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Conformationally-restricted vigabatrin analogs as irreversible and reversible inhibitors of γ-aminobutyric acid aminotransferase

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Abstract—Compounds that inhibit γ-aminobutyric acid aminotransferase exhibit anticonvulsant activity; vigabatrin is a known irreversible inhibitor of this enzyme and anticonvulsant drug. Conformationally-restricted, five-membered- and six-membered-ring vigabatrin analogs were synthesized and tested as inhibitors of γ-aminobutyric acid aminotransferase. Two monofluorinated compounds, **4** and **5**, are time-dependent inhibitors of the enzyme, and their potencies are comparable to that of vigabatrin. Compounds **6** and **7** are weak reversible inhibitors.
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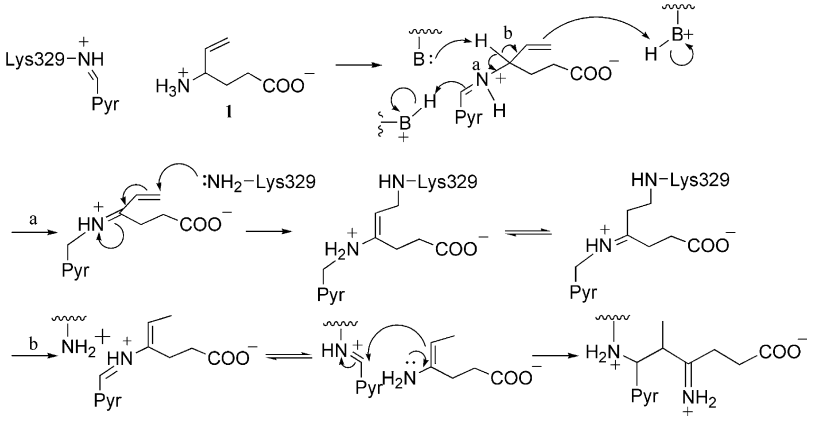
1. Introduction

Epilepsy refers to an etiologically and clinically diverse group of neurological disorders characterized by spontaneous, recurring, cerebral discharges called seizures.[1] Convulsions arise from a variety of reasons including heredity, head trauma, brain tumors, heat stroke, acute intoxication, and labor.[2,3] When the level of γ-aminobutyric acid (GABA) in the brain falls below a threshold level, convulsions occur.[4] Raising the level of GABA in the brain has an anticonvulsant effect,[5,6] as seizures were stopped when GABA was directly injected into the brain of convulsing animals.[7] However, because GABA does not cross the blood–brain barrier,[8] it cannot be used as an anticonvulsant drug.[9] γ-Aminobutyric acid aminotransferase (GABA-AT, E.C. 2.6.1.19) is the enzyme that degrades GABA, and inhibition of this enzyme increases the availability of GABA in the brain for therapeutic applications.

Vigabatrin (**1**) is a potent irreversible inhibitor of GABA-AT[10] and is used in the treatment of epilepsy.[11] Vigabatrin inactivates GABA-AT through two mechanistic pathways (Scheme 1): a Michael addition mechanism (pathway a) and an enamine mechanism



Scheme 1. Inactivation of GABA-AT by vigabatrin.

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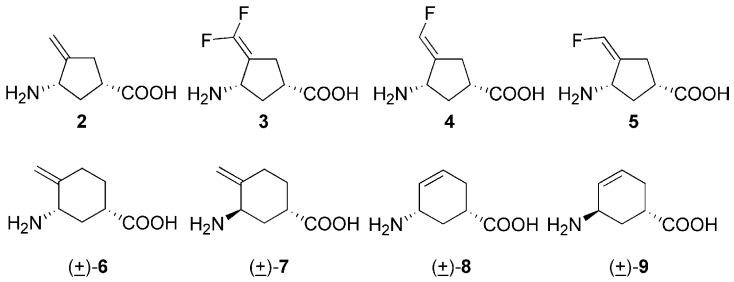


Figure 1. Conformationally-restricted vigabatrin analogs.

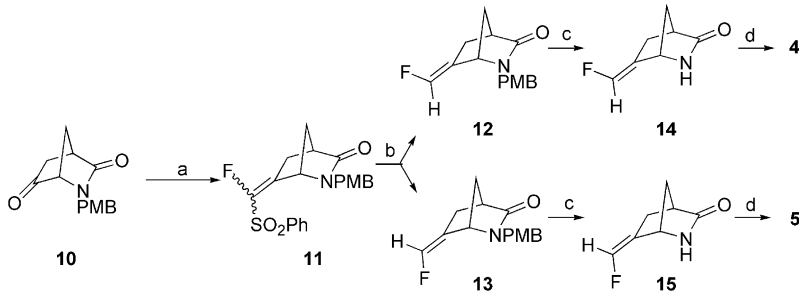
(pathway b).[12] The advantages of conformationally-restricted compounds are that conformation restriction may increase the potency by stabilizing a biologically active conformer (therefore reducing the entropic penalty on binding to the enzyme), decrease degradation by eliminating metabolized conformers, and improve selectivity by eliminating bioactive conformers that give undesired biological responses.[13] Previously, our group reported two conformationally-restricted five-membered-ring vigabatrin analogs (**2** and **3**, Fig. 1).[14] Compound **2** showed time-dependent inhibition of GABA-AT only in the absence of 2-mercaptoethanol, which is an antioxidant used in enzyme assays; no inactivation occurred when 2-mercaptoethanol was present, probably because it trapped the released electrophilic species responsible for inactivation. To increase the electrophilicity of the reactive species with the goal of getting a reaction to occur with GABA-AT prior to its release into the medium, fluorines were substituted at the terminus of the double bond. Although fluorine substitution decreases the potency of vigabatrin,[15] the difluorinated analog of **2**, that is, **3**, is a very potent irreversible inactivator of GABA-AT, even more effective than vigabatrin in vitro.[14] Here we report the *E*- and *Z*-monofluorinated analogs of **2** (compounds **4** and **5**). Another strategy we utilized to optimize lead compound **2** was to synthesize **6** and **7**, both of which are six-membered-ring vigabatrin analogs with an exocyclic double bond. Because the orientation of the double bond is very important to the activity and potency of GABA-AT inactivators,[16] these compounds, with the exocyclic double bond oriented differently from that of **2**, may be covalently attached to the enzyme before being released from the active site. We are also interested in **6** and **7** because the vigabatrin analogs containing a six-membered-ring with an endocyclic double bond (**8** and **9**) are inhibitors; **8** is a weak irreversible inhibitor (K_i 2.3 mM, k_{inact} 0.01 min^{-1}, k_{inact}/K_i 0.004 $mM^{-1} min^{-1}$), and **9** is a reversible GABA-AT inhibitor (K_i 100 μM).[17] It would be intriguing if the different orientation of the double bond resulted in increased potency.

2. Results and discussion

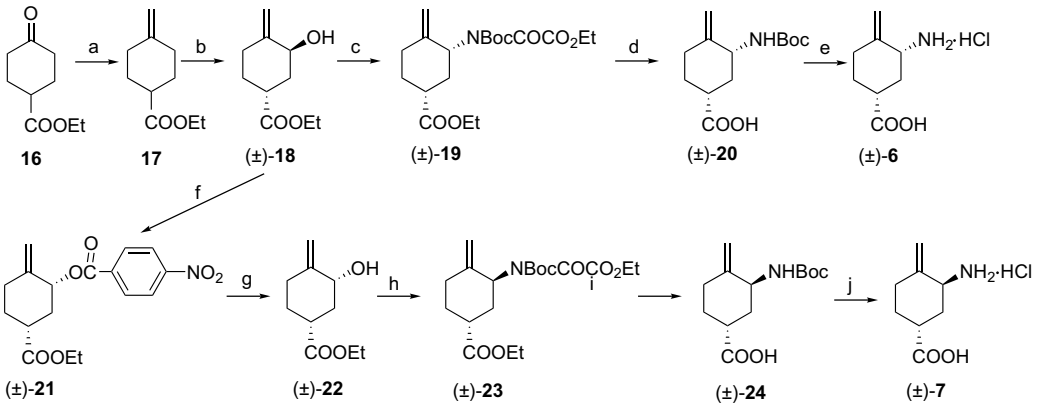
2.1. Chemistry

The syntheses of the monofluorinated vigabatrin analogs (**4** and **5**) started from **10** (Scheme 2).[18,19] The fluorosulfone[20,21] **11** was prepared in an 82% yield as an inseparable mixture of the *E*- and *Z*-isomers. Although aluminum amalgam has been used to reductively remove the phenylsulfone moiety,[22,23] it was unreactive toward **11**. Magnesium combined with mercury chloride,[24] however, produced the *E*-(**12**) and *Z*-(**13**) monofluoro alkenes smoothly, the stereochemistry of which was assigned based on the presence or absence of a nuclear Overhauser effect (NOE) between H-1 and the alkene proton. For **12**, a NOE was observed between H-1 and the alkene proton but for **13** it was not observed. Subsequent oxidative deprotection of the PMB group with ceric ammonium nitrate (CAN) and hydrolysis of the lactam gave the corresponding conformationally-restricted, monofluorinated vigabatrin analogs **4** and **5**.

The syntheses of **6** and **7** (Scheme 3) started with a Wittig reaction, which converted **16** to the alkene **17**. Compound **17** was then oxidized with *tert*-butyl hydroperoxide under the catalysis of selenium dioxide to give the *trans*-allylic alcohol **18** as the major product (*trans*:*cis* 14:1, the configurations were assigned by 2D



Scheme 2. Syntheses of **4** and **5**. Reagents and conditions: (a) $PhSO_2CH_2F$, $(EtO)_2POCl$, LiHMDS, 82%; (b) Mg, $HgCl_2$, 64% for **12** and 23% for **13**; (c) CAN, 42%; (d) HCl, 75%.



Scheme 3. Syntheses of **6** and **7**. Reagents and conditions: (a) Ph_3PCH_3Br, LiHMDS, 87%; (b) *t*-BuOOH, SeO_2, 90%; (c) $BocNHCOCO_2Et$, DIAD, PPh_3, 64%; (d) LiOH, 52%; (e) TFA, CH_2Cl_2, then HCl, 85%; (f) *p*-nitrobenzoic acid, DIAD, PPh_3, 58%; (g) NaN_3, MeOH, 40 °C, 64%; (h) $BocNHCOCO_2Et$, DIAD, PPh_3, 55%; (i) LiOH, 36%; (j) TFA, CH_2Cl_2, then HCl, 80%.

NOESY experiments; for **22** there is a NOE between H-1 and H-3).[25] A Mitsunobu reaction between **18** and *N*-Boc ethyl oxamate gave **19**,[26] which was unstable and hard to completely purify at this stage. Crude **19** was subjected to hydrolysis to produce the Boc-protected amino acid **20**. Deprotection of the Boc group with TFA furnished **6**. To synthesize **7**, a Mitsunobu reaction with *p*-nitrobenzoic acid followed by methanolysis in the presence of sodium azide[27] was used to carry out the inversion of configuration of **18** to give the *cis*-alcohol **22**. Compound **7** was synthesized from **22** using steps similar to those used for **6**.

2.2. Enzyme inhibition results

Both **4** and **5** exhibited concentration- and time-dependent inhibition of pig brain GABA-AT in the presence of 2-mercaptoethanol (Fig. 2). Remarkably, substitution with only one fluorine atom changed the activity of **2** dramatically. The K_i values for **4** (K_i 250 μM) and **5** (K_i 530 μM) are lower (better binding) than that for (*S*)-vigabatrin (K_i 1.3 mM), but the rate constants for inactivation of **4** (k_{inact} 0.25 min^{-1}) and **5** (k_{inact} 0.74 min^{-1}) also are lower than that for vigabatrin (k_{inact} 2.2 min^{-1}), therefore, the efficiency constants for **4** (k_{inact}/K_I 1.0 $mM^{-1}min^{-1}$) and **5** (k_{inact}/K_i 1.4 $mM^{-1}min^{-1}$) are comparable to that of vigabatrin (k_{inact}/K_i is 1.7 $mM^{-1}min^{-1}$). The inactivation mechanisms of **4** and **5** are under investigation. Despite the similarities in structure and potency between **4** and **5**, the inactivation mechanisms may be different, like the difference in mechanism that was reported for **25** and **26** (Fig. 3).[28]

Unfortunately, neither **6** nor **7** inactivated GABA-AT. Also, no substrate activity was observed. Compound **6** was tested as a reversible inhibitor, and the IC_{50} was 1.7 mM, while **7** was even weaker. It is possible that the exocyclic methylene group affects the binding of these compounds and as a result, the γ-proton of **6** and **7** is too far away from Lys329 for abstraction, which is necessary for both substrate and inactivator properties.

3. Conclusion

By fluorine substitution of **2**, we discovered two potent, conformationally-restricted, time-dependent inhibitors of GABA-AT, although they are not as potent as the difluorinated analog **3**. Currently the inhibition mechanisms are under investigation. The fact that none of **2**,

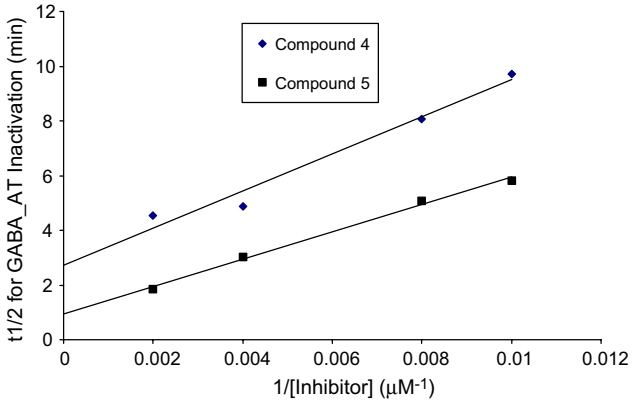


Figure 2. Determination of the inactivation constants for **4** and **5** at pH 8.5, 25 °C.

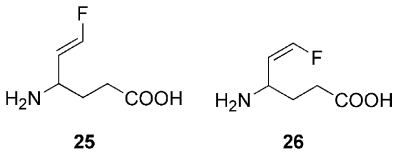


Figure 3. Previously prepared monofluorovigabatrin analogs.

6, and **7** inactivated GABA-AT suggests that the orientation of the double bond still requires further tuning for these conformationally-restricted vigabatrin analogs.

4. Experimental

All NMR spectra were recorded with a Varian Mercury 400 MHz or a Varian Inova 500 MHz NMR spectrometer. ^{1}H NMR chemical shifts are reported as δ values in ppm downfield from Me_4Si as the internal standard in $CDCl_3$. For samples run in D_2O, the HOD resonance was set at 4.80 ppm. For samples run in CD_3OD, the HCD resonance was set at 3.31 ppm. ^{13}C chemical shifts are listed in ppm with the $CDCl_3$ carbon peak set to 77.23 ppm. For samples run in D_2O, DSS was used as the external standard. For samples run in CD_3OD, the CD_3OD carbon peak was set to 49.15 ppm. ^{19}F chemical shifts are listed in ppm downfield from $CFCl_3$ as the external standard for samples in $CDCl_3$ and TFA as the external standard for samples in D_2O. Mass spectra were obtained on a VG70-250SE (EI) or a Micromass Quattro II (ESI) mass spectrometer. Column chromatography was carried out with Merck silica gel 60 (230–400 mesh). TLC was run with EM Science silica gel 60 F254 preloaded glass plates. Melting points were obtained with a Fisher–Johns melting point apparatus and are not corrected. An Orion Research 702 pH meter with a general combination electrode was used for pH measurements. All enzyme assays were recorded with a Perkin–Elmer Lambda 10 UV/vis spectrometer. Fluoromethyl phenylsulfone was purchased from TCI America. Vigabatrin was purchased from Sigma Chemical Co. All other reagents were purchased from Aldrich Chemical Co. and were used without purification. All solvents were purchased from Fisher Scientific. Anhydrous THF was distilled from sodium metal under nitrogen.

4.1. *E*/*Z*-(1*S*,4*S*)-6-(1′-Fluoro-1′-phenylsulfonyl)methylenyl-2-(4′-methoxybenzyl)-azabicyclo[2.2.1]heptan-3-one (11)

To anhydrous THF (20 mL) under nitrogen was added fluoromethyl phenylsulfone (1.18 g, 6.77 mmol) and diethyl chlorophosphate (0.98 mL, 6.78 mmol). At −78 °C, lithium bis(trimethylsilyl)amide (1.0 M in THF, 13.1 mL, 13.1 mmol) was slowly added over 0.5 h. After 1 h, a solution of **10** (1.07 g, 4.37 mmol) in anhydrous THF (10 mL) was slowly added via cannula. The solution was then warmed to room temperature and stirred overnight. After being quenched with saturated aqueous ammonium chloride solution (30 mL), THF was evaporated, and the resulting solution was extracted with ethyl acetate (3 × 30 mL). The organic layers were combined, washed with brine (2 × 30 mL), and dried over anhydrous Na_2SO_4. This solution was concentrated under reduced pressure and purified by flash column chromatography (hexanes–EtOAc 1:0–1:2), giving an inseparable mixture of the *E*- and *Z*-isomers (3:1, 1.44 g, 82%) as a colorless oil. ^{1}H NMR for the major product (400 MHz, $CDCl_3$) δ 7.94 (d, 2H, *J* 8.0 Hz), 7.72 (t, 1H, *J* 7.4 Hz), 7.61 (t, 2H, *J* 7.6 Hz), 7.33 (d, 2H, *J* 8.4 Hz), 6.90 (d, 2H, *J* 8.8 Hz), 5.24 (s, 1H), 4.77 (d, 1H, *J* 14.8 Hz), 3.82 (s, 3H), 3.79 (d, 1H, *J* 14.8 Hz), 3.00 (s, 1H), 2.49–2.66 (m, 2H), 2.10 (d, 1H, *J* 9.2 Hz), 1.63 (d, 1H, *J* 8.8 Hz); HRMS (EI) M calcd for $C_{21}H_{20}NO_4FS$ 401.1092, found 401.1088.

4.2. *E*-(1*S*,4*S*)-6-Fluoromethylenyl-2-(4′-methoxybenzyl)-azabicyclo[2.2.1]heptan-3-one (12) and *Z*-(1*S*,4*S*)-6-fluoromethylenyl-2-(4′-methoxybenzyl)-azabicyclo-[2.2.1]heptan-3-one (13)

Compound **11** (1.70 g, 4.24 mmol) was dissolved in anhydrous methanol (40 mL) under nitrogen and cooled in an ice–salt bath. Magnesium turnings (1.00 g, 41.7 mmol) and mercury(II) chloride (150 mg, 0.55 mmol) were added. The solution was stirred for 2 h, warmed to room temperature and stirred overnight. To the reaction mixture was added HCl (1 N, 30 mL). Methanol was evaporated under reduced pressure, and the resulting aqueous solution was extracted with ethyl acetate (3 × 30 mL). The organic layers were combined, washed with saturated aqueous $NaHCO_3$ solution (2 × 30 mL) and brine (2 × 30 mL), and dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the residue was purified by flash column chromatography (hexanes–EtOAc 3:1), giving **12** (0.70 g, 64%) and **13** (0.25 g, 23%), both as white solids.

For **12**: mp 108.0–110.0 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.15 (d, 2H, *J* 8.5 Hz), 6.87 (d, 2H, *J* 8.5 Hz), 6.65 (d, 1H, *J* 82.9 Hz), 4.66 (d, 1H, *J* 15.0 Hz), 3.83 (s, 1H), 3.81 (s, 3H), 3.72 (d, 1H, *J* 15.0 Hz), 2.98 (s, 1H), 2.55 (dd, 1H, *J* 16.0, 2.5 Hz), 2.36 (dd, 1H, *J* 16.0, 1.5 Hz), 2.02 (d, 1H, *J* 8.0 Hz), 1.53 (d, 1H, *J* 9.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.60, 158.97, 142.67 (d, *J* 255.4 Hz), 129.19, 128.54, 120.40 (d, *J* 7.3 Hz), 113.98, 59.37 (d, *J* 9.2 Hz), 55.20, 44.89, 43.53, 40.77, 27.93; HRMS (EI) M calcd for $C_{15}H_{16}NO_2F$ 261.1160, found 261.1159.

For **13**: mp 51.0–53.0 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.21 (d, 2H, *J* 8.5 Hz), 6.87 (d, 2H, *J* 8.5 Hz), 6.54 (d, 1H, *J* 84.9 Hz), 4.67 (d, 1H, *J* 15.0 Hz), 4.36 (s, 1H), 3.81 (s, 3H), 3.67 (d, 1H, *J* 15.0 Hz), 2.96 (s, 1H), 2.43 (d, 1H, *J* 14.0 Hz), 2.21 (d, 1H, *J* 15.0 Hz), 1.97 (d, 1H, *J* 9.5 Hz), 1.48 (d, 1H, *J* 9.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.80, 159.11, 142.53 (d, *J* 250.9 Hz), 129.77, 128.79, 120.59 (d, *J* 11.9 Hz), 114.04, 57.46 (d, *J* 2.7 Hz), 55.38, 45.30, 44.35, 39.74, 27.45 (d, *J* 4.6 Hz); HRMS (EI) M calcd for $C_{15}H_{16}NO_2F$ 261.1160, found 261.1155.

4.3. *E*-(1*S*,4*S*)-6-Fluoromethylenyl-2-azabicyclo[2.2.1]-heptan-3-one (14)

Compound **12** (482 mg, 1.85 mmol) was dissolved in acetonitrile (10 mL). To this solution was added a

solution of ceric ammonium nitrate (3.04 g, 5.55 mmol) in water (5 mL). After being stirred at room temperature for 1.5 h, the reaction mixture was diluted with ethyl acetate (100 mL), washed with saturated aqueous $NaHCO_3$ solution (2×40 mL) and brine (2×40 mL), and dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the residue was purified by column chromatography (hexanes–EtOAc 1:1) to give **14** as a white solid (110 mg, 42%). Mp 119.0–121.0 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 6.83 (d, 1H, *J* 83.2 Hz), 5.48 (br s, 1H), 4.15 (s, 1H), 2.90 (s, 1H), 2.60 (d, 1H, *J* 16.8 Hz), 2.39 (d, 1H, *J* 15.6 Hz), 2.15 (d, 1H, *J* 9.2 Hz), 1.61 (d, 1H, *J* 9.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.06, 142.66 (d, *J* 254.6 Hz), 122.93 (d, *J* 7.3 Hz), 56.62 (d, *J* 10.1 Hz), 44.70, 42.72, 27.59; ^{19}F NMR (376 MHz, $CDCl_3$) δ −2.75 (d, *J* 83.6 Hz); HRMS (EI) M calcd for C_7H_8NOF 141.0584, found 141.0582.

4.4. *Z*-(1*S*,4*S*)-6-Fluoromethylenyl-2-azabicyclo[2.2.1]-heptan-3-one (15)

This was prepared as a colorless oil using the same procedure as **14** in 43% yield from **13**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.47 (d, *J* 85.6 Hz, 1H), 5.93 (br s, 1H), 4.61 (s, 1H), 2.88 (s, 1H), 2.47 (dd, 1H, *J* 15.0, 2.0 Hz), 2.25 (d, 1H, *J* 15.0 Hz), 2.12 (d, 1H, *J* 9.5 Hz), 1.58 (d, 1H, *J* 9.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.64, 141.77 (d, *J* 251.3 Hz), 122.74 (d, *J* 11.2 Hz), 54.42 (d, *J* 4.4 Hz), 44.74, 41.66, 26.80 (d, *J* 5.4 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ −0.35 (d, *J* 84.7 Hz); HRMS (EI) M calcd for C_7H_8NOF 141.0584, found 141.0585.

4.5. *E*-(1*S*,3*S*)-3-Amino-4-fluoromethylenyl-1-cyclopentanoic acid, hydrochloride (4)

To compound **14** (38.5 mg, 27.3 μmol) was added HCl (4 N, 4 mL). The solution was heated to 70 °C and stirred for 10 h. It was cooled, washed with ethyl acetate (2×4 mL), and evaporated under reduced pressure to give a white solid, which was recrystallized with ethanol and ethyl ether to give white crystals (40 mg, 75%). Mp 200.0–202.0 °C (dec); ^{1}H NMR (400 MHz, D_2O) δ 6.96 (d, 1H, *J* 80.4 Hz), 4.36 (s, 1H), 3.08 (t, 1H, *J* 7.6 Hz), 2.93 (d, 1H, *J* 17.2 Hz), 2.72 (d, 1H, *J* 16.0 Hz), 2.51 (t, 1H, *J* 6.8 Hz), 2.01–2.08 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 181.42, 149.82 (d, *J* 257.3 Hz), 123.57 (d, *J* 12.8 Hz), 53.20 (d, *J* 9.2 Hz), 44.02, 36.78, 32.03 (d, *J* 1.9 Hz); ^{19}F NMR (376 MHz, D_2O) δ −48.59 (d, *J* 78.7 Hz); HRMS (EI) M calcd for $C_7H_{10}NO_2F$ 159.0690, found 159.0687. Anal. Calcd for $C_7H_{11}NO_2FCl$: C, 42.98; H, 5.67; N, 7.16. Found: C, 42.65; H, 5.36; N, 6.81.

4.6. *Z*-(1*S*,3*S*)-3-Amino-4-fluoromethylenyl-1-cyclopentanoic acid, hydrochloride (5)

This was prepared as white crystals using the same procedure as **4** in 75% yield from **15**. Mp 212.0–214.0 °C (dec); ^{1}H NMR (400 MHz, D_2O) δ 6.84 (d, *J* 82.4 Hz, 1H), 4.51 (s, 1H), 3.01 (q, 1H, *J* 8.0 Hz), 2.71 (dd, 1H, *J* 16.0, 8.0 Hz), 2.61–2.63 (m, 1H), 2.51 −2.58 (m, 1H), 1.96–2.03 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 180.67, 149.14 (d, *J* 254.5 Hz), 122.07 (d, *J* 7.3 Hz), 51.97, 44.51, 36.37, 32.27 (d, *J* 5.4 Hz); ^{19}F NMR (376 MHz, D_2O) δ −50.47 (d, *J* 82.5 Hz); HRMS (EI) M calcd for $C_7H_{10}NO_2F$ 159.0690, found 159.0688. Anal. Calcd for $C_7H_{11}NO_2FCl$: C, 42.98; H, 5.67; N, 7.16. Found: C, 42.61; H, 5.40; N, 6.95.

4.7. Ethyl 4-methylenyl-1-cyclohexanecarboxylate (17)

To a 500 mL, three-neck flask was added methyltriphenylphosphonium bromide (18.4 g, 51.5 mmol) and anhydrous THF (240 mL) under N_2. At 0 °C, LiHMDS (1.0 M in THF, 56.0 mL, 56.0 mmol) was slowly added over 1 h. The solution was then stirred for 0.5 h before **16** (7.95 g, 46.8 mmol) was slowly added. The resulting solution was warmed to room temperature and stirred overnight. A saturated aqueous ammonium chloride solution (100 mL) was added, THF was evaporated, and the aqueous solution was extracted with EtOAc (3×100 mL). The organic layers were combined, washed with brine (2×100 mL), and dried over anhydrous Na_2SO_4. After being concentrated under reduced pressure, the residue was purified with a silica gel plug (hexanes–EtOAc 7:1) to give a light yellow oil (6.8 g, 87%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.50 (s, 2H), 3.99 (q, 2H, *J* 6.8 Hz), 2.27–2.33 (m, 1H), 2.20 (d, 2H, *J* 13.6 Hz), 1.84–1.95 (m, 4H), 1.44 (dq, 2H, *J* 12.4, 4.4 Hz), 1.11 (t, 3H, *J* 6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.15, 147.46, 107.85, 60.13, 42.58, 33.66, 30.17, 14.25.

4.8. *trans*-Ethyl 3-hydroxy-4-methylenyl-1-cyclohexanecarboxylate (18)

To CH_2Cl_2 (250 mL) was added selenium dioxide (0.30 g, 2.7 mmol) and *tert*-butyl hydroperoxide (5.0–6.0 M solution in decane, 20 mL). A solution of **17** (6.8 g, 40 mmol) in CH_2Cl_2 (100 mL) was slowly added, and the resulting solution was stirred at room temperature for 7 h. After CH_2Cl_2 was evaporated, the solution was concentrated under high vacuum to remove excess *t*-BuOOH and decane. The residue was purified by flash column chromatography (hexanes–EtOAc 3:1) to give a light yellow oil (6.7 g, 90%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.82 (s, 1H), 4.72 (s, 1H), 4.29 (t, 1H, *J* 3.6 Hz), 4.06 (q, 2H, *J* 7.6 Hz), 2.80–2.86 (m, 1H), 2.38 (dt, 1H, *J* 12.4, 4.0 Hz), 2.13 (dt, 1H, *J* 13.6, 4.4 Hz), 1.95–2.00 (m, 1H), 1.87–1.91 (m, 1H), 1.74–1.77 (m, 1H), 1.55 (dq, 1H, *J* 10.8, 4.4 Hz), 1.18 (t, 3H, *J* 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 175.64, 149.01, 109.52, 71.22, 60.55, 37.77, 36.74, 29.92, 29.60, 14.43; HRMS (EI) M calcd for $C_{10}H_{16}O_3$ 184.1094, found 184.1097.

4.9. *cis*-Ethyl 3-(N-Boc-N-ethyloxamate)-4-methylenyl-1-cyclohexanecarboxylate (19)

Compound **18** (0.81 g, 4.4 mmol), triphenylphosphine (1.73 g, 6.6 mmol), and N-Boc ethyl oxamate (1.43 g, 6.59 mmol) were added to anhydrous THF (20 mL) under nitrogen. At 0 °C, DIAD (1.28 mL, 6.50 mmol) was slowly added over 5 min. The solution was then heated to reflux for 36 h. After being cooled, the solution was concentrated under reduced pressure, and the residue

was purified by flash column chromatography (hexanes–EtOAc 5:1) to give the product as a colorless oil (1.08 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.97 (s, 1H), 4.85 (s, 1H), 4.64 (s, 1H), 4.35 (q, 2H, J 7.6 Hz), 4.14 (q, 2H, J 7.6 Hz), 2.44–2.57 (m, 3H), 2.05–2.19 (m, 4H), 1.50 (s, 9H), 1.37 (t, 3H, J 7.6 Hz), 1.26 (t, 3H, J 7.6 Hz); MS (CI) M + H calcd for $C_{19}H_{30}NO_7$ 384, found 384.2, 324.2, 284.2, 167.1.

4.10. *cis*-3-(Boc-amino)-4-methylenyl-1-cyclohexanoic acid (20)

Compound **19** (1.08 g, 4.22 mmol) was dissolved in THF (15 mL) and cooled to 0 °C. An aqueous solution of LiOH (0.40 g in 15 mL water) was added, and the resulting solution was stirred at room temperature for 4 h. THF was evaporated, and the aqueous solution was washed with EtOAc (2 × 10 mL), acidified with HCl (1 N) to pH 1, and extracted with EtOAc (3 × 10 mL). The organic layers were combined and dried over anhydrous Na_2SO_4. After concentration under reduced pressure, the residue was purified by column chromatography (hexanes–EtOAc–HOAc 6:1:0.7) to give a colorless oil (0.37 g, 52%). ^{1}H NMR (400 MHz, CD_3OD) δ 4.99 (s, 1H), 4.77 (s, 1H), 3.99 (d, 1H, J 7.6 Hz), 2.60 (t, 1H, J 12.0 Hz), 2.47–2.50 (m, 1H), 1.96–2.16 (m, 4H), 1.52 (d, 1H, J 4.0 Hz), 1.45 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 178.32, 157.90, 149.10, 106.23, 80.22, 53.54, 44.07, 37.92, 34.81, 31.52, 28.94; HRMS (CI) M + H calcd for $C_{13}H_{22}NO_4$ 256.1543, found 256.1540.

4.11. *cis*-3-Amino-4-methylenyl-1-cyclohexanoic acid, hydrochloride (6)

To **20** (78 mg, 0.31 mmol) was added CH_2Cl_2 (4 mL) and TFA (4 mL). The solution was stirred at room temperature for 4 h. The solvent was then evaporated under reduced pressure, and the residue was dissolved in HCl (1.0 N, 5 mL). After evaporation under high vacuum, HCl (1.0 N, 5 mL) was added and evaporated two more times to give a white solid. The solid was recrystallized from ethanol/ethyl ether to give the product as white crystals (50 mg, 85%). Mp 241.5–243.5 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 5.00 (s, 1H), 4.81 (s, 1H), 3.81 (d, 1H, J 11.6 Hz), 2.71 (dt, 1H, J 12.4, 3.2 Hz), 2.54 (d, 1H, J 14.0 Hz), 2.41 (d, 1H, J 12.4 Hz), 2.14–2.26 (m, 2H), 1.55 (q, 1H, J 12.4 Hz), 1.44 (dq, 1H, J 12.4, 4.4 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 177.23, 145.44, 107.79, 53.14, 42.64, 35.76, 34.62, 31.29; HRMS (EI) M calcd for $C_8H_{13}NO_2$ 155.0941, found 155.0940. Anal. Calcd for $C_8H_{14}NO_2Cl$: C, 50.13; H, 7.37; N, 7.31. Found: C, 49.91; H, 7.00; N, 7.08.

4.12. *cis*-Ethyl 3-(4′-nitrobenzoyloxy)-4-methylenyl-1-cyclohexanecarboxylate (21)

Compound **18** (1.19 g, 6.47 mmol), triphenylphosphine (2.20 g, 8.40 mmol), and *p*-nitrobenzoic acid (1.43 g, 8.56 mmol) were added to anhydrous THF (30 mL) under nitrogen. At 0 °C, DIAD (1.65 mL, 8.38 mmol) was slowly added over 15 min. The solution was then warmed to room temperature and stirred for 36 h. After concentration under reduced pressure, the residue was purified by flash column chromatography (hexanes–EtOAc 7:1) to give the product as a colorless oil (1.25 g, 58%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.29 (dd, 4H, J 22.8, 8.4 Hz), 5.52 (dd, 1H, J 10.4, 4.4 Hz), 4.92 (s, 1H), 4.90 (s, 1H), 4.03–4.17 (m, 2H), 2.66–2.73 (m, 1H), 2.60 (dt, 1H, J 14.0, 3.6 Hz), 2.42 (d, 1H, J 12.4 Hz), 2.21 (dt, 1H, J 12.8, 3.6 Hz), 2.05–2.08 (m, 1H), 1.92 (q, 1H, J 11.6 Hz), 1.66 (dd, 1H, J 12.0, 4.4 Hz), 1.23 (t, 3H, J 7.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.65, 163.38, 150.52, 144.44, 135.44, 130.71, 123.54, 107.07, 74.17, 60.59, 40.96, 35.14, 32.34, 29.45, 14.23; HRMS (EI) M calcd for $C_{17}H_{19}O_6N$ 333.1207, found 333.1205.

4.13. *cis*-Ethyl 3-hydroxy-4-methylenyl-1-cyclohexanecarboxylate (22)

Compound **21** (4.00 g, 21.7 mmol) was dissolved in methanol (100 mL). Sodium azide (5.65 g, 86.9 mmol) was added, and the solution was heated to 40 °C for 5.5 h. Water (100 mL) was added, and methanol was evaporated under reduced pressure. The resulting aqueous solution was extracted with EtOAc (3 × 80 mL). The organic layers were combined, washed with brine (2 × 80 mL), and dried over Na_2SO_4. After being concentrated under reduced pressure, the residue was purified by column chromatography (hexanes–EtOAc 6:1) to give a colorless oil (1.42 g, 64%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.99 (s, 1H), 4.81 (s, 1H), 4.06–4.16 (m, 3H), 2.77 (br s, 1H), 2.56 (tt, 1H, J 10.8, 3.6 Hz), 2.47 (dt, 1H, J 13.2, 2.8 Hz), 2.31 (d, 1H, J 12.4 Hz), 1.97–2.08 (m, 2H), 1.45–1.56 (m, 2H), 1.25 (t, 3H, J 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.73, 149.69, 105.44, 71.17, 60.66, 42.01, 38.70, 32.62, 29.98, 14.28; HRMS (EI) M calcd for $C_{10}H_{16}O_3$ 184.1094, found 184.1091.

4.14. *trans*-Ethyl 3-(N-Boc-N-ethyloxamate)-4-methylenyl-1-cyclohexanecarboxylate (23)

This was prepared using the same procedure as **19** in 55% yield from **22**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.49 (s, 1H), 4.74 (s, 1H), 4.53 (s, 1H), 4.28 (q, 2H, J 7.2 Hz), 4.09 (q, 2H, J 7.6 Hz), 2.84 (s, 1H), 2.43 (td, 1H, J 12.0, 5.6 Hz), 2.33 (d, 1H, J 15.2 Hz), 2.12–2.21 (m, 2H), 1.94–1.98 (m, 2H), 1.43 (s, 9H), 1.30 (t, 3H, J 7.2 Hz), 1.19 (t, 3H, J 7.2 Hz); MS (CI) M + H calcd for $C_{19}H_{30}NO_7$ 384, found 384.2, 324.2, 284.2, 167.1.

4.15. *trans*-3-(Boc-amino)-4-methylenyl-1-cyclohexanoic acid (24)

This was prepared using the same procedure as **20** in 36% yield from **23**. ^{1}H NMR (400 MHz, CD_3OD) δ 4.79 (s, 1H), 4.75 (s, 1H), 4.20 (s, 1H), 2.78 (t, 1H, J 4.4 Hz), 2.30–2.34 (m, 1H), 2.20–2.25 (m, 1H), 2.00–2.05 (m, 1H), 1.85–1.89 (m, 1H), 1.71–1.78 (m, 2H), 1.45 (s, 9H); ^{13}C NMR (100 MHz, CD_3OD) δ 178.58, 157.86, 149.30, 108.25, 80.22, 52.59, 40.04, 36.40, 31.94, 30.64, 28.92; HRMS (CI) M + H calcd for $C_{13}H_{22}NO_4$ 256.1543, found 256.1541.

4.16. *trans*-3-Amino-4-methylenyl-1-cyclohexanoic acid, hydrochloride (7)

This was prepared using the same procedure as **6** in 80% yield from **24**. Mp 203.0–205.0 °C; ^{1}H NMR (400 MHz, CD_3OD) 5.08 (s, 1H), 4.90 (s, 1H), 4.01–4.04 (m, 1H), 2.91 (t, 1H, *J* 4.8 Hz), 2.38–2.43 (m, 1H), 2.25–2.36 (m, 2H), 2.01–2.05 (m, 1H), 1.78–1.84 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 177.18, 145.09, 110.48, 52.36, 39.36, 34.30, 31.89, 30.22; HRMS (EI) M calcd for $C_8H_{13}NO_2$ 155.0941, found 155.0940. Anal. Calcd for $C_8H_{14}NO_2Cl$: C, 50.13; H, 7.37; N, 7.31. Found: C, 49.95; H, 7.07; N, 7.00.

4.17. Time-dependent inhibition of GABA-AT by 4 and 5

GABA-AT was isolated and purified from pig brain by a modified procedure.[29] Incubation solutions (100 μL) contained the enzyme (20 μL, 1.84 mg/mL), potassium pyrophosphate buffer (60 μL, 50 mM, pH 8.5), α-ketoglutarate (10 μL, 16 mM in 50 mM potassium pyrophosphate buffer, pH 8.5), 2-mercaptoethanol (2 mM), and **4** or **5**. The concentrations of **4** and **5** were 100, 125, 250, and 500 μM. At timed intervals, aliquots (20 μL) from the incubation solution were added to the assay solution (575 μL, 50 mM potassium pyrophosphate buffer containing 5.3 mM of α-ketoglutarate, 11 mM of GABA, 1.1 mM of $NADP^+$, and 4.8 mM of 2-mercaptoethanol) with excess succinic semialdehyde dehydrogenase (SSDH). Rates were measured spectrophotometrically at 340 nm, and the logarithm of the remaining activity (percentage) was plotted against time for each concentration to determine the half-life. Then a secondary plot of half-life versus the reciprocal of the inhibitor concentration was obtained to determine the k_{inact}/K_i.

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