1.75 electron per molecule. Treatment of the electrolysis solution afforded, after semipreparative reversed-phase HPLC, 6-NAC-THMA in 27% yield.

**Enzymatic Procedure for the Synthesis of 6-NAC-THMA 5.** To ensure that 6-NAC-THMA 5 could be formed enzymatically, THMA was oxidized with mushroom tyrosinase in the presence of 5 equiv of NAC (see the Experimental Procedures section), a method previously reported for the synthesis of thioether conjugates of HHMA.\(^{18,25,42}\) Because of the rapid degradation of the solution at \(37^\circ C\), the reaction was stopped after only 10 min by acidification of the reaction mixture to pH 2.0 with concentrated HCl. After semipreparative reversed-phase HPLC, 6-NAC-THMA 5 was isolated in 21% yield. Although 6-NAC-THMA 5 can be prepared enzymatically, this procedure was not adapted for routine synthesis because it was too expensive and not suitable for yielding substantial amounts of thioether conjugates. In this respect, the electro synthesis performed under acidic conditions proved to be particularly attractive for routine synthesis, leading to 6-NAC-THMA 5 in high yield (71%).

**Biological Studies of THMA 4.** THMA 4 administered into the right lateral ventricle of rats produced a significant lasting depletion of 5-HT and 5-HIAA in the cortex and hippocampus (Figure 2). 5-HT and 5-HIAA levels were also reduced in the striatum, but the reductions did not achieve statistical significance. In contrast, significant effects on DA were evident in the striatum (Figure 3). Taken together, these results confirm previous reports that THMA has the potential to produce lasting effects on brain 5-HT and DA neurons.\(^ {23,24}\)

The lasting effect of THMA on brain 5-HT neurons could be blocked with the 5-HT uptake inhibitor fluoxetine (Figure 4). This observation indicates that the lasting effect of THMA on brain 5-HT neurons, like that of MDMA, is dependent on intact 5-HT transporter function.\(^ {30,43}\) Further, it suggests that THMA could play a role in MDMA’s long-term effects on brain 5-HT neurons.

If THMA is, in fact, the metabolite that mediates MDMA neurotoxicity, its presence in the brain would be anticipated. We therefore sought to identify THMA in the brain of MDMA-treated animals. While other O-demethylated MDMA metabolites of MDMA (THMA and HMMA) could be readily and reliably detected in the brains of rats treated with a high neurotoxic dose of MDMA (80 mg/kg), THMA could not be detected in either the cortex or hippocampus, at either 1.5 or 3 h after MDMA administration (Figure 5).

Recognizing that our inability to detect THMA in the brain of MDMA-treated rats might be due to the inherent instability of the THMA molecule, we next carried out studies to determine the stability of THMA in vivo. In particular, we administered THMA to rats intracerebroventricularly, then attempted to measure THMA in various rat brain regions at several times after THMA administration. Figure 6 shows time—concentration profiles of THMA in the cortex, hippocampus, and striatum of rats treated in manner identical to those whose lasting 5-HT and 5-HIAA depletions are shown in Figure 2. THMA levels peaked approximately 0.5—1 h after THMA administration and could be measured up to 4 h. These results demonstrate that it is feasible to detect and reliably measure THMA (and its indoline formed via cyclization; not shown) in rat brain tissue for several hours after its intracerebroventricular administration. Thus, it appears that our inability to identify THMA in the rat brain after peripheral MDMA administration is not related to instability of the THMA molecule. The absence of detectable levels of THMA after MDMA administration casts doubt on the view that THMA, at least in its free (unconjugated) form, mediates MDMA’s lasting effects on brain 5-HT neurons. At this time, it cannot be stated with absolute certainty that other conjugated forms of THMA, such as sulfate or glucuronic conjugates, are not present in the brains of MDMA-treated rats because the present THMA analyses were done without performing conjugate cleavage (because the cleavage procedure results in THMA degradation and disappearance).

Notably, inspection of Figures 2 and 6 shows that there was no correlation between THMA concentrations and lasting 5-HT deficits in various rat brain regions. That is, higher concentrations of THMA were not associated with greater 5-HT deficits. This may be yet another indication that THMA does not mediate the long-term effects of MDMA on brain 5-HT neurons because, in general, there is an excellent correlation between brain MDMA concentrations and lasting 5-HT deficits.\(^ {25}\) In considering the lack of correlation between THMA concentrations and regional brain 5-HT deficits, it is also important to take into account that 5-HT terminals in different brain regions may differ in their susceptibility to various neurotoxicants, including THMA. Further, it is possible that 5-HT terminals in the striatum are less affected because some THMA in this brain region may be sequestered within DA terminals, effectively reducing the amount of THMA to which 5-HT terminals are exposed.

**Biological Studies of 6-NAC-THMA 5.** Given reports implicating thioether conjugates of O-demethylated metabolites of...
MDMA as mediators of MDMA neurotoxicity, we next assessed the neurotoxic potential of 6-NAC-THMA, a thioether conjugate of THMA. These studies involved a direct injection of 6-NAC-THMA into cortical tissue, using the known 5-HT neurotoxin, 5,7-DHT, as a positive control. As expected, 5,7-DHT produced lasting depletions of both 5-HT and 5-HIAA in the brain (Figure 7). In contrast, 6-NAC-THMA was without long-term effects on brain 5-HT neuronal markers. These results suggest that 6-NAC-THMA lacks significant 5-HT neurotoxic potential and do not support the view that this particular thioether conjugate of THMA is responsible for mediating MDMA-induced 5-HT neurotoxicity.

**CONCLUSIONS**

THMA produces lasting effects on brain 5-HT neurons. The lasting effect of THMA on brain 5-HT neurons, like that of MDMA, is dependent upon intact 5-HT transporter function. In contrast to other O-demethylated MDMA metabolites (HHMA and HMMA), THMA was not detected in the brain tissue of rats treated peripherally with a high neurotoxic dose of MDMA. Inability to detect THMA in the brain after peripheral MDMA administration was not due to the instability of the THMA molecule because exogenous THMA administered centrally could be readily detected in rat brains for several hours after intracerebroventricular administration. The thioether conjugate of THMA, 6-NAC-THMA, lacks 5-HT neurotoxic potential, as evidenced by the fact that it fails to produce lasting effects on brain 5-HT axonal markers. Taken together, these observations suggest that neither THMA nor 6-NAC-THMA is directly responsible for MDMA neurotoxicity but leave open the possibility that forms of THMA different from those measured here (e.g., different THMA conjugates or oxidized cyclic forms) could be involved. Additional research is required to test these possibilities.

**ASSOCIATED CONTENT**

**Supporting Information.** ¹H and ¹³C NMR spectra of THMA and its synthetic intermediates, together with 6-(N-acetylcystein-S-yl)-THMA; analytical HPLC chromatograms of THMA and of 6-(N-acetylcystein-S-yl)-THMA. This material is available free of charge via the Internet at http://pubs.acs.org.

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