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Functionalized Amino Acid Anticonvulsants: Synthesis and Pharmacological Evaluation of Conformationally Restricted Analogues

Arnaud LeTiran,[a] James P. Stables[b] and Harold Kohn[c,*]

[a]*Department of Chemistry, University of Houston, Houston, TX 77204-5641, USA*
[b]*Epilepsy Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Federal Building, Room 114, Bethesda, MD 20892-9020, USA*
[c]*Division of Medicinal Chemistry and Natural Products, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7360, USA*

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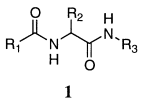
Abstract—Proven conformationally restricted analogues of anticonvulsant functionalized amino acids (FAAs) were prepared using short-range cyclizations and evaluated in pharmacological assays providing new information concerning the structural requirements for FAA function.

Introduction

We have described a new class of anticonvulsant agents, termed functionalized amino acids (FAAs, **1**).[1] *N*-Benzyl-2-acetamidopropionamide (**2**) is the parent compound in this series, and (*R*)-*N*-benzyl-2-acetamido-3-methoxypropionamide[1i] (**3**) is the lead antiepileptic agent, currently in phase II clinical trials. Structure–activity relationships studies have determined the correct stereochemical orientation of the C(2) substituent and the key units within **1** that are necessary for activity. However, we have neither identified the site of action of FAAs nor have we learned the bioactive conformation for this class of compounds.



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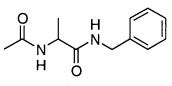

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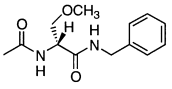

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There has been considerable effort in recent years to determine the relationship of three-dimensional, conformational structure to biological activity for small molecules.[2] Understanding the preferred structure provides new leads for therapeutic agents with improved pharmacological properties. One approach to this is to minimize the flexibility of the lead compound through the use of conformationally constrained (semirigid) analogues.[3] It is proposed that appropriate structural constraints will restrict a residue or group of residues to a sufficiently small region of conformational space thereby permitting the drug to bind, with high affinity, to its designated receptor.[3] An expectation of this approach is that the semirigid analogue will have improved biological activity through the elimination of bioactive conformers that give unwanted biological responses.[4] This strategy is limited because it is impossible to search all accessible conformations for most biomolecules. We tried to determine in this study whether we could learn the preferred bioactive FAA conformer(s) through the use of a select set of proven, constrained peptidomimetics.

Results and Discussion

Selection of compounds

Introduction. The interconnecting network of single bonds within FAAs leads to a spectrum of possible conformations. Using **2** as the prototype the four torsion (dihedral) angles, ω_1 [CH_3–C(1)–N(1)–C(2)], ϕ [C(1)–N(1)–C(2)–C(3), ψ (N(1)–C(2)–C(3)–N(2)] and ω_2 [C(2)–C(3)–N(2)–CH_2], can be used to define the compound's structural backbone (Fig. 1). Resonance theory predicts that ω_1 and ω_2 will be either *cis* (0°) or *trans* (180°). Greater conformational freedom exists for the ϕ

*Corresponding author. Tel.: +1-919-966-2680; fax: +1-919-843-7835; e-mail: harold_kohn@unc.edu

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and ψ angles. Nonetheless, studies have shown that preferred angles exist for ϕ and ψ, and two dimensional probability distribution functions have been reported for these dihedral angles (Ramachadran map) in vacuo and in solvent.[5,6]

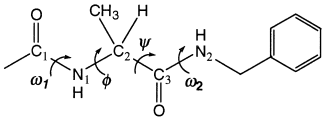
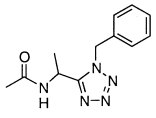


Figure 1. The backbone dihedral angles ϕ, ψ, ω_1, and ω_2 within **2**.

In our study, four conformationally restricted classes of peptidomimetic compounds of the parent FAA **2** were evaluated. Conformational constraints were enforced using short-range cyclizations.[7] The first analogue selectively limited the ω_2 dihedral angle while the second and third set of compounds constrained the ψ and ω_2, and the ϕ and ψ angles, respectively. The final class of agents restricted the ω_1, ϕ, and ψ angles. For these four sets of peptidomimetics, we elected to prepare the racemates and to compare their biological activities with the corresponding acyclic racemates.

Selection of compounds. The preferred dihedral angles for ω_1 and ω_2 are *cis* (0°) and *trans* (180°). Of these two, the *trans* arrangement in peptides is the energetically more stable,[8] unless the peptide is incorporated in a cyclic array or unusual steric and/or hydrogen-bonding interactions exist that favor the *cis* conformer.[9] While we expected the FAA ω_2 dihedral angle to be 180° in the absence of the receptor, we were anxious to learn if a *cis*-amide-type bond ($\omega_2 = 0°$) would show equal or improved bioactivity. The 1,5-disubstituted tetrazole (Ψ[CN_4]) ring system has been employed as a peptidomimetic unit for the *cis*-amide bond that exhibited bond lengths and bond angles close to those observed for the *cis*-peptide conformer.[10] Accordingly, we prepared the 1,5-disubstituted tetrazole **4**.



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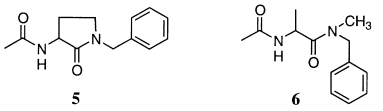
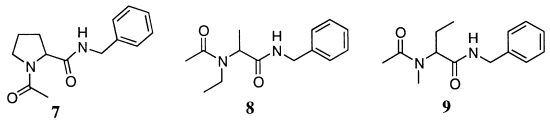
Freidinger[11] and others[12] have demonstrated that bridging the C(2) and N(2) terminal amide units through the use of a γ-lactam constrains the ψ torsion angle and restricts ω_2 to 180°. Conformational energy minimization studies and X-ray structure analyses for 3-amino-2-pyrrolidones indicated that the most stable conformations have ψ angles in a restricted region around $-130 \pm 15°$ for the (*S*)-enantiomer and $+130 \pm 15°$ for the corresponding (*R*)-enantiomer.[12b,c] We adopted this short-range cyclization strategy and prepared **5**. This approach converts one of the two secondary amide groups [N(2)] in **2** to a tertiary amide unit. Removal of the N(H) moiety may affect the binding of the drug candidate to the putative receptor and so we selected **6** as the reference compound for **5**.



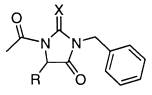
5 **6**

Incorporation of an L-proline unit within the peptide framework constrains the two central torsion angles, ϕ and ψ. Studies document that ϕ is restricted to $-65 \pm 15°$,[7,9c] and the peptide adopts a conformation that affords ψ angles of either $+150 \pm 15°$ or $-40 \pm 15°$.[13] Finally, the proline tertiary amide bond (ω_1) exists in both the *trans*-(180°) and the *cis*-(0°) conformations, with the *trans*-conformer being predominant. Using this conformational constraint, we synthesized **7** and the two acyclic reference compounds, **8** and **9**; the N(1) amide moiety was further modified.



7 **8** **9**

Simultaneous restriction of the three adjoining ϕ, ψ, and ω_2 torsion angles can be achieved by bridging the N(1), N(2) sites in dipeptides[14,15] with a carbonyl unit to give substituted hydantoins. X-ray diffraction studies of 5,5-disubstituted hydantoins showed the ring to be nearly planar with ψ and ω_2 torsion angles at 0° and 180°, respectively.[16,17] We prepared hydantoin **10** and the corresponding thiohydantoin **11**, to study the effect of restriction of the ϕ, ψ and ω_2 dihedral angles on anticonvulsant activity. This investigation was expanded to the 5-phenyl **12** and the 5-methoxymethylene **13** derivatives.

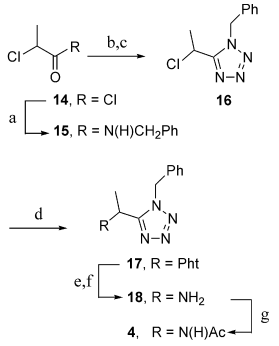


10 X = O, R = CH_3 **11** X = S, R = CH_3

12 X = O, R = Ph **13** X = S, R = CH_2OCH_3

Synthesis and structural evaluation

1,5-Disubstituted tetrazole 4. We prepared tetrazole **4** using the von Braun[18] procedure (Scheme 1). Beginning with **14**, treatment with benzylamine gave *N*-benzyl-α-



Scheme 1. Synthesis of tetrazole analogue **4**. Reagents: (a) $PhCH_2NH_2$, TEA, THF, 0 °C to rt, 3 h, 73%; (b) PCl_5, benzene, rt, 12 h; (c) HN_3, benzene, 65 °C, 5 h, 80%; (d) potassium phthalimide, KI, DMF, 110 °C, 3 h, 74%; (e) N_2H_4, DMF, rt, 2 h; (f) 1 N NaOH, rt, 99%; (g) Ac_2O, TEA, THF, rt, 45 min, 84%.

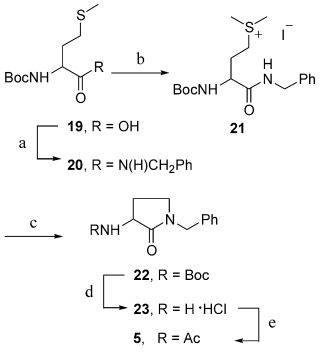
chloro amide **15**,[19] which was sequentially treated with PCl_5 and hydrazoic acid[20] to give 5-α-chloroalkyltetrazole **16**. Substitution of the α-chloro group in **16** with potassium phthalimide and KI yielded **17**, which upon hydrazinolysis gave the free amine **18**. Acetylation [Ac_2O, TEA, DMAP (cat)] of the amine afforded **4** (36% overall yield from **14**).

X-ray crystallographic analyses of **4** confirmed the restriction of the ω_2 dihedral angle to near 0° [(*R*)-isomer: $\omega_2 = -1.9(6)°$; (*S*)-isomer: $\omega_2 = -0.1(6)°$] (data not shown).[21]

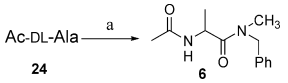
Lactam 5 and *N*-methyl-*N*-benzylamide 6. The Freidinger lactam **5** required the cyclization of the N(2) amide group with the C(2) side chain. Commercially available *t*Boc-DL-methionine (**19**) was treated with benzylamine to provide **20**, using the standard, mixed anhydride coupling methodology (Scheme 2).[1i,22] Treatment of **20** with MeI gave sulfonium salt **21** and then we cyclized it to the γ-lactam **22** with NaH[11] in DMF. Acid deprotection[23] of the *t*Boc group in **22** afforded the corresponding hydrochloride salt of **23**, which was then acylated to provide **5** in excellent yield (60% overall yield from **19**).

The reference compound **6** was prepared using commercially available *N*-acetyl-DL-alanine (**24**) (83% yield) (Scheme 3).

The γ-lactam ring in **5** restricted the ω_2 torsional angle to *trans* ($\omega_2 = 180°$) and reduced the conformational freedom (ψ) for this molecule. Only one conformer was observed by ^1H NMR for **5** and synthetic precursors **22** and **23**, while two conformers (65:35 ratio) were detected for **6**. This finding suggested that a single ω_1-conformation existed for **6** and for pyrrolidinone derivatives **5**, **22**, and **23**.



Scheme 2. Synthesis of lactam **5**. Reagents: (a) NMM, IBCF, benzylamine, THF, −78 °C to rt, 1 h, 86%; (b) CH_3I, rt, 12 h, 100%; (c) NaH, DMF, 0 °C to rt, 2 h, 74%; (d) 3 N HCl, EtOAc, rt, 2 days, 99%; (e) Ac_2O, TEA, DMAP (cat), THF, rt, 10 h, 96%.

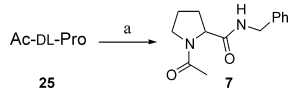


Scheme 3. Synthesis of **6**. Reagents: (a) EDCI, NMM, HOBt, *N*-methylbenzylamine, THF, −78 °C to rt, 1 h, 83%.

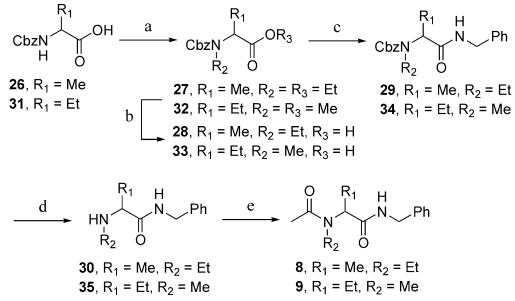
Proline 7 and N(1)-alkyl FAA analogues 8 and 9. Proline **7** was prepared from commercially available *N*-acetyl-DL-proline **25** by mixed anhydride coupling with benzylamine (64% yield) (Scheme 4).

The two reference compounds, **8** and **9**, were synthesized according to the procedure of Olsen[24] using Cbz-protected amino acids (Scheme 5). Commercially available Cbz-DL-Ala (**26**) was treated with EtI and Ag_2O in DMF to produce the *N*-ethylamino ethyl ester **27** as an oil. Similarly, using readily available Cbz-DL-Abu[25] (**31**) and MeI, we produced **32** in 70% yield. Saponification of the esters in aqueous alcoholic 2 N KOH solution followed by acid workup gave the desired carboxylic acids, **28** and **33**, which were then converted to their *N*-benzylamides **29** and **34**, respectively, using the mixed anhydride coupling procedure. Hydrogenolysis (H_2, Pd/C in MeOH)[23b,26] of the Cbz protecting groups produced the amines **30** and **35**, which were then acylated [Ac_2O, TEA, DMAP (cat)] to give the reference N(1)-alkyl FAA analogues **8** and **9** in 62 and 44% overall yields, respectively.

The ^1H and ^{13}C NMR spectra of **7** showed two sets of peaks corresponding to the expected *cis*-($\omega_1 = 0°$) and *trans*-($\omega_1 = 180°$) isomers. Our ^{13}C NMR assignment of isomer geometry was made on the basis of chemical shift values for the pyrrolidinone carbons in $CDCl_3$.[27] We measured the *trans*- to *cis*-amide ratio to be 3:1 by ^1H NMR integration of the N(1)H proton signals (*trans*: δ 8.35; *cis*: δ 8.66–8.68) in DMSO-d_6; a value substantiated by previous data for N(1)-acetylprolyl amides.[28] The ^1H and ^{13}C NMR data for reference compounds **8** and **9** and their synthetic precursors (**27**–**29**, **32**–**34**) also showed the presence of two major conformations in $CDCl_3$, and we have arbitrarily assigned the major conformer as the ω_1-*trans* isomer, based on those findings.[28]



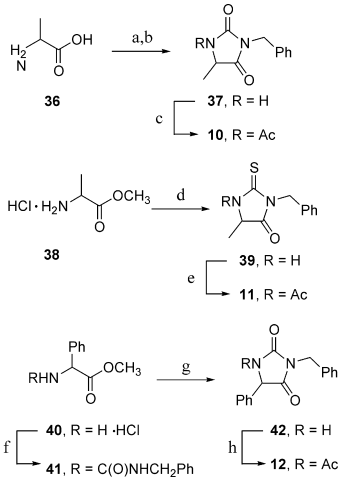
Scheme 4. Synthesis of **7**. Reagents: (a) NMM, IBCF, benzylamine, THF, −78 °C to rt, 1 h, 64%.



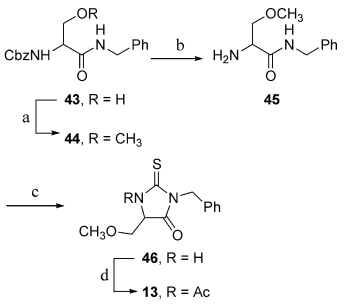
Scheme 5. Synthesis of the *N*-alkyl FAAs **8** and **9**. Reagents: (a) Ag_2O, RI (R = Et or Me), DMF, 50 °C (R = Et) or 35 °C (R = Me), 1–2 days, 88 and 70% (R = Et and Me, respectively); (b) 2 N aq KOH, ROH (R = Et or Me), 35 °C, 1–3 h, 99%; (c) NMM, IBCF, benzylamine, THF, −78 °C to rt, 1 h, 83 and 90% (**29** and **34**, respectively); (d) H_2 (1 atm), Pd/C, MeOH, rt, 12 h, 96 and 99% (**30** and **35**, respectively); (e) Ac_2O, TEA, DMAP (cat), THF, rt, 16–24 h, 89 and 76% (**8** and **9**, respectively).

(Thio)hydantoins 12–15. Using established procedures we prepared **37**, **39**, and **41** in moderate-to-good yield (55–82%) by treating **36**, **38**, and **40**, respectively, with either benzyl isocyanate or benzyl isothiocyanate and triethylamine (Scheme 6). Cyclization of the intermediate phenylglycine urea derivative **41** to the hydantoin **42** required an additional equivalent of triethylamine (40 °C, 4 h). Acetylation [Ac_2O, TEA and DMAP (cat)] of the hydantoin N(1) ring nitrogen provided (thio)hydantoins (**10**, **11**, and **12**) (41–81% overall yield).

The final thiohydantoin **13** was prepared beginning with *N*-benzyl-2-*N*-(Cbz)amino-3-hydroxypropionamide[29] (**43**) (Scheme 7). Methylation (CH_3I, Ag_2O) followed by Cbz-deprotection (H_2, Pd/C) and treatment with di-2-pyridylthiocarbonate (DPT) afforded thiohydantoin **46**. N(1)-Acetylation (Ac_2O, TEA, DMAP) gave **13** (51% overall yield from **43**).



Scheme 6. Synthesis of (thio)hydantoins **10**, **11**, and **12**. Reagents: (a) aq KOH, $PhCH_2NCO$, 65 °C, 20 min; (b) 6 N HCl, 75 °C, 12 h, 55%; (c) Ac_2O, TEA, DMAP (cat), THF, rt, 1.5 days, 84%; (d) $PhCH_2NCS$, TEA, CH_2Cl_2, reflux, 30 min, 82%; (e) Ac_2O, TEA, DMAP (cat), THF, rt, 1 h, 99%; (f) $PhCH_2NCO$, TEA, THF, 60 °C, 10 min, 72%; (g) TEA, THF, 40 °C, 4 h, 84%; (h) Ac_2O, TEA, DMAP (cat), THF, rt, 16 h, 67%.



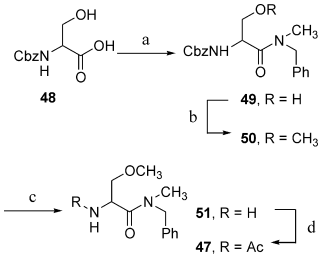
Scheme 7. Synthesis of thiohydantoin **13**. Reagents: (a) CH_3I, Ag_2O, CH_3CN, rt, 3 days, 93%; (b) H_2 (1 atm), Pd/C, MeOH, rt, 4 h, 99%; (c) DPT, THF, rt, 24 h; (d) Ac_2O, TEA, DMAP (cat), THF, rt, 6 h, 55% (2 steps).

Pharmacological evaluation

Introduction. All new compounds and appropriate reference compounds were tested for anticonvulsant activity using the procedures described by Stables and Kupferberg,[30] and these results were compared with the findings previously reported for FAAs **2** and **3**,[1b,i] and appropriate anticonvulsant agents[31,32] (Tables 1–3). All compounds were administrated intraperitoneally (ip) to mice and, when appropriate, orally (po) to rats. For specific compounds, we report the ED_{50} values required to prevent tonic extension of the hind limbs in mice and in rats in the MES-induced test and the neurological toxicity (TD_{50}) values using the rotorod test.[33]

Tetrazole 4. Tetrazole **4** exhibited diminished anticonvulsant activity (mice: $ED_{50} > 100$, < 300 mg/kg) compared with its parent analogue **2** (mice: $ED_{50} = 77$ mg/kg). This finding suggests that constraining ω_2 to a *cis* conformation ($\omega_2 = 0°$) does not enhance drug function. Several factors prohibit further conclusions concerning this conformational constraint. First, only one compound was tested. Second, the *cis*-amide bond offers a unique arrangement of an adjacent hydrogen-bond donor and acceptor interactions that does not exist in tetrazole **4**. Finally, the tetrazole unit is sterically larger than the *cis*-amide bond and this structural feature may adversely impact anticonvulsant activity.

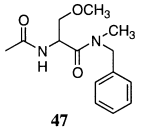
Freidinger lactams 5. The pharmacological data for γ-lactam **5** (mice: $ED_{50} > 300$ mg/kg; rats: $ED_{50} > 30$ mg/kg) and the acyclic N(2)-methyl reference compound **6** (mice: $ED_{50} > 100$, < 300 mg/kg; rats: $ED_{50} > 30$ mg/kg) showed that both compounds exhibited weak in vivo anticonvulsant activity. Comparison of these findings with the MES activity for parent FAA **2** (mice: $ED_{50} = 77$ mg/kg; rats: $ED_{50} = 48$ mg/kg) indicated that N(2)-alkylation of the *N*-benzylamide moiety caused a significant decrease in anticonvulsant activity. To verify this trend, we synthesized **47** and compared the pharmacological activity of **47** with **3**.[1i] Compound **47** was prepared in four steps beginning with Cbz-serine (**48**) (Scheme 8). Conversion of **48** to the *N*-benzyl-*N*-methylamide **49** followed by methylation (CH_3I, Ag_2O) and Cbz-deprotection (H_2, Pd/C) permitted acetylation (Ac_2O, TEA) of the terminal amine to give **47**.



Scheme 8. Synthesis of **47.** Reagents: (a) EDCI, NMM, HOBt, *N*-methylbenzylamine, THF, −78 °C to rt, 1 h, 49%; (b) Ag_2O, CH_3I, CH_3CN, rt, 3 days, 97%; (c) H_2 (1 atm), Pd/C, MeOH, rt, 12 h, 91%; (d) Ac_2O, TEA, DMAP (cat), THF, rt, 12 h, 96%.

Once again, we saw a large drop in activity. The MES ED_{50} values for **47** and **3** in mice were > 100, < 300 mg/kg and 8.3 mg/kg,[1i] respectively. These results may indicate that the secondary benzylamide unit serves as a key hydrogen bond donor upon binding to the putative receptor, and this hydrogen bond is lost upon N(2)-

alkylation.[34] Alternatively, the incorporation of a γ-lactam ring (**5**) within the FAA backbone and/or N(2) methylation (**6**) may prevent adoption of the conformation needed for maximal anticonvulsant activity. Finally, we note that the steric size of the terminal amine unit for lactam **5** and for the N(2)-methyl compound **6** is larger than FAAs **2** and **3**. This factor may prevent binding to the putative receptor and may be responsible for the observed drop of anticonvulsant activity.



47

Proline 7 and *N*-alkyl amino acid derivatives 8 and 9. The pharmacological data for proline **7** (mice: $ED_{50} > 100$, < 300 mg/kg; rats: $ED_{50} > 30$ mg/kg) and the two standard compounds **8** (mice: $ED_{50} = 85$ mg/kg; rats: $ED_{50} = 69$ mg/kg) and **9** (mice: $ED_{50} > 30$, < 100 mg/kg; rats: $ED_{50} \sim 30$ mg/kg) showed that incorporation of a proline unit in the FAA backbone leads to appreciable loss of anticonvulsant activity. Significantly, both reference compounds **8** and **9** exhibited anticonvulsant activity comparable with the parent FAA **2** (mice: $ED_{50} = 77$ mg/kg; rats: $ED_{50} = 48$ mg/kg). The finding that **7** was less active than its reference compounds **8** and **9** suggested that the proline-induced ϕ_1 and/or ψ_1 dihedral angles constraints[7,9] impacted the anticonvulsant function of these FAA peptidomimetics. Recently, Paruszewski

Table 1. Pharmacological data for the conformationally constrained derivatives and their reference compounds

Compd	Mice (ip)[a]			Rats (po)[b]		
	MES,[c] ED_{50}	Tox,[d] TD_{50}	PI[e]	MES,[c] ED_{50}	Tox,[d] TD_{50}	PI[e]
2	77 [1] (67–89)	450 [0.5] (420–500)	5.9	48 [1] (32–72)	—[f]	>20
3	8.3 [0.5] (7.9–9.8)	43 [0.25] (38–47)	5.2	3.8 [2] (2.9–5.5)	390 [1] (320–520)	101
4	>100, <300	>300	>1.0	~60[g]	>30	—
5	>300	>300	>1.0	>30	>30	—
6	>100, <300	~300	—	>30	>30	—
7	>100, <300	>300	>1.0	>30	>30	—
8	85 [0.25] (74–99)	180 [0.25] (160–200)	2.1	69 [6] (35–290)	>240	>3.5
9	>30, <100	>300	>3.0	~30[g,h]	>30	—
Phenytoin[i]	9.5 [2] (8.1–10)	66 [2] (53–72)	6.9	30 [4] (22–39)	—[i]	>100

[a]The compounds were administered intraperitoneally. ED_{50} and TD_{50} values are in mg/kg. Numbers in parentheses are 95% confidence intervals. The dose effect data was obtained at the 'time of peak effect' (indicated in hours in the square brackets).
[b]The compounds were administered orally.
[c]MES = maximal electroshock seizure test.
[d]Tox = neurologic toxicity determined from rotorod test.
[e]PI = protective index (TD_{50}/ED_{50}).
[f]No ataxia observed up to 1000 mg/kg.
[g]2/4 animals were protected at this dose.
[h]$ED_{50} < 30$ mg/kg by ip administration in rats (4/4 animals were protected at 30 mg/kg after 0.25 h).
[i]Ref 31.
[j]No ataxia observed up to 3000 mg/kg.

Table 2. Pharmacological data for (thio)hydantoin derivatives and their acyclic FAA analogues

Compd	Mice (ip)[a]			Rats (po)[b]		
	MES,[c] ED_{50}	Tox,[d] TD_{50}	PI[e]	MES,[c] ED_{50}	Tox,[d] TD_{50}	PI[e]
10	>30, <100 [0.5]	>300[f]	—	27 [0.5] (17–41)	>500	>18
11	100 [0.5] (89–120)	120 [0.5] (96–150)	1.2	≥40[g]	>50	—
12	>300	>300	—	>30	>30	—
13	>300	>100, <300	<1.0	—[h]	—[h]	—
2	77 [1] (67–89)	450 [0.5] (420–500)	5.9	48 [1] (32–72)	—[i]	>20
52	20 [0.5] (17–25)	97 [0.5] (80–120)	4.8	48 [4] (31–68)	—[i]	—
3	8.3 [0.5] (7.9–9.8)	43 [0.25] (38–47)	5.1	3.8 [2] (2.9–5.5)	390 [1] (320–520)	100
Phenytoin[j]	9.5 [2] (8.1–10)	66 [2] (53–72)	6.9	30 [4] (22–39)	—[k]	>100
Phenobarbital[l]	22 [1] (15–23)	69 [0.5] (63–73)	3.2	9.1 [5] (7.6–12)	61 [0.5] (44–96)	6.7

[a]The compounds were administered intraperitoneally. ED_{50} and TD_{50} values are in mg/kg. Numbers in parentheses are 95% confidence intervals. The dose effect data was obtained at the 'time of peak effect' (indicated in hours in the square brackets).
[b]The compounds were administered orally.
[c]MES = maximal electroshock seizure test.
[d]Tox = neurologic toxicity determined from rotorod test.
[e]PI = protective index (TD_{50}/ED_{50}).
[f]2/4 animals were protected at this dose.
[g]No linear dose response was obtained in rats by po for this compound. $ED_{50} = 12$ [0.5] (7.9–17) mg/kg by ip administration in rats.
[h]Not determined.
[i]No ataxia observed up to 1000 mg/kg.
[j]Ref 31.
[k]No ataxia observed up to 3000 mg/kg.
[l]Ref 32.

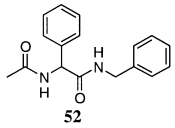
Table 3. Subcutaneous Metrazol® (scMet) seizure threshold test for selected (thio)hydantoin derivatives and their acyclic FAA analogues

Compd	Mice (ip)[a]			Rats (po)[b]		
	scMet,[c] ED_{50}	Tox,[d] TD_{50}	PI[e]	scMet,[c] ED_{50}	Tox,[d] TD_{50}	PI[e]
10	>100, <300	~300[f]	>1.0	>250	>500	—
11	46 [0.5] (36–60)	120 [0.5] (96–150)	2.5	>50	>50	—
12	>300	>300	—	—[g]	>30	—
13	>100, <300	>100, <300	<1.0	—[g]	—[g]	—
2	>800[f]	450 [0.5] (420–500)	<0.6	>1000	—[h]	—
52	>120	97 [0.5] (79–120)	<0.8	>1000	—[h]	—
3	>300	43 [0.25] (38–47)	<0.2	>260	390 [1] (320–520)	<1.5
Phenytoin[i]	—[j]	66 [2] (53–72)	—	—[j]	—[k]	—
Phenobarbital[l]	13 [1] (5.9–16)	69 [0.5] (63–73)	5.2	12	61 [0.5] (44–96)	5.3

[a]The compounds were administered intraperitoneally. ED_{50} and TD_{50} values are in mg/kg. Numbers in parentheses are 95% confidence intervals. The dose effect data was obtained at the 'time of peak effect' (indicated in hours in the square brackets).
[b]The compounds were administered orally.
[c]scMet = subcutaneous Metrazol® seizure threshold test.
[d]Tox = neurologic toxicity determined from rotorod test.
[e]PI = protective index (TD_{50}/ED_{50}).
[f]2/4 animals were protected at this dose.
[g]Not determined.
[h]No ataxia observed up to 1000 mg/kg.
[i]Ref 31.
[j]Not effective.
[k]No ataxia observed up to 3000 mg/kg.
[l]Ref 32.

and co-workers reported the anticonvulsant activity for optically pure (*R*)-**7** (ED_{50} = 67 mg/kg) in mice (ip).[35] The increased potency for (*R*)-**7**, compared with **7**, was in agreement with the C(2) stereochemical requirements of FAAs.[1c–e,g,i] Finally, we note that our finding that N(1)-alkylation did not affect activity contrasted with the discovery that N(2)-alkylation leads to loss of anticonvulsant activity (e.g., **6**; Table 1).

3.5(Thio)hydantoins 10–13. The in vivo anticonvulsant activities for (thio)hydantoins **10**–**13**, the corresponding FAAs **2**, **3**, and **52**,[1b,i] and the potent antiepileptic agents, phenytoin,[31] and phenobarbital,[32] are summarized in Tables 2 and 3.



52

These results provided several important findings. First, no significant differences in anticonvulsant activities were observed for hydantoin **10** and thiohydantoin **11**. Both compounds provided protection in the MES-induced seizure test in mice and rats. The ED_{50} values for compound **10** in rats (po) was 27 mg/kg, while compound **11** protected half of the rats tested at 40 mg. We observed that **11** did not give a linear dose-response (4/8 rats were protected at 40 mg/kg but only 5/16 at 80 mg/kg). Accordingly, we tested ip administration of **11** in rats to eliminate potential absorption problems. The ED_{50} (ip) was impressive at 12 mg/kg in the MES-seizure test. Second, the SAR patterns for FAAs[1] were not useful for predicting the activities of newly prepared (thio)hydantoins. For FAAs, improved anticonvulsant activities in the MES-seizure assay were observed by either incorporating a heteroatom one atom removed from the C(2) site (i.e., **3** vs **2**) or by placing a small aromatic moiety at C(2) (i.e., **52** vs **2**). This trend was *not* maintained in our (thio)hydantoins. We found that replacement of the methyl group in **10** (mice: ED_{50} >30, <100 mg/kg) by a phenyl moiety to give **12** (mice: ED_{50} >300 mg/kg) led to a significant drop in anticonvulsant activity. Similarly, the C(5) methyl thiohydantoin **11** (mice: ED_{50} = 100 mg/kg) was considerably more potent than the C(5) methoxymethylene analogue **13** (mice: ED_{50} >300 mg/kg). Third, hydantoin **10** and thiohydantoins **11** and **13** possessed moderate-to-good activity in the scMet-seizure test[30] in mice (Table 3). The most active compound was **11** (ED_{50} = 46 mg/kg). This finding contrasted with the lack of scMet-activity exhibited by FAA analogues[1] **2**, **3**, and **52** and phenytoin.[31] Previous SAR studies have shown that hydantoin N(3) alkylation confers significant sc-Met activity without appreciable loss in MES-activity.[36,37]

We concluded that FAAs and their monosubstituted hydantoin analogues exhibited different anticonvulsant profiles. To gain additional insight into the possible mechanism of action of these hydantoins, we examined the activities of **10**, **2**, and (*R*)-**3** in the voltage-sensitive Na^+ channel test using patch-clamp electrophysiology techniques.[38] Table 4 documents that hydantoin **10** exhibits a large voltage-dependant blockage of Na^+ channels (35% blockage at −60 mV), while the two FAAs [**2** and (*R*)-**3**] had no significant effect on peak current at 100 μM. For comparison, two prototypical antiepileptic Na^+ channel blockers, phenytoin and lamotrigine, provide 48 and 53% blockage at −60 mV, respectively.[39] These findings indicate that **10** expresses its anticonvulsant activity, in part, by acting at the Na^+ channel. Together, the divergent SAR and the different electrophysiological findings for (thio)hydantoins and FAAs provide evidence that these two classes of compounds function by different mechanisms.

Table 4. Effect of **2**, (*R*)-**3** and **10** on voltage sodium channels in NI E-115 neuroblastoma cells at 100 μM

Compd	No. of cells	% Control (±SEM)[a]	
		−90 mV	−60 mV
2	5, 5	100±1	83±4
(*R*)-**3**	6	102±5	—[b]
10	6, 9	93±3	65±3
Phenytoin[c]	5, 5	78±2	52±4
Lamotrigine[c]	7, 9	67±5	47±7

[a]The results from several individual cells were averaged, the SEM calculated and statistical significance was determined using the Student's *t*-test. Significance was taken to be in the range of *p* values: $p < 0.05$.
[b]Not determined.
[c]Ref 39.

Conclusions

We have synthesized and evaluated a series of proven, conformationally constrained analogues and compared their anticonvulsant activities with their corresponding reference FAAs. We designed these compounds to learn the preferred bioactive FAA conformer(s). No improvement in pharmacological activity was observed upon conformational constraint thus preventing our identifying new lead compounds. Important new information on the SAR of FAAs was obtained. We showed that FAA N(1)-alkylation did not reduce anticonvulsant activity while N(3)-alkylation led to appreciable activity loss. We also learned that structurally similar (thio)hydantoins function by pathway(s) distinct from FAAs. This finding is important since the mode of action of FAAs has not been determined. Finally, we observed that hydantoin **10** and thiohydantoin **11**, upon po and ip administration, respectively, displayed activities comparable with phenytoin in the MES-seizure test in rats and that both compounds displayed significant protection against sc-Met-induced seizures.

Experimental

General methods

Nuclear magnetic resonance spectra were measured at 300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR. Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and coupling constants (*J* values) are in Hertz. In cases where conformational isomers were observed the number in parenthesis in the NMR assignments correspond to the minor conformer and signals not detected are believed to overlap with those of the major conformer. Yields reported are for purified products and were not optimized. Reactions involving water- or air-sensitive compounds were conducted in dried glassware and carried out under a positive pressure of Ar. Mass spectral studies were conducted by Dr. M. Moini (Unversity of Texas, Austin, TX, USA) and the X-ray crystallographic determination of **4** was performed by Dr. James Korp (University of Houston, Houston, TX, USA). Microanalyses were provided by Atlantic Microlab, Inc. (Northcross, GA, USA).

General procedure for the preparation of *N*-benzylamide amino acids derivatives using the mixed anhydride coupling (MAC) (method A)[1i,22]

A dry THF solution (~0.5–2 mmol carboxylic acid/mL of THF) containing the carboxylic acid was cooled to −78 °C under Ar, and 4-methylmorpholine (1.1–2.2 equiv) was added. After stirring (2 min), isobutyl chloroformate (1.1–1.25 equiv) was added leading to the precipitation of a white solid. The reaction was allowed to proceed for an additional 2 min, and then benzylamine (1.1–1.25 equiv) was added at −78 °C. The reaction mixture was allowed to stir at room temperature (30 min–3 h), and then the insoluble salts filtered. The organic layer was concentrated in vacuo and the product purified by column chromatography on SiO_2 gel.

General procedure for the preparation of *N*-alkylbenzylamide-substituted amino acids (method B)

To a dry DMF or THF solution (~0.1–0.5 mmol carboxylic acid/mL of solvent) of the carboxylic acid were successively added under Ar EDCI (1.1–1.8 equiv), HOBt (1.1–1.8 equiv), and 4-methylmorpholine (1.1–1.8 equiv). After stirring (5 min), the amine (0.8–1.2 equiv) was added dropwise and the reaction was stirred at room temperature (12–24 h). The mixture was diluted with 1 M HCl and extracted with EtOAc. The organic layers were combined, washed with brine, concentrated in vacuo, and the product purified by column chromatography on SiO_2 gel.

General procedure for the preparation of N(1)-acetyl-substituted amino acids (method C)

To a dry THF solution of amine or ammonium hydrochloride salt (~0.1–1 mmol amine/mL of THF) were successively added TEA (1–2 equiv), Ac_2O (1–2 equiv), and a catalytic amount of DMAP. The reaction was allowed to stir at room temperature (1 h–1 day). The organic layer was concentrated in vacuo and the product purified by column chromatography on SiO_2 gel or by recrystallization.

1-Benzyl-5-(1-chloroethyl)-1*H*-tetrazole (16). Phosphorous pentachloride (6.79 g, 32.6 mmol) was added with stirring and cooling (5–10 °C) to a suspension of **15**[19] (5.85 g, 29.7 mmol) in dry benzene (45 mL). The reaction was exothermic and was accompanied by the evolution of HCl. The reaction was stirred overnight at room temperature and then concentrated in vacuo by approximately one half. A benzene solution (92 mL) of hydrazoic acid[20] (**CAUTION**) (~4 M) was then added in portions with vigorous stirring at 5–10 °C. The mixture was allowed to warm to room temperature and stirred at 65 °C (5 h). The solvent was evaporated in vacuo and iced cooled H_2O (100 mL) was added. The product was extracted from the cooled suspension with benzene (3×60 mL). The organic layers were combined, dried (Na_2SO_4), and the solvent evaporated in vacuo. The residue was purified by column chromatography (SiO_2; 1:99, EtOAc/hexanes to 1:9, EtOAc/hexanes) to yield 5.29 g (80%) of pure **16** as a clear oil: R_f 0.43 (3:1,

hexanes/EtOAc); IR (neat) 3067, 3035, 2990, 1498 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.98 (d, $J=6.8$ Hz, CH_3), 4.97 (q, $J=6.8$ Hz, CH), 5.55 (d, $J=15.3$ Hz, CHH′Ph), 5.83 (d, $J=15.3$ Hz, CHH′Ph), 7.26–7.29 (m, 2 PhH), 7.35–7.41 (m, 3 PhH); ^{13}C NMR ($CDCl_3$) δ 22.4, 44.4, 51.7, 127.9, 129.4, 129.5, 132.9, 154.7; MS (+CI) (rel intensity) 225 (33), 223 ($M^+ + 1$, 100); M_r (+CI) 223.075 67 [$M^+ + 1$] (calcd for $C_{10}H_{12}{}^{35}ClN_4$ 223.075 05). Anal. calcd for $C_{10}H_{11}ClN_4$: C, 53.94%; H, 4.98%; N, 25.16%. Found: C, 54.18%; H, 5.07%; N, 24.96%.

2-[1-(1-Benzyl-1*H*-tetrazole-5-yl)-ethyl]-isoindole-1,3-dione (17).[20a] To a DMF solution (60 mL) of **16** (3.92 g, 17.6 mmol) were added potassium phthalimide (3.92 g, 21.2 mmol) and KI (0.35 g, 1.8 mmol). The mixture was stirred at 110 °C (3 h), and the solvent was evaporated in vacuo to give crude **17** as a pale-yellow solid. The residue was recrystallized from toluene (2×) to yield 4.33 g (74%) of pure **17** as a white solid: mp 148–149 °C (lit.[20a] mp 146–147 °C); R_f 0.35 (3:1, hexanes/EtOAc); ^1H NMR (DMSO-d_6) δ 1.83 (d, $J=7.1$ Hz, CH_3), 5.33 (d, $J=16.7$ Hz, CHH′Ph), 5.79 (d, $J=16.7$ Hz, CHH′Ph), 5.89 (q, $J=7.1$ Hz, CH), 6.71–6.80 (m, 3 PhH), 6.91 (t, $J=7.5$ Hz, 2 PhH), 7.60 (dd, $J=3.0$, 6.0 Hz, 2 Pht$H$), 7.72 (dd, $J=3.0$, 6.0 Hz, 2 Pht$H$).

1-(1-Benzyl-1*H*-tetrazole-5-yl)-ethylamine (18)[20a] and *N*-acetyl-[1-(1-benzyl-1*H*-tetrazole-5-yl)ethyl]-ethylamine (4). To a DMF solution (30 mL) of **17** (3.00 g, 9.0 mmol) was added hydrazine (570 μL, 18.0 mmol) and the solution was stirred at room temperature (2 h). The solvent was evaporated in vacuo and the residue was redissolved in a binary mixture of an aqueous 1 N NaOH solution (60 mL) and CH_2Cl_2 (100 mL) and then stirred (30 min). The organic layer was separated and then extracted with an aq 1 N HCl solution (6×50 mL). The aqueous layers were combined, adjusted to pH 13 with an aq 5 N NaOH solution and extracted with CH_2Cl_2 (6×60 mL). The organic layers were combined, dried (Na_2SO_4), evaporated in vacuo to yield 1.85 g (99%) of **18** as an oil: R_f 0.29 (1:19, MeOH/$CHCl_3$); ^1H NMR ($CDCl_3$) δ 1.45 (d, $J=6.8$ Hz, CH_3), 1.65 (s, NH_2), 4.26 (q, $J=6.8$ Hz, CH), 5.72 (s, CH_2Ph), 7.23–7.26 (m, 2 PhH), 7.33–7.39 (m, 3 PhH).

Utilizing method C and using crude **18** (187 mg, 0.92 mmol), THF (10 mL), TEA (150 μL, 1.10 mmol) and Ac_2O (100 μL, 1.10 mmol) gave crude **4** as an oil after 45 min. The product was purified by column chromatography (SiO_2; 1:19, MeOH/$CHCl_3$) to give 250 mg (84%) of pure **4** as a white solid: mp 110–112 °C; R_f 0.50 (1:19, MeOH/$CHCl_3$); IR (KBr) 3242, 3066, 2994, 1639, 1554, 1511 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.42 (d, $J=6.9$ Hz, CH_3), 1.94 (s, CH_3C(O)), 5.36–5.41 (m, CH), 5.75 (s, CH_2Ph), 7.25–7.27 (m, 2 PhH), 7.35–7.39 (m, 3 PhH), 7.57 (d, $J=7.8$ Hz, NH); ^{13}C NMR ($CDCl_3$) 19.2, 22.8, 38.7, 51.1, 127.7, 129.0, 129.3, 133.9, 156.7, 170.4; MS (+CI) (rel intensity) 247 (11), 246 ($M^+ + 1$, 100); M_r (+CI) 246.135 68 [$M^+ + 1$] (calcd for $C_{12}H_{16}N_5O$ 246.135 49). Anal. calcd for $C_{12}H_{15}N_5O$: C, 58.76%; H, 6.16%; N, 28.55%. Found: C, 58.50%; H, 6.15%; N, 28.41%.

***N*-Benzyl-2-*N*-(*tert*-butoxycarbonyl)amino-4-(thiomethyl)-butanamide (20).** Utilizing method A and *N*-*t*Boc-DL-methionine (**19**) (2.00 g, 8.0 mmol), THF (80 mL), 4-methylmorpholine (1.1 mL, 9.63 mmol), isobutyl chloroformate (1.3 mL, 9.63 mmol) and benzylamine (1.1 mL, 9.63 mmol) gave 2.32 g (86%) of pure **20** as a white solid after purification by column chromatography (SiO_2; 2:1, hexanes/EtOAc): mp 95–96 °C; R_f 0.30 (2:1, hexanes/EtOAc); IR (KBr) 3336, 3305, 2970, 2924, 1682, 1655, 1570, 1523 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.41 (s, (CH_3)$_3$), 1.89–1.98 (m, CHH′CH), 2.05–2.15 (m, CHH′CH), 2.06 (s overlapping with CHH′CH, SCH_3), 2.49–2.60 (m, CH_2SCH$_3$), 4.25–4.33 (m, CH), 4.37–4.50 (m, CH_2Ph), 5.35 (d, $J=8.4$ Hz, NHCH), 6.81–6.85 (m, NHCH$_2$), 7.20–7.36 (m, 5 PhH); ^{13}C NMR ($CDCl_3$) δ 15.5, 28.5, 30.4, 31.9, 43.7, 53.7, 80.0, 127.7, 127.8, 128.9, 138.1, 155.8, 171.6; MS (+CI) (rel intensity) 339 ($M^+ + 1$, 27), 283 (100), 239 (42); M_r (+CI) 339.175 12 [$M^+ + 1$] (calcd for $C_{17}H_{27}N_2O_3S$ 339.174 24). Anal. calcd for $C_{17}H_{26}N_2O_3S$: C, 60.33%; H, 7.74%; N, 8.28%. Found: C, 60.19%; H, 7.79%; N, 8.33%.

***N*-Benzyl-2-*N*-(*tert*-butoxycarbonyl)amino-4-(*S*,*S*-dimethyl sulfonium)-butanamide iodide (21) and 1-benzyl-3-(*tert*-butoxycarbonyl)amino-pyrrolidin-2-one (22).** Compound **20** (1.49 g, 4.4 mmol) was dissolved in CH_3I (10 mL, 160 mmol) and stirred at room temperature (12 h). Evaporation of the solvent in vacuo gave 2.10 g (100%) of **21** as a pale-yellow solid which was used without further purification: mp 103–110 °C; R_f 0.26 (1:9, MeOH/$CHCl_3$); IR (KBr) 3328, 3270, 2976, 1704, 1674, 1563 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.40 (s, (CH_3)$_3$), 1.95–2.16 (m, CH_2CH), 2.90 (s, S^+CH_3), 2.91 (s, S^+CH_3), 3.27–3.35 (m, CH_2S(CH$_3$)$_2$), 4.03–4.12 (m, CH), 4.27 (dd, $J=5.7$, 15.2 Hz, CHH′Ph), 4.36 (dd, $J=5.9$, 15.2 Hz, CHH′Ph), 7.17–7.34 (m, 5 PhH and NHCH$_2$), 8.47 (t, $J=5.7$ Hz, NH).

The crude sulfonium salt **21** (5.14 g, 10.7 mmol) was dissolved in DMF (50 mL) under Ar and cooled (0 °C) and then sodium hydride (428 mg (60% dispersion in mineral oil), 10.7 mmol) was added all at once. The mixture was stirred at 0 °C (10 min) and then allowed to warm to room temperature (2 h). The reaction mixture was quenched with H_2O (50 mL) at 0 °C. The solution was concentrated in vacuo and the residue purified by column chromatography (SiO_2; 1:1, hexanes/EtOAc) to give 2.30 g (74%) of pure **22** as a white solid: mp 126–128 °C; R_f 0.48 (1:1, hexanes/EtOAc); IR (KBr) 3294, 2978, 1712, 1666, 1512 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.38 (s, (CH_3)$_3$), 1.68–1.82 (m, CHH′CH), 2.48–2.56 (2.73–2.78) (m, CHH′CH (CH_2CH)), 3.11–3.16 (m, CH$_2$CH_2N), 4.06–4.20 (m, CH), 4.34–4.50 (s, CH_2Ph), 5.15–5.24 (br s, NHCH), 7.13–7.28 (m, 5 PhH), the ^1H NMR structural assignments were in agreement with the ^1H–^1H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 28.3, 28.4 (28.5), 43.5 (43.1), 47.2 (47.3), 52.7, 79.9, 127.9, 128.2, 128.9, 136.0, 156.0, 172.3, the ^{13}C NMR structural assignments were in agreement with the HETCOR experiment; MS (+CI) (rel intensity) 291 ($M^+ + 1$, 22), 235 (100); M_r (+CI) 291.170 00 [$M^+ + 1$] (calcd for $C_{16}H_{23}N_2O_3$ 291.170 87). Anal. calcd for

$C_{16}H_{22}N_2O_3$: C, 66.18%; H, 7.64%; N, 9.65%. Found: C, 66.23%; H, 7.64%; N, 9.64%.

3-Amino-1-benzyl-pyrrolidin-2-one hydrochloride (23) and 3-acetylamino-1-benzyl-pyrrolidin-2-one (5). Compound **22** (2.28 g, 7.86 mmol) was dissolved in 3 M aq HCl/EtOAc (20/60 mL) and stirred at room temperature. After 2 days, the solution was concentrated in vacuo to yield a green hygroscopic oil. The crude hydrochloride salt was redissolved in water (50 mL) and extracted with EtOAc (2×25 mL). The aqueous layer was concentrated in vacuo to give 1.77 g (99%) of crude **23** as a thick colorless, highly hygroscopic oil, which was used without further purification: ^{1}H NMR (DMSO-d_6 + 1 drop D_2O) δ 1.87–1.97 (m, C*H*H′CH), 2.37–2.41 (m, CH*H*′CH), 3.26–3.30 (m, CHH′CH_2N), 4.06 (t, J = 9.3 Hz, C*H*), 4.38 (d, J = 15.0 Hz, C*H*H′Ph), 4.47 (d, J = 15.0 Hz, CH*H*′Ph), 7.24–7.38 (m, 5 Ph*H*), the structural assignments were in agreement with the ^{1}H–^{1}H COSY experiment; ^{13}C NMR (DMSO-d_6 + 1 drop D_2O) δ 23.9, 43.7, 46.3, 50.2, 127.8, 127.9, 129.0, 136.1, 169.4.

Utilizing method C and using crude **23** (2.00 g, 8.85 mmol), THF (100 mL), TEA (2.5 mL, 17.7 mmol), Ac_2O (920 μL, 9.73 mmol) and DMAP (~200 mg) gave crude **5** after 10 h. The product was purified by column chromatography (SiO_2; 1:19, MeOH/$CHCl_3$) to obtain 1.97 g (96%) of pure **5** as a white solid: mp 130–132 °C; R_f 0.34 (1:19, MeOH: $CHCl_3$); IR (KBr) 3267, 3082, 2993, 2939, 1697, 1639, 1558 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.76–1.84 (m, C*H*H′CH), 2.05 (s, CH_3C(O)), 2.65–2.72 (m, CH*H*′CH), 3.21–3.26 (m, CHH′CH_2N), 4.32–4.50 (m, C*H*), 4.44 (d, J = 14.7 Hz, C*H*H′Ph), 4.53 (d, J = 14.7 Hz, CH*H*′Ph), 6.69 (d, J = 4.5 Hz, N*H*CH), 7.21–7.37 (m, 5 Ph*H*), the structural assignments were in agreement with the ^{1}H–^{1}H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 23.2, 28.1, 43.9, 47.4, 52.1, 128.0, 128.2, 129.0, 135.8, 171.1, 172.6; MS (+CI) (rel intensity) 233 (M^+ + 1, 27), 215 (10), 191 (13); M_r (+CI) 233.128 61 [M^+ + 1] (calcd for $C_{13}H_{17}N_2O_2$ 233.129 00). Anal. calcd for $C_{13}H_{16}N_2O_2 \cdot 0.1H_2O$: C, 66.70%; H, 6.98%; N, 11.97%. Found: C, 66.56%; H, 6.86%; N, 11.86%.

***N*-Benzyl-*N*-methyl-2-acetamidopropionamide (6).**[40] Utilizing method B and using **24** (1.43 g, 10.9 mmol), THF (150 mL), EDCI (2.51 g, 13.09 mmol), 4-methylmorpholine (1.4 mL, 13.09 mmol), HOBt (1.77 g, 13.09 mmol) and *N*-methylbenzylamine (1.1 mL, 8.73 mmol) gave crude **6** after 24 h. The product was purified by column chromatography (SiO_2; 1:33, MeOH/$CHCl_3$) to give 1.70 g (83%) of pure **6** as a clear oil: R_f 0.57 (1:19, MeOH/$CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 1.37 (1.32) (d, J = 6.9 (4.5) Hz, CHCH_3), 2.01 (1.97) (s, CH_3C(O)), 2.99 (2.92) (s, NCH_3), 4.50–4.69 (m, CH_2Ph), 4.94–5.05 (m, C*H*), 7.00 (d, J = 5.4 Hz, N*H*), 7.18–7.35 (m, 5 Ph*H*), ^{1}H NMR analysis indicated the major and minor conformational isomers existed in a 65:35 ratio in $CDCl_3$.

***N*-Benzyl-1-acetyl-2-pyrrolidinecarboxamide (7).**[41] Utilizing method A and using **25** (1.10 g, 7.02 mmol), THF (90 mL), 4-methylmorpholine (960 μL, 8.77 mmol), isobutyl chloroformate (1.10 mL, 8.77 mmol), and benzylamine (950 μL, 8.77 mmol) gave crude **7**. The product was purified by column chromatography (SiO_2; 1:19, MeOH/$CHCl_3$) to obtain 1.10 g (64%) of pure **7** as a clear oil, which crystallized upon standing (24 h, in vacuo): mp 96–97 °C; R_f 0.61 (1:9, MeOH/$CHCl_3$); ^{1}H NMR (DMSO-d_6) δ 1.86–2.24 (m, CH_3C(O), C(3)*HH*′ and C(4)H_2), 3.42–3.55 (m, C(5)*H*H′), 3.58–3.66 (m, C(5)H*H*′), 4.31–4.40 (m, CH_2Ph and C*H*), 7.25–7.40 (m, 5 Ph*H*), 8.35 (8.66–8.68) (t (m), J = 6.0 Hz, N*H*), ^{1}H NMR analysis indicated the major and minor conformational isomers existed in a 75:25 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 22.1, 24.5 (22.8), 28.2 (31.8), 42.8, 47.9 (46.3), 59.4 (61.6), 126.7, 127.0, 127.2, 128.1, 138.3, 138.4, 170.2 (169.9), 171.5 (171.8); MS (+CI) (rel intensity) 248 (16), 247 (M^+ + 1, 100); M_r (+CI) 247.144 32 [M^+ + 1] (calcd for $C_{14}H_{19}N_2O_2$ 247.144 65). Anal. calcd for $C_{14}H_{18}N_2O_2$: C, 68.27%; H, 7.37%; N, 11.37%. Found: C, 68.40%; H, 7.36%; N, 11.51%.

2-(*N*-Benzyloxycarbonyl-*N*-ethyl)aminopropionic acid ethyl ester (27). To a DMF (80 mL) solution of **26** (5.34 g, 23.9 mmol) was successively added Ag_2O (22.16 g, 95.7 mmol) and EtI (15.3 mL, 107 mmol) at room temperature. The reaction mixture was stirred at 50 °C (2 days), filtered and the filtrate evaporated in vacuo. The oily residue was purified by column chromatography (SiO_2; 4:1, hexanes/EtOAc) to give 5.86 g (88%) of pure **27** as a clear oil: R_f 0.25 (4:1, hexanes/EtOAc); IR (neat) 2983, 2941, 1741, 1702 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.10–1.25 (m, NCH_2CH_3 and OCH_2CH_3), 1.46 (d, J = 7.2 Hz, CHCH_3), 3.42–3.50 (3.12–3.20) (m, NCH_2CH_3), 4.14–4.17 (3.94–4.06) (m, OCH_2CH_3), 4.53–4.63 (4.25–4.33) (m, C*H*), 5.12 (5.13) (s, OCH_2Ph), 7.27–7.35 (m, 5 Ph*H*), ^{1}H NMR analysis indicated the major and minor conformational isomers existed in a 55:45 ratio in $CDCl_3$, the structural assignments were in agreement with the ^{1}H–^{1}H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 14.2, 15.2, 16.1 (17.0), 40.6 (41.8), 55.2, 61.1, 67.2, 127.8, 127.9, 128.2, 128.5, 136.7, 136.8, 156.2 (155.4), 172.2; MS (+CI) (rel intensity) 281 (14), 280 (M^+ + 1, 100), 236 (16); M_r (+CI) 280.155 17 [M^+ + 1] (calcd for $C_{15}H_{22}NO_4$ 280.154 88). Anal. calcd for $C_{15}H_{21}NO_4$: C, 64.50%; H, 7.58%; N, 5.01%. Found: C, 64.59%; H, 7.64%; N, 5.09%.

2-(*N*-Benzyloxycarbonyl-*N*-ethyl)aminopropionic acid (28). Compound **27** (4.57 g, 16.39 mmol) was saponified with 2 M KOH/EtOH solution (20 /40 mL) at 35 °C (3 h). The solution was partially concentrated in vacuo and the remaining aqueous solution was washed with Et_2O (20 mL), cooled (0 °C), and brought to pH 2 with aqueous 4 N HCl. The mixture was extracted with EtOAc (3×50 mL), and then the combined extracts were dried (Na_2SO_4) and evaporated in vacuo to give 4.10 g (99%) of pure **28** as a clear oil: R_f 0.57 (1:9, MeOH/$CHCl_3$); IR (neat) 3381–2900 (br), 1699 (br) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.03–1.07 (m, NCH_2CH_3), 1.36 (d, J = 6.9 Hz, CHCH_3), 3.01–3.17 (3.33–3.40) (m, NCH_2CH_3), 4.48 (4.24) (q, J = 6.9 (6.9) Hz, C*H*), 5.05 (5.02) (s, OCH_2Ph), 7.13–7.23 (m, 5 Ph*H*), 10.20 (s, C(O)O*H*), ^{1}H NMR analysis indicated the major and minor conformational isomers existed in a 55:45 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 15.0 (14.1), 15.2 (15.8),

40.9 (41.9), 55.1 (55.0), 67.5, 127.7, 128.0, 128.1, 128.4, 136.2, 136.4, 156.4 (155.6), 176.9; MS (+CI) (rel intensity) 252 ($M^+ + 1$, 78), 208 (100); M_r (+CI) 252.123 75 [$M^+ + 1$] (calcd for $C_{13}H_{18}NO_4$ 252.123 58).

***N*-Benzyl-2-(*N*-benzyloxycarbonyl-*N*-ethylamino)propionamide (29).** Utilizing method A and using **28** (3.80 g, 15.1 mmol), THF (80 mL), 4-methylmorpholine (2.0 mL, 18.2 mmol), isobutyl chloroformate (2.4 mL, 18.2 mmol), and benzylamine (2.0 mL, 18.2 mmol) gave crude **29**. The product was purified by column chromatography (SiO_2; 2:1, hexanes/EtOAc) to obtain 4.27 g (83%) of pure **29** as a white solid: mp 88–89 °C; R_f 0.39 (2:1, hexanes/EtOAc); IR (KBr) 3304, 3067, 3031, 2980, 1683, 1649, 1531 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.11 (t, $J = 6.8$ Hz, NCH_2CH_3), 1.41 (d, $J = 7.2$ Hz, $CHCH_3$), 3.21–3.39 (m, NCH_2CH_3), 4.36 (d, $J = 4.2$ Hz, CH_2Ph), 4.62–4.67 (m, CH), 5.11 (5.15) (s, OCH_2Ph), 7.17–7.32 (m, 10 PhH), the structural assignments were in agreement with the ^1H–^1H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 15.1 (14.8), 15.2 (15.3), 39.3 (39.4), 43.6, 55.1 (55.3), 67.6, 127.4, 127.9, 128.3, 128.7, 136.5, 138.3, 157.0, 171.7; MS (+CI) (rel intensity) 342 (22), 341 ($M^+ + 1$, 100), 233 (21); M_r (+CI) 341.186 01 [$M^+ + 1$] (calcd for $C_{20}H_{25}N_2O_3$ 341.186 52). Anal. calcd for $C_{20}H_{24}N_2O_3$: C, 70.57%; H, 7.11%; N, 8.23%. Found: C, 70.64%; H, 7.08%; N, 8.34%.

***N*-Benzyl-2-ethylaminopropionamide (30).** A methanolic solution of **29** (2.09 g, 6.15 mmol) was hydrogenated (1 atm) in the presence of 10% Pd/C (~200 mg) at room temperature (12 h). The mixture was carefully filtered through a bed of Celite® 521 and the clear filtrate was evaporated in vacuo to obtain a pale-yellow oil. Purification of the product by column chromatography (SiO_2; 1:9, MeOH/$CHCl_3$) gave **30** (1.22 g, 96%) as a clear oil: R_f 0.54 (1:9, MeOH/$CHCl_3$); IR (neat) 3303 (br), 3064, 2969, 1656, 1525 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.03 (dt, $J = 2.0$, 7.2 Hz, $NHCH_2CH_3$), 1.19–1.25 (br s, $NHCH_2CH_3$), 1.32 (d, $J = 7.1$ Hz, $CHCH_3$), 2.50–2.68 (m, $NHCH_2CH_3$), 3.21 (q, $J = 7.1$ Hz, CH), 4.44 (d, $J = 5.7$ Hz, CH_2Ph), 7.24–7.35 (m, 5 PhH), 7.60–7.63 (br s, C(O)NH); ^{13}C NMR ($CDCl_3$) δ 15.6, 20.1, 43.1, 43.2, 58.5, 127.5, 127.7, 128.8, 138.9, 175.4; MS (+CI) (rel intensity) 208 (12), 207 ($M^+ + 1$, 100); M_r (+CI) 207.149 72 [$M^+ + 1$] (calcd for $C_{12}H_{19}N_2O$ 207.149 74). Anal. calcd for $C_{12}H_{18}N_2O{\cdot}0.4H_2O$: C, 67.51%; H, 8.88%; N, 13.12%. Found: C, 67.52%; H, 8.81%; N, 13.17%.

***N*-Benzyl-2-(*N*-acetyl-*N*-ethyl)aminopropionamide (8).** Compound **8** was prepared utilizing method C and using **30** (1.60 g, 7.76 mmol), THF (100 mL), TEA (1.2 mL, 8.54 mmol), Ac_2O (800 μL, 8.54 mmol), and a catalytic amount of DMAP (~100 mg). The reaction was stirred at room temperature (16 h), and then the solvent was evaporated in vacuo to provide a pale-yellow residue. The product was purified by column chromatography (SiO_2; 1:33, MeOH/$CHCl_3$) to give 1.71 g (89%) of pure **8** as a white solid: mp 60–63 °C; R_f 0.42 (1:19, MeOH/$CHCl_3$); IR (KBr) 3309, 2977, 2917, 1673, 1626, 1526 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.29 (t, $J = 7.2$ Hz, NCH_2CH_3), 1.37 (1.49) (d, $J = 7.2$ (6.0) Hz, $CHCH_3$), 2.07 (2.03) (s, $CH_3C(O)$), 3.34 (q, $J = 7.2$ Hz, NCH_2CH_3), 4.37 (d, $J = 5.7$ Hz, CH_2Ph), 5.03 (q, $J = 7.2$ Hz, CH), 7.10–7.14 (br s, C(O)NH), 7.20–7.31 (m, 5 PhH), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 90:10 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 14.4, 15.5, 21.7, 40.0, 43.3, 53.3, 127.2, 127.5, 128.5, 138.5, 171.7, 171.8; MS (+CI) (rel intensity) 249 ($M^+ + 1$, 21), 142 (100); M_r (+CI) 249.160 54 [$M^+ + 1$] (calcd for $C_{14}H_{21}N_2O_2$ 249.160 30). Anal. calcd for $C_{14}H_{20}N_2O_2$: C, 67.71%; H, 8.12%; N, 11.28%. Found: C, 67.83%; H, 8.16%; N, 11.31%.

2-(*N*-Benzyloxycarbonyl-*N*-methyl)aminobutyric acid methyl ester (32). Utilizing a procedure comparable for the synthesis of **27** and using **31**[33] (86 mg, 0.36 mmol), DMF (2.5 mL), Ag_2O (336 mg, 1.45 mmol), and MeI (0.2 mL, 2.90 mmol) at 35 °C (1 d) gave pure **32** (67 mg, 70%) as a clear oil after purification by column chromatography (SiO_2; 2:1, hexanes/EtOAc): R_f 0.57 (2:1, hexanes/EtOAc); IR (neat) 2970, 2955, 1744, 1703 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.93 (0.91) (t, $J = 7.2$ Hz, CH_2CH_3 (m, CH_2CH_3 minor conformer, the signal overlaps the major conformer peak)), 1.66–1.78 (m, $CHH'CH_3$), 1.94–2.06 (m, $CHH'CH_3$), 2.88 (2.89) (s, NCH_3), 3.70 (3.65) (s, $C(O)OCH_3$), 4.75 (4.53) (dd, $J = 5.1$ (5.0), 10.5 (10.7) Hz, CH), 5.17 (5.15) (s, OCH_2Ph), 7.26–7.47 (m, 5 PhH), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 60:40 ratio in $CDCl_3$, the structural assignments were in agreement with the ^1H–^1H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 10.8, 22.1 (22.4), 30.4 (30.9), 52.1, 60.0 (60.3), 67.5, 127.8, 128.1, 128.6, 136.8, 157.2 (156.5), 172.2 (172.0); MS (+CI) (rel intensity) 267 (15), 266 ($M^+ + 1$, 100); M_r (+CI) 266.139 29 [$M^+ + 1$] (calcd for $C_{14}H_{20}NO_4$ 266.139 23). Anal. calcd for $C_{14}H_{19}NO_4$: C, 63.38%; H, 7.22%; N, 5.28%. Found: C, 63.29%; H, 7.23%; N, 5.38%.

2-(*N*-Benzyloxycarbonyl-*N*-methylamino)butyric acid (33). Utilizing a procedure comparable for the synthesis of **28** and using **32** (3.22 g, 12.1 mmol) and aq 2 M KOH/MeOH solution (20/40 mL) at 35 °C (1 h) gave 3.06 g (99%) of pure **33** as a clear oil: R_f 0.59 (1:9, MeOH/$CHCl_3$); IR (neat) 3112 (br), 2972, 2939, 1741, 1705, 1680 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.93 (0.90) (t, $J = 7.2$ Hz, CH_2CH_3 (m, CH_2CH_3 minor conformer, the signal overlaps the major conformer peak)), 1.69–1.79 (m, $CHH'CH_3$), 1.97–2.10 (m, $CHH'CH_3$), 2.89 (2.90) (s, NCH_3), 4.76 (4.57) (dd, $J = 5.2$ (4.8), 11.0 (10.8) Hz, CH), 5.17 (s, OCH_2Ph), 7.26–7.47 (m, 5 PhH), 10.60 (s, C(O)OH), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 55:45 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 10.9, 21.9 (22.2), 30.4 (30.8), 60.1, 67.8, 127.9, 128.1, 128.6, 136.5, 157.6 (156.8), 176.9 (176.7); MS (+CI) (rel intensity) 253 (14), 252 ($M^+ + 1$, 100), 208 (61); M_r (+CI) 252.123 55 [$M^+ + 1$] (calcd for $C_{13}H_{18}NO_4$ 252.123 58). Anal. calcd for $C_{13}H_{17}NO_4{\cdot}0.2H_2O$: C, 61.48%; H, 6.87%; N, 5.51%. Found: C, 61.48%; H, 6.83%; N, 5.47%.

***N*-Benzyl-2-(*N*-benzyloxycarbonyl-*N*-methyl)aminobutanamide (34).** Utilizing method A and using **33** (3.38 g, 13.4 mmol), THF (100 mL), 4-methylmorpholine

(1.8 mL, 16.2 mmol), isobutyl chloroformate (2.1 mL, 16.2 mmol), and benzylamine (1.8 mL, 16.2 mmol) gave crude **34**. The product was purified by column chromatography (SiO_2; 3:1, hexanes/EtOAc) to obtain 4.12 g (90%) of pure **34** as a clear oil: R_f 0.40 (2:1, hexanes/EtOAc); IR (KBr) 3327, 3064, 3032, 2968, 2936, 1694, 1674, 1531 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.97 (t, $J=7.2$ Hz, CH_2CH_3), 1.74–1.84 (m, $CHH'CH_3$), 2.02–2.18 (m, $CHH'CH_3$), 2.95 (s, NCH_3), 4.46 (br d, $J=4.8$ Hz, $NHCH_2Ph$), 4.62–4.70 (m, CH), 5.17 (s, OCH_2Ph), 6.82–6.94 (6.56–6.60) (br s, NH), 7.27–7.39 (m, 10 PhH), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 75:25 ratio in $CDCl_3$, the structural assignments were in agreement with the ^1H–^1H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 10.5, 21.2 (20.8), 29.6 (29.7), 43.3, 60.3 (60.4), 67.5, 127.3, 127.5, 127.6, 128.1, 128.5, 128.6, 136.4, 138.4, 157.4 (156.4), 170.6, the structural assignments for the ^1H and ^{13}C NMR were in agreement with the HETCOR and DEPT experiments; MS (+CI) (rel intensity) 342 (17), 341 ($M^+ + 1$, 100); M_r (+CI) 341.187 12 [$M^+ + 1$] (calcd for $C_{20}H_{25}N_2O_3$ 341.186 52). Anal. calcd for $C_{20}H_{24}N_2O_3$: C, 70.56%; H, 7.11%; N, 8.23%. Found: C, 70.41%; H, 7.13%; N, 8.25%.

***N*-Benzyl-2-*N*-methylaminobutanamide (35).** Utilizing the procedure for **30** and using **34** (3.70 g, 10.8 mmol), MeOH (50 mL), 10% Pd/C (~300 mg) and H_2 (1 atm) gave after 1 day pure **35** (2.30 g, 99%) as a clear oil: R_f 0.53 (1:9, MeOH/$CHCl_3$); IR (neat) 3301 (br), 2966, 2934, 1650, 1525 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (t, $J=7.5$ Hz, CH_2CH_3), 1.32 (br s, $NHCH_3$), 1.58–1.68 (m, $CHH'CH_3$), 1.75–1.86 (m, $CHH'CH_3$), 2.37 (s, NCH_3), 2.95 (dt, $J=1.5$, 7.2 Hz, CH), 4.47 (d, $J=5.7$ Hz, CH_2Ph), 7.26–7.33 (m, 5 PhH), 7.49 (br s, NH); ^{13}C NMR ($CDCl_3$) δ 10.4, 26.8, 35.8, 43.2, 66.7, 127.5, 127.9, 128.9, 138.9, 174.2; MS (+CI) (rel intensity) 208 (13), 207 ($M^+ + 1$, 100); M_r (+CI) 207.149 37 [$M^+ + 1$] (calcd for $C_{12}H_{19}N_2O$ 207.149 74). Anal. calcd for $C_{12}H_{18}N_2O{\cdot}0.4H_2O$: C, 67.51%; H, 8.88%; N, 13.12%. Found: C, 67.56%; H, 8.73%; N, 13.08%.

***N*-Benzyl-2-(*N*-acetyl-*N*-methyl)aminobutanamide (9).** Compound **9** was prepared utilizing method C and using **35** (2.15 g, 10.4 mmol), THF (120 mL), TEA (1.6 mL, 11.5 mmol), Ac_2O (1.1 mL, 11.5 mmol), and a catalytic amount of DMAP (~200 mg). The reaction was stirred at room temperature (1 day) and then the solvent was evaporated in vacuo to provide a pale-yellow residue. The product was purified by column chromatography (SiO_2; 1:33, MeOH/$CHCl_3$) to give 1.94 g (76%) of pure **9** as a thick clear oil: R_f 0.58 (1:19, MeOH/$CHCl_3$); IR (neat) 3315 (br), 2927, 1683, 1647, 1526 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, $J=7.5$ Hz, CH_2CH_3), 1.60–1.72 (m, $CHH'CH_3$), 1.91–1.99 (m, $CHH'CH_3$), 2.07 (2.17) (s, $CH_3C(O)$), 2.91 (2.75) (s, NCH_3), 4.37–4.41 (m, $NHCH_2Ph$), 4.95 (dd, $J=7.2$, 8.4 Hz, CH), 6.83 (7.01) (br s, NH), 7.20–7.33 (m, 5 PhH), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 90:10 ratio in $CDCl_3$, the structural assignments were in agreement with the ^1H–^1H COSY experiment; ^{13}C NMR ($CDCl_3$) δ 10.6, 21.0, 22.1, 31.4, 43.3, 57.6, 127.4, 127.6, 128.7, 138.5, 170.6, 172.2; MS (+CI) (rel intensity) 250 (13), 249 ($M^+ + 1$, 100), 142 (97); M_r (+CI) 249.159 93 [$M^+ + 1$] (calcd for $C_{14}H_{21}N_2O_2$ 249.160 30). Anal. calcd for $C_{14}H_{20}N_2O_2$: C, 67.71%; H, 8.12%; N, 11.28%. Found: C, 67.82%; H, 8.11%; N, 11.17%.

3-Benzyl-5-methyl-imidazolidin-2,4-dione (37).[36,42] DL-Alanine (**36**) (1.52 g, 17.06 mmol) was dissolved in an aqueous KOH solution (1.15 g, 20.5 mmol, 31 mL H_2O) at 0 °C and then benzyl isocyanate (2.5 mL, 20.5 mmol) was added over a 20 min period. The reaction was warmed to 65 °C for 20 min and then the precipitate was filtered, and the filtrate acidified to pH 2 with aqueous 3 N HCl leading to the precipitation of a white solid. The solid was collected by filtration and then suspended in an aqueous 6 N HCl solution (14 mL) and the reaction mixture heated at 75 °C (12 h). The resulting solution was allowed to return to room temperature leading to a precipitation of a solid that was filtered, and recrystallized (EtOH) to give 1.88 g (55%) of pure **37** as a white solid: mp 112–113 °C (lit.[42] mp 112–114 °C); R_f 0.60 (EtOAc); ^1H NMR ($CDCl_3$) δ 1.43 (d, $J=6.9$ Hz, $CHCH_3$), 4.08 (q, $J=6.9$ Hz, CH), 4.65 (s, CH_2Ph), 6.30 (s, NH), 7.26–7.39 (m, 5 PhH).

1-Acetyl-3-benzyl-5-methyl-imidazolidin-2,4-dione (10). Utilizing method C and using **37** (1.58 g, 7.74 mmol), THF (30 mL), TEA (2.6 mL, 18.58 mmol), Ac_2O (880 μL), and DMAP (~150 mg) gave crude **10** after 1.5 days. The product was purified by column chromatography (SiO_2; 1:1, hexanes/EtOAc) to give 1.60 g (84%) of pure **10** as a white solid: mp 64–65 °C; R_f 0.64 (1:1, hexanes/EtOAc); IR (KBr) 3066, 2935, 1793, 1705 (br) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.57 (d, $J=6.9$ Hz, $CHCH_3$), 2.56 (s, $CH_3C(O)$), 4.53 (q, $J=6.9$ Hz, CH), 4.69 (s, CH_2Ph), 7.31–7.42 (m, 5 PhH); ^{13}C NMR ($CDCl_3$) δ 16.6, 25.3, 42.9, 55.3, 128.5, 128.9, 129.1, 135.3, 153.5, 169.2, 171.8; MS (+CI) (rel intensity) 248 (12), 247 ($M^+ + 1$, 100); M_r (+CI) 247.108 71 [$M^+ + 1$] (calcd for $C_{13}H_{15}N_2O_3$ 247.108 27). Anal. calcd for $C_{13}H_{14}N_2O_3$: C, 63.40%; H, 5.73%; N, 11.38%. Found: C, 63.36%; H, 5.74%; N, 11.29%.

3-Benzyl-5-methyl-2-thioxo-imidazolidin-4-one (39). To a solution of **38** (3.13 g, 22.4 mmol) in CH_2Cl_2 (70 mL) was added TEA (3.1 mL, 22.4 mmol) and benzyl isothiocyanate (3.0 mL, 22.4 mmol). The solution was stirred at room temperature (30 min) and then heated to reflux (30 min). The solvent was evaporated in vacuo, the residue redissolved in CH_2Cl_2 (50 mL), and washed with H_2O (20 mL). The organic phase was concentrated in vacuo to yield crude **39**. The solid was recrystallized (EtOH) to obtain 4.00 g (82%) of pure **39** as a white solid: mp 149–150 °C; R_f 0.52 (1:1, hexanes/EtOAc); IR (KBr) 3170, 3012, 1743, 1535 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.46 (d, $J=7.2$ Hz, $CHCH_3$), 4.15 (q, $J=7.2$ Hz, CH), 4.95 (d, $J=14.6$ Hz, $CHH'Ph$), 5.02 (d, $J=14.6$ Hz, $CHH'Ph$), 7.26–7.45 (m, 5 PhH), 7.80 (s, NH); ^{13}C NMR ($CDCl_3$) δ 17.0, 44.7, 55.2, 128.1, 128.7, 128.9, 135.8, 174.8, 183.7; MS (+CI) (rel intensity) 221 ($M^+ + 1$, 100); M_r (+CI) 221.075 25 [$M^+ + 1$] (calcd for $C_{11}H_{13}N_2OS$ 221.074 86). Anal. calcd for $C_{11}H_{12}N_2O_2$:

C, 59.97%; H, 5.49%; N, 12.72%. Found: C, 59.96%; H, 5.48%; N, 12.72%.

1-Acetyl-3-benzyl-5-methyl-2-thioxo-imidazolidin-4-one (11). Utilizing method C and using **39** (1.50 g, 6.82 mmol), THF (60 mL), TEA (1.1 mL, 8.18 mmol), Ac_2O (770 μL, 8.18 mmol) and DMAP (~100 mg) gave crude **11** after 1 h. The product was recrystallized (EtOH) to give 1.78 g (99%) of pure **11** as a white solid: mp 76–77 °C; R_f 0.69 (1:1, hexanes/EtOAc); IR (KBr) 3034, 2928, 1759, 1701 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.59 (d, J = 6.9 Hz, CHCH_3), 2.80 (s, CH_3C(O)), 4.68 (q, J = 6.9 Hz, CH), 5.00 (d, J = 14.4 Hz, CHH′Ph), 5.08 (d, J = 14.4 Hz, CHH′Ph), 7.28–7.42 (m, 5 PhH); ^{13}C NMR ($CDCl_3$) δ 17.0, 28.2, 45.1, 57.4, 128.2, 128.7, 128.9, 135.1, 170.4, 172.8, 180.6; MS (+CI) (rel intensity) 264 (12), 263 (M^+ + 1, 100); M_r (+CI) 263.085 17 [M^+ + 1] (calcd for $C_{13}H_{15}N_2O_2S$ 263.085 43). Anal. calcd for $C_{13}H_{14}N_2O_2S$: C, 59.52%; H, 5.38%; N, 10.68%. Found: C, 59.64%; H, 5.33%; N, 10.61%.

2-(3-Benzylureido)-2-phenylacetic acid methyl ester (41).[43] Utilizing a similar procedure for **39** and using **40** (1.50 g, 7.43 mmol), THF (70 mL), TEA (2.1 mL, 14.87 mmol), and benzyl isocyanate (1.80 mL, 14.8 mmol) gave crude **41** after 10 min at 60 °C. The residue was recrystallized (EtOH) to give 3.17 g (72%) of pure **41** as a white solid: mp 105–107 °C; R_f 0.43 (1:1, hexanes/EtOAc); ^1H NMR (DMSO-d_6) δ 3.62 (s, OCH_3), 4.22 (d, J = 5.9 Hz, CH_2Ph), 5.31 (d, J = 7.8 Hz, CH), 6.54 (t, J = 5.9 Hz, NHCH$_2$), 7.05 (d, J = 7.8 Hz, NHCH), 7.24–7.42 (m, 10 PhH).

3-Benzyl-5-phenylimidazolidin-2,4-dione (42).[36] To a methanolic solution (40 mL) of **41** (3.00 g, 10.07 mmol) was added TEA (1.4 mL, 10.07 mmol), and the reaction solution was stirred at 40 °C (4 h). The solvent was evaporated in vacuo to yield crude **42**. The residue was recrystallized (*i*PrOH–H_2O) to give 2.25 g (84%) of pure **42** as a white solid: mp 171–172 °C (lit.[36a] mp 171–173 °C); ^1H NMR (DMSO-d_6) δ 4.54 (d, J = 15.6 Hz, CHH′Ph), 4.59 (d, J = 15.6 Hz, CHH′Ph), 5.31 (s, CH), 7.24–7.44 (m, 10 PhH), 8.79 (s, NH).

1-Acetyl-3-benzyl-5-phenylimidazolidin-2,4-dione (12). Utilizing method C and using **42** (1.42 g, 5.30 mmol), THF (30 mL), TEA (890 μL, 6.40 mmol), Ac_2O (600 μL, 6.40 mmol), and DMAP (~150 mg) gave after 16 h crude **12**. The product was recrystallized (EtOH) to give 1.10 g (67%) of pure **12** as a white solid: mp 112–113 °C; R_f 0.67 (1:1, hexanes/EtOAc); IR (KBr) 3035, 2966, 1782, 1716 (br) cm^{-1}; ^1H NMR ($CDCl_3$/DMSO-d_6) δ 2.50 (s, CH_3C(O)), 4.64 (d, J = 15.2 Hz, CHH′Ph), 4.70 (d, J = 15.2 Hz, CHH′Ph), 5.58 (s, CH), 7.26–7.35 (m, 10 PhH); ^{13}C NMR ($CDCl_3$) δ 24.9, 43.1, 62.3, 126.5, 128.4, 128.8, 128.9, 129.1, 133.5, 135.2, 153.7, 168.2, 169.2, one of the aromatic carbon peaks was not detected and is believed to overlap with the observed peaks; MS (+CI) (rel intensity) 310 (19), 309 (M^+ + 1, 100), 121 (46); M_r (+CI) 309.123 16 [M^+ + 1] (calcd for $C_{18}H_{17}N_2O_3$ 309.123 92). Anal. calcd for $C_{18}H_{16}N_2O_3 \cdot 0.45H_2O$: C, 68.32%; H, 5.38%; N, 8.85%. Found: C, 68.23%; H, 5.22%; N, 8.80%.

***N*-Benzyl-2-*N*-(benzyloxycarbonyl)amino-3-methoxy-propionamide (44).** To a CH_3CN (250 mL) solution of **43**[29] (6.72 g, 20.4 mmol) was successively added Ag_2O (23.70 g, 0.15 mol) and CH_3I (12.75 mL, 0.30 mol) at room temperature. The reaction was maintained at room temperature (3 days), filtered and the solvent evaporated in vacuo. The residue was purified by column chromatorgraphy (SiO_2; 1:9, MeOH/$CHCl_3$) to give 6.50 g (93%) of **44** as a white solid: mp 140–141 °C; R_f 0.31 (1:1, hexanes/EtOAc); IR (KBr) 3304, 3062, 2954, 1690, 1546 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.36 (s, OCH_3), 3.50 (dd, J = 6.6, 9.2 Hz, CHH′OCH$_3$), 3.85 (dd, J = 3.9, 9.2 Hz, CHH′OCH$_3$), 4.30–4.36 (m, CH), 4.47 (d, J = 5.7 Hz, CH_2NH), 5.11 (s, CH_2OC(O)), 5.60–5.70 (m, NH), 6.65–6.72 (m, NH), 7.26–7.36 (m, 10 PhH); ^{13}C NMR ($CDCl_3$) δ 43.8, 54.6, 59.3, 67.5, 72.2, 127.7, 128.4, 128.5, 128.8, 128.9, 136.3, 138.1, 156.3, 170.1; MS (+CI) (rel intensity) 344 (22), 343 (M^+ + 1, 100); M_r (+CI) 343.165 81 [M^+ + 1] (calcd for $C_{19}H_{23}N_2O_4$ 343.165 78). Anal. calcd for $C_{19}H_{22}N_2O_4$: C, 66.65%; H, 6.48%; N, 8.18%. Found: C, 66.93%; H, 6.55%; N, 8.15%.

***N*-Benzyl-2-amino-3-methoxypropionamide (45).** Utilizing the procedure for **30** and using **44** (4.00 g, 11.7 mmol) and 10% Pd/C (~400 mg) gave 2.43 g (99%) of **45** after purification by flash column chromatography (SiO_2; 1:9, MeOH/$CHCl_3$) as a pale-yellow oil: R_f 0.33 (1:9, MeOH/$CHCl_3$); IR (liquid film) 3357, 3312, 3063, 2927, 2896, 2826, 1655, 1527 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.74 (br s, NH_2), 3.34 (s, OCH_3), 3.53–3.61 (m, CHCH$_2$), 4.36–4.48 (m, CH_2NH), 7.22–7.33 (m, 5 PhH), 7.75–7.86 (m, NH); ^{13}C NMR ($CDCl_3$) δ 43.1, 54.9, 58.8, 74.6, 127.3, 127.6, 128.6, 138.4, 172.8; MS (+CI) (rel intensity) 210 (12), 209 (M^+ + 1, 100); M_r (+CI) 209.129 15 [M^+ + 1] (calcd for $C_{11}H_{17}N_2O_2$ 209.129 00). Anal. calcd for $C_{11}H_{16}N_2O_2 \cdot 0.2H_2O$: C, 62.36%; H, 7.80%; N, 13.22%. Found: C, 62.43%; H, 7.78%; N, 13.11%.

1-Acetyl-3-benzyl-5-methoxymethyl-2-thioxo-imidazolidin-4-one (13). To a THF solution (100 mL) of **45** (2.50 g, 12.0 mmol), was added di-2-pyridyl thionocarbonate (2.66 g, 11.4 mmol) and then the solution was stirred at room temperature (24 h). The reaction was concentrated in vacuo and the residue redissolved in MeOH, stirred at room temperature (2 h), and then the solvent was evaporated in vacuo to yield a dark orange oil. The crude was used without further purification and was dissolved in THF (125 mL) and then TEA (1.9 mL, 13.6 mmol), Ac_2O (1.3 mL, 13.6 mmol), and DMAP (~200 mg) were added. The reaction was stirred at room temperature (6 h), and the solvent evaporated in vacuo to give crude **13**. The product was purified by column chromatography (SiO_2; 1:1, hexanes/EtOAc) to yield 1.94 g (55%) of pure **13** as a pale-orange solid: mp 98–99 °C; R_f 0.41 (4:1, hexanes/EtOAc); IR (KBr) 2931, 2827, 1759, 1697 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.84 (s, CH_3C(O)), 3.25 (OCH_3), 3.86 (dd, J = 1.2, 9.9 Hz, CHH′OCH$_3$), 4.10 (dd, J = 2.1, 9.9 Hz, CHH′OCH$_3$), 4.71–4.74 (br s, CH), 5.03 (d, J = 14.9 Hz, CHH′Ph), 5.12 (d, J = 14.9 Hz, CHH′Ph), 7.26–7.39 (m, 5 PhH); ^{13}C NMR ($CDCl_3$) δ 28.1, 45.2, 59.6, 62.2, 69.3, 128.0,

128.4, 128.6, 134.9, 170.7, 170.8, 181.4; MS (+CI) (rel intensity) 294 (14), 293 ($M^+ + 1$, 100); M_r (+CI) 293.095 60 [$M^+ + 1$] (calcd for $C_{14}H_{17}N_2O_3S$ 293.095 99). Anal. calcd for $C_{14}H_{16}N_2O_3S$: C, 57.52%; H, 5.52%; N, 9.58%. Found: C, 57.61%; H, 5.45%; N, 9.54%.

***N*-Benzyl-*N*-methyl-2-(benzyloxycarbonyl)amino-3-hydroxypropionamide (49).** Utilizing method B and using carbobenzyloxy-DL-serine (**48**) (12.60 g, 52.7 mmol), DMF (100 mL), EDCI (16.10 g, 84.3 mmol), 4-methylmorpholine (9.3 mL, 84.3 mmol), HOBt (10.60 g, 79 mmol) and *N*-methylbenzylamine (8.2 mL, 63.2 mmol) gave after 16 h crude **49** as a yellow oil. The product was purified by column chromatography (SiO_2; 1:1, hexanes/EtOAc) to give 8.76 g (49%) of pure **49** as a white solid: mp 89–91 C; R_f 0.53 (1:19, MeOH/$CHCl_3$); IR (liquid film) 3433, 3309, 3032, 2931, 1712, 1628 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.01 (2.91) (s, NC*H*$_3$), 3.42 (br s, O*H*), 3.70–3.86 (m, C*H*$_2$OH), 4.50–4.70 (m, C*H*$_2$Ph), 4.74–4.84 (m, C*H*), 5.10 (5.03–5.05) (s (m), OC*H*$_2$Ph), 6.05–6.14 (m, N*H*), 7.18–7.34 (m, 10 Ph*H*), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 60:40 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 35.0 (34.2), 51.4 (53.4), 52.7 (51.9), 64.1, 67.3, 126.2, 127.7, 128.0, 128.1, 128.3, 128.6, 128.9, 129.0, 136.1, 136.3, 136.4, 156.5, 170.9 (171.6), the ^{13}C NMR assignments for the aliphatic carbons signals were in agreement with the DEPT experiment; MS (+CI) (rel intensity) 343 ($M^+ + 1$, 100), 299 (22), 235 (18); M_r (+CI) 343.165 70 [$M^+ + 1$] (calcd for $C_{19}H_{23}N_2O_4$ 343.165 78). Anal. calcd for $C_{19}H_{22}N_2O_4 \cdot 0.7H_2O$: C, 64.28%; H, 6.64%; N, 7.89%. Found: C, 64.17%; H, 6.33%; N, 7.89%.

***N*-Benzyl-*N*-methyl-2-(benzyloxycarbonyl)amino-3-methoxypropionamide (50).** To a CH_3CN (250 mL) solution of **49** (8.70 g, 25.4 mmol) was successively added Ag_2O (29.00 g, 0.13 mol) and MeI (16.0 mL, 0.25 mmol) at room temperature. The reaction was maintained at room temperature (3 days), filtered and the solvent evaporated in vacuo. The residue was purified by column chromatography (SiO_2; 1:1, hexanes/EtOAc) to give 8.41 g (97%) of pure **50** as a pale-yellow 0:1: R_f 0.29 (1:1, hexanes/EtOAc); IR (liquid film) 3292, 3032, 2931, 1712, 1643 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.00 (2.90) (s, NC*H*$_3$), 3.33 (3.26) (s, OC*H*$_3$), 3.48–3.65 (m, C*H*$_2$OCH$_3$), 4.44 (4.57) (d, $J = 14.7$ (16.8) Hz, C*H*H′Ph), 4.77 (4.71) (d, $J = 14.7$ (16.8) Hz, CH*H*′Ph), 4.91–4.98 (br q, $J = 6.7$ Hz, C*H*), 5.10 (5.07) (s, OC*H*$_2$Ph), 5.94 (m, N*H*), 7.19–7.33 (m, 10 Ph*H*), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 65:35 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 35.0 (34.0), 50.7, 51.4 (53.3), 59.3, 66.9, 73.4, 126.9, 127.5, 127.9, 128.0, 128.1, 128.5, 128.7, 128.8, 136.2, 136.5, 136.7, 156.0, 170.6, the ^1H NMR and ^{13}C NMR assignments for the aliphatic carbons signals were in agreement with the HETCOR and DEPT experiments; MS (+CI) (rel intensity) 358 (19), 357 ($M^+ + 1$, 100), 249 (15); M_r (+CI) 357.181 69 [$M^+ + 1$] (calcd for $C_{20}H_{25}N_2O_4$ 357.181 43). Anal. calcd for $C_{20}H_{24}N_2O_4$: C, 67.40%; H, 6.79%; N, 7.86%. Found: C, 67.24%; H, 6.88%; N, 7.80%.

***N*-Benzyl-*N*-methyl-2-amino-3-methoxypropionamide (51).** Utilizing the procedure for **30** and employing **50** (3.66 g, 10.2 mmol) and 5% Pd/C (~350 mg) gave 2.08 g (91%) of **51** as a pale-yellow oil after purification by flash column chromatography (SiO_2; 1:9, MeOH/$CHCl_3$): R_f 0.45 (1:9, MeOH/$CHCl_3$); IR (liquid film) 3474, 3368, 3299, 2980, 2929, 2894, 1640 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.72 (br s, N*H*$_2$), 2.98 (2.95) (s, NC*H*$_3$), 3.36 (3.31) (s, OC*H*$_3$), 3.47–3.55 (3.40–3.46) (m, C*H*$_2$OCH$_3$), 4.00 (3.92) (t, $J = 6.6$ (6.6) Hz, C*H*), 4.51 (d, $J = 14.6$ Hz, C*H*H′Ph), 4.73 (d, $J = 14.6$ Hz, CH*H*′Ph), 7.18–7.37 (m, 5 Ph*H* and N*H*), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 60:40 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 34.5 (34.1), 51.1 (52.8), 51.2, 59.0, 76.1 (76.2), 126.4, 127.2, 127.5, 127.8, 128.5, 128.8, 136.6, 136.9, 173.4 (173.9), the ^1H and ^{13}C NMR assignments for the aliphatic carbons signals were in agreement with the HETCOR and DEPT experiments; MS (+CI) (rel intensity) 224 (13), 223 ($M^+ + 1$, 100), 122 (11); M_r (+CI) 223.144 45 [$M^+ + 1$] (calcd for $C_{12}H_{19}N_2O_2$ 223.144 65). Anal. calcd for $C_{12}H_{18}N_2O_2 \cdot 0.32H_2O$: C, 63.20%; H, 8.16%; N, 12.28%. Found: C, 63.29%; H, 8.15%; N, 12.13%.

***N*-Benzyl-*N*-methyl-2-acetamido-3-methoxypropionamide (47).** Utilizing method C and employing **51** (360 mg, 1.62 mmol), THF (10 mL), TEA (250 μL, 1.78 mmol), Ac_2O (170 μL, 1.78 mmol) and a catalytic amount of DMAP gave crude **47** after 3 h at room temperature. The product was purified by column chromatography (SiO_2; 1:19, MeOH/$CHCl_3$) to obtain 412 mg (96%) of pure **47** as a colorless oil: R_f 0.44 (1:19, MeOH/$CHCl_3$); IR (neat) 3302 (br), 3062, 2931, 1766, 1635 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.99 (1.96) (s, C*H*$_3$C(O)), 3.00 (2.90) (s, NC*H*$_3$), 3.32 (3.25) (s, OC*H*$_3$), 3.43–3.63 (m, C*H*$_2$OCH$_3$), 4.44 (4.56) (d, $J = 14.9$ (17.0) Hz, C*H*H′Ph), 4.57 (4.71) (d, $J = 14.9$ (17.0) Hz, CH*H*′Ph), 5.13–5.20 (m, C*H*), 6.52–6.62 (m, N*H*), 7.18–7.37 (m, 5 Ph*H*), ^1H NMR analysis indicated the major and minor conformational isomers existed in a 65:35 ratio in $CDCl_3$; ^{13}C NMR ($CDCl_3$) δ 23.4, 35.2 (34.2), 49.3, 51.6 (53.4), 58.4, 73.2, 127.0, 127.7, 127.9, 128.9, 129.1, 136.2, 136.7, 169.9, 170.8, the ^{13}C NMR assignments were in agreement with the DEPT experiment; MS (+CI) (rel intensity) 265 ($M^+ + 1$, 100), 122 (53); M_r (+CI) 265.154 95 [$M^+ + 1$] (calcd for $C_{14}H_{21}N_2O_3$ 265.155 22). Anal. calcd for $C_{14}H_{20}N_2O_3 \cdot 0.2H_2O$: C, 62.76%; H, 7.67%; N, 10.46%. Found: C, 62.75%; H, 7.76%; N, 10.32%.

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)]. The mice weighed between 18 and 25 g while rats were between 100 and 150 g. All animals had free access to feed and water except during actual testing period. Housing, handling and feeding were all in accordance with recommendations contained in the 'Guide for the Care and Use of Laboratory Animals'. All of the test

compounds were administrated in suspensions of 0.5% (w/v) of methylcellulose in water. The volumes administered were 0.01 mL/g of body weight for mice and 0.2 mL/10 g for rats. Anticonvulsant activity was established using the maximal electroshock (MES) test.[30,44] For the MES test, a drop of electrolyte solution with an anesthetic (0.5% butacaine hemisulfate in 0.9% sodium chloride) was placed in the eyes of the animals prior to positioning the corneal electrodes and delivery of the non-lethal current. A 60-cycle alternating current was administered for 0.2 s in both species, utilizing 50 mA in mice and 150 mA in rats. Protection endpoints were defined as the abolition of the hind limb tonic extensor component of the induced seizure.[45] The subcutaneous pentylenetetrazole (Metrazol®) seizure threshold test (scMet) entailed administration of either 85 mg/kg of pentylenetetrazole in mice (Carworth Farms No. 1) or 70 mg/kg in rats (Sprague–Dawley) as a 0.5% solution subcutaneously in the posterior midline. This amount of pentylenetetrazole produces clonic seizures in 97% (CD_{97}) of animals tested. The animal is observed for 30 min. Protection is defined as the failure to observe even a threshold seizure (a single episode of clonic spasms of at least 5-s duration). In mice, effects of compounds on forced spontaneous motor activity were determined using the rotorod test. The inability of experimental mice to maintain their balance for 1 min on a 1-in diameter knurled rod rotating at 6 rpm in three successive trials was interpreted as a demonstration of motor impairment. Under these conditions, mice can normally maintain their balance indefinitely. Motor impairment in rats was assessed by observing the overt evidence of ataxia, abnormal gait and stance, and/or loss of placing response and muscle tone. In the mouse identification screens, all compounds were administered at three dose levels (30, 100, 300 mg/kg) and two time periods (0.5 and 4 h). Typically, in the MES seizure test one animal was used at 30 and 300 mg/kg, and three animals at 100 mg/kg. In the rotorod toxicity test four animals were used at 30 and 300 mg/kg, and eight animals at 100 mg/kg (Tables 1–3). Oral rat identification screening was performed using four animals at a fixed dose of 30 mg/kg for both the MES and the rotorod toxicity tests over five time periods ranging from one quarter to 4 h post drug administration. The quantitative determination of the median effective (ED_{50}) and toxic doses (TD_{50}) were conducted at previously calculated time of peak effect using ip route in mice and oral route in rats. Groups of at least eight animals were tested using different doses of test compound until at least two points were determined between 100 and 0% protection and minimal motor impairment. The dose of the candidate substance required to produce the desired endpoint (abolition of hindlimb tonic extensor component) in 50% of the animals in each test, and 95% confidence interval were calculated by a computer program based on methods described by Finney.[46]

Voltage-dependent sodium currents in N1 E-115 neuroblastoma

The effect of the identified compound on voltage-gated Na^+ channels was assessed using N1 E-115 neuroblastoma cells and whole-cell voltage-clamp recording techniques. The N1 E-115 neuroblastoma cell line was maintained at 35 °C in Dulbecco's modified Eagles Medium supplemented with 5% fetal calf serum, 20 mM HEPES, 80 μg/mL gentamicin and 4 mM glutamine. Prior to electrophysiological studies, cells were plated and incubated for 3–5 days in a differentiation medium similar to above with reduced (2.5%) fetal calf serum and 2% DMSO. Recordings were carried out at room temperature in a bathing solution containing 130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 5 mM glucose, 5 mM HEPES. The buffer also contained 0.1 mM $CdCl_2$ and 25 mM tetraethylammonium chloride to block voltage-gated Ca^{2+} and K^+ channels, respectively. Whole cell recordings were obtained using patch electrodes (1–2 MΩ) filled with the intracellular solution described above. The currents were filtered at 10 KHz and acquired on computer using PClamp 6 (Axon Instruments). Series resistance and capacitive currents were compensated using the internal clamp circuitry. The series resistance was 3–5 MΩ, and 75–85% of the series resistance was compensated. Cells were initially voltage clamped at −60 mV and in separate experiments the test compound was applied using the perfusion system noted above. To activate voltage-gated sodium channels, cells were hyperpolarized to −90 mV for 90 ms and then depolarized to 0 mV for five trials in control solution then again in the solution containing test compound. The data from the multiple trials were averaged. The process was then repeated but this time hyperpolarizing at −60 mV. Drug was applied using a gravity fed perfusion system coupled to a piezoelectric stepper controlled electronically.

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