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Lacosamide Isothiocyanate-based Agents: Novel Agents to Target and Identify Lacosamide Receptors

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Abstract

(*R*)-Lacosamide ((*R*)-**2**, (*R*)-*N*-benzyl 2-acetamido-3-methoxypropionamide), has recently gained regulatory approval for the treatment of partial-onset seizures in adults. Whole animal pharmacological studies have documented that (*R*)-**2** function is unique. A robust strategy is advanced for the discovery of interacting proteins associated with function and toxicity of (*R*)-**2** through the use of (*R*)-**2** analogs, **3**, that contain "affinity bait (AB)" and "chemical reporter (CR)" functional groups. In **3**, covalent modification of the interacting proteins proceeds at the AB moiety, and detection or isolation of the selectively captured protein occurs through the bioorthogonal CR group upon reaction with an appropriate probe. We report the synthesis, pharmacological evaluation, and interrogation of the mouse soluble brain proteome using **3** where the AB group is an isothiocyanate moiety. One compound, (*R*)-*N*-(4-isothiocyanato)benzyl 2-acetamido-3-(prop-2-ynyloxy) propionamide ((*R*)-**9**), exhibited excellent seizure protection in mice and, like (*R*)-**2**, anticonvulsant activity principally resided in the (*R*)-stereoisomer. Several proteins were preferentially labeled by (*R*)-**9** compared with (*S*)-**9**, including collapsin response mediator protein 2.

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Supporting Information Available: Synthetic procedures for the preparation of (*R*)- and (*S*)-**13, 15, 16, 27–30, 42, 44–46, 52-55**, and **21, 39, 40, 49, 50, 56–59, 61, 63–65**, elemental analyses, ^{1}H and ^{13}C NMR spectra of compounds reported in this study, and MS table. This material is available free of charge via the internet at http://pubs.acs.org.

Introduction

Epilepsy is a major neurological disorder that affects all populations.[1] Epilepsy describes the types of recurrent seizures produced by paroxysmal excessive neuronal discharges in the brain.[2,3] The mainstay of treatment has been the long-term and consistent administration of anticonvulsant drugs.[4-6] Unfortunately, current medications are ineffective for approximately one-third of patients with epilepsy.[7] Many continue to have seizures, while others experience disturbing side effects (*e.g.*, drowsiness, dizziness, nausea, liver damage).[8] The shortcomings of current regimens highlight the need for new agents with novel mechanisms of action. The ability to identify specific nervous system pathways and protein target sites responsible for seizures would accelerate the development of new and safer therapeutic agents.

In 1985, we discovered a novel class of anticonvulsant agents, termed functionalized amino acids (FAA[a], **1**).[9-18] The lead compound, (*R*)-lacosamide ((*R*)-**2**, (*R*)-*N*-benzyl 2-acetamido-3-methoxypropionamide[18]), has recently gained regulatory approval for adjunctive therapy for partial-onset seizures in adults.[19] Whole animal pharmacological studies have documented that (*R*)-**2** function is unique and prevents seizure spread by mechanisms different from current clinical agents.[18,20] Radioligand displacement assays using more than 100 potential binding sites, which include both ligand- and voltage-gated receptors, failed to identify high-affinity binding targets,[21] suggesting that either the target site(s) are novel or the binding is weak or both. Only recently has (*R*)-**2** function been reported to be correlated to the modulation of both the slow inactivation gate of sodium channels[22] and collapsin response mediator protein 2 (CRMP2).[21] The full spectrum of pharmacological pathways for (*R*)-**2** remains elusive.

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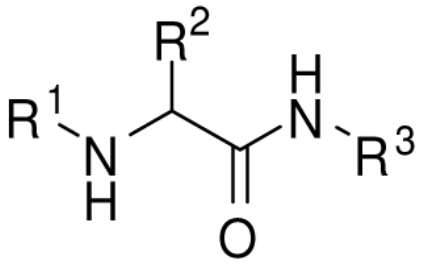
[a]Abbreviations:

FAA	functionalized amino acids
AB	affinity bait
CR	chemical reporter
CRMP2	collapsin response mediator protein 2
NMDA	*N*-methyl D-aspartate
GABA	gamma-aminobutyric acid
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
SAR	structure-activity relationship
MES	maximal electroshock
IBCF	isobutyl chloroformate
NMM	*N*-methyl morpholine
DMTMM	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
DPT	di(2-pyridyl) thionocarbonate
DITC	1,1′-thiocarbonyldiimidazole
scMet	subcutaneous metrazol
TCEP	tris(2-carboxyethyl)phosphine
iTRAQ	isobaric tag for relative and absolute quantitation
GST	glutathione-S-transferase
CNS	central nervous system



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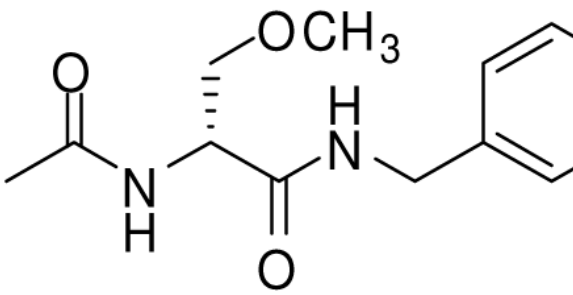


(*R*)-**2**

These findings have led us to suggest that (*R*)-**2** may interact with several targets and that (*R*)-**2** drug efficacy is a summation of multiple beneficial interactions, similar to other neurological agents.[23] Using two antiepileptic agents as examples,[24] carbamazepine interacts with sodium channels, reduces sodium and calcium influx through *N*-methyl D-aspartate (NMDA)-activated channels at glutamate receptor sites, blocks the reuptake of norepinephrine into presynaptic terminals, and has adenosine receptor antagonistic activity. Similarly, topiramate blocks sodium channels, enhances gamma-aminobutyric acid $(GABA)_A$-mediated chloride influx, acts as an antagonist to α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) and kainate receptors, and inhibits carbonic anhydrase.

A major objective of our laboratories is to identify and characterize the target(s) and mechanism (s) of (*R*)-**2** function and to advance molecular tools useful to elucidate the pathways leading to seizure control. Here, we report three modified (*R*)-**2** agents each with an appended isothiocyanate moiety that have been designed to search the brain proteome for site(s) of drug binding. These three compounds were used to interrogate soluble mouse brain lysate. We show that CRMP2 along with other potential lacosamide target(s) are selectively captured.

Results

a. The Proposed Method

Central to our efforts is the expansion of traditional and contemporary methods of identifying small-molecular-weight ligand protein target sites where binding is modest and where moderate-to-extensive ligand structural change abolishes target binding. Accordingly, we have designed (*R*)-**2** analogs that contain an "affinity bait (AB)" and a "chemical reporter (CR)"[25] group. These compounds are defined as lacosamide AB&CR agents (*R*)-**3**. In Scheme 1, we diagram the proposed pathway for (*R*)-**3** function. We anticipate that (*R*)-**3** reversibly binds to the (*R*)-**2** receptor(s) **4** to give **5**, provided that the AB and CR moieties do not perturb binding. This interaction is followed by an irreversible, covalent modification wherein receptor capture occurs through the AB moiety to give **6**. In most instances, the AB group is either an electrophilic or a photoaffinity agent. Both groups have been extensively utilized to capture biomolecular targets. Removal of the selectively captured protein **6** occurs through the bioorthogonal[25a] CR group upon reaction with a probe (**P, 7**) to provide **8**. While similar target discovery approaches have been reported,[26] few, if any, have been designed to identify receptors of small-molecular-weight ligands (drugs) where binding does not occur at the enzyme catalytic site. The captured (*R*)-**2**-bound target **8** in the reaction mixture (*e.g.*, cell lysate) is either detected by in-gel fluorescence through the use of a reporter group tethered to

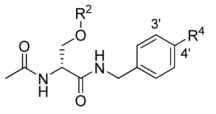
7 or is selectively removed from the mixture using affinity-based chromatographic methods (*e.g.*, biotin-(strept)avidin).

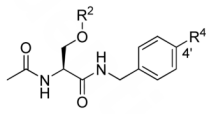
b. Structural and Chemical Criteria for Lacosamide AB&CR Probes ((*R*)-3)

Six criteria have been established for our AB&CR ((*R*)-**3**) agents. First, the compound must conform to the structure-activity relationship (SAR) observed for **1**.[9-18] In this case, we must limit the size of the AB and the CR groups because of **1**'s SAR.[9-18] Second, the reactive AB group must have documented success in previous target adduction studies.[27] In this report, we describe (*R*)-**3** agents into which an electrophilic affinity bait has been installed. For the electrophilic AB, we expect that this group must be correctly aligned with the target-based nucleophile if covalent modification is to take place.[27] Third, the CR group must be inert under the conditions necessary to bait the target protein yet must be prone to react with probe **P**. Fourth, syntheses of both enantiomeric forms of the lacosamide AB&CR agents **3** are necessary. A distinguishing feature of **1** is that its anticonvulsant activity resides principally in the D-configuration.[11-13,15,18] Accordingly, preferential enantioselective irreversible inactivation with the AB&CR agents serves as an important measure of target site specificity.[28,29] Fifth, the electrophilic (photoaffinity) labels should be incorporated at different sites within the (*R*)-**2** structure to capture all its possible binding partners. Sixth, the (*R*)-**3** agents used to identify target sites should ideally demonstrate significant anticonvulsant activity in *in vivo* assays (*e.g.*, maximal electroshock (MES)-seizure test[30]). Documentation of similar pharmacological activity provides preliminary evidence that the mode of action of (*R*)-**3** is comparable with (*R*)-**2**. We recognize that promising pharmacological data do not guarantee that these compounds will serve as site-selective agents since the ability of the AB unit to efficiently modify the protein targets is a composite of variables, some of which are unrelated to function. Correspondingly, compounds inactive *in vivo* may still function as *in vitro* affinity labels since chemical and cellular (*e.g.*, transport, metabolic) processes may prevent ligand-target site binding. Nonetheless, demonstrating significant and stereospecific *in vivo* anticonvulsant activity provides an initial stringent test for our agents.

c. Compound Selection

In this study, we selected (*R*)- and (*S*)-**9–11** as our initial AB&CR agents where AB is the electrophilic isothiocyanate moiety.[31-42] We have chosen small AB and CR groups to minimize adverse binding of our agents with possible receptor sites. In **9** and **11**, we have incorporated the isothiocyanate moiety at the 4′ *N*-benzyl position. Earlier studies showed that *N*-benzyl 4′-substituted **1** compounds exhibited enhanced anticonvulsant activities compared with their corresponding 3′-isomers.[43] In **9**, the CR group is an alkyne, while in **11** it is an azide. In compound **10**, the sites of AB (isothiocyanate) and CR (alkyne) incorporation have been interchanged from that in **9**.





(*R*)-**9** $R^2 = CH_2C{\equiv}CH$, $R^4 = N{=}C{=}S$ (*S*)-**9** $R^2 = CH_2C{\equiv}CH$, $R^4 = N{=}C{=}S$

(*R*)-**10** $R^2 = CH_2CH_2N{=}C{=}S$, $R^4 = C{\equiv}CH$ (*S*)-**10** $R^2 = CH_2CH_2N{=}C{=}S$, $R^4 = C{\equiv}CH$

(*R*)-**11** $R^2 = CH_2CH_2N_3$, $R^4 = N{=}C{=}S$ (*S*)-**11** $R^2 = CH_2CH_2N_3$, $R^4 = N{=}C{=}S$

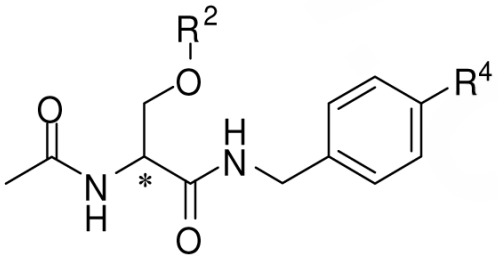
The isothiocyanate AB group displays an excellent balance between stability and reactivity; it does not readily react with hydroxylic solvents (permitting its dissolution in aqueous solutions) but does react with nucleophiles (*e.g.*, thiols (cysteines), amines (lysines)).[27] Significantly, isothiocyanate electrophilic units have proven effective in selectively modifying target proteins

(*e.g.*, GABA,[37] opioid[31,34,35]) within brain membrane fractions and have been used for *in vivo* modification studies after intracerebroventricular administration.[31c] Nonetheless, the isothiocyanate AB unit has limitations. First, only lysine and cysteine residues are likely to be modified. This restriction may prevent covalent adduction of some receptors. Fortunately, the extended C(α) methylene chain within lysines gives this nucleophilic amino acid considerable conformational flexibility to reach a bound AB&CR agent. Another concern is that hydrophobic drug binding pockets may not contain nearby lysine residues. Considerable support exists for the selected CR moieties (*i.e.*, alkyne, azide) groups and for the notion that protein capture with **P** occurs through a Cu(I)-mediated cycloaddition reaction ("click chemistry").[25]

d. Synthesis of Agents

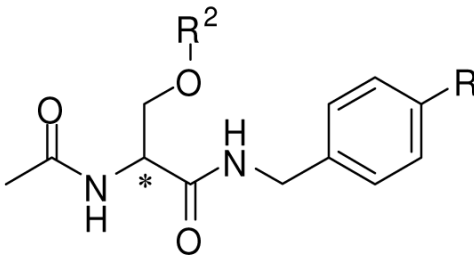
The synthetic pathways used to prepare **9–11** are provided in Scheme 2. Care was taken to install the AB unit in the last step to avoid possible isothiocyanate-related reactions during the synthesis and reaction workup. In general, commercially available D- or L-serine methyl ester hydrochloride (**12**) was converted to the corresponding (*R*)- and (*S*)-methyl 1-acetylaziridine-2-carboxylates (**16**), followed by stereospecific ring opening with the appropriate alcohol using catalytic amounts of BF_3•Et_2O to give the desired (*R*)- and (*S*)-methyl 2-acetamido-3-(alkoxy)propanoates (**17, 18**).[44,45] The esters **17** and **18** were converted to the free acids **19** and **20**, respectively, using stoichiometric amounts of LiOH at 0 °C. Under these conditions, we detected little or no racemization. The free acids were coupled with the appropriate substituted benzylamine using either the mixed anhydride coupling method[46a] (*i.e.*, isobutyl chloroformate (IBCF), *N*-methyl morpholine (NMM)) or 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM)[46b] to give the lacosamide analogs **23–25**. The syntheses of **9–11** concluded with AB isothiocyanate moiety installation. For **9** and **10**, we used di(2-pyridyl) thionocarbonate (DPT)[47a] and for **11**, 1,1′-thiocarbonyldiimidazole (DITC).[47b] The spectral and analytical data for **9–11** and all intermediates were in agreement with their proposed structures. We documented the enantiopurity of the final compounds using the chiral resolving agent (*R*)-mandelic acid and ^{1}H NMR spectroscopy.[48]

In order to assess the individual effects of the AB (isothiocyanate) and CR (alkyne, azide) units on anticonvulsant activities, the corresponding lacosamide analogs that contained only the AB (**27**) or the CR (**28–30**) units have also been prepared (Supplementary Schemes S1-S3 in the Supporting Information). We have previously reported the synthesis and anticonvulsant activity for the lacosamide isothiocyanate (AB) probes (*R*)-**31** and (*S*)-**31**.[43a]



27 $R^2 = CH_2CH_2N{=}C{=}S$, $R^4 = H$

31 $R^2 = CH_3$, $R^4 = N{=}C{=}S$



28 $R^2 = CH_2C{\equiv}CH$, $R^4 = H$

29 $R^2 = CH_3$, $R^4 = C{\equiv}CH$

30 $R^2 = CH_2CH_2N_3$, $R^4 = H$

e. Pharmacological Evaluation

Initial criteria we established for our lacosamide AB&CR agents are that they prove effective in preventing MES-induced seizures in animal models[30] and that the principal activity resides in the (*R*)-stereoisomer (D-configuration). In the MES test, tonic hindlimb seizures are induced by corneal electrical stimulation. This assay is an established method to identify compounds effective against generalized tonic-clonic seizures[30] and was the initial assay used to discover (*R*)-**2**. Table 1 lists the MES anticonvulsant activities obtained for the lacosamide AB&CR compounds (**9, 11**), the analogs into which only an AB (**27, 31**) or a CR (**28–30**) moiety was installed in the lacosamide framework, (*R*)-**2**,[18] and other clinical antiepileptic agents. The compounds were also evaluated in the subcutaneous metrazol (scMet) seizure model but provided no protection (data not shown). The absence of seizure protection in this assay is a hallmark of **1** activity.[9-18]

We found that we could incorporate a small moiety in the 4′ position of the *N*-benzylamide unit of (*R*)-**2** without appreciable loss of MES anticonvulsant activity compared with unmodified (*R*)-**2** (MES ED_{50} = 4.5 mg/kg) in mice (ip). For example, excellent seizure protection was observed for CR (*R*)-**29** (MES ED_{50} = >3, <10 mg/kg) and AB (*R*)-**31** (MES ED_{50} = 24 mg/kg).[43] We also found that some modifications of the C(2) side chain in (*R*)-**2** led to excellent activity retention in the MES seizure test, while others did not. The MES ED_{50} values for the CR *O*-propargyl ((*R*)-**28**), the CR *O*-2-azidoethyl ((*R*)-**30**), and the AB *O*-2-isothiocyanatoethyl ((*R*)-**27**) lacosamide analogs were 16, 100-300, and >300 mg/kg, respectively. We do not know if transport, efflux or metabolic factors are responsible for the diminished activity for (*R*)-**27** and (*R*)-**30** in the MES test. Interestingly, (*R*)-**30** showed appreciable activity in the psychomotor seizure test[49] (6 Hz ED_{50} = 44 mg/kg). Consistent with the pharmacological pattern observed for **2**[18] and other FAAs (**1**),[11-13,15] we found that for **28, 29**, and **31**, the (*R*)-stereoisomer was consistently more potent than the (*S*)-stereoisomer.

The findings for the lacosamide AB and the lacosamide CR analogs guided our investigation of **9–11** where both an AB and a CR moiety were installed in the molecule. Here, we found that **9**, containing a *N*-benzyl 4′-isothiocyanate moiety and an *O*-propargyl moiety, provided significant seizure protection (MES ED_{50} = 45 mg/kg) and that activity principally resided in the (*R*)-stereoisomer. For **11**, we observed that both stereoisomers gave only minimal seizure protection, a finding that mirrored the anticonvulsant activity for the corresponding CR-only

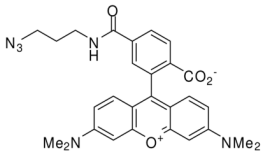
analogs (*R*)-**30** and (*S*)-**30**. Finally, we did not evaluate (*R*)-**10** and (*S*)-**10** in animal models since the corresponding AB compounds (*R*)-**27** and (S)-**27** were inactive at 300 mg/kg.

f. Chemical Evaluation of the AB and CR Units in 3

The AB and CR moieties have been installed within **9–11** for specific chemical functions. The isothiocyanate AB unit serves as an electrophilic agent to trap either lysine or cysteine residue (s) situated near the (*R*)-**2** binding site, and either the alkyne or azide CR group acts as a bioorthogonal unit that can be selectively captured by the corresponding azide- or alkyne-containing probe via a Cu(I)-mediated cycloaddition reaction.[50,51] Prior to conducting our studies we evaluated the chemical reactivity of these groups using model reactions (Figure 1). Treating AB (*R*)-**31** with $EtNH_2$ gave the expected thiourea (*R*)-**32** in high yield (94%).[43] Substituting the aliphatic isothiocyanate **33** for (*R*)-**31** gave the expected thiourea **34** (77% yield).[43a] Correspondingly, when (*R*)-**9** was reacted with 1.0 equiv of the biotin-containing azide probe **35**,[52] $CuSO_4$ and sodium ascorbate,[50] we isolated the desired product, (*R*)-**36**, in 51% yield. No modification of the isothiocyanate moiety was observed under these Cu(I)-mediated cycloaddition conditions. Finally, we treated aliphatic azide (*R*)-**30** with alkyne **37** under similar conditions to obtain (*R*)-**38** (96% yield).

g. Using Mouse Brain Soluble Lysates to Identify Lacosamide Targets

The utility of the lacosamide AB&CR agents **9–11** to identify drug binding targets was first assessed using mouse brain soluble lysates prepared from male (2 months) ICR mice (Rockland Immunochemicals, Gilbertsville, PA). The lysates were treated with (*R*)- and (*S*)-**9–11** (5 μM) for 0.5 h at room temperature, and the lysate mixture reacted with an appropriate rhodamine-containing probe (**39**,[53] **40**) under Cu(I)-mediated cycloaddition conditions ($CuSO_4$, tris(2-carboxyethyl)phosphine (TCEP)). The reaction samples were separated by electrophoresis using a 10% SDS-PAGE gel and then analyzed for proteins labeled by the AB&CR agents by in-gel fluorescence using a typhoon scanning laser (emission 532 nm, absorption 580 nm) (Figure 2a). Gels were Coomassie-stained to visualize the proteins (Figure 2c).



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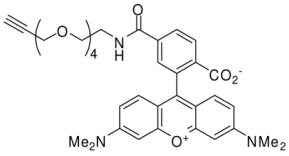

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Figure 2a showed that AB&CR **9** efficiently labeled many proteins in the lysate while **10** did not. The in-gel fluorescence pattern for **11** suggested the labeling of many of the proteins seen with **9**, along with additional background proteins. Significantly, the rhodamine-based probe used for **9**- and **10**-modified reactions was different from that used for **11**-modified reaction. The alkyne CR moieties in **9** and **10** required the use of the azide probe **39** for the Cu(I)-mediated cycloaddition step while the azide CR group in **11** required the alkyne-based probe **40**. Cravatt and co-workers have previously reported higher levels of background protein adduction with alkyne probes compared with their azide counterparts.[53] This observation was consistent with the gel patterns observed for **11** versus **9** and **10** (Figure 2a) that showed more proteins modified by the probe **40** compared with probe **39** under Cu(I)-mediated cycloaddition conditions. Similarly, we observed more labeled proteins with **40** compared with **39** in the *absence* of the AB&CR agent (Figure 2a, no drug control lanes).

Analysis of the (*R*)-**9**- and (*S*)-**9**-modified lysate gel patterns showed that some proteins were more extensively modified by (*R*)-**9** than (*S*)-**9** (Figure 2a, see bands labeled by arrow and

asterisk). One of these bands appeared to be ~62-kDa (arrow), the approximate molecular weight of CRMP2.[54] To confirm that the ~62-kDa band contained CRMP2, we performed a western blot analysis using an anti-CRMP2 antibody and observed prominent bands consistent with full-length and truncated forms of CRMP2[54] (Figure 2b). In addition to the ~62-kDa band, we observed another protein band (Figure 2a) with stronger intensity for the (*R*)-**9** lane than for (*S*)-**9** (Figure 2a, see band labeled by asterisk). The identity of this protein(s) is currently under investigation.

We recognized that both the increased fluorescence of the ~62 kDa band for the (*R*)-**9** treated lysate compared with the (*S*)-**9** reaction, and the identification of CRMP2 by western blot analysis do not demonstrate, by themselves, the preferential modification of CRMP2 by (*R*)-**9**. We suspect that the ~62 kDa band consists of several proteins, and thus the observed fluorescence pattern may be due to a protein(s) other than CRMP2. Accordingly, additional studies were conducted to verify the selective modification of CRMP2 by (*R*)-**9**. First, the (*R*)-**9** lysate adduction was repeated. The modified lysate was treated with biotinyl CR probe **35** using Cu(I)-mediated cycloaddition conditions. Substitution of probe **35** for **39** permits the selective isolation of the (*R*)-**9** captured proteins from the lysate. The biotinylated proteins were captured with streptavidin beads, the beads stringently washed to remove background proteins adsorbed on the resin, and then the protein adducts released with SDS treatment. SDS-PAGE electrophoresis showed bands at ~62 kDa upon Coomassie blue staining. The 62 kDa band was excised, trypsinized, and analyzed by mass spectrometry (MS). Analysis of the tryptic fragments indicated the presence of several proteins (*e.g.*, CRMP2, CRMP1, serum albumin) whose molecular weights were consistent with their electrophoretic mobility. The protein with the most number of identified fragments in the (*R*)-**9** treated reaction was CRMP2 (17 peptides including 11 peptides unique to CRMP2 and 6 peptides common to other CRMP proteins, 45% coverage), followed by CRMP1 (6 peptides including 4 peptides unique to CRMP1 and 2 peptides common to other CRMP proteins, 17% coverage), and serum albumin (4 peptides, 10% coverage), indicating that CRMP2 was a likely major constituent in the ~62 kDa band. Correspondingly, CRMP2 and other proteins were not observed in ~62 kDa band in the no drug control reaction. We made a quantitative comparison of the 62 kDa trypsin digests using differential stable isotope labeling (isobaric tag for relative and absolute quantitation (iTRAQ)).[55] The trypsin digests from the (*R*)-**9**, (*S*)-**9**, the no drug control reaction, and iTRAQ buffer alone were each separately treated with distinct iTRAQ isotope labels, the samples combined, and then analyzed by LC-MS/MS (Supplementary Table S1 in the Supporting Information). There was no difference in observed iTRAQ ratio between fragments unique to CRMP2 and those common to other CRMP proteins. The peptide fragments showed, on average, an ~1.3:1 increase in the relative amounts of each tryptic fragment from the (*R*)-**9** treated sample compared with (*S*)-**9**, after normalization to the tubulin fragments, based on the assumption of no stereochemical preference for lacosamide binding to tubulin. Minor amounts (<6% compared with the (*R*)-**9** sample) of CRMP2 fragments were observed in the no drug sample. Analysis of the CRMP1 fragments also showed a 1.3:1 increase in the (*R*)-**9** reaction compared with the (*S*)-**9** reaction. This differential amount in tryptic fragment abundances was in agreement with the observed ratio for the fluorescence intensities of the ~62 kDa band in the (*R*)-**9** reaction versus the (*S*)-**9** experiment (Figure 2a), substantiating the identification of CRMP1 and CRMP2 as the relevant components of the gel bands from which they were derived. Finally, we overexpressed CRMP2 as a glutathione-S-transferase (GST)-fusion protein.[56] Attachment of the GST-tag permitted protein purification and increased the size of the CRMP2 protein by ~25 kDa. The purified recombinant GST-CRMP2 protein (10 μg) was included in the lysate (50 μL, 2 mg/mL) and the AB&CR adduction reaction repeated with (*R*)-**9**, (*S*)-**9**, and no drug. The reaction products were treated with rhodamine azide **39** under Cu(I)-mediated cycloaddition conditions and then separated by 10% SDS-PAGE (Figure 3). The electrophoretic mobility of the GST-CRMP2 placed it in a region (~82 kDa) that was relatively free of other soluble lysate proteins. We observed an approximate 1.5-fold increase

in fluorescent intensity for the (*R*)-**9** modified GST-CRMP2 protein compared with the corresponding (*S*)-**9** modified recombinant protein (double arrowhead, Figure 3a). By comparison, the (*R*)-**9**-modified endogenous 62-kDa band was approximately 1.8-fold more intense than the corresponding (*S*)-**9**-modified band (arrow, Figure 3a). Western blot analysis of the ~82 kDa band was in agreement with the presence of CRMP2 and GST (Figure 3b). Collectively, these studies show that CRMP2 is preferentially modified in the lysate by lacosamide AB&CR agent (*R*)-**9**, and that (*R*)-**9** leads to higher CRMP2 adduction levels than the corresponding (*S*)-**9** derivative.

Discussion

A robust strategy is advanced for the discovery of proteins that interact (bind) with small-molecular-weight ligands (drugs) that explicate function. Our target discovery approach could be general. As a test case, we have searched for the central nervous system (CNS) binding partners for (*R*)-**2**, a therapeutic agent recently approved for the treatment of partial-onset seizures.[19] Whole animal pharmacological studies indicated that (*R*)-**2** function is unique.[20]

Our strategy requires the installation of AB and CR units within the drug molecule (Scheme 1). The AB group in **3** covalently modifies the drug target to give **6** rendering permanently a reversible binding interaction. The CR moiety in **3** is a biologically inert group that specifically reacts with the bioorthogonal probe **7** designed to either detect or isolate the modified protein adduct. Specific criteria have been established for the designed AB&CR units. These requirements are stringent and have been adopted to minimize false positive results. In this study, we sought lacosamide AB&CR agents that exhibited potent anticonvulsant activity in rodent models for epilepsy and where the pharmacological activity principally resided in the enantiomer corresponding to D-serine, similar to that seen for (*R*)-**2**. This stereochemical selectivity for (*R*)-**2** function and related **1**s is a distinguishing feature for this class of compounds,[11-13,15,18] and we now show that this enantiospecificity may extend to receptor tagging as well.

An efficient synthesis was devised to permit the preparation of enantiomerically pure samples of the AB&CR agents **9–11** and the corresponding AB **27** and **31**, and CR **28–30** compounds (Scheme 1, Schemes S1-S3 in the Supporting Information). Key to our route was the BF_3•Et_2O catalyzed ring opening of aziridine **16**.[44] We found this method versatile, and it reliably gave optically pure 2-alkoxypropionamide derivatives. The isothiocyanate moiety was chosen as the AB moiety in **9–11** because of its relative stability in aqueous solutions and its reactivity with amines and thiols.[27] We installed this group from the corresponding amine in the final step of the synthesis to minimize product loss. For amines **23** and **26**, we used di(2-pyridyl) thionocarbonate,[47a] and for amine **25** we employed 1,1′-thiocarbonyldiimidazole [47b] for this step. We further tested the reactivity of the different AB and CR groups to validate their use in our proteomic target search (Figure 1).

An important design component for our AB&CR agents is the use of small AB and CR units in order to minimize possible adverse interactions that could either prevent or alter target binding. In the case of the C(2) site in (*R*)-**2**, for example, we demonstrated that progressive size increases of the oxygen substituent led to a steady loss in seizure protection in the MES test (mice, ip).[57] A similar structure-activity relationship has been found for the 4′ benzyl position in (*R*)-**2** derivatives.[58] Thus, in (*R*)-**9**, we incorporated an isothiocyanate group at the 4′ *N*-benzyl site for the AB moiety and a propargyl unit at C(2) alkoxy site for the CR moiety. We found that (*R*)-**9** exhibited notable anticonvulsant activity in mice (ip) (MES ED_{50} = 45 mg/kg) while the corresponding (*S*)-**9** did not (MES ED_{50} = >300 mg/kg). The anticonvulsant activities of the corresponding AB (*R*)-**31** (MES ED_{50} = 24 mg/kg)[43a] and CR (*R*)-**28** (MES ED_{50} = 16 mg/kg) derivatives exceeded that of the AB&CR agent (*R*)-**9** (MES ED_{50} = 45 mg/

kg). The loss of activity observed for (*R*)-**9** is consistent with the cumulative adverse effects that may have occurred when two groups were appended to the lacosamide framework. We observed only modest MES seizure protection for AB&CR agent (*R*)-**11** (MES ED_{50} = 100-300 mg/kg). Moreover, the animal behavioral studies did not differentiate the activities of (*R*)-**11** and its stereoisomer (*S*)-**11**. The 6 Hz psychomotor seizure test[49] for (*R*)-**30** (mice, ip; 32 mA) provided evidence to support the use of the 2-azidoethoxy group in this lacosamide AB&CR agent. The absence of detectable anticonvulsant activity of (*R*)-**27** in mice (>300 mg/kg) in the MES-seizure test (Table 1) discouraged our preparation and evaluation of the structural isomer of (*R*)-**11** where the positions of the isothiocyanate and azide moieties are reversed.

Collectively, the whole animal pharmacological data showed that incorporating small AB and CR units at either the 4′ *N*-benzyl site or the C(2)-alkoxy position did not markedly perturb anticonvulsant function and likely did not impede binding to the drug's cognate receptors. Our data demonstrated that the seizure protection provided by (*R*)-**9** and (*S*)-**9** mirrored that of (*R*)-**2** and (*S*)-**2**, respectively. Correspondingly, the whole animal pharmacological data for **10** and **11** provided neither support nor evidence against their use for *in vitro* proteomic searches. Placement of either an isothiocyanate or azido group at the C(2) aliphatic site may diminish their activity, in part, presumably by preventing these compounds from reaching the CNS due to chemical, transport, efflux, and metabolic processes. We chose to interrogate the mouse brain lysates with all three AB&CR agents (**9–11**) to gain further information about the suitability of different AB and CR units for receptor identification, with the understanding that only animal pharmacological data supported the use of **9**.

We first chose to examine the soluble proteome from Charles River male ICR mouse brains. Comparison of AB&CR agents (*R*)- and (*S*)-**9–11** showed that (*R*)-**9** and (*S*)-**9** provided the cleanest in-gel fluorescent patterns while still showing appreciable protein labeling (Figure 2a). This is particularly apparent when the lanes for **9** are compared with that for **11**. These sets of agents contain the same AB unit (4′-isothiocyanate *N*-benzyl moiety) but have different CR moieties. Consistent with results reported in the literature, we observed that the number of proteins modified by using alkyne-based probe **40** were considerably higher than with azide-based probe **39**. We have attributed this, in part, to non-specific lysate modification by the rhodamine alkyne **40** probe.[53] This unwanted reaction complicates receptor identification. When the protein band pattern for lacosamide AB&CR agent **10** was compared with that for **9**, we observed a marked decrease in the extent of protein modification. Compounds **9** and **10** can be considered functional isomers where the structural positions of the AB and CR units have been reversed. Together, our findings indicate that AB&CR agent **9** may be best suited for lacosamide target identification.

This study showed that CRMP2 was preferentially modified in the lysate by lacosamide AB&CR agent (*R*)-**9**, and that (*R*)-**9** leads to higher CRMP2 adduction levels than the corresponding reaction with the (*S*)-**9** derivative. Interrogation of the mouse brain soluble lysate is complicated by the complexity of the proteome. Thus, several findings were used to validate CRMP2 labeling. First, western blot analysis verified the presence of CRMP2 in the ~62 kDa band after lysate modification by (*R*)-**9** and treatment with **39** (Figure 2b). Second, MS analysis of the trypsin digest of the modified proteins in the ~62 kDa band indicated that CRMP2 was among the most abundant proteins present in this gel slice. Third, the enhanced adduction by (*R*)-**9** compared with (*S*)-**9** was confirmed using iTRAQ analysis of the tryptic fragments from the ~62 kDa band. A modest increase (1.3-fold) in the abundance of CRMP2 fragments was observed in the (*R*)-**9** adduction experiment compared to (*S*)-**9** reaction when compared to an internal protein (tubulin alpha chain) (Table S1 in the Supporting Information). The increased (*R*)-**9** adduction levels are important since whole animal pharmacological studies have demonstrated that the principal anticonvulsant activity for **2** resided in the (*R*)-stereoisomer. Finally, we overexpressed GST-CRMP2 and then added a small amount of the fusion protein

to the mouse lysate. Repetition of the **9** adduction experiments followed by Cu(I)-mediated cycloaddition with probe **39** and SDS-PAGE showed increased fluorescent levels (1.5–1.8-fold) for both the exogenous ~82 kDa and the endogenous ~62 kDa bands in the (*R*)-**9** reaction compared with the (*S*)-**9** experiment (Figure 3a).

The relative ratios of (*R*)-**9** versus (*S*)-**9** modification of CRMP2 proteins (1.3–1.8-fold) were modest compared with the observed MES ED_{50} values for (*R*)-**2** and (*S*)-**2** in this seizure model in mice (>22-fold).[18] Several factors likely contributed to this finding. First, the MES ED_{50} values for (*R*)-**9** and (*S*)-**9** in rodents represent a composite value that encompasses numerous pharmacological factors that include binding to target(s), metabolism, transport, efflux, and nonspecific binding to abundant cellular proteins. Therefore, the *in vitro* modification data is not likely to fully recapitulate the *in vivo* pharmacological results. Second, different (*R*)-**2** targets likely display different stereochemical preferences for the **9** enantiomers, and this stereochemical preference may be further influenced by the AB and CR groups that are incorporated within the lacosamide framework to facilitate *in vitro* analysis. Finally, we expect that the conditions of adduction such as drug and target concentration, temperature, and time will affect the levels of (*R*)-**9** and (*S*)-**9** CRMP2 adduction. For our experiments, we used relatively high amounts of **9** and moderate reaction conditions to provide substantial protein adduction levels.

Our results support a report by Beyreuther and coworkers that CRMP2 may interact with (*R*)-**2**.[21] In their study, (*R*)-**2**-like derivatives (precise structures not disclosed) that contained an appended isothiocyanate group and a biotin moiety were prepared and then reacted with mouse brain lysates. Subsequent treatment of the modified lysate with streptavidin removed the biotinylated tagged proteins. Differentiating Beyreuther and colleagues' study from ours was their use of significantly larger (*R*)-**2**-type agents and the absence of whole animal pharmacological data to support their use. Knowing these collective findings, detailed studies are warranted to determine the site of (*R*)-**3** CRMP2 adduction and to validate this protein as an (*R*)-**2** target.

We observed other possible (*R*)-**2** binding target(s) (Figure 2a, asterisk band). The identity of this band is under investigation. Only mouse soluble brain proteome was used in this study. Future studies will examine the membrane-bound fraction. In this regard, (*R*)-**2** has been reported to selectively enhance the slow inactivation of voltage-gated sodium channels.[22] The combined use of our AB&CR (*R*)-**3** agents, enzymatic digestion, and MS of the protein fragments may help reveal the site(s) of drug binding.

Conclusions

We advance a strategy to identify molecular targets for ligands (*e.g.*, drugs) whose binding to their cognate receptors is modest and where moderate-to-extensive structural change abolishes target binding. Our method requires the installation of AB and CR units within the ligand where the AB group covalently modifies the target and the CR group is used to either identify or isolate the ligand-modified receptor. Strict guidelines for the use of the AB&CR strategy have been established to increase the success of this method and to minimize the isolation of false positive sites. Among these guidelines are that the AB and CR groups must conform to the SAR for the ligand and that the pharmacological profile for the AB&CR agent mirror that of the ligand. Using this approach we interrogated the mice brain soluble proteome for (*R*)-**2** binding sites. We identified several potential drug targets including CRMP2, a protein previously suggested to interact with (*R*)-**2**. Further studies will be needed to validate these targets and to identify the sites of drug interaction.

Experimental Section

General Methods

Melting points were determined in open capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. Infrared spectra (IR) were run on an ATI Mattson Genesis FT-IR spectrometer. Absorption values are expressed in wavenumbers (cm^{-1}). Optical rotations were obtained on a Jasco P-1030 polarimeter at the sodium D line (589 nm) using a 1 dm path length cell. NMR spectra were obtained at 300 MHz (^{1}H) and 75 MHz (^{13}C) using TMS as an internal standard. Chemical shifts (δ) are reported in parts per million (ppm) from tetramethylsilane. Low-resolution mass spectra were obtained with a BioToF-II-Bruker Daltonics spectrometer by Drs. Matt Crowe and S. Habibi at the University of North Carolina Department of Chemistry. The high-resolution mass spectrum was performed on a Bruker Apex-Q 12 Telsa FTICR spectrometer by Drs. Matt Crowe and S. Habibi. Microanalyses were performed by Atlantic Microlab, Inc. (Norcross, GA). Reactions were monitored by analytical thin-layer chromatography (TLC) plates (Aldrich, Cat # Z12272-6) and analyzed with 254 nm light. The reaction mixtures were purified by MPLC (CombiFlash Rf) with self-packed columns (silica gel from Dynamic Adsorbents Inc., Cat # 02826-25) or by flash column chromatography using silica gel (Dynamic Adsorbents Inc., Cat # 02826-25). All chemicals and solvents were reagent grade and used as obtained from commercial sources without further purification. THF was distilled from blue sodium benzophenone ketyl. Yields reported are for purified products and were not optimized. All compounds were checked by TLC, ^{1}H and ^{13}C NMR, MS, and elemental analyses. The analytical results are within +0.40% of the theoretical value. The TLC, NMR and the analytical data confirmed the purity of the products was ≥95%.

General procedure for the aziridine ring opening of (*R*)/(*S*)-16 with alcohols. Method A

To a cooled CH_2Cl_2 solution (ice bath) containing the *N*-acetylaziridine methyl ester (*R*)/(*S*)-**16** ([C] ~ 0.5–1 M) and the appropriate alcohol (1–3 eq.) was added BF_3•Et_2O (0.1–1 equiv) dropwise while stirring. After addition, the mixture was warmed to room temperature and stirred (90 min—2 h), and then an equal volume of saturated aqueous $NaHCO_3$ was added. The reaction was vigorously stirred (15 min) and the organic layer separated. The aqueous layer was extracted with CH_2Cl_2 until no product could be detected (TLC analysis) and then all the organic layers were combined, dried (Na_2SO_4), and concentrated in vacuo to yield a residue that was purified by flash column chromatography.

General procedure for the ester hydrolysis of (*R*)/(*S*)-17 or (*R*)/(*S*)-18 with LiOH. Method B

To a THF solution of methyl ester (2 volumes, [C] ~ 0.1 M) was added an aqueous LiOH (1 equiv) solution (1 volume). The reaction was stirred at room temperature (90 min—12 h), after which time the aqueous layer was washed with Et_2O (2 volumes). The aqueous layer was acidified (pH ~ 1) by the dropwise addition of aqueous concentrated HCl at 0 °C, saturated with NaCl, and extracted with EtOAc until no further product was detected (TLC analysis). The combined organic layers were combined, dried (Na_2SO_4), and evaporated to an oily residue that was used directly for the next step, or recrystallized when needed from EtOAc and hexanes to provide an analytical sample.

General procedure for the mixed anhydride coupling reaction. Method C

To a cooled THF solution (−78 °C, dry ice acetone bath) of acid (*R*)/(S)-**19** ([C] ~ 0.1 M) were successively added NMM (1.0–1.1 equiv), stirred for 2 min, IBCF (1.0–1.2 equiv), stirred for 5 min, and then the desired benzylamine (1.0–1.2 equiv). Upon addition the reaction mixture was allowed to warm to room temperature and further stirred (2–3 h). The salts were filtered and rinsed with THF and the filtrate was concentrated in vacuo. The residue obtained was

purified by flash chromatography, followed by recrystallization from EtOAc and hexanes when necessary.

General procedure for the DMTMM amide coupling reaction. Method D

To a THF solution of acid (*R*)/(*S*)-**20** ([C] ~ 0.1 M) at room temperature was added the desired benzylamine (1.2 equiv). The solution was stirred (5–10 min) until the benzylammonium carboxylate precipitated. While stirring, DMTMM (1.2 equiv) was added all at once, and the resulting suspension was stirred at room temperature (3–12 h). In those cases where a salt did not precipitate, DMTMM was added after 15 min to the solution. The salts were removed by filtration, washed with THF, and the solvent was removed in vacuo. The residue obtained was purified by flash column chromatography to afford the benzylamide, and then recrystallized from EtOAc and hexanes.

(*R*)-Methyl 2-Acetamido-3-(prop-2-ynyloxy)propionate ((*R*)-17)

Utilizing Method A, (*R*)-methyl 1-acetylaziridine-2-carboxylate[44] ((*R*)-**16**) (1.50 g, 10.49 mmol), propargyl alcohol (0.92 mL, 15.74 mmol) and BF_3•Et_2O (1.39 mL, 11.01 mmol) gave 1.43 g (68%) of (*R*)-**17** as a white solid after recrystallization with cold hexanes: mp 58.5–59.5 °C; $[\alpha]^{25}_{D}$ −64.6° (*c* 1.0, $CHCl_3$); R_f = 0.35 (3/1 EtOAc/hexanes); IR (nujol mull) 3301, 2919, 2114, 1750, 1639, 1542, 1457 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.06 (s, C**H**$_3$C(O)), 2.46 (t, *J* = 2.3 Hz, CH_2C**C**H), 3.78 (s, OC**H**$_3$), 3.79 (dd, *J* = 3.3, 9.6 Hz, C**H**H'OCH_2), 3.98 (dd, *J* = 2.9, 9.6 Hz, CH**H'**OCH_2), 4.09–4.21 (m, OC**H**$_2$C), 4.77–4.82 (m, C**H**), 6.33–6.35 (br d, *J* = 7.2 Hz, N**H**CH); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H_3C(O)), 52.6 (**C**H), 52.8 (O**C**H_3), 58.7 (**C**H_2CCH), 69.6 (**C**H_2OCH_2), 75.3 (CH_2C**C**H), 79.0 (CH_2**C**CH), 170.1, 170.8 (2 **C**(O)); M_r (+ESI) 200.0916 $[M+H]^+$ (calcd for $C_9H_{13}NO_4H^+$ 200.0923 $[M+H]^+$). Anal. ($C_9H_{13}NO_4$): C, H, N.

(*S*)-Methyl 2-Acetamido-3-(prop-2-ynyloxy)propionate ((*S*)-17)

Utilizing the preceding procedure and using (*S*)-**16**[44] (1.50 g, 10.49 mmol), propargyl alcohol (0.92 mL, 15.74 mmol) and BF_3•Et_2O (1.39 mL, 11.01 mmol) gave 1.32 g (63%) of (*S*)-**17** as a white solid: mp 59.0–59.5 °C; $[\alpha]^{25}_{D}$ +65.3° (*c* 1.0, $CHCl_3$); R_f = 0.35 (3/1 EtOAc/hexanes); IR (nujol mull) 3296, 2924, 2116, 1740, 1641, 1543, 1457 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.06 (s, C**H**$_3$C(O)), 2.46 (t, *J* = 2.4 Hz, CH_2C**C**H), 3.78 (s, OC**H**$_3$), 3.79 (dd, *J* = 3.2, 9.4 Hz, C**H**H'OCH_2), 3.98 (dd, *J* = 3.0, 9.4 Hz, CH**H'**OCH_2), 4.12 (1/2**H**H'$_q$, *J* = 2.4, 16.2 Hz, OC**H**H'C), 4.18 (1/2H**H'**$_q$, *J* = 2.4, 16.2 Hz, OCH**H'**C), 4.77–4.82 (m, C**H**), 6.32–6.34 (br d, *J* = 7.2, N**H**CH); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H_3C(O)), 52.6 (**C**H), 52.8 (O**C**H_3), 58.7 (**C**H_2CCH), 69.6 (**C**H_2OCH_2), 75.3 (CH_2C**C**H), 79.0 (CH_2**C**CH), 170.1, 170.7 (2 **C**(O)); M_r (+ESI) 200.0917 $[M+H]^+$ (calcd for $C_9H_{13}NO_4H^+$ 200.0923 $[M+H]^+$)). Anal. ($C_9H_{13}NO_4$) C, H, N.

(*R*)-2-Acetamido-3-(prop-2-ynyloxy)propionic Acid ((*R*)-19)

Utilizing Method B, (*R*)-**17** (1.00 g, 5.03 mmol), LiOH (0.6 M, 8.4 mL, 5.03 mmol) gave 0.87 g of (*R*)-**19** (94%) as a yellow oil: R_f = 0.35 (15/5/1 EtOAc/hexanes/AcOH); IR (nujol mull) 3264, 2924, 2120, 1726, 1642, 1548, 1459 cm^{-1}; ^1H NMR (CD_3OD) δ 2.01 (s, C**H**$_3$C(O)), 2.88 (t, *J* = 2.6 Hz, CH_2C**C**H), 3.77 (dd, *J* = 3.6, 9.7 Hz, C**H**H'OCH_2), 3.92 (dd, *J* = 5.0, 9.7 Hz, CH**H'**OCH_2), 4.18 (d, *J* = 2.6 Hz, OC**H**$_2$C), 4.61–4.64 (m, C**H**); ^{13}C NMR (CD_3OD) δ 22.5 (**C**H_3C(O)), 54.1 (**C**H), 59.3 (**C**H_2CCH), 70.4 (**C**H_2OCH_2), 76.5 (CH_2C**C**H), 80.2 (CH_2**C**CH), 173.1, 173.5 (2 **C**(O)); M_r (+ESI) 186.0761 $[M+H]^+$ (calcd for $C_8H_{11}NO_4H^+$ 186.0766 $[M+H]^+$). Anal. ($C_8H_{11}NO_4$•$0.14H_2O$): C, H, N.

(*S*)-2-Acetamido-3-(prop-2-ynyloxy)propionic Acid ((*S*)-19)

Utilizing the preceding procedure and using (*S*)-**17** (1.00 g, 5.03 mmol) and LiOH (0.6 M, 8.4 mL, 5.03 mmol) gave 0.86 g (93%) of (*S*)-**19** as a yellow oil: R_f = 0.35 (15/5/1 EtOAc/hexanes/

AcOH); IR (nujol mull) 3270, 2924, 2120, 1729, 1641, 1547, 1459 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.87 (s, C**H**$_3$C(O)), 3.48 (t, J = 2.4 Hz, CH$_2$C**CH**), 3.62 (dd, J = 4.1, 9.6 Hz, C**H**H'OCH$_2$), 3.74 (dd, J = 2.4, 9.6 Hz, CH**H'**OCH$_2$), 4.16 (d, J = 2.4 Hz, OC**H**$_2$C), 4.41–4.47 (m, C**H**), 8.21–8.23 (d, J = 8.4 Hz, N**H**CH); ^{13}C NMR (DMSO-d_6) δ 22.4 (**C**H$_3$C(O)), 52.1 (**C**H), 57.8 (**C**H$_2$CCH), 69.2 (**C**H$_2$OCH$_2$), 77.6 (CH$_2$**C**CH), 79.9 (CH$_2$**C**CH), 169.6, 171.5 (2 **C**(O)); M_r (+ESI) 186.0760 $[M+H]^+$ (calcd for $C_8H_{11}NO_4H^+$ 186.0766 $[M+H]^+$). Anal. ($C_8H_{11}NO_4$•0.25H_2O) C, H, N.

(*R*)-Methyl 2-Acetamido-3-(2-azidoethoxy)propionate ((*R*)-18)

Using Method A, compound (*R*)-**16**[44] (2.35 g, 16.4 mmol), 2-azidoethanol (4.5 mL, 65.6 mmol) and BF_3•Et_2O (1 mL, 8.2 mmol) gave 1.56 g (41%) of (*R*)-**18** after purification: $[\alpha]^{25}_D$ +49.1° (*c* 1.0; EtOAc); R_f = 0.47 (EtOAc); IR (neat) 3302, 2948, 2107, 1746, 1668, 1534, 1443 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.06 (s, C**H**$_3$C(O)NH), 3.23–3.42 (m, C**H**$_2$N$_3$), 3.31–3.64 (m, OC**H**$_2$CH$_2$N$_3$, CHC**H**H'OCH$_2$), 3.78 (s, OC**H**$_3$), 3.96 (dd, J = 3.0, 9.1 Hz CHCH**H**'OCH$_2$), 4.76–4.81 (m, C**H**CH$_2$O), 6.30–6.41 (br d, CH C(O)N**H**); ^{13} 3 C NMR ($CDCl_3$) δ 23.2 (**C**H$_3$C(O)), 50.0 (OCH$_2$**C**H$_2$N$_3$), 52.6 (O**C**H$_3$ or **C**HCH$_2$O), 52.8 (**C**HCH$_2$O or OC**H**$_3$), 70.9 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 71.1 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 170.1, 170.6 (**C**(O)OCH$_3$, CH$_3$**C**(O)NH); M_r (+ESI) 253.0906 $[M+Na]^+$ (calcd for $C_8H_{14}N_4O_4Na^+$ 253.0913 $[M+Na]^+$).

(*S*)-Methyl 2-Acetamido-3-(2-azidoethoxy)propionate ((*S*)-18)

Utilizing the preceding procedure, and using (*S*)-**16**[44] (3.30 g, 23 mmol), 2-azidoethanol (6.3 mL, 92 mmol), and BF_3•Et_2O (1.4 mL, 11.5 mmol) in CH_2Cl_2 (115 mL) gave 1.67 g (31%) of a colorless residue: $[\alpha]^{25}_D$ −48.0° (*c* 1.0; EtOAc); R_f = 0.47 (EtOAc); IR (neat) 3302, 2948, 2107, 1746, 1667, 1534, 1443 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.06 (s, C**H**$_3$C(O)NH), 3.23–3.42 (m, C**H**$_2$N$_3$), 3.31–3.64 (m, OC**H**$_2$CH$_2$N$_3$, CHC**H**H'OCH$_2$), 3.78 (s, OC**H**$_3$), 3.96 (dd, J = 3.0, 9.1 Hz CHCH**H'**OCH$_2$), 4.76–4.81 (m, C**H**CH$_2$O), 6.30–6.41 (br d, CH$_3$C(O)N**H**); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H$_3$C(O)), 50.1 (OCH$_2$**C**H$_2$N$_3$), 52.7 (O**C**H$_3$ or **C**HCH$_2$O), 52.9 (**C**HCH$_2$O or O**C**H$_3$), 71.0 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 71.2 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 170.2, 170.7 (CH$_3$**C**(O)NH and **C**(O)OCH$_3$); M_r (+ESI) 253.0906 $[M+Na]^+$ (calcd for $C_8H_{14}N_4O_4Na^+$ 253.0913 $[M+Na]^+$).

(*R*)-2-Acetamido-3-(2-azidoethoxy)propionic Acid ((*R*)-20)

Utilizing Method B, and using compound (*R*)-**18** (1.56 g, 6.78 mmol) in THF (60 mL) and LiOH (195 mg, 8.14 mmol) in H_2O (30 mL) gave 750 mg (51%) of (*R*)-**20** as a white solid. An analytical sample was obtained by recrystallization from EtOAc and hexanes: mp 99–100 °C; $[\alpha]^{25}_D$ −12.6° (*c* 1.8; MeOH); R_f = 0.21 (1/9 MeOH/$CHCl_3$); IR (nujol mull) 3356, 2119, 1735, 1624, 1547 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.07 (s, C**H**$_3$C(O)NH), 3.23– 3.42 (m, C**H**$_2$N$_3$), 3.60–3.80 (m, OC**H**$_2$CH$_2$N$_3$, CHC**H**H'O), 4.01 (dd, J = 3.0, 9.1 Hz, CHCH**H'**O), 4.76–4.80 (m, C**H**CH$_2$O), 6.00–7.00 (br, CO$_2$**H**), 6.48 (d, J = 7.5 Hz, CH$_3$C(O)N**H**), no signal could be detected for the COO**H** proton; ^{13}C NMR (DMSO-d_6) δ. 22.3 (**C**H$_3$C(O)), 49.9 (OCH$_2$**C**H$_2$N$_3$), 52.2 (**C**HCH$_2$O), 69.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 70.1 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 169.4 (CH$_3$**C**(O)NH or **C**(O)OH), 171.5 (**C**(O)OH or CH$_3$**C**(O)NH); M_r (+ESI) 239.0750 $[M+Na]^+$ (calcd for $C_7H_{12}N_4O_4Na^+$ 239.0756, $[M+Na]^+$). Anal. ($C_7H_{12}N_4O_4$•0.07EtOAc): C, H, N.

(*S*)-2-Acetamido-3-(2-azidoethoxy)propionic Acid ((*S*)-20)

Utilizing the preceding procedure and using (*S*)-**18** (1.67 g, 7.26 mmol) in THF (60 mL), and LiOH (209 mg, 8.71 mmol) in H_2O (30 mL) gave 862 mg (55%) of (*S*)-**20** as a white solid upon work-up and evaporation. An analytical sample was obtained by recrystallization from EtOAc and hexanes: mp 99–100 °C; $[\alpha]^{25}_D$ +12.4° (*c* 1.3; MeOH); R_f = 0.21 (1/9 MeOH/

$CHCl_3$); IR (nujol mull) 3356, 2119, 1735, 1624, 1547 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.86 (s, C**H$_3$**C(O)NH), 3.30–3.45 (m, C**H$_2$**N_3), 3.50–3.78 (m, OC**H$_2$**CH_2N_3, CHC**H$_2$**OCH_2), 4.39–4.48 (m, C**H**CH_2O), 8.13 (d, J = 8.1 Hz, $CH_3C(O)N$**H**), no signal could be detected for the COO**H** proton; ^{13}C NMR (DMSO-d_6) δ 22.3 (**C**$H_3C(O)$), 49.9 (OCH_2**C**H_2N_3), 52.2 (**C**HCH_2O), 69.4 (CH**C**H_2O or O**C**$H_2CH_2N_3$), 70.1 (O**C**$H_2CH_2N_3$ or CH**C**H_2O), 169.4 (CH_3**C**(O)NH or **C**(O)OH), 171.5 (**C**(O)OH or CH_3**C**(O)NH); M_r (+ESI) 239.0750 [M+Na]$^+$ (calcd for $C_7H_{12}N_4O_4Na^+$ 239.0756 [M+Na]$^+$). Anal. ($C_7H_{12}N_4O_4$•0.07EtOAc): C, H, N.

(*R*)-N-(4-Amino)benzyl 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-23)

Utilizing Method C, (*R*)-**19** (0.25 g, 1.37 mmol), NMM (0.23 mL, 2.06 mmol), IBCF (0.23 mL, 1.73 mmol), and 4-aminobenzylamine (**22**) (0.21 mL, 1.85 mmol) gave 0.24 g (61%) of (*R*)-**23** as a light yellow solid: mp 97.5–98.5 °C; $[\alpha]^{25}_D$ +4.2° (*c* 1.0, MeOH); R_f = 0.40 (1/9 MeOH/$CHCl_3$); IR (nujol mull) 3279, 2930, 2111, 1628, 1536, 1459 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.00 (s, C**H$_3$**C(O)), 2.44 (t, J = 2.4 Hz, CH_2C**C**H), 3.62 (dd, J = 6.9, 9.3 Hz, C**H**H'OCH_2), 3.56–3.72 (br m, N**H$_2$**), 3.88 (dd, J = 4.1, 9.3 Hz, CH**H'**OCH_2), 4.12 (1/2**H**H'$_q$, J = 2.4, 15.9 Hz, OC**H**H'C), 4.19 (1/2H**H'**$_q$, J = 2.4, 15.9 Hz, OCH**H'**C), 4.32 (d, J = 5.7 Hz, C**H$_2$**Ar), 4.54–4.60 (m, C**H**), 6.55–6.58 (br d, J = 7.2 Hz, N**H**CH), 6.60–6.65 (m, 2 Ar**H**), 6.65-6.71 (br m, N**H**CH_2), 7.03-7.06 (m, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 23.4 (**C**H_3C(O)), 43.5 (**C**H_2Ph), 52.7 (**C**H), 58.8 (**C**H_2CCH), 69.4 (**C**H_2OCH_2), 75.5 (CH_2C**C**H), 79.1 (CH_2**C**CH), 115.4, 127.7, 129.1, 146.1 (Ar**C**), 169.6, 170.5 (2 **C**(O)); M_r (+ESI) 290.1500 [M+H]$^+$ (calcd for $C_{15}H_{19}N_3O_3H^+$ 290.1505 [M+H]$^+$). Anal. ($C_{15}H_{19}N_3O_3$): C, H, N.

(*S*)-N-(4-Amino)benzyl 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*S*)-23)

Utilizing the preceding procedure and using (*S*)-**19** (0.25 g, 1.37 mmol), NMM (0.20 mL, 1.78 mmol), IBCF (0.19 mL, 1.44 mmol), and **22** (0.16 mL, 1.44 mmol) gave 0.30 g (77%) of (*S*)-**23** as a light yellow solid: mp 97.0–98.0 °C; $[\alpha]^{25}_D$ −4.1° (*c* 1.0, MeOH); R_f = 0.40 (1/9 MeOH/$CHCl_3$); IR (nujol mull) 3282, 2924, 2112, 1630, 15372, 1458 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.03 (s, C**H$_3$**C(O)), 2.45 (t, J = 2.4 Hz, CH_2C**C**H), 3.62 (dd, J = 7.1, 9.1 Hz, C**H**H'OCH_2), 3.62–3.74 (br m, N**H$_2$**), 3.92 (dd, J = 4.2, 9.1 Hz, CH**H'**OCH_2), 4.14 (1/2**H**H'$_q$, J = 2.4, 15.9 Hz, OC**H**H'C), 4.22 (1/2H**H'**$_q$, J = 2.4, 15.9 Hz, OCH**H'**C), 4.35 (d, J = 6.0, C**H$_2$**Ar), 4.53–4.59 (m, C**H**), 6.42–6.44 (br d, J = 6.3 Hz, N**H**CH), 6.48–6.56 (br m, N**H**CH_2), 6.62–6.67 (m, 2 Ar**H**), 7.03–7.08 (m, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 23.6 (**C**H_3C(O)), 43.8 (**C**H_2Ph), 52.9 (**C**H), 59.0 (**C**H_2CCH), 69.7 (**C**H_2OCH_2), 75.7 (CH_2C**C**H), 79.4 (CH_2**C**CH), 115.6, 128.0, 129.4, 146.3 (**C$_6$**H_4), 169.9, 170.7 (2 **C**(O)); M_r (+ESI) 290.1499 [M+H]$^+$ (calcd for $C_{15}H_{19}N_3O_3H^+$ 290.1505 [M+H]$^+$). Anal. ($C_{15}H_{19}N_3O_3$): C, H, N.

(*R*)-*N*-(4-Isothiocyanato)benzyl 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*R*)-9)

To a dry THF solution (20 mL) of (*R*)-**23** (0.17 g, 0.60 mmol) was added dropwise a solution of DPT (0.21 mL, 0.89 mmol) in THF (8 mL). The reaction solution was stirred under Ar at room temperature (2 h). The solvent was removed in vacuo, and the product was purified by silica gel column chromatography (1/9 acetone/EtOAc) to give 0.15 g (77%) of (*R*)-**9** as a white solid: mp 157–159 °C; $[\alpha]^{25}_D$ −10.9° (*c* 1.5, $CHCl_3$); R_f = 0.40 (1/9 acetone/EtOAc); IR (nujol mull) 3270, 2924, 2084, 1634, 1553, 1459 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.03 (s, C**H$_3$**C(O)), 2.48 (t, J = 2.5 Hz, CH_2C**C**H), 3.65 (dd, J = 7.1, 9.3 Hz, C**H**H'OCH_2), 3.92 (dd, J = 4.2, 9.3 Hz, CH**H'**OCH_2), 4.15 (1/2**H**H'$_q$, J = 2.5, 15.9 Hz, OC**H**H'C), 4.22 (1/2H**H'**$_q$, J = 2.5, 15.9 Hz, OCH**H'**C), 4.40 (1/2**H**H'$_q$, J = 5.9, 15.5 Hz, C**H**H'Ar), 4.49 (1/2H**H'**$_q$, J = 5.9, 15.5 Hz, CH**H'**Ar), 4.60–4.66 (m, C**H**), 6.52 (br d, J = 6.9 Hz, N**H**CH), 6.96 (br t, J = 5.9 Hz, N**H**CH_2), 7.16–7.19 (m, 2 Ar**H**), 7.24–7.27 (m, 2 Ar**H**), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**9** gave only a single signal for the acetyl methyl protons and the alkyne proton, addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**9** and

(*S*)-**9** (1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.019 (*R*) and 2.033 (*S*) (Δppm = 0.014)), and two signals for the alkyne proton (δ 2.424 (*S*) and 2.462 (*R*) (Δppm = 0.038)); ^{13}C NMR ($CDCl_3$) δ 23.4 (**C**H_3C(O)), 43.1 (**C**H_2Ph), 52.7 (**C**H), 58.9 (**C**H_2CCH), 69.3 (**C**H_2OCH$_2$), 75.6 (CH_2C**C**H), 79.0 (CH_2**C**CH), 126.1, 128.8, 130.6 (3 Ar**C**), 135.7 (N**C**S), 137.5 (1 Ar**C**), 170.0, 170.6 (2 **C**(O)); M_r (+ESI) 332.1065 $[M+H]^+$ (calcd for $C_{16}H_{17}N_3O_3SH^+$ 332.1069 $[M+H]^+$). Anal. ($C_{16}H_{17}N_3O_3S$): C, H, N, S.

(*S*)-*N*-(4-Isothiocyanato)benzyl 2-Acetamido-3-(prop-2-ynyloxy)propionamide ((*S*)-9)

Utilizing the preceding procedure, and using (*S*)-**23** (0.30 g, 1.02 mmol), and DPT (0.32 g, 1.38 mmol) gave 0.25 g (75%) of pure (*S*)-**9** as a white solid: mp 157–158 °C; $[\alpha]^{25}_D$ +10.6° (*c* 1.5, $CHCl_3$); R_f = 0.40 (1/9 MeOH/$CHCl_3$); IR (nujol mull) 3272, 2924, 2084, 1633, 1553, 1459 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* 2.04 (s, C**H$_3$**C(O)), 2.48 (t, *J* = 2.5 Hz, CH_2C**CH**), 3.65 (dd, *J* = 7.1, 9.2 Hz, C**H**H'OCH$_2$), 3.94 (dd, *J* = 4.1, 9.2 Hz, CH**H'**OCH$_2$), 4.16 (1/2**H**H'$_q$, *J* = 2.5, 16.0 Hz, OC**H**H'C), 4.24 (1/2H**H'**$_q$, *J* = 2.5, 16.0 Hz, OCH**H'**C), 4.42 (1/2**H**H'$_q$, *J* = 6.3, 15.5 Hz, C**H**H'Ar), 4.50 (1/2H**H'**$_q$, *J* = 6.3, 15.5 Hz, CH**H'**Ar), 4.57–4.63 (m, C**H**), 6.41–6.43 (br d, *J* = 6.6 Hz, N**H**CH), 6.81 (br t, *J* = 6.3 Hz, N**H**CH_2), 7.17–7.20 (m, 2 Ar**H**), 7.25-7.27 (m, 2 Ar**H**), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*S*)-**9** gave only a single signal for the acetyl methyl protons and the alkyne proton, addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**9** and (*S*)-**9** (1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.019 (*R*) and 2.033 (*S*) (Δppm = 0.014)), and two signals for the alkyne proton (δ 2.424 (*S*) and 2.462 (*R*) (Δppm = 0.038)); ^{13}C NMR ($CDCl_3$) *δ* 23.3 (**C**H_3C(O)), 43.1 (**C**H_2Ph), 52.7 (**C**H), 58.8 (**C**H_2CCH), 69.3 (**C**H_2OCH$_2$), 75.6 (CH_2C**C**H), 79.0 (CH_2**C**CH), 126.1, 128.7, 130.5 (3 Ar**C**), 135.7 (N**C**S), 137.5 (1 Ar**C**), 170.0, 170.7 (2 **C**(O)); M_r (+ESI) 332.1063 $[M+H]^+$ (calcd for $C_{16}H_{17}N_3O_3SH^+$ 332.1069 $[M+H]^+$). Anal. ($C_{16}H_{17}N_3O_3S$): C, H, N, S.

(*R*)-*N*-(4-Isothiocyanato)benzyl 2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-11)

Utilizing Method D, and using acid (*R*)-**20** (1130 mg, 5.2 mmol), 4-aminobenzylamine (**22**) (760 mg, 6.3 mmol), and DMTMM (1730 mg, 6.3 mmol) in THF (100 mL) gave 715 mg (42%) of a dark brown residue. The residue was directly dissolved in dry acetonitrile (40 mL) and DITC (90%, 510 mg, 2.57 mmol) was added all at once. The reaction solution was stirred at room temperature (1 h), the solvent was removed in vacuo, and the residue purified using flash chromatography (5/95 MeOH/$CHCl_3$) and then recrystallized from EtOAc to yield 502 mg (27%, 2 steps) of (*R*)-**11** as an off-white solid: mp 142–143 °C; $[\alpha]^{25}_D$ −28.6° (*c* 0.81; $CHCl_3$); R_f = 0.45 (1/9 acetone/EtOAc); IR (nujol mull) 3276, 2181, 2114, 1631, 1547, 1459, 1374, 1285 cm^{-1}; ^{1}H NMR ($CDCl_3$) *δ* 2.05 (s, C**H$_3$**C(O)NH), 3.30–3.50 (m, C**H$_2$**N_3), 3.55 (dd, *J* = 7.5, 9.3 Hz, CHC**H**H'OCH$_2$), 3.62–3.80 (m, OC**H$_2$**CH_2N_3), 3.95 (dd, *J* = 4.0, 9.3 Hz, CHCH**H'**OCH$_2$), 4.46 (d, *J* = 6.0 Hz, C(O)NHC**H$_2$**Ph), 4.54–4.60 (m, C**H**CH_2O), 6.45 (br d, *J* = 6.2 Hz, CH_3C(O)N**H**), 6.84–6.92 (br t, C(O)N**H**CH_2Ph), 7.18–7.22 (m, 2 Ar**H**), 7.23–7.29 (m, 2 Ar**H**), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**11** gave only one signal for the acetyl protons, addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*S*)-**11** and (*R*)-**11** (1:2 ratio) gave two signals for the acetyl methyl protons (*δ* 2.033 (*S*) and 2.020 (*R*) (Δppm = 0.013); ^{13}C NMR ($CDCl_3$) $^{\delta}$. 23.2 (**C**H_3C(O)), 41.0 (NH**C**H_2Ph), 50.7 (**C**H_2N_3), 52.4 (**C**HCH$_2$O), 70.0 (O**C**$H_2CH_2N_3$ or CH**C**H_2O), 70.3 (CH**C**H_2O or O**C**$H_2CH_2N_3$), 125.9, 128.6, 130.5 (3 Ar**C**), 135.6 (N**C**S), 137.4 (1 Ar**C**), 169.8, 169.2 (CH_3**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 363.1236 $[M+H]^+$ (calcd for $C_{15}H_{18}N_6O_3SH^+$ 363.1239 $[M+H]^+$). Anal. ($C_{15}H_{18}N_6O_3S$): C, H, N, S.

(*S*)-*N*-(4-Isothiocyanato)benzyl 2-Acetamido-3-(2-azidoethoxy)propionamide ((*S*)-11)

Utilizing the preceding procedure, and using acid (*S*)-**20** (1.61 g, 7.52 mmol), **22** (1.02 mL, 9.03 mmol) and DMTMM (2.50 g, 9.03 mmol) in anhydrous THF (200 mL) followed by DITC

(90%, 1.58 g, 7.90 mmol) in acetonitrile (20 mL) gave 706 mg (26%, two steps) of (*S*)-**11** as an off-white solid after flash chromatography and recrystallization: mp 142–143 °C; $[\alpha]^{25}_{D}$ +28.5° (*c* 0.95; $CHCl_3$); R_f = 0.45 (1/9 acetone/EtOAc); IR (nujol mull) 3280, 2177, 2108, 1637, 1548, 1452, 1374, 1293 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.01 (s, C**H**$_3$C(O)NH), 3.28–3.48 (m, C**H**$_2$N$_3$), 3.55 (dd, *J* = 7.2, 9.3 Hz, CHC**H**H'OCH$_2$), 3.62-3.78 (m, OC**H**$_2$CH$_2$N$_3$), 3.91 (dd, *J* = 4.0, 9.3 Hz, CHCH**H'**OCH$_2$), 4.43 (d, *J* = 6.0 Hz, C(O)NHC**H**$_2$Ph), 4.56–4.62 (m, C**H**CH$_2$O), 6.56 (br d, *J* = 6.2 Hz, CH$_3$C(O)N**H**), 6.84–6.92 (br t, *J* = 6.0 Hz, C(O) N**H**CH$_2$Ph), 7.14–7.20 (m, 2 Ar**H**), 7.22–7.28 (m, 2 Ar**H**), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*S*)-**11** gave only one signal for the acetyl protons, addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*S*)-**11** and (*R*)-**11** (1:2 ratio) gave two signals for the acetyl methyl protons (δ 2.033 (*S*) and 2.020 (*R*) (Δppm = 0.013); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H$_3$C(O)), 43.2 (NH**C**H$_2$Ph), 50.9 (**C**H$_2$N$_3$), 52.7 (**C**HCH$_2$O), 70.3 (O**C**H$_2$CH$_2$N$_3$ or CH**C**H$_2$O), 70.4 (CH**C**H$_2$O or O**C**H$_2$CH$_2$N$_3$), 126.1, 128.8, 130.6 (3 Ar**C**), 135.8 (N**C**S), 137.6 (1 Ar**C**), 170.0, 170.7 (CH$_3$**C**(O)NH, **C**(O)NHCH$_2$); M_r (+ESI) 363.1241 $[M+H]^+$ (calcd for $C_{15}H_{18}N_6O_3SH^+$ 363.1239 $[M+H]^+$). Anal. ($C_{15}H_{18}N_6O_3S$): C, H, N, S.

(*R*)-*N*-(4-Ethynyl)benzyl 2-Acetamido-3-(2-azidoethoxy)propionamide ((*R*)-24)

Using Method C, (*R*)-2-acetamido-3-(2-azidoethoxy)propionic acid ((*R*)-**20**) (0.31 g, 1.4 mmol), NMM (189 μL, 1.7 mmol), IBCF (228 μL, 1.7 mmol), 4-ethynylbenzylamine (**21**) (225 mg, 1.7 mmol), and THF (20 mL) gave 0.26 g (55%) of (*R*)-**24** as a white solid: R_f = 0.18 (EtOAc); mp 80–87 °C (dec); $[\alpha]^{25}_{D}$ +4.8° (*c* 0.5, DMSO); IR (nujol mull) 3282, 2103, 1636, 1548, 1458, 1375, 1303, 1258, 1124, 982, 821, 721 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.03 (s, C**H**$_3$C (O)), 3.08 (s, **H**C≡C), 3.27-3.46 (m, C**H**$_2$N$_3$), 3.56 (dd, *J* = 7.2, 9.6 Hz, OC**H**H'), 3.62–3.77 (m, CH$_2$C**H**$_2$O), 3.92 (dd, *J* = 3.6, 9.6 Hz, OCH**H'**), 4.46 (d, *J* = 5.7 Hz, C**H**$_2$N), 4.56–4.62 (m, C**H**), 6.53 (d, *J* = 6.9 Hz, N**H**), 6.88–7.01 (br t, CH$_2$N**H**), 7.22 (d, *J* = 8.1 Hz, 2 Ar**H**), 7.45 (d, *J* = 8.1 Hz, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 23.1 (**C**H$_3$CO), 43.3 (**C**H$_2$N), 50.7 (**C**H$_2$N$_3$), 52.5 (C**H**), 70.1 (**C**H$_2$O), 70.3 (**C**H$_2$O), 77.4 (**C**≡C), 83.3 (C≡**C**), 121.3, 127.4, 132.4, 138.7 (4 Ar**C**), 169.8, 170.5 (2 **C**(O)); M_r (+ESI) 330.1569 $[M+H]^+$ (calcd for $C_{16}H_{19}N_5O_3H^+$ 330.1566 $[M+H]^+$).

(*S*)-*N*-(4-Ethynyl)benzyl 2-Acetamido-3-(2-azidoethoxy)propionamide ((*S*)-24)

Utilizing the preceding procedure, and using THF (20 mL), (*S*)-**20** (0.31 g, 1.4 mmol), NMM (189 μL, 1.7 mmol), IBCF (225 μL, 1.7 mmol) and **21** (225 mg, 1.7 mmol) gave 165 mg of a white solid (35%): R_f = 0.18 (EtOAc); mp 107–109 °C; $[\alpha]^{25}_{D}$ −6.0° (*c* 0.5, DMSO); IR (nujol mull) 3274, 2100, 1635, 1548, 1457, 1375, 1301, 1257, 1121, 980, 820, 719 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.02 (s, C**H**$_3$C(O)), 3.08 (s, **H**C≡C), 3.27–3.46 (m, C**H**$_2$N$_3$), 3.56 (dd, *J* = 7.5, 9.6 Hz, OC**H**H'), 3.61–3.76 (m, CH$_2$C**H**$_2$O), 3.91 (dd, *J* = 3.6, 9.6 Hz, OCH**H'**), 4.45 (d, *J* = 6.0 Hz, C**H**$_2$N), 4.56–4.63 (m, C**H**), 6.54 (d, *J* = 6.0 Hz, N**H**), 6.95–7.05 (br t, CH$_2$N**H**), 7.22 (d, *J* = 7.9 Hz, 2 Ar**H**), 7.45 (d, *J* = 7.9 Hz, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 23.1 (**C**H$_3$C(O)), 43.2 (**C**H$_2$N), 50.6 (C**H**$_2$N$_3$), 52.4 (C**H**), 70.1, 70.2 (2 **C**H$_2$O), 77.3 (**C**≡C), 83.2 (C≡**C**), 121.2, 127.4, 132.3, 138.7 (4 Ar**C**), 169.8, 170.4 (2 **C**(O)); M_r (+ESI) 352.1387 $[M+Na]^+$ (calcd for $C_{16}H_{19}N_5O_3Na^+$ 352.1386 $[M+Na]^+$).

(*R*)-*N*-(4-Ethynyl)benzyl) 2-Acetamido-3-(2-isothiocyanato-ethoxy)propionamide ((*R*)-10)

Triphenylphosphine polymer bound (Fluka, cat # 93094) (1.6 mol/g, 3.0 mmol) was added to an aqueous (180 μL, 10.0 mmol)/THF (10 mL) solution of (*R*)-**24** (329 mg, 1.0 mmol). The mixture was shaken until the starting material was no longer evident by TLC analysis (18 h). The triphenylphosphine-based support was filtered, washed with CH_2Cl_2, and the filtrate was concentrated in vacuum to obtain the free amine (*R*)-**26**: ^1H NMR ($CDCl_3$) δ 2.03 (s, C**H**$_3$C (O)), 2.76-2.92 (m, C**H**$_2$NH$_2$), 3.07 (s, **H**C≡C), 3.48–3.62 (m, OC**H**H', C**H**$_2$O), 3.84 (dd, *J* = 4.2, 10.2 Hz, OCH**H'**), 4.39–4.52 (m, C**H**$_2$N), 4.54–4.60 (m, C**H**), 6.71–6.79 (br d, CH$_3$C(O)

N**H**), 7.23 (d, *J* = 7.9 Hz, 2 Ar**H**), 7.45 (d, *J* = 7.9 Hz, 2 Ar**H**), 7.82–7.91 (br m, N**H**CH_2); ^{13}C NMR (CD_3CN) δ 23.1 (**C**H_3CO), 42.5, 43.2 (2 **C**H_2N), 54.7 (**C**H), 71.2, 74.2 (2 **C**H_2O), 78.7 (**C**≡C), 84.2 (C≡**C**), 121.5, 128.3, 133.0, 141.7 (Ar**C**), 171.2, 171.4 (2 **C**(O)).

Anhydrous CH_2Cl_2 (5 mL) was added to the free amine (*R*)-**26** and DPT (232 mg, 1.0 mmol) was added. The yellow solution was stirred at room temperature (18 h). The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography (EtOAc to 90/10 EtOAc/MeOH) to obtain (*R*)-**10** as a white solid (80 mg, 23%); R_f = 0.45 (EtOAc); mp 124–126 °C; $[\alpha]^{25}_{D}$ +8.0° (*c* 0.5, DMSO); IR (nujol mull) 3266, 2571, 2201, 2103, 1631, 1538, 1458, 1375, 1304, 1120, 820, 725 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.08 (s, C**H$_3$**C(O)), 3.07 (s, **H**C≡C), 3.55–3.77 (m, C**H$_2$**NCS, OC**H**H', CH_2C**H$_2$**O), 4.01 (dd, *J* = 3.3, 9.3 Hz, OCH**H'**), 4.43–4.57 (m, C**H$_2$**N), 4.58–4.64 (m, C**H**), 6.54 (d, *J* = 6.3 Hz, CH_3C(O)N**H**), 6.79–6.88 (br t, N**H**CH_2), 7.25 (d, *J* = 8.6 Hz, 2 Ar**H**), 7.46 (d, *J* = 8.6 Hz, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 23.3 (**C**H_3CO), 43.3 (**C**H_2N), 45.4 (**C**H_2NCS), 52.7 (**C**H), 69.3, 70.0 (2 **C**H_2O), 77.4 (**C**≡C), 83.3 (C≡**C**), 121.3, 127.5, 132.4, 138.8 (4 Ar**C**), 169.7, 170.6 (2 **C**(O)), the signal for the isothiocyanate carbon resonance was not detected under the conditions used for the acquisition of the NMR spectrum; M_r (+ESI) 368.1048 $[M+Na]^+$ (calcd for $C_{17}H_{19}N_3O_3SNa^+$ 368.1045 $[M+Na]^+$). Anal. ($C_{17}H_{19}N_3O_3S$•0.2H_2O): C, H, N, S.

(*S*)-*N*-(4-Ethynyl)benzyl) 2-Acetamido-3-(2-isothiocyanato-ethoxy)propionamide ((*S*)-10)

Utilizing the preceding procedure, polymer bound (Fluka, cat # 93094) (1.6 mol/g, 3.3 mmol), H_2O (205 μL, 11.4 mmol)/THF (10 mL) solution, and (*S*)-**24** (375 mg, 1.1 mmol) gave the free amine (*S*)-**26**; ^{1}H NMR ($CDCl_3$) δ 2.01 (s, C**H$_3$**C(O)), 2.77–2.91 (m, C**H$_2$**NH_2), 3.07 (s, **H**C≡C), 3.49–3.57 (m, OC**H**H', C**H$_2$**O), 3.80 (dd, *J* = 4.5, 9.9 Hz, OCH**H'**), 4.38–4.51 (m, C**H$_2$**N), 4.56–4.62 (m, C**H**), 6.88 (d, *J* = 6.9 Hz, CH_3C(O)N**H**), 7.23 (d, *J* = 8.7 Hz, 2 Ar**H**), 7.45 (d, *J* = 8.7 Hz, 2 Ar**H**), 7.88–8.01 (br t, N**H**CH_2); M_r (+ESI) 304.1662 $[M+H]^+$ (calcd for $C_{16}H_{21}N_3O_3H^+$ 304.1661 $[M+H]^+$).

Utilizing the preceding procedure, the free amine (*S*)-**26** was coupled with DPT (125 mg, 32%) to give (S)-**10**: R_f = 0.45 (EtOAc); mp 127 °C; $[\alpha]^{25}_{D}$ −9.6° (*c* 0.5, DMSO); IR (nujol mull) 3277, 2730, 2208, 2107, 1635, 1549, 1458, 1375, 1307, 1130, 819, 726 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.07 (s, C**H$_3$**C(O)), 3.07 (s, **H**C≡C), 3.53–3.77 (m, C**H$_2$**NCS, OC**H**H', CH_2C**H$_2$**O), 4.00 (dd, *J* = 3.3, 9.3 Hz, OCH**H'**), 4.42–4.56 (m, C**H$_2$**N), 4.59–4.64 (m, C**H**), 6.55 (d, *J* = 6.9 Hz, CH_3C(O)N**H**), 6.81-6.85 (br t, N**H**CH_2), 7.24 (d, *J* = 8.4 Hz, 2 Ar**H**), 7.46 (d, *J* = 8.4 Hz, 2 Ar**H**); ^{13}C NMR ($CDCl_3$) δ 23.2 (**C**H_3CO), 43.3 (**C**H_2N), 45.4 (**C**H_2NCS), 52.7 (**C**H), 69.3, 70.0 (2 **C**H_2O), 77.3 (**C**≡C), 83.3 (C≡**C**), 121.3, 127.5, 132.4, 138.7 (4 Ar**C**), 169.7, 170.6 (2 **C**(O)), the signal for the isothiocyanate carbon resonance was not detected under the conditions used for the acquisition of the NMR spectrum; M_r (+ESI) 368.1046 $[M+Na]^+$ (calcd for $C_{17}H_{19}N_3O_3SNa^+$ 368.1045 $[M+Na]^+$). Anal. ($C_{17}H_{19}N_3O_3S$•0.16CH_3OH): C, H, N, S.

Preparative Reaction of (*R*)-*N*-(4-Isothiocyanato)benzyl) 2-Acetamido-3-(prop-2-ynyloxy) propionamide ((*R*)-9) and *N*-2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl Biotin Amide (35) to Give (*R*)-36

(*R*)-**9** (50 mg, 0.15 mmol) and **35** (81 mg, 0.18 mmol) were dissolved in *t*-BuOH/H_2O (1:2, 3 mL) and then a freshly prepared aqueous 1 M sodium ascorbate solution (15 μL, 15.0 mol) was added, followed by a freshly prepared aqueous 0.1 M $CuSO_4$·5H_2O solution (15 μL, 1.5 μmol). The reaction mixture was vigorously stirred at room temperature (15 h), evaporated in vacuo, and then purified by column chromatography (SiO_2; 1/9 MeOH/$CHCl_3$) to yield 60 mg (51%) of (*R*)-**36** as a white sticky foam: R_f = 0.30 (1/9 MeOH/$CHCl_3$); IR (nujol) 3284, 2925, 2108, 1657, 1544, 1459 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.37-1.44 (m, C(6)**H$_2$**), 1.55-1.67 (m, C**H$_2$**C**H$_2$**C(6)), 2.02 (s, C**H$_3$**C(O)), 2.14 (t, *J* = 7.5 Hz, C(10)C**H$_2$**), 2.70 (d, *J* = 12.6 Hz, C(5)

HH'), 2.89 (dd, *J* = 4.8, 12.6 Hz, C(5)H**H'**), 3.09-3.15 (m, C(2)**H**), 3.38-3.41 (m, $OCH_2C\mathbf{H_2}NHC(O)$), 3.52-3.62 (m, 2 $OC\mathbf{H_2}C\mathbf{H_2}O$, triazole-$CH_2C\mathbf{H_2}O$), 3.67 (dd, *J* = 6.5, 9.8 Hz, CHC**H**H'OCH_2), 3.86-3.94 (m, $OC\mathbf{H_2}CH_2NHC(O)$, CHCH**H'**OCH_2), 4.25-4.30 (m, C(3)**H**), 4.40-4.49 (m, $C\mathbf{H_2}Ar$, C(4)**H**), 4.55 (t, *J* = 5.0 Hz, triazole-$C\mathbf{H_2}CH_2O$), 4.62-4.68 (m, C**H**CH_2OCH_2-triazole, $CHCH_2OC\mathbf{H_2}$-triazole), 5.57, 6.52 (s, N(1′)**H**, N(3′)**H**), 6.84 (t, *J* = 5.4 Hz, $OCH_2CH_2N\mathbf{H}C(O)$), 7.10-7.15 (m, 2 Ar**H**), 7.24 (d, *J* = 8.7 Hz, 2 Ar**H**), 7.40 (d, *J* = 7.5 Hz, $CH_3C(O)N\mathbf{H}CH$), 7.80 (s, C**H** triazole), 7.83 (t, *J* = 6.0 Hz, $N\mathbf{H}CH_2Ar$); ^{13}C NMR (CD_3OD) δ 23.3 (**C**$H_3C(O)$), 25.7 (**C**(6)), 28.3 (**C**(7)), 28.4 (**C**(8)), 35.9 (**C**(9)), 39.3 ($OCH_2\mathbf{C}H_2NHC(O)$), 40.8 (**C**(5)), 43.0 (**C**H_2Ar), 50.4 (triazole-**C**H_2CH_2O), 53.2 (**C**HCH_2OCH_2-triazole), 55.8 (**C**(2)), 60.3 (**C**(3)), 62.1 (**C**(4)), 64.6 ($CHCH_2O\mathbf{C}H_2$-triazole), 69.5 ($CH\mathbf{C}H_2OCH_2$-triazole), 70.0, 70.1, 70.2, 70.4, 70.5, 70.6 (3 $\mathbf{C}H_2O\mathbf{C}H_2$ in PEG linker), 124.3 (triazole **C**H), 126.0, 128.7, 130.1, 138.1 ($\mathbf{C_6}H_4$), 135.4 (N**C**S), 144.3 (triazole **C**), 164.1, 170.6, 171.2, 173.6 (4 **C**(O)); HRMS (ESI) 776.3215 [M + H^+] (calcd. for $C_{34}H_{50}N_9O_8S_2$ 776.3224).

(*R*)- *N*-Benzyl 2-Acetamido-3-(2-(4-(methoxymethyl)-1*H*-1,2,3-triazol-1-yl)ethoxy) propionamide ((*R*)-38)

Compound (*R*)-**30** (400 mg, 1.3 mmol) was dissolved in a THF:H2O mixture (1:1, 50 mL) and while stirring, propargyl methyl ether (1 mL, 11.8 mmol), sodium ascorbate (25 mg, 0.13 mmol), and $CuSO_4$ (3 mg, 0.01 mmol), were successively added. The reaction was stirred at room temperature (24 h), and saturated aqueous $NaHCO_3$ (100 mL) was added. The aqueous layer was extracted with CH_2Cl_2 (2 × 100 mL), the combined organic layers were washed with brine (100 mL) and filtered through a Celite® bed. Removal of solvents in vacuo gave an off-white solid that was purified by flash chromatography (10/90 MeOH/CH_2Cl_2) to yield (*R*)-**38** (470 mg, 96%) as a crystalline solid: mp 127-129 °C; $[\alpha]^{25}_{D}$ +1.8° (c 0.34; MeOH); R_f = 0.49 (10/90 MeOH/CH_2Cl_2); IR (nujol mull) 3296, 2861, 1641, 1545, 1457, 1377, 1143, 1095 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.00 (s, C$\mathbf{H_3}$C(O)), 3.38 (s, $CH_2OC\mathbf{H_3}$), 3.50 (dd, *J* = 6.5, 9.6 Hz, C**H**H'OCH_2CH_2), 3.76–3.85 (m, $OC\mathbf{H_2}CH_2N(N)CH$), 3.92 (dd, *J* = 4.2, 9.6 Hz, CH**H'**OCH_2CH_2), 4.34–4.51 (m, NHC$\mathbf{H_2}$Ph, $OCH_2C\mathbf{H_2}N(N)CH$, C$\mathbf{H_2}OCH_3$), 6.63 (d, *J* = 6.6 Hz, N**H**CHC(O)), 7.00–7.10 (br t, N**H**CH_2Ph), 7.18–7.35 (m, $C_6\mathbf{H_5}$), 7.52 (s, NC**H**C(N)), addition of excess (*R*)-(−)-mandelic acid to a $CDCl_3$ solution of (*R*)-**38** gave only one signal for the acetyl protons; ^{13}C NMR ($CDCl_3$) δ .. 23.2 (**C**$H_3C(O)$), 43.7 (NH**C**H_2Ph), 50.0 (**C**$H_2N(N)CH$), 52.9 (**C**HC(O)NH), 58.6 ($CH_2O\mathbf{C}H_3$), 66.0 ($O\mathbf{C}H_2CH_2N$), 69.4, 70.5 ($CH\mathbf{C}H_2OCH_2$, **C**H_2OCH_3), 123.8 (N**C**HC(N)), 127.6, 127.7, 128.8, 138.3 ($\mathbf{C_6}H_5$), 145.3 (NCH**C**(N)), 169.7, 170.8 ($CH_3\mathbf{C}(O)$, CH**C**(O)NH); M_r (+ESI) 398.1806 $[M+Na]^+$ (calcd for $C_{18}H_{25}N_5O_4Na^+$ 398.1804 $[M+Na]^+$). Anal. ($C_{18}H_{25}N_5O_4$) C, H, N.

Preparation of Mouse Soluble Brain Proteosomes

Mouse brains (male, 2 months, ICR mice [Rockland Immunochemicals, Gilbertsville, PA]) were Dounce-homogenized in 50 mM HEPES buffer (pH 7.4). The lysate was centrifuged at slow speed (1200 × g for 12 min at 4 °C) to remove debris. The supernatant was then centrifuged at high speed (100,000 × g for 1 h at 4 °C). The resulting supernatant was collected and stored at −80 °C until use. The total protein concentration was determined by using the Bradford assay.

Three NCS Probe Labeling, Cycloaddition Reaction, and In-Gel Fluorescence Scanning

Mouse brain lysate (1 mL, 50 mM HEPES buffer (pH 7.4)) was passed through a NAP-10 column (GE Healthcare) to exchange buffer to an aqueous 50 mM HEPES buffer (pH 8.0). Lysate aliquots (50 μL of 2.0 mg/mL protein in 50 mM HEPES buffer (pH 8.0)) were treated with lacosamide AB (NCS) & CR (alkyne, N_3) compounds (5 μM) at room temperature (30 min). The modified lysates were sequentially treated with rhodamine reporter tag (50 μM)

(either rhodamine-azide (Rho-N_3, **39**) or rhodamine-alkyne (Rho-PEG-≡, **40**)), TCEP (1 mM), TBTA (200 μM) and $CuSO_4$ (1 mM). Samples were shaken and allowed to rotate using Roto-shake (8 rpm, Scientific Industries Inc., Model No. SI-1100, Bohemia, NY) at room temperature (1 h). Proteins were separated by SDS-PAGE after addition of 4× SDS-PAGE loading buffer and visualized by in-gel fluorescence using a typhoon 9400 scanner (GE Healthcare/Amersham Bioscience) with excitation at 555 nm and detection at 580 nm.

Probe Labeling of GST-CRMP2 in Mouse Lysate, Cycloaddition, and In-Gel Fluorescence Scanning

Utilizing the previous method, lysate aliquots (150 μL, pH 8.0) were prepared after passage through a NAP-10 column. Overexpressed GST-CRMP2 (30 μg) was added to the lysate (150 μL) and divided into three equal aliquots. The aliquots (50 μL) were treated with (*R*)- and (*S*)-**9** (5 μM) at room temperature (30 min). Using the previous method, the modified lysates were then treated with **39** under Cu(I)-mediated cycloaddition conditions, followed by SDS-PAGE separation, and in-gel fluorescence.

Pharmacology

Compounds were screened under the auspices of the National Institutes of Health's Anticonvulsant Screening Program. Experiments were performed in male rodents [albino Carworth Farms No. 1 mice (intraperitoneal route, ip), albino Spague-Dawley rats (oral route, po)]. Housing, handling, and feeding were in accordance with recommendations contained in the 'Guide for the Care and Use of laboratory Animals'. Anticonvulsant activity was established using the MES test[59,60] and the scMet test,[59] using previously reported methods.[43a]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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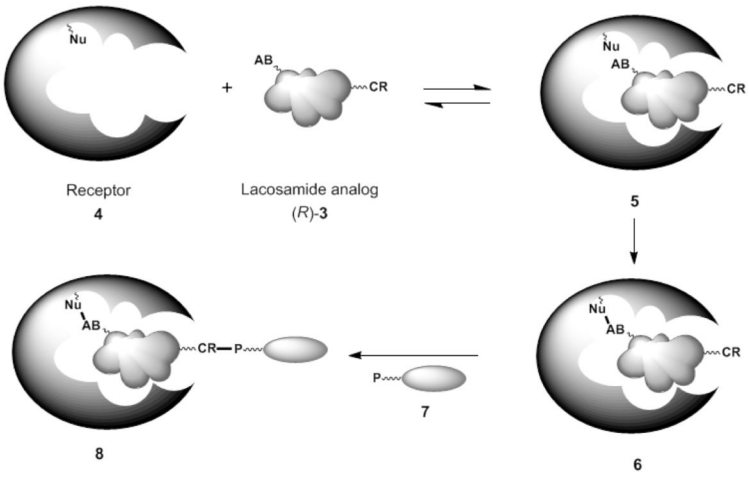
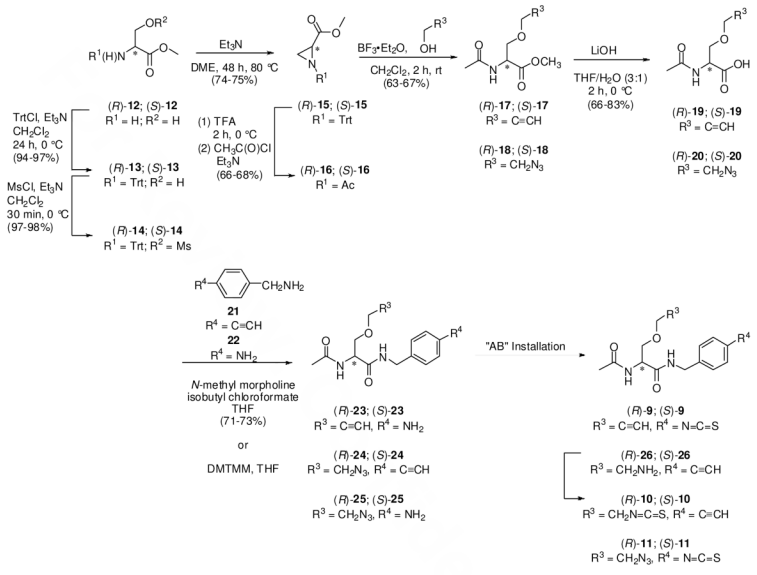


Scheme 1.
"Affinity Bait" and "Chemical Reporter" (AB&CR) Strategy to Scan the Brain Proteome for (*R*)-Lacosamide Target(s)



Scheme 2.
General Synthetic Pathway to Prepare Lacosamide AB&CR Agents **9–11**

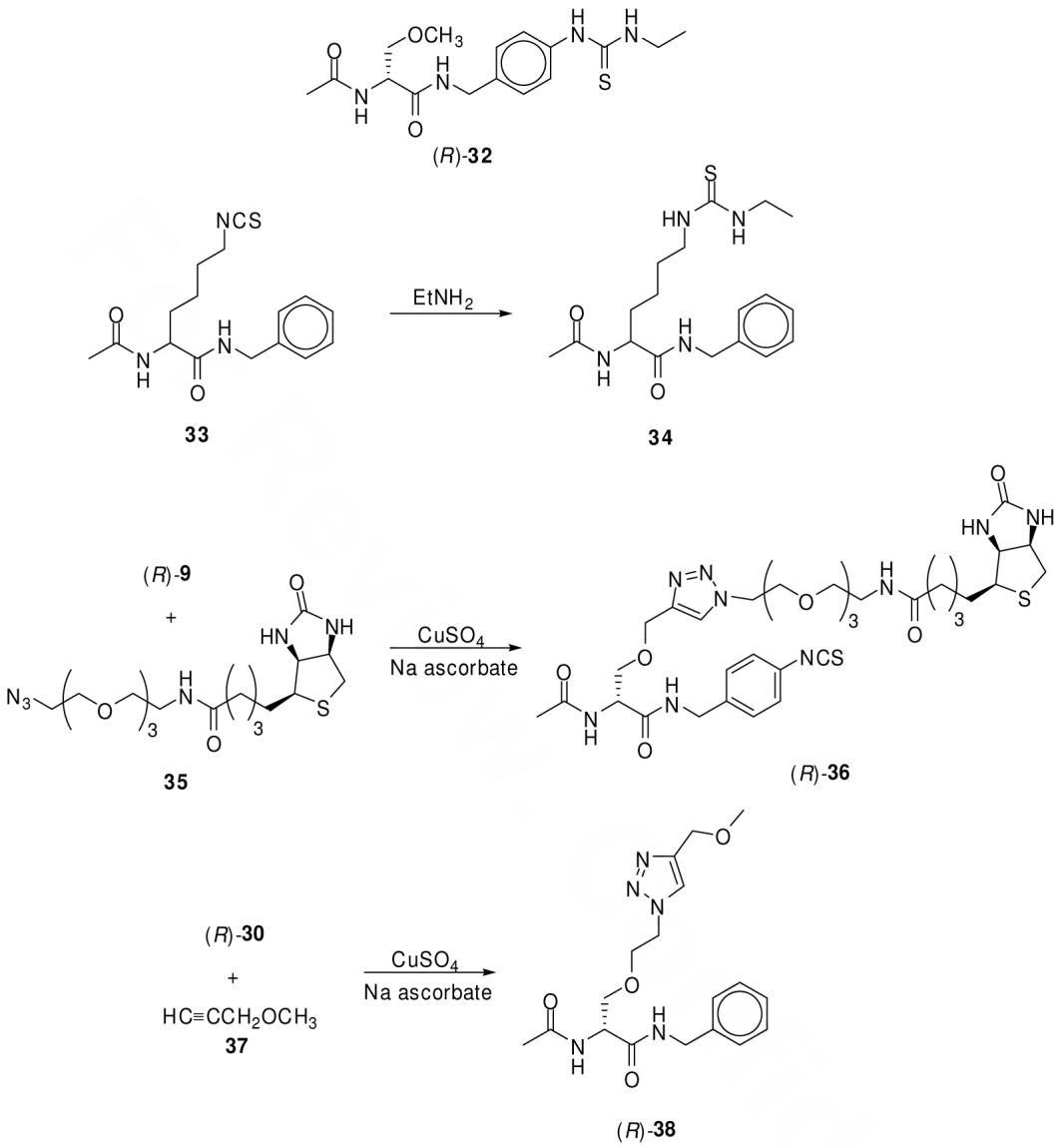


Figure 1.
Chemical evaluation of the AB and the CR units in **3**

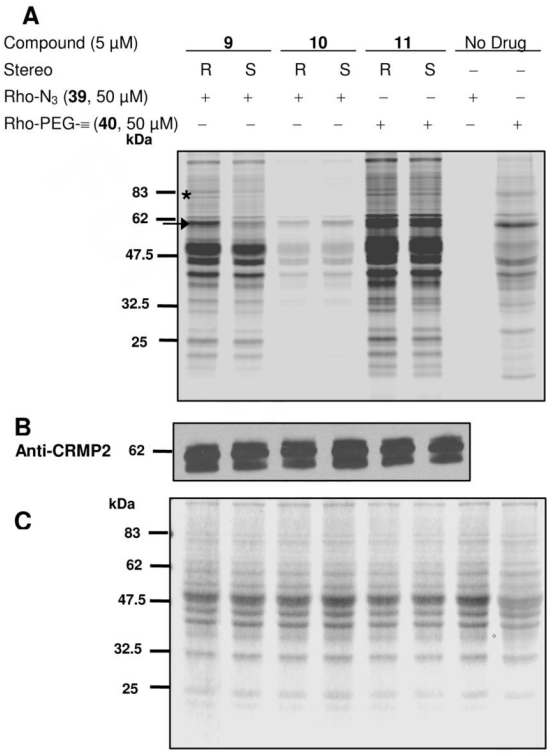


Figure 2.

Proteome reactivity profiles of three different isothiocyanate (NCS) agents **9–11**. Figure 2A; AB&CR agent-labeled proteins were detected by in-gel fluorescence scanning after Cu(I)-mediated cycloaddition to either a rhodamine-azide reporter probe **39** or a rodamine-alkyne reporter probe **40**. Figure 2B; CRMP2 protein was detected by western blot using anti-CRMP2. Figure 2C; All proteins in lysate were visualized by Coomassie blue staining after in-gel fluorescence scanning. (All images shown in gray scale).

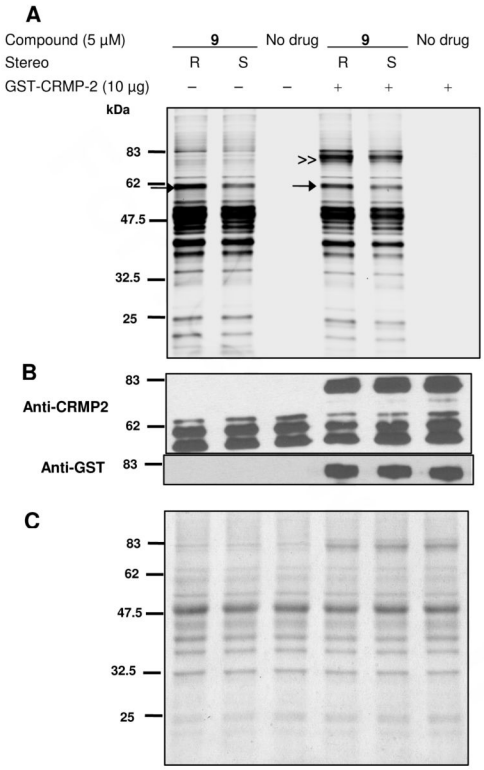


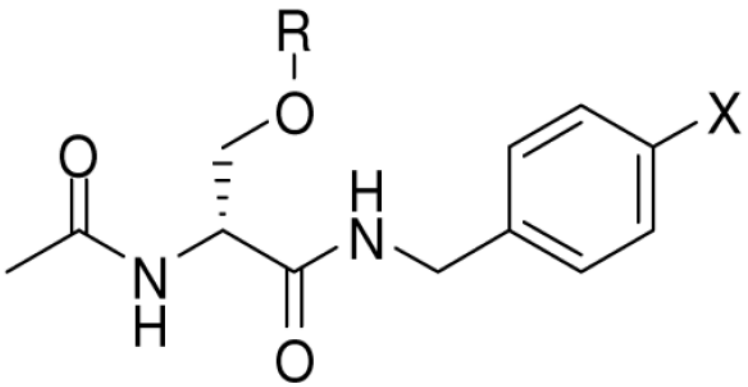
Figure 3.

In vitro labeling of externally added GST-CRMP2 and endogenous CRMP2 in mixture of mouse lysate and overexpressed GST-CRMP2.

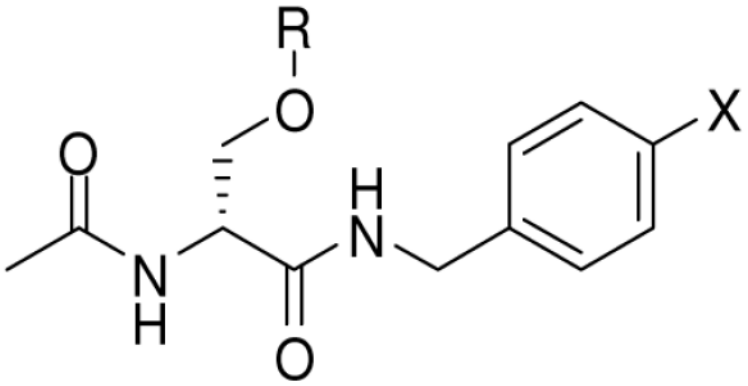
Figure 3A; AB&CR agent-labeled proteins were detected by in-gel fluorescence scanning after Cu(I)-mediated cycloaddition to rhodamine-azide reporter probe **39**. Figure 3B; CRMP2 and GST proteins were detected by western blot using anti-CRMP2 and anti-GST antibodies. Figure 3C; All proteins in lysate were visualized by Coomassie blue staining after in-gel fluorescence scanning. (All images shown in gray scale).

Table 1

Pharmacological Evaluation of Lacosamide AB&CR Agents



Compd. No.	R	X	Mice (ip)[a] MES[c], ED_{50} (mg/kg)	Mice (ip)[a] Tox[d], TD_{50} (mg/kg)	Mice (ip)[a] PI[e]	Rat (po)[b] 6Hz, ED_{50} (mg/kg) (32 mA)	Rat (po)[b] MES, ED_{50} (mg/kg)	Rat (po)[b] Tox, TD_{50}[f] (mg/kg)	Rat (po)[b] PI
(*R*)-**9**	$CH_2C{\equiv}CH$	NCS	45 [1] (41 – 48)	110 [0.25] (76 – 150)	2.4	53 [0.5] (42 – 68)	> 30	> 30	
(*S*)-**9**	$CH_2C{\equiv}CH$	NCS	> 300	> 300		ND[g]	ND	ND	
(*R*)-**28**	$CH_2C{\equiv}CH$	H	16 [0.25] (13 – 19)	59 [0.25] (55 – 66)	3.7	29 [0.5] (21 – 40)	7.9 [0.5] (4.7 – 11)	> 500	>63
(*S*)-**28**	$CH_2C{\equiv}CH$	H	> 300	> 300		> 30	> 30	> 30	
(*R*)-31[h]	CH_3	NCS	24 [0.5] (21 – 27)	47 [0.25] (43 – 50)	2.0	ND	4.2 [4] (2.4 – 8.0)	> 250	> 59
(*S*)-**31**	CH_3	NCS	> 100, < 300	> 30, < 100		ND	> 180	> 30	
(*R*)-**10**	CH_2CH^2NCS	C≡CH	ND	ND		ND	ND	ND	
(*S*)-**10**	CH_2CH_2NCS	C≡CH	ND	ND		ND	ND	ND	
(*R*)-**29**	CH_3	C≡CH	> 3, < 10 [0.5]	>10, <30 [0.5]		ND	< 30 [0.5 –4.0]	< 30 [0.5 – 4.0]	
(*S*)-**29**	CH_3	C≡CH	> 300 [0.5]	> 300 [0.5]		ND	ND	ND	
(*R*)-**27**	CH_2CH_2NCS	H	>300 [0.5]	> 30, < 100		ND	< 30 [4.0]	>30 [4.0]	
(*S*)-**27**	CH_2CH_2NCS	H	> 300 [0.5]	> 30, < 100 [0.5]		ND	< 30 [4.0]	ND	
(*R*)-**11**	$CH_2CH_2N_3$	NCS	> 100, < 300 [4.0]	> 100, < 300 [0.5]		ND	ND	ND	
(*S*)-**11**	$CH_2CH_2N_3$	NCS	> 100, < 300 [0.25]	> 300		ND	ND	ND	
(*R*)-**30**	$CH_2CH_2N_3$	H	> 100, < 300 [4.0]	> 100, < 300 [4.0]		44 [0.5] (32 – 65)	>40 [0.25]	>30	
(*S*)-**30**	$CH_2CH_2N_3$	H	> 300	> 300		ND	ND	ND	
(*R*)-**31**	CH_3	NCS	24 [0.5] (21 – 27)	47 [0.25] (43 – 50)	2.0	ND	4.2 [4] (2.4 – 8.0)	> 250	> 59
(*S*)-**31**	CH_3	NCS	> 100, < 300	> 30, < 100		ND	> 180	> 30	



Compd. No.	R	X	Mice (ip)[a] MES[c], ED_{50} (mg/kg)	Mice (ip)[a] Tox[d], TD_{50} (mg/kg)	Mice (ip)[a] PI[e]	Mice (ip)[a] 6Hz, ED_{50} (mg/kg) (32 mA)	Rat (po)[b] MES, ED_{50} (mg/kg)	Rat (po)[b] Tox, TD_{50}[f] (mg/kg)	Rat (po)[b] PI
(*R*)-**2**[i]			4.5 [0.5] (3.7-5.5)	27 [0.25] (38-47)	6.0	10	3.9 [0.5] (2.9-5.5)	>500 [0.5]	> 130
phenytoin[j]			9.5 [2] (8.1 – 10)	66 [2] (53 – 72)	6.9		30 [4] (22 – 39)	*k*	> 100
phenobarbital			22 [1] (15 – 23)	69 [0.5] (63 – 73)	3.2		9.1 [5] (7.6 – 12)	61 [0.5] (44 – 96)	6.7
valproate			270 [0.25] (250 – 340)	430 [0.25] (370 – 450)	1.6		490 [0.5] (350 – 730)	280 [0.5] (190 – 350)	0.6

[a] The compounds were administered intraperitoneally. Numbers in parentheses are 95% confidence intervals. The dose effect data was obtained at the "time of peak effect" (indicated in hours in the 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] Tox = behavioral toxicity.

[g] ND = not determined.

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[k] No ataxia observed up to 3000 mg/kg.