Drug Testing and Analysis (accepted, uncorrected)

Synthesis, characterization and monoamine transporter activity of the new psychoactive substance 3',4'-methylenedioxy-4-methylaminorex (MDMAR).

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Abstract

The recent occurrence of deaths associated with the psychostimulant *cis*-4,4'-dimethylaminorex (4,4'-DMAR) in Europe indicated the presence of a newly emerged psychoactive substance on the market. Subsequently, the existence of 3,4-methylenedioxy-4-methylaminorex (MDMAR) has come to the authors' attention and this study describes the synthesis of *cis*- and *trans*-MDMAR followed by extensive characterization by chromatographic, spectroscopic, mass spectrometric platforms and crystal structure analysis. MDMAR obtained from an online vendor was subsequently identified as predominantly the *cis*-isomer (90%). Exposure of the *cis*-isomer to the mobile phase conditions (acetonitrile/water 1:1 with 0.1% formic acid) employed for high performance liquid chromatography analysis showed an artificially induced conversion to the *trans*-isomer, which was not observed when characterized by gas chromatography. Monoamine release activities of both MDMAR isomers were compared with the non-selective monoamine releasing agent (+)-3,4-methylenedioxymethamphetamine (MDMA) as a standard reference compound. For additional comparison, both *cis*- and *trans*-4,4'-DMAR, were assessed under identical conditions. *cis*-MDMAR, *trans*-MDMAR, *cis*-4,4'-DMAR and *trans*-4,4'-DMAR were more potent than MDMA in their ability to function as efficacious substrate-type releasers at the dopamine (DAT) and norepinephrine (NET) transporters in rat brain tissue. While *cis*-4,4'-DMAR, *cis*-MDMAR and *trans*-MDMAR were fully efficacious releasing agents at the serotonin transporter (SERT), *trans*-4,4'-DMAR acted as a fully efficacious uptake blocker. Currently, little information is available about the presence of MDMAR on the market but the high potency of ring-substituted methylaminorex analogs at all three monoamine transporters investigated here might be relevant when assessing the potential for serious side-effects after high dose exposure.

Keywords: New psychoactive substances; aminorex; psychostimulants; monoamine transporters; synaptosomes; 4,4'-DMAR; MDMAR; forensic; clinical

Introduction

Since the end of 2012, several European Member States have reported the identification of 4,4'-dimethylaminorex (4,4'-DMAR) (Figure 1A) via the European Union 'Early Warning System' on New Psychoactive Substances' (EU Early Warning System).[1] In addition, the recent emergence of at least 27 deaths associated with the consumption of 4,4'-DMAR[1,2] led to the request of the European Commission to carry out a risk assessment in order to assess control measures across EU Member States.[3] 4,4'-DMAR is a ring-substituted analog of 4-methylaminorex (4-MAR or U4Euh), a stimulant drug of abuse that was popular in the 1980s.[4] Like 4-methylaminorex, 4,4'-DMAR contains two chiral centers which yield the potential for four stereoisomers and two racemic mixtures (i.e. *cis*- and *trans*-racemates). A recently carried out study involving the characterization of 4,4'-DMAR obtained from online vendors and case samples revealed the presence of *cis*-4,4'-DMAR.[2,5]

Aminorex (2-amino-5-phenyl-oxazoline) and its analog 4-MAR (Figure 1A) were both first synthesized by McNeil Laboratories in the 1960s to evaluate their potential as appetite suppressants.[6,7] Aminorex was introduced as a prescription drug for weight loss in Europe in 1965 under the trade names Menocil and Apiquel but withdrawn shortly afterwards due to fatal complications related to pulmonary hypertension.[8,9] While 4,4'-DMAR is currently not a controlled substance, 4-MAR (*cis*-racemate) is classified as a Schedule I substance under the United Nations Convention on Psychotropic Substances 1971, while aminorex is classified as a Schedule IV substance.[10]

Clinical observations related to 4,4'-DMAR intoxication included a range of adverse effects such as agitation, hyperthermia, foaming at the mouth, breathing problems and cardiac arrest.[11] Little information is available regarding the biological mechanism of action for these substances. The related compound aminorex is a substrate for monoamine transporter proteins, which evokes transporter-mediated release of the monoamine neurotransmitters dopamine,

norepinephrine, and serotonin (5-HT) in the central nervous system.[12-14] A previous study revealed that *cis*-4,4'-DMAR is a powerful monoamine-releasing agent that displays low nanomolar potency at all three monoamine transporters.[5]

The work presented in this study describes the synthesis and analytical characterization of 3',4'-methylenedioxy-4-methylaminorex (MDMAR) (Figure 1A). Currently, chemical, pharmacological and clinical information on MDMAR appears to be absent in the scientific literature. There is also little evidence to suggest MDMAR is being used as a NPS. However, similar to the work described previously on 4,4'-DMAR, this investigation was also instigated by the donation from an online vendor. Therefore, its appearance on the recreational market could not be fully excluded. Both *cis* and *trans* isomeric forms of MDMAR (Figure 1B) were synthesized and subjected to analytical characterization using LC-MS, GC-MS, NMR and HR-MS platforms. This was followed by the characterization of the donated vendor MDMAR sample and the identification of the corresponding isomer. Previously published work on the releasing effects of the *cis*-4,4'-DMAR included the comparison with *d*-amphetamine, aminorex and *cis*-4-MAR.[5] In the present study, the monoamine transporter activity of *cis*-MDMAR, *trans*-MDMAR, *cis*-4,4'-DMAR and *trans*-4,4'-DMAR have been evaluated. In these experiments, the non-selective monoamine releasing agent (+)-3,4-methylenedioxymethamphetamine (MDMA) was included as a standard reference compound with known pharmacology.[15]

Experimental

Reagents and standards

All reagents and dry solvents used in the syntheses were obtained from Sigma Aldrich Ltd. (Arklow, Ireland). LC-MS grade solvents were obtained from Fisher Scientific (Dublin, Ireland). A sample subsequently characterized as (±)-*cis*-3',4'-methylenedioxy-4-methylaminorex (*cis*-MDMAR) was donated by Scientific Supplies Ltd. (London, UK).

Syntheses

Unless otherwise stated, the synthesis procedures reported here were essentially adapted from the method reported previously by Brandt *et al*. for the preparation of *cis*- and *trans*-4,4'-DMAR isomers (Figure 1C).[5]

3',4'-Methylenedioxynorephedrine

3',4'-Methylenedioxynorephedrine was prepared from 3,4-methylenedioxypropiophenone.[5] The reaction yielded 3',4'-methylenedioxynorephedrine as a beige powder (7.64 g, 39 mmol, 39%): m.p. 112-114 ^{o}C. ^{1}H NMR (DMSO) δ 6.86 (doublet; *J* = 7.4 Hz; 1H; Ar-H), 6.76 (double doublet; *J* = 8.0, 1.6 Hz; 1H; Ar-H), 5.98 (Singlet; *J* = 7.5 Hz; 2H; CH_2), 4.21 (doublet; *J* = 5.4 Hz; 1H; C<u>H</u>(OH)), 2.83 (doublet quartet; *J* = 12.2, 6.2 Hz; 1H; C<u>H</u>(CH_3)), 0.90 (doublet; *J* = 6.8 Hz; 3H; CH(C<u>H</u>$_3$)); ^{13}C NMR (DMSO) δ 146.77 (Ar C4), 145.67 (Ar C3), 137.69 (Ar C1), 119.73 (Ar C6), 107.41 (Ar C5), 106.99 (Ar C2), 100.55 (CH_2), 77.44 (<u>C</u>H(OH)), 52.32 (<u>C</u>H(CH_3)), 18.45 (CH(<u>C</u>H$_3$)); HR-ESIMS found 196.0965 (theory $[M+H]^+$: $C_{10}H_{13}NO_3$. 196.0929)

(±)-cis-3',4'-Methylenedioxy-4-methylaminorex

Cyanogen bromide (0.583 g, 5.5 mmol) in methanol (2 mL) was added dropwise to a solution of 3',4'-methylenedioxynorephedrine (0.975 g, 5.0 mmol) in methanol (5 mL). The mixture was stirred for 2 h in an ice bath. Following removal of solvent, saturated sodium carbonate (25 mL) was added and this was shaken until a white precipitate formed. The mixture was filtered to yield a colorless powder. Purification by recrystallization in methanol, followed by a further recrystallization step with ethanol, afforded a colorless powder (78 mg, 0.35 mmol, 7%): m.p. 198-200 ^{o}C. ^{1}H NMR (DMSO) δ 6.90 (doublet; *J* = 7.9 Hz; 1H; Ar-<u>H</u>6'), 6.72 (doublet; *J* = 7.9 Hz; 1H; Ar-<u>H</u>2'); 6.69 (doublet; *J* = 1.8 Hz; 1H; Ar-<u>H</u>5'), 5.91 (singlet; *J* = 7.5 Hz; 2H; C<u>H</u>$_2$); 5.43 (doublet; *J* = 8.5 Hz; 1H; H-5), 4.14 (double quartet; *J* = 8.5, 6.8 Hz; 1H; H-4), 0.60 (doublet; *J* = 6.8Hz; 3H; CH_3); ^{13}C NMR (DMSO) δ 159.59 (C-2); 147.39 (Ar C3'); 146.69 (Ar C4'); 132.36 (Ar C1'); 119.48 (Ar C6'); 108.19 (Ar C2'); 101.23 (CH_2); 82.52 (C-5); 63.00 (C-4); 18.90 (CH_3); HR-ESIMS found 221.0916 (theory $[M+H]^+$: $C_{11}H_{12}N_2O_3$, 221.0881).

(±)-trans-3',4'-*Methylenedioxy-4-methylaminorex*

A mixture of 3',4'-methylenedioxynorephedrine hydrochloride (prepared from 0.96 g (4.92 mmol) of the free base and ethereal hydrogen chloride) and potassium cyanate (410 mg) and ethanol (50 mL) was refluxed for 10 h. Removal of solvent was followed by the addition of thionyl chloride (500 µL), dichloromethane (10 mL) and a stirring period of 4 h at room temperature. Following removal of solvent, ethanol (5 mL) was added and the mixture was refluxed for 4 h. Solvent removal yielded crude *trans*-MDMAR followed by the addition of distilled water and diethyl ether (50:50). The aqueous layer was collected and made alkaline with 25% sodium hydroxide. This mixture was shaken until a white precipitate formed and then stirred for a further 20 min. The mixture was filtered yielding a beige solid, which was left to air dry. Purification by flash chromatography (dichloromethane/methanol, 7/3) afforded a colorless powder (21 mg, 0.1 mmol, 2 %): m.p. 148-150 ^{o}C. ^{1}H NMR (DMSO) δ 6.88 (doublet; *J* = 1.9 Hz; 1H; Ar-H6'); 6.82 (doublet; *J* = 8.0Hz; 1H; Ar-H2'); 6.77 (doublet; J = 1.9 Hz; 1H; Ar-H5'); 5.93 (singlet; *J* = 7.5 Hz; 2H; CH_2); 4.75 (doublet; *J*= 6.5 Hz; 1H; H-5); 4.16 (double quartet; *J* = 6.5, 6.4 Hz; 1H; H-4); 1.12 (doublet; *J* = 6.4 Hz; 3H; CH_3); ^{13}C NMR (DMSO) δ 158.87 (C-2); 147.46 (Ar C3'); 146.85 (Ar C4'); 134.85 (Ar C1'); 119.08 (Ar C6'); 108.16 (Ar C5'); 105.81 (Ar C2'); 100.98 (CH_2); 86.33 (C-5); 68.08 (C-4); 21.93 (CH_3); HR-ESIMS found 221.0915 (theory $[M+H]^+$: $C_{11}H_{12}N_2O_3$, 221.0881).

Instrumental analysis

Gas chromatography quadrupole mass spectrometry

Samples were prepared to give a 1 mg/mL solution in methanol and analyzed on an Agilent 6890N GC coupled to 5975 Mass Selective Detector. A HP-ULTRA 1 column (12 m × 0.2 mm × 0.33 µm) was used with helium carrier gas at a constant flow of 1 mL/min and a split ratio of 50:1. The injector was set at 250 °C and the transfer line at 280 °C. The initial oven temperature was 60 °C, held for 2 minutes then ramped at 25 °C/minute to 295 °C with a hold time of 3 minutes.

The mass spectra were collected after a 1.5 min solvent delay time. The ionization energy was set at 70 eV and the mass range was *m/z* 40-450. The total run time was 14.40 minutes.

Liquid chromatography electrospray single quadrupole mass spectrometry

LC-MS analyses were performed on an Agilent 1100 LC system. Separation was obtained on an Allure PFP Propyl column (5 µm, 50 mm x 2.1 mm) Restek (Bellefonte, PA, USA). Mobile phase A consisted of 0.1% formic acid in water, whereas, mobile phase B consisted of 0.1% formic acid in acetonitrile. The Aligent LC-MSD settings were as follows: positive electrospray mode, capillary voltage 3500 V, drying gas (N_2) 12 L/min at 350 ^oC, nebulizer gas (N_2) pressure 60 psi, SIM *m/z* 221 and 178, fragmentor voltage 70 V. Samples for LC-MS analysis were dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) at a concentration of 10 µg/mL. The injection volume was 1 µL, flow rate was 1 mL/min and the column temperature was 30 ^oC. The total run time was 40 min. The following gradient elution program was used: 0-4 min 2% B, followed by an increase to 30% within 30 min, reaching 80% within 33 min before returning to 2% within 40 min.

Nuclear magnetic resonance spectroscopy

For NMR analysis, the MDMAR standards and the vendor sample were prepared in deuterated dimethyl sulfoxide (DMSO-d_6). ^1H (600 MHz) and ^{13}C NMR spectra were recorded on a Bruker AV600 NMR spectrometer using a 5 mm TCI cryoprobe. ^1H NMR spectra were referenced to an external TMS reference at δ = 0 ppm.

High resolution electrospray mass spectrometry

HR-ESI mass spectra were recorded by direct injection into a LTQ Orbitrap Discovery (Thermo Fisher, UK). Samples were dissolved in acetonitrile/water (1:1, containing 0.1% formic acid) and infused at a rate of 5 µL/min. Full accurate high-resolution (30000) mass scans were performed in positive electrospray mode. Measured accurate masses were within ± 5ppm of the theoretical masses. The following conditions were used: drying gas (N_2) 10

L/min, capillary temperature 310 °C, spray voltage 4 V, capillary voltage 22 V and tube lens 77 V.

X-ray crystallography

Intensity data were collected at 100(2) K using a MiTeGen micromount on a Bruker APEX Duo CCD diffractometer equipped with an Oxford Cobra cryosystem. Data were collected using ω and φ scans, corrected for Lorentz and polarization effects, and integrated using the Bruker APEX program suite.[16] Structures were solved by direct methods and refined with least squares procedures.[17] All non-hydrogen atoms were refined anisotropically and hydrogen atoms were placed geometrically in the calculated positions using a riding model except for the amino group where hydrogen atoms were located and refined.

Data collected using Cu Kα radiation (1.54178Å) for a colorless plate crystal 0.35 × 0.16 × 0.02 mm^3, $C_{11}H_{12}N_2O_3$, M = 220.23, triclinic, $P\bar{1}$, a = 6.1800*(2)*, b = 6.4291*(3)*, c = 14.0726*(6)*Å, α = 80.360*(2)*, β = 78.857*(2)* γ=72.431*(2)*°, V =519.43*(4)* Å^3, Z = 2, μ = 0.868 mm^{-1}, 1932 unique data *(θ_{max}* = 69.91°*)*, S = 1.058, R_1 = 0.0369 (1730 reflections with $I > 2\sigma(I)$), wR_2 = 0.0960.* CCDC deposition number 1006741. (* $R_1 = \Sigma||F_o|-|F_c|| / \Sigma |F_o|$ and $wR_2 = \Sigma w(|F_o|^2-|F_c|^2)^2 / \Sigma w|F_o|^2)^{1/2}$)

Monoamine transporter assays

Male Sprague-Dawley rats (250-300 g, Charles River Laboratories, Wilmington, MA, USA) were housed 2 per cage and maintained on a 12-hour light-dark cycle. Food and water were provided *ad libitum*. Animal use procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and the Animal Care and Use Committee of the Intramural Research Program of the National Institute on Drug Abuse (Baltimore, MD, USA). Rats were euthanized by CO_2 narcosis and brains were processed to yield synaptosomes as previously described.[18,19] For release assays, 9 nM [^{3}H]-1-methyl-4-phenylpyridinium ([^{3}H]MPP$^+$) was used as the radiolabeled substrate

for dopamine transporters (DAT) and norepinephrine transporters (NET), while 5 nM [^{3}H]5-HT was used as the radiolabeled substrate for 5-HT transporters (SERT). All buffers used in the release assay methods contained 1 µM reserpine to block vesicular uptake of substrates. The selectivity of release assays was optimized for a single transporter by including unlabeled blockers to prevent the uptake of [^{3}H]MPP$^+$ or [^{3}H]5-HT by competing transporters. Synaptosomes were preloaded with radiolabeled substrate in Krebs-phosphate buffer for 1 h (steady state). Release assays were initiated by adding 850 µL of preloaded synaptosomes to 150 µL of test drug. Release was terminated by vacuum filtration and retained radioactivity was quantified by scintillation counting. Time-course evaluation revealed that *cis*-MDMAR was subject to isomerization in aqueous assay buffer after approximately 40 min. Thus, for this compound, drug dilutions were prepared immediately prior to assay initiation to avoid the potential for isomerization.

Results and discussion

The presence of (±)-*cis*-4,4'-DMAR on the European market became apparent following a number of notifications to the EU Early Warning System and from reports published in the scientific literature,[1,2] which triggered an in-depth chemical and analytical characterization and evaluation of its monoamine transporter activity.[5] While *cis*-4,4'-DMAR has also been advertised under the name 'Serotoni',[20] data about availability, prevalence of use and properties of MDMAR are currently unavailable. However, since this substance was donated from an online vendor it was deemed important to chemically profile this substance as a potential new psychoactive substance as it seemed possible that it might also potentially become available on the drug market. A number of ring-substituted aminorex analogs have been occasionally discussed, for example on the now defunct Hive forum or in the literature,[21] and it appeared that some discussion has also occurred on MDMAR ('MDMAminorex') on an online forum.[22] So far, however, it is unclear whether MDMAR is available for purchase from online retailers.

Synthesis

The synthesis employed for the preparation of the racemic isomers of MDMAR was based on the synthetic pathways outlined in the literature.[5,6,23,24] The synthesis involved bromination of the 3,4-methylenedioxypropiophenone precursor (**a**), yielding α-bromo-3,4-methylenedioxypropiophenone (**b**). This was reacted with sodium diformylamide (Gabriel Reaction) to give a methylenedioxy-*N*,*N*-diformylamide derivative (**c**). Hydrolysis under acidic conditions provided the methylenedioxycathinone species (**d**) and reduction to an alcohol provided the methylenedioxynorephedrine intermediate (**e**). This was subsequently converted to either (±)-*cis*-, via the cyanogen bromide route, or (±)-*trans*-, via the potassium cyanate route, 3',4'-MDMAR (Figure 1C). The preliminary synthesis of the *trans* isomer, using the literature based methods, yielded a cyanamide intermediate and not the *trans* MDMAR isomer (supplementary data). This compound failed to undergo cyclization to form the oxazoline ring, which is necessary for the formation of the MDMAR isomer. The synthesis of the *trans*-MDMAR isomer was then conducted using an alternative synthetic route. This synthetic route involved reacting 3',4'-methylenedioxynorephedrine hydrochloride with potassium cyanate in the presence of ethanol. The urea intermediate formed was then reacted with thionyl chloride, which spontaneously cyclized[25] to form the desired *trans*-MDMAR isomer.

X-Ray Crystallography

The structure of the synthesized *cis*-MDMAR isomer is shown in Figure 1D. After repeated crystallization from propanol, a single colorless very thin plate crystal was used for analysis. The structure in the triclinic space group Pī, confirms the 4S,5R-*cis* arrangement of the oxazoline moiety. Bond lengths and angles in the oxazoline moiety are similar to those found in (4S,5R)-(-)-*cis*-4-methyl-5-phenyloxazoline-2-amine.[25] The angle of the oxazoline plane to benzodioxole plane (64°, torsion angle C7-C6-C10-O11 = 29.54(16) °) is more acute than in the phenyloxazoline (104°, torsion angle C9-C4-C3-O1 = 34.4°). *cis*-MDMAR is linked

in the solid state by hydrogen bonds between the amino group and neighboring oxazoline groups (supplementary data). Short contacts are also formed between neighboring dioxoles.

(Insert Figure 1 here)

Gas chromatography mass spectrometry

The chromatographic method used was able to achieve separation between the two isomers obtaining a retention time of 7.97 minutes for the *cis* isomer (Figure 2A) and a retention time of 7.88 minutes for the *trans* isomer (Figure 2B). The EI mass spectra obtained for the synthesized *cis* and *trans* racemates were similar as expected. The base peak was observed at *m/z* 70 and indicated the loss of 3,4-methylenedioxybenzaldehyde, which gave rise to a radical cation. The suggested species, with molecular formula $C_3H_6N_2$, which may be the 3-methylaziridin-2-imine fragment, was consistent with the EI-MS data reported for 4-MAR[26] and 4,4'-DMAR.[5] The loss of a methyl group (*m/z* 205) from the molecular ion was observed while the presence of an acylium ion was represented by the ion at *m/z* 149, followed by a loss of CO to give *m/z* 121. The observed species at *m/z* 176 was due to the presence of an aziridinium ion. Overall, it was found that the main principles of fragmentation were in agreement with the EI spectra reported previously on 4,4'-DMAR.[5] The proposed electron ionization fragmentation pattern for 3',4'-methylenedioxy-4-methylaminorex by GC-MS is outlined in Figure 2C.

(Insert Figure 2 here)

Liquid chromatography mass spectrometry

The HPLC method successfully separated and distinguished between both isomers of MDMAR. A retention time of 15.25 minutes was observed for the *cis* isomer, while a retention time of 16.29 minutes was observed for the *trans* isomer. The EI mass spectra of the synthesized *cis* and *trans* racemates were

identical, sharing highly abundant fragments, i.e. *m/z* 221 for the protonated molecule and m/z 178 (supplementary data). The mass spectrum indicated the loss of 43 Da, which represented HNCO, resulting in a species with *m/z* 178 (Figure 3A). The loss of 43 Da (HNCO) was described previously in studies involving mass spectrometric analysis of 4,4'-DMAR[5] and 4-MAR.[27] However, due to the mass difference between MDMAR, 4,4'-DMAR and 4-MAR, the equivalent loss of 43 Da resulted in ions at *m/z* 148 and *m/z* 134, respectively. An identical fragmentation pattern was obtained from high resolution mass spectrometric analysis (supplementary data). An unexpected phenomenon occurred when analyzing the *cis* isomer by LC, i.e. the *cis* isomer underwent an isomerization process in solution (acetonitrile/water 1:1 with 0.1% formic acid) that led to the increasing formation of the *trans* isomer. The *cis* isomer was analyzed twenty times in succession to monitor the *cis* to *trans* conversion. A ratio of *cis* and *trans* peak area was calculated and is presented graphically in Figure 3B. It was considered possible that the *cis* isomer reacted with the H_2O in the LC mobile phase, hence, resulting in the change of configuration from the *cis* isomer to its *trans* equivalent (Figure 3C). The isomerization of *cis*-MDMAR to *trans*-MDMAR was examined under three conditions: acetonitrile only, LC grade water only and aqueous assay buffer conditions. Isomerization did not occur in acetonitrile only conditions. Isomerization began to occur in water only conditions and the aqueous assay buffer conditions after approximately 40 minutes (supplemental data). This *cis* to *trans* conversion was unique to *cis*-MDMAR and had not been encountered in previous analyses of 4-MAR or 4,4'-DMAR.

(Insert Figure 3 here)

Nuclear magnetic resonance spectrometry

The NMR spectra associated with both racemic MDMAR isomers shared some key characteristics, which were consistent with those reported previously for *cis* and *trans* 4,4'-DMAR and 4-MAR.[5] However, there were also differences in chemical shifts present that facilitated the differentiation between the *cis* and *trans* MDMAR isomers. The significant differences occurred at the chemical

environments around the chiral carbons. In the ^{1}H NMR, the protons of the CH_3 attached to the carbon at position four of the oxazoline ring in the *trans* isomer produced a signal at 1.12 ppm while the protons on the CH_3 at the same position in the *cis* isomer was shifted upfield and produced a signal at 0.60 ppm. Again, the proton attached to the carbon at position five of the oxazoline ring in the *trans* isomer was represented by a signal at 4.75 ppm whereas the same proton was shifted downfield in the *cis* isomer and was represented by a signal at 5.43 ppm. The chiral carbons in both isomers were in different environments and produced different resonances. The carbon at position four of the oxazoline ring in the *trans* isomer produced a signal at 68.08 ppm, while the same carbon in the *cis* isomer was represented by a signal at 63.00 ppm. The second chiral carbon at position five of the oxazoline ring in the *trans* isomer produced a signal at 86.33 ppm and a signal at 82.52 ppm in the *cis* isomer. The carbon of the methyl group gave a signal at 21.93 ppm in the *trans* isomers compared to a signal at 18.90 ppm in the *cis* isomer.

The *cis* to *trans* isomerization conversion highlighted by LC-MS analysis was further investigated using NMR. The composition of the LC mobile phase was recreated using deuterated solvents (acetonitrile-d_3:D_2O [1:1] with 0.1% formic acid-d_2). Both, *cis* and *trans* isomers were dissolved in the deuterated mobile phase and analyzed at t = 0 h, t = 6 h, t = 12 h and t = 24 h. From the ^{1}H NMR time study, it was evident that the *cis* to *trans* conversion process was occurring when the compound was in solution (Figure 4). The signal representing the protons on the methyl group in the *cis* isomer (1.28 ppm) reduced over time whereas the signal representing the same protons in the *trans* isomer (1.78 ppm) increased. The signal response related to the proton attached to the carbon at position four of the oxazoline ring (4.94 ppm) was seen to decrease in the *cis* isomer while increasing in the case of the *trans* isomer (4.60 ppm). The signal representing the proton attached to the carbon at position five of the oxazoline ring in the *trans* isomer (5.85 ppm) was increasingly formed while signal intensity representing the same proton in the *cis* isomer (6.48 ppm) decreased.

(Insert Figure 4 here)

Monoamine transporter activity

Figure 5 shows the dose-response effects of *cis*-DMAR, *trans*-DMAR, *cis*-MDMAR and *trans*-MDMAR on transmitter release at DAT, NET and SERT. Table 1 summarizes potency values as concentrations producing 50% of maximal release (EC_{50} concentration) for the test drugs based on data depicted in Figure 5. All of the ring-substituted 4-methylaminorex analogs displayed potent substrate-type releasing activity at DAT, with EC_{50} values ranging from 10.2 ± 1.2 nM for *cis*-MDMAR to 36.2 ± 3.6 nM for *trans*-MDMAR. The drugs were also potent releasers at NET, with EC_{50} values ranging from 11.8 ± 2.0 nM for *cis*-DMAR to 38.9 ± 4.7 nM for *trans*-MDMAR. All of the 4-methylaminorex analogs were more potent than MDMA as substrates at DAT and NET (see Table 1). Activity at SERT varied from 17.7 ± 2.3 nM for *cis*-DMAR to 73.4 ± 12.0 nM for *trans*-MDMAR. The test drugs were fully efficacious in their ability to evoke release at DAT, NET and SERT (i.e., drug effects achieved 100% of maximal release), with the exception of *trans*-DMAR, which functioned as a "partial" releaser at SERT (75% maximal release). Subsequent experiments revealed that *trans*-DMAR is fully efficacious as an uptake blocker at SERT (data not shown), suggesting this drug displays "hybrid" activity across monoamine transporters. It is proposed that *trans*-DMAR acts as a substrate-type releaser at DAT and NET, but an uptake blocker at SERT. When considering the transporter selectivity of the test compounds, all were essentially non-selective, with DAT/SERT ratios ranging from 0.6 for MDMA to 4.3 for *cis*-DMAR.

(Insert Figure 5 and Table 1 here)

cis-DMAR, *trans*-DMAR, *cis*-MDMAR and *trans*-MDMAR are potent and efficacious substrate-type releasers at DAT and NET in rat brain tissue. Importantly, all of the ring-substituted 4-methylaminorex analogs are more potent than MDMA as catecholamine releasers. *cis*-DMAR, *cis*-MDMAR and *trans*-MDMAR are fully efficacious releasing agents at SERT as well, while *trans*-DMAR displays partial releasing activity at this transporter. It is hypothesized that *trans*-DMAR displays

the unusual profile of a catecholamine releaser with 5-HT uptake blocking properties, but further experiments are needed to confirm this proposal. The potencies for *cis* and *trans* racemates of DMAR and MDMAR were generally similar at each transporter, indicating a minimal influence of stereoselectivity in determining drug-transporter interactions across this group of structures. It seems worth mentioning that all of the 4-methylaminorex analogs tested here had a profile of transporter releasing activity that mimics the effects of MDMA (i.e. non-selective transporter releaser), but the methylaminorex compounds were more potent.

Confirmation of *cis*-MDMAR in vendor sample

The sample donated from an online vendor and thought to contain MDMAR was subjected to identical analytical conditions. GC-MS studies showed that the online vendor product consisted predominantly of the *cis* MDMAR isomer (90%). The retention time (7.97 minutes) and fragmentation pattern of the vendor sample was congruent with that of the *cis* MDMAR standard. LC-MS studies, which included the comparison with the synthesized *cis* standard, also confirmed the presence of *cis* MDMAR in the vendor sample. Inspection of the LC chromatogram derived from the donated sample also suggested that the product contained a significant amount of the *trans* isomer. However, based on the observations described and investigated above, it was determined that this detection of the *trans* isomer was formed artificially following exposure to the water-containing mobile phase. Analysis by GC-MS, on the other hand, did not result in isomerization and detected the *trans* species at a much less significant level (10%). The ^{1}H and ^{13}C NMR spectra were in agreement with the GC and LC data confirming the presence of *cis* MDMAR in the vendor product.

Conclusion

The preparation and analytical characterization of *cis*- and *trans*-3',4'-methylenedioxy-4-methylaminorex (MDMAR) demonstrated facile differentiation between both isomers but also showed a potential for

misinterpretation under HPLC conditions when it was observed that the aqueous mobile phase caused isomerization of the *cis*- to the *trans* form. The high potency of ring-substituted methylaminorex analogs and their ability to be fully efficacious substrate-type releasers might contribute to the possibility of a range of serious side effects after high dose exposure and/or when combined with other substances that act on similar targets. Psychotic symptoms, agitation and hyperthermia could result from overstimulation of central dopamine and 5-HT systems, whereas dangerous cardiovascular effects could be produced by excessive norepinephrine release in the periphery. [28,29] The comparison between the monoamine transporter activity of *cis*- and *trans*-4,4'-dimethylaminorex pointed towards the possibility that the *trans*-species might display "hybrid" activity across monoamine transporters. This is the first report on the characterization of MDMAR, which demonstrates the continuous need to remain vigilant on the availability of newly emerging psychoactive substances.[30]

Acknowledgement

Portions of this research were generously supported by the National Institute on Drug Abuse, National Institutes of Health, U.S.A.

References

[1] EMCDDA-Europol. Joint Report on a new psychoactive substance: 4,4′-DMAR (4-methyl-5-(4-methylphenyl)-4,5- dihydrooxazol-2-amine), Joint Reports, Publications Office of the European Union, Luxembourg, **2014**. Available at: http://www.emcdda.europa.eu/attachements.cfm/att_229825_EN_TDAS14006ENN.pdf [21 July 2014].

[2] S. Cosbey, S. Kirk, M. McNaul, L. Peters, B. Prentice, A. Quinn, S.P. Elliott, S.D. Brandt, R.P. Archer. Multiple fatalities involving a new designer drug: *para*-methyl-4-methylaminorex. *J. Anal. Toxicol.* **2014**, *38*, 383.

[3] Council of the European Union. Request for risk assessment on new psychoactive substance 4,4'-DMAR (4-methyl-5-(4-methylphenyl)-4,5-dihydrooxazol-2-amine). 10 June **2014**. Available at: http://register.consilium.europa.eu/doc/srv?l=EN&f=ST 10619 2014 INIT [21 July 2014].

[4] F.T. Davis, M.E. Brewster. A fatality involving U4Euh, a cyclic derivative of phenylpropanolamine. *J. Forensic Sci.* **1988**, *33*, 549.

[5] S.D. Brandt, M.H. Baumann, J.S. Partilla, P.V. Kavanagh, J.D. Power, B. Talbot, B. Twamley, J. O'Brien, O. Mahony, S.P. Elliott, R.P. Archer, J. Patrick, K. Singh, N.M. Dempster, S.H. Cosbey. Characterization of a novel and potentially lethal designer drug, (±)-*cis*-*para*-methyl-4-methylaminorex (4,4'-DMAR, or 'Serotoni'). *Drug Test. Anal.* **2014**, *6*, 684.

[6] G.I. Poos, J.R. Carson, J.D. Rosenau, A.P. Roszkowski, N.M. Kelley, J. McGowin. 2-Amino-5-aryl-2-oxazolines. Potent new anorectic agents. *J. Med. Chem.* **1963**, *6*, 266.

[7] J.R. Carson, G.I. Poos, H.R. Almond, Jr. 2-Amino-5-aryl-2-oxazolines. Tautomerism, stereochemistry, and an unusual reaction. *J. Org. Chem.* **1965**, *30*, 2225.

[8] F. Follath, F. Burkart, W. Schweizer. Drug-induced pulmonary hypertension? *Br. Med. J.* **1971**, *1*, 265.

[9] H.P. Gurtner. Aminorex and pulmonary hypertension. A review. *Cor Vasa* **1985**, *27*, 160.

[10] United Nations. Convention on psychotropic substances, 1971. Available at http://www.unodc.org/pdf/convention_1971_en.pdf [25 March 2014].

[11] EMCDDA-Europol. Dangerous synthetic drugs hit the EU market. Available at: http://www.emcdda.europa.eu/news/2014/europol-emcdda1 [05 March 2014].

[12] R.B. Rothman, M.A. Ayestas, C.M. Dersch, M.H. Baumann. Aminorex, fenfluramine, and chlorphentermine are serotonin transporter substrates. Implications for primary pulmonary hypertension. *Circulation* **1999**, *100*, 869.

[13] R.B. Rothman, M.H. Baumann. Monoamine transporters and psychostimulant drugs. *Eur. J. Pharmacol.* **2003**, *479*, 23.

[14] T. Hofmaier, A. Luf, A. Seddik, T. Stockner, M. Holy, M. Freissmuth, G.F. Ecker, R. Schmid, H.H. Sitte, O. Kudlacek. Aminorex, a metabolite of the cocaine adulterant levamisole, exerts amphetamine like actions at monoamine transporters. *Neurochem. Int.* **2014**, in press; doi: 10.1016/j.neuint.2013.11.010.

[15] M.H. Baumann, X. Wang, R.B. Rothman. 3,4-Methylenedioxymethamphetamine (MDMA) neurotoxicity in rats: a reappraisal of past and present findings. *Psychopharmacology* **2007**, *189*, 407.

[16] APEX2 program suite, Bruker AXS Inc., Madison, Wisconsin, USA, **2012**.

[17] G.M. Sheldrick. A short history of SHELX. *Acta Crystallogr. A* **2008**, *64*, 112.

[18] R.B. Rothman, N. Vu, J.S. Partilla, B.L. Roth, S.J. Hufeisen, B.A. Compton-Toth, J. Birkes, R. Young, R.A. Glennon. In vitro characterization of ephedrine-related stereoisomers at biogenic amine transporters and the receptorome reveals selective actions as norepinephrine transporter substrates. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 138.

[19] M.H. Baumann, M.A. Ayestas Jr, J.S. Partilla, J.R. Sink, A.T. Shulgin, P.F. Daley, S.D. Brandt, R.B. Rothman, A.E. Ruoho, N.V. Cozzi. The designer methcathinone analogs, mephedrone and methylone, are substrates for monoamine transporters in brain tissue. *Neuropsychopharmacology* **2012**, *37*, 1192.

[20] Serotoni. Available at: http://serotoni.info [22 July 2014].

[21] D.A. Cooper, Future synthetic drugs of abuse. In *Proceedings of the international symposium on the forensic aspects of controlled substances: March 28 - April 1, 1988*, (Ed.: R.T. Castonguay), Laboratory Division, Federal Bureau of Investigation, U.S. Dept. of Justice, Washington, D.C., **1988**, pp. 79.

[22] Bluelight. Thread: Substituted Aminorex Series. March **2008**. Available at: http://www.bluelight.org/vb/threads/367053-Substituted-Aminorex-Series [Accessed 28 May 2014].

[23] G. Fodor, K. Koczka. 155. The stereochemical course of the conversion of 2-ureidoalcohols into oxazolidines. Part I. *J. Chem. Soc.* **1952**, 850.

[24] W.R. Rodriguez, R.A. Allred. Synthesis of *trans*-4-methylaminorex from norephedrine and potassium cyanate. *Microgram J.* **2005**, *3*, 154.

[25] A. Cruz, I.I. Padilla-Martínez, E.V. García-Báez, R. Contreras. Reactivity of chlorodeoxypseudoephedrines with oxo-, thio-, and selenocyanates. *Tetrahedron: Asymmetry* **2007**, *18*, 123.

[26] R.F.X. Klein, A.R. Sperling, D.A. Cooper, T.C. Kram. The stereoisomers of 4-methylaminorex. *J. Forensic Sci.* **1989**, *34*, 962.

[27] G.L. Henderson, M.R. Harkey, Y.T. Chueh. Metabolism of 4-methylaminorex ("EU4EA") in the rat. *J. Anal. Toxicol.* **1995**, *19*, 563.

[28] S.L. Hill, S.H. Thomas. Clinical toxicology of newer recreational drugs. *Clin Toxicol (Phila)* **2011**, *49*, 705.

[29] L.J. Schep, R.J. Slaughter, D.M. Beasley. The clinical toxicology of metamfetamine. *Clin Toxicol (Phila)* **2010**, *48*, 675.

[30] S.D. Brandt, L.A. King, M. Evans-Brown. The new drug phenomenon. *Drug Test Anal*. **2014**, *6*, 587.

Figure 1

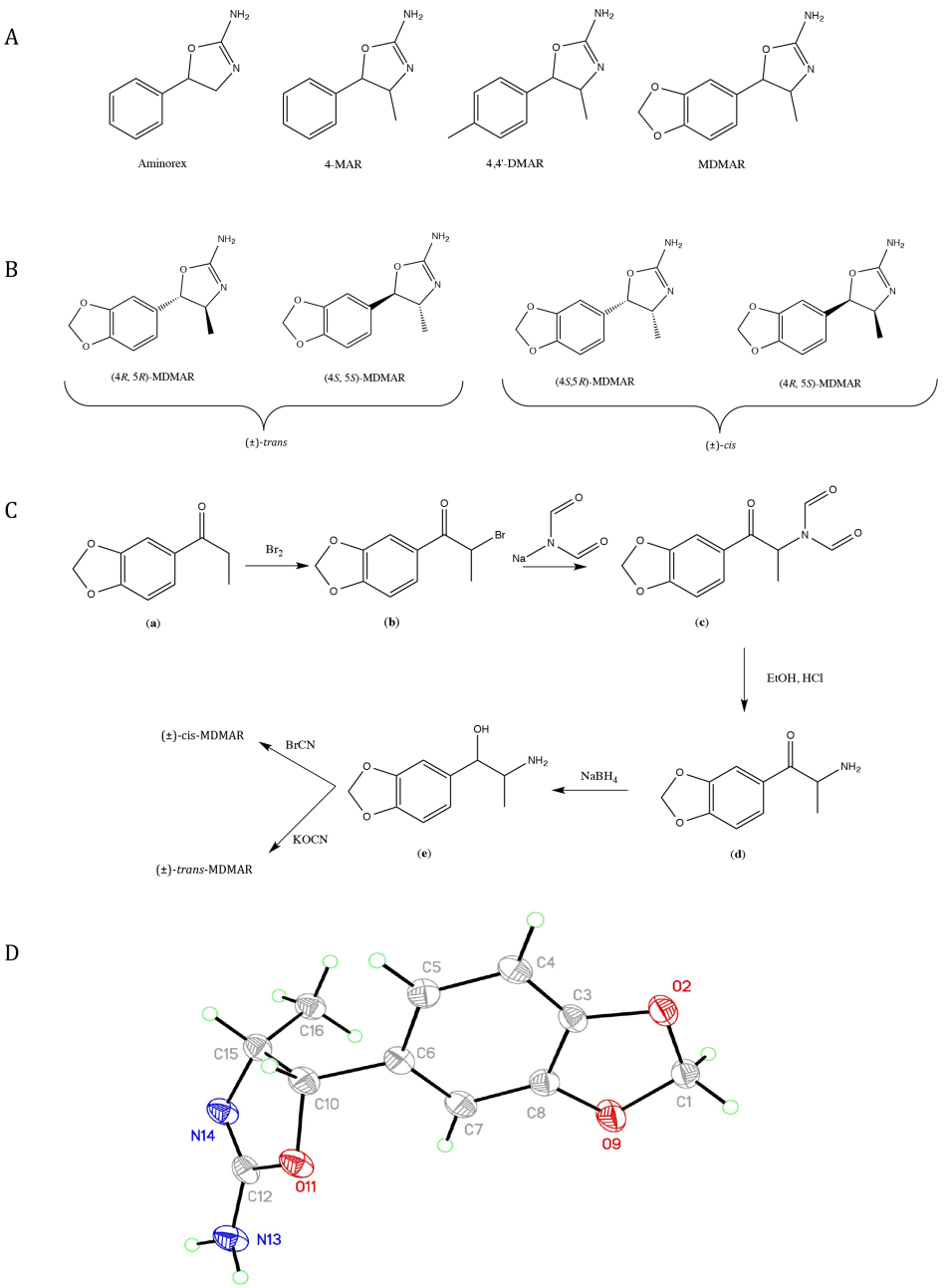
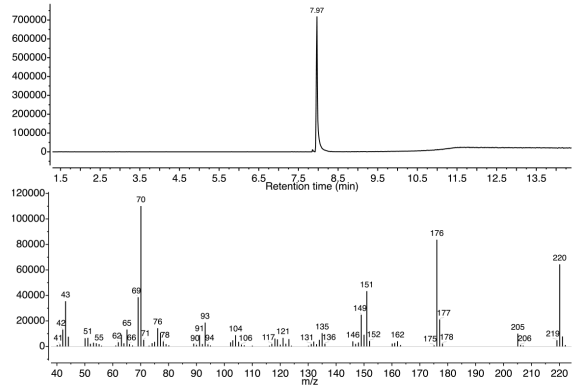


Figure 1. A: Chemical structures of the three psychostimulants aminorex, 4-methylaminorex (4-MAR), *para*-methyl-4-methylaminorex (4,4'-DMAR) and the new potential psychoactive substance 3',4'-methylenedioxy-4-methylaminorex (MDMAR). B: Structural representation of all four MDMAR enantiomers. C: Synthetic route to both (±)-*cis*- and (±)-*trans*- MDMAR. Both isomers were prepared from the same 3',4'-methylenedioxynorephedrine prescursor (**e**) using cyanogen bromide or potassium cyanate to yield the (±)-*cis* and (±)-*trans*-MDMAR product, respectively. D: Molecular structure of synthesized *cis* MDMAR (50% displacement ellipsoids).

Figure 2

A



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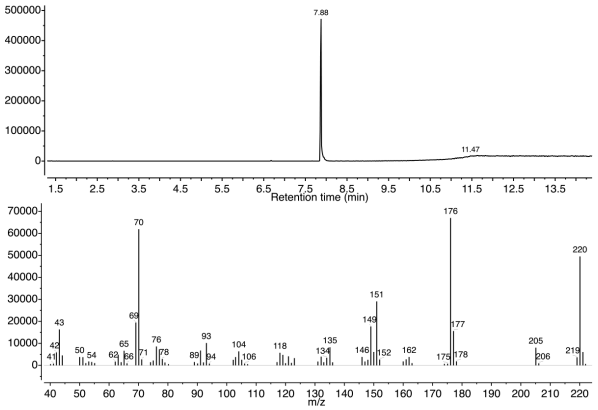

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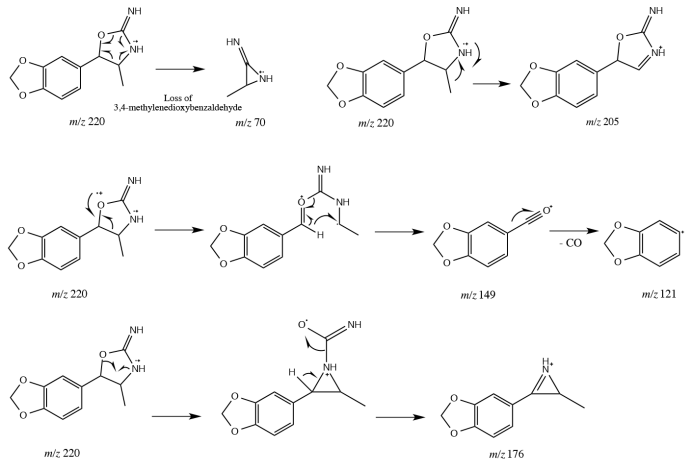

Figure 2. A: GC-MS data obtained for the (±)-*cis*-MDMAR isomer. B: GC-MS data obtained for the (±)-t*rans*-MDMAR isomer. C: A proposed EI-MS fragmentation pattern for the (±)-*cis*- and (±)-t*rans*-MDMAR

Figure 3

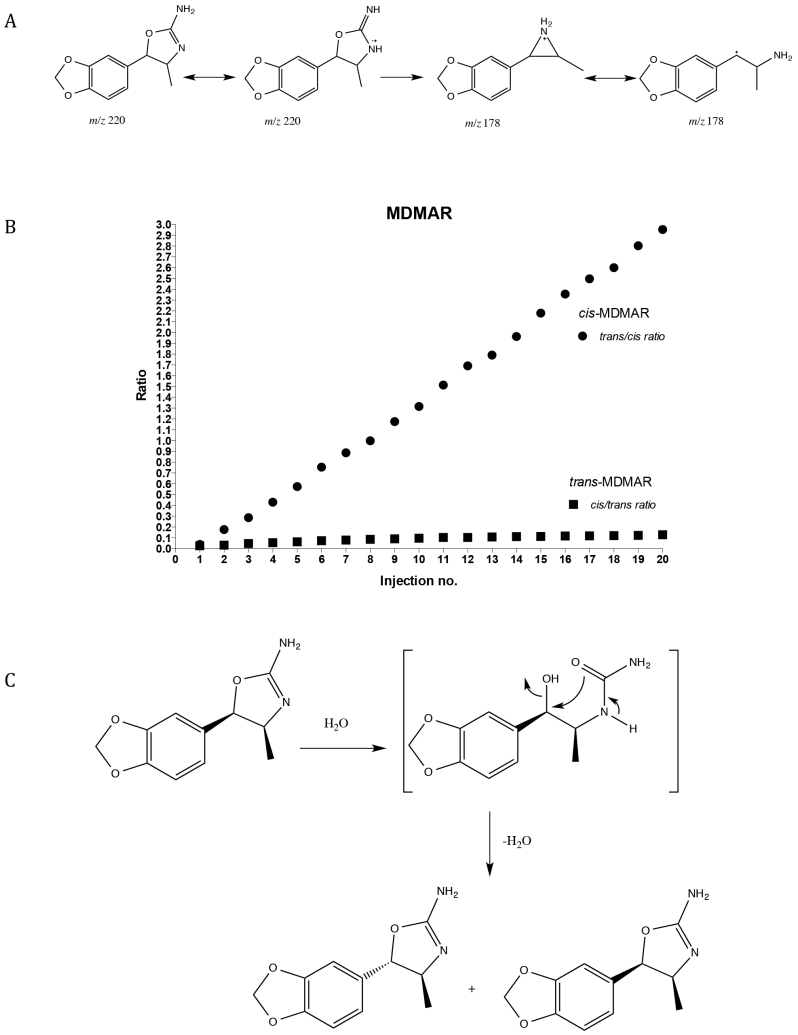

Figure 3. A: Suggested mechanism for the formation of the m/z 178 ion following loss of HCNO. B: Graph representing the *cis* to *trans* isomerization conversion overtime. C: Suggested mechanism to explain the *cis* to *trans* conversion – *cis* isomer reacts with the H_2O in the LC mobile phase resulted in a change of configuration in the *cis* isomer converting it to the *trans* equivalent.

Figure 4

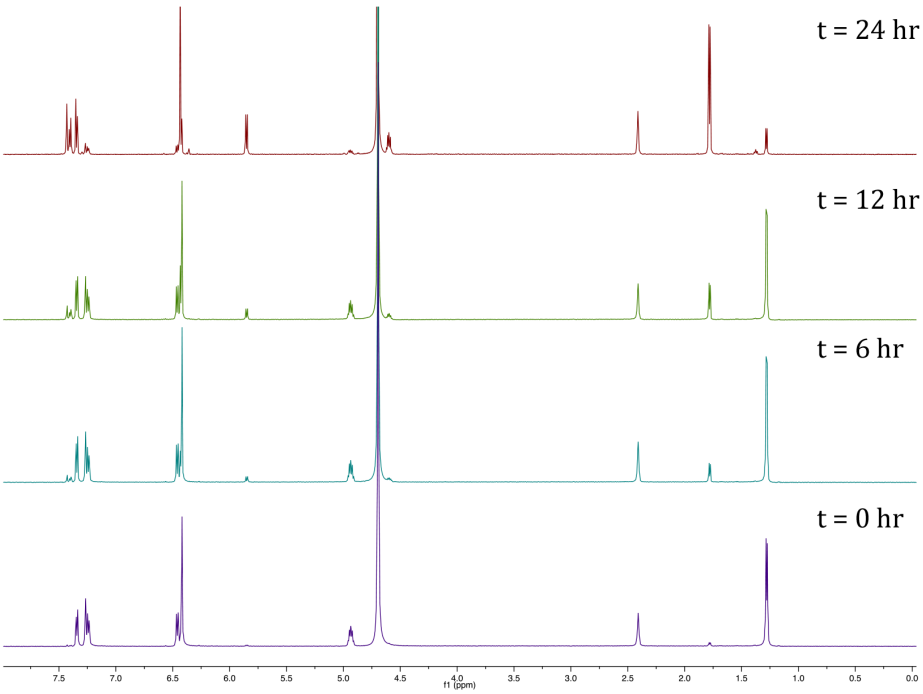

Figure 4. ^{1}H NMR data obtained for the *cis* to *trans* conversion - monitored over 24 hrs.

Figure 5

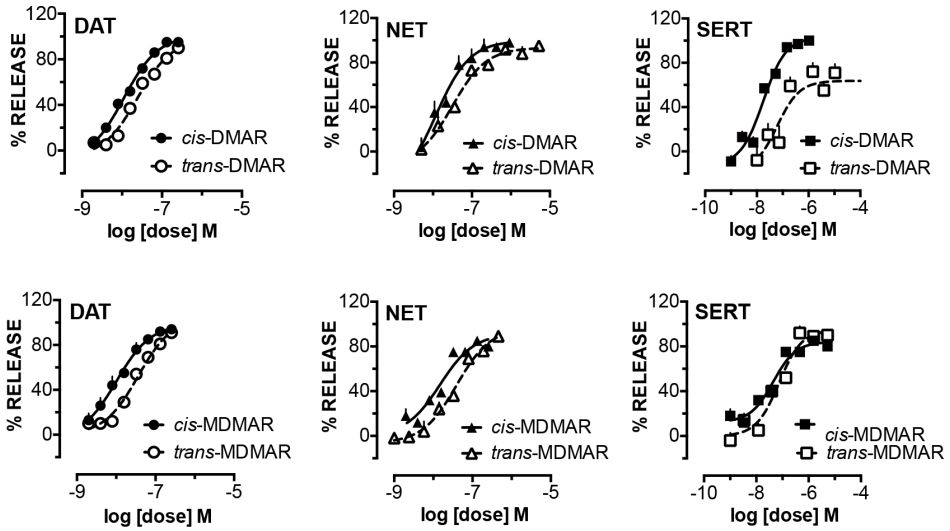

Figure 5. Dose-response effects of *cis*-DMAR, *trans*-DMAR, *cis*-MDMAR and *trans*-MDMAR on transmitter release at DAT, NET and SERT. Data are mean ± SD for N=3-4 experiments performed in triplicate.

Drug	[a] Release at DAT EC_{50} (nM)	[a] Release at NET EC_{50} (nM)	[a] Release at SERT EC_{50} (nM)	[b] DAT/SERT ratio
(+)-MDMA	143±16	98.3±15.0	85.0±13.3	0.6
Cis-DMAR	10.9±0.7	11.8±2.0	17.7±2.3	1.6
Trans-DMAR	24.4±2.7	31.6±4.6	59.9±17.2	2.5
Cis-MDMAR	10.2±1.2	14.8±2.7	43.9±6.7	4.3
Trans-MDMAR	36.2±3.6	38.9±4.7	73.4±12.0	2.0

[a] Data are expressed as mean ± SD for N=3-4 experiments performed in triplicate

[b] DAT/SERT ratio calculated by $(EC_{50}$ at DAT$)^{-1}$/ $(EC_{50}$ at SERT$)^{-1}$; higher value indicates greater DAT selectivity

Table 1. Summary of the potency values (EC_{50} concentration) for the test drugs based on data depicted in Figure 5.