Synthesis, Anticonvulsant Activity and Metabolism of 4-chlor-3-methylphenoxyethylamine Derivatives of *Trans*-2-aminocyclohexan-1-ol

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ABSTRACT In this study, we report the synthesis, spectral characterization, antiepileptic activity and biotransformation of three new, chiral, N-aminoalkyl derivatives of *trans* – 2 aminocyclohexan-1-ol: **1** (R enantiomer), **2** (S enantiomer) and **3** (racemate). Antiepileptic activity of the titled compounds was studied using MES and scMet. Moreover, in this study, the biotransformation of **1**, **2** and **3** in microbial model (*Cunninghamella*), liver microsomal assay as well as *in silico* studies (MetaSite) was evaluated.

Studies have indicated that **1**, **2** and **3** have good antiepileptic activity *in vivo,* comparable to valproate. Biotransformation assays showed that the most probable metabolite (indicated in every tested assays) was **M1**. The microbial model as well as *in silico* study showed no difference in biotransformation between tested enantiomers. However, in a rat liver microsomal study compound **1** and **2** (R and S enantiomer) had different main metabolite – **M2** for **1** and **M1** for **2**. MS/MS fragmentation allowed us to predict the structures of obtained metabolites, which were in agreement with 1°alcohol (**M1**) and carboxylic acid (**M2**).

Our research has shown that microbial model, microsomal assay, and computational methods can be included as useful and reliable tools in early ADME-Tox assays in the process of developing new drug candidates. *Chirality 27:163–169, 2015*.

KEY WORDS: Cunninghamella; Biotransformation; Anticonvulsant activity; Liver microsomes; Enantiomers

INTRODUCTION

The phenomenon of chirality plays a key role in the life of every living organism. All proteins, amino acids, enzymes, carbohydrates, and nucleosides are chiral compounds, which means that they are not superimposable to their mirror images. They exist in nature under a single enantiomeric form. In contrast, in pharmaceutical industries almost 90% of chiral drugs are marketed as racemates consisting of an equimolar mixture of two enantiomers. This is due to the fact that obtaining the enantiomers is much more difficult and more expensive.

Enantiomers show completely identical physicochemical properties, when they are in an achiral environment. But because the human body is a chiral environment, two different enantiomers of one compound can display the pharmacokinetic processes – such as absorption, distribution, metabolism, and excretion – as well as pharmacology and toxicology, in a stereoselective manner.[1]

It is known that metabolizing enzymes often display a preference for one enantiomer of a chiral drug over another, resulting in enantioselectivity.[2] Therefore, enantiomers in a racemic drug should be treated as two different compounds, and studies on every enantiomer – not only racemates – must be carried out.[3] Antiepileptic drugs are no exception.

Epilepsy is one of the most common chronic neurological problems characterized by seizures. Antiepileptic drugs (AEDs) exert their action by various mechanisms: they can influence inhibitory or excitatory neurotransmitter systems such as GABA or glutamic and aspartic acid, respectively, or the ion transport across cell membranes. Although there are a number of antiepileptic drugs, there is a significant group of patients (up to 30%) who are resistant to the available AEDs.[4] Therefore, there is an unmet need to develop AEDs with lower toxicity and higher selectivity. In order to make this development more rational, structural fragments that may enhance anticonvulsant activity were identified.

While searching for new substances with anticonvulsant activities, we directed our attention to derivatives of aminocyclohexanol. Many pharmacological activities of aminocyclohexanol have been described (such as secretolytic,[5] analgetic,[6] and antidepressant).[7] Our former research proved that aminoalkanol derivatives can also act as an anticonvulsant - they provide protection against seizures, mainly MES-induced seizures and at the same time exhibited low neurotoxicity.[8–11] We have previously reported some derivatives of 6-methoxy-2-methylxanthone with anticonvulsant activities: (R,S)- and (S)-1-amino- propan-2-ol as well as (R,S)-1-aminobutan-2-ol which showed values of ED_{50} in MES (mice, ip) of 37.4, 33.89, and 41.2 mg/kg, respectively.[11]

Considering our former findings we decided to continue research in the group of aminoalkanol derivatives. Herein we report the synthesis, *in vivo* anticonvulsant activities and the *in vitro* biotransformation of the *trans*-2-aminocyclohexan-1-ol derivatives – compound **1**, **2,** and **3** (two enantiomers and the racemate, respectively). Those compounds were found to have anticonvulsant activity against a MES test in

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studies carried out at the National Institute of Health (Rockville, USA). Rat hepatic microsomal fractions in the presence of an NADPH-generating system were used to evaluate the metabolism of titled compounds. The results obtained from liver fraction applications were compared to the results from the *in silico* study as well as to the microbiological biotransformation assay in which the *Cunninghamella* model was involved.

MATERIALS AND METHODS

Chemicals and Reagents

Chemical synthesis. Reagents: cyclohexene oxide, L-tartaric acid, 4-chlor-3-methylphenol, K_2CO_3 were purchased from Sigma Aldrich. Solvents were commercially available materials of reagent grade.

Melting points (mp) are uncorrected and were determined using a Büchi SMP-20 apparatus (Büchi Labortechnik, Flawil, Switzerland).

Elemental analyses were performed on a Elementar Vario EL III (Elementar Analysensysteme, Hanau, Germany).

The purity of the obtained compounds was confirmed by thin-layer chromatography (TLC), carried out on precoated aluminum sheets (Silica Gel, 60 F-254 Merck, Darmstadt, Germany) using the solvents indicated below. Spots were visualized by UV light. Specific rotation was measured on a Jasco P-2000 polarimeter (1% w/v solutions in chloroform, sodium light 589 nm).

^{1}H NMR spectra were obtained in $CDCl_3$ or DMSO-d_6 with a Varian Mercury-VX 300 NMR spectrometer (Varian Inc., Palo Alto, CA, USA), a Bruker Avance II spectrometer (Brucker, Karlsruhe, Germany) with 500.13 MHz, or a Brucker Avance III spectrometer (Brucker, Karlsruhe, Germany) with 600.2 MHz with TMS or DMSO as an internal standard, respectively.

The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were carried out using an Acquity UPLC BEH (bridged ethyl hybrid) C_{18} column; 2.1 × 100 mm, and 1.7 μm particle size. The column was maintained at 40 °C, and eluted under gradient conditions using from 95% to 0% of eluent A over 10 min, at a flow rate of 0.3 ml min^{-1}. Eluent A: water/formic acid (0.1%, v/v); eluent B: acetonitrile/formic acid (0.1%, v/v). 10 μL of each sample were injected.

Chromatograms were recorded using a Waters λ PDA detector. Spectra were analyzed in 200-700 nm range with 1.2 nm resolution and a sampling rate 20 points/s.

MS detection settings of the Waters TQD mass spectrometer were as follows: source temperature 150 °C, desolvation temperature 350 °C, desolvation gas flow rate 600 L h^{-1}, cone gas flow 100 L h^{-1}, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 m/z in 0.5 s time intervals; 8 scans were summed up to get the final spectrum.

Collision activated dissociation (CAD) analyses were carried out with energy of 20 eV, and all the fragmentations were observed in the source. Consequently, the ion spectra were obtained by scanning from 50 to 500 m/z range. The data acquisition software was MassLynx V 4.1 (Waters).

Biological biotransformation. *Cunninghamella blakesleeana* DSM 1906 (which was a gift from A.J. Carnell, University of Liverpool, UK), *was* propagated on a Potato-Dextrose-Agar (PDA) plate at 30 °C for 7 days. A fermentation basal medium (20 g D-glucose and 20 g corn step liquor in 1000 ml of water pH 5.0) was seeded with a 200 μl spore suspension gained by wetting colonies on solid medium PDA with sterile water. The media required for the growth of *Cunnighamella* strains were purchased from BioShop (Canada) (Potato Dextrose Agar) and from Sigma-Aldrich (St Louis, MO, USA) (CSL, Corn Steep Liquor).

Rat liver microsomal fractions as well as components of NADPH regenerating fraction (NADP$^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase) were obtained from Sigma Aldrich.

General Methods

***Procedure for the synthesis of* 2-(4-chlor-3-methylphenoxy)ethyl] amino}cyclohexan-1-ol.** In the aminolysis reaction, 2.5 g (0.01 mol) of 1-bromo- 2-(4'-chloro-3'-methylphenoxy)ethan, 1.2 g (0.01 mol) of 1RS,2RS-*trans*-2-aminocyclohexan-1-ol, 1.4 g (0.01 mol) of K_2CO_3, and 20 ml of DMF were used.

The mixture was heated for 5 h. The precipitated KBr was separated. The filtrate was concentrated and 15 ml of 10%HCL was added. To separate the free base –10%NaOH was added to the filtrated. The base was crystallized from n-hexane (mp. 94-96 °C) with a 48% yield (3). To obtain R and S enantiomers R or S *trans*-2-aminocyclohexan-1-ol were used respectively. The S-2-(4-chlor-3-methylphenoxy)ethyl]amino}cyclohexan-1-ol (compound **2**) (mp. 72-74 °C) was obtained with a 42.5% yield and R-2-(4-chlor-3-methylphenoxy)ethyl]amino}cyclohexan-1-ol (compound **1**) (mp. 72-74 °C) with a 52.5% yield. **1** and **2** were used as hydrochlorides and their melting points were 163-165 °C and 161-163 °C respectively.

1R,2R trans-{[2-(4-chlor-3-methylphenoxy)ethyl]amino}cyclohexan-1-ol (compound 1). Anal. Calcd for $C_{15}H_{22}ClNO_2$: C, 63,47; H, 7,83; N, 4,93. Found: C, 63,85; H, 7,83; N, 4,93. ^{1}H NMR (300 MHz, chloroform-*d*) δ ppm 1.04 - 1.20 (m, 2 H) 1.20 - 1.34 (m, 1 H) 1.59 - 1.79 (m, 3 H) 1.91 (d, *J* = 14.62 Hz, 2 H) 2.12 (d, *J* = 12.82 Hz, 1 H) 2.31 (s, 3 H) 2.87 - 2.97 (m, 1 H) 3.28 (br. s., 1 H) 3.46 (br. s., 1 H) 3.74 (tt, *J* = 10.13, 5.39 Hz, 1 H) 4.26 - 4.42 (m, 2 H) 5.00 (d, *J* = 6.41 Hz, 1 H) 6.74 (dd, *J* = 8.72, 3.08 Hz, 1 H) 6.85 (d, *J* = 2.82 Hz, 1 H) 7.19 (d, *J* = 8.72 Hz, 1 H). R_f =0.8 (toluene/methanol 1:1). $[\alpha]_{20}^{546}$ = +5.2 [C 0.5; $CHCl_3$]

1S,2S trans-{[2-(4-chlor-3-methylphenoxy)ethyl]amino}cyclohexan-1-ol (compound 2). Anal. Calcd for $C_{15}H_{22}ClNO_2$: C, 63,47; H, 7,83; N, 4,93. Found: C, 63,42; H, 7,80; N, 4,97. ^{1}H NMR (300 MHz, chloroform-*d*) δ ppm 1.05 - 1.34 (m, 3 H) 1.60 - 1.81 (m, 3 H) 1.86 - 1.96 (m, 1 H) 2.12 (d, *J* = 12.57 Hz, 1 H) 2.31 (s, 3 H) 2.86 - 2.98 (m, 1 H) 3.21 - 3.31 (m, 1 H) 3.49 (dt, *J* = 12.76, 5.03 Hz, 1 H) 3.74 (t, *J* = 10.13 Hz, 1 H) 4.25 - 4.42 (m, 2 H) 4.97 (br. s., 1 H) 6.74 (dd, *J* = 8.72, 3.08 Hz, 1 H) 6.85 (d, *J* = 2.82 Hz, 1 H) 7.19 (d, *J* = 8.72 Hz, 1 H). R_f =0.8 (toluene/methanol 1:1). $[\alpha]_{20}^{546}$ = - 5.2 [C 0.5; $CHCl_3$]

1RS,2RS trans-{[2-(4-chlor-3-methylphenoxy)ethyl]amino}cyclohexan-1-ol (compound 3). Anal. Calcd for $C_{15}H_{22}ClNO_2$: C, 63,47; H, 7,83; N, 4,93. Found: C, 63,38; H, 7,96; N, 4,93.^{1}H NMR (300 MHz, chloroform-*d*) δ ppm 0.92 - 1.08 (m, 1 H) 1.19 - 1.33 (m, 3 H) 1.68 - 1.78 (m, 2 H) 2.01 - 2.14 (m, 2 H) 2.20 - 2.30 (m, 1 H) 2.33 (s, 3 H) 2.83 (dt, *J* = 12.57, 4.87 Hz, 1 H) 3.19 (dt, *J* = 12.31, 5.90 Hz, 2 H) 4.01 (t, *J* = 5.26 Hz, 2 H) 6.67 (dd, *J* = 8.72, 2.82 Hz, 1 H) 6.78 (d, *J* = 2.82 Hz, 1 H) 7.21 (d, *J* = 8.72 Hz, 1 H). R_f =0.8 (toluene/methanol 1:1).

Pharmacology. Pharmacological tests were performed at the Epilepsy Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health (Rockville, USA).

All compounds were tested using MES, scMet and neurotoxicity assays, after intraperitoneal (ip) injection into male albino mice (Carworth Farms No. 1) as suspensions in 0.5% methylcellulose at doses of 30, 100, and 300 mg/kg. Observations were carried out after 0.5 h and 4 h after compound's administration.[12–14]

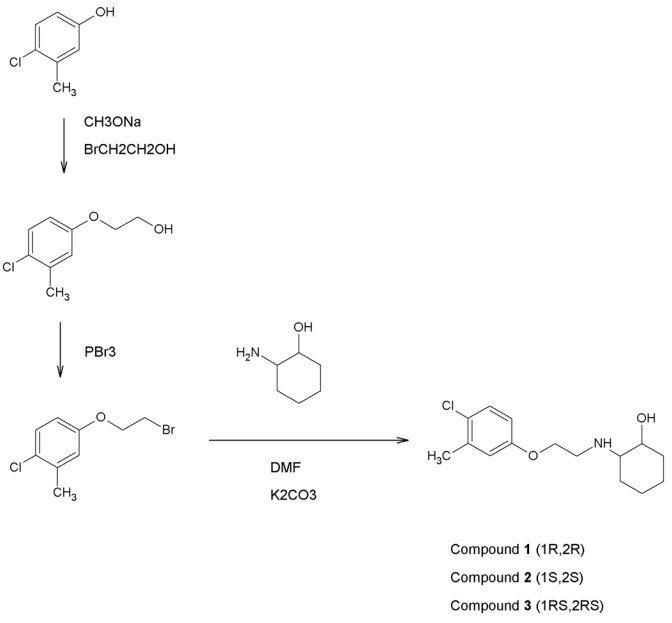
The maximal electroshock test (MES). Seizures were elicited by 60 Hz alternating current at 50 mA (mice) or 150 mA (rats) delivered for 0.2 s via corneal electrodes. A drop of 0.9% NaCl solution was placed into each eye prior to applying the electrodes. Protection in the MES test is defined as the abolition of the hindlimb tonic extension component of the seizure.[12–14]

The subcutaneous pentetrazole seizure test (scMet). The scMet was conducted by subcutaneous administration of pentetrazole dissolved in 0.9% NaCl solution at the dose of 85 mg/kg (mice) or 70 mg/kg (rats) into animals. A minimal time of 30 min subsequent to sc administration of pentetrazole was used for seizure detection. A failure to observe even a threshold seizure (a single episode of clonic spasm of at least 5 s in duration) was regarded as protection.[12–14]

Neurotoxicity (TOX). Neurological deficit was measured in mice by the rotorod test. The mouse was placed on a 1 in. diameter knurled plastic rod rotating at 6 rpm. Neurotoxicity was indicated by the inability of the animal to maintain equilibrium on the rod for at least 1 min in each of the three trials. In rats, the neurological deficit was indicated by ataxia and loss of placing response and muscle tone.[12–14]

Anticonvulsant quantification. Anticonvulsant quantification, that is, the dose of drug required to produce the biological responses in 50% of animals (ED_{50}), and the respective 95% confidence intervals, were determined for selected compounds displaying sufficient antiepileptic activity and low neurotoxicity in the primary evaluations. The details of these procedures have been published elsewhere.[12–14]

Microbiological transformation. Biotransformation was carried out in a 50 ml fermentation medium, which was inoculated with a 200 µl suspension of corresponding *Cunninghamella* species. Microorganisms were cultured in 30 °C for 48 h. Flasks were shaken for the whole time. After 2 days 250 µl of the stock solution, which was prepared by dissolving 100 mg of **1, 2,** or **3** in 1 ml H_2O, was added to the fermentation medium.



Scheme 1. The synthesis of compounds 1, 2 and 3.

The biotransformation was carried out for 7 days and its progress was followed using TLC and LC-MS/MS.

Rat liver microsomal transformation. Mixture reactions contained substrate (20 µM **1, 2,** or **3** in 100 mM potassium phosphate buffer, pH 7.4), microsomes (0.5 mg/ml rat liver microsomes), NADPH-regenerating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase in 100 mM potassium phosphate buffer, pH 7.4), and potassium phosphate buffer (100 mM, pH 7.4). Incubation mixtures containing microsomes, substrate, and buffer were pre-incubated at 37 °C for 15 min before the addition of the NADPH-regenerating system. The resulting mixture was incubated for 60 min at 37 °C in a Thermoblock (Eppendrof). The reaction was stopped by the addition of perchloric acid (69–72%, by volume). Proteins were sedimented by centrifugation.[15]

After the biotransformation (either microbiological or using liver fractions), samples were extracted with ethyl acetate. The water phase was separated from the organic phase. In the following experiment, only the organic phase was used, which was then dried over anhydrous sodium sulfate and concentrated in vacuo.

For the identification of compounds **1, 2,** and **3** and their possible biotransformation products TLC and UPLC-MS/MS were applied. TLC was used only for microbiological assay and was performed using Merck aluminum plates coated with silica gel with a thickness of 0.2 mm. It was concluded, as a result of many experimental studies, that the most preferred developing system is a mixture of toluene and methanol (1:1). Observation of the chromatograms was carried out under UV light and after exposure to iodine vapors.

***In silico* studies.** Computational simulation of metabolism of the title compound was carried out by using MetaSite software, demo version 2.1.0. (2005), Molecular Discovery Ltd. MetaSite is a computational procedure to predict metabolism issues related to cytochrome-mediated reactions in phase I metabolism. The methodology uses 3-D maps of interaction energies between the protein, chemical probes, and the 3-D structure of compounds to be analyzed. The MetaSite procedure is completely automated and does not require any user assistance. All the work can be handled and submitted in a batch queue. The basic concept of MetaSite is to compare the interaction patterns inside the protein with the 3-D structure of the ligand. The information obtained from a friendly user interface can be easily translated into decisions in the drug discovery process.[16]

RESULTS AND DISCUSSION

Chemistry. Title compounds **1**, **2,** and **3** were obtained by N-alkylation of *trans*-2-amino-1-cyclohexanol with 3-methyl-4-chlorphenoxyethyl bromide in the presence of DMF and K_2CO_3.

TABLE 1. The results of anticonvulsant activity evaluation in mice (ip)

		MES[a]		scMet[b]		TOX		
Compd.no.	Dose mg/kg	0.5 h	4 h	0.5 h	4 h	0.5 h	4 h	*ASP class
1	30	1/1	0/1	0/1	0/1	0/4	0/2	1
	100	3/3	0/3	0/1	0/1	1/8	0/4	
	300	–	–	–	–	4/4	–	
2	30	0/1	0/1	0/1	0/1	0/4	0/2	1
	100	3/3	1/3	0/1	0/1	3/8	0/4	
	300	–	–	–	–	4/4	–	
3	30	1/1	0/1	0/1	0/1	0/4	0/2	1
	100	3/3	1/3	0/1	0/1	2/8	0/4	
	300	–	–	–	–	4/4	–	

MES, maximal electroshock seizure.
[a]Number of animals protected/number of animals tested in the MES; ScMet, subcutaneous pentylenetetrazole seizure;
[b]Number of animals protected/number of animals tested in the scMet tests; TOX toxicity screen: the minimum dose of compound whereby toxicity was exhibited.
*The ASP classification (Anticonvulsant Screening Project) is as follows: 1, anticonvulsant activity at 100 mg/kg or less; 2,– anticonvulsant activity at doses grater than 100 mg/kg; 3, compound inactive at 300 mg/kg; 4, toxicity at doses 30 mg/kg.
The compound was not tested.

TABLE 2. The results of anticonvulsant quantification in *in vivo* study

Compd. no.	Dose mg/kg	TD_{50}[1]	$ED_{50(MES)}$[2]	$PI_{(MES)}$
1	30	66.03[a]	38.34[a]	1.722[a]
	100	66.03[a]$_{scMet}$	>90.00[a]$_{scMet}$	<1.722[a]$_{scMet}$
3	30	71.04[a]	25.05[a]	2.839[a]
	100	71.04[a]$_{scMet}$	>150[a]$_{scMet}$	<0.474[a]$_{scMet}$
	300	>250[b]	99.25[b]	2.519[b]
PHT		34.45[a]	6.48[a]	6.60[a]
		>500[b]	32.2[b]	>22[b]
CBZ		47.8[a]	9.85[a]	4.90[a]
		361[b]	3.57[b]	101[b]
VPA		483[a]	287[a]	1.70[a]
		859[b]	395[b]	2.2[b]

[1]Dose of the compound which produces toxicity in 50% of tested animals; [2]Dose of the compound which gives protection against seizures toxicity in 50% of tested animals.
[a]Values for mice after i.p. administration.
[b]Values for rats after p.o. administration.
not determined,
PHT: phenytoin, BZ: carbamazpine, VPA: valproate.

2-(4-chlor-3-methylphenoxy)ethanol was synthesized from 4-chlor-3-methylphenol by O-alkylation with 2-bromoethanol in the presence of methanolate sodium. 3-methyl-4-chlorophenoxyethyl bromide was synthesized by bromination of 2-(4-chlor-3-methylphenoxy)ethanol by tribromo phosphine (Scheme 1).

Racemic *trans*-2-amino-1-cyclohexanol was obtained from cyclohexene oxide in reaction with 25% ammonia. The racemic *trans*-2-amino-1-cyclohexanol was separated into its optical active S-(+) and R-(-) enantiomers, using diastereoisomeric pairs of salts (hydrogen (+)-tartrates). The process was possible due to the difference in solubility of the both isomers in ethanol and methanol.[17]

Anticonvulsant screening. The new compounds (**1**, **2**, **3**) were tested *in vivo* by using three screens: MES, ScMet (anticonvulsant test), and TOX (neurotoxicity) (Table 1). The most interesting anticonvulsant results were observed for **1** and **3**. For these compounds advanced quantitative tests (ED_{50} and TD_{50}) were performed. Compound **1** displayed anti-MES activity with a protective index (TD_{50}/ED_{50}) of 1.72 (mice, *i.p.*), and compound **3** – 2.83 (mice, *i.p.*) corresponding with PI for valpronate of 1.7 (mice, *i.p.*) (Table 2).[18]

Since compounds **1** and **2** are enantiomers of **3**, it could be expected that one of the enantiomers should be chosen for further development. The screening results in MES reveal that enantiomer **1** (1R,2R) is more active (in the dose of 30 mg/kg) and less neurotoxic (in the dose of 100 mg/kg) 0.5 h after administration, giving promises for the choice of **1** for development. However, further quantitative analysis of TD_{50}, ED_{50} and PI showed that **3** (racemate) has the most favorable ED_{50} and PI (Table 2). Moreover, the costs of providing the racemate is much lower than the costs of synthesizing the enantiomer.

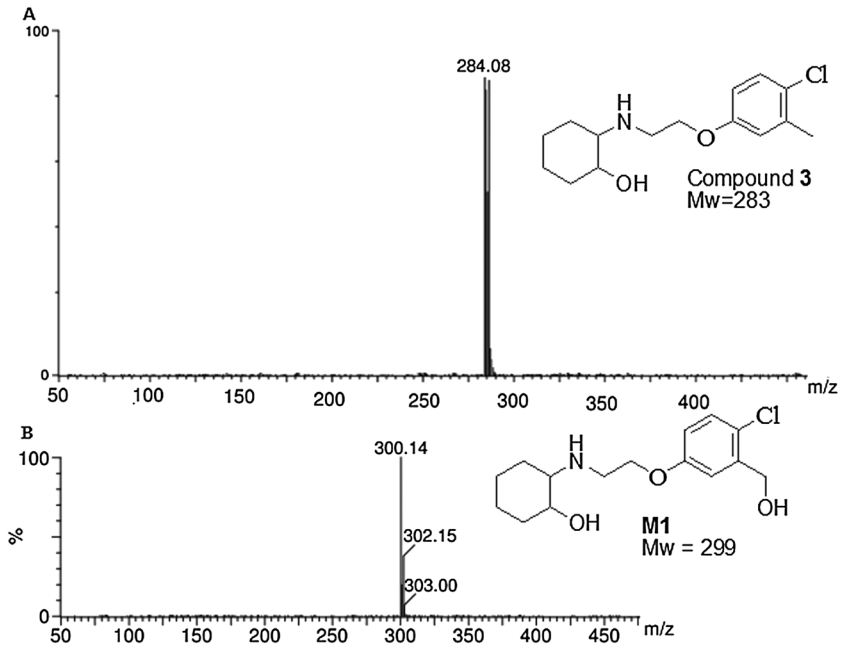


Fig. 1. A: The LC-MS/MS spectrum of 3. MS spectra from 3 (m/z 284) eluted at 4.38 minutes. B: The LC-MS/MS spectrumof mtabolite of 1, 2 and 3 (and its proposed structure) obtained after biotransformation carried out by *Cunninghamella blakesleeana* DSM 1906. MS spectra from metabolite (m/z 300) eluted at 3.17 minutes.

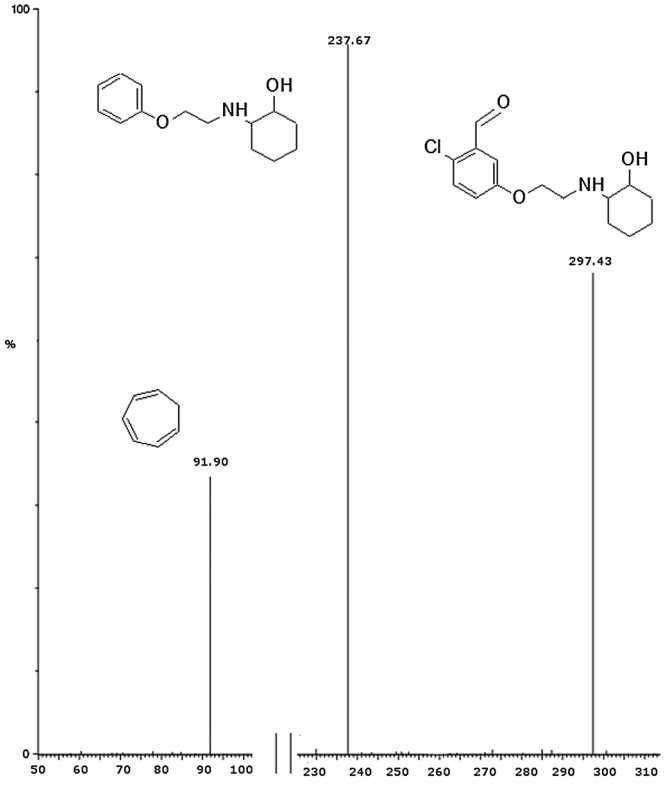


Fig. 2. MS/MS spectrum and fragmentation analysis for metabolite M1.

Microbiological transformation study. In our biotransformation study the filamentous fungus *Cunninghamella blakesleeana* DSM 1906 was used. During the study, evidence that the strain is able to carry out the biotransformation of all the tested compounds, was obtained. The samples from all cultures were taken in subsequent days of biotransformation and TLC was performed.

It was noticed that on the seventh day of biotransformation no new derivatives appeared. After completion of the reaction samples were taken to investigate the structure of the obtained derivatives of **1**, **2,** and **3**. UPLC-MS/MS spectra were carried out. In every mass spectrum of **1**, **2.** and **3** the protonated molecule $[M+H]^+$ at *m/z* 284 (Figure 1 A) was detected, which corresponds to the molecular weight (283) of the parent molecule. In addition, in every analyzed spectra of the biotransformation products the protonated ion $[M+H]^+$ at *m/z* 300 (**M1**) was noticed (Figure 1 B), which is in agreement with the molecular formula $C_{15}H_{22}ClNO_3$. MS/MS spectrum and fragmentation analysis for metabolite **M1** is shown in Figure 2. The proposed hydroxylation at the methylphenyl moiety is in agreement with the main metabolite of mexiletine – hydroxymethylmexiletine.[19] Comparison with mexiletine is due to the structural similarity of this drug and the tested compounds.

M1 was absent in the spectra from the samples obtained in "the zero day" of biotransformation. The ion at *m/z* 300 appeared predominantly in the spectra obtained after biotransformation of every tested compound (Figure 3).

Liver microsomes biotransformation study. The biotransformation of **1**, **2,** and **3** by rat liver microsomes was investigated. The presence of possible metabolites was investigated in all incubations using UPLC-MS/MS. The protonated ion $[M+H]^+$ at *m/z* 300 (**M1**) was present in every mass spectra of analyzed samples. However, incubations of two different enantiomers with the same concentrations of rat liver microsomes exhibited a distinct pattern of metabolism.

The presented studies indicate that the most of the tested compound disappeared from the reaction mixture during the biotransformation assay. When compound **1** was used (R enantiomer) the protonated ion $[M+H]^+$ at *m/z* 314 (retention time 2.8 min) appeared predominantly (**M2**). Ion *m/z* 314 is in agreement with the molecular formula $C_{15}H_{20}ClNO_4$ and structure of carboxylic acid. MS/MS spectrum and fragmentation analysis for metabolite **M2** is shown in Figure 4. The protonated ion $[M+H]^+$ at *m/z* 342 (**M4**) was also present.

When compound **2** (S enantiomer) was analyzed in rat liver microsomes model, two metabolites were detected. First, with the protonated ion $[M+H]^+$ at *m/z* 300 (**M1**, retention time 3.16 min), second - **M3** - with the protonated ion $[M+H]^+$ at *m/z* 320 (retention time 2.5 min). **M3** was produced in majority. There were no **M2** and **M4** metabolites.

In the case of the biotransformation of compound **3** (racemate), metabolites **M1**-**M4** were present. **M3** was the predominant metabolite. The parent molecule was also present.

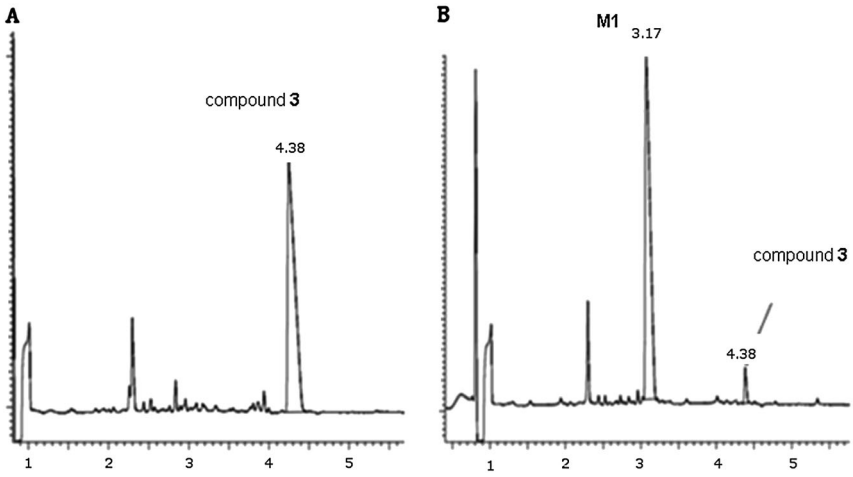


Fig. 3. Chromatograms obtained in the microbiological model. Analysis of *Cunninghamella* biotransformation samples incubated with 3 at time A) 0 and B) after 7 days of biotransformation, using a LC-MS/MS operating in scan mode. For 1 and 2 chromatogram profiles are similar.

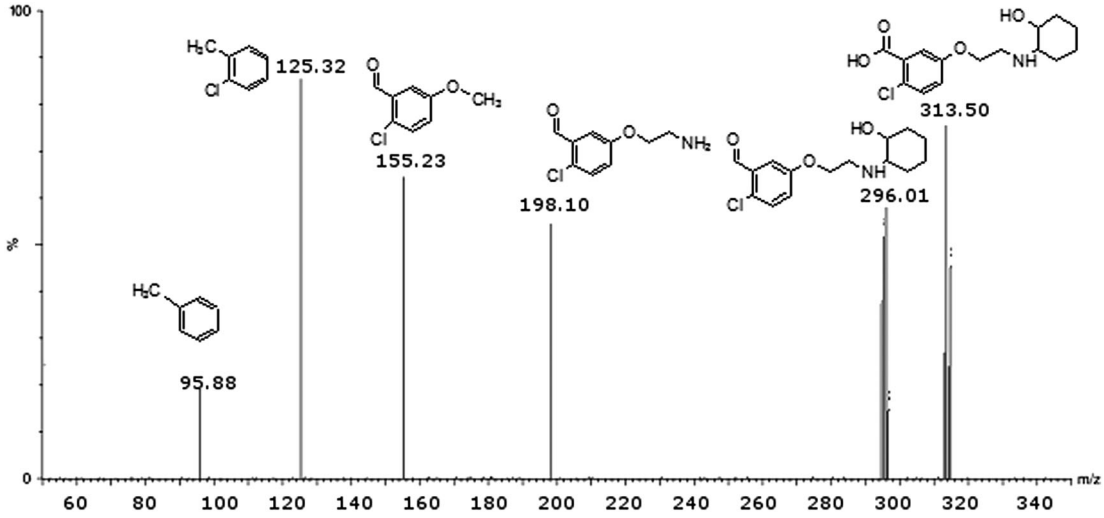


Fig. 4. MS/MS spectrum and fragmentation analysis for metabolite M2.

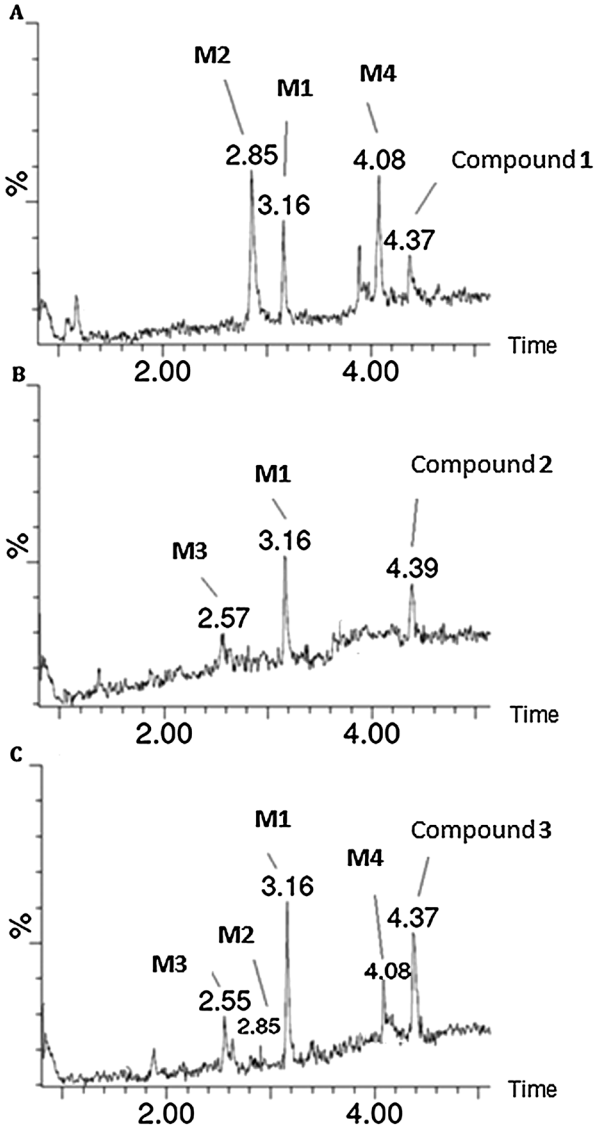


Fig. 5. Chromatograms obtained in the microsomal biotransformation model. Analysis of rat liver microsomes biotransformation samples incubated with A: 1, B: 2 and C: 3 after 60 min of biotransformation, using a LC-MS/MS operating in scan mode. Obtained metabolities (M1-M4) as well as parent mplecules (1, 2 and 3) are indicated.

In the rat microsomes model, the obtained metabolites were different depending on the used enantiomer (Figure 5). This difference was not noticeable when the *Cunninghamella* model was used.

***In silico* study.** In our *in silico* study, MetaSite software was used to predict **1**, **2,** and **3** metabolic transformation and in particular to help in the identification of the metabolite structure. MetaSite is able to predict human regioselective metabolism using only the 3-D structure of the given compound.

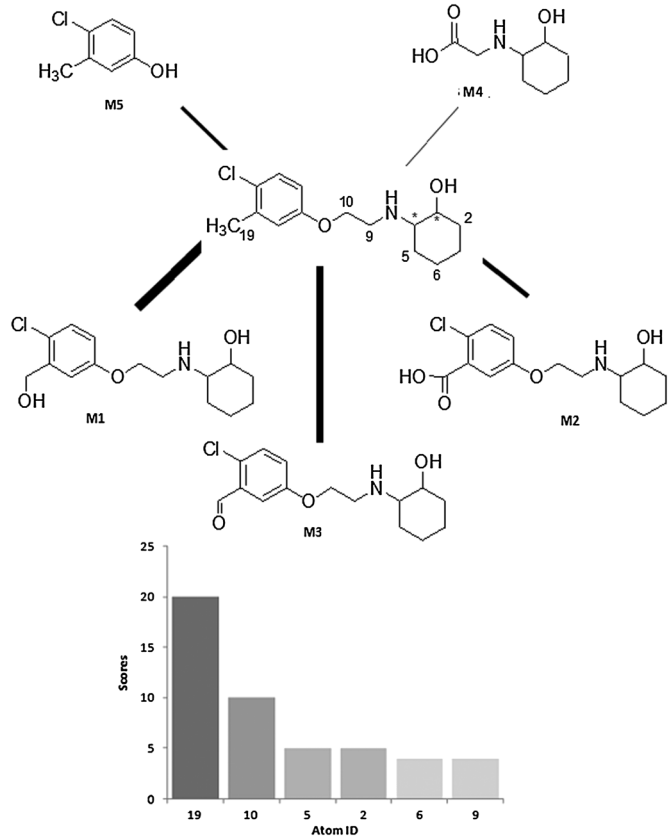


Fig. 6. Metabolic simulation of tested compounds performed by MetaSite.

The recognition of the site of metabolism could be a significant step in designing new compounds with a better pharmacokinetic profile. Labile compounds can be stabilized when the site of metabolism is known by adding stable groups at metabolically susceptible positions.

The most probable pathways of the CYP2D6 metabolism predicted by the MetaSite program are shown in the Figure 6. CYP2D6 was chosen because mexiletine, the structure of which is similar to the structure of tested compounds, is mainly metabolized by CYP2D6.[20]

There was no difference in metabolism between the enantiomers.

MetaSite also gave the most probable structures of the metabolites, which are shown in Figure 6. Among them, we were able to find **M1** and **M2** which were also identified using the *Cunninghamella* model (**M1**) and rat liver microsomes (**M1** and **M2**). Unlike the microsomes model – MetaSite predicted the same metabolic pathway for **1** and **2** (R – and S – enantiomer, respectively).

CONCLUSION

Synthesis, antiepileptic activity and biotransformation of **1**, **2,** and **3** were studied. The structure of the obtained products was confirmed using ^{1}H NMR spectra. It was shown in a MES test that **1** (R enantiomer) has more promising antiepileptic activity with less neurotoxicity than **2** (S enantiomer). The difference between these two stereoisomers was also studied using biotransformation assays with rat liver microsomes.

The analysis of UPLC-MS/MS spectra allowed us to propose the metabolites for **1**, **2,** and **3** based on hydroxylation reaction. Moreover, we were able to show in an *in vitro* microsomal model that different enantiomers (**1**, **2**) undergo different biotransformation process. R enantiomer (**1**) was metabolized mainly to **M2** (structure of carboxylic acid) while S enantiomer (**2**) to **M1** (structure of 1° alcohol). These could be the reason for the different antiepileptic activity of tested compounds in the MES test in studies carried out at NIH. Knowledge about the differences in the metabolism of optically active compounds is an important step in preclinical studies. These differences can affect the therapeutic effect of tested substances as well as its toxicity.

The differences in metabolism between **1** and **2** were not detectable in the *in silico* and microbial model. However, **M1** was present in every assay.

Our study showed that *trans*-2-aminocyclohexan-1-ol derivatives with chiral carbon atoms are promising structures with antiepileptic activity. Moreover, we proved that alternative biotransformation studies using liver microsomes are good and valuable methods for studing the metabolism of stereoisomers.

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