

N-Substituted amino acid *N'*-benzylamides: synthesis, anticonvulsant, and metabolic activities

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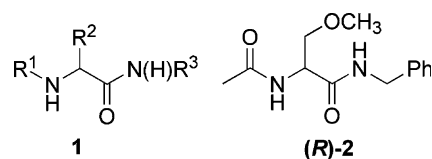
Abstract—Amino acid amides (AAA) were prepared and evaluated in seizure models. The AAA displayed moderate-to-excellent activity in the maximal electroshock seizure (MES) test and were devoid of activity in the subcutaneous Metrazol-induced (scMet) seizure test. The AAA anticonvulsant activity was neither strongly influenced by the C(2) substituent nor by the degree of terminal amine substitution. An in vitro metabolism study suggested that the structure–activity relationship pattern was due, in part, to metabolic processes that occurred at the N-terminal amine unit.

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1. Introduction

Amino acids that are functionalized at both the N- and C-termini are proven potent anticonvulsant agents.^{1–3} These compounds are termed functionalized amino acids (FAA, **1**). We conducted a SAR study of >250 compounds¹ that identified (*R*)-*N*-benzyl-2-acetamido-3-methoxypropionamide ((*R*)-**2**) as the lead compound.^{1j} (*R*)-**2** has entered phase II clinical trials for the treatment of epilepsy and neuropathic pain under Schwarz Pharma sponsorship. An important FAA structural unit is the N-terminus. In the initial design of FAA, the N-terminal amine was protected as an amide to provide compounds with increased lipophilicity.^{1a} Subsequent studies demonstrated the importance of the acetamido unit ($R^1 = C(O)CH_3$) for potent anticonvulsant activity and showed that either a decrease^{1k} (i.e., $R^1 = C(O)H$) or increases^{1b} (i.e., $R^1 = C(O)CH_2CH_3$, $C(O)C(H)(CH_3)_2$, $C(O)C(CH_3)_3$) in the size of this moiety led to reduced activity. Furthermore, when the acetamido

($CH_3C(O)N(H)$) unit in **1** was replaced with methyl, methoxy, hydroxy, acetoxy, or halogen we obtained compounds with diminished anticonvulsant activity.^{4,5}

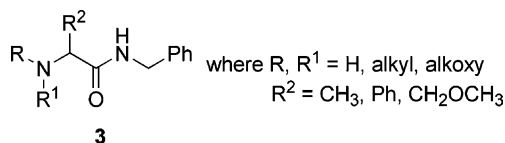


The importance of the intact acetamido group in FAA has recently been reexamined by our^{1k,6} and Paruszewski's^{2a,b,d} research groups. Both reported that substituted amino acid derivatives, in which the acetyl ($C(O)CH_3$) substructure within the acetamido ($CH_3C(O)N(H)$) moiety in FAA was replaced with either a hydrogen or a small alkyl unit to give primary and secondary amines, respectively, prevented seizures in the animal models. The finding for *N,N*-dialkyl amino acid derivatives was similar. Significantly, conversion of the acetamido unit in **1** to an amino moiety provided amino acid amides (AAA, **3**) that are likely to have increased water solubility compared with their FAA (**1**) counterparts. The hydrophilicity of the terminal amino unit may permit

Keywords: Amino acid *N'*-benzylamides; Synthesis; Anticonvulsants; Metabolism.

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the introduction of lipophilic groups at the other sites of the molecule while still retaining the necessary physicochemical properties for drug administration. In this study, we present an in-depth inspection of the N-terminus requirements within substituted AAA (**3**) for anticonvulsant activity, demonstrate that AAA are potent anticonvulsants, and document that the terminal amino group is prone to metabolic change.



2. Results and discussion

2.1. Choice of compounds and methods of pharmacological evaluation

AAA structural sites 1 and 2 in AAA (Fig. 1) were systematically altered to provide **4–30** (Fig. 2). We varied the AAA N-terminus (site 1) by incorporating acyclic (primary, secondary) and cyclic alkylamino, and alkoxyamino units at this position. The second was the R^2 moiety (site 2). Here, we restricted our choice to the methyl (CH_3), phenyl (C_6H_5), or methoxymethyl (CH_2OCH_3) units to give AAA series A–C, respectively. All three units were evaluated in the FAA series, thus

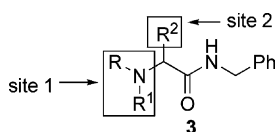


Figure 1. Sites of AAA modification.

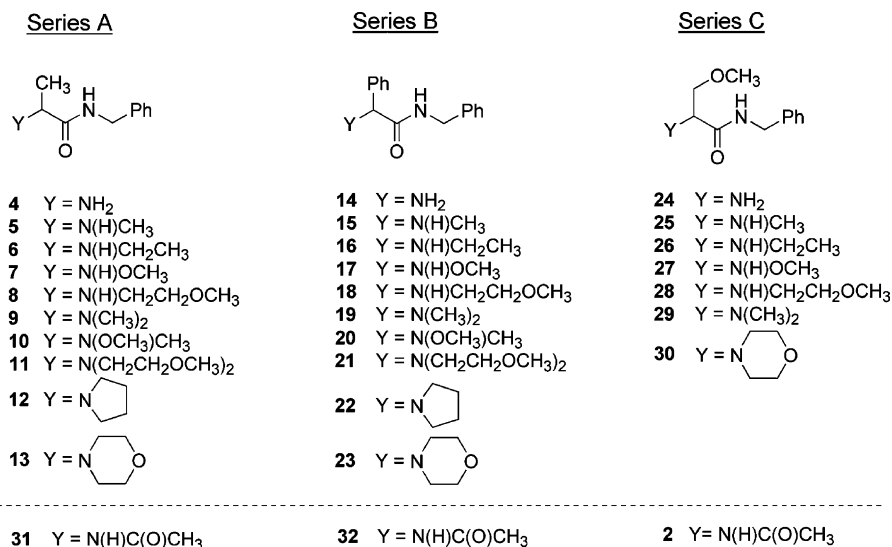
31,^{1b} **32**,^{1b} **2**,^{1j} served as our reference compounds. For these FAA, we found a significant improvement in anticonvulsant activities in maximal electroshock-induced seizure test (MES) in mice and rats as we progressed from **31** to **32** to **2**.

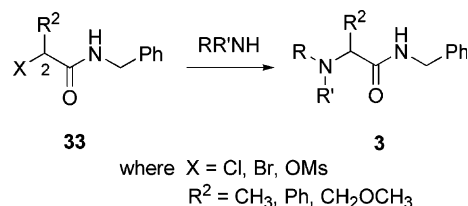
For most compounds, we prepared the racemic AAA, since we did not know if either isomer would exhibit preferential activity. This choice obviated the need to prepare optically pure amines. To test if AAA, like FAA, exhibited a stereochemical preference for anticonvulsant activity^{1c–e,g,j,l} we synthesized the individual (*R*)- or (*S*)-enantiomers or both for **4**, **24**, and **26**.

The anticonvulsant activities for AAA **4–30** were evaluated in rodents at the NIH's Anticonvulsant Screening Program (ASP) (Rockville, MD). In one of the initial tests, the compound was administered to mice intraperitoneally (ip), followed by the determination of its anticonvulsant properties measured by means of the MES and the subcutaneous Metrazol-induced convulsion (scMet) models.^{7,8} In the second test, the compound was administered to rats orally (po) and then evaluated in the MES-seizure model. Neurological toxicity (Tox) was also determined. For mice it was accomplished using the rotorod model,⁹ and in rats by the observation of motor impairment utilizing assessment of muscle tone, positional sense, righting, and gait and stance. Selected compounds were further evaluated in the MES test in mice (po) and rats (ip) under a more strict monitoring of dosages and activity time spans.

2.2. Synthesis

The AAA were prepared from the corresponding 2-halogen or 2-mesylate of the 2-substituted benzyl amide **33** followed by either amine or hydroxylamine displacement (Scheme 1). In series A, 2-chloropropionyl chloride (**34**) and 2-bromopropionyl bromide (**35**) served as our starting materials, and in series B, 2-chloro-2-

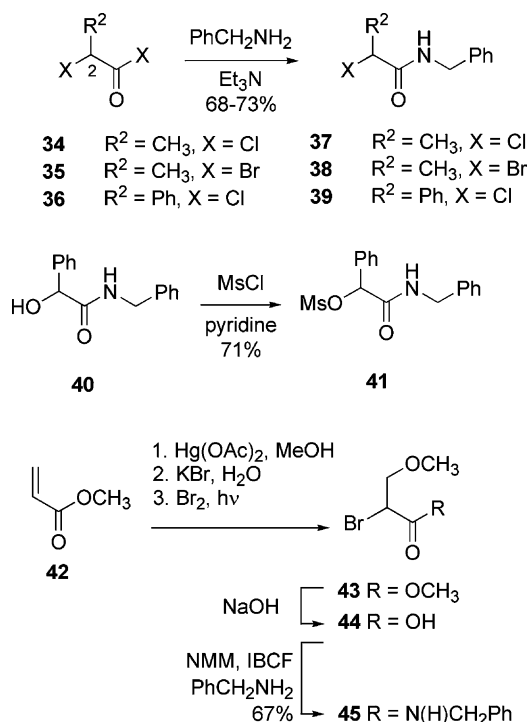
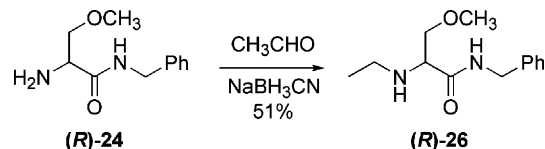




Scheme 1. General synthetic route to AAA.

phenylacetyl chloride (**36**) was employed in most syntheses (Scheme 2). Treatment of **34–36** with an equivalent of benzylamine gave **37**,¹⁰ **38**, and **39**, respectively. We found it necessary to use mesylate **41** in place of **39** for reactions involving hydroxylamines. Mesylate **41**⁵ was prepared from alcohol **40**⁵ with mesyl chloride and pyridine (Scheme 2). For series C AAA, we synthesized **45** from methyl acrylate (**42**) using the protocol of Carter and West (Scheme 2).¹¹ Intermediates **37–39**, **41**, and **45** were then treated with commercially available amines and hydroxylamines to afford compounds **5–13**, **15–23**, and **25–30** in 35–99% yields (Fig. 2). Racemic amines **4**,^{1a} **6**,¹¹ **9**,^{2a} **14**,^{1b} **15**,¹² **23**,¹³ **24**,¹⁰ and optically active amines (*R*)-**4**¹⁴ and (*S*)-**4**¹⁵ have been reported. Optically pure (*R*)-**26** was synthesized in 51% yield by condensation of amine (*R*)-**24** with acetaldehyde followed by imine reduction with sodium cyanoborohydride (Scheme 3).

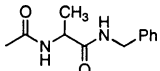
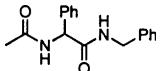
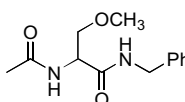
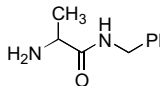
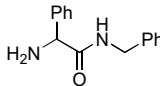
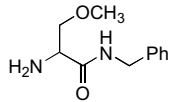
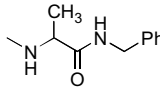
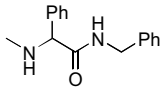
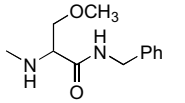
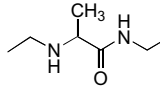
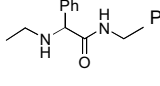
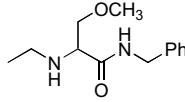
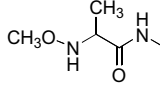
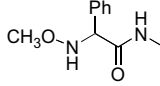
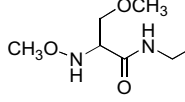
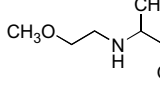
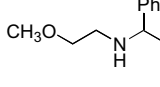
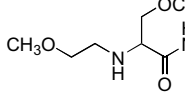
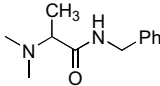
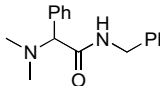
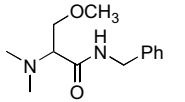
Spectral data (IR, ¹H NMR, ¹³C NMR, low-resolution mass, high-resolution mass) consistent with their proposed structures were obtained for the compounds prepared in this study. Satisfactory elemental analyses were obtained for all compounds other than **28**.

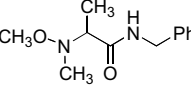
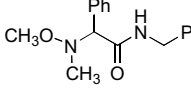
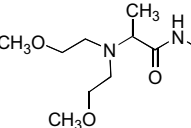
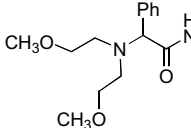
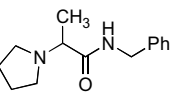
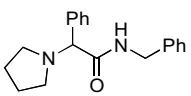
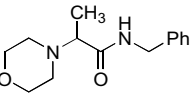
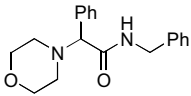
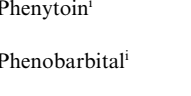
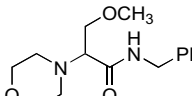
Scheme 2. Synthesis of *N*-benzyl-2-substituted amides.Scheme 3. Synthesis of (*R*)-*N*-benzyl-2-ethylamino-3-methoxypropionamide.

2.3. Pharmacological evaluation

The in vivo anticonvulsant activities for racemic amines **4–30** are summarized in Tables 1 and 2, along with MES activities of the

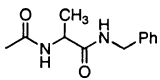
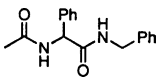
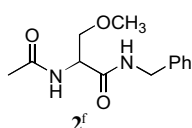
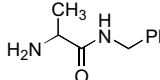
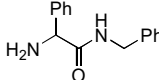
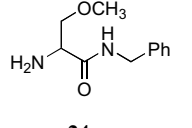
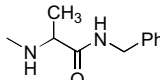
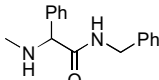
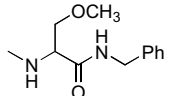
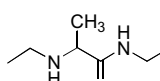
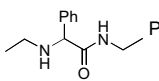
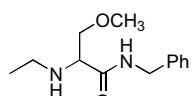
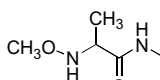
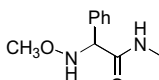
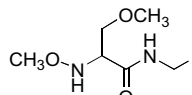
Table 1. Selected pharmacological data for AAA and FAA in mice^a

Series A				Series B				Series C			
Compd structure	MES, ^b ED ₅₀	Tox, ^c TD ₅₀	PI ^d	Compd structure	MES, ^b ED ₅₀	Tox, ^c TD ₅₀	PI ^d	Compd structure	MES, ^b ED ₅₀	Tox, ^c TD ₅₀	PI ^d
 31^e	76 [1] (67–89)	454 [0.5] (420–500)	5.9	 32^e	20 [0.5] (17–25)	97 [0.5] (80–118)	4.8	 2^f	8.3 [0.5] (7.9–9.8)	43 [0.25] (38–47)	5.2
 4	>100, <300	>300	—	 14	>100, <300	>100, <300	—	 24	84 [0.25] (65–97)	290 [0.25] (240–320)	3.5
 5^g	31 (21–41)	99 (75–121)	3.2	 15	46 [0.25] (34–59)	83 [0.25] (64–104)	1.8	 25	68 [0.25] (55–96)	290 [0.25] (250–330)	4.2
 6	>100	>100, <300	—	 16	<30	>30, <100	—	 26	51 [0.25] (42–61)	150 [0.25] (120–180)	2.9
 7	94 [0.25] (76–125)	410 [0.5] (290–590)	4.3	 17	>100, <300	>100, <300	—	 27	>30, <100	>100, <300	—
 8	>30, <100	>100, <300	—	 18	>30, <100	>30, <100	—	 28	>30, <100	>100, <300	—
 9^g	74 [0.25] (61–90)	160 [0.25] (140–180)	2.1	 19	36 [0.25] (30–46)	72 [0.25] (57–86)	2.0	 29	>300	~300</	

	>30, <100	>100, <300	—		>100, <300	>100, <300	—	— ^h
10				20				
	>30, <100	>100, <300	—		>30, <100	>100, <300	—	— ^h
11				21				
	>100, <300	>100, <300	—		>30, <100	>30, <100	—	— ^h
12				22				
	>30, <100	~300	—		>100, <300	>100, <300	—	
13				23				
								
				30			>100, <300	~300
Phenytoin ⁱ	9.5 [2] (8.1–10)	66 [2] (53–72)	6.9					
Phenobarbital ⁱ	22 [1] (15–23)	69 [0.5] (63–73)	3.2					
Valproate ⁱ	270 [0.25] (250–340)	430 [0.25] (370–450)	1.6					

^a The compounds were administered intraperitoneally.^b MES = maximal electroshock seizure test in mg/kg. Numbers in parenthesis are 95% confidence intervals. The dose effect data was obtained at the ‘time of peak effect’ (indicated in hours in the brackets).^c Tox = neurologic toxicity determined from rotorod test in mg/kg. Numbers in parenthesis are 95% confidence intervals. The dose effect data was obtained at the ‘time of peak effect’ (indicated in hours in the brackets).^d PI = protective index (TD₅₀/ED₅₀).^e Ref. 1b.^f Ref. 1j.^g Ref. 2a.^h Not prepared.ⁱ Ref. 17.

Table 2. Selected pharmacological data for AAA and FAA in rats^a

Series A				Series B				Series C			
Compd structure	MES, ^b ED ₅₀	Tox, ^c TD ₅₀	PI ^d	Compd structure	MES, ^b ED ₅₀	Tox, ^c TD ₅₀	PI ^d	Compd structure	MES, ^b ED ₅₀	Tox, ^c TD ₅₀	PI ^d
 31^c	48 [1] (32–72)	>1000	>21	 32^c	48 [4] (31–68)	>1000	>21	 2^f	3.8 [2] (2.9–5.5)	390 [1] (320–520)	102
 4	14 [1] (7–22)	>500	>36	 14	>30, <100	>30	—	 24	29 [1] (18–45)	>450	>16
 5^g	27 (16–48)	>500	>18	 15	20 [0.25] (8–39)	>63, <500	>3.1	 25	28 [2] (19–40)	>400	>14
 6	32 [4] (19–46)	>125	>3.9	 16	20 [0.25] (14–32)	>125, <250	—	 26	22 [0.25] (13–33)	>500	>23
 7	37 [2] (25–52)	>500	>13.5	 17	>30	>30	—	 27	22 [0.5] (14–29)	>500	>23

^a The compounds were administered orally.

^b MES = maximal electroshock seizure test in mg/kg. Numbers in parenthesis are 95% confidence intervals. The dose effect data was obtained at the ‘time of peak effect’ (indicated in hours in the brackets).

^c Tox = neurologic toxicity determined from the observation of motor impairment in mg/kg. Numbers in parenthesis are 95% confidence intervals. The dose effect data was obtained at the ‘time of peak effect’ (indicated in hours in the brackets).

^d PI = protective index (TD₅₀ /ED₅₀).

^e Ref. 1b.

^f Ref. 1j.

^g Ref. 2a.

^h Not prepared.

ⁱ Ref. 17.

^j No ataxia observed up to 3000 mg/kg.

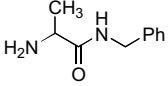
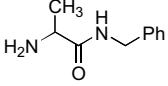
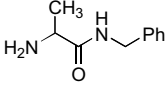
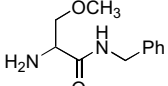
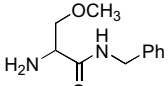
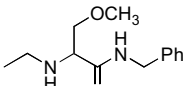
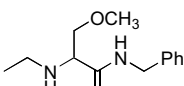
most C(2) phenyl AAA (**15**, **16**, **18**, **19**, **22**) displayed excellent activity in rats ($ED_{50} < 30$ mg/kg), and the most potent compound, **19** ($ED_{50} = 7.1$ mg/kg), was seven times more potent than the corresponding FAA, **32** ($ED_{50} = 48$ mg/kg).^{1b} The anticonvulsant activity of **19** exceeded that of phenytoin ($ED_{50} = 30$ mg/kg) and phenobarbital ($ED_{50} = 9.1$ mg/kg).^{16,17} The remaining analogues (**14**, **20**, **21**, and **23**), excluding **17**, showed moderate activity ($ED_{50} = 30$ – 100 mg/kg). Finally, in the C(2) methoxymethyl series (**24**–**30**) a narrow range of activity was observed in rats ($ED_{50} = 22$ – 35 mg/kg), except for the tertiary amine **30** in which a slightly lower anticonvulsant activity was found ($ED_{50} = 30$ – 100 mg/kg). The most active AAA in this subset were **26** and **27** ($ED_{50} = 22$ mg/kg), which were nearly six times less potent than **2**.^{1j} The neurological toxicities for AAA in rats were generally low. We found increased toxicities for the C(2) phenyl series (**14**–**23**) compared with the C(2) methyl (**4**–**13**) and C(2) methoxymethyl (**24**–**30**) analogues. However, our most active compound, **19**, provided a high protective index (PI = 33) that compared favorably with both phenytoin and phenobarbital.^{16,17}

Little differences in AAA activity were observed among the three classes of compounds [C(2) methyl, C(2) phenyl, C(2) methoxymethyl], and the observed potencies of the compounds were not measurably affected by the degree and type of terminal amine substitution. For their FAA counterparts we observed distinctive SAR patterns.¹ Why the absence of similar trends for AAA? We suggest that several factors contributed to the observed activity profile of AAA in rodents. AAA are basic compounds and the N-terminal substituents are prone to metabolism. By comparison, FAA are neutral and (*R*)-**2** was shown to undergo little metabolic change either in rodents or dogs. In phase I human trials (*R*)-**2** had nearly 100% oral bioavailability.¹⁸ Thus, AAA and FAA are likely to have different pharmacokinetic and metabolic properties that affect drug concentrations in the brain. In Section 2.4, we test this hypothesis, in part, by monitoring the in vitro metabolism of a series of C(2) phenyl AAA.

Optically pure AAA **4**, **24**, and **26** were evaluated using the MES test and their ED_{50} values were compared with the corresponding racemic mixtures (Table 3). For **4** in mice (ip), we observed an increase in anticonvulsant activity proceeding from (*S*)-**4** ($ED_{50} > 300$ mg/kg) to (*R,S*)-**4** ($ED_{50} = 100$ – 300 mg/kg) to (*R*)-**4** ($ED_{50} = 10$ – 30 mg/kg). This trend is similar to that previously reported for FAA **31**.^{1d} The preference for (*R*) versus (*S*) stereochemistry was also observed in rats (po) where (*R*)-**4** ($ED_{50} = 19$ mg/kg) was more active than (*S*)-**4** ($ED_{50} > 80$ mg/kg). We found that (*R*)-**4** and (*R,S*)-**4** ($ED_{50} = 14$ mg/kg) displayed nearly identical seizure protection. The stereochemical preference for the (*R*)-isomer was also found for **24** where (*R*)-**24** showed enhanced activity compared with (*R,S*)-**24** in both mice and rats. The ED_{50} values in mice for (*R*)-**24** and (*R,S*)-**24** were 48 and 84 mg/kg, respectively, while in rats the ED_{50} values for (*R*)-**24** and (*R,S*)-**24** were 18 and 29 mg/kg, respectively. Surprising to us was the finding that the *N*-ethyl analogue of **24**, compound **26**, did not follow the pattern observed for **4**, **24**, and FAA.^{1c–e,g,j,l} Here, we found

in mice (ip) that (*R,S*)-**26** ($ED_{50} = 51$ mg/kg) was more effective in controlling seizures than (*R*)-**26** ($ED_{50} > 100$ mg/kg) while both compounds displayed comparable activities in rats (po) [ED_{50} values (mg/kg): (*R,S*)-**26**, 22 mg/kg; (*R*)-**26**, ~30 mg/kg]. At this stage, we do not have sufficient data to provide a rationale for this finding since we have not prepared and tested (*S*)-**26**. We do note that both C(2) substituents (i.e., CH_2OCH_3 , $N(H)CH_2CH_3$) in **26**

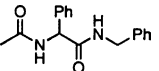
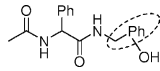
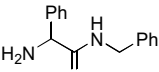
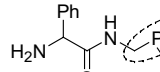
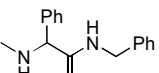
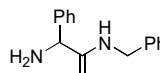
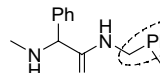
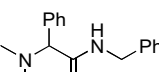
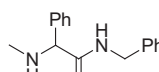
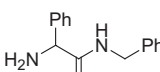
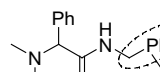
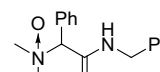
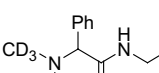
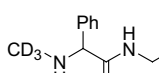
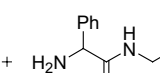
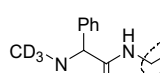
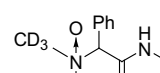
Table 3. Effect of stereochemistry at the C(2) position on AAA anticonvulsant activity

Compd	C(2)	Mice (ip) ^a			Rat (po) ^b		
		MES, ^c ED ₅₀	Tox, ^d TD ₅₀	PI ^e	MES, ^c ED ₅₀	Tox, ^d TD ₅₀	PI ^e
	(<i>R,S</i>)- 4	>100, <300	>300	—	14 [1] (7–22)	>500	>36
	(<i>R</i>)- 4	>10, <30	>100, <300	—	19 [2] (13–25)	>30	>1.5
	(<i>S</i>)- 4	>300	>300	—	>80	>80	—
	(<i>R,S</i>)- 24	84 [0.25] (65–97)	290 [0.25] (240–320)	3.5	29 [1] (18–45)	>450	>16
	(<i>R</i>)- 24	48 [0.25] (40–61)	>30, <100	—	18 [4] ^f	>500	>28
	(<i>R,S</i>)- 26	51 [0.25] (42–61)	150 [0.25] (120–180)	2.9	22 [0.25] (13–33)	>500	>23
	(<i>R</i>)- 26	>100	>100, <300	—	~30 [0.5]	>30	—

^a The compounds were administered intraperitoneally.^b The compounds were administered orally.^c MES = maximal electroshock seizure test in mg/kg. Numbers in parenthesis are 95% confidence intervals. The dose effect data was obtained at the 'time of peak effect' (indicated in hours in the brackets).^d Tox = neurologic toxicity determined from rotorod test in mice and the observation of motor impairment in rats in mg/kg. Numbers in parenthesis are 95% confidence intervals. The dose effect data was obtained at the 'time of peak effect' (indicated in hours in the brackets).^e PI = protective index (TD₅₀/ED₅₀).^f Ref. 6.

ions in the LC–MS profile were obtained for the substrates and most metabolites. We characterized most metabolites formed after 60 min microsomal incubation. Calibration curves were obtained using UV detection for the AAA and **32** and were linear over a 5–500 μ M range, thus permitting us to quantify the amount of substrate depletion over time. We recognized that without a complete set of metabolite calibration curves we would not be able to provide precise percentages of each metabolite produced. Thus, our assignment of the major and minor metabolic adducts rested upon the assumption that these compounds displayed

Table 4. Proposed metabolism profile for *N*-substituted C(2) phenyl amino acid *N'*-benzylamides^a

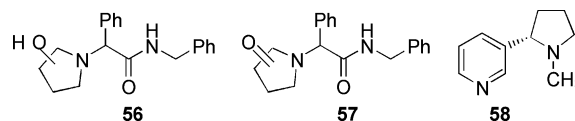
Substrate	Compd	Substrate depletion ^b , %	N-Dealkylation metabolites (type 1)	Minor metabolites (type 2)	Minor metabolites (type 3)
 (12.0)	32	~10	—	 47 (5.3)	—
 (6.5)	14	~25	—	 48 (3.2)	—
 (7.1)	15	~32	 14 (6.6)	 49 (3.4)	—
 (11.0)	19	~75	 +  15 (7.1) 14 (6.6)	 50 (4.6)	 51 (7.3)
 (10.2)	46	— ^c	 +  52 (7.0) 14 (6.6)	 53 (4.3)	 54 (7.3)

those metabolites were characterized by the loss of 106 and 151 (data not shown). The 151 Da unit corresponded to the elimination of an ‘oxygenated’ 135 Da fragment previously observed for substrates **19**, **22**, and **46** (data not shown). This finding suggested the presence of an OH group within the *N*-benzylamide unit. Further supporting this contention was the detection of a second fragment ion that equated to the loss of 106 from the molecular ion. This fragmentation pattern suggests that hydroxylation occurred either at the methylene position of the benzylamide unit or within the aromatic unit. The early retention times in the HPLC are consistent with either metabolite and there is prior literature precedence for both.²¹ Significantly, all of the type 2 metabolic processes observed for our C(2) phenyl AAA corresponded to *N*-benzylamide hydroxylation. Under our assay conditions, we did not detect C(2) phenyl hydroxylation (phenolic)-derived products.

Type 3 metabolites were observed for only **19** and **46**. The fragmentation patterns for type 3 metabolites were consistent with the formation of *N*-oxides **51** and **54**. The major MS/MS fragments were the loss of 60 for metabolite **51** and 66 for the deuteriated adduct **54** to provide *m/z* 225 and suggested the molecular cleavage of the *N*-oxide units. Significantly, the detection of fragmentation ions at *m/z* = 107 for these type 3 metabolites indicated the presence of an unmodified *N*-benzylamine unit. The LC–MS/MS analysis of a synthetic sample of *N*-oxide **51** confirmed the identity of type 3 metabolite (data not shown).

We observed that the in vitro metabolism profiles for **14**, **15**, **19**, **22**, **32**, and **46** were similar for both mice and rats microsomes. Incubation of **14**, **15**, **19**, **22**, and **32** with the microsomes in the absence of NADPH gave very little or no consumption of substrate (<2%) and suggested little nonoxidative metabolism for this class of compounds. Addition of NADPH (1 mM) to the incubation medium led to increased levels of metabolism and the extent of reaction was substrate dependent. FAA **32** showed little change (~10%) over the 60-min reaction time course. The principal metabolite for **32** resulted from *N*-benzylamide hydroxylation. Our finding that **32** underwent little in vitro metabolism was in agreement with previous results obtained for FAA (*R*)-**2**.¹⁸ Removal of the acetyl unit in **32** to give AAA **14** led to a threshold increase in metabolism (60 min: est. 25% substrate depletion) with *N*-benzylamide hydroxylation accounting for the majority of the product. Successive incorporation of methyl units at the AAA N-terminal amine site to provide **15** and **19** led to a steady, further increase in the extent of metabolism (60 min: **15**: est. 32%, **19**: est. 75%). The major pathway for AAA **15**, **19**, and **46** consumption was N-dealkylation. For **15**, **19**, and **46** *N*-benzylamide hydroxylation (type 2) was observed; while for **19** and **46**, *N*-oxide formation (type 3) accounted for a substantial portion of the depleted substrate. *N*-Oxide formation is a common metabolism pathway for tertiary amines^{22,23} and we were surprised that we did not detect any *N*-oxide product for AAA **22**. Inclusion of the N-terminal group within a cyclic pyrrolidine ring to give **22** led to pyrrolidine ring hydrox-

ylation to give **56**. The two peaks in the HPLC (10.6, 10.9 min) may correspond to a pair of diastereoisomers where hydroxylation occurred at one of the pyrrolidine ring carbons, or the two peaks may correlate to two metabolic products where hydroxylation occurred at different positions within the pyrrolidine ring. Furthermore, enzymatic oxidation proceeded to give metabolite **57** (HPLC: 13.6 min). The MS/MS data did not allow us to identify the position of the hydroxyl or the carbonyl unit in the oxidized pyrrolidine ring. These observations were in general agreement with those previously reported for the metabolism of nicotine (**58**).²⁴ For **58**, hydroxylation proceeded selectively at the methylene group adjacent to the *N*-methyl position.



When we monitored the rate of substrate depletion we found little differences for the mice (Fig. 3A) and the rats (Fig. 3B). Accordingly, we suspect that the agent(s) responsible for the control of MES-induced seizures is the same in both mice (ip) and rats (po). In agreement with this notion, we observed the same order of anti-convulsant activity (**19** > **15**, **22** > <

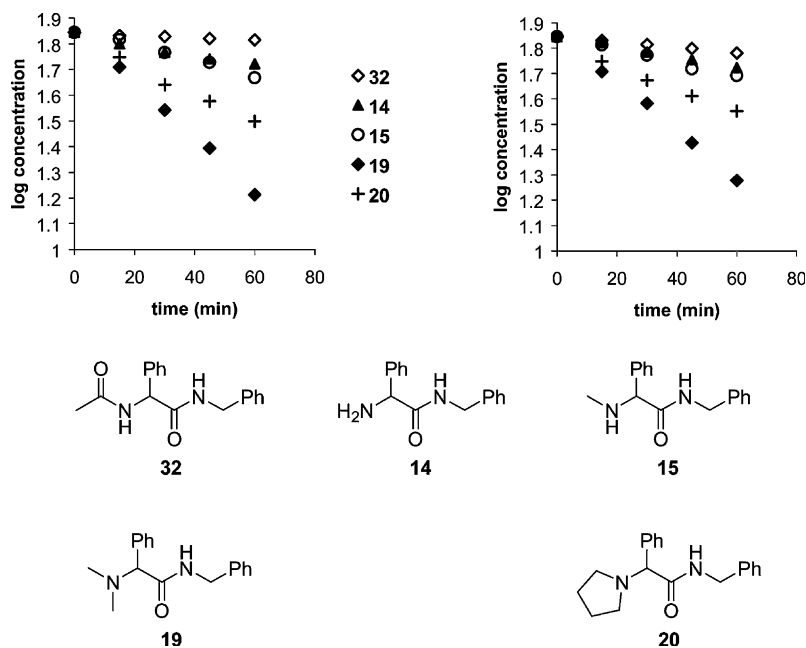


Figure 3. In vitro metabolism rate of substrate consumption of C(2) phenyl AAA. Incubations were performed with 1 mg microsomal protein, 1 mM NADPH, and 100 μ M substrate at room temperature for 60 min.

C(2)-phenyl AAA both the anticonvulsant activities and the extent of metabolism increased in proceeding from primary (**14**) to secondary (**15**) to tertiary (**19**, **32**) amines. These trends diminished the beneficial effects of *N*-alkyl substitution on biological activity and contributed, in part, to the absence of a distinctive SAR trend for this class of compounds.

4. Experimental

4.1. General methods

Melting points were determined with a Thomas–Hoover melting point apparatus and are uncorrected. Infrared spectra (IR) were run on a ATI Mattson Genesis Series FTIRTM spectrometer. Absorption values are expressed in wave-numbers (cm^{-1}). Optical rotations were obtained on a Jasco P-1030 polarimeter. Proton (^1H NMR) and carbon (^{13}C NMR) nuclear magnetic resonance spectra were taken either on General Electric QE-300 NMR or Varian Gemini 2000 spectrometers. Low resolution mass spectra (CI+) were obtained with a Varian MAT CH-5 spectrometer by Dr. M. Moini at the University of Texas—Austin. The high-resolution chemical ionization mass spectrum was performed on a Finnigan MAT TSQ-70 by Dr. M. Moini at the University of Texas—Austin. Microanalysis were provided by Atlantic Microlab, Inc. (Norcross, GA).

4.2. Synthesis of *N'*-alkyl-substituted *N*-benzyl amino acid amides. Method A

A solution of *N*-benzyl-2-haloamino acid amide (1 equiv) in amine (excess) was stirred in a Schlenk flask. In select cases, THF was added. The white pre-

cipitate that formed during the reaction was filtered and the filtrate was evaporated and purified by column chromatography (SiO_2 ; MeOH– CHCl_3 or EtOAc–hexanes) to obtain the desired product.

4.3. Synthesis of *N*-benzyl-substituted amino acid amide. Method B

A THF (45–125 mL) solution of carboxylic acid (1 equiv) was cooled (-78°C) and then 4-methylmorpholine (1.25 equiv) was added and the solution was stirred (2 min). Isobutyl chloroformate (1.25 equiv) was then added and the reaction was stirred (2 min) followed by the addition of benzylamine (1.25 equiv). The reaction was stirred at -78°C (15 min), allowed to warm to room temperature and then stirred (1.5 h). The reaction mixture was filtered and the filtrate evaporated. The residue was purified by column chromatography (SiO_2 ; 1:49, MeOH– CHCl_3) to obtain the desired product.

4.4. Synthesis of (*R,S*)-*N*-benzyl-2-bromo-3-methoxypropionamide (**45**)

Compound **45** (1.17 g, 73%) was prepared as a white solid from **44**¹¹ (1.06 g, 5.89 mmol), 4-methylmorpholine (810 μL , 7.37 mmol), isobutyl chloroformate (960 μL , 7.37 mmol), and benzylamine (805 μL , 7.37 mmol) utilizing method B: mp $71\text{--}73^\circ\text{C}$; R_f 0.72 (1:49, MeOH– CHCl_3); ^1H NMR (CDCl_3) δ 3.43 (s, $\text{O$

($M^+ + 1$, 100), 102 (17); M_r (+CI) 272.02884 [$M^+ + 1$] (calcd for $C_{11}H_{15}^{79}BrNO_2$ 272.02861). Anal. Calcd for $C_{11}H_{14}BrNO_2$: C, 48.55; H, 5.18; N, 5.15. Found: C, 48.68; H, 5.14; N, 5.13.

4.5. Synthesis of (*R,S*)-*N*-benzyl-2-(*N*-methoxyamino)-propionamide (7)

Compound **7** (975 mg, 64%) was prepared as a pale-yellow oil from **38**²⁶ (1.77 g, 7.31 mmol), *O*-methoxyhydroxylamine (4.0 mL, 76.5 mmol), and THF (5 mL) at 45 °C (4 d) utilizing method A. Purification was by column chromatography (SiO₂; 1:49, MeOH–CHCl₃): R_f 0.36 (1:49, MeOH–CHCl₃); IR (neat) 3291 (br), 3087, 3031, 1655, 1537 cm⁻¹; ¹H NMR (CDCl₃) δ 1.27 (d, J = 7.2 Hz, CH₃), 3.49 (s, OCH₃), 3.63 (dq, J = 5.4, 7.2 Hz, CH), 4.44 (dd, J = 5.7, 15.0 Hz, CHH'Ph), 4.54 (dd, J = 6.3, 15.0 Hz, CHH'Ph), 5.64 (d, J = 5.4 Hz, NH), 6.98 (br s, NH), 7.25–7.38 (m, 5PhH); ¹³C NMR (CDCl₃) 15.7, 43.2, 60.2, 62.0, 127.4, 127.6, 128.7, 138.2, 173.3 ppm; MS (+CI) (rel intensity) 210 (14), 209 ($M^+ + 1$, 100); M_r (+CI) 209.13004 [$M^+ + 1$] (calcd for $C_{11}H_{17}N_2O_2$ 209.12900). Anal. Calcd for $C_{11}H_{16}N_2O_2 \cdot 0.1H_2O$: C, 62.90; H, 7.77; N, 13.34. Found: C, 62.75; H, 7.69; N, 13.20.

4.6. Synthesis of (*R,S*)-*N*-benzyl-2-(2-methoxyethyl-amino)propionamide (8)

Compound **8** (1.25 g, 95%) was prepared as an oil from **37**¹⁰ (1.10 g, 5.58 mmol) and 2-methoxyethylamine (20 mL, 230 mmol) at 50 °C (2 d) utilizing method A. Purification was by column chromatography (SiO₂; 1:24, MeOH–CHCl₃): R_f 0.39 (1:24, MeOH–CHCl₃); IR (neat) 3313 (br), 3064, 3031, 1656, 1525 cm⁻¹; ¹H NMR (CDCl₃) δ 1.35 (d, J = 6.9 Hz, CH₃), 2.18 (br s, NH), 2.63–2.71 (m, NHCHH'CH₂), 2.80–2.88 (m, NHCHH'CH₂), 3.28 (q, J = 6.9 Hz, CH), 3.29 (s, OCH₃), 3.35–3.48 (m, CH₂OCH₃), 4.44 (d, J = 6.0 Hz, CH₂Ph), 7.25–7.32 (m, 5PhH), 7.77 (t, J = 6.0 Hz, NHCH₂Ph); ¹³C NMR (CDCl₃) 19.6, 42.9, 47.8, 58.1, 58.6, 71.4, 127.2, 127.5, 128.5, 138.5, 174.6 ppm; MS (+CI) (rel intensity) 238 (15), 237 ($M^+ + 1$, 100); M_r (+CI) 237.16040 [$M^+ + 1$] (calcd for $C_{13}H_{21}N_2O_2$ 237.16030). Anal. Calcd for $C_{13}H_{20}N_2O_2 \cdot 0.31H_2O$: C, 64.55; H, 8.59; N, 11.58. Found: C, 64.87; H, 8.55; N, 11.72.

4.7. Synthesis of (*R,S*)-*N*-benzyl-2-(*N*-methoxy-*N*-meth-ylamino)propionamide (10)

(m, CH_2NCH_2), 3.60–3.72 (m, CH_2OCH_2), 3.06 (q, $J = 6.9$ Hz, CH), 4.39–4.52 (m, CH_2Ph), 7.24–7.35 (m, 5PhH), 7.48 (br s, NH); ^{13}C NMR (CDCl_3) 12.1, 42.9, 50.2, 64.4, 66.9, 127.3, 127.4, 128.6, 138.5, 173.3 ppm; MS (+CI) (rel intensity) 250 (15), 249 ($\text{M}^+ + 1$, 100); M_r (+CI) 249.16088 [$\text{M}^+ + 1$] (calcd for $\text{C}_{14}\text{H}_{21}\text{N}_2\text{O}_2$ 249.16030). Anal. Calcd for $\text{C}_{14}\text{H}_{20}\text{N}_2\text{O}_2$: C, 67.72; H, 8.12; N, 11.28. Found: C, 67.48; H, 8.18; N, 11.36.

4.11. Synthesis of (*R,S*)-*N*-benzyl-2-ethylamino-2-phenylacetamide (16)

Compound **16** (1.60 g, 78%) was prepared as a thick oil from **39** (2.00 g, 7.71 mmol) and ethylamine (2.0 M in THF, 25 mL, 50 mmol) at 45 °C (5 d) utilizing method A. Purification was by column chromatography (SiO_2 ; 1:49, MeOH– CHCl_3): R_f 0.18 (1:49, MeOH– CHCl_3); IR (neat) 3296 (br), 3063, 3034, 1661, 1521 cm^{-1} ; ^1H NMR (CDCl_3) δ 1.17–1.25 (m, CH_2CH_3), 2.70–2.84 (m, CH_2CH_3), 4.34 (s, CH), 4.58 (d, $J = 5.7$ Hz, CH_2Ph), 7.33–7.53 (m, 10PhH), 7.73 (br s, NH), the signal for the remaining NH proton was not detected; ^{13}C NMR (CDCl_3) 15.3, 43.0, 43.2, 67.8, 127.1, 127.3, 127.5, 128.0, 128.6, 128.7, 138.4, 138.7, 172.3 ppm; MS (+CI) (rel intensity) 270 (16), 269 ($\text{M}^+ + 1$, 100); M_r (+CI) 269.16475 [$\text{M}^+ + 1$] (calcd for $\text{C}_{17}\text{H}_{21}\text{N}_2\text{O}$ 269.16539). Anal. Calcd for $\text{C}_{17}\text{H}_{20}\text{N}_2\text{O} \cdot 0.2\text{H}_2\text{O}$: C, 75.80; H, 7.56; N, 10.30. Found: C, 74.96; H, 7.28; N, 10.24.

4.12. Synthesis of (*R,S*)-*N*-benzyl-2-(*N*-methoxylamino)-2-phenylacetamide (17)

Compound **17** (327 mg, 35%) was prepared as a pale-yellow oil from **41** (1.10 g, 3.45 mmol) and *O*-methylhydroxylamine (4.0 mL, 76.5 mmol) at 50 °C (2 d) utilizing method A. Purification was by column chromatography (SiO_2 ; 1:1, EtOAc–hexanes): R_f 0.52 (1:1, EtOAc–hexanes); IR (neat) 3296 (br), 3066, 1662, 1534 cm^{-1} ; ^1H NMR (CDCl_3) δ 3.51 (s, OCH_3), 4.46 (dd, $J = 5.5$, 15.0 Hz, $\text{CHH}'\text{Ph}$), 4.56 (dd, $J = 6.0$, 15.0 Hz, $\text{CHH}'\text{Ph}$), 4.62 (s, CH), 6.85 (br s, NH), 7.23–7.40 (m, 10PhH), the signal for the remaining NH proton was not detected; ^{13}C NMR (CDCl_3) 43.2, 61.9, 69.1, 127.4, 127.5, 127.8, 128.6, 128.8, 135.4, 138.0, 170.5 ppm, one aromatic carbon signal was not detected and is believed to overlap with nearby peaks; MS (+CI) (rel intensity) 272 (17), 271 ($\text{M}^+ + 1$, 100), 267 (14), 240 (18), 239 (94); M_r

(2 d). EtOAc (100 mL) was added and then the reaction mixture was extracted with an aqueous 0.1 M KH_2PO_4 solution (5×20 mL). The organic layer was dried (Na_2SO_4) and evaporated. The residue was purified by column chromatography (SiO_2 ; 1:49, MeOH– CHCl_3) to obtain 1.43 g (69%) of pure **21** as a pale-yellow oil: R_f 0.24 (1:49, MeOH– CHCl_3); IR (neat) 3309 (br), 3033, 1663, 1524 cm^{-1} ; ^1H NMR (CDCl_3) δ 2.51–2.59 (m, $2\text{CHH}'\text{CH}_2\text{OCH}_3$), 2.72–2.81 (m, $2\text{CHH}'\text{CH}_2\text{OCH}_3$), 3.19 (s, 2OCH_3), 3.31–3.44 (m, $2\text{CH}_2\text{OCH}_3$), 4.48 (dd, $J = 5.2$, 14.9 Hz, $\text{CHH}'\text{Ph}$), 4.54 (dd, $J = 6.3$, 14.9 Hz, $\text{CHH}'\text{Ph}$), 4.62 (s, CH), 7.25–7.35 (m, 10PhH), 8.52 (br s, NH); ^{13}C NMR (CDCl_3) 43.5, 51.4, 58.7, 70.9, 71.1, 127.1, 127.6, 127.9, 128.2, 129.7, 136.1, 138.8, 172.2 ppm, one aromatic carbon signal was not detected and is believed to overlap with nearby peaks; MS (+CI) (rel intensity) 358 (22), 357 ($\text{M}^+ + 1$, 100); M_r (+CI) 357.21801 [$\text{M}^+ + 1$] (calcd for $\text{C}_{21}\text{H}_{29}\text{N}_2\text{O}_3$ 357.21782). Anal. Calcd for $\text{C}_{21}\text{H}_{28}\text{N}_2\text{O}_3$: C, 70.76; H, 7.92; N, 7.86. Found: C, 70.49; H, 7.94; N, 7.77.

4.17. Synthesis of (*R,S*)-*N*-benzyl-2-pyrrolidin-1-yl-2-phenylacetamide (**22**)

Compound **22** (2.47 g, 99%) was prepared as a white solid from **39** (2.20 g, 8.48 mmol) and pyrrolidine (20 mL, 240 mmol) at 40°C (18 h) utilizing method A. Purification was by column chromatography (SiO_2 ; 1:49, MeOH– CHCl_3); mp $96\text{--}97^\circ\text{C}$; R_f 0.36 (SiO_2 ; 1:49, MeOH– CHCl_3); IR (KBr) 3328, 2952, 1655, 1517 cm^{-1} ; ^1H NMR (CDCl_3) δ 1.71–1.75 (m, CH_2CH_2), 2.42–2.54 (m, CH_2NCH_2), 3.85 (s, CH), 4.42 (dd, $J = 5.6$, 15.0 Hz, $\text{CHH}'\text{Ph}$), 4.49 (dd, $J = 6.1$, 15.0 Hz, $\text{CHH}'\text{Ph}$), 7.19–7.41 (m, 10PhH), the signal for the NH proton was not detected; ^{13}C NMR (CDCl_3) 23.3, 43.1, 52.6, 75.6, 127.3, 127.6, 127.9, 128.1, 128.4, 128.6, 138.2, 138.4, 171.8 ppm; MS (+CI) (rel intensity) 296 (19), 295 ($\text{M}^+ + 1$, 100); M_r (+CI) 295.18071 [$\text{M}^+ + 1$] (calcd for $\text{C}_{19}\text{H}_{23}\text{N}_2\text{O}$ 295.18104). Anal. Calcd for $\text{C}_{19}\text{H}_{22}\text{N}_2\text{O}$: C, 77.52; H, 7.53; N, 9.52. Found: C, 77.75; H, 7.66; N, 9.57.

4.18. Synthesis of (*R,S*)-*N*-benzyl-2-methylamino-3-methoxypropionamide (**25**)

Compound **25** (1.42 g, 87%) was prepared as an oil from **45** (2.01 g, 7.39 mmol) and methylamine (2.0 M in TH

(s, OCH₃), 3.31–3.40 (m, CH, OCH₃, and CH₂OCH₃), 3.52–3.57 (m, CHCHH'OCH₃), 3.62–3.67 (m, CHCHH'OCH₃), 4.42 (d, *J* = 6.0 Hz, CH₂Ph), 7.22–7.32 (m, 5PhH), 7.96 (br s, NH); ¹³C NMR (CDCl₃) 42.5, 47.6, 58.1, 58.2, 62.1, 71.4, 72.3, 126.7, 127.0, 128.0, 138.0, 171.5 ppm; MS (+CI) (rel intensity) 268 (17), 267 (M⁺+1, 100), 208 (26); *M_r* (+CI) 267.17120 [M⁺+1] (calcd for C₁₄H₂₃N₂O₃ 267.17087).

4.22. Synthesis of (*R,S*)-*N*-benzyl-2-dimethylamino-3-methoxypropionamide (**29**)

Compound **29** (3.15 g, 97%) was prepared as a pale-yellow oil from **45** (3.75 g, 13.79 mmol) and dimethylamine (2.0 M in THF, 25 mL, 50 mmol) at room temperature (18 h) utilizing method A. Purification was by column chromatography (SiO₂; 1:49, MeOH–CHCl₃): *R_f* 0.27 (1:49, MeOH–CHCl₃); IR (neat) 3322 (br), 1662, 1523 cm⁻¹; ¹H NMR (CDCl₃) δ 2.35 (s, N(CH₃)₂), 3.05 (dd, *J* = 3.6, 5.7 Hz, CH), 3.24 (s, OCH₃), 3.71 (dd, *J* = 5.7, 10.5 Hz, CHH'OCH₃), 3.82 (dd, *J* = 3.6, 10.5 Hz, CHH'OCH₃), 4.43 (dd, *J* = 5.3, 14.4 Hz, CHH'Ph), 4.50 (dd, *J* = 5.3, 14.4 Hz, CHH'Ph), 7.22–7.35 (m, 5PhH), 7.57 (br s, NH); ¹³C NMR (CDCl₃) 42.8, 43.0, 58.8, 69.6, 70.4, 127.2, 127.5, 128.5, 138.5, 171.4 ppm; MS (+CI) (rel intensity) 238 (17), 237 (M⁺+1, 100), 102 (41); *M_r* (+CI) 237.15994 [M⁺+1] (calcd for C₁₃H₂₁N₂O₂ 237.16030). Anal. Calcd for C₁₃H₂₀N₂O₂: C, 66.07; H, 8.53; N, 11.86. Found: C, 65.89; H, 8.69; N, 11.81.

4.23. Synthesis of (*R,S*)-*N*-benzyl-2-morpholin-4-yl-3-methoxypropionamide (**30**)

Compound **30** (950 mg, 80%) was prepared as a clear thick oil from **45** (1.17 g, 4.30 mmol) and morpholine (20 mL, 229 mmol) at 45 °C (2 d) utilizing method A. Purification was by column chromatography (SiO₂; 1:49, MeOH–CHCl₃): *R_f* 0.35 (1:49, MeOH–CHCl₃); IR (neat) 3319 (br), 1662, 1521 cm⁻¹; ¹H NMR (CDCl₃) δ 2.54–2.73 (m, CH₂NCH₂), 3.11 (dd, *J* = 3.5, 5.6 Hz, CH), 3.32 (s, OCH₃), 3.60–3.74 (m, CH₂OCH₂ and CHH'OCH₃), 3.83 (dd, *J* = 3.5, 10.4 Hz, CHH'OCH₃), 4.42 (dd, *J* = 6.0, 14.9 Hz, CHH'Ph), 4.50 (dd, *J* = 6.3, 14.9 Hz, CHH'Ph), 7.24–7.35 (m, 5PhH), 7.63 (br s, NH); ¹³C NMR (CDCl₃) 42.9, 50.8, 58.7, 66.9, 68.7, 69.6, 127.1, 127.2, 128.4, 138.2, 170.6 ppm; MS (+CI) (rel intensity) 280 (16), 279 (M⁺+1, 100

4.27. Pharmacology

Compounds were screened under the auspices of the National Institutes of Health's Anticonvulsant Screening Project. Experiments were performed in male rodents [albino Carworth Farms No. 1 mice (intraperitoneal route, ip), albino Sprague-Dawley rats (oral route, po)]. Housing, handling, and feeding were all in accordance with recommendations contained in the 'Guide for the Care and Use of Laboratory Animals'. Anticonvulsant activity was established using the MES test,^{7,16} the scMet test,⁷ and the timed intravenous pentylenetetrazole (iv Metrazol) seizure threshold test,⁷ using previously reported methods.^{11,10}

4.28. Microsomal studies. General methods

Male rat (Catalog No. 452501) and mouse (Catalog No. 452701) liver microsomes were purchased from GEN-TEST Corp (Woburn, MA). β -Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was purchased from Sigma/RBI (St. Louis, MO). All other reagents were of best commercial grade available and were used without further purification. Microsomal incubations were performed at room temperature in Corning centrifuge tubes containing the substrate (100 μ M), NADPH (1 mM), the buffer (100 mM phosphate buffer at pH 7.4 containing 3.3 mM MgCl_2), and the rodent liver microsomal protein (1 mg/mL) in a total volume of 2 mL. The substrates were dissolved in a MeOH in such concentrations that the total organic solvent content did not exceed 1% when added to microsomal incubations. The total incubation time was 1 h. Incubations in the absence of NADPH were used as controls, and incubations without any substrate were used as blanks. Samples (300 μ L) were taken at 15 min interval points into Eppendorf tubes containing MeCN (150 μ L). The incubation mixtures were then centrifuged for 10 min (14,000 rpm) in an Eppendorf centrifuge 5415C. An aliquot of the supernatant was analyzed by HPLC–UV and HPLC–MS/MS.

4.29. LC–UV analysis of metabolic products

To determine the substrate metabolic profile, the microsomal incubations at various time intervals were analyzed by LC–UV (256 nm). A Waters Alliance 2690 HPLC was interfaced with a photodiode array detector (Waters Photodiode Array, model 996). Samples (50 μ L) were injected onto an Xterra Phenyl column (3.9 $\times$ 150 mm, Waters Corp., Catalog No. 186001184). A gradient mobile phase was employed and was first held for 4 min at 80:20 ratio of solvent A (10 mM ammonium acetate buffer adjusted to pH 5.5) and solvent B (MeCN) and then successively brought to a 40:60 A–B composition over a 10 min period and then a 20:80 A–B composition over a 2 min interval using a flow rate of 0.8 mL/min. A 3 min recycling period was then employed. Calibration curves using compounds **14**, **15**, **16**, **19**, **20**, and **32** were used for the quantification of the substrates and major metabolites.

4.30. LC–MS analysis of metabolic products

The metabolic profiles were analyzed using LC–MS/MS on an Agilent 1100 LC/MSD Trap system at LCMS Limited (Raleigh, NC). Samples (5 μ L) were injected onto a Xterra Phenyl column (2.0 $\times$ 150 mm, Waters Corp., Catalog No. 186001181). A gradient mobile phase was employed and was first held for 4 min at 80:20 ratio of solvent A (10 mM ammonium acetate buffer adjusted to pH 5.5) and solvent B (MeCN) and then successively brought to a 40:60 A–B composition over a 10 min period and then a 20:80 A–B composition over a 2 min interval using a flow rate of 0.2 mL/min. A 3 min recycling period was then employed. Positive ion electrospray over the range m/z 70–400 was used for detection. Some of the key electrospray MS operating conditions are as follows: capillary voltage, 3000 V; capillary exit voltage, 60 V; trap drive, m/z 43; drying gas temperature, 350 °C; drying gas flow rate, 9.0 L/min; nebulization pressure, 45 psi. The ion trap was also operated in auto-MS/MS mode to gain structural information on the metabolites. Parent ion isolated using the auto-MS/MS routine selects the base peak in the mass spectrum for collision-induced decomposition (CID), corresponding to the $[\text{M}+\text{H}]^+$ ion for the metabolites in this paper. The product ions were generated by CID of the parent ion with He at a fragmentation voltage linear ramp from 0.3–2 V over 30 ms duration and a cutoff mass of 35% of the parent ion m/z value. The product ion spectra were acquired from m/z 70 to 400.

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