RESEARCH ARTICLE

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2018, 9, 1933

Synthesis and anticonvulsant activity of phenoxyacetyl derivatives of amines, including aminoalkanols and amino acids†

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A series of 17 new phenoxyacetamides has been prepared *via* multistep chemical synthesis as a continuation of the research carried out by our group on di- and tri-substituted phenoxyalkyl and phenoxyacetyl derivatives of amines. The obtained compounds vary in an amide component, for example aminoalkanol or (un)modified amino acid moieties were introduced. The structures of selected products were confirmed by means of crystallographic methods. All 17 compounds were the subject of preliminary screening for potential anticonvulsant activity (MES, 6 Hz and/or scMET tests) and neurotoxicity (rotarod) in mice after intraperitoneal administration, while several active compounds were subsequently examined in additional models (e.g. MES and rotarod – rats, *p.o.* or *i.p.*, hippocampal kindling – rats, *i.p.*). Finally, safety studies (cytotoxicity and cell proliferation assays on astrocytes, metabolic stability assessment, mutagenicity evaluation) were performed for several active compounds, including the most promising one (*R*-(–)-2-(2,6-dimethylphenoxy)-*N*-(1-hydroxypropan-2-yl)acetamide, MES ED₅₀ = 12.00 mg per kg b.w., rats, *p.o.*).

Received 29th August 2018,
Accepted 20th September 2018

DOI: 10.1039/c8md00430g

rsc.li/medchemcomm

1. Introduction

Epilepsy is a neurological condition which affects approximately 65–70 million people worldwide.¹ Despite the availability of a huge number of anticonvulsants on the market, characterized by various mechanisms of action, still about 40% of patients do not respond to treatment.² Such pharmacoresistance of epilepsy, as well as frequently observed adverse effects,³ is a great premise for searching for new anticonvulsant compounds.

Within our previous research we investigated the anticonvulsant activity of many phenoxyalkyl and phenoxyethoxyethyl derivatives of amino alcohols. Among them, there were many

active phenoxyethyl derivatives di- and tri-substituted with methyl groups in the phenyl ring and general conclusions derived from our studies indicate that this type of structural modification might be particularly favorable.^{4–7} Additionally, SAR analysis showed that incorporation of 2-amino-propan-1-ol as an amine moiety might be advantageous (e.g. reference compound I, active in animal models of convulsions, showing high affinity towards the receptors sigma, 5-HT_{1A}, alpha-2 and 5-HT transporter, Fig. 1B).⁶ We also investigated some phenoxyacetyl derivatives of aminoalkanols, as an amide moiety is a scaffold known to be present in many anticonvulsants currently available on the market as well as compounds under research (Fig. 1A). In the case of compounds disubstituted in the phenyl ring with chlorine and a methyl group (in positions 2,5 and 4,2 or 4,3, respectively) the amides lacked the desired activity.^{8,9} However, other types of substitution seem to be promising, e.g. in the case of the 2,6-dimethylphenoxyethyl derivative of 2-amino-1-cyclohexanol, incorporation of an amide group resulted in an active compound (although slightly less potent than the amine analog) (Fig. 1C, reference compound II).⁵

In the currently presented research, we decided to synthesize phenoxyacetyl derivatives which are di- or tri-substituted in the phenyl ring with methyl groups and investigate their anticonvulsant properties. The amine component of the designed compounds varies in structure. Apart from amines

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† Electronic supplementary information (ESI) available. CCDC 1832675–1832677. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c8md00430g

Fig. 1 Chemical structures of anticonvulsants containing an amide moiety (A) and reference compounds I (B)⁶ and II (C).⁵

with/without an additional hydroxyl substituent (particularly derivatives of 2-aminopropan-1-ol) that were already within our interests, we decided to include an (un)modified amino acid group in the structure of several compounds. Such modification resulted in compounds that may be the metabolic products of aminoalkanol derivatives. Moreover, there are examples of the anticonvulsant activity of amino acid derivatives in the literature as well as drugs available on the market (*e.g.* gabapentin, pregabalin, vigabatrin) (Fig. 2).^{10–13}

2. Results and discussion

2.1. Chemistry

17 phenoxyacetamide derivatives of phenethylamine, aminoalkanol or amino acids (Table 1) were designed and obtained by means of chemical synthesis. Compounds 5–7, 10–12 and 16 were subject to patent protection¹⁴ and compounds 1, 2, 4, 9, 13 and 15 possess CAS numbers (however, their physicochemical properties are unknown). The integral

Fig. 2 Chemical structures of anticonvulsants containing an amino acid scaffold.

part of the design of structures 1–17 was calculation of several physicochemical parameters by means of the Molinspiration online toolkit,¹⁵ including molecular weight (MW), log *P*, number of hydrogen bond acceptors (nON) and donors (nOHNH), number of rotatable bonds (nrotb) as well as topological polar surface area (TPSA) (particular values are presented in the ESI†). None of the compounds violated more than one component of the Lipinski “Rule of 5” (MW ≤ 500, log *P* ≤ 5, H-bond donors ≤ 5, H-bond acceptors ≤ 10, nrotb ≤ 10), which is favorable for potential medicines.¹⁶ The calculated values are also beneficial in terms of blood–brain penetration (according to van de Waterbeemd *et al.*, the favorable parameters are MW < 450, TPSA < 90 Å²).¹⁷

All the title compounds were obtained by multistep chemical synthesis (Scheme 1). Firstly, 2,3-dimethyl-, 2,6-dimethyl- and 2,4,6-trimethylacetic chlorides (**Ia–Ic**) were obtained according to a previously published procedure.^{8,18} In the next step, the chlorides were used as acylating agents in the reaction with appropriate amines in order to obtain the final compounds 1, 3–7, 12, 13, 15, 16 (reaction carried out in a biphasic environment) and 2, 14 (reaction carried out in toluene). For compounds 8–11 and 17 the acetic chlorides were treated with amino acid methyl ester hydrochlorides in a biphasic environment to obtain compounds **IIa–IIc**. The ester groups of the obtained products were subsequently hydrolysed (compounds 8 and 9) or were subjected to aminolysis (compounds 10, 11 and 17).

Table 1 Structures and physicochemical properties of compounds 1–17

Compd	R	Z
1	2,3-(CH ₃) ₂	<i>trans</i>
2	2,6-(CH ₃) ₂	<i>R</i> -(-)
3	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
4	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
5	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
6	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
7	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
8	2,6-(CH ₃) ₂	<i>S</i> -(-)
9	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
10	2,6-(CH ₃) ₂	<i>trans</i>
11	2,6-(CH ₃) ₂	<i>R</i> -(-)

Table 1 (continued)

Compd	R	Z
12	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
13	2,6-(CH ₃) ₂	<i>S</i> -(+)
14	2,6-(CH ₃) ₂	<i>R</i> , <i>S</i>
15	2,4,6-(CH ₃) ₃	<i>R</i> , <i>S</i>
16	2,4,6-(CH ₃) ₃	<i>R</i> , <i>S</i>
17	2,4,6-(CH ₃) ₃	<i>S</i> -(-)

The final compounds were purified by crystallization from organic solvents (hexane, heptane, and toluene). Their identity and purity were confirmed by means of chromatographic and spectral analysis (*e.g.* LCMS, HPLC, IR, ¹H NMR, ¹³C NMR). For chiral compounds the optical rotation was measured. In the case of compounds 9 and 13, the plane of rotation of polarized light was rotated in the opposite direction compared to the appropriate amino alcohols used for their synthesis.

2.2. Crystallography

The crystal structures of enantiomers 6 and 7 and racemic compound 12 were studied in order to confirm their structures and gain information for future design of their potentially active derivatives. An overview of the asymmetric unit of 6, 7 and 12 with the atom numbering is presented in Fig. 3A, B and C, respectively.

The crystal structures of 6 and 7 confirm *R* and *S* configuration at C2, respectively. Both enantiomers crystallize in the *P*₂₁₂₁₂₁ space group with one molecule in the

Scheme 1 Synthesis of compounds 1–17.

Fig. 3 The molecular structures showing the atom numbering scheme: (A) compound **6**; (B) compound **7**; (C) compound **12**. For enantiomer *S* the bonds at C1b are in green. Displacement ellipsoids are drawn at the 30% probability level.

asymmetric unit. The values of bond distances and angles are almost the same in both enantiomers. Also in both enantiomers the disorder of methyl substituents at the benzene ring as well as the carbon atoms C8 and C10 of the benzene ring is observed.

The crystal structure of **12** confirms its racemic mixture. This compound crystallizes in the *Pna*2₁ space group with one molecule in the asymmetric unit, and for atoms C1, H1, O1, and H1O two positions (A for configuration *R* and B for configuration *S*) with an occupancy factor of 0.5 are observed.

The conformations of molecules are very similar in all the studied compounds. Two weak intramolecular hydrogen bonds, N–H···O, in which oxygen atoms O1 and O3 are engaged, seem to have the main influence on the geometry of the compounds. These hydrogen bonds are observed in all the presented structures (Table 2). The same type of interactions is present in the similar crystal structures reported earlier.^{6,19} The investigated molecules differ only in the substituents at N1, 1-hydroxyisopropyl in the enantiomers (**6** and **7**) and 2-hydroxybutyl in **12**. Due to a longer hydrocarbon chain in **12**, weak interactions of oxygen atom O1 with C–H of the aromatic ring of the other molecules in the crystal lattice take place. These interactions appear to provide some stabilization of the benzene ring with methyl groups in this crystal structure and no disorder is observed. Similar stabilization effects are not observed in the crystal structures of **6** and **7**, and therefore disorder can be observed. The packing of the molecules in the unit cell can be characterized by the intermolecular interaction listed in Table 2. The arrangement of all molecules is dominated by O–H···O hydrogen bonds. The crystal structures are also stabilized by weak C–H···O interactions. The nitrogen atom is not involved in any intermolecular interactions.

2.3. Pharmacology

Pharmacological tests for the title compounds were carried out within the Epilepsy Therapy Screening Program (ETSP),

Table 2 Intra- and intermolecular interactions of **6**, **7** and **12**

Compd	D-H...A	H...A (Å)	D...A (Å)	D-H-A (°)	Symmetry codes
6	N1-H1N...O1	2.25	2.691	113	
	N1-H1N...O3	2.20	2.596	109	
	O1-H1O...O2	1.83	2.697	163	$x - 1, y, z$
	C9-H9...O2	2.45	3.372	165	$-x + 1, y + 0.5, -z + 1.5$
7	N1-H1N...O1	2.24	2.688	110	
	N1-H1N...O3	2.16	2.591	108	
	O1-H1O...O2	1.89	2.688	166	$x + 1, y, z$
	C9-H9...O2	2.46	3.369	166	$-x + 1, y - 0.5, -z + 0.5$
12	N1-H1N...O1A	2.47	2.782	101	
	N1-H1N...O1B	2.42	2.682	97	
	N1-H1N...O3	2.14	2.614	112	
	O1A-H1OA...O2	1.89	2.735	180	$x, y, z - 1$
	O1B-H1OB...O2	1.92	2.769	180	$x, y, z - 1$
	C9-H9...O1B	2.44	3.228	142	$-x + 0.5, y - 0.5, z + 0.5$
	C11-H11...O1A	2.70	3.563	155	$-x + 0.5, y + 0.5, z + 0.5$

Epilepsy Branch, National Institute of Neurological and Communicative Disorders and Stroke, NIH, Rockville, MD, USA.²⁰ All achieved compounds were the subject of preliminary evaluation of their anticonvulsant activity and neurological toxicity (rotarod) in mice after *i.p.* administration, including MES and scMET (compounds 2–13 and 15–17, Table 3) or 6 Hz (44 mA (ref. 21)) and MES (compounds 1, 14, Table 4) as models of convulsions. Among all tested compounds, **1**, **4–6**, **11** and **12** showed activity in preliminary screening in either the MES or the 6 Hz test and some of them were subjected to further pharmacological studies (Tables 5–7). None of the tested compounds was active in scMET, which evaluates the ability of protecting from clonic, forebrain seizure²² and the positive results may correlate with potential utility against absence seizures.²³

Previously, we obtained and screened for anticonvulsant activity amine analogs of compounds **3–7**, **12**, **13** and **16**.^{4–7} Their amide derivatives presented in this paper showed weaker activity in all cases. However, some of them still possessed notable pharmacological profiles (**4**, **5**, **6**, **12**) and they were also generally less neurotoxic in rotarod compared to their amine analogs. For instance, the most promising compound among the achieved amides, compound **6**, possessed a lower value of protective index in the MES test performed on mice after *i.p.* administration than its amine analog (1.43 and 5.52, respectively) but higher in the rat model after *p.o.* administration (>41.64 and >17.48, respectively). The outcomes indicate that although the amine moiety seems to be preferential in terms of anticonvulsant activity, in the case of obtaining a potent amine showing high neurotoxicity in animal tests, it might be reasonable to explore the pharmacological activity of its amide derivatives.

Most of the active compounds possessed aminoalkanol as an amide moiety. The exception was compound **11** containing an *N*-methylamide derivative of alanine. These results

prove an important role of the aminoalkanol substituent for anticonvulsant activity. They also encourage further research on amino acid derivatives, with the suggestion that the carboxyl group should be significantly modified.

Among active phenoxyacetyl derivatives of aminoalkanols, aminobutanol (**12** – *R,S*-1-aminobutan-2-ol derivative) and aminopropanol (**4–7**) as an amine moiety seem to be beneficial for activity in MES. Large substituents containing an aryl ring (compounds **2**, **3**, **14**) seem unprofitable. Among the derivatives of aminopropanol, both compounds containing *R,S*-1-aminopropan-2-ol (**4**) and *R,S*-2-aminopropan-1-ol (**5**) moieties exhibited comparable activity in the MES test; however, compound **5** was more toxic, especially after 4 h. Studies on its enantiomers resulted in compound **6** (derivative of *R*-2-aminopropan-1-ol), exhibiting the best pharmacological profile not only among compounds **4–7**, but also among all tested compounds. Our previous studies on phenoxyalkylamines showed that in the case of aminoalkanols it is difficult to predict which enantiomer is the more active one. Thus, it is reasonable to investigate pharmacological properties of racemic mixture first and then, in case of observed anticonvulsant activity, synthesize the enantiomers.

Compound **1** was more active in MES at a higher dose (300 mg kg^{−1}) than in the 6 Hz test (100 mg kg^{−1}). The compound differs from compounds **4–7** both with the phenyl ring substitution (2,3 instead of 2,6) and the presence of a cyclic aminoalkanol moiety (*trans*-4-aminocyclohexanol). Both modifications might be responsible for the change in the mechanism of action. Moreover, compounds **6** and **8** were additionally evaluated in the 6 Hz test (mice, *i.p.*) and compound **6** exhibited moderate (2/4) activity after 15 min at a dose of 120 mg per kg b.w. (see the ESI†). It proves that phenoxyacetyl derivatives of aminoalkanols may be active in both the MES and the 6 Hz tests and suggests including the 6 Hz test in future studies as a part of preliminary screening.

The active compounds **4–6**, **11** and **12** were subjected to further pharmacological studies on rats (MES, neurotoxicity, Table 5). Compound **6** remained the most promising and for that reason it was chosen for quantitative tests (ED₅₀ = 12.00 mg kg^{−1}, PI >41.640, rats, *p.o.*) as well as additional screening for anticonvulsant activity in hippocampal kindling in rats. Results of the preliminary screening (presented in the ESI†) encouraged further studies (Table 6). The compound exhibited activity at a dose of 100 mg kg^{−1}; however, neurotoxicity was also observed with this dose.

Previously performed receptor studies for an amine analog of compound **6** (reference compound **I**, Fig. 1 (ref. 6)) suggested that modulation of the function of the sigma receptor, serotonergic receptors 5-HT_{1A} and 5-HT transporter, and alpha-adrenergic receptors might be the possible mechanism of anticonvulsant activity observed within phenoxyalkyl derivatives of amino alcohols. There is a possibility that this is true also for their amide analogs, as available pharmacophore models established for binding to the receptors of interest do not exclude the presence of an amide moiety.^{26–28}

Table 3 Anticonvulsant properties of compounds 1–17 in MES (50 mA) and scMET as well as their neurotoxicity in the rotarod test (mice, *i.p.*)

Compd	Dose [mg kg ⁻¹]	MES ^a				scMET ^b				TOX ^b			
		Time [h]											
		0.25	0.5	1.0	4.0	0.25	0.5	1.0	4.0	0.25	0.5	1.0	4.0
2	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	/	0/3	/	0/3	/	0/1	/	0/1	/	0/8	/	0/4
	300	/	0/1	/	0/1	/	0/1	/	0/1	/	1/4	/	0/2
3	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	/	0/3	/	0/3	/	0/1	/	0/1	/	1/8	/	0/4
	300	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
4	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	2/3	0/3	0/3	0/3	/	0/1	/	0/1	1/3	0/8	0/3	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	4/4	/	0/2
5	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	2/3	0/3	0/3	0/3	/	0/1	/	0/1	1/3	2/8	0/3	2/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	4/4	/	0/2
6	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	2/3	0/3	0/3	0/3	/	0/1	/	0/1	0/3	0/8	0/3	0/4
	110	4/4	0/4	0/4	0/4	/	/	/	/	/	/	/	/
	220	/	/	/	/	/	/	/	/	8/8	1/8	0/8	0/8
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	2/4	/	0/2
TPE [h] = 0.25													
MES ED ₅₀ = 97.93 (78.524–112.599) mg kg ⁻¹													
TD ₅₀ = 139.96 (128.762–156.811) mg kg ⁻¹													
PI = 1.429													
7	30	/	0/1	/	0/1	/	0/1	/	0/1	/	1/4	/	0/4
	100	1/3	0/3	1/3	0/3	/	0/1	/	0/1	1/3	3/8	1/3	1/4
	300	/	1/1	/	1/1	/	0/1	/	0/1	/	1/4	/	1/2
8	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	/	0/3	/	0/3	/	0/1	/	0/1	/	0/8	/	0/4
	300	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
9	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	/	0/3	/	0/3	/	0/1	/	0/1	/	0/8	/	0/4
	300	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
10	30	/	0/1	/	0/1	/	0/1	/	1/5	/	0/4	/	0/2
	100	0/3	0/3	0/3	0/3	/	0/1	/	0/1	0/3	2/8	1/3	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	1/4	/	0/2
11	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	/	2/3	/	0/3	/	0/1	/	0/1	/	1/8	/	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	4/4	/	0/2
12	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	3/3	0/3	0/3	0/3	/	0/1	/	0/1	1/3	0/8	0/3	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	4/4	/	0/2
13	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	1/3	0/3	0/3	0/3	/	0/1	/	0/1	1/3	0/8	0/3	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	4/4	/	0/2
15	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	0/3	0/3	0/3	0/3	/	0/1	/	0/1	0/3	1/8	0/3	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	2/4	/	0/2
16	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	/	0/3	/	0/3	/	0/1	/	0/1	/	0/8	/	0/4
	300	/	0/1	/	0/1	/	0/1	/	0/1	/	4/4	/	0/2
17	30	/	0/1	/	0/1	/	0/1	/	0/1	/	0/4	/	0/2
	100	0/3	0/3	0/3	0/3	/	0/1	/	0/1	0/3	0/8	0/3	0/4
	300	/	1/1	/	0/1	/	0/1	/	0/1	/	1/4	/	0/2
CBZ ²⁴	—	ED ₅₀ = 7.81 (6.32–8.45) mg kg ⁻¹				—				TD ₅₀ = 45.4 (32.9–54.4) mg kg ⁻¹			
Allopregnanolone ²⁵	—	—				TPE = 0.17 [h] ED ₅₀ = 13.7 (10.1–18.7) mg kg ⁻¹				—			

^a Number of animals protected/number of animals tested. ^b Number of animals exhibiting toxicity/number of animals tested in the rotarod test. / not tested; — no data.

2.4. *In vitro* cytotoxicity studies

Compound 6, possessing the most favorable pharmacological profile, was also subjected to further toxicity studies, includ-

ing *in vitro* cytotoxicity tests on astrocytes. The astrocyte model was chosen due to the previously observed influence of known anticonvulsants (used in higher concentrations) on cells originating from the nervous system.²⁹ Compound 6

Table 4 Anticonvulsant properties of compounds **1** and **14** in 6 Hz (44 mA) and MES (50 mA) as well as their neurotoxicity in the rotarod test (mice, *i.p.*)

Compd	Dose [mg kg ⁻¹]	6 ^a Hz		MES ^a		TOX ^b	
		Time [h]					
		0.5	2.0	0.5	2.0	0.5	2.0
1	30	0/4	0/4	0/4	0/4	0/8	0/8
	100	2/4	0/4	0/4	0/4	0/8	0/8
	300	3/4	4/4	4/4	1/4	1/8	0/8
14	30	0/4	0/4	0/4	0/4	0/8	0/8
	100	0/4	0/4	1/4	0/4	0/8	0/8
	300	0/4	1/4	0/4	1/4	0/8	0/8
CBZ ²⁴	—	Max. 75% protection at 40 and 80 mg kg ⁻¹		ED ₅₀ = 7.81 (6.32–8.45) mg kg ⁻¹		TD ₅₀ = 45.4 (32.9–54.4) mg kg ⁻¹	

^a Number of animals protected/number of animals tested. ^b Number of animals exhibiting toxicity/number of animals tested in the rotarod test. — no data.

showed no cytotoxicity in concentrations ranging from 10 to 200 μ M in the MTT assay (both mice and human astrocytes) and neutral red test (mice astrocytes). Moreover, the compound did not significantly affect mice astrocyte proliferation (CV assay). Taking the above into account, it can be claimed that it is completely safe for cells that are derived from the nervous system. For a graphical interpretation of the presented results, see the ESI.†

2.5. Metabolic stability

According to the results presented in Table 7, compound **6** can be classified as metabolically stable, as its rat intrinsic clearance is lower than 10 μ l mg⁻¹ min⁻¹, while compound **12** exhibited moderate *in vitro* metabolic stability. Both

Table 6 Activity in hippocampal kindling for compound **6** and its neurotoxicity in rats, *i.p.*

Compd	Dose [mg kg ⁻¹]	Time [h]	Seizure score $\pm$ SEM ^a	Duration [s] $\pm$ SEM	Test results (after 0.25 h)
6	50	0	4.4 $\pm$ 0.2	72 $\pm$ 5	0/7 ^{c,d}
		15	4.9 $\pm$ 0.1	71 $\pm$ 5	
		45	4.9 $\pm$ 0.1	73 $\pm$ 4	
		75	5.0 $\pm$ 0.0 ^b	75 $\pm$ 6	
		105	4.9 $\pm$ 0.1	77 $\pm$ 7	
		135	4.9 $\pm$ 0.1	72 $\pm$ 7	
	100	0	5.0 $\pm$ 0.0	68 $\pm$ 5	2/7 ^c
		15	3.6 $\pm$ 7 ^b	48 $\pm$ 10	
		45	4.7 $\pm$ 0.2	86 $\pm$ 8	
		75	4.9 $\pm$ 0.1	80 $\pm$ 6	
		105	4.7 $\pm$ 0.2	77 $\pm$ 6	
		135	4.9 $\pm$ 0.1	76 $\pm$ 6	

^a Racine's scale. ^b Significantly different from control. ^c No. animals protected/tested. ^d Number of animals exhibiting toxicity/number of animals tested.

compounds demonstrated values of Cl_{int} lower than those reported for imipramine³⁰ or propranolol.³¹ It is worth noting that compound **12**, which was the more lipophilic one (log *P* = 2.33), possessed a higher Cl_{int} value than that of the more hydrophilic compound **6**.³²

In both compounds (**6** and **12**) the predominant transformation is hydroxylation of the parent compound (Table 8). Both metabolites have a protonated molecular ion [M + H]⁺ with a molecular weight 16 Da higher than those of the parent compounds.

2.6. Mutagenicity

2.6.1. Assessment of mutagenic properties of test compounds. As a part of our broader studies on mutagenicity in

Table 5 Anticonvulsant activity in MES (150 mA) of compounds **4–6**, **11** and **12** and their neurotoxicity evaluation performed on rats

Compd	Rats, <i>p.o.</i> (dose = 30 mg kg ⁻¹)						Rats, <i>i.p.</i>						
	Test	Time [h]					Test	Dose [mg kg ⁻¹]	Time [h]				
		0.25	0.5	1.0	2.0	4.0			0.25	0.5	1.0	2.0	4.0
4	MES	0/4	0/4	0/4	0/4	1/4	—						
	TOX	0/4	0/4	0/4	0/4	0/4							
5	MES	2/4	2/4	0/4	0/4	2/4	—						
	TOX	0/4	0/4	0/4	0/4	0/4							
6	MES	2/4	4/4	1/4	0/4	1/4	TOX	30	1/2	0/2	0/2	0/2	0/2
	TOX	0/4	0/4	0/4	0/4	0/4		100	1/2	0/2	0/2	0/2	0/2
	Quantitative assay							300	2/2	2/2	2/2	2/2	2/2
	MES ED ₅₀ = 12.00 (7.098–20.671)												
	TD ₅₀ > 500.00												
	PI > 41.640												
	TPE [h] = 0.50												
11	MES	1/4	1/4	0/4	0/4	0/4	—						
	TOX	0/4	0/4	0/4	0/4	0/4							
12	MES	3/4	4/4	1/4	0/4	0/4	TOX	100	4/4	4/4	0/4	/	/
	TOX	0/4	0/4	0/4	0/4	0/4							
CBZ ²⁴	MES	ED ₅₀ = 5.35 (3.26–7.62) mg kg ⁻¹					—						
	TOX	TD ₅₀ = 364 (223–500) mg kg ⁻¹											

Results presented as: number of animals protected/number of animals tested (MES) or number of animals exhibiting toxicity/number of animals tested (TOX). / not tested; — no data.

Table 7 Stability ($t_{1/2}$ and Cl_{int}) of **6** and **12** in the rat liver microsomal system

Compd	Protein concentration (mg ml ⁻¹)	clog <i>P</i>	$t_{1/2}$ (min)	Cl_{int} (μl mg ⁻¹ min ⁻¹)
6	0.4	1.80	182.4	9.5
12	0.4	2.33	30.4	57
Propranolol	0.5 (ref. 31)	2.75	—	79 (ref. 31)
Imipramine	0.5 (ref. 30)	5.04	4.6 (ref. 30)	302.0 (ref. 30)

Table 8 Summary of **6** and **12** metabolites generated in rat liver microsomes after 60 min

Compd	Metabolite	Mass shift (Da)	<i>m/z</i>	Retention time (min)
6	Parent	0	238	4.73
	M1	+16	254	3.38
12	Parent	0	252	5.15
	M1	+16	268	3.73

a group of phenoxyalkyl and phenoxyacetyl derivatives of amines,^{6,33} compounds **3**, **6**, **12** and **13** were examined in the Ames test. They did not produce any mutagenic effect in the employed *S. typhimurium* TA100 and TA1535 strains. At the concentrations of 500, 250, 100 and 50 μg per plate of test compounds, the mutagenicity index (MI) ranged from 0.7 to 1.7, suggesting a lack of mutagenic activity of the studied compounds both with and without metabolic activation. The mean number of revertants ± standard deviation and mutagenicity indices for all tested concentrations are available in the ESI.†

2.6.2. Antimutagenicity evaluation of test compounds. In the antimutagenicity assay performed with *S. typhimurium* TA100 and TA1535 strains, compound **3** exhibited the highest antimutagenic effect against sodium azide mutagenicity at various test concentrations (Table 9). This compound demonstrated strong inhibition rates of NaN₃ mutagenicity (41–58%) for three test concentrations in strain TA100 and two concentrations in strain TA1535. Compound **6** exhibited high or medium antimutagenic activities (inhibition rates from 29% to 57%) on NaN₃-induced mutagenesis in all tested concentrations in both *Salmonella* strains. Compound **13** reduced strongly or moderately sodium azide mutagenicity only in the TA100 strain; in TA1535, low antimutagenic effect was observed.

3. Conclusions

Within the presented research we designed and synthesized 17 substituted phenoxyacetamides, including mainly amide analogs of the previously achieved centrally active amines^{4–7} and several derivatives of (un)modified amino acids. The anticonvulsant activity of the obtained compounds was evaluated within the Epilepsy Therapy Screening Program (ETSP)²⁰ with use of the following animal models: MES, 6 Hz, scMET, hippocampal kindling, neurotoxicity (rotarod) performed on mice and/or rats. The results indicated compound **6** as the one possessing the most favorable pharmacological profile and allowed us to draw several conclusions.

In general, amide derivatives di- or trisubstituted with methyl groups in the phenyl ring seem to be less promising compared to their amine analogs. However, due to their

Table 9 Antimutagenicity results of compounds **3**, **6**, **12** and **13** in *Salmonella typhimurium* assay

Compd	Concentration (μg per plate)	Number of revertants			
		<i>S. typhimurium</i>			
		TA 100		TA 1535	
		Mean ± S.D.	Inhibition (%)	Mean ± S.D.	Inhibition (%)
Negative control	—	102 ± 8	—	10 ± 4	—
Positive control	5	533 ± 36	—	397 ± 25	—
3	500	355 ± 11	41 ^c	345 ± 26	13 ^a
	250	313 ± 17	51 ^c	261 ± 21	35 ^b
	100	340 ± 14	45 ^c	222 ± 11	45 ^c
	50	460 ± 12	17 ^a	171 ± 5	58 ^c
6	500	360 ± 29	40 ^b	276 ± 18	31 ^b
	250	371 ± 27	38 ^b	217 ± 21	47 ^c
	100	346 ± 20	43 ^c	231 ± 14	43 ^c
	50	288 ± 15	57 ^c	285 ± 9	29 ^b
12	500	332 ± 13	47 ^c	292 ± 13	27 ^b
	250	484 ± 25	11 ^a	182 ± 4	56 ^c
	100	422 ± 34	26 ^b	198 ± 10	51 ^c
	50	443 ± 17	21 ^a	234 ± 12	42 ^c
13	500	348 ± 17	43 ^c	305 ± 2	24 ^a
	250	413 ± 19	28 ^b	323 ± 24	19 ^a
	100	385 ± 22	34 ^b	313 ± 4	22 ^a
	50	420 ± 25	26 ^b	350 ± 4	12 ^a

*Mean ± S.D. – mean value ± standard deviation and mutagenicity inhibition are given; negative control – dimethyl sulfoxide (DMSO); positive control – sodium azide (NaN₃, 5 μg per plate).^a No antimutagenic effect (<25% inhibition). ^b Moderate effect (25–40% inhibition). ^c Strong antimutagenic effect (>40% inhibition).

relative safety, in some cases it might be reasonable to investigate the amide analogs of potent amines. Incorporation of the amino acid moiety did not result in increase of activity. Modification of a carboxyl group might result in better pharmacological properties of the designed compounds; however, further studies on this subject should be conducted in order to draw proper conclusions. For future studies, inclusion of the 6 Hz test instead of the scMET test as preliminary screening of anticonvulsant activity seems to be reasonable in the case of the studied group of compounds.

4. Experimental protocols

4.1. Chemistry

The reagents and solvents for synthetic purposes were commercially available materials of reagent grade and were purchased from Alfa Aesar (purchased from Chemat, Gdansk, Poland), Sigma-Aldrich (purchased from Sigma-Aldrich, Poznan, Poland) or Merck Sp. z o.o. (Warszawa, Poland). The solvents used for LCMS and HPLC were of LCMS grade and were purchased from Avantor Performance Materials Poland S.A., Gliwice, Poland.

The physicochemical parameters for the designed structures were calculated *in silico* using the ChemBioDraw Ultra 14.0 program. Melting points (mp) are uncorrected and were determined using a Büchi SMP-20 apparatus.

Analyses of C, H and N were within $\pm 0.4\%$ of the theoretical values. The IR spectra were recorded on a Jasco FT/IR 410 spectrometer (KBr pellets).

The LCMS system consisted of a Waters Acquity UPLC system coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). All the analyses were carried out using an Acquity UPLC BEH C18, 1.7 μm , 2.1×100 mm column. A flow rate of 0.3 ml min^{-1} and a gradient of 5–95% B over 10 min and then 100% B over 2 min was used. Eluent A: water/0.1% HCO_2H ; eluent B: acetonitrile/0.1% HCO_2H . LCMS data were obtained by scanning the first quadrupole in 0.5 s in a mass range from 50 to 1000 Da; eight scans were summed up to produce the final spectrum.

Analytical HPLC analysis was carried out with an LC Ultimate 3000 system consisting of a pump, a degasser, an autosampler and a column heater, equipped with a Corona charged aerosol detector (Corona CAD). Nitrogen from an NG-4-1 nitrogen generator set at 35 psi was introduced to the detector. Data were processed with Chromeleon 6.8 software. The following columns were used: (A) Allure PFP (150 mm $\times$ 4.6 mm; 5 μm particle size); (B) Hypersil Gold C18 (150 mm $\times$ 4.6 mm; 5 μm particle size). The isocratic elution was performed with 30% water (solvent A) and 70% methanol (solvent B) at the temperature of 20 °C. The flow rate was set at 1.0 ml min^{-1} , and the injection volume was 20 μl .

NMR spectra were recorded at the Faculty of Pharmacy, Jagiellonian University Medical College (Cracow, Poland) with a Varian Mercury-VX 300 NMR spectrometer operating at 300 MHz (^1H NMR for 1, 5, 6, 8–10, 13–17) or 75 MHz (^{13}C NMR for 1, 4–6, 8–17) at the Faculty of Chemistry, Jagiellonian

University (Cracow, Poland) using a Bruker Avance II spectrometer at 500.13 MHz (^1H NMR for 2–4, 12), 300 MHz (^1H NMR for 11) and 125.17 MHz (^{13}C NMR for 2 and 3), or at Jagiellonian Centre of Innovation (NMR Laboratory, Cracow, Poland) using a Bruker Avance III HD spectrometer operating at 400.17 MHz and 100 MHz (^1H and ^{13}C NMR for 7, respectively). The following abbreviations are used in spectra description: singlet (s), doublet (d), triplet (t), multiplet (m), and broad signal (br. s.).

Measurement of optical rotation ($[\alpha]_{589}^{20.0}$) for chiral compounds was carried out using a Jasco 2000 system (2% methanol solutions).

4.1.1. General procedure for preparation of compounds 1–17. Compounds 1–17 were obtained *via* multistep chemical synthesis. In the first step, the appropriately substituted phenoxyacetic chlorides (**1a–1c**) were obtained according to a previously published procedure.^{8,18} 2,3-Dimethyl-, 2,6-dimethyl- or 2,4,6-trimethylphenol (0.2 mol) was dissolved in 100 ml of 2 M NaOH aqueous solution and heated in a round bottom flask under a reflux condenser while an equimolar amount of 2-chloroacetic acid dissolved in 100 ml of 2 M NaHCO_3 aqueous solution was added dropwise. After 1 hour of heating, activated carbon was added and the product was obtained by filtering the reaction mixture using fluted filter paper and subsequently acidifying the obtained filtrate. Then, the phenoxyacetic acids were transformed into chlorides by heating in a round bottom flask under a reflux condenser for about 1 h with an excess of thionyl chloride (molar ratio 1:1.5) and in benzene solution. The unreacted thionyl chloride and solvent were then distilled off under vacuum and the acid chloride remaining in the flask was diluted with toluene. The obtained products **1a** and **1b** are known compounds of reagent grade, while compound **1c** was previously described.³⁴

In the next step, the appropriate aminoalkanol (**1**, 3–7, 12, 13, 16), amine (**2**) or amino acid methyl ester (**11a–11d**) (0.02 mol) was dissolved in 50 ml of 1 M K_2CO_3 aqueous solution and subsequently about 30 ml of toluene was added. The mixture was placed on a magnetic stirrer at 10–12 °C and an equimolar amount of the appropriate acid chloride in toluene was added dropwise for 2 h. All reagents were stirred for an additional 10 min after adding the whole amount of acid chloride, and then heated to the boiling point and left to cool. The precipitated amide deposit was filtered off, stirred with a 10% solution of NaHCO_3 and (after drying) recrystallized from a heptane–toluene (1:1) mixture. Compounds **11a–11d** are semi-products for synthesis compounds 8–11 and 17. The physicochemical parameters of **11d** are presented below, while compounds **11a–11c** were used as raw products after washing with a 10% solution of NaHCO_3 . Compound **11c** is commercially available (CAS 1994257-51-0).

For compound **15**, the reaction with the appropriate aminoalkanol was carried out in toluene, in the presence of K_2CO_3 as a proton acceptor. The reagents (0.03 mol of *trans*-4-aminocyclohexan-1-ol, 0.06 mol of 2,6-dimethylphenoxyacetic acid chloride, and 0.03 mol of K_2CO_3) were

heated in a round bottom flask under a reflux condenser. After about 6 h the inorganic salt was filtered off, the remaining mixture was concentrated and the obtained product was crystallized from hexane.

In order to obtain compounds 8–11 and 17, the ester groups of compounds **Ila–IId** were hydrolysed with 10% NaHCO₃ (8 and 9) at room temperature (6–8 h) or were subjected to aminolysis with use of NH₃(aq conc) (**10**, **17**) or methylamine (**11**) with the addition of methanol. The precipitated final products were recrystallized from a hexane–toluene (1:1) mixture.

The purity and identity of the obtained compounds were confirmed by means of LCMS, HPLC, IR, ¹H NMR and ¹³C NMR.

Characterisation of semi-product

R,S-Methyl 2-(2-(mesityloxy)acetamido)butanoate (**IId**). (80%); mp 64–66 °C; C₁₅H₂₁NO₄; LCMS, *m/z*: 294.14 (M⁺ + 1), 100.00%.

Characterisation of final products

4.1.1.1. *trans*-2-(2,3-Dimethylphenoxy)-*N*-(4-hydroxycyclohexyl)acetamide (**1**). (60%); mp 177–179 °C; C₁₆H₂₃NO₃; δ ¹H NMR (CDCl₃): 6.99–7.11 (1H, m, Ar-H5), 6.85 (1H, d, *J* = 7.57 Hz, Ar-H4), 6.64 (1H, d, *J* = 8.21 Hz, Ar-H6), 6.41 (1H, d, *J* = 8.21 Hz, NH), 4.44 (2H, s, CH₂), 3.77–3.97 (1H, m, CH-OH), 3.55–3.72 (1H, m, CH-N), 2.29 (3H, s, Ar-CH₃), 2.18 (3H, s, Ar-CH₃), 1.93–2.12 (4H, m, cyclohex-H3,5), 1.56 (1H, br s, OH), 1.35–1.52 (2H, m, cyclohex-H2,6), 1.18–1.35 (2H, m, cyclohex-H2,6); δ ¹³C NMR (CDCl₃): 168.0 (>C=O), 155.3, (Ar-C1), 138.4 (Ar-C3), 126.2 (Ar-C5), 125.0 (Ar-C4), 123.8 (Ar-C2), 109.7 (Ar-C6), 69.5 (>CH-OH), 68.1 (–CH₂–CO–), 47.1 (–NH–CH<), 33.7 ((CH₂)₂ > CH-OH), 30.7 ((CH₂)₂ > CH-NH–), 20.1 (Ar-CH₃(3)), 11.9 (Ar-CH₃(2)); LCMS, *m/z* 278.112 (M⁺ + 1, 97.70%); HPLC, RT (B) = 3.63 min, 99.15%. CAS 1157017-49-6.

4.1.1.2. 2-(2,6-Dimethylphenoxy)-*N*-phenethylacetamide (**2**). (54%); mp 84–86 °C; C₁₈H₂₁NO₂; δ ¹H NMR (DMSO-*d*₆): 8.20 (1H, t, *J* = 5.6 Hz, NH), 7.27–7.33 (2H, m, Ar'-H2,6), 7.18–7.27 (3H, m, Ar'-H3,4,5), 7.04 (2H, dddd, *J* = 8.2 Hz; 6.8 Hz; 1.4 Hz; 0.8 Hz, Ar-H3,5), 6.95 (1H, dd, *J* = 8.2 Hz; 6.8 Hz, Ar-H4), 4.16 (2H, s, Ar-O-CH₂), 3.43 (2H, dt, *J* = 5.6 Hz; 7.5 Hz, –NH-CH₂), 2.81 (2H, t, *J* = 7.5 Hz, –CH₂-Ar), 2.19 (6H, s, Ar-(CH₃)₂); δ ¹³C NMR (DMSO-*d*₆): 167.66 (>C=O), 154.57 (Ar-C1), 139.33 (Ar'-C1), 130.35 (Ar-C2,6), 128.80 (Ar'-C3,5), 128.68 (Ar-C3,5), 128.29 (Ar'-2,6), 126.09 (Ar'-C4), 124.22 (Ar-C4), 70.47 (–CH₂–CO–), 39.76 (–NH–CH₂–), 35.01 (–CH₂-Ar'), 15.83 (Ar-(CH₃)₂); LCMS, *m/z* 284.17 (M⁺ + 1, 97.57%); HPLC, RT (B) = 6.67 min, 97.08%. CAS 447437-41-4.

4.1.1.3. *R,S*-2-(2,6-Dimethylphenoxy)-*N*-(2-hydroxy-2-phenylethyl)acetamide (**3**). (49%); mp 113–114; C₁₈H₂₁NO₃; IR (KBr) ν = 3396, 3317, 2939, 2871, 1635, 1556, 1479, 1196, 1030, 762 cm^{–1}; δ ¹H NMR (DMSO-*d*₆): 7.96 (1H, t, *J* = 5.6 Hz, NH), 7.31–7.40 (4H, m, Ar'-H2,3,5,6), 7.18–7.27 (1H, m, Ar'-H4), 7.03 (1H, d, *J* = 7.0, Ar-H), 6.95 (1H, d, *J* = 7.0 Hz, Ar-H), 6.93 (1H, d, *J* = 7.0 Hz, Ar-H), 5.57 (1H, d, *J* = 4.5 Hz, OH), 4.72–4.78 (1H, m, CH-OH), 4.17 (2H, s, Ar-O-CH₂), 3.41–3.48 (1H, m, N-CHH), 3.29–3.34 (1H, m, N-CHH), 2.19 (6H, s,

Ar-(CH₃)₂); δ ¹³C NMR (DMSO-*d*₆): 167.79 (>C=O), 154.52 (Ar-C1), 143.45 (Ar'-C1), 130.33 (Ar-C2,6), 128.82 (Ar'-C3,5), 128.00 (Ar-C3,5), 127.09 (Ar'-2,6), 126.07 (Ar'-C4), 124.25 (Ar-C4), 70.98 (>CH-Ar'), 70.39 (–CH₂–CO–), 46.21 (–NH–CH₂–), 15.87 (Ar-(CH₃)₂); LCMS, *m/z*: 300.19 (M⁺ + 1, 98.26%); HPLC, RT (A) = 8.56 min, 97.62%; found: C 72.20, H 7.07, N 4.70, calc. for C₁₈H₂₁NO₃: C 72.10, H 7.21, N 4.46.

4.1.1.4. *R,S*-2-(2,6-Dimethylphenoxy)-*N*-(2-hydroxypropyl)acetamide (**4**). (62%); mp 106–108; C₁₃H₁₉NO₃; IR (KBr) ν = 3421, 3340, 2962, 2922, 2875, 1655, 1541, 1473, 1194, 1140, 1051, 972, 791, 665, 579, 436 cm^{–1}; δ ¹H NMR (DMSO-*d*₆): 7.99 (1H, t, *J* = 5.6 Hz, NH), 7.03 (2H, d, *J* = 7.5 Hz, Ar-H), 6.95 (1H, t, *J* = 7.5 Hz, Ar-H), 4.79 (1H, d, *J* = 5.1 Hz, OH), 4.21 (2H, s, Ar-O-CH₂–), 3.71–3.79 (1H, m, >CH-OH), 3.15–3.22 (1H, m, –CHH-NH–), 3.08–3.14 (1H, m, –CHH-NH–), 2.26 (6H, s, Ar-(CH₃)₂), 1.06 (3H, d, *J* = 6.2 Hz, >CH-CH₃). δ ¹³C NMR (DMSO-*d*₆): 168.3 (>C=O), 155.1 (Ar-C1), 130.8 (Ar-C2,6), 129.3 (Ar-C3,5), 124.7 (Ar-C4), 71.0 (–CH₂–CO–), 65.4 (>CH–), 46.4 (–CH₂–CH<), 21.5 (>CH-CH₃), 16.4 (Ar-(CH₃)₂); LCMS, *m/z*: 238.12 (M⁺ + 1, 100%); HPLC, RT (A) = 4.93 min, 97.19%. CAS 11567-97-47-5.

4.1.1.5. *R,S*-2-(2,6-Dimethylphenoxy)-*N*-(1-hydroxypropan-2-yl)acetamide (**5**). (60%); mp 98–100; C₁₃H₁₉NO₃; δ ¹H NMR (CDCl₃): 6.92–7.13 (4H, m, Ar-H3,4,5, NH), 4.35 (1H, s, OH), 4.30 (1H, s, Ar-O-CHH–), 4.29 (1H, s, Ar-O-CHH–), 4.14–4.25 (1H, m, –CH<), 3.59–3.81 (2H, m, –CH₂-OH), 2.67–2.75 (1H, m, OH), 2.26 (6H, s, Ar-(CH₃)₂), 1.29 (3H, d, *J* = 7.0 Hz); δ ¹³C NMR (CDCl₃): 169.3 (>C=O), 154.4 (Ar-C1), 130.4 (Ar-C2,6), 129.1 (Ar-C3,5), 124.7 (Ar-C4), 70.4 (–CH₂–CO–), 66.6 (–CH₂-OH), 47.4 (>CH–), 17.1 (>CH-CH₃), 16.3 (Ar-(CH₃)₂); LCMS, *m/z*: 238.18 (M⁺ + 1, 99.08%); HPLC, RT (A) = 5.40 min, 100%.

4.1.1.6. *R*-(–)-2-(2,6-Dimethylphenoxy)-*N*-(1-hydroxypropan-2-yl)acetamide (**6**). (62%); mp 122–124; C₁₃H₁₉NO₃; δ ¹H NMR (DMSO-*d*₆): 7.75 (1H, d, *J* = 8.2 Hz, NH), 6.98–7.04 (2H, m, Ar-C3,5), 6.88–6.95 (1H, m, Ar-C4), 4.75 (1H, t, *J* = 5.6 Hz, OH), 4.17 (1H, s, Ar-O-CHH–), 4.16 (1H, s, Ar-O-CHH–), 3.90 (1H, dd, *J* = 7.6; 6.4 Hz, >CH–), 3.32–3.45 (2H, m, –CH₂-OH), 2.20 (6H, s, Ar-(CH₃)₂), 1.08 (3H, d, *J* = 7.0 Hz, >CH-CH₃); δ ¹³C NMR (DMSO-*d*₆): 167.7 (>C=O), 155.4 (Ar-C1), 130.8 (Ar-C3,5), 129.3 (Ar-C2,6), 124.6 (Ar-C4), 71.2 (–CH₂–CO–), 64.6 (–CH₂-OH), 46.7 (>CH–), 17.5 (>CH-CH₃), 16.4 (Ar-(CH₃)₂); LCMS, *m/z*: 238.22 (M⁺ + 1, 100%); HPLC, RT (A) = 5.40 min, 99.78%; [α]₅₈₉^{20.0} = –1.4104° (2% concentration in MeOH).

4.1.1.7. *S*-(+)-2-(2,6-dimethylphenoxy)-*N*-(1-hydroxypropan-2-yl)acetamide (**7**). (60%); mp 121–123; C₁₃H₁₉NO₃; δ ¹H NMR (CDCl₃): 7.08–7.16 (1H, m, NH), 6.96–7.08 (3H, m, Ar-H3,4,5), 4.26–4.37 (2H, m, Ar-O-CH₂–), 4.22 (1H, m, –CH<), 3.77 (1H, dd, *J* = 10.76; 3.13 Hz, –CHH-OH), 3.66 (1H, dd, *J* = 11.00; 5.92 Hz, –CHH-OH), 3.00 (1H, br s, OH), 2.28 (6H, s, Ar-(CH₃)₂), 1.31 (3H, d, *J* = 6.85 Hz, >CH-CH₃); δ ¹³C NMR (CDCl₃): 169.39 (>C=O), 154.41 (Ar-C1), 130.38 (Ar-C3,5), 129.17 (Ar-C2,6), 124.77 (Ar-C4), 70.41 (–CH₂–CO–), 66.95 (–CH₂-OH), 47.58 (>CH–), 17.06 (>CH-CH₃), 16.28 (Ar-(CH₃)₂); LCMS, *m/z*: 238.18 (M⁺ + 1, 100%); HPLC, RT (A) = 5.38 min, 99.50%; [α]₅₈₉^{20.0} = 1.2198° (2% concentration in MeOH).

4.1.1.8. *R,S*-(2-(2,6-Dimethylphenoxy)acetyl)alanine (8). (70%), mp 180–182; $C_{13}H_{17}NO_4$; δ 1H NMR ($CDCl_3$): 7.49 (1H, d, J = 7.6 Hz, NH), 6.94–7.05 (3H, m, Ar-H3,4,5), 4.76 (1H, m, >CH-), 4.26–4.41 (2H, m, Ar-O-CH₂-), 2.27 (6H, s, Ar-(CH₃)₂), 1.58 (3H, d, J = 7.0 Hz, >CH-CH₃); δ ^{13}C NMR ($CDCl_3$): 176.4 (–COOH), 169.2 (>C=O), 154.3 (Ar-C1), 130.4 (Ar-C2,6), 129.2 (Ar-C3,5), 124.8 (Ar-C4), 70.1 (–CH₂-CO-), 47.7 (>CH-), 18.2 (>CH-CH₃), 16.3 (Ar-(CH₃)₂); LCMS, m/z : 252.14 (M^+ + 1, 98.99%); HPLC, RT (C) = 2.252 min.

4.1.1.9. *S*-(–)-(2-(2,6-Dimethylphenoxy)acetyl)alanine (9). (68%), mp 188–190; $C_{13}H_{17}NO_4$; δ 1H NMR ($CDCl_3$): 7.47 (1H, d, J = 7.0 Hz, NH), 6.93–7.06 (3H, m, Ar-H3,4,5), 4.76 (1H, m, >CH-), 4.26–4.41 (2H, m, Ar-O-CH₂-), 2.27 (6H, s, Ar-(CH₃)₂), 1.58 (3H, d, J = 7.0 Hz, >CH-CH₃); δ ^{13}C NMR ($CDCl_3$): 176.2 (–COOH), 169.2 (>C=O), 154.3 (Ar-C1), 130.4 (Ar-C2,6), 129.2 (Ar-C3,5), 124.8 (Ar-C4), 70.2 (–CH₂-CO-), 47.7 (>CH-), 18.2 (>CH-CH₃), 16.3 (Ar-(CH₃)₂); LCMS, m/z : 252.14 (M^+ + 1, 98.61%); $[\alpha]_{589}^{20.0}$ = –9.8930° (2% concentration in MeOH). CAS 1046053-08-0.

4.1.1.10. *R*-(–)-(2-(2-(2,6-Dimethylphenoxy)acetamido)propanamide (10). (60%), mp 177–178; $C_{13}H_{18}N_2O_3$; δ 1H NMR ($CDCl_3$): 7.46 (1H, d, J = 6.4 Hz, –NH-CH-), 6.93–7.06 (3H, m, Ar-H), 6.22–6.36 (1H, m, –NH), 5.51–5.62 (1H, m, –NH), 4.67 (1H, m, >CH-), 4.31 (2H, s, –O-CH₂-CO-), 2.26 (6H, s, Ar-(CH₃)₂), 1.51 (3H, d, J = 7.0 Hz, >CH-CH₃); δ ^{13}C NMR ($CDCl_3$): 173.7 (–CO-NH₂), 169.0 (–CO-NH-), 154.3 (Ar-C1), 130.4 (Ar-C2,6), 129.2 (Ar-C3,5), 124.8 (Ar-C4), 70.3 (–CH₂-CO-), 47.9 (>CH-), 18.0 (>CH-CH₃), 16.3 (Ar-(CH₃)₂); LCMS, m/z : 251.14 (M^+ + 1, 98.60%); HPLC, RT (B) = 2.92 min, 100%; $[\alpha]_{589}^{20.0}$ = –2.6710° (2% concentration in MeOH).

4.1.1.11. *R,S*-(2-(2-(2,6-Dimethylphenoxy)acetamido)-*N*-methylpropanamide (11). (55%), mp 129–131; $C_{14}H_{20}N_2O_3$; δ 1H NMR ($DMSO-d_6$): 8.02 (1H, d, J = 8.5 Hz, NH), 7.95 (1H, q, J = 4.6 Hz, –NH-CH₃), 6.98–7.07 (2H, m, Ar-H3,5), 6.89–6.98 (1H, m, Ar-H4), 4.38 (1H, dq, J = 7.6 Hz; 4.7 Hz, >CH-), 4.27 (1H, d, J = 14.3 Hz, Ar-O-CHH-), 4.21 (1H, d, J = 14.3 Hz, Ar-O-CHH-), 2.62 (3H, d, J = 4.7 Hz, –NH-CH₃), 2.23 (6H, s, Ar-(CH₃)₂), 1.28 (3H, d, J = 7.1 Hz, >CH-CH₃); δ ^{13}C NMR ($CDCl_3$): 167.6 (>CH-(C=O)-NH-), 164.1 (–CH₂-CO-), 149.7 (Ar-C1), 125.7 (Ar-C2,6), 124.4 (Ar-C3,5), 120.0 (Ar-C4), 65.6 (–CH₂-CO-), 43.6 (>CH-), 21.6 (–NH-CH₃), 13.7 (Ar-(CH₃)₂), 11.6 (–CH-CH₃); LCMS, m/z : 265.17 (M^+ + 1, 97.24%); HPLC, RT (A) = 5.53 min, 99.84%.

4.1.1.12. *R,S*-(2-(2,6-Dimethylphenoxy)-*N*-(2-hydroxybutyl)acetamide (12). (60%), mp 96–97; $C_{14}H_{21}NO_3$; IR (KBr) ν = 3420, 3336, 2969, 2923, 2878, 1655, 1540, 1455, 1353, 1266, 1196, 1053, 791, 668, 570, 435, 418 cm^{-1} ; δ 1H NMR ($DMSO-d_6$): 7.95 (1H, t, J = 5.6 Hz, NH), 6.90–7.12 (3H, m, Ar-H), 4.76 (1H, d, J = 5.2 Hz, OH), 4.21 (2H, s, –O-CH₂-), 3.48–3.50 (1H, m, >CH-), 3.23–3.28 (1H, m, –NH-CHH-), 3.08–3.13 (1H, m, –NH-CHH-), 2.23 (6H, s, Ar-(CH₃)₂), 1.39–1.48 (1H, m, >CH-CHH-), 1.26–1.35 (1H, m, >CH-CHH-), 0.88 (3H, t, J = 7.4 Hz, –CH₂-CH₃); δ ^{13}C NMR ($DMSO-d_6$): 168.3 (>C=O), 155.1 (Ar-C1), 130.8 (Ar-C2,6), 129.3 (Ar-C3,5), 124.7 (Ar-C4), 71.0 (>CH-), 70.7 (–CH₂-CO-), 44.6 (–NH-CH₂-), 27.8 (–CH₂-CH₃), 16.4 (Ar-(CH₃)₂), 10.3 (–CH₂-

CH₃); LCMS, m/z : 252.21 (M^+ + 1, 100.00%); HPLC, RT (A) = 5.94 min, 100%.

4.1.1.13. *S*-(–)-(2-(2,6-Dimethylphenoxy)-*N*-(1-hydroxybutan-2-yl)acetamide (13). (70%), mp 76–78; $C_{14}H_{21}NO_3$; δ 1H NMR ($CDCl_3$): 6.90–7.10 (3H, m, Ar-H3,4,5), 4.23–4.39 (2H, m, Ar-O-CH₂-), 3.99 (m, 1H, –CH<), 3.65–3.83 (2H, m, –CH₂-OH), 2.56–2.64 (1H, m, impurity), 2.27 (6H, s, Ar-(CH₃)₂), 1.58–1.76 (2H, m, –CH₂-CH₃), 1.03 (3H, t, J = 7.6 Hz, –CH₂-CH₃); δ ^{13}C NMR ($CDCl_3$): 169.5 (>C=O), 154.3 (Ar-C1), 130.4 (Ar-C2,6), 129.1 (Ar-C3,5), 124.7 (Ar-C4), 70.4 (–CH₂-CO-), 64.8 (–CH-OH), 53.0 (>CH-), 24.2 (–CH₂-CH₃), 16.2 (Ar-(CH₃)₂), 10.5 (–CH₂-CH₃); LCMS, m/z : 252.14 (M^+ + 1, 97.35%); HPLC, RT (A) = 5.58 min, 99.05%; $[\alpha]_{589}^{20.0}$ = –14.1980° (2% concentration in MeOH). CAS 1983486-85-6.

4.1.1.14. *trans*-(2-(2-(2,6-Dimethylphenoxy)acetamido)cyclohexyl)-2-(2,6-dimethylphenoxy)acetate (14). (52%), mp 170–171; $C_{26}H_{33}NO_5$; δ 1H NMR ($CDCl_3$): 6.92–7.06 (6H, m, Ar-H), 6.82 (1H, d, J = 8.2 Hz, NH), 4.93 (1H, tt, J = 10.6, 4.0 Hz, CH-O), 4.41 (2H, s, CO-CH₂-O-Ar), 4.28 (2H, s, CH₂-CO-NH), 3.87–4.06 (1H, m, NH-CH), 2.31 (6H, s, Ar-CH₃ (amide)), 2.26 (6H, s, Ar-CH₃ (ester)), 2.14 (4H, ddd, J = 13.3 Hz, 9.6 Hz, 3.8 Hz, 4H, cyclohex), 1.60–1.71 (2H, m, J = 3.1 Hz, cyclohex), 1.59 (overlap – H₂O from $CDCl_3$), 1.36–1.52 (2H, m, J = 13.6 Hz, cyclohex); δ ^{13}C NMR ($CDCl_3$): 168.7 (–O-CO-), 168.1 (–CO-NH-), 155.4 (Ar'-C1), 154.4 (Ar-C1), 130.6 (Ar'-C2,6), 130.3 (Ar-C2,5), 129.2 (Ar'-C3,5), 129.0 (Ar-C3,5), 124.8 (Ar'-C4), 124.4 (Ar-C4), 72.7 ((CH₂)₂ > CH-O-), 70.5 (–CH₂-CO-NH-), 69.3 (–O-CO-CH₂-), 46.7 (–NH-CH-(CH₂)₂<), 30.5 ((CH₂)₂ > CH-O-), 29.9 (–NH-CH-(CH₂)₂<), 16.3 (Ar-(CH₃)₂, Ar'-(CH₃)₂); LCMS, m/z : 440.22 (M^+ + 1, 100.00%); HPLC, RT (B) = 14.21 min, 99.26%.

4.1.1.15. *R,S*-(1-Hydroxybutan-2-yl)-2-(mesityloxy)acetamide (15). (58%), mp 87–88; $C_{15}H_{23}NO_3$; IR (KBr) ν = 3413, 3330, 2965, 2923, 1651, 1536, 1212, 1146, 1085, 1054, 986, 856, 661, 586, 574 cm^{-1} ; δ 1H NMR ($CDCl_3$): 6.99–7.09 (1H, m, NH), 6.83 (2H, s, Ar-H), 4.20–4.35 (2H, m, Ar-O-CH₂-CO-), 3.92–4.03 (1H, m, >CH-), 3.75–3.81 (1H, m, –CHH-OH), 3.65–3.73 (1H, m, –CHH-OH), 2.24 (3H, s, Ar-CH₃(4)), 2.22 (6H, s, Ar-(CH₃)₂(2,6)), 1.54–1.77 (2H, m, –CH₂-CH₃), 0.97–1.07 (3H, s, –CH₂-CH₃); δ ^{13}C NMR ($CDCl_3$): 169.7 (–CO-NH-), 152.1 (Ar-C1), 134.1 (Ar-C4), 129.9 (Ar-C3,5), 129.7 (Ar-C2,6), 70.5 (–CH₂-CO-), 65.1 (–CH₂-OH), 53.1 (–CH<), 24.2 (–CH₂-CH₃), 20.6 (Ar-CH₃(4)), 16.2 (Ar-(CH₃)₂(2,6)), 10.5 (–CH₂-CH₃); LCMS, m/z : 266.23 (M^+ + 1, 97.42%); HPLC, RT (A) = 10.06 min, 99.55%. CAS 1156896-52-4.

4.1.1.16. *R,S*-(2-(2-Hydroxy-2-phenylethyl)-2-(mesityloxy)acetamide (16). (60%), mp 121–123; $C_{19}H_{23}NO_3$; IR (KBr) ν = 3357, 2975, 2937, 2921, 2873, 1653, 1536, 1483, 1208, 1148, 1097, 1065, 1043, 854, 752, 702, 593, 573, 528, 412 cm^{-1} ; δ 1H NMR ($CDCl_3$): 7.27–7.45 (5H, m, Ar'-H, NH), 6.82 (2H, s, Ar-H3,5), 4.96 (1H, m, OH), 4.26 (1H, s, Ar-O-CHH-CO-), 4.25 (1H, s, Ar-O-CHH-CO-), 3.84 (1H, ddd, J = 13.8, 6.7, 3.5 Hz, 1H, >CH-), 3.47–3.61 (1H, m, –NH-CHH-CH<), 2.92–3.31 (1H, m, –NH-CHH-CH<), 2.24 (3H, s, Ar-CH₃(4)), 2.17 (6H, s, Ar-(CH₃)₂(2,6)); δ ^{13}C NMR ($CDCl_3$): 170.1 (>C=O), 152.0 (Ar-C1), 141.7 (Ar'C1), 134.1, (Ar-C4),

130.0 (Ar-C2,6), 129.6 (Ar'-C2,6), 128.5 (Ar-C3,5), 127.9 (Ar'-C4), 125.9 (Ar'-C3,5), 73.4 (>CH-OH), 70.3 (-Ar-O-CH₂-), 46.7 (-NH-CH₂-), 20.7 (Ar-CH₃(4)), 16.1 (Ar-(CH₃)₂(2,6)); LCMS, *m/z*: 314.21 (*M*⁺ + 1, 100.00%); HPLC, RT (B) = 5.76 min, 99.10%.

4.1.1.17. *R,S*-2-(2-(Mesityloxy)acetamido)butanamide (17). (60%); mp 156–158; C₁₅H₂₂N₂O₃; IR (KBr) ν = 3368, 3198, 2963, 2930, 1656, 1536, 1484, 1424, 1210, 1147, 1062, 852, 674, 650, 637, 434, 420, 407 cm⁻¹; δ ¹H NMR (DMSO-*d*₆): 7.84 (1H, d, *J* = 8.2 Hz, -NH-CH<), 7.50 (1H, br s, -CO-NHH), 7.14 (1H, br s, -CO-NHH), 6.81 (2H, s, Ar-H3,5), 4.24–4.33 (1H, m, >CH-), 4.21 (1H, s, Ar-O-CHH-), 4.18 (1H, s, Ar-O-CHH-), 2.17 (9H, s, Ar-(CH₃)₃), 1.57–1.82 (2H, m, -CH₂-CH₃), 0.84 (3H, t, *J* = 7.3 Hz, -CH₂-CH₃); δ ¹³C NMR (DMSO-*d*₆): 173.4 (-CONH₂), 167.9, (-CONH-), 153.0, (Ar-C1), 133.4 (Ar-C4), 130.3 (Ar-C2,6), 129.8 (Ar-C3,5), 71.1 (-CH₂-CO-), 53.3 (>CH-), 25.9 (-CH₂-CH₃), 20.7 (Ar-CH₃(4)), 16.3 (Ar-(CH₃)₂(2,6)), 10.1 (-CH₂-CH₃); LCMS, *m/z*: 279.19 (*M*⁺ + 1, 100.00%); HPLC, RT (B) = 4.18 min, 99.74%.

4.2. Crystallography

Crystals suitable for X-ray diffraction analysis were obtained by slow evaporation of the solvent at room temperature from acetonitrile for 6 and 7 and from a mixture of acetone and decane (1:1) for 12. Colourless crystals appeared within two weeks.

Diffraction data for single crystals of 6 and 7 were collected at 130 K using an Oxford Diffraction SuperNova four circle diffractometer equipped with a Cu (1.54184 Å) K α radiation source, graphite monochromator and Oxford CryoJet system for measurements at low temperature. Diffraction data for 12 were collected at 100 K on a Bruker-Nonius Kappa CCD four circle diffractometer equipped with a Mo (0.71069 Å) K α radiation source. The structures were solved by direct methods using SIR-97.³⁵ All non-hydrogen atoms were refined anisotropically using weighted full-matrix least-squares on *F*². Refinement and further calculations were carried out using SHELXL.³⁶ For molecular graphics ORTEP³⁷ was used.

Compound 6. C₁₃H₁₉NO₃, *M_r* = 237.29, crystal size = 0.56 × 0.27 × 0.14 mm³, orthorhombic, space group *P*2₁2₁2₁, *a* = 7.3307(4) Å, *b* = 20.5418(9) Å, *c* = 8.5970(4) Å, *V* = 1294.6(1) Å³, *Z* = 4, *T* = 130(2) K, 19 025 reflections collected, 2497 unique reflections (*R*_{int} = 0.0581), *R*₁ = 0.0952, *wR*₂ = 0.2371 [*I* > 2 σ (*I*)], Flack parameter -0.21(11).

Compound 7. C₁₃H₁₉NO₃, *M_r* = 237.29, crystal size = 0.34 × 0.22 × 0.10 mm³, orthorhombic, space group *P*2₁2₁2₁, *a* = 7.3182(8) Å, *b* = 20.524(2) Å, *c* = 8.597(1) Å, *V* = 1291.2(3) Å³, *Z* = 4, *T* = 130(2) K, 18 683 reflections collected, 2497 unique reflections (*R*_{int} = 0.0741), *R*₁ = 0.0913, *wR*₂ = 0.2315 [*I* > 2 σ (*I*)], Flack parameter 0.12(18).

Compound 12. C₁₄H₂₀NO₃, *M_r* = 250.31, crystal size = 0.35 × 0.24 × 0.05 mm³, orthorhombic, space group *P*na2₁, *a* = 21.431(1) Å, *b* = 8.483(2) Å, *c* = 7.516(3) Å, *V* = 1366.4(6) Å³, *Z* = 4, *T* = 100(2) K, 6398 reflections collected, 2386 unique reflections (*R*_{int} = 0.0982), *R*₁ = 0.0851, *wR*₂ = 0.1979 [*I* > 2 σ (*I*)].

CCDC 1832675–1832677 contain the supplementary crystallographic data.

4.3. Pharmacology

Antiepileptic activity and neurological toxicity assays were carried out by the Epilepsy Therapy Screening Program (ETSP, previously known as the Anticonvulsant Screening Program, ASP), Epilepsy Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health in Rockville, MD, USA.²⁰ For all experiments male Carworth Farms no. 1 (CF1) mice (18–30 g) and male Sprague Dawley rats (100–150 g for MES and 250–300 g for hippocampal kindling) were used.

For compounds 1 and 14 testing was performed in a manner consistent with a protocol approved by the Institutional Animal Care and Use Committee at the University of Utah (protocol 15-10007, PI: Wilcox), while for other compounds with a protocol “Evaluation and Characterization of Novel Therapeutics for the Treatment and Prevention of Epilepsy and Countermeasures” (protocol 12-11011).

4.3.1. Maximal electroshock seizure (MES) test. The MES were elicited by 60 Hz alternating current (50 mA in mice and 150 mA in rats) delivered for 0.2 s *via* corneal electrodes, primed with an electrolyte solution containing an anesthetic agent (0.5% tetracaine HCl). Protection in the MES test was defined as the abolition of the hindlimb tonic extension component of the seizure.²²

4.3.2. Subcutaneous pentylenetetrazole (scMET) test. The scMET test was conducted by administration of pentylene-tetrazole (85 mg kg⁻¹) dissolved in 0.9% NaCl solution to mice into the posterior midline of its neck. A minimal time of 30 min subsequent to subcutaneous administration of pentylenetetrazole was used for seizure detection. Animals not displaying fore and/or hind limb clonus, jaw chomping, or vibrissae twitching (3 to 5 seconds) were classified as protected.²²

4.3.3. 6 Hz model test. Corneal stimulation at 6 Hz (44 mA) was delivered for 3 s to mice by a constant current device to induce a psychomotor seizure. The animals then exhibited a ‘stunned’ posture associated with rearing, forelimb automatic movements and clonus, twitching of the vibrissae, and Straub tail. The experimental endpoint was protection against the seizure. The animal was considered to be protected if it did not display this behavior.²¹

4.3.4. Hippocampal kindling test. A bipolar stimulating electrode was stereotactically implanted in the ventral hippocampus (AP -3.6, ML 4.9, DV -5.0 from dura, incisor bar +5) of adult male Sprague Dawley rats (250–300 g) under ketamine-xylazine anesthesia. Three anchor screws were attached to the skull with dental acrylic cement. After the incision was closed with sutures, the animal received a single dose of Bicillin (60 000 units, i.m.) and was returned to his home cage in the animal quarters. Animals were kindled according to the procedure of Lothman and Williamson.³⁸ After 1 week animals were stimulated with suprathreshold trains for 200

μA for 10 s, 50 Hz, every 30 min for 6 h on alternate days until they were fully kindled. One week later, a single dose of test substance (50 mg kg^{-1} , *i.p.*) on the behavioral seizure score and afterdischarge duration was assessed in a single group of kindled rats respectively at 15 or 0, 15, 45, 75, 105 and 135 min after drug administration. Results obtained at the various points were compared with the last control stimulus delivered 15 min prior to drug administration. Thus, each animal served as its own control. When a drug treatment was observed to significantly lower seizure score and decrease afterdischarge, a dose–response study was initiated.

4.3.5. Neurotoxicity. Neurological deficit was measured in mice by means of the rotarod 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, which is defined as falling off the rod at least three times. In rats, neurological deficit was indicated by ataxia and a range of additional symptoms: a circular or zigzag gait, abnormal body posture and spread of the legs, tremors, hyperactivity, lack of exploratory behavior, somnolence, stupor, catalepsy, loss of placing response, and changes in muscle tone. A rat is considered impaired if it displays two or more of these abnormal behaviors.²²

4.3.6. Quantification of effective dose and toxic dose. The ED_{50} and TD_{50} were calculated for MES and rotarod tests performed in rats, respectively, by means of a computer program using probit analysis.²²

4.4. In vitro studies on astrocytes

4.4.1. Cell culture

Mouse astrocytes. An astrocyte cell line (ATCC, CRL-2541) was used in the study. The cells were cultured under standard conditions (37°C , 5% CO_2) in DMEM medium supplemented with 10% FBS and antibiotics.

Human astrocytes. The iPSC cells were kindly provided by Damian Ryszawy, PhD (Department of Cell Biology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Cracow, Poland). The cells were cultured on 100 mm Petri dishes in Essential 8 Medium (ThermoFisher: A1517001) supplemented with 1% pen–strep solution and Y-27632 ROCK inhibitor (SCM075, MERCK Millipore) at a concentration of $10 \mu\text{M}$. The culture medium was changed each day. Before culture the dishes were covered with vitronectin at a concentration $0.5 \mu\text{g cm}^{-2}$ according to the manufacturer's protocol (ThermoFisher: A14700). To obtain the neural stem cells the iPSC cells were cultured for 7 days in Gibco PSC Neural Induction Medium (ThermoFisher: A1647801) supplemented with 1% pen–strep solution. The medium was changed each day. We used astrocyte growth medium (Sigma-Aldrich: 821-500) supplemented with 1% pen–strep to differentiate the NSC cells. The medium was changed each day for 17 days until the appropriate confluence of astrocyte culture has been achieved. The astrocyte phenotype was then confirmed by GFAP staining. The purity of the astrocyte population was about 92% (glial fibrillary

acidic protein (GFAP)-positive astrocytes); the rest (8%) consisted of Tuj-1 positive neurons (neuronal class III β -tubulin). However, in the next passages the population of neurons gradually decreased leading to a pure population of GFAP-positive astrocytes.

One day before the experiment, the cells were seeded at a density of 10 000 cells per well in 96-well plates in a medium containing a reduced amount of serum (1% FBS). The astrocytes were treated with compound 1 (dissolved in ddH_2O) at concentrations of from 10 to $200 \mu\text{M}$ for 24 hours. The control cultures were supplemented with the same amount of an appropriate vehicle.

4.4.2. MTT assay. Cells were seeded at a density of 1×10^4 cells per well in 96 well plates. Following overnight culture, the cells were then treated with increasing doses of compound 6 and incubated for 24 h. Following cell exposure to each drug for 24 h in 96-well plates, $10 \mu\text{l}$ MTT reagent (Cayman) was added to each well and after 4 hours of incubation (37°C , 5% CO_2), the medium was aspirated and the formazan produced in the cells appeared as dark crystals at the bottom of the wells. Next, Crystal Dissolving Solution (Cayman) was added to each well. Then the optical density (OD) of each well was determined at 570 nm on a plate reader (BIOTEK).

4.4.3. NR assay. Cells were seeded at a density of 1×10^4 cells per well in 96-well plates. After 24 hours they were treated with compound 6 at a concentration of 10– $200 \mu\text{M}$. After 24 h the medium was removed from the cells and replaced with $100 \mu\text{l}$ of medium containing NR. The medium containing NR ($4 \mu\text{g mL}^{-1}$) was prepared by mixing NR solution in PBS (4 mg mL^{-1}) with EMEM supplemented with FBS (5%). After 3 h of incubation and microscopic examination of the cells, the medium was removed and the cells were washed with PBS.

Then, $150 \mu\text{l}$ of solubilization solution (50–96% ethanol, 49% distilled water and 1% glacial acetic acid) was added to each well, and the plates were shaken on a microtiter plate shaker for 10 minutes. Absorbance was determined at 540 nm (A_{540}) and cytotoxicity was calculated as: cytotoxicity (%) = $(1 - (\text{experiment } A_{540} / \text{control } A_{540})) \times 100$.

4.4.4. CV assay. Cells were seeded at a density of 1×10^4 cells per well in 96-well plates. After 24 hours they were treated with compound 6 at a concentration of 10– $200 \mu\text{M}$. After 72 hours, cells were fixed for 15 min in a solution of formaldehyde (3.7%), washed with PBS and subsequently stained with $500 \mu\text{l}$ of 0.01% crystal violet solution for 10 minutes. The dye that stained the cells on the plates was eluted by $500 \mu\text{l}$ CH_3OH solution (25% V/V) of citric acid (1.33% m/V) and sodium citrate (1.09% m/V), and the optical density of the extracted dye was read with a spectrophotometer at 540 nm.

4.5. Metabolic stability

In vitro tests with liver microsomes isolated from male rats (Sigma, Saint Louis, MO, USA) were designed according to Słoczyńska *et al.*³³ with minor modifications. The

compounds, tested at a concentration of 20 μM , were preincubated with rat liver microsomes (0.4 mg mL^{-1}) in 100 mM potassium phosphate buffer (pH 7.4) for 15 min. The proper experiment started with the addition of a regeneration system consisting of 3.4 mM of NADP^+ , 7.7 mM glucose 6-phosphate and 0.6 U mL^{-1} glucose 6-phosphate dehydrogenase in potassium phosphate buffer (pH 7.4). Experiments were carried out for 5 to 60 min at 37 °C in duplicate. Reactions were stopped by adding cooled perchloric acid, followed immediately by spiking samples with an internal standard. Then, the samples were centrifuged at 9000 rpm for 10 min at 4 °C. The supernatant was analyzed by LC-MS/MS analysis (UPLC/MS, Waters Corporation, Milford, MA, USA). *In vitro* half-life ($t_{1/2}$) and intrinsic clearance (Cl_{int}) of the studied compounds in liver microsomes were determined according to literature procedures.^{39,40}

4.6. Mutagenicity

4.6.1. Bacterial strains. *Salmonella typhimurium* strains TA100 and TA1535 were kindly provided by Dr. T. Nohmi (Division of Genetics and Mutagenesis, National Institute of Hygienic Sciences, Tokyo, Japan).

4.6.2. Chemicals and reagents. Magnesium sulfate, citric acid monohydrate, potassium phosphate dibasic, sodium ammonium phosphate, L-histidine, D-biotin, sodium phosphate dibasic, monobasic sodium phosphate, magnesium chloride, potassium chloride, sodium azide (NaN_3), 2-aminoanthracene (2AA), nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and liver S9 fraction were purchased from Sigma-Aldrich. D-Glucose and DMSO were supplied by Chempur, nutrient broth no. 2 was obtained from Oxoid and agar was purchased from Merck.

4.6.3. Mutagenicity testing. Mutagenicity was assessed using the plate incorporation method with and without metabolic activation. 100 μL of an overnight grown *Salmonella* (TA100 or TA1535) culture, 50 μL of a test compound solution (in DMSO), and 500 μL of a buffer solution (or S9 mix) were mixed and preincubated (37 °C, 30 min). Next, 2 ml of top agar were added to the tubes. Positive control plates (containing NaN_3 in the assay without metabolic activation and 2AA in the assay with metabolic activation) and negative control plates (vehicle) were performed alongside. The contents of the tubes were mixed and poured onto the top of glucose minimal agar plates and allowed to solidify. The plates were incubated (37 °C, 48 h), and revertants growing per plate in triplicate experiments were recorded.^{41,42} The mutagenicity index (MI), the ratio between the number of revertants per plate with a test compound and the number of revertants observed in a negative control, was calculated. Mutagenic activity results may be considered as positive when at least a two-fold increase in the number of revertant colonies ($\text{MI} \geq 2$) is observed with at least one concentration and a dose-response relationship is observed.^{42–44}

4.6.4. Antimutagenicity testing. 0.1 ml of an overnight culture of *S. typhimurium* TA100 or TA1535, 0.05 ml of a test

substance solution (in DMSO), 0.05 ml of a mutagen (NaN_3) and 0.5 ml of a phosphate buffer were mixed and preincubated (37 °C, 30 min). 2 ml of top agar were added and the mixture was poured onto the top of glucose minimal agar plates. Revertant colonies per plate were counted after the incubation (37 °C, 48 h). Each determination was made in triplicate. The inhibition percentage of mutagenicity was calculated using the following formula: $[1 - ((S1 - S0)/(S2 - S0))] \times 100$, where $S0$ is the number of spontaneous revertants, $S1$ is the number of revertant colonies per plate in the presence of both mutagen and test compound, and $S2$ is the number of revertant colonies per plate in the presence of mutagen only.^{42,44,45} The antimutagenic effect was defined as weak or absent (inhibition up to 25%), moderate (25–40% inhibition) or strong (40% or more inhibition).^{46,47}

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors thank Jeff Jiang, Ph.D., James Stables, Ph.D., Prof. Steve White, Harvey Kupferberg, Ph.D., John Kehne, Ph.D., Tracy Chen, Ph.D. and Shamsi Raeisi, Ph.D., from the National Institute of Neurological Disorders and Stroke (NIH, Rockville, MD, USA) for providing the results of anticonvulsant evaluation, and Prof. Katarzyna Kieć-Kononowicz for coordination of the cooperation between NINDS and the Faculty of Pharmacy at Jagiellonian University Medical College. This work was supported by the National Science Centre, Poland, decision on grant no. DEC-2015/17/N/NZ7/00966 and statutory funds K/DSC/003524, K/ZDS/007882 and K/ZDS/007884.

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