Journal of Medicinal Chemistry

Article

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jmedchem.8b01775 • Publication Date (Web): 04 Feb 2019

ACS Publications

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Development of an N-acyl amino acid that selectively inhibits the glycine transporter 2 to produce analgesia in a rat model of chronic pain

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Key words: Glycine Transport Inhibition, N-acylamino acid, analgesia, GlyT2

Abstract

Inhibitors that target the glycine transporter 2, GlyT2, show promise as analgesics but may be limited by their toxicity through complete or irreversible binding. Acyl-glycine inhibitors, however, are selective for GlyT2 and have been shown to provide analgesia in animal models of pain with minimal side effects, but are comparatively weak GlyT2 inhibitors. Here, we modify the simple acyl-glycine by synthesising lipid analogues with a range of amino acid head groups in both L- and D-configurations, to produce nanomolar affinity, selective GlyT2 inhibitors. The potent inhibitor oleoyl-D-lysine (**33**) is also resistant to degradation in both human and rat plasma and liver microsomes, and is rapidly absorbed following an intraperitoneal injection to rats and readily crosses the blood brain barrier. We demonstrate that **33** provides greater analgesia at lower doses, and does not possess the severe side effects of the very slowly reversible GlyT2 inhibitor, ORG25543 (**2**).

Introduction

Disease or injury to the somatosensory nervous system can cause long-term neuropathic pain, characterised by the associated hyperalgesia (amplified pain sensation) and allodynia (pain response to innocuous stimuli), which produce distinctive symptoms of numbness, burning, shooting, and electric-like pain in sufferers.[1] Changes within the central nervous system (CNS) produce disordered synaptic pathways and central sensitisation which allow for symptoms to persist long after the site of injury has healed.[2-3] There is a great need to develop new drugs to treat chronic neuropathic pain as a large number of cases are refractory to conventional analgesics[4], perhaps due to these adaptations within the CNS. Additionally, many current analgesics, such as opioids, have an unacceptable side effect profile and potential for abuse.[5]

Recently, it has been shown in rodents suffering from neuropathic pain that there are reduced inhibitory glycinergic inputs within the spinal cord dorsal horn,[6] where primary sensory information is relayed before transmission to the brain for cognitive processing.[7] A reduction in inhibitory control may allow for hyperexcitability and disinhibition of pain signalling.[3,8] Inhibition of glycine transporters to increase glycine at inhibitory synapses may therefore restore this control and reduce spontaneous nerve activity and associated pain symptoms.

There are two subtypes of glycine transporters within the CNS, GlyT1 and GlyT2.[9] GlyT1 is expressed by astrocytes surrounding both inhibitory glycinergic and excitatory glutamatergic synapses throughout the brain and spinal cord, whereas GlyT2 is located on presynaptic inhibitory glycinergic neurons predominantly in the spinal cord, including within the dorsal horn.[10] Conflicting reports have been made concerning the effectiveness of GlyT1 inhibitors, with one study showing an initial increase in pain due to NMDA receptor stimulation before analgesics effects are apparent[11], whilst other studies have shown analgesic effects.[12] On the other hand, selective inhibition of GlyT2 could selectively increase glycinergic neurotransmission in the ascending pain pathway and provide effective relief from pain symptoms.[3] This has been demonstrated using partial knockdown of GlyT2 via targeted siRNA, which ameliorates pain symptoms in a sciatic nerve injury model of neuropathic

pain.[11] The role of glycine in the development of pain and its utility in producing analgesia has driven the development of a number of glycine transport inhibitors (Figure 1) for the treatment of neuropathic pain.

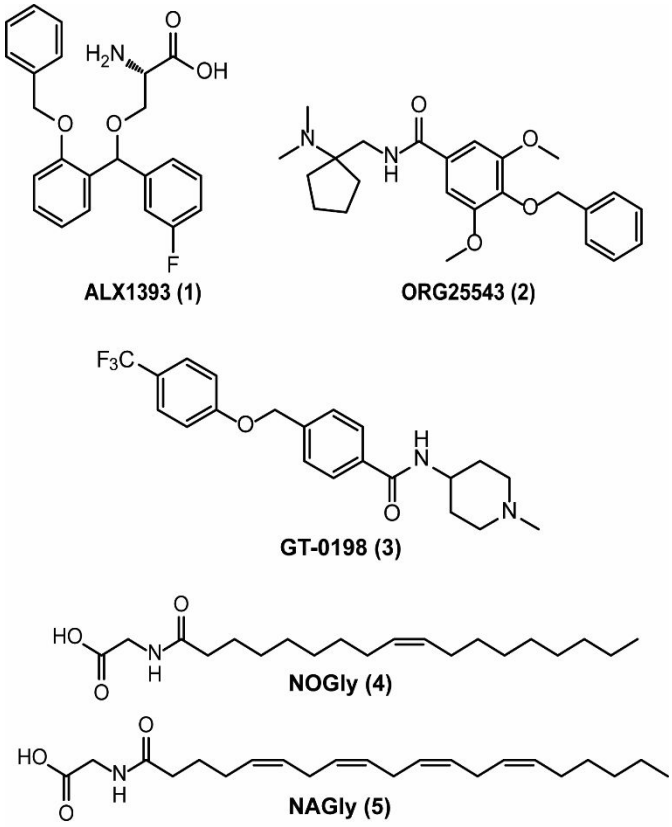


Figure 1: Chemical structures of selected GlyT2 inhibitors.

ALX1393[12] (**1**) and ORG25543[14] (**2**) were among the first potent inhibitors of GlyT2 to be developed (Figure 1). **1** inhibits GlyT2 with an IC_{50} of 100 nM[15] and has been shown to be anti-allodynic when intrathecally or intravenously injected into rodents suffering neuropathic pain.[11-13,16] However, **1** does not readily cross the blood brain barrier (BBB) and following IV administration the ratio of unbound drug in brain and plasma (Kp,uu) is only 0.05. For this reason, **1** is generally delivered directly into the CNS to produce analgesia in rodent models of pain,[17] although analgesia has been reported following IV delivery.[11]

2 is a potent inhibitor of GlyT2 (IC_{50} 16 nM), and unlike **1**, has no appreciable activity at GlyT1.[14] **2** ameliorates hyperalgesia and allodynia,[11,15] but has also been shown to produce tremors, seizures, and

death in rodents,[15] likely due to inhibition of vesicular refilling and reduced recycling of glycine into the synapse, proposed to be via irreversible, or very slowly reversible, binding to GlyT2. **2** analogues have since been synthesised to improve reversibility.[15] More recently, Hayashi and colleagues synthesised a series of phenoxymethylbenzamides which are potent inhibitors of GlyT2.[18] One derivative, GT-0198 (**3**) (Figure 1), is a potent and reversible inhibitor of GlyT2 (IC_{50} 105 nM), permeates the BBB following oral administration, and is analgesic in a partial nerve ligation model of pain.[19]

Endogenous lipids have also been identified as novel agents capable of inhibiting glycine transporters. N-arachidonyl glycine (NAGly) (**5**) is a partial, reversible, non-competitive inhibitor of GlyT2 (IC_{50} ~9 μM) with little activity at GlyT1.[20] Other endogenous N-acyl amino acids also inhibit GlyT2; with an order of potency of oleoyl L-carnitine (340 nM) > N-oleoyl glycine (NOGly) (**4**) (500 nM) ≥ palmitoyl L-carnitine (600 nM) >> **5** ~ N-arachidonyl L-alanine (9 μM) ≥ N-arachidonyl GABA (12 μM).[20-21] These lipid inhibitors of GlyT2 are amphipathic; comprised of a long flexible lipid tail conjugated to a charged or polar head group (Figure 1).

5 is very similar in structure to the endogenous cannabinoid anandamide but has no activity on the cannabinoid receptors. It has been suggested that the analgesic effects of **5** are due to inhibition of Fatty Acid Amide Hydrolase (FAAH), which would elevate levels of anandamide to stimulate cannabinoid receptors.[22] While the analgesic activity of **5** may in part be due to inhibition of FAAH, it has also been shown that **5** directly increases glycinergic synaptic transmission within the dorsal horn through inhibition of GlyT2, providing a cellular mechanism for its analgesic actions. **5** enhances the actions of both exogenously applied and endogenous glycine at inhibitory glycinergic synapses of lamina II of the superficial dorsal horn.[23] **5** also prolongs the decay phase of evoked inhibitory post-synaptic currents, but has no effect on evoked N-methyl-D-aspartate mediated excitatory postsynaptic currents; suggesting **5** exerts its effects by selectively blocking GlyT2 and increasing glycine exclusively at inhibitory synapses. Application of **5** to rodents suffering from either inflammatory or neuropathic pain reduces mechanical allodynia and thermal hyperalgesia.[24-25] **5** is also analgesic in a formalin-induced pain model, where the second phase of pain thought to depict afferent input and

central sensitisation in the dorsal horn is reduced.[26] In contrast to **1** and **2**, no adverse effects are produced following intrathecal injection of **5**.[24] The lack of apparent side effects may be a consequence of partial and reversible inhibition of GlyT2 by **5**, which would allow for sufficient glycine re-uptake into presynaptic terminals for loading of vesicles, but also slow the removal of glycine to increase transient concentrations at inhibitory glycinergic synapses while minimising spill-over into excitatory glutamatergic synapses.

The *in vivo* efficacy and lack of side effects of **5** make it a promising lead compound for analgesic drug development. To improve GlyT2 inhibitory potency we prepared a series of acyl-glycine analogues incorporating lipid tails of varied chains lengths and double bond positions.[26] We showed that the lipid tail constituent is critical in their activity as inhibitors of GlyT2, and that the optimal chain is 18 carbons in length with a *cis*-double bond in the ω-8, -9 or -10 positions. Thus, **4** was identified as among most potent analogues in the series, and partially inhibited (max. 67%) GlyT2 with an IC_{50} of 500 nM and had no effect on GlyT1 at concentrations up to 10 μM.

In this study we report on the development of a second series of N-acyl amino acids as GlyT2 inhibitors. The optimal oleoyl tail (C18 ω9) was retained and conjugated to amino acids and amino acid derivatives in both the L- and D- configurations. Inhibitory activity at GlyT1 and GlyT2 was assessed, and select compounds were also screened for metabolic stability, with the most potent, stable compound, oleoyl-D-Lys (**33**) tested for BBB permeability, selectivity against a range of targets, and ability to reduce allodynia in a nerve ligation rat model of neuropathic pain.

Results and Discussion

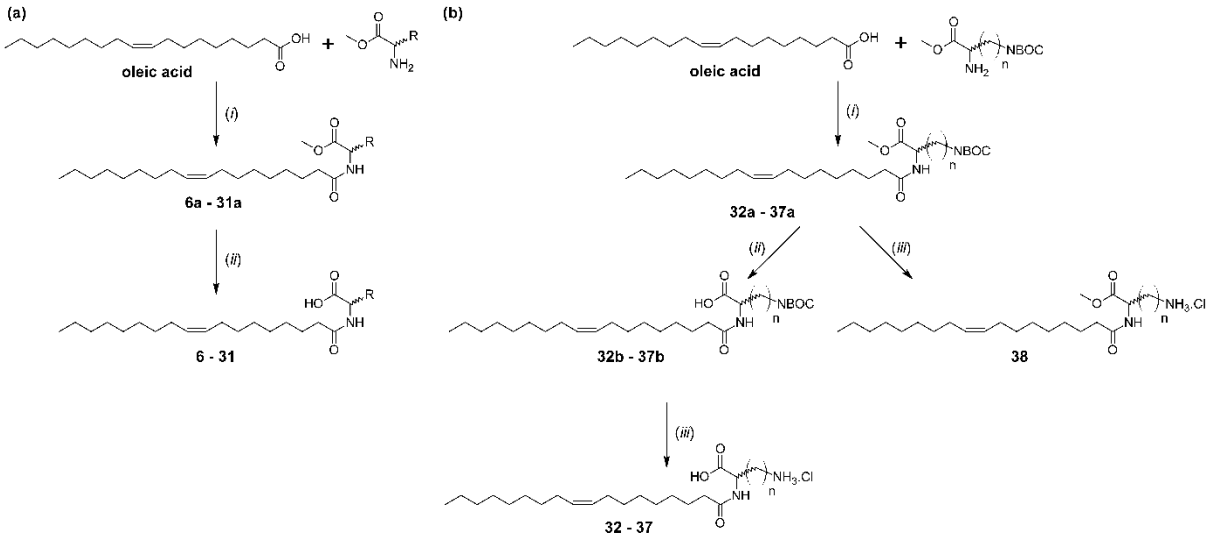
Library design and Synthesis

We have previously shown that oleic acid tails impart optimal GlyT2 inhibitory activity to N-acyl amino acids containing glycine head groups.[27] To further investigate the structure activity relationship for N-acyl amino acids as GlyT2 inhibitors, we synthesised a series of analogues in which an oleoyl tail was conjugated to a number of amino acid head groups. A range of aliphatic, aromatic, polar,

negatively charged, and positively charged amino acids were used, in both the L- and D-configurations.

N-acyl amino acids **6** – **31**(Table 1), which lack a primary amine on the head group, were synthesised in two steps (Scheme 1a). In the first step ester protected and enantiomerically pure D- and L-amino acids were conjugated via amide bonds to oleic acid using the peptide coupling agent EDCI. The resulting intermediates **6a** – **31a** were readily purified by silica gel chromatography and isolated in good yields. The ester groups in **6a** – **31a** were then hydrolysed under basic conditions, and acidification of the reaction mixtures precipitated **6** – **31**, which were isolated in high yields by filtration.

Compounds **32** – **38**, which possess primary amine groups on the head group, were synthesised using ester and BOC protected head groups (Scheme 1b). BOC protection was used to prevent amide bond formation between the side arm amine and oleic acid. EDCI couplings to oleic acid afforded the BOC-protected intermediates **32a** – **37a**. De-esterification by alkaline hydrolysis afforded **32b** – **37b**, followed by removal of the BOC-protecting groups using HCl yielded the final products **32** – **37** as hydrochloride salts. The ester **38** was prepared by removal of the BOC group in **32a** using HCl in ether.



Scheme 1. Synthesis of N-acyl amino acids without (a) or with (b) primary amine-substituted head

groups. Reagents and conditions: (*i*) EDCI, HOBt, NEt_3, rt, 18h; (*ii*) NaOH, 40 ^oC, 3 h, then HCl; (iii) HCl, rt, 4 h.

Inhibitory Activity at GlyT2 and GlyT1.

Application of glycine to oocytes expressing human GlyT1b or GlyT2a (herein referred to as GlyT1 and GlyT2) generates inward transport currents, which are reduced by co-application of the synthesised N-acyl amino acids **6** – **38** (Figure 2A, B). Application of increasing concentrations of N-acyl amino acid generated cumulative inhibition responses (representative trace shown, Figure 2C). Concentration responses curves for selected compounds are shown in Figure 3 and the % max inhibition and apparent IC_{50} values for each N-acyl amino acid at GlyT1 and GlyT2 shown in Table 1. The specificity of inhibition of glycine transport was also measured using [^{3}H]-glycine uptake by oocytes expressing GlyT2 and GlyT1 in the presence of 100 nM and 1 μM **32** (Figure S2), confirming the observations with electrophysiological measurements.

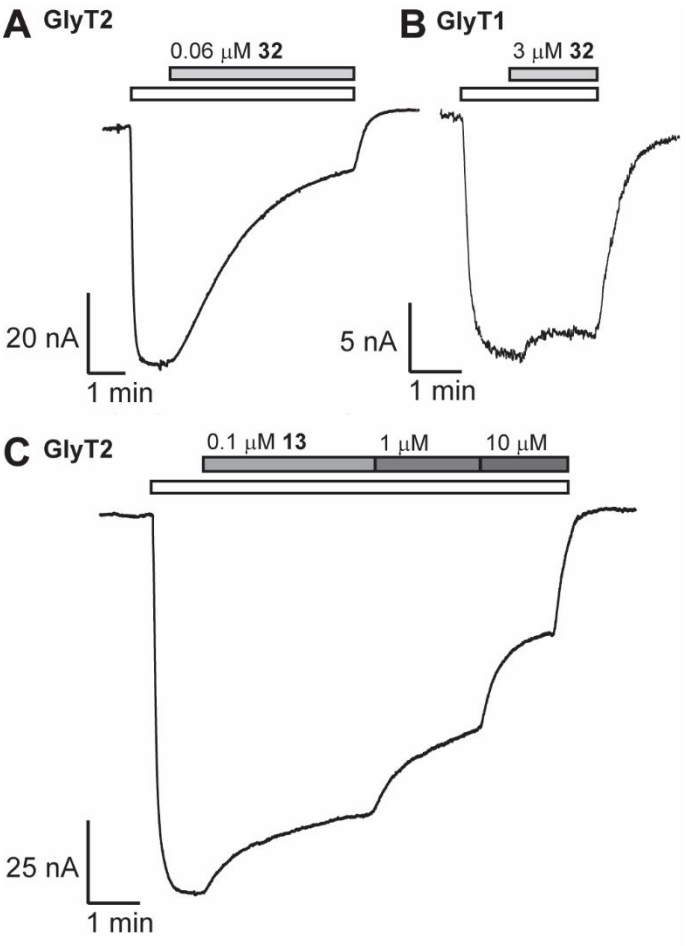


Figure 2. Representative current traces from oocytes expressing glycine transporters, clamped at -60 mV. **A.** The EC_{50} concentration of glycine (30 μM) was applied to an oocyte expressing GlyT2 (open bar) to produce an inward current, which was reduced by application of 0.06 μM **32** (grey bar). **B.** GlyT1 mediated glycine currents were not reduced by **32**, even at 3μM. **C.** Cumulative concentration response curves were generated by applying increasing concentrations of N-acyl amino acid (**13** in this example, grey segmented bar) to reduce glycine induced currents at GlyT2.

Prolonged application of >10 μM N-acyl amino acids to oocytes expressing transporters or uninjected oocytes did not produce any currents, with the exception of acyl-serine (**15** and **16**) and positively charged N-acyl amino acid compounds. Concentration response curves for these inhibitors were therefore limited to 3 μM to circumvent non-specific membrane effects.

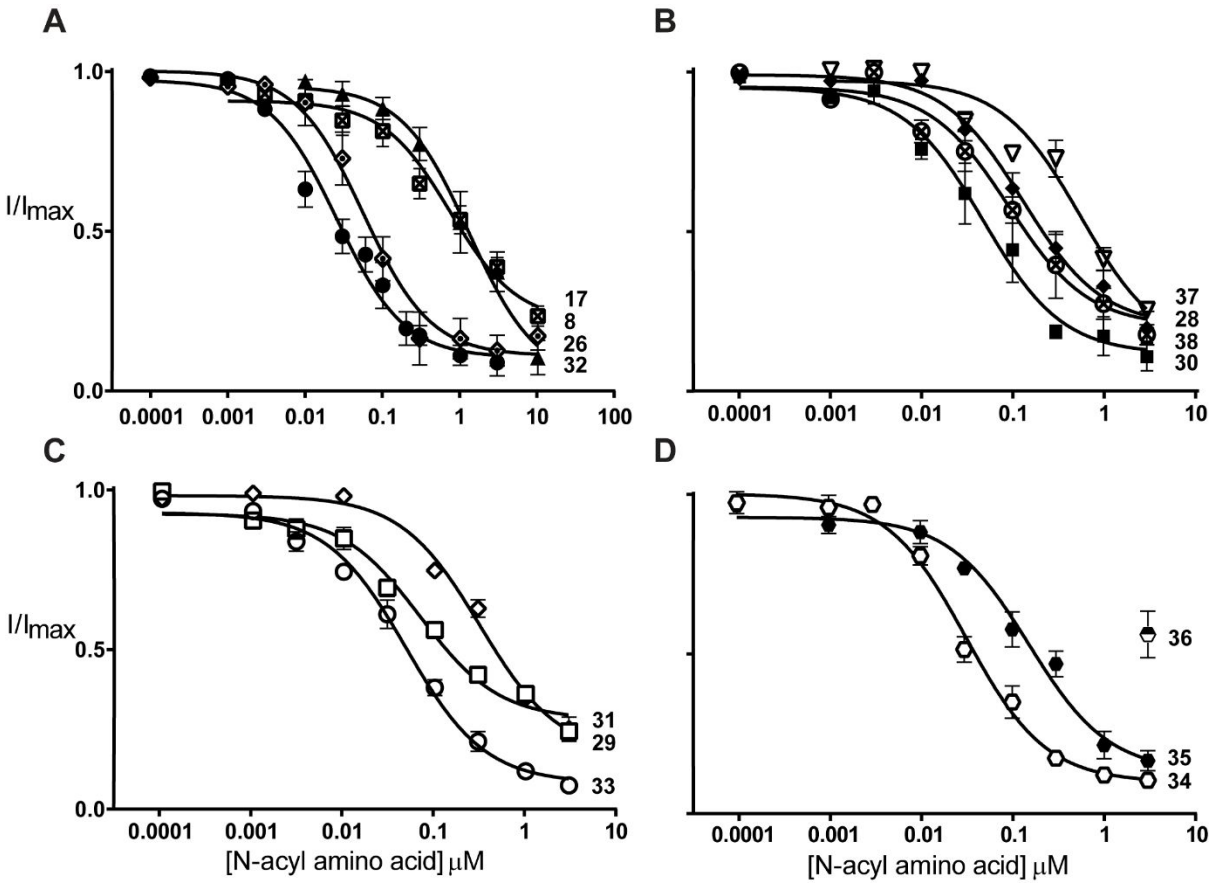


Figure 3. N-acyl amino acids inhibit glycine transport currents of GlyT2 expressing *Xenopus laevis* oocytes. 30 μM glycine transport currents were measured in the presence of N-acyl amino acids to generate concentration inhibition curves **A.** Representative N-acyl amino acids from aliphatic (**8**),

acidic (**17**), aromatic (**26**), and positively charged (**32**) groups. **B.** Positively charged oleoyl L-amino acids (**28** and **30**) and the backbone modified oleoyl N-acyl compounds (**37** and **38**). **C.** Positively charged oleoyl D-amino acids (**29, 31,** and **33**). **D.** "Lysine-like" oleoyl L-amino acids, where the pendant NH_3^+ is linked to the amino acid backbone with 3 (**34**), 2 (**35**), or 1 (**36**) carbons. Only 1 data point is presented for **36** at the maximal concentration (3 μM) as the data could not be reliably fit. The responses are normalized mean values ± SEM (n ≥ 3 cells) fit using least-squares analysis. ▲ L-Val (**8**); ⊠ L-Asp (**17**); ◈ L-Trp (**26**) ◆ L-His (**28**); ◇ D-His (**29**); ■ L-Arg (**30**); □ D-Arg (**31**); ● L-Lys (**32**); ○ D-Lys (**33**); ⬡ L-C3-Lys (**34**); ⬢ L-C2-Lys (**35**); ⬣ L-C1-Lys (**36**); ▽ C5-NH_3 (**37**); ⊗ L-Lys OMe (**38**).

N-acyl amino acid with head groups in the L-configuration all reduced GlyT2 transport currents, with IC_{50} values ranging from 25.5 nM – 4.35 μM (Table 1). For compounds containing small aliphatic side chains (L-Ala (**6**) and L-Val (**8**)) weak inhibition of GlyT2 was observed with IC_{50} values above 1 μM, whereas the bulkier *iso*-butyl group in the L-Leu analogue **10** produced a large improvement in potency (IC_{50} 143 nM). The *iso*-butyl branched side chain in **10** appears to be beneficial to activity, as the straight chain *n*-butyl analogue **14** was 2-fold less potent than **10**. Interestingly, although methionine is often grouped with aliphatic amino acids, the L-Met analogue **12**, which is structurally analogous to **14** but contains a side chain sulphur, is a potent, near complete inhibitor (29.2 nM, 91.2%). This ~9-fold improvement in activity indicates the sulfur is providing additional interactions with GlyT2. The methionine sulfur atom may also behave as an electrophile; the σ-hole of the S possibly interacting with an oxygen atom, or with aromatic π electrons in the binding site.[28-27]

For acidic amino acids, where their side chains would be negatively-charged under the assay conditions, conjugation to the oleoyl tail produces compounds which are all relatively low potency inhibitors of GlyT2 (865 nM (**17**) and 4.35 μM (**19**)). Interestingly, the IC_{50} of the amide derivative of **17**, oleoyl L-Asn (**21**), is not largely changed (613 nM) compared to **17**, which suggests that the negative charge is not providing any significant binding or repulsive interactions with GlyT2.

The N-acyl amino acids with aromatic side chains (**22, 24,** and **26**) are all relatively potent inhibitors of GlyT2. The L-Phe analogue **19** inhibited GlyT2 with an IC_{50} of 214 nM, and lengthening the alkyl chain between the aromatic ring and amino acid back bone marginally improved potency (**24**; IC_{50} 151 nM). Extending the side chain, as in the L-Trp analogue **26** further improved potency (IC_{50} 54.6 nM).

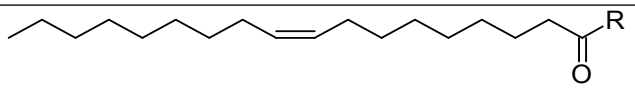
The most potent N-acyl amino acids in the series were those with positively ionisable groups on the side chain. Thus, **28** (L-His), **30** (L-Arg) and **32** (L-Lys) inhibited GlyT2 with IC_{50}s of 130, 47.9 and 25.5 nM, respectively. Encouraged by the activity of **32**, we prepared a series of derivatives of **32** to gain additional structure-activity insights. Inhibitory activity decreased as the butyl side chain in **32** was shortened to propyl (**34**), ethyl (**35**) and methyl (**36**). These findings are consistent with aromatic N-acyl amino acids, where extension of the side chain improves potency. The importance of the carboxylic acid group to activity was examined through two lysine derivatives; **37**, where the carboxylic acid is removed, and **38** which instead contains a methyl ester. In both cases, the compounds were less active than **32** (IC_{50} 560 nM and 91.3 nM respectively), with complete removal of the carboxylic acid reducing potency by ~22-fold.

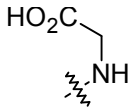
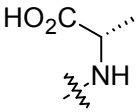
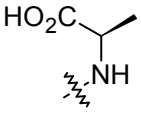
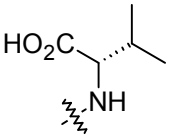
Pairwise comparison of D- and L- enantiomers showed that with the exception of serine analogues **15** and **16**, the L-enantiomers were equal, and in most cases, superior GlyT2 inhibitors. The IC_{50} values obtained for L-analogues with less bulky or flexible head group side chains were between 2-9 fold lower than their corresponding the D-enantiomers. For example, **33** (D-Lys) inhibits GlyT2 with an IC_{50} of 48.3 nM compared with 25.5 nM for **32**. Interestingly, the decrease in activity of D-enantiomers was much greater when the head group side chain was bulkier and/or less flexible; the D-enantiomers **11** (D-Leu), **23** (D-Phe), **25** (D-C2-Phe), and **27** (D-Trp) were inactive at the highest assay concentration of 10 μM, compared with their L-enantiomers that are all nanomolar inhibitors (IC_{50}s from 54.6 – 214 nM).

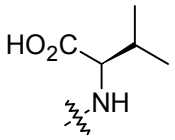
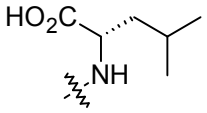
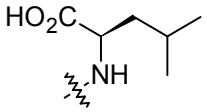
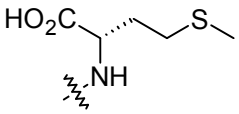
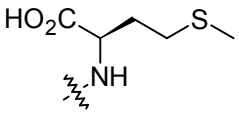
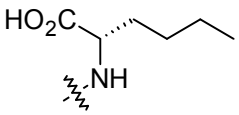
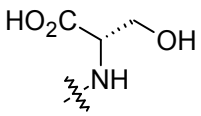
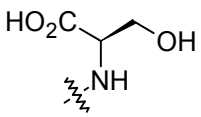
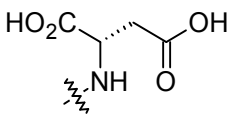
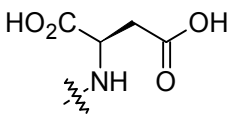
Given the wide range of head groups that are tolerated, the binding region must be large and/or flexible to accommodate a number of different chemical groups and conformations. The chemical

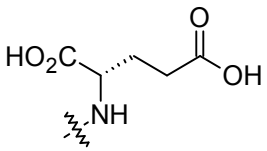
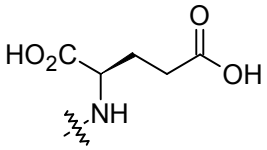
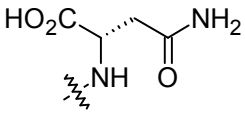
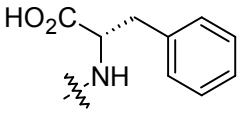
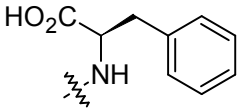
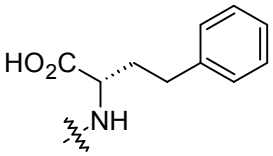
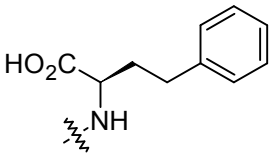
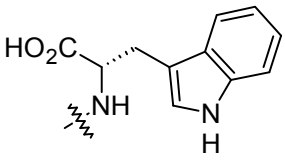
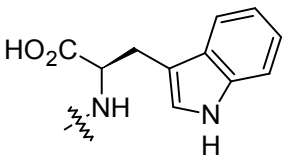
nature of the binding site can also be inferred through structure activity data. We propose that the head group binding site should be comprised of residues that strongly coordinate a positively charged or aromatic group, but do not repel a negatively charged group. It is therefore unlikely that ionic interactions mediate binding, but rather aromatic residues that would generate similar energies for cation-π and π-π interactions for the most potent N-acyl amino acids.

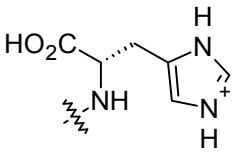
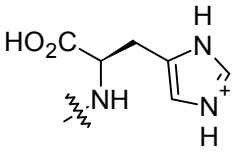
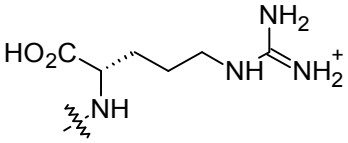
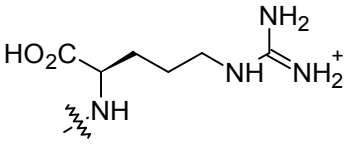
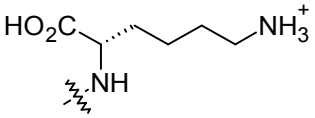
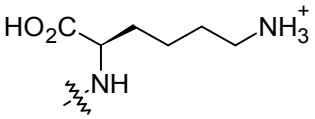
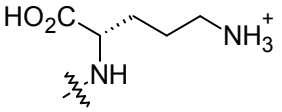
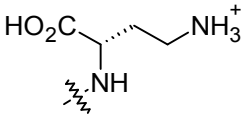
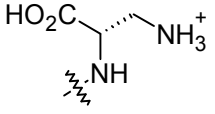
Table 1: Inhibitory activity of N-acyl amino acids **2, 4, 6 – 38** at GlyT2 and GlyT1*

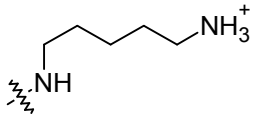
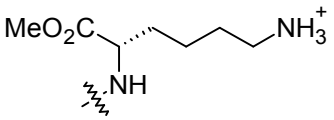


Compound	Head group	R	GlyT2 IC_{50} (nM)	GlyT1 IC_{50} (μM)	Max. GlyT2 inhibition (%)
2		(ORG25543)	3.8 (2.4-6.0)	n.d	93.00 ± 0.04
4 (NOGly)[26]	Gly		500	>30	66.8 ± 2.9
6	L-Ala		1230 (649 – 2340)	>30[a]	89.8 ± 5.8
7	D-Ala		2330 (1.26 – 4.34)	>30	81.7 ± 6.2
8	L-Val		1170 (643 – 2120)	>30	90.1 ± 5.9

9	D-Val		1000 (448 – 2570)	>30	56.7 ± 5.1
10	L-Leu		143 (70.7 – 287)	>10	72.2 ± 3.8
11	D-Leu		>30 μM[a]	>30	35.6 ± 2.6
12	L-Met		29.2 (18.6 – 45.7)	>3	91.2 ± 2.7
13	D-Met		259 (79.6 – 845)	>3	68.6 ± 8.1
14	L-NorLeu		259 (166 – 372)	>10	64.5 ± 6.9
15	L-Ser		496 (308 – 799)	>3	73.1 ± 5.1
16	D-Ser		64.8 (31.1 – 135)	>3	73.6 ± 4.4
17	L-Asp		865 (468 – 1600)	>10	76.7 ± 5.3
18	D-Asp		1810 (1050 – 3140)	>10	92.5 ± 6.9

19	L-Glu		4350 (2350 – 8070)	>10	100 ± 14.0
20	D-Glu		3750 (2240 – 6260)	>10	91.6 ± 7.9
21	L-Asn		613 (265 – 1420)	>10	92.7 ± 7.2
22	L-Phe		214 (86.4 – 530)	>10	54.7 ± 3.8
23	D-Phe		>10 μM[a]	>10	42.3 ± 2.8
24	L-C2-Phe		151 (88.8 – 257)	>10	75.1 ± 2.9
25	D-C2-Phe		>10 μM[a]	>10	10.2 ± 1.2
26	L-Trp		54.6 (31.4 – 95.8)	>10	86.2 ± 3.8
27	D-Trp		>10 μM[a]	>10	14.2 ± 3.5

28	L-His		130 (84.4 - 199)	>3	78.4 ± 3.4
29	D-His		313 (229 – 889)	>3	82.1 ± 3.3
30	L-Arg		47.9 (27.2 – 84.2)	>3	87.2 ± 3.7
31	D-Arg		69.2 (48.0 – 99.9)	>3	70.9 ± 2.2
32	L-Lys		25.5 (17.2 – 37.7)	>3	86.8 ± 2.4
33	D-Lys		48.3 (37.2 – 62.7)	>3	91.0 ± 1.9
34	L-C3-Lys		31.6 (23.1 – 43.3)	>3	86.0 ± 2.2
35	L-C2-Lys		152 (100 - 231)	>3	82.7 ± 3.3
36	L-C1-Lys		182[b] (27.3 – 1210)	>3	58.2 ± 7.6

37	C5-NH_3		560 (312 – 1010)	>3	86.7 ± 7.2
38	L-Lys-OMe		91.3 (37.5 - 222)	>3	78.8 ± 5.8

* Compounds were tested for inhibition of 30 μM glycine transport by GlyT2 and GlyT1, expressed in *Xenopus laevis* oocytes. n ≥ 3 with measurements taken from at least 2 batches of oocytes. Data presented are mean and 95 % confidence intervals or mean ± SEM.

a. Where significant inhibition was not reached, IC_{50} are presented as greater than the maximum concentration of each compound applied.

b. Curve could not reliably be fit from the data

The GlyT2 inhibitor **2** produces adverse side effects that have been attributed to its full and slowly reversible inhibition of GlyT2.[14] We therefore assessed the maximum level of GlyT2 inhibition for all of our N-acyl amino acids. Positively charged N-acyl amino acids all inhibited glycine transport in the range 71 – 95% (Table 1). Similarly, all negatively charged N-acyl amino acids produced a high % max inhibition (77 – 100%). For aliphatic and aromatic N-acyl amino acids, however, the level of inhibition was influenced by the size and configuration of the side chain. For these compounds, the L-isomer possessed a higher % max inhibition compared to the D- for all cases (for example **8** and **9** inhibited 90.1 ± 5.9 and 56.7 ± 5.1, respectively). This was particularly apparent for aromatic N-acyl amino acids, where all of the D- compounds displayed low level or no inhibition at GlyT2. The differences in level of inhibition by each compound may be a reflection on the stability of GlyT2 in its inhibited conformation.

Mechanism of binding

GlyT2 possesses the smallest substrate (S1) binding site of the NSS family and is only able to transport the smallest amino acid, glycine,[30,31] which suggests much larger bioactive lipids are unlikely to bind in the S1 central binding site. To determine the mechanism of inhibition for synthetic

N-acyl amino acids, glycine concentration responses were measured in the presence of increasing concentrations of **32**. The I_{max} of transport was significantly reduced for all concentrations of **32** (% I_{max} [10 nM] = 82.8 ± 4.0%*; [30 nM] = 62.4 ± 5.8%***; [1 μM] = 30.2 ± 4.4%***) (Figure 4). Conversely the EC_{50} values for glycine were unchanged compared to glycine transport in the absence of any inhibitor. This indicates that **32** acts as a non-competitive inhibitor of GlyT2, however an Eadie-Hofstee plot of the data suggests that the mechanism may not be purely non-competitive (Figure 3S). Lipid inhibitors may therefore display a mixed mechanism of action where binding at an allosteric site influences glycine binding at the substrate site.

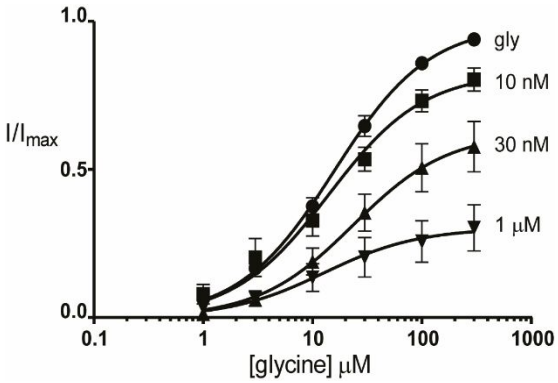


Figure 4. 32 reduces concentration-dependent glycine currents. Concentration-dependent glycine transport currents with increasing concentrations of **32**. EC_{50} (absence of inhibitor) = 15.8 ± 1.3 μM ; EC_{50} [10 nM **32**] = 14.0 ± 2.7 μM; [30 nM **32**] = 23.5 ± 8.0 μM; [1 μM **32**] = 12.8 ± 7.6 μM) ANOVA tests were performed for EC_{50} and I_{max} values. ● gly, ■ 10 nM **32**, ▲ 30 nM **32**, ▼ 1 μM **32**.

In vitro metabolism

32 emerged as a potent GlyT2 inhibitor that causes a maximum inhibition of 91% and is suitable for further development. N-acyl amino acids may be metabolised through several pathways that can limit their in vivo half-lives and therapeutic potential. Degradation occurs primarily through amide bond hydrolysis carried out by fatty acid amide hydrolase (FAAH) and other hydrolytic enzymes, as well as

oxidations of the lipid chains and head groups.[33-34] Thus, the stability of **32** and its D-enantiomer **33** were assessed in human and rat plasma and liver microsomes.

Table 2. Degradation half-lives of N-acyl amino acids in human and rat plasma.

Compound	Head group	Degradation half-life ($t_{1/2}$; min)	
		Human plasma	Rat plasma
4	Gly	> 1022[a]	27
32	L-Lys	> 1022[a]	86
33	D-Lys	> 1022[a]	> 1022[a]

a. Minimal degradation (<15%) was observed over the course of the 240 minute incubation period.

All compounds were resistant to degradation in human plasma (see Table 2 and Figure S1 in Supporting Information). In rat plasma **4** was rapidly metabolised with a degradation half-life of 27 minutes. The degradation half-life of **32** improved ~ 3-fold relative to **4**, and interestingly **33**, the D-enantiomer of **32**, did not exhibit measurable degradation in either species. A similar pattern of stability was observed in liver microsomes (Table 3). Thus, **32** and **33** were both stable in human liver microsomes, and in rat liver microsomes **32** was rapidly metabolised ($t_{1/2}$ = 54 minutes) while **33** was minimally degraded over the course of the assay.

The improved stability of **32** relative to **4** may arise from the lysine side chain of **32**. The side chain is adjacent to the amide bond and can act as a steric shield to protect the amide from hydrolysis. This phenomenon is well-known in medicinal chemistry and has been used to develop stabilised analogues of anandamide, a structurally related fatty acid amide.[35-36] The remarkable stability of **33** relative to **32** is likely to result of the unnatural head group orientation in **33**, which hinders its ability to fit within the active site of hydrolytic enzymes. Indeed, incorporation of D-amino acids into synthetic peptides is employed to produce peptide bonds that are resistant to the actions of proteases and other endogenous enzymes.[37]

Table 3. Degradation half-lives of 32 and 33 in human and rat liver microsomes.

Compound	Head group	Degradation half-life ($t_{1/2}$; min)	
		Human	Rat
32	L-Lys	> 255[a]	54
33	D-Lys	> 255 [a]	> 255 [a]

a. Minimal degradation (<15%) was observed over the course the 60 minute incubation period.

Taken together, these studies show that incorporation of unnatural head group configuration can improve stability to N-acyl amino acids, and that **33** is a metabolically stable candidate for in vivo evaluation.

Selectivity of 33

As shown in Table 1, all N-acyl amino acids were selective for GlyT2 over the closely related GlyT1. For **33**, GlyT2 was potently inhibited (IC_{50} 48.3 nM), while little inhibition of GlyT1 was seen at maximal concentrations (3 μM). We further explored the selectively of **33** at 1 and 10 μM against other targets, including transporters, GPCRs, and ion-channels (full panel results in Tables 2S, 3S, 4S). Three additional targets were identified as potential binding sites for **33**. 10 μM **33** caused greater than 50% displacement of radioactive ligands for the Adrenergic α2A receptor, norepinephrine transporter, μ opioid receptor (μOR). As μORs are a well established target for analgesics, we further examined the mechanism of interaction by **33** in AtT20 cells expressing μORs (see Supporting Information). We found **33** had no activity as a μOR agonist or antagonist at 1 μM, which suggests **33** is unlikely to produce analgesia via this mechanism.

We also tested **33** at Na_v, Ca_v channels, and α7 nicotinic acetylcholine receptors (Table 3S) which are known pain targets[37] to rule out analgesia through this mechanism, and no significant inhibition was

observed. The cannabinoid receptors, CB_1 and CB_2, as well as the sphingosine-1-phosphate receptor are targeted by similar lipid based ligands, such as anandamide, and so these receptors were also screened for **33** activity (Table 4S), but no activity as either an agonist or antagonist was observed for all three receptors.

In vivo pharmacokinetics

GlyT2 is predominantly expressed in the dorsal horn of the spinal cord and for GlyT2 inhibitors to show *in vivo* efficacy they must cross the BBB. Structurally related fatty acid amides such as methanandamide[39] and DHA-dopamine (NMI 8739)[40] are both BBB permeable, and we anticipated that **33** would act similarly. We tested this directly by studying the pharmacokinetics and brain penetration of **33** in vivo.

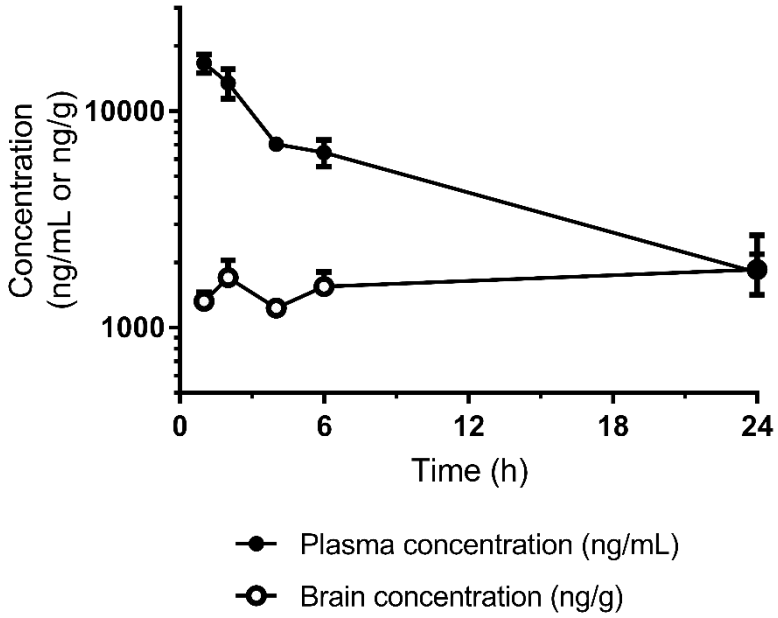


Figure 5. Plasma and brain concentrations of **33** in male Sprague Dawley rats following ip administration at 27.5 mg/kg.

Table 4. Pharmacokinetic parameters of **33** after ip administration of a single 27.5 mg/kg dose in Sprague Dawley rats.

Parameter	Plasma	Brain
C_{max} (ng/mL, ng/g)	16690	1854
T_{max} (h)	1	24
$t_{1/2}$ (h)	10	c.n.c.
AUC_{0-24} (ng*h/mL, ng*h/g)	122473	38479
Time averaged B/P ratio	0.31	

c.n.c – Could not calculate as the flat profile precluded an estimation of half life

Male Sprague Dawley rats received a single intraperitoneal dose of **33** (27.5 mg/kg) and plasma and brain concentrations were measured over a 24-hour period (Figure 5). The C_{max}, T_{max}, terminal half-life ($t_{1/2}$), AUC_{0-24h}, and total brain-to-plasma ratio (B/P) calculated from the data are presented in Table 4. Following administration **33** was rapidly adsorbed with maximum plasma concentrations observed at 1 hour. This was followed by a clearly defined terminal phase where **33** was slowly cleared from plasma with a terminal half-life ($t_{1/2}$) of 10 hours. **33** was also rapidly taken up into the brain, however unlike in plasma, there was no clear terminal phase and brain concentrations of **33** remained relatively constant over the 24 hour sampling period. Accordingly, the B/P ratio increased over the sampling period from 0.08 at 1 hour to 1.1 at 24 hours, and a time averaged B/P ratio of 0.31 was calculated from the AUC_{0-24h} values.

Encouragingly, C_{max} for **33** in brain was 1854 ng/g (approximately 4.5 μM), which although well above the IC_{50} for 28 at GlyT2, is comprised of both bound and unbound fractions of **33**. Given that **33** it is likely to be highly bound within the CNS, we assessed drug binding in vitro. Rat brain homogenate was spiked with **33** and incubated for 4.2 hours, with ultracentrifugation used to separate bound and unbound fractions. From these studies **33** was estimated to be 99.98% bound in rat brain.

Thus, at C_{max} concentrations of **33** in brain, the concentration of unbound drug ($C_{unbound}$) is approximately 90 nM (cf. IC_{50} = 48.3 nM). Together, these studies indicate that systemic administration of **33** can produce unbound concentrations of **33** within the CNS required for analgesia. The high degree of binding may also explain the slow rate of elimination of **33** from the brain. It is likely that **33** is mostly sequestered to brain tissue once it crosses the BBB, and therefore unable to rapidly leave the CNS.

Analgesia

Based on the pharmacokinetic findings, the analgesic efficacy of **33** was assessed in the same species. Male Sprague Dawley rats received a single intraperitoneal bolus of **33** and the efficacy in reversing allodynia in a neuropathic pain model was assessed over 6 h. A significant main interaction effect of treatment over time was observed ($F(18, 138) = 1.765$, $p < 0.05$), and the 30 mg/kg dose of **33** produced significant analgesia over the first 90 min after injection compared to vehicle ($p < 0.001$ at 15 and 30 min, $p < 0.01$ at 60 and 90 min; Bonferroni's multiple comparisons test; Figure 6 A). A lower dose of 3 mg/kg alleviated allodynia in a manner similar to the positive control (**2**, 30 mg/kg). Mild side effects were observed in one **33**-treated (30 mg/kg) animal up to 15 min post injection, where localised pain (abdominal contraction) was evident at the ip site of drug delivery ($p > 0.05$ compared to vehicle treated group). In contrast, more severe side effects were observed in 4 out of the 6 animals treated with **2** (30 mg/kg; $p < 0.01$ compared to vehicle). **2**-treated animals remained recumbent and exhibited acute abdominal constriction for up to 60 min, with two animals exhibiting writhing behaviour. No side effects were seen in the vehicle treated animals so these behaviours are likely due to on/off-target effects of the administered drugs (Figure 6 B). Thus, at the equivalent dose, **33** was able to offer significantly greater analgesia, with milder side effects, than the GlyT2 inhibitor **2**.

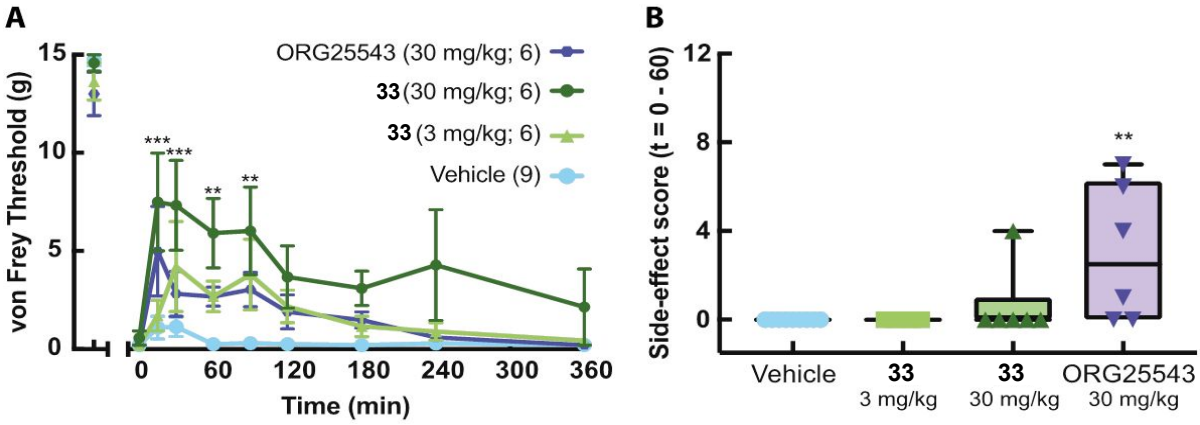


Figure 6. ***In vivo*** **33 reverses allodynia with fewer side effects than 2 in a neuropathic pain model in male Sprague Dawley rats. A.** Significant reversal of allodynia was observed after ip administration of **33** (30 mg/kg) compared to vehicle. Non-significant reversal of allodynia was observed for **33** at 3 mg/kg and **2** at 30 mg/kg. **B.** Side effects for **33** were less severe than for **2** at equal doses. Side effects were scored as described in the methods.

Reversibility of 2 and 33

The toxicity produced by **2** has previously been suggested to be due to irreversible binding at GlyT2.[14] As **33** displayed considerably less side effects, we measured the relative reversibility of **2** and **33** in an electrophysiology washout assay (Figure 5S). The reversibility of inhibition was investigated by first applying an IC_{50} concentration of inhibitor and once a stable level of inhibition was observed, washing the recording chamber with buffer. Glycine was then reapplied at 5 minute intervals throughout the wash time course to determine transport recovery, which is indicative of the reversibility of an inhibitor. For **2**, we observed 35% recovery of transport currents after 30 minutes which suggests **2** is a slowly reversible, but not irreversible, inhibitor. Conversely, transport following inhibition by **33** was completely restored after 25 minutes.

Conclusions

By conjugating a broad range of amino acids to the oleoyl tail, a large pool of inhibitors of GlyT2 have been identified, most of which have similar or improved potency compared with **4** and other acyl-glycine compounds. It has been determined that acyl-amino acids are active with the order of

potency: positively charged > aromatic >> aliphatic > negatively charged, and that L- isomers are more active than D-isomers in most cases. While the most potent compound identified, **32** (IC_{50} 25.5 nM), is ~353-*fold* more potent than **4**, it is limited by its metabolic instability. The D-enantiomer **33**, however, is also a potent GlyT2 inhibitor (IC50 48.3 nM), is metabolically stable, and is rapidly absorbed into the CNS following ip administration. It has also been demonstrated that ip injection of **33** reduces allodynia in the chronic nerve constriction model of neuropathic pain at concentrations that have minimal overt side effects. In contrast, the very slowly reversible GlyT2 inhibitor, **2**, was less effective as an analgesic and caused more substantial side effects. At this stage it is not possible to establish whether short-term analgesic effects of **33** without overt side effects is a consequence of partial occupancy of the transporter or full occupancy with partial inhibition. However, with high protein binding it is unlikely that free concentrations of the lipids *in vivo* are likely to saturate the binding site on GlyT2 and so this question may become mute. Further investigations into the availability of free lipids over a longer time frame and their potential for toxic side effects will be needed to assess the efficacy of this class of compounds for long term treatment of chronic pain. These novel bioactive lipids provide a promising lead in the development of a new class of analgesics.

Experimental

1. General Chemistry. Ester protected valine, alanine and phenylalanine in L- and D- configurations were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). All other protected amino acids were purchased from Fluorochem (Derbyshire, United Kingdom). General reagents and anhydrous solvents were purchased from Sigma Aldrich and Astatech (Bristol, USA). Reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F_{254} plates. TLC plates were visualised with UV light and potassium permanganate TLC stain. Reaction products were purified by dry column vacuum chromatography on silica gel using gradient elutions. ^{1}H and ^{13}C NMR spectra were recorded on an Agilent 500 MHz NMR. Spectra were referenced internally to residual solvent ($CDCl_3$; ^{1}H δ 7.26, ^{13}C δ 77.10. DMSO-d_6; ^{1}H δ 2.49, ^{13}C δ 39.52). High resolution mass spectra (HRMS) were recorded on an Agilent Technologies 6510 Q-TOF LCMS. The purity of all test compounds was confirmed to be >95% by absolute quantitative NMR (see Supporting Information for further details).

2. Synthesis

General procedure A for EDCI coupling. To a solution of oleic acid (2.0 mmol) in anhydrous DMF (10 mL) was added hydroxybenzotriazole hydrate (2.40 mmol), and EDCI (2.80 mmol). The mixture was stirred at room temperature for 1 h, then the amino acid or amino acid derivative (1.0 mmol) and triethylamine (6.0 mmol) were added. The reaction mixture was stirred for 18 h, then diluted with water (50 mL). The crude product was extrated with ethyl acetate (3 x 25 mL) and purified on silica gel by stepwise gradient elution with chloroform/isopropanol (100:0 to 90:10).

General procedure B for ester hydrolysis. To a solution of the ester (0.50 mmol) in ethanol (30 mL) was added 1M NaOH (10 mL), and the resulting solution was stirred at 40°C for 3 h. The reaction volume was reduced ethanol was removed under reduced pressure, and the aqueous residue was adjusted to pH 2 with 0.5M HCl. The resulting suspension was filtered and the solid product washed with water (10 mL) and ethanol (5 mL) and dried in vacuo.

General procedure C for BOC removal. The BOC-protected intermediate was dissolved in dichloromethane (2 mL) and 2M HCl in diethyl ether (2 mL) was added. The solution was stirred at room temperature for 4 hours and then concentrated in vacuo. The resulting solid was triturated with diethyl ether and dried in vacuo.

(2S)-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid (6). General procedure B. White solid, yield = 51%. Mp = 48 – 50 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.00 (d, *J*=8.0Hz, 1H), 5.32-5.30 (m, 2H), 4.16-4.13 (m, 1H) 2.06 (t, *J*=8.0Hz, 2H), 1.98-1.95 (m, 4H), 1.45 (p, *J*=7.0Hz, 2H), 1.28-1.23 (m, 23H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.31, 171.94, 129.63, 129.62, 47.30, 34.98, 31.28, 29.12, 29.10, 28.84(2C), 28.69, 28.61, 28.59, 28.57, 26.61, 26.57, 25.18, 22.09, 17.21, 13.93. $[\alpha]^{23}_{D}$ -11.1 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{39}NO_3$, 354.3003; found, 354.3009.

Methyl (2S)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (6a). General procedure A. White solid, yield = 70%. Mp = 28 – 30 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.17 (d, *J*=7.0Hz, 1H), 5.32-5.30 (m, 2H), 4.24-4.21 (m, 1H) 3.59 (s, 3H), 2.07 (t, *J*=7.5Hz, 2H), 1.99-1.96 (m, 4H), 1.45 (*p*, *J*=7.5Hz,

2H), 1.30-1.23 (m, 23H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.23, 172.08, 129.61(2C), 51.70, 47.39, 34.86, 31.27, 29.11, 29.09, 28.82, 28.68, 28.66, 28.58, 28.55, 28.54, 26.60, 26.56, 25.13, 22.08, 16.94, 13.93. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{41}NO_3$, 368.3159; found, 368.3154.

(2R)-2-[[(Z)-octadec-9-enyl] amino]propanoic acid (7). General procedure B. White solid, yield = 51%. Mp = 44 – 46 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.02 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.18-4.15 (m, 1H) 2.06 (t, *J*=7.5Hz, 2H), 1.99-1.95 (m, 4H), 1.45 (p, *J*=7.5Hz, 2H), 1.28-1.23 (m, 23H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.28, 171.93, 129.61, 129.60, 47.30, 34.98, 31.28, 29.13, 29.11, 28.84(2C), 28.69, 28.61, 28.59, 28.57, 26.61, 26.56, 25.18, 22.09, 17.21, 13.93. $[\alpha]^{23}_D$ +14.3 (*c* = 2.2, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{39}NO_3$, 354.3003; found, 354.3003.

Methyl (2S)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (7a). General procedure A. White solid, yield = 70%. Mp = 26 – 28 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.11 (d, *J*=6.5Hz, 1H), 5.32-5.30 (m, 2H), 4.21-4.17 (m, 1H), 3.60 (s, 3H), 2.07 (t, *J*=7.5Hz, 2H), 1.99-1.96 (m, 4H), 1.45 (*p*, *J*=7.0Hz, 2H), 1.30-1.23 (m, 23H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.26, 172.10, 129.63(2C), 51.71, 47.40, 34.86, 31.27, 29.11, 29.10, 28.83, 28.68, 28.66, 28.58, 28.55(2C), 26.60, 26.56, 25.13, 22.08, 16.94, 13.93. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{41}NO_3$, 368.3159; found, 368.3154.

(2S)-3-methyl-2-[[(Z)-octadec-9-enoyl]amino]butanoic acid (8). General procedure B. White solid, yield = 33%. Mp = 58 – 60 °C. ^{1}H NMR (500 MHz DMSO-d_6): δ 7.85 (d, *J*=8.5Hz, 1H), 5.32-5.30 (m, 2H), 4.50-4.47 (m, 1H) 2.18-2.09 (m 2H), 2.04-1.95 (m, 5H), 1.48-1.44 (m, 2H), 1.28-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.25, 172.36, 129.59(2C), 57.07, 34.98, 31.28, 29.78, 30.66, 29.83, 29.10, 28.82(2C), 28.69(2C), 28.65, 28.59, 26.61, 26.56, 25.37, 22.08, 19.17, 18.02, 13.92. $[\alpha]^{23}_D$ -2.0 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{43}NO_3$, 382.3316; found, 382.3319.

Methyl (2S)-3-methyl-2-[[(Z)-octadec-9-enoyl]amino]butanoate (8a). General procedure A. Colourless liquid, yield = 53%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.03 (d, *J*=8.0Hz, 1H), 5.32-5.30 (m, 2H), 4.17-4.14 (m, 1H) 3.61 (s, 3H), 2.18-2.08 (m 2H), 2.02-1.95 (m, 5H), 1.48-1.44 (m, 2H) 1.28-1.23 (m, 20H), 0.87 (dt, *J*=7.0, 9.50 Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.56, 172.23, 129.59(2C), 57.25, 51.51, 34.82, 31.28, 29.78, 29.10, 28.83, 28.68(2C), 28.62(2C), 28.59, 28.57, 28.55, 26.60, 26.56, 25.69, 22.08, 18.97, 18.16, 13.93. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{45}NO_3$, 396.3472; found, 396.3469.

(2R)-3-methyl-2-[[(Z)-octadec-9-enoyl]amino]butanoic acid (9). General procedure B. White solid, yield = 56%. Mp = 61 – 63 ^{o}C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.85 (d, *J*=9.0Hz, 1H), 5.32-5.30 (m, 2H), 4.13-4.10 (m, 1H) 2.16-2.10 (m 2H), 2.00-1.95 (m, 5H), 1.48-1.44 (m, 2H) 1.28-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.96, 172.14, 129.38(2C), 56.80, 34.72, 31.28, 30.43, 29.53, 28.84, 28.83(2C), 28.56, 28.42, 28.38, 28.32, 28.31, 26.34, 26.30, 25.11, 21.83, 18.93, 17.79, 13.92. $[\alpha]^{23}_{D}$ +1.8 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{43}NO_3$, 382.3316; found, 382.3318.

Methyl (2R)-3-methyl-2-[[(Z)-octadec-9-enoyl]amino]butanoate (9a). General procedure A. Colourless liquid, yield = 65%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.03 (d, *J*=8.0Hz, 1H), 5.32-5.30 (m, 2H), 4.16-4.13 (m, 1H) 3.60 (s, 3H), 2.17-2.07 (m 2H), 2.01-1.95 (m, 5H), 1.47- 1.44 (m, 2H) 1.28-1.23 (m, 20H), 0.87 (dt, *J*=7.0, 9.50 Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.57, 172.25, 129.60(2C), 57.26, 51.51, 34.82, 31.28, 29.78, 29.10, 28.83, 28.68(2C), 28.62(2C), 28.59, 28.57, 28.55, 26.60, 26.56, 25.69, 22.09, 18.97, 18.16, 13.93. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{45}NO_3$, 396.3472; found, 396.3467.

(2S)-4-methyl-2-[[(Z)-octadec-9-enoyl]amino]pentanoic acid (10). General procedure B. White solid, yield = 60%. Mp = 66 – 68 ^{o}C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.98 (d, *J*=8.0Hz, 1H), 5.32-5.30 (m, 2H), 4.20-4.18 (m, 1H), 2.09-2.06 (m, 2H), 1.99-1.97 (m, 4H), 1.62-1.58 (m, 1H), 1.50-1.43 (m, 4H), 1.30-1.17 (m, 20H), 0.83 (t, *J*=7.0Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.32, 172.19, 129.62(2C), 50.00, 35.02, 31.26, 29.08, 28.81, 28.67, 28.62, 28.59(3C), 28.57, 28.49, 26.62,

26.57, 25.25, 24.32, 22.89, 22.08, 21.17, 13.94. $[\alpha]^{23}_{D}$ -10.9 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{45}NO_3$, 396.3472; found, 396.3472.

Methyl (2S)-4-methyl-2-[[(Z)-octadec-9-enoyl]amino]pentanoate (10a). General procedure A. Colourless liquid, yield = 86%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.80 (d, *J*=8.5Hz, 1H), 5.34-5.32 (m, 2H), 4.66-4.64 (m, 1H), 3.73 (s, 3H), 2.20 (t, *J*=7.5Hz, 2H), 2.02-1.99 (m, 4H), 1.66-1.62 (m, 5H), 1.53-1.50 (m, 1H), 1.30-1.26 (m, 20H), 0.95-0.93 (m, 6H), 0.87 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCL_3$): δ 173.12, 172.99, 130.13, 129.89, 52.19, 50.65, 41.95, 36.73, 32.04, 29.91, 29.84, 29.67, 29.46, 29.45, 29.37, 29.33, 29.28, 27.36, 27.31, 25.70, 25.03, 22.94, 22.82, 22.13,14.25. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{47}NO_3$, 410.3629; found, 410.3628.

(2R)-4-methyl-2-[[(Z)-octadec-9-enoyl]amino]pentanoic acid (11). General procedure B. White solid, yield = 70%. Mp = 64 – 66 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.98 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.21-4.17 (m, 1H), 2.10-2.06 (m, 2H), 1.98-1.96 (m, 4H), 1.63-1.58 (m, 1H), 1.52-1.42 (m, 4H), 1.27-1.20 (m, 20H), 0.84 (t, *J*=7.0Hz, 9H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 174.31, 172.18, 129.61(2C), 49.99, 35.01, 31.27, 29.09, 28.82, 28.67, 28.63, 28.60(3C), 28.58, 28.50, 26.62, 26.56, 25.25, 24.31, 22.87, 22.08, 21.15, 13.93. $[\alpha]^{23}_{D}$ +11.5 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{45}NO_3$, 396.3472; found, 396.3470.

Methyl (2R)-4-methyl-2-[[(Z)-octadec-9-enoyl]amino]pentanoate (11a). General procedure A. Colourless liquid, yield = 80%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 5.79 (d, *J*=8.5Hz, 1H), 5.35-5.32 (m, 2H), 4.66-4.64 (m, 1H), 3.73 (s, 3H), 2.20 (t, *J*=7.5Hz, 2H), 2.03-1.99 (m, 4H), 1.66-1.62 (m, 5H), 1.53-1.50 (m, 1H), 1.30-1.26(m, 20H), 0.95-0.93(m, 6H), 0.87 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.12, 172.99, 130.13, 129.89, 52.19, 50.65, 41.95, 36.73, 32.04, 29.91, 29.84, 29.67, 29.46, 29.45, 29.37, 29.33, 29.28, 27.36, 27.31, 25.70, 25.03, 22.94, 22.82, 22.13,14.25. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{47}NO_3$, 410.3629; found, 410.3627.

(2S)-4-methylsulfanyl-2-[[(Z)-octadec-9-enoyl]amino]butanoic acid (12). General procedure B. White solid, yield = 59%. Mp = 28 – 30 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.04 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.29-4.26 (m, 1H), 2.47-2.40 (m, 2H), 2.11-2.07 (m, 2H), 2.02 (s, 3H), 1.99-1.97

(m, 4H), 1.93-1.90 (m, 1H), 1.83-1.80 (m, 1H), 1.46 (p, *J*=7.0Hz, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.48, 172.34, 129.60(2C), 50.75, 35.04, 31.28, 30.70, 29.74, 29.12, 29.10(2C), 28.84, 28.68, 28.67, 28.59, 28.56, 26.62, 26.57, 25.22, 22.09, 14.57, 13.92. $[\alpha]^{23}_{D}$ -7.4 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{43}NO_3$, 414.3036; found, 414.3038.

Methyl (2S)-4-methylsulfanyl-2-[[(Z)-octadec-9-enoyl] amino] butanoate (12a). General procedure A. Colourless liquid, yield = 89%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.14 (d, *J*=7.5Hz, 1H), 5.34-5.32 (m, 2H), 4.74-4.70 (m, 1H), 3.75 (s, 3H), 2.52-2.48 (m, 2H), 2.22 (t, *J*=7.5Hz, 2H). 2.19-2.14 (m, 1H), 2.08 (s, 3H), 1.99-1.94 (m, 5H), 1.63 (p, *J*=7.0Hz, 2H), 1.32-1.24 (m, 20H), 0.87 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.06, 172.77, 130.12, 129.86, 52.64, 51.53, 36.72, 32.02, 31.90, 30.11, 29.89, 29.83, 29.64, 29.44, 29.43, 29.36, 29.34, 29.26, 27.37, 27.30, 25.70, 22.80, 15.63, 14.24. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{45}O_3S$, 428.3193; found, 428.3198.

(2R)-4-methylsulfanyl-2-[[(Z)-octadec-9-enoyl]amino]butanoic acid (13). General procedure B. White solid, yield = 98%. Mp = 30 – 32 ^{o}C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.04 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.29-4.26 (m, 1H), 2.47-2.42 (m, 2H), 2.11-2.07 (m, 2H), 2.02 (s, 3H), 1.99-1.97 (m, 4H), 1.93-1.90 (m, 1H), 1.83-1.80 (m, 1H), 1.46 (p, *J*=7.0Hz, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.48, 172.34, 129.60(2C), 50.75, 35.04, 31.28, 30.70, 29.74, 29.12, 29.10(2C), 28.84, 28.68, 28.67, 28.59, 28.56, 26.62, 26.57, 25.22, 22.09, 14.57, 13.92. $[\alpha]^{23}_{D}$ +7.2 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{43}NO_3$, 414.3036; found, 414.3039.

Methyl (2R)-4-methylsulfanyl-2-[[(Z)-octadec-9-enoyl] amino] butanoate (13a). General procedure A. Colourless liquid, yield = 56%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.12 (d, *J*=7.5Hz, 1H), 5.34-5.32 (m, 2H), 4.74-4.70 (m, 1H), 3.75 (s, 3H), 2.52-2.48 (m, 2H), 2.22 (t, *J*=7.5Hz, 2H), 2.19-2.14 (m, 1H), 2.08 (s, 3H), 1.99-1.94 (m, 5H), 1.63 (p, *J*=7.0Hz, 2H), 1.32-1.24 (m, 20H), 0.87 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.06, 172.77, 130.12, 129.86, 52.64, 51.53, 36.72,

32.02, 31.90, 30.11, 29.89, 29.83, 29.64, 29.44, 29.43, 29.36, 29.34, 29.26, 27.37, 27.30, 25.70, 22.80, 15.63, 14.24. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{45}O_3S$, 428.3193; found, 428.3195.

(2S)-2-[[(Z)-octadec-9-enoyl]amino]hexanoic acid (14). General procedure B. White solid, yield = 87%. Mp = 50 – 52 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.97 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.14-4.01 (m, 1H), 2.11-2.09 (m, 2H), 1.98-1.95 (m, 4H), 1.61-1.53 (m, 2H), 1.47-1.43 (m, 2H), 1.31-1.23 (m, 24H), 0.84 (t, *J*=7.0Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.92, 172.21, 129.62, 129.61, 51.55, 34.99, 31.27, 30.72, 29.10, 29.09, 28.82, 28.67, 28.66, 28.59, 28.58, 28.53, 27.59, 26.61, 26.57, 25.27, 22.08, 21.69, 13.93, 13.78. $[\alpha]^{23}_D$ -4.5 (*c* = 1.9, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{46}NO_3$, 396.3472; found, 396.3470.

Methyl (2S)-2-[[(Z)-octadec-9-enoyl]amino]hexanoate (14a). General procedure A. Colourless liquid, yield = 72%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.12 (d, *J*=7.5Hz, 1H), 5.31-5.30 (m, 2H), 4.20-4.17 (m, 1H), 3.59 (s, 3H), 2.12-2.06 (m, 2H), 1.99-1.97 (m, 4H), 1.68-1.62 (m, 1H), 1.60-1.54 (m, 1H), 1.46 (p, *J*=6.5Hz, 2H), 1.29-1.22 (m, 24H), 0.84 (t, *J*=7.0Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.87, 172.35, 129.59, 129.58, 51.65, 51.62, 34.88, 31.28, 30.56, 29.10, 28.83, 28.68, 28.63(3C), 28.59, 28.49, 27.51, 26.61, 26.56, 25.21, 22.08, 21.64, 13.90, 13.71. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{47}NO_3$, 410.3629; found, 410.3622.

(2S)-3-hydroxy-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid (15). General procedure B. White solid, yield = 98%. Mp = 96 – 98 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.87 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.23-4.21 (m, 1H), 3.65 (dd, *J*=5.5, 11.0Hz, 1H), 3.56 (dd, *J*=4.5, 11.0Hz, 1H), 2.10 (t, *J*=7.5Hz, 2H), 1.98-1.97 (m, 4H), 1.46 (p, *J*=7.0Hz, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.37, 171.38, 129.63, 129.59, 40.82, 35.09, 31.36, 29.08, 29.00(2C), 28.94, 28.85, 28.81, 28.62, 28.57, 26.55, 26.30, 25.19, 21.70, 13.81. $[\alpha]^{23}_D$ + 5.2 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{39}NO_4$, 370.2952; found, 370.2953.

Methyl (2S)-3-hydroxy-2-[[(Z)-octadec-9-enoyl]amino]propanoate (15a). General procedure A. Colourless liquid, yield = 74%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.40 (d, *J*=7.5Hz, 1H), 5.35-5.32 (m, 2H), 4.70-4.67 (m, 1H), 3.99-3.91 (m, 2H), 3.79 (s, 3H), 2.46 (t, *J*=6.0Hz, 3H). 2.26 (t, *J*=7.5Hz, 2H),

2.04-1.99 (m, 4H), 1.65 (p, *J*=7.0Hz, 2H), 1.34-1.24 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.86, 171.15, 130.13, 129.86, 63.83, 54.81, 52.92, 36.64, 32.03, 29.90, 29.84, 29.66, 29.46, 29.45, 29.37, 29.33, 29.26, 27.35, 27.31, 25.67 22.81, 14.24. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{41}NO_4$, 384.3108, found, 384.3108.

(2R)-3-hydroxy-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid (16). General procedure B. White solid, yield = 90%. Mp = 94 – 96 °C. ^{1}H NMR (500 MHz DMSO-d_6): δ 7.88 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.24-4.22 (m, 1H), 3.65 (dd, *J*=5.5, 11.0Hz, 1H), 3.57 (dd, *J*=4.5, 11.0Hz, 1H), 2.11 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 4H), 1.46 (p, *J*=7.0Hz, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.27, 172.16, 129.65, 129.63, 61.46, 54.52, 35.03, 31.27, 29.13, 29.10, 28.83, 28.72, 28.69, 28.64, 28.59, 28.57, 26.62, 26.57, 25.21, 22.09, 13.95. $[\alpha]^{23}_D$ -4.8 (*c* = 1.9, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{39}NO_4$, 370.2952; found, 370.2955.

Methyl (2R)-3-hydroxy-2-[[(Z)-octadec-9-enoyl]amino]propanoate (16a). General procedure A. Colourless liquid, yield = 67%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.36 (d, *J*=7.5Hz, 1H), 5.35-5.32 (m, 2H), 4.70-4.67 (m, 1H), 3.99-3.91 (m, 2H), 3.79 (s, 3H), 2.46 (t, *J*=6.0Hz, 3H). 2.26 (t, *J*=7.5Hz, 2H), 2.03-1.99 (m, 4H), 1.65 (p, *J*=7.0Hz, 2H), 1.31-1.27 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.86, 171.15, 130.17, 129.89, 63.96, 54.85, 52.96, 36.66, 32.05, 29.92, 29.86, 29.67, 29.48, 29.47, 29.38, 29.34, 29.27, 27.37, 27.32, 25.68, 22.83, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{41}NO_4$, 384.3108; found, 384.3106.

(2S)-2-[[(Z)-octadec-9-enoyl]amino]butanedioic acid (17). General procedure B. White solid, yield = 75%. Mp = 73 – 75 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.05 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.49-4.47 (m, 1H), 2.65 (dd, *J*=5.5, 16.5Hz, 1H), 2.53 (dd, *J*=5.5, 16.5Hz, 1H), 2.05 (t, *J*=7.5Hz, 2H), 1.99-1.96 (m, 4H), 1.46-1.43 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.56, 172.03, 171.67, 129.65, 129.62, 48.48, 35.02, 31.26, 29.12, 29.09, 28.82(2C), 28.68, 28.67, 28.57, 28.55, 28.53, 26.61, 26.56, 25.19, 22.08, 13.94. $[\alpha]^{23}_D$ -1.6 (*c* = 2.2, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{39}NO_5$, 398.2901, found, 398.2909.

Dimethyl (2S)-2-[[(Z)-octadec-9-enoyl]amino]butanedioate (17a). General procedure A. White solid, yield = 76%. Mp = 48 – 50 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.44 (d, *J*=8.5Hz, 1H), 5.35-5.33 (m, 2H), 4.88-4.86 (m, 1H), 3.76 (s, 3H), 3.69 (s, 3H), 3.04 (dd, *J*=4.0, 17.0Hz, 1H), 2.86 (dd, *J*=4.0, 17.0Hz, 1H), 2.22 (t, *J*=7.0Hz, 2H), 2.02-1.99 (m, 4H), 1.65 (p, *J*=7.0Hz, 2H), 1.33-1.26 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.04, 171.83, 171.45, 130.14, 129.89, 52.95, 52.16, 48.45, 36.66, 36.27, 32.05, 29.91, 29.85, 29.67(2C), 29.47, 29.46, 29.39, 29.30, 27.37, 27.33, 25.66, 22.83, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{43}NO_5$, 426.3214; found, 426.3209.

(2R)-2-[[(Z)-octadec-9-enoyl]amino]butanedioic acid (18). General procedure B. White solid, yield = 70%. Mp = 75 – 77 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.07 (d, *J*=8.0Hz, 1H), 5.32-5.30 (m, 2H), 4.50-4.47 (m, 1H), 2.64 (dd, *J*=5.5, 16.0Hz, 1H), 2.52 (dd, *J*=5.5, 16.0Hz, 1H), 2.05 (t, *J*=7.5Hz, 2H), 1.99-1.96 (m, 4H), 1.45-1.43 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.64, 172.22, 171.77, 129.72, 129.69, 48.58, 36.15, 35.11, 31.36, 29.22, 29.18, 28.92, 28.78, 28.77, 28.67, 28.65, 28.62, 26.70, 26.65, 25.28, 22.17, 14.01. $[\alpha]^{23}_D$ +1.6 (*c* = 2.4, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{39}NO_5$, 398.2901, found, 398.2901.

Dimethyl (2R)-2-[[(Z)-octadec-9-enoyl]amino]butanedioate (18a). General procedure A. White solid, yield = 73%. Mp = 48 – 50 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.44 (d, *J*=8.5Hz, 1H), 5.35-5.33 (m, 2H), 4.88-4.86 (m, 1H), 3.76 (s, 3H), 3.69 (s, 3H), 3.04 (dd, *J*= 4.5, 17.0Hz, 1H), 2.85 (dd, *J*=4.5, 17.0Hz, 1H), 2.22 (t, *J*=7.5Hz, 2H), 2.02-1.99 (m, 4H), 1.63 (p, *J*=7.0Hz, 2H), 1.34-1.26 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.03, 171.82, 171.45, 130.15, 129.89, 52.94, 52.16, 48.45, 36.66, 36.27, 32.05, 29.91, 29.85, 29.67(2C), 29.47, 29.46, 29.39, 29.30, 27.37, 27.33, 25.66, 22.83, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{43}NO_5$, 426.3214; found, 426.3219

(2S)-2-[[(Z)-octadec-9-enoyl] amino]pentanedioic acid (19). General procedure B. White solid, yield = 62%. Mp = 82 – 84 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.00 (d, *J*=7.5Hz, 1H), 5.32-5.30 (m, 2H), 4.21-4.16 (m, 1H), 2.27-2.23 (m, 2H), 2.09-2.07 (m, 2H), 1.98-1.95 (m, 5H), 1.78-1.72 (m, 1H), 1.46-1.43 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.75, 173.50, 172.40, 129.64, 129.60, 51.06, 35.09, 31.37, 30.13, 29.23, 29.20(2C), 28.94, 28.79,

28.69(3C), 26.70, 26.65, 26.41, 25.30, 22.18, 13.95. $[\alpha]^{23}_{D}$ -3.5 (*c* = 2.3, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{41}NO_5$, 412.3058; found, 412.3061.

Dimethyl (2S)-2-[[(Z)-octadec-9-enoyl]amino]pentanedioate (19a). General procedure A. White solid, yield = 79%. Mp = 28 – 30 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.12 (d, *J*=7.5Hz, 1H), 5.35-5.33 (m, 2H), 4.66-4.61 (m, 1H), 3.75 (s, 3H), 3.68 (s, 3H), 2.45-2.33 (m, 2H), 2.23-2.19 (m, 3H), 2.03-1.98 (m, 5H), 1.63 (p, *J*=7.0Hz, 2H), 1.30-1.26 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO d_6): δ 173.49, 173.20, 172.65, 130.15, 129.89, 52.67, 51.99, 51.64, 36.70, 30.05, 30.21, 29.91, 29.86, 29.67, 29.47, 29.46, 29.58, 29.36, 29.28, 27.54, 27.37 27.33, 25.68, 22.83, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{45}NO_5$, 440.3371, found, 440.3372.

(2R)-2-[[(Z)-octadec-9-enoyl]amino]pentanedioic acid (20). General procedure B. White solid, yield = 69%. Mp = 82 – 84 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.00 (d, *J*=8.0Hz, 1H), 5.32-5.30 (m, 2H), 4.20-4.15 (m, 1H), 2.26-2.23 (m, 2H), 2.09-2.07 (m, 2H), 1.98-1.95 (m, 5H), 1.78-1.72 (m, 1H), 1.47-1.45 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.71, 173.46, 172.33, 129.63(2C), 50.99, 35.02, 31.27, 30.11, 29.12 (2C), 29.09, 28.82, 28.67(3C), 28.58, 26.61, 26.56, 26.34, 25.22, 22.08, 13.94. $[\alpha]^{23}_{D}$ +3.4 (*c* = 2.3, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{41}NO_5$, 412.3058; found, 412.3060.

Dimethyl (2R)-2-[[(Z)-octadec-9-enoyl]amino]pentanedioate (20a). General procedure A. White solid, yield = 77%. Mp = 29 – 31 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.20 (d, *J*=7.5Hz, 1H), 5.33-5.31 (m, 2H), 4.63-4.59 (m, 1H), 3.73 (s, 3H), 3.66 (s, 3H), 2.43-2.31 (m, 2H), 2.22-2.17 (m, 3H), 2.01-1.95 (m, 5H), 1.61 (p, *J*=7.0Hz, 2H), 1.30-1.25 (m, 20H), 0.86 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.43, 173.19, 172.61, 130.08, 129.84, 52.59, 51.92, 51.59, 36.61, 31.99, 30.17, 29.86, 29.81, 29.62, 29.42, 29.41, 29.34, 29.32, 29.23, 27.46, 27.31, 27.28, 25.64, 22.78, 14.21. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{45}NO_5$, 440.3371; found, 440.3367

(2S)-4-amino-2-[[(Z)-octadec-9-enoyl]amino]-4-oxo-butanoic acid (21). General procedure B. White solid, yield = 95%. Mp = 115 – 117 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.95 (d, *J*=8.0Hz, 1H), 7.31 (s, 1H), 6.86 (s, 1H), 5.32-5.30 (m, 2H), 4.47-4.45 (m, 1H), 2.54-2.38 (m, 2H), 2.06 (t,

J=8.0Hz, 2H), 2.00-1.94 (m, 4H), 1.46 (p, *J*=7.0Hz, 2H), 1.32-1.18 (m, 20H), 0.84 (d, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.97, 171.95, 171.21, 129.63, 129.60, 48.67, 36.78, 35.06, 31.26, 29.13(2C), 29.08, 28.81, 28.70, 28.66(2C), 28.57, 26.61, 26.56, 25.21, 22.07, 13.94. $[\alpha]^{23}_{D}$ -2.4 (*c* = 1.9, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{40}N_2O_4$, 397.3061; found, 397.3065

Methyl (2S)-4-amino-2-[[(Z)-octadec-9-enoyl]amino]-4-oxo-butanoate (21a). General procedure A. White solid, yield = 63%. Mp = 90 – 92 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.10 (d, *J*=8.0Hz, 1H), 7.35 (s, 1H), 6.88 (s, 1H), 5.34-5.28 (m, 2H), 4.55-4.50 (m, 1H), 3.57 (s, 3H), 2.55-2.40 (m, 2H), 2.06 (t, *J*=7.5Hz, 2H), 2.00-1.94 (m, 4H), 1.45 (p, *J*=7.0Hz, 2H), 1.32-1.18 (m, 20H), 0.84 (d, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.09, 171.99, 170.87, 129.62(2C), 51.76, 48.69, 36.71, 34.94, 31.27, 29.13, 29.09(2C), 28.82, 28.67(2C), 28.58, 28.51, 26.61, 26.56, 25.18, 22.08, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{42}N_2O_3$, 411.3217; found, 411.3214.

(2S)-2-[[(Z)-octadec-9-enoyl]amino]-3-phenyl-propanoic acid (22). General procedure B. White solid, yield = 92%. Mp = 87 – 89 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.77 (d, *J*=8.0Hz, 1H), 7.20-7.12 (m,5H), 5.32-5.30 (m, 2H), 4.27-4.25 (m, 1H), 3.05 (dd, *J*=5.5, 14.0Hz, 1H), 2.82 (dd, *J*=6.0, 14.0Hz, 1H), 1.98-1.95 (m, 6H), 1.36 (p, *J*=7.0Hz, 2H), 1.30-1.17 (m, 18H), 1.11-2.09 (m, 2H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.62, 171.46, 138.69, 129.65, 129.60, 129.27, 127.76, 125.83, 54.34, 37.24, 35.41, 31.29, 29.16, 29.12, 28.85(2C), 28.75, 28.70(2C), 28.61, 28.58, 28.55, 26.65, 26.59, 25.30, 22.10, 13.94. $[\alpha]^{23}_{D}$ +17.8 (*c* = 2.2, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{27}H_{43}NO_3$, 430.3315; found, 430.3317.

Methyl (2R)-2-[[(Z)-octadec-9-enoyl]amino]-3-phenyl-propanoate (22a). General procedure A. Colourless liquid, yield = 58%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.32-7.26 (m, 3H), 7.11 (d, *J*=7.0Hz, 2H), 5.88 (d, *J*=7.5Hz, 1H), 5.37-5.35 (m, 2H), 4.94-4.91 (m, 1H), 3.75 (s, 3H), 3.18 (dd, *J*=6.0, 14.0Hz, 1H) 3.11 (dd, *J*=5.5, 14.0Hz, 1H), 2.20 (t, *J*=7.5Hz, 2H), 2.04-2.00 (m, 4H), 1.60 (p, *J*=7.0Hz, 2H), 1.30-1.17 (m, 20H), 0.89 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$). δ 172.76, 172.29, 135.97, 130.13, 129.88, 129.38, 128.68, 127.24, 52.98, 52.40, 38.05, 36.68, 31.99, 29.90(2C),

29.84, 29.65, 29.46, 29.44(2C), 29.37, 29.31, 29.27, 27.32, 27.28, 25.63, 22.78, 14.21. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{45}NO_3$, 444.3472; found, 444.3472.

(2R)-2-[[(Z)-octadec-9-enoyl]amino]-3-phenyl-propanoic acid (23). General procedure B. White solid, yield = 82%. Mp = 88 – 90 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.77 (d, *J*=8.0Hz, 1H), 7.20-7.12 (m,5H), 5.32-5.30 (m, 2H), 4.27-4.25 (m, 1H), 3.05 (dd, *J*=5.5, 14.0Hz, 1H), 2.85 (dd, *J*=6.0, 14.0Hz, 1H), 1.99-1.95 (m, 6H), 1.36 (p, *J*=7.0Hz, 2H), 1.30-1.17 (m, 18H), 1.14-1.08 (m, 2H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.62, 171.46, 138.69, 129.65, 129.60, 129.27, 127.76, 125.83, 54.34, 37.24, 35.41, 31.29, 29.16, 29.12, 28.85(2C), 28.75, 28.70(2C), 28.61, 28.58, 28.55, 26.65, 26.59, 25.30, 22.10, 13.94. $[\alpha]^{23}_D$ -17.8 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{27}H_{43}NO_3$, 430.3315; found, 430.3319.

Methyl (2R)-2-[[(Z)-octadec-9-enoyl]amino]-3-phenyl-propanoate (23a). General procedure A. Colourless liquid, yield = 90%. Mp = XX – YY °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.32-7.26 (m, 3H), 7.11 (d, *J*=7.0Hz, 2H), 5.87 (d, *J*=7.5Hz, 1H), 5.37–5.35 (m, 2H), 4.94-4.91 (m, 1H), 3.75 (s, 3H), 3.18 (dd, *J*=6.0, 14.0Hz, 1H) 3.10 (dd, *J*=5.5, 14.0Hz, 1H), 2.20 (t, *J*=7.5Hz, 2H), 2.03-2.00 (m, 4H), 1.60 (p, *J*=7.0Hz, 2H), 1.30-1.17 (m, 20H), 0.89 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$). δ 172.76, 172.32, 136.02, 130.13, 129.88, 129.38, 128.68, 127.24, 53.02, 52.43, 38.05, 36.68, 32.03, 29.90(2C), 29.84, 29.65, 29.46, 29.44(2C), 29.37, 29.31, 29.25, 27.36, 27.31, 25.66, 22.81, 14.24. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{45}NO_3$, 444.3472; found, 444.3470.

(2S)-2-[[(Z)-octadec-9-enoyl]amino]-4-phenyl-butanoic acid (24). General procedure B. White solid, yield = 70%. Mp = 65 – 66 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.10 (d, *J*=8.0Hz, 1H), 7.28-7.24 (m, 2H), 7.18-7.14 (m, 3H), 5.33-5.26 (m, 2H), 4.16-4.10 (m, 1H), 2.66-2.52 (m, 2H), 2.20-2.08 (m, 2H), 1.98-1.80 (m, 6H), 1.49 (d, *J*=7.0Hz, 2H), 1.32-1.18 (m, 20H), 0.84 (d, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.74, 172.32, 170.28, 141.04, 129.59, 129.58, 129.28, 125.87, 59.73, 51.20, 35.06, 32.86, 32.50, 31.27, 29.12, 29.09, 28.83, 28.68, 28.61(2C), 28.58, 26.62, 26.56, 25.28, 22.09, 20.74, 14.07, 13.92. $[\alpha]^{23}_D$ + 3.5 (*c* = 2.6, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{45}NO_3$, 443.3472; found, 443.3469.

Ethyl (2S)-2-[[(Z)-octadec-9-enoyl]amino]-4-phenyl-butanoate (24a). General procedure A. Colourless liquid, yield = 75%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.24-8.21 (m, 1H), 7.28-7.24 (m, 2H), 7.18-7.16 (m, 3H), 5.31-5.29 (m, 2H), 4.16-4.13 (m, 1H), 4.06-4.03 (m, 2H), 2.66-2.52 (m, 2H), 2.14-2.12 (m, 2H), 2.00-1.80 (m, 6H), 1.49 (d, *J*=7.0Hz, 2H), 1.30-1.20 (m, 20H), 1.15 (t, *J*=7.0Hz, 3H), 0.83 (d, *J*=7.0Hz, 3H). ^{13}C NMR (500 MHz, DMSO d_6): δ 172.42, 172.14, 140.87, 129.57, 129.55, 128.28, 125.91, 60.29, 51.37, 34.95, 32.71, 31.37, 31.27, 29.10, 29.09, 28.81, 28.67(4C), 28.58(3C), 28.54, 26.60, 26.55, 25.23, 22.08, 14.02, 13.90. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{49}NO_3$, 472.3785; found, 472.3779.

(2R)-2-[[(Z)-octadec-9-enoyl]amino]-4-phenyl-butanoic acid (25). General procedure B. White solid, yield = 73%. Mp = 68 – 70 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.09 (d, *J*=8.0Hz, 1H), 7.28-7.24 (m, 2H), 7.18-7.14 (m, 3H), 5.31-5.29 (m, 2H), 4.12-4.10 (m, 1H), 2.62-2.55 (m, 2H), 2.15-2.10 (m, 2H), 1.97-1.84 (m, 6H), 1.49 (d, *J*=7.0Hz, 2H), 1.28-1.22 (m, 20H), 0.84 (d, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.74, 172.31, 141.09, 129.62, 129.61, 128.30, 125.89, 51.15, 35.02, 32.88, 31.49, 31.26, 29.10, 29.07, 28.81, 28.66(4C), 28.59(3C), 28.57, 26.60, 26.55, 25.27, 22.08, 13.94. $[\alpha]^{23}_{D}$ -3.3 (*c* = 2.4, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{45}NO_3$, 443.3472; found, 443.3475.

Ethyl (2R)-2-[[(Z)-octadec-9-enoyl]amino]-4-phenyl-butanoate (25a). General procedure A. Colourless liquid, yield = 73%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.22 (d, *J*=7.5Hz, 1H), 7.28-7.24 (m, 2H), 7.18-7.15 (m, 3H), 5.31-5.28 (m, 2H), 4.16-4.15 (m, 1H), 4.06-4.03 (m, 2H), 2.63-2.58 (m, 2H), 2.12 (dp, *J*=7.0, 4.0Hz, 2H), 1.98-1.51 (m, 6H), 1.49 (d, *J*=7.0Hz, 2H), 1.28-1.18 (m, 20H), 1.15 (t, *J*=7.0Hz, 3H), 0.83 (d, *J*=7.0Hz, 3H).^{13}C NMR (125 MHz, DMSO-d_6): δ 172.42, 172.14, 140.87, 129.57, 129.55, 128.28, 125.91, 60.29, 51.37, 34.95, 32.71, 31.37, 31.27, 29.10, 29.09, 28.81, 28.67(4C), 28.58(3C), 28.54, 26.60, 26.55, 25.23, 22.08, 14.02, 13.90. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{49}NO_3$, 472.3785; found, 472.3780.

(2S)-3-(1H-imidazol-5-yl)-2-[[(Z)-octadec-9-enoyl] amino]propanoic acid.2HCl (26). General procedure B. White solid, yield = 95%. Mp = 66 – 68 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.80 (s,

1H), 8.28 (d, *J*=8.0Hz, 1H), 7.27 (s, 1H), 5.32-5.30 (m, 2H), 4.50-4.47 (m, 1H), 3.12-3.08 (m, 2H), 2.98-2.92 (m, 1H), 2.05 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 4H), 1.42-1.40 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.36, 172.32, 133.73, 129.67, 129.63, 116.74, 51.26, 36.72, 31.28, 29.16, 29.02, 28.84(2C), 28.68(2C), 28.59, 28.56, 28.51, 26.63, 26.58, 26.45, 25.14, 22.10, 13.97. $[\alpha]^{23}_{D}$ -5.6 (*c* = 1.7, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{41}N_3O_3$, 420.3221; found, 420.3224.

Methyl (2S)-3-(1H-imidazol-5-yl)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (26a). General procedure A. Colourless liquid, yield = 60%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.56 (s, 1H), 7.10 (s, 1H), 6.80 (s, 1H), 5.35-5.33 (m, 2H), 4.83-4.80 (m, 1H), 3.69 (s, 3H), 3.15-3.08 (m, 2H), 2.23 (t, *J*=7.5Hz, 2H), 2.01-1.98 (m, 4H), 1.64-1.60 (m, 2H), 1.30-1.26 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.44, 172.30, 134.60, 130.12, 129.91, 52.47, 52.36, 36.72, 32.04, 29.91, 29.89, 29.67, 29.49, 29.47(2C), 29.46, 29.41, 29.32, 29.31(2C), 27.37, 27.34, 25.69, 22.83, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{43}N_3O_3$, 434.3377; found, 434.3375.

(2R)-3-(1H-imidazol-5-yl)-2-[[(Z)-octadec-9-enoyl] amino]propanoic acid.2HCl (27). General procedure B. White solid, yield = 60%. Mp = 68 – 70 ^{o}C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.86 (s, 1H), 8.22 (d, *J*=8.0Hz, 1H), 7.30 (s, 1H), 5.32-5.30 (m, 2H), 4.50-4.47 (m, 1H), 3.12-3.08 (m, 2H), 2.98-2.92 (m, 1H), 2.05 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 4H), 1.42-1.40 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.36, 172.32, 133.73, 129.67, 129.63, 116.74, 51.26, 36.72, 31.28, 29.16, 29.02, 28.84, 28.68(2C), 28.59, 28.56, 28.51, 28.47, 26.63, 26.58, 26.45, 25.14, 22.10, 13.97. $[\alpha]^{23}_{D}$ +5.8 (*c* = 2.2, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{41}N_3O_3$, 420.3221; found, 420.3220.

Methyl (2R)-3-(1H-imidazol-5-yl)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (27a). General procedure A. Colourless liquid, yield = 55%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.75 (s, 1H), 7.42 (s, 1H), 7.16 (s, 1H), 5.34-5.33 (m, 2H), 4.83-4.80 (m, 1H), 3.76 (s, 3H), 3.40-3.38 (m, 1H), 3.25-3.21 (m, 1H), 2.30 (t, *J*=7.5Hz, 2H), 2.01-1.98 (m, 4H), 1.64-1.60 (m, 2H), 1.30-1.26 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.44, 172.30, 134.60, 130.12, 129.91, 52.47, 52.36,

36.72, 32.04, 29.91, 29.89, 29.67, 29.49, 29.47(2C), 29.46, 29.41, 29.32, 29.31(2C), 27.37, 27.34, 25.69, 22.83, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{43}N_3O_3$, 434.3377; found, 434.3373.

(2S)-3-(1H-indol-3-yl)-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid.HCl (28). General procedure B. White solid, yield = 89%. Mp = 76 – 78 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.80 (s, 1H), 7.90 (d, *J*=8.0Hz, 1H), 7.51 (d, *J*=7.5Hz, 1H), 7.30 (d, *J*=8.0Hz, 1H), 7.10 (d, *J*=2.0Hz, 1H), 7.03 (t, *J*=7.0Hz, 1H), 6.95 (t, *J*=7.0Hz, 1H), 5.32-5.30 (m, 2H), 4.45-4.44 (m, 1H), 3.15 (dd, *J*=5.5, 14.0Hz, 1H), 2.96 (dd, *J*=8.5, 14.0Hz, 1H), 2.06-2.04 (m, 2H), 1.98-1.96 (m, 4H), 1.39-1.37 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.58, 172.10, 136.06, 129.66, 129.60, 127.23, 123.45, 120.82, 118.25, 118.17, 110.10, 110.06, 52.86, 35.08, 31.26, 29.12, 29.09, 28.82, 28.67, 28.58, 28.54, 28.52, 27.13(2C), 26.62, 26.57, 25.27, 22.08, 13.94. $[\alpha]^{23}_D$ -5.2 (*c* = 1.5, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{29}H_{44}N_2O_3$, 469.3425; found, 469.3429.

Methyl (2S)-3-(1H-indol-3-yl)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (28a). General procedure A. Waxy solid, yield = 84%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.14 (s, 1H), 7.53 (d, *J*=8.0Hz, 1H), 7.35 (d, *J*=8.0Hz, 1H), 7.20 (t, *J*=7.5Hz, 1H), 7.12 (t, *J*=7.5Hz, 1H), 6.98 (d, *J*=7.5Hz, 1H), 5.95 (d, *J*=7.5Hz, 1H), 5.35-5.33 (m, 2H), 4.99-4.95 (m, 1H), 3.70 (s, 3H), 3.36-3.28 (m, 4H), 2.14 (t, *J*=7.5Hz, 2H), 2.02-1.98 (m, 4H), 1.59-1.56 (m, 2H), 1.32-1.25 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.89, 172.67, 136.23, 130.14, 129.93, 127.89, 122.75, 122.42, 119.85, 118.76, 111.38, 110.37, 52.99, 52.46, 36.76, 32.04, 29.92, 29.86, 29.66, 29.47, 29.46, 29.38, 29.34, 29.27, 27.80, 27.36, 27.33, 25.60, 22.82, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{46}N_2O_3$, 483.3581; found, 483.3578.

(2R)-3-(1H-indol-3-yl)-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid.HCl (29). General procedure B. White solid, yield = 60%. Mp = 77 – 79 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.80 (s, 1H), 7.99 (d, *J*=8.0Hz, 1H), 7.51 (d, *J*=7.50Hz, 1H), 7.31 (d, *J*=8.0Hz, 1H), 7.11 (d, *J*=2.0Hz, 1H), 7.03 (t, *J*=7.0Hz, 1H), 6.95 (t, *J*=7.0Hz, 1H), 5.32-5.30 (m, 2H), 4.45-4.44 (m, 1H), 3.15 (dd, *J*=5.5, 14.0Hz, 1H), 2.96 (dd, *J*=8.5, 14.0Hz, 1H), 2.06-2.04 (m, 2H), 1.97-1.95 (m, 4H), 1.39-1.37 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.58, 172.10,

136.06, 129.66, 129.60, 127.23, 123.45, 120.82, 118.25, 118.17, 110.10, 110.06, 52.86, 35.08, 31.26, 29.12, 29.09, 28.82, 28.67, 28.58, 28.54, 28.52, 27.13(2C), 26.62, 26.57, 25.27, 22.08, 13.94. $[\alpha]^{23}_{D}$ +5.3 (*c* = 2.5, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{29}H_{44}N_2O_3$, 469.3425; found, 469.3420.

Methyl (2R)-3-(1H-indol-3-yl)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (29a). General procedure A. Waxy solid, yield = 92%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.84 (s, 1H), 8.18 (d, *J*=8.0Hz, 1H), 7.47 (d, *J*=8.0Hz, 1H), 7.31 (t, *J*=7.5Hz, 1H), 7.11 (t, *J*=7.5Hz, 1H), 7.03 (d, *J*=7.5Hz, 1H), 6.96 (d, *J*=7.5Hz, 1H), 5.35-5.33 (m, 2H), 4.49-4.47 (m, 1H), 3.70 (s, 3H), 3.12 (dd, *J*=6.0, 14.5Hz, 2H), 3.00 (dd, *J*=8.5, 14.5Hz, 2H), 2.13 (t, *J*=7.5Hz, 2H), 2.02-1.98 (m, 4H), 1.59-1.56 (m, 2H), 1.32-1.25 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.55, 172.24, 136.07, 129.63, 129.59, 127.06, 123.56, 120.89, 118.32, 117.97, 111.38, 109.57, 52.99, 51.69, 34.95, 31.27, 29.12, 29.10, 28.82, 28.67, 28.66, 28.59, 28.53, 28.50, 27.08, 26.61, 26.57, 25.10, 22.08, 14.26 HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{46}N_2O_3$, 483.3581; found, 483.3578.

(2S)-5-guanidino-2-[[(Z)-octadec-9-enoyl]amino]pentanoic acid.2HCl (30). General procedure B. White solid, yield = 68%. Mp = 182 – 184 °C. ^{1}H NMR (500 MHz, DMSO d_6): δ 8.05 (d, *J*=7.5Hz, 1H), 7.71 (t, *J*=5.5Hz, 1H), 7.42-7.13 (m, 3H), 5.32-5.30 (m, 2H), 4.18-4.13 (m, 1H), 3.01 (t, *J*=6.5Hz, 2H), 2.11-2.09 (m, 2H), 1.98-1.95 (m, 4H), 1.72-1.69 (m, 2H), 1.58-1.55 (m, 2H), 1.48-1.44 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (500 MHz, DMSO d_6): δ 175.98, 170.99, 157.32, 129.64, 129.60, 53.50, 35.03, 31.28, 29.77, 29.15, 29.10(2C), 28.83, 28.77, 28.74, 28.68, 28.61, 28.58, 26.63, 26.57, 25.42, 25.16, 22.09, 13.94. $[\alpha]^{23}_{D}$ +4.12 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{46}N_4O_3$, 439.3643; found,439.3644.

Methyl (2S)-5-guanidino-2-[[(Z)-octadec-9-enoyl]amino]pentanoate (30a). General procedure A. Waxy solid, yield = 54%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.64 (m, 1H), 7.38 (d, *J*=7.5Hz, 1H), 6.99-6.92 (m, 3H), 5.35-5.31 (m, 2H), 4.45-4.40 (m, 1H), 3.74 (s, 3H), 3.32-3.20 (m, 2H), 2.28 (t, *J*=7.5Hz, 2H), 2.00-1.96 (m, 4H), 1.90-1.76 (m, 2H), 1.68-1.58 (m, 4H), 1.31-1.23 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.81, 173.03, 157.55, 130.14, 129.85, 52.84, 51.83, 41.07,

36.41, 32.06, 29.91, 29.70, 29.53(2C), 29.49, 29.47(2C), 29.41, 28.42, 27.40, 27.38, 25.85, 25.19, 22.84, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{48}N_4O_3$, 453.3799; found, 453.3802.

(2R)-5-guanidino-2-[[(Z)-octadec-9-enoyl]amino]pentanoic acid.2HCl (31). General procedure B. White solid, yield = 60%. Mp = 185 – 187 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.30 (s, 1H), 7.71 (s, 1H), 7.47 (d, *J*=7.5Hz, 1H), 7.31 (s, 2H), 5.32–5.30 (m, 2H), 3.93-3.89 (m, 1H), 3.01 (t, *J*=6.5Hz, 2H), 2.09-2.04 (m, 2H), 1.98-1.95 (m, 4H), 1.72-1.69 (m, 2H), 1.58-1.55 (m, 2H), 1.45-1.36 (m, 2H), 1.30-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 175.98, 170.99, 157.32, 129.64, 129.60, 53.50, 35.03, 31.28, 29.77, 29.15, 29.10(2C), 28.83, 28.77, 28.74, 28.68, 28.61, 28.58, 26.63, 26.57, 25.42, 25.16, 22.09, 13.94. $[\alpha]^{23}_D$ -2.2 (*c* = 1.7, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{46}N_4O_3$, 439.3643; found,439.3644.

Methyl (2R)-5-guanidino-2-[[(Z)-octadec-9-enoyl]amino]pentanoate (31a). General procedure A. Waxy solid, yield = 71%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.83 (m, 1H), 7.20 (d, *J*=7.5Hz, 1H), 7.05-7.03 (m, 3H), 5.34-5.32 (m, 2H), 4.45-4.40 (m, 1H), 3.74 (s, 3H), 3.36-3.20 (m, 2H), 2.28 (t, *J*=7.5Hz, 2H), 1.99-1.96 (m, 4H), 1.90-1.76 (m, 2H), 1.68-1.58 (m, 4H), 1.31-1.23 (m, 20H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.81, 173.03, 157.55, 130.14, 129.85, 52.84, 51.83, 41.07, 36.41, 32.06, 29.91, 29.70, 29.53(2C), 29.49, 29.47(2C), 29.41, 28.42, 27.40, 27.38, 25.85, 25.19, 22.84, 14.26. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{48}N_4O_3$, 453.3799; found, 453.3804.

(2S)-6-amino-2-[[(Z)-octadec-9-enoyl]amino]hexanoic acid.HCl (32). General procedure C. Light yellow solid, yield = 87%. Mp = 68 – 70 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.05 (d, *J*=7.5Hz, 1H), 7.90 (s, 3H), 5.32-5.30 (m, 2H), 4.14-4.12 (m, 1H), 2.74-2.71 (m, 2H), 2.10 (t, *J*=7.5Hz, 2H), 1.98-1.90 (m, 4H), 1.67-1.65 (m, 1H), 1.57-1.44 (m, 5H), 1.31-1.23 (m, 22H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.69, 172.31, 129.60(2C), 51.49, 38.38, 34.99, 31.24, 30.33, 29.10, 29.06, 28.79, 28.66(2C), 28.64, 28.59, 28.55, 26.59, 26.54, 26.44, 25.21, 22.40, 22.06, 13.92. $[\alpha]^{23}_D$ -4.5 (*c* = 2.2, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{46}N_2O_3$, 411.3581; found, 411.3589.

Methyl(2S)-6-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]hexanoate (32a). General procedure A. Waxy solid, yield = 80%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.11 (d, *J*=7.5Hz, 1H), 6.74 (t, *J*=6.0Hz, 1H), 5.35-5.33 (m, 2H), 4.19-4.14 (m, 1H), 3.60 (s, 3H), 2.87-2.85 (m, 2H), 2.10-2.07 (m, 2H), 1.99-1.95 (m, 4H), 1.64-1.62 (m, 1H), 1.57-1.53 (m, 1H), 1.48-1.42 (m, 2H), 1.36-1.31 (m, 9H), 1.30-1.23 (m, 24H), 0.85 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.72, 172.37, 155.55, 129.61(2C), 77.36, 59.73, 51.65, 34.90, 31.27, 29.11, 29.09, 28.82, 28.67(2C), 28.64, 28.58(2C), 28.55, 28.54(2C), 28.23, 26.60, 26.56, 26.00, 25.20, 22.71 22.74, 14.07, 13.92. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{56}N_2O_5$, 525.4262; found, 525.4261.

(2S)-6-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]hexanoic acid (32b). General procedure B. White solid, yield = 86%. Mp = 62 – 64 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.95 (d, *J*=7.5Hz, 1H), 6.74 (t, *J*=5.5Hz, 1H), 5.32-5.30 (m, 2H), 4.12-4.11 (m, 1H), 2.88-2.84 (m, 2H), 2.10-2.08 (m, 2H), 1.98-1.96 (m, 4H), 1.65-1.62 (m, 1H), 1.54-1.51 (m, 1H), 1.49-1.44 (m, 2H), 1.35-1.33 (m, 9H), 1.28-1.22 (m, 24H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.89, 172.32, 155.60, 129.67(2C), 77.36, 51.73, 34.90, 31.31, 30.77, 29.15, 29.12(4C), 28.85(3C), 28.71(2C), 28.61(2C), 28.29, 26.64, 26.59, 25.30, 22.84, 22.12, 13.98. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{29}H_{54}N_2O_5$, 511.4106; found, 511.4108.

(2R)-6-amino-2-[[(Z)-octadec-9-enoyl]amino]hexanoic acid.HCl (33). General procedure C. Light yellow solid, yield = 87%. Mp = 68 – 70 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.05 (d, *J*=7.5Hz, 1H), 7.92 (s, 3H), 5.32-5.30 (m, 2H), 4.13-4.12 (m, 1H), 2.74-2.70 (m, 2H), 2.10 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 4H), 1.66 (m, 1H), 1.57-1.44 (m, 5H), 1.31-1.23 (m, 22H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.72, 172.36, 129.63(2C), 51.53, 38.42, 35.03, 31.27, 30.33, 29.14, 29.10, 28.83, 28.70(2C), 28.68, 28.63, 28.59, 26.62, 26.58, 26.48, 25.25, 22.44, 22.09, 13.96. $[\alpha]^{23}_{D}$ +5.8 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{24}H_{46}N_2O_3$, 411.3581; found, 411.3579.

Methyl(2R)-6-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]hexanoate (33a). General procedure A. Waxy solid, yield = 85%. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.04 (d, *J*=7.0Hz, 1H), 5.35-5.33 (m, 2H), 4.60-4.57 (m, 2H), 3.74 (s, 3H), 3.11-3.08 (m, 2H), 2.21 (t, *J*=7.5Hz, 2H),

2.02-1.98 (m, 4H), 1.86-1.80 (m, 1H), 1.70-1.66 (m, 1H), 1.64-1.60 (m, 2H), 1.52-1.45 (m, 2H), 1.44-1.42 (m, 9H), 1.34-1.25 (m, 22H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 173.25, 173.12, 156.24, 130.13, 129.90, 79.89, 52.50, 51.93, 40.16, 36.70, 32.23, 32.04, 29.90(2C), 29.86, 29.75, 29.66, 29.46, 29.45, 29.38(2C), 29.37, 29.29, 28.56, 27.36, 27.32, 25.72, 22.82, 22.51, 14.25. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{30}H_{56}N_2O_5$, 525.4262; found, 525.4258.

(2R)-6-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]hexanoic acid (33b). General procedure B. White solid, yield = 97%. Mp = 64 – 66 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.95 (d, *J*=8.0Hz, 1H), 6.73 (t, *J*=5.5Hz, 1H), 5.32-5.30 (m, 2H), 4.13-4.10 (m. 1H), 2.88-2.84 (m, 2H), 2.10-2.06 (m, 2H), 1.98-1.94 (m, 4H), 1.65-1.63 (m, 1H), 1.54-1.51 (m, 1H), 1.49-1.44 (m, 2H), 1.35-1.33 (m, 9H), 1.28-1.22 (m, 24H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.87, 172.24, 155.55, 129.63(2C), 77.29, 51.70, 35.03, 31.27, 30.73, 29.13, 29.10(4C), 28.83(3C), 28.68(2C), 28.58(2C), 28.26, 26.61, 26.57, 25.26, 22.82, 22.09, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{29}H_{54}N_2O_5$, 511.4106; found, 511.4111.

(2S)-5-amino-2-[[(Z)-octadec-9-enoyl]amino]pentanoic acid.HCl (34). General procedure C. Light yellow waxy solid, yield = 95%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.10 (d, *J*=8.0Hz, 1H), 7.94 (s, 3H), 5.32-5.30 (m, 2H), 4.17-4.16 (m, 1H), 2.75-2.73 (m, 2H), 2.10 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 4H), 1.76-1.74 (m, 1H), 1.60-1.56 (m, 3H), 1.47-1.45 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.44, 171.37, 129.64(2C), 51.23, 38.34, 35.06, 31.28, 29.15, 29.09(2C), 28.82, 28.72(2C), 28.68, 28.58, 28.00, 26.63, 26.57, 25.25, 22.80, 22.09, 13.96. $[\alpha]^{23}_D$ -3.5 (*c* = 2.1, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{44}N_2O_3$, 397.3425; found, 397.3428.

Methyl(2S)-5-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]pentanoate (34a). General procedure A. Waxy solid, yield = 83%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.11 (d, *J*=8.0Hz, 1H), 6.78-6.76 (m, 1H), 5.32-5.30 (m, 2H), 4.18-4.17 (m, 1H), 3.60 (s, 3H), 2.88 (q, *J*=6.5Hz, 2H), 2.10-2.08 (m, 2H), 1.98-1.96 (m, 4H), 1.65-1.62 (m, 1H), 1.52-1.44 (m, 3H), 1.41-1.38 (m, 2H), 1.35-1.33 (m, 9H), 1.28-1.22 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.73,

172.37, 155.58, 129.61(2C), 77.36, 51.59(2C), 35.03, 31.27, 29.13, 29.09, 28.82, 28.68, 28.64, 28.58(2C), 28.55(2C), 28.54(2C), 28.25, 26.60, 26.56, 26.17, 25.26, 22.08, 21.05, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{29}H_{54}N_2O_5$, 511.4105; found, 511.4102.

(2S)-5-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]pentanoic acid (34b). General procedure B. Waxy solid, yield = 96%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.96 (d, *J*=8.0Hz, 1H), 6.78-6.77 (m, 1H), 5.32-5.30 (m, 2H), 4.14-4.11 (m, 1H), 2.88 (q, *J*=6.5Hz, 2H), 2.10-2.06 (m, 2H), 1.98-1.96 (m, 5H), 1.65-1.62 (m, 1H), 1.51-1.38 (m, 4H), 1.35-1.33 (m, 9H), 1.28-1.22 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 173.75, 172.21, 155.58, 129.63, 129.62, 77.35, 51.59, 35.03, 31.27, 29.13, 29.09, 28.82, 28.68, 28.60, 28.58, 28.57(2C), 28.47(2C), 28.25, 26.60, 26.56, 26.17, 25.26, 22.08, 21.05, 20.75, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{52}N_2O_5$, 497.3949; found, 497.3948.

(2S)-4-amino-2-[[(Z)-octadec-9-enoyl]amino]butanoic acid.HCl (35). General procedure C. Light yellow waxy solid, yield = 98%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.22 (d, *J*=7.5Hz, 1H), 7.97 (s, 3H), 5.32-5.30 (m, 2H), 4.27-4.26 (m, 1H), 2.79-2.77 (m, 2H), 2.10 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 5H), 1.88-1.86 (m, 1H), 1.48-1.46 (m, 2H), 1.31-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.77, 172.54, 129.63(2C), 49.68, 36.15, 35.18, 31.26, 29.14, 29.08, 28.97(2C), 28.82, 28.71, 28.67, 28.62, 28.58, 26.62, 26.56, 25.19, 22.08, 13.94. $[\alpha]^{23}_D$ -4.3 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{22}H_{42}N_2O_3$, 383.3268; found, 383.3273.

Methyl-(2S)-4-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]butanoate (35a). General procedure A. Waxy solid, yield = 90%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.14 (d, *J*=8.0Hz, 1H), 6.79-6.77 (m, 1H), 5.32-5.30 (m, 2H), 4.24-4.20 (m. 1H), 3.60 (s, 3H), 2.96-2.90 (m, 2H), 2.10-2.07 (m, 2H), 1.98-1.96 (m, 4H), 1.84-1.78 (m, 1H), 1.69-1.64 (m, 1H), 1.49-1.45 (m, 2H), 1.40-1.34 (m, 9H), 1.32-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.61, 172.44, 155.47, 129.63, 129.61 77.54, 51.75, 49.72, 36.81, 34.95, 31.27, 30.88, 29.13, 29.09, 28.82(2C), 28.68, 28.58(2C), 28.56, 28.54(2C), 28.21, 26.62, 26.56, 25.17, 22.08, 13.93. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{52}N_2O_5$, 497.3949; found, 497.3952.

(2S)-4-(tert-butoxycarbonylamino)-2-[[(Z)-heptadec-9-enoyl]amino]butanoic acid (35b). General procedure B. Waxy solid, yield = 93%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.00 (d, *J*=8.0Hz, 1H), 6.76-6.74 (m, 1H), 5.32-5.30 (m, 2H), 4.17-4.14 (m. 1H), 2.99-2.88 (m, 2H), 2.09 (t, *J*=7.0Hz, 2H), 1.98-1.96 (m, 4H), 1.84-1.79 (m, 1H), 1.66-1.60 (m, 1H), 1.48-1.45 (m, 2H), 1.38-1.34 (m, 9H), 1.28-1.22 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.27, 171.03, 155.58, 129.61(2C), 78.01, 51.82, 41.06, 35.02, 34.95, 31.26, 29.11, 29.08, 28.81(2C), 28.66(2C), 28.57, 28.56, 28.54(2C), 28.11, 26.60, 26.56, 25.18, 22.08, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{27}H_{50}N_2O_5$, 483.3792; found, 483.3790.

(2S)-3-amino-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid.HCl (36). General procedure C. Light yellow waxy solid, yield = 96%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.31 (d, *J*=8.0Hz, 1H), 8.00 (s, 3H), 5.32-5.30 (m, 2H), 4.47-4.44 (m, 1H), 3.19-3.17 (m, 1H), 2.99-2.97 (m, 1H), 2.17-2.11 (m, 2H), 1.98-1.95 (m, 4H), 1.50-1.47 (m, 2H), 1.28-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.92, 170.81, 129.63(2C), 49.82, 35.18, 31.26, 29.14, 29.08(2C), 28.82(2C), 28.74, 28.67, 28.62, 28.58, 26.62, 26.56, 24.88, 22.08, 13.95. $[\alpha]^{23}_{D}$ -8.3 (*c* = 2.0, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{21}H_{40}N_2O_3$, 369.3112; found, 369.3109.

Methyl (2S)- 3-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]propanoate (36a). General procedure A. Waxy solid, yield = 80%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.02 (d, *J*=7.5Hz, 1H), 6.85-6.83 (m, 1H), 5.32-5.30 (m, 2H), 4.27-4.25 (m, 1H), 3.58 (s, 3H), 3.24-3.22 (m, 2H), 2.09 (t, *J*=7.5Hz, 2H), 1.98-1.95 (m, 4H), 1.47-1.45 (m, 2H), 1.37-1.35 (m, 9H), 1.29-1.23 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.16, 171.94, 155.58, 129.63(2C), 77.91, 52.27, 51.87, 35.19, 31.26, 29.13, 29.08, 28.81(2C), 28.69(2C), 28.67(2C), 28.57(2C), 28.54(2C), 28.16, 26.61, 26.56, 25.13, 22.08, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{27}H_{50}N_2O_5$, 483.3792; found, 483.3788.

(2S)-3-(tert-butoxycarbonylamino)-2-[[(Z)-octadec-9-enoyl]amino]propanoic acid (36b). General procedure B. White solid, yield = 95%. Mp = 50 – 51 °C. ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.86 (d, *J*=8.0Hz, 1H), 6.76-6.73 (m, 1H), 5.32-5.30 (m, 2H), 4.24-4.20 (m, 1H), 3.24-3.22 (m, 2H), 2.07 (t,

J=7.0Hz, 2H), 1.98-1.95 (m, 4H), 1.48-1.45 (m, 2H), 1.38-1.34 (m, 9H), 1.28-1.22 (m, 20H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.16, 171.94, 155.58, 129.63(2C), 77.91, 52.27, 41.25, 35.19, 31.26, 29.13, 29.08, 28.81, 28.69, 28.67(2C), 28.57(2C), 28.54(2C), 28.16, 26.61, 26.56, 25.13, 22.08, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{26}H_{48}N_2O_5$, 469.3636; found, 469.3633.

(Z)-N-(5-aminopentyl)octadec-9-enamide.HCl (37). General procedure C. Waxy solid, yield = 98%. ^{1}H NMR (500 MHz DMSO-d_6): δ 7.93(s, 3H), 7.78 (t, *J*=5.5Hz, 1H), 5.32-5.30 (m, 2H), 3.00 (q, *J*= 7.0Hz, 2H), 2.74-2.70 (m, 2H), 2.02 (t, *J*=8.0Hz, 2H), 1.98-1.95 (m, 4H), 1.55-1.50 (p, *J*=7.5Hz, 2H), 1.46-1.43 (m, 2H), 1.38-1.34 (m, 2H), 1.30-1.23 (m, 22H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 172.97, 129.62(2C), 38.99, 38.60, 38.09, 35.39, 31.26, 29.11, 29.08, 28.82(2C), 28.66(2C), 28.57(2C), 28.54, 26.59, 26.55, 25.31, 23.21, 22.09, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{23}H_{46}N2O$, 367.3683; found, 367.3688.

Tert-butyl N-[5-[[(Z)-octadec-9-enoyl]amino]pentyl]carbamate (37a). General procedure A. White solid, yield = 74%. Mp = 35 – 37 ^{o}C. ^{1}H NMR (500 MHz $CDCl_3$): δ 5.58 (s, 1H), 5.34-5.32 (m, 2H), 4.58 (s, 1H), 3.23 (q, *J*= 7.0Hz, 2H), 3.12-3.09 (m, 2H), 2.14 (t, *J*=8.0Hz, 2H), 2.01-1.97 (m, 4H), 1.60 (p, *J*=7.5Hz, 2H), 1.49-1.47 (m, 4H), 1.45-1.42 (m, 9H), 1.34-1.25 (m, 22H), 0.88 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 172.97, 129.62(2C), 38.99, 38.60, 38.09, 35.39, 31.26, 29.11, 29.08, 28.82(2C), 28.66(2C), 28.57(2C), 28.54, 26.59, 26.55, 25.31, 23.21, 22.09, 13.94. HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{28}H_{54}N_2O_3$, 467.4207; found, 467.4211.

Methyl (2S)-6-amino-2-[[(Z)-octadec-9-enoyl]amino]hexanoate.HCl (38). General procedure C. Waxy solid, yield = 98%. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.20 (d, *J*=7.5Hz, 1H), 7.86 (s, 3H), 5.32-5.30 (m, 2H), 4.19-4.17 (m, 1H), 3.60 (s, 3H), 2.75-2.71 (m, 2H), 2.10 (t, *J*=7.5Hz, 2H), 1.99-1.95 (m, 4H), 1.68-1.50 (m, 4H), 1.48-1.45 (m, 2H), 1.28-1.23 (m, 22H), 0.84 (t, *J*=7.0Hz, 3H). ^{13}C NMR (125 MHz DMSO-d_6): δ 172.71, 172.47, 129.64, 129.62, 51.73, 51.63, 38.39, 34.66, 32.26, 30.21, 29.11, 29.08(2C), 28.81(2C), 28.66(2C), 28.56, 26.60, 26.56, 26.43, 25.18, 22.31, 22.08, 13.94. $[\alpha]^{23}_D$ -5.6 (*c* = 2.2, MeOH). HRMS (ESI) *m/z* $[M+H]^+$ calcd for $C_{25}H_{48}N_2O_3$, 425.3738; found, 425.3740.

3. Glycine Transporter Expression in *Xenopus laevis* oocytes. Human GlyT1b or GlyT2a cDNA were sub-cloned into the plasmid oocyte transcription vector. Plasmid DNA were linearised with SpeI (New England Biolabs (Genesearch) Arundel, Australia) and RNA transcribed by T7 RNA polymerase using the mMessagemMachine kit (Ambion, TX, USA). Oocytes were extracted from female *Xenopus laevis* as previously described[37] and detached from follicle cell containing lobes by digestion with 2 mg/mL collagenase A (Boehringer, Mannheim, Germany). Defoliculated stage V-VI oocytes were injected with 4.6 ng of cRNA encoding GlyT1 or GlyT2 (Drummond Nanoinject, Drummond Scientific Co., Broomall, PA, USA). The oocytes were stored at 16-18 ºC for 2-5 days in ND96 solution (96 mM NaCl, 2 mM KCL, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.55), supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 μg/mL gentamicin and 100 μM/mL tetracycline.

4. Two electrode voltage clamp electrophysiology. Glycine transport currents from oocytes expressing either GlyT1 or GlyT2 were measured at -60 mV using Geneclamp 500 amplifier (Axon Instruments, Foster City, CA, USA) with a Powerlab 2/20 chart recorder (ADInstruments, Sydney, Australia) and chart software (ADInstruments). Inhibitor concentration responses were performed using cumulative application. The EC_{50} concentration of glycine was applied until a stable level of transport was reached. Increasing concentrations of N-acyl amino acid were then co-applied with glycine, with each concentration producing a distinct plateau in response. Currents at each of these plateaus were measured and compared to glycine currents in the absence of any inhibitor. All data was subsequently analysed using GraphPad Prism 7.02 (GraphPad Software, San Diego, CA). Inhibitor concentration responses were fit by the method of least squares using: **Y=Bottom + (Top-Bottom)/(1+10(X-LogIC50))** where X is log[acyl amino acid] (μM), Y is current normalised to glycine in the absence of inhibitor and Top and Bottom are the maximal and minimal plateau responses respectively. This equation was constrained to have the bottom value > 0, but not = 0, as to capture partial levels of inhibition, and the standard hill slope -1.0. Concentration response curves were thus able to generate IC_{50} values as well as % maximum (max.) inhibition values. IC_{50} values are presented as mean and 95% confidence intervals, and % max inhibition are presented as mean ± SEM.

Data are from n ≥ 3 cells from at least two batches of oocytes. Where significant inhibition was not reached, the IC_{50} value is recorded as greater than the highest concentration of N-acyl amino acid used.

To determine the mechanism of binding, glycine concentration responses were performed on oocytes expressing GlyT2 first in the absence of inhibitor, and then in the presence of 10 nM, 30 nM, or 1 μM **32**. **32** is not immediately reversible so glycine concentration responses were generated by first applying ND96 with **32** for 2 minutes and then adding increasing concentrations of glycine and measuring the plateau response for each value. Currents were normalised to the I_{max} at the maximal glycine concentration (300 μM) in the absence of inhibitor for each individual cell. EC_{50} values were determined using the modified Michaelis-Menten equation **I = ([Gly].Imax)/EC50 + [Gly])**. Data was then transformed using an Eadie-Hofstee plot. One way ANOVA analysis with Dunnett's post-hoc tests were performed for both EC_{50} and I_{max} values, and presented as $p < 0.05$ *, $p < 0.01$ **, $p < 0.001$ *** etc.

5. In vitro microsomal stability. Human or rat liver microsomes (Xenotech, LLC, Lenexa, KS) were suspended in 0.1M phosphate buffer (pH 7.4) at a final protein concentration of 0.4 mg/mL and incubated with test compound (1 μM) at 37°C. An NADPH-regenerating system (1 mg/mL NADP, 1 mg/mL glucose-6-phosphate, 1 U/mL glucose-6-phosphate dehydrogenase) and $MgCl_2$ (0.67 mg/mL) was added to initiate the metabolic reactions. At various time points over a 60 minute period the samples were quenched with ice cold acetonitrile. Samples were then subjected to centrifugation, and the concentration of parent compound remaining in the supernatant was monitored by LC-MS (Waters Xevo G2 QTOF coupled to a Waters Acquity UPLC). The first-order rate constant for substrate depletion was determined by fitting the data to an exponential decay function.

6. Stability studies in human and rat plasma. Human plasma (pooled; n=3 donors) was separated from blood collected from non-identifiable volunteer donors (Australian Red Cross Blood Service under a supply agreement approved by the Monash Human Research Ethics Committee) and rat plasma (pooled, multiple rats) from male Sprague Dawley rats was procured from Animal Resources Centre (ARC; Perth, Australia), then stored frozen at -80°C. Esterase activity of the plasma has

verified using procaine (human plasma) and enalapril (rat plasma). On the day of the experiment, plasma was thawed in a water bath maintained at 37°C. An aliquot of plasma was spiked with test compound prepared in DMSO/acetonitrile/water (20:40:40) at a nominal final compound concentration of 1000 ng/mL and final DMSO and acetonitrile concentrations of 0.2% and 0.4% (v/v), respectively. Plasma samples were then vortex mixed and aliquots (50 µL) were transferred to fresh microcentrifuge tubes and incubated at 37°C. At various time points over the 4 h incubation period, plasma samples were removed and snap-frozen in dry ice (n=3 per time point). Plasma samples were frozen at -80°C prior to analysis by LC-MS. Plasma sample aliquots were processed for analysis by protein precipitation using a two-fold excess of acetonitrile, followed by centrifugation. Analysis of the supernatant was conducted using a Waters (Milford, MA) Acquity UPLC coupled to a Waters Xevo TQ mass spectrometer operated in negative electrospray ionisation multiple-reaction monitoring. The cone voltage and collision energy were 50V and 25V. Processed samples (3 µL) were injected onto a Supelco Ascentis Express RP Amide column (50x2.1 mm, 2.7 µm) and analytes eluted using a water/acetonitrile gradient with 0.005M ammonium formate over 4min with a flow rate of 0.4 mL/min. Plasma concentrations were obtained by comparison of the peak area ratio (using leucine enkephalin as the internal standard) to that for a calibration curve prepared using blank plasma of the same species. The limit of quantitation ranged from 50 ng/mL to 5 ng/mL.

7. Drug binding in rat brain. Brains from male Sprague Dawley rats were harvested in-house and homogenised (gentleMACSTM Dissociator, Miltenyi Biotec GmbH, Germany) in 8 parts of isotonic pH 7.4 PBS (100 mM sodium phosphate buffer and 40 mM NaCl) to 1 part of brain tissue (assuming 1 g brain tissue is equal to 1 mL) to achieve a fine suspension with a tissue concentration of 0.11 g tissue/mL (9-fold dilution). An aliquot of rat brain homogenate was spiked with **33**, immediately vortex mixed and aliquots (n = 4) were transferred to ultracentrifuge tubes. The tubes were then transferred to the ultracentrifuge rotor, sealed and subjected to ultracentrifugation at 37°C (Beckman Rotor type 42.2 Ti; 223,000 x g) for 4.2 h to separate proteins. Additional aliquots of spiked matrix were maintained in ultracentrifuge tubes (but not spun) at 37°C for 0.5, 2.0 and 4.2 h to serve as controls for the assessment of stability and to obtain a measure of the total compound concentration in

brain homogenate (C_{total}). Following ultracentrifugation, an aliquot of protein-free supernate was taken from each of the ultracentrifuge tubes (n = 4) to obtain measures of the unbound concentration ($C_{unbound}$). The contents of the non-centrifuged tubes were mixed and aliquots (n = 4) were taken for measurement of the total concentration (C_{total}). Samples were analysed by LC-MS and the unbound fraction and % bound in brain were calculated as previously described.[42]

8. In vivo pharmacokinetic study. Animal studies were conducted using established procedures in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the study protocols were reviewed and approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Rat plasma and brain exposure studies were conducted in male Sprague-Dawley rats (250−306 g) over a 24h period (n=3 rats per time point). An intraperitoneal suspension formulation of **33** (containing 10% v/v DMSO, 1% solutol HS-15 in 50 mM phosphate buffered saline, pH7.4) was administered via 27G 1/2" needle. Blood samples (1 mL) were collected under isoflurane anaesthesia via terminal cardiac puncture into tubes containing heparin, followed by collection of the whole brain. The brain samples were blotted, weighed and frozen on dry-ice. Blood samples were centrifuged at 4°C and plasma was separated and stored at -80°C. Plasma sample aliquots were processed by protein precipitation using a two-fold excess of acetonitrile, followed by centrifugation. Whole brains were homogenised in 3-volume/weight of stabilisation mixture (composed of 0.1M EDTA and 4 g/L potassium fluoride in water) using a gentleMACS dissociator (Miltenyi Biotec). Extraction of **33** from the resulting tissue homogenate was conducted using protein precipitation with methanol (3-fold volume ratio), followed by centrifugation and separation of the supernatant. Analysis of the supernatant was conducted using a Waters (Milford, MA) Acquity UPLC coupled to a Waters Xevo TQ mass spectrometer operated in positive electrospray ionisation multiple-reaction monitoring. The cone voltage and collision energy were 40V and 20V, respectively and the m/z transition was 411.17 > 129.09. Processed samples (5 µL) were injected onto a Supelco Ascentis Express RP Amide column (50x2.1 mm, 2.7 µm) and analytes eluted using a water/acetonitrile gradient with 0.05% formic acid over 4min with a flow rate of 0.4 mL/min. Plasma and brain concentrations were obtained by comparison of the peak area ratio (using diazepam

as the internal standard) to that for a calibration curve prepared using blank rat plasma or brain homogenate matrix. The limit of quantitation was 0.5 ng/mL in plasma and 5 ng/mL in brain homogenate. Plasma and brain pharmacokinetic parameters were calculated using non-compartmental methods (PK Solver, Version 2.0).

9. In vivo analgesia model

Analgesia studies were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). The study protocols were reviewed and approved by the University of Sydney Animal Ethics Committee. Experiments were conducted on 27 male Sprague Dawley rats (ARC WA, Perth Australia) weighing 320 - 400 g on the day of testing. Rats were housed in groups of 4 under standard conditions with a 12 h light-dark cycle and free access to food and water. Animals were allowed 7 days acclimatisation to the research facility prior to commencement of any procedures.

Baseline measurements of mechanical paw withdrawal threshold (PWT) were taken using the von Frey test. Animals were acclimatised to Perspex testing chambers with wire mesh floors, and a series of calibrated von Frey filaments 0.4 – 15.1 g (Stoelting, USA) were presented. Pain thresholds were calculated using the up-down method.[42] Response to all filaments were scored as the minimum threshold of 0.2 g, while response to none of the filaments were scored as the maximum threshold of 15 g.

Partial nerve ligation of the left sciatic nerve was then performed on each animal according to the method of Bennett & Xie.[44] Briefly, under isoflurane anaesthesia (2% in saturated oxygen), the left sciatic nerve was isolated from the surrounding connective tissue using blunt dissection. One third to one half of the nerve was ligated with 6-0 silk suture (Ethicon, USA). The muscle was closed using silk sutures (4-0, Ethicon, USA) and the skin incision was closed using tissue adhesive (3M Vetbond). Animals were allowed to recover from surgery and neuropathic pain allowed to develop for 14 days before being tested.

On the day of the experiment, animals were acclimatised to the chambers before pre-injection testing (t = 0). Animals were then given intra-peritoneal (ip injections) of **33** (30 or 3 mg/kg), **2** (30 mg/kg) or vehicle (1% DMSO, 10% Solutol HS15 in 0.9% saline) and retested on the von Frey test at 15, 30, 60, 90, 120, 180, 240 and 360 minutes post-injection.

Data are presented as raw scores, averaged across treatment groups ± standard error of the mean. A repeated measures two-way ANOVA was performed and, where main effects were observed, multiple comparisons with post hoc Bonferroni correction was used. Significance was set at $\alpha = 0.05$.

Supporting Information

Information on test compound purity analysis, supplementary figures and supplementary methods.

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Author Contributions

TR, SNM and RV designed and directed the chemistry; SNM, ZJF, SS, AY, IR conducted experiments and analysed data for activity of transporters, ion channels and receptors; SM and MJC designed and directed the analgesia experiments; SNM, TR, RV, MJC and RR analysed data and wrote the manuscript.

Acknowledgements

This work was supported by the Australian National Health and Medical Research Council Project Grant APP1144429, the University of Sydney Commercial Development and Industry Partners Fund, and the Austrian Science Fund, Erwin-Schrödinger-Fellowship Program Project No: J3982-B27. We are grateful for the services provided by Monash Centre for Drug Candidate Optimization. We are grateful for administrative support provided by Cheryl Handford and discussions and comments throughout the course of the study from Josep Font, Casey Gallahger, Diba Sheipouri, Qianyi Wu, Natasha Friedman, Ichia Chen.

Abbreviations

GlyT1 Glycine transporter 1

GlyT2 Glycine transporter 2

NAGly N-arachidonyl glycine

NOGly N-oleoyl glycine

BBB Blood Brain Barrier

FAAH Fatty Acid Amide Hydrolase

μOR mu opioid receptor

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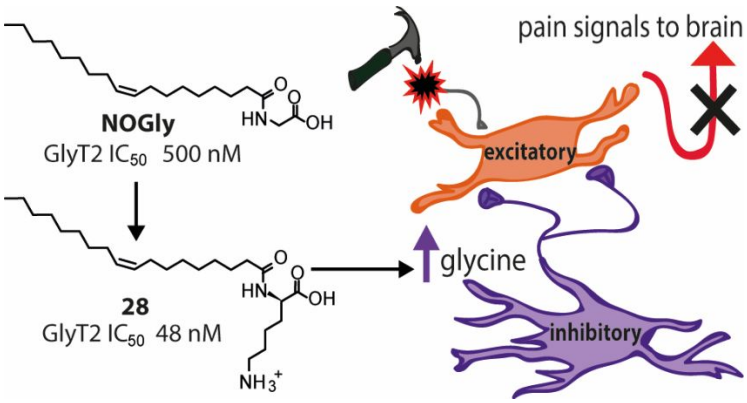

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