Available online at www.sciencedirect.com
SCIENCE DIRECT
Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 14 (2006) 2242–2252

Syntheses and evaluation of fluorinated conformationally restricted analogues of GABA as potential inhibitors of GABA aminotransferase

Zhiyong Wang[†] and Richard B. Silverman[*]

Department of Chemistry, Department of Biochemistry, Molecular Biology, and Cell Biology, and the Center for Drug Discovery and Chemical Biology, Northwestern University, Evanston, IL 60208-3113, USA

Received 11 October 2005; revised 1 November 2005; accepted 2 November 2005
Available online 28 November 2005

Abstract—Inhibition of γ-aminobutyric acid aminotransferase (GABA-AT) could raise the concentration of GABA, an inhibitory neurotransmitter in the human brain, and could have therapeutic applications for a variety of neurological diseases including epilepsy. Four fluorine-containing analogues of GABA with conformations restricted by a cyclohexane ring system were designed and synthesized, but unlike some of their five-membered ring counterparts, minimal inhibition of GABA-AT was observed. It is likely that the rigid chair conformation of these compounds cannot be accommodated well in the enzyme's active site.

1. Introduction

γ-Aminobutyric acid aminotransferase[1] (GABA-AT, E.C. 2.6.1.19) is the enzyme responsible for the degradation of γ-aminobutyric acid (GABA), one of the major inhibitory neurotransmitters in the mammalian central nervous system,[2] to succinic semialdehyde. Inhibition of this enzyme results in an increased concentration of GABA in the brain and could have therapeutic applications in neurological disorders including epilepsy,[3] Parkinson's disease,[4] Huntington's chorea,[5] and Alzheimer's disease.[6] It has also been found that an increase in the availability of GABA can block the effects of drug addiction.[7]

GABA-AT utilizes pyridoxal 5′-phosphate (PLP) as its cofactor, and one general strategy to design mechanism-based inactivators of this type of enzyme is to incorporate a good leaving group in the β-position of an amino acid that acts as a substrate for the targeted enzyme.[8] To this end, 4-amino-5-fluoropentanoic acid (**1**, Fig. 1) was synthesized and found to be a potent irreversible inhibitor of GABA-AT.[9]

Restriction of conformation is a strategy that has been widely used in modern drug design to create potent and selective enzyme inhibitors, especially in the case of peptides.[10–12] 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] A series of five-membered ring analogues of **1** were prepared in our laboratory, and the best among them, **2**, was

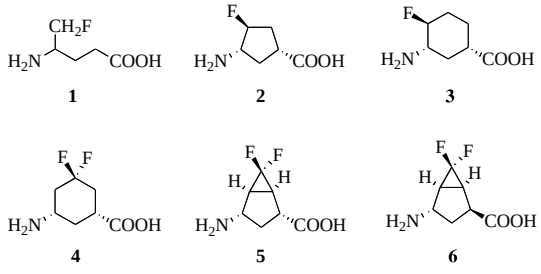


Figure 1. Fluorinated analogues of GABA.

Keywords: GABA; GABA-AT; Enzyme inhibition; Fluorinated compounds; Conformationally restricted compounds.
* Corresponding author. Tel.: +1 847 491 5653; fax: +1 847 491 7713; e-mail: Agman@chem.northwestern.edu
† Present address: Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA.

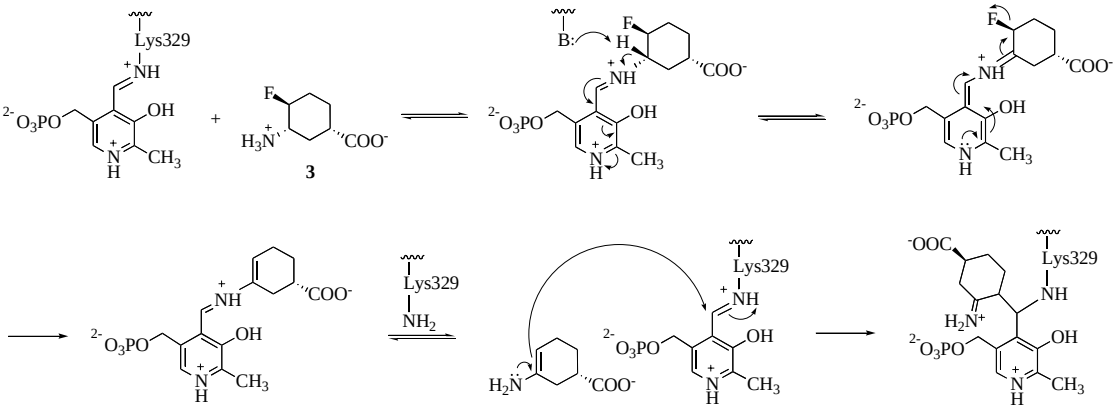
0968-0896/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2005.11.010

found to be 17 times less potent toward GABA-AT than **1**.[14] Because the inactivation mechanisms require strict orbital overlap, constraint of **1** with a cyclopentane ring seems to have had a detrimental effect on the inactivation rate. A cyclohexane ring, however, has a distinct preferred chair conformation, which might be better for binding to GABA-AT. Compounds **3**–**6** were thus designed as potential mechanism-based inactivators of GABA-AT; each of them has a unique inactivation mechanism. Compound **3** could inactivate the enzyme through an enamine mechanism (Scheme 1), similar to those reported for **1**[9b] and **2**.[14b]

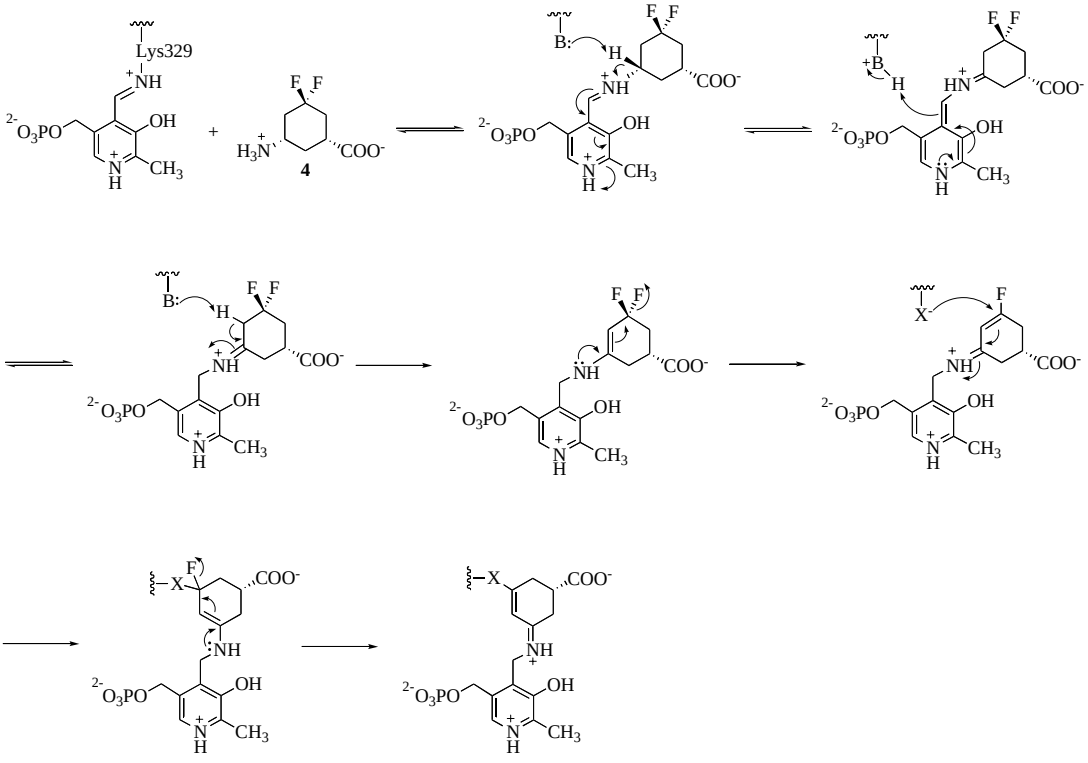
The difluorinated **4** could potentially inactive GABA-AT through an unprecedented novel mechanism (Scheme 2), and **5** could inactivate through an aromatization mechanism by covalently modifying the PLP cofactor of the enzyme (Scheme 3), reminiscent of the inactivation of GABA-AT by gabaculine.[15] *Trans* compound **6** was expected to be a reversible inhibitor of GABA-AT, in accordance with our previous experience with the cyclopentane series of compounds.[14,16]

2. Results and discussion

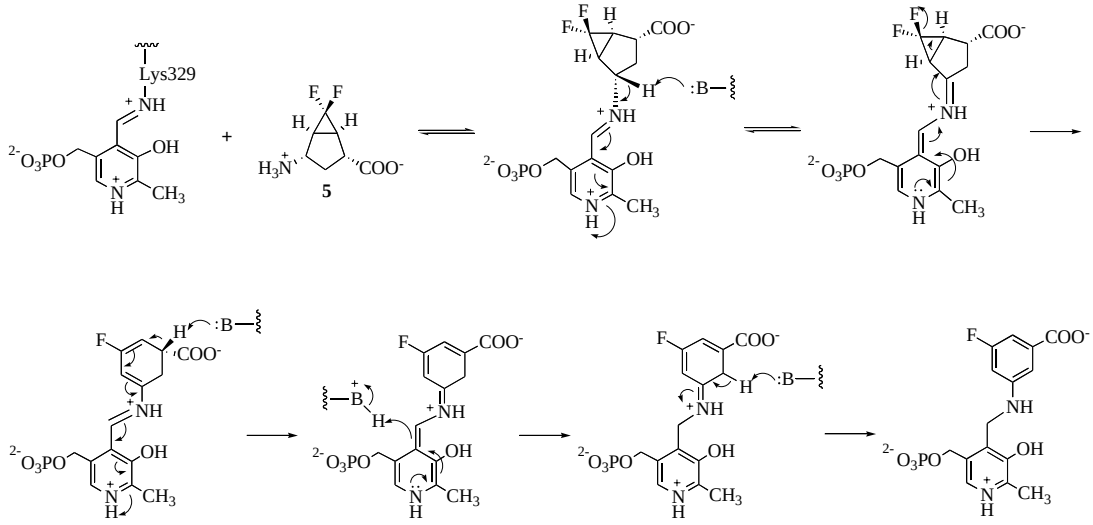
Compound **3** was synthesized in racemic form from **7** (Scheme 4). High-pressure hydrogenation of **7** with rhodium on alumina[17] followed by amino acid protection gave **10**, which was treated with (diethylamino)sulfur trifluoride (DAST)[18] to afford **12**. An elimination byproduct (**11**) was also formed in an equal amount. The *trans*



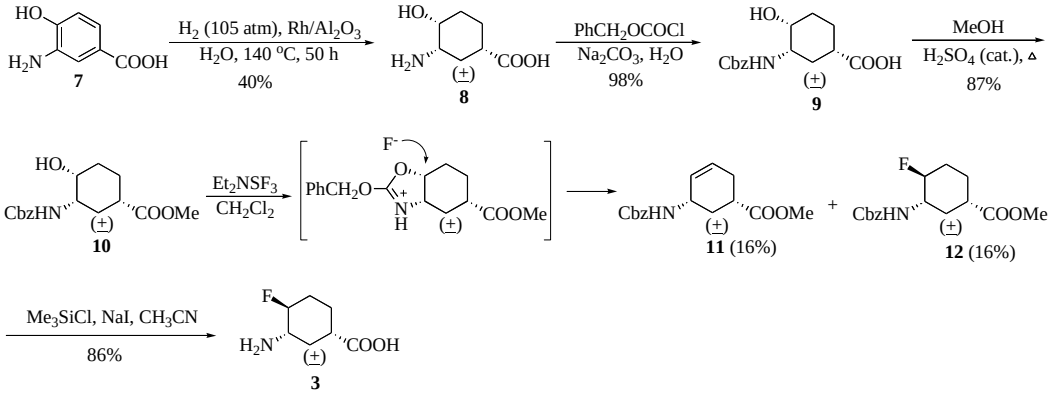
Scheme 1. Potential inactivation mechanism of GABA-AT by **3**.



Scheme 2. Potential inactivation mechanism of GABA-AT by **4**.



Scheme 3. Potential inactivation mechanism of GABA-AT by **5**.

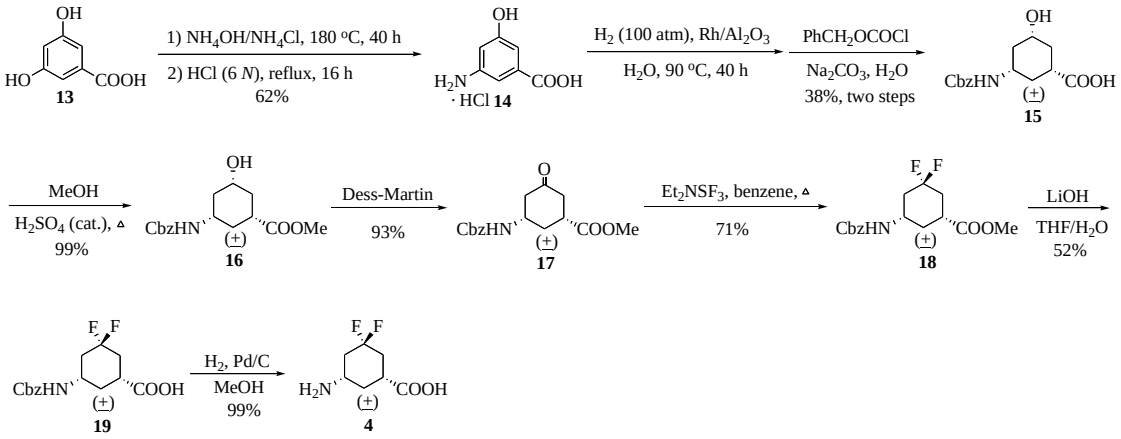


Scheme 4. Synthesis of **3**.

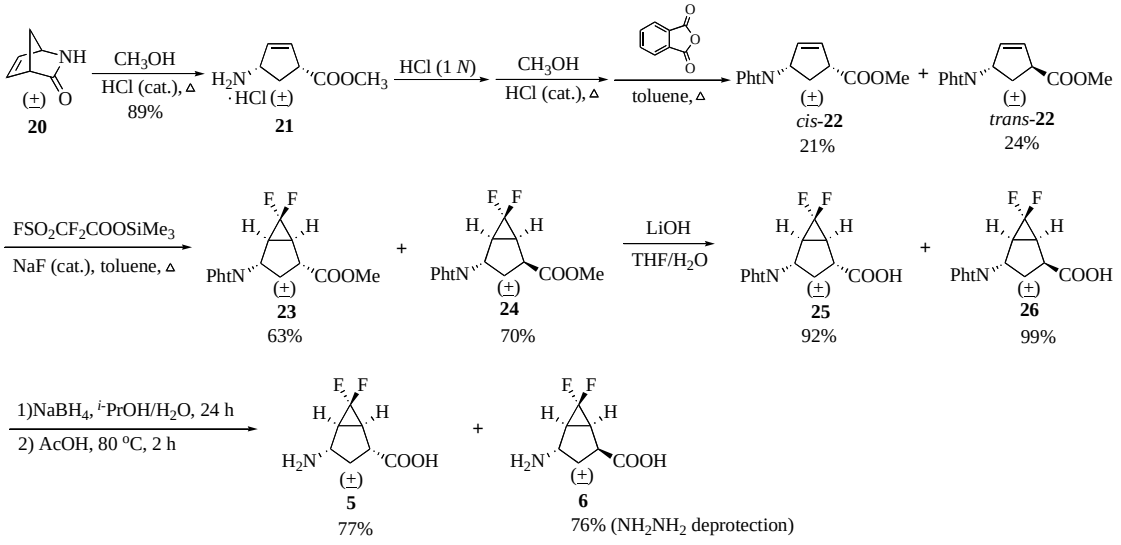
relationship of the fluorine and carbamate groups in **12** was supported by 1D NOESY NMR analysis, as irradiation of H-3 resulted in a nuclear Overhauser effect (NOE)[19] of 1.6% for the Cbz benzyl and only 0.2% for H-4. Likely participation of the neighboring group (benzyloxycarbamate) was involved in the fluorination step.[20] The Cbz-protecting group is likely causing the low yield, and a phthalimide-protecting group might be desired for large-scale preparations. One-pot deprotection of **12** with trimethylsilyl iodide (TMSI) generated in situ[21] then afforded **3**.

Compound **4** was synthesized by a similar strategy (Scheme 5). Direct amination of **13** afforded **14**,[22] which was hydrogenated and protected as above to give **16**. Oxidation was effected by Dess–Martin periodinane[23] and fluorination of **17** followed by deprotection afforded **4**.

Compounds **5** and **6** were synthesized from a common starting material (**20**, Scheme 6). Direct protection of the amino group in **21** as a phthalimide gave *cis*-**22** in 58% yield, and it was accidentally discovered that hydrolysis of **21** under acidic conditions caused epimerization at C-1, whereas no epimerization took place if **20** were directly hydrolyzed under heating acidic conditions. Thus, a mixture of *cis* and *trans*-**22** could be prepared from the same starting material (**20**), and they were easily separated by flash chromatography. Our preparation of *cis* and *trans*-**22** from **20** is milder than the procedures developed by Allan and Fong,[24] in which **20** is directly hydrolyzed to the corresponding amino acid and then allowed to react with phthalic anhydride at over 200 °C to effect epimerization. Addition of difluorocarbene[25] to **22** turned out to be a sluggish reaction, and the *gem*-difluorocyclopropane was formed exclusively *trans* to the phthalimide group due to steric effects. Even when excessive reagents were employed, the reaction could not be driven to completion. Thus, the reaction was worked up and chromatographed to give an inseparable mixture of starting material and product, which was resubjected to the reaction. This process was repeated three times to give about 90% conversion of the starting material, and the remaining **22** was oxidized by $KMnO_4$ to give the corresponding dicarboxylic acid, which could be easily removed from the mixture by basic washing. Hydrolysis and deprotec-



Scheme 5. Synthesis of **4**.



Scheme 6. Syntheses of **5** and **6**.

tion then afforded **5** and **6**; hydrazine[26] was found to be better than sodium borohydride[27] at unmasking phthalimide for larger-scale reactions.

Enzymatic testing showed that neither **3** nor **4** was an inhibitor of GABA-AT at a concentration of 10 mM. Also no substrate activity was detected. It is surprising that these fluorinated compounds completely lose their inhibitory activity toward GABA-AT in going from a five-membered ring (**2**) to a six-membered ring (**3** and **4**). Possibly the rigid chair conformation of the cyclohexane ring is not good for binding to the active site of GABA-AT, as evidenced by the lack of substrate activity for **3** and **4**.

To see whether the conformation of the cyclohexane ring is solely responsible for poor binding of **3** and **4** to GABA-AT, the non-fluorinated 3-aminocyclohexanecarboxylic acid (purchased from TCI America as a 1.6:1 mixture of *cis* and *trans* isomers) was tested against GABA-AT. Neither substrate nor inhibitor activity was found. Although its *cis* isomer is a substrate for the neuronal GABA uptake process,[28] both isomers apparently do not fit appropriately into the active site of GABA-AT, which is known to be very narrow.[29]

Enzymatic testing of **5** and **6** showed a similarly weak reversible inhibitory activity ($K_i > 10$ mM) toward GABA-AT, but neither time-dependent inactivator nor substrate activity was found. The rigid bicyclic structure of these molecules, apparently, is also not accommodated well in the active site of GABA-AT.

In conclusion, four fluorinated conformationally rigid analogues of GABA (**3**–**6**) were synthesized. Compounds **3** and **4** have no inhibitory activity toward GABA-AT, and **5** and **6** were found to be poor reversible inhibitors. Unlike their five-membered ring analogues, these compounds bind poorly to GABA-AT. Because all of our proposed mechanisms rely on them to bind to GABA-AT and act as substrates, it is likely that the initial α-proton removal did not take place, preventing the following inactivation steps that lead to the covalent attachment of either an enzyme active-site res-

idue (Schemes 1 and 2) or its cofactor (Scheme 3) to the inactivator from occurring. Our results suggest that a saturated six-membered ring system is disfavored for binding to the crowded active site of GABA-AT, and this information will be of help for the future design of conformationally restricted inhibitors of GABA-AT.

Testing of compounds **3**–**6** with the related GABA transport and receptor systems is currently under investigation. These compounds could also serve as novel fluorinated amino acid building blocks.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 400 MHz and Inova 500 MHz NMR spectrometers. Chemical shifts are reported as δ values in parts per million (ppm) as referenced to chloroform (7.27 ppm for ^{1}H and 77.23 ppm for ^{13}C) or to methanol (4.87 ppm for CD_3OH and 49.15 ppm for ^{13}C). For compounds that are soluble only in deuterium oxide, the ^{1}H chemical shifts are referenced to DOH (4.80 ppm), and the ^{13}C chemical shifts are referenced to an external standard of 3-(trimethylsilyl)-1-propanesulfonic acid-d_6 sodium salt (from Aldrich) in deuterium oxide. All ^{19}F chemical shifts are referenced to an external standard of fluorotrichloromethane in deuterated chloroform. Mass spectra were obtained on Finnigan MAT900XL (EI) and VG70-250SE (ESI) mass spectrometers in the Analytical Service Laboratory at Northwestern University and on a 70-SE-4F mass spectrometer (FAB) in the Mass Spectrometry Laboratory at University of Illinois. Elemental analyses were performed by Atlantic Microlab, Inc. Flash chromatography was carried out with standard silica gel (230-400 mesh) from Sorbent Technologies, Inc. TLC was run with EM Science silica gel 60 F254 precoated glass plates. Cation exchange chromatography was carried out with Dowex 50WX8-200 ion-exchange resin (100–200 mesh). Melting points were measured on a Buchi B-540 melting point apparatus and are uncorrected. All reactions involving moisture sensitive reagents were conducted in oven-dried glassware under a nitrogen atmosphere. Enzyme assays were recorded on a Perkin-Elmer Lambda 10 UV/vis spectrophotometer. Radioactivity was measured by liquid scintillation counting using a Packard Tri-Carb 2100TR counter and Packard Ultima Gold XR scintillation cocktail.

All common reagents and solvents were purchased from either Aldrich Chemical Co. or Fisher Scientific without further purification, except for anhydrous ether and tetrahydrofuran, which were distilled over sodium metal under nitrogen, and anhydrous dichloromethane, which was distilled over calcium hydride.

3-Aminocyclohexanecarboxylic acid was purchased from TCI America and tested against GABA-AT as received. The *cis*/*trans* ratio (1.6:1) was determined by ^{1}H NMR integration.[30]

3.2. Chemistry

3.2.1. (*cis*,*cis*)-3-Amino-4-hydroxycyclohexanecarboxylic acid (8). A solution of **7** (1.5 g, 9.8 mmol) and 5 wt% rhodium on alumina (0.2 g) in water (60 mL) was hydrogenated in a Parr apparatus for 50 h at 140 °C and 105 atm. The mixture was cooled to room temperature and filtered to give a clear solution. Upon evaporation of solvent at reduced pressure a red oil was obtained, which was crystallized in methanol and washed with acetone to yield an off-white solid (0.62 g, 40%); mp 237.2–240.0 °C. ^{1}H NMR (D_2O, 400 MHz) δ 1.54–1.80 (m, 4H, H-5 and H-6), 1.91–1.95 (m, 2H, H-2), 2.26–2.33 (tt, 1H, J = 11.6 Hz, 3.6 Hz, H-1), 3.33–3.38 (dt, 1H, J = 11.6 Hz, 3.6 Hz, H-3), 4.07 (s, 1H, H-4). ^{13}C NMR (D_2O, 100 MHz) δ 22.60 (C-6), 27.54 (C-2), 30.11 (C-5), 44.14 (C-1), 52.17 (C-3), 64.99 (C-4), 183.52 (COOH). HRMS (ESI) calcd for $C_7H_{14}NO_3$ $(M+H)^+$ 160.0974, found 160.0969. Irradiation of H-3 resulted in a NOE of 3.9% and 3.3% for H-1 and H-4, respectively. Thus, the three functional groups on the cyclohexane ring are *cis* to each other.

3.2.2. (*cis*,*cis*)-3-(Benzyloxycarbonylamino)-4-hydroxycyclohexanecarboxylic acid (9). To a solution of **8** (0.52 g, 3.3 mmol) in sodium carbonate solution (20 mL, pH 11) was added dropwise benzyl chloroformate (0.7 mL, 5.0 mmol), and the mixture was stirred at room temperature overnight. The solution was then extracted with ethyl acetate (2 × 20 mL), and the organic phase was discarded. The separated aqueous phase was then adjusted to pH 1 with concentrated hydrochloric acid, extracted with ethyl acetate (3 × 20 mL), washed with brine (20 mL), dried with sodium sulfate, evaporated, and crystallized from ethyl acetate/hexanes to afford an off-white solid (0.94 g, 98%); mp 159.0–160.0 °C. ^{1}H NMR (CD_3OD, 400 MHz) δ 1.53–1.90 (m, 6H, ring CH_2), 2.34–2.42 (m, 1H, H-1), 3.51–3.54 (d, 1H, J = 10.8 Hz, H-3), 3.90 (s, 1H, H-4), 5.06 (s, 2H, $PhCH_2O$), 7.27–7.34 (m, 5H, ArH). ^{13}C NMR (CD_3OD, 100 MHz) δ 23.10 (C-6), 29.82 (C-5), 32.36 (C-2), 43.32 (C-1), 54.00 (C-3), 67.59 ($PhCH_2O$), 67.89 (C-4), 129.02 (Ar), 129.12 (Ar), 129.60 (Ar), 138.48 (Ar), 158.20 (OCONH), 178.81 (COOH). HRMS (CI) calcd for $C_{15}H_{20}NO_5$ $(M+H)^+$ 294.1336, found 294.1331; calcd for $C_{15}H_{18}NO_4$ $(M-OH)^+$ 276.1230, found 276.1231.

3.2.3. (*cis*,*cis*)-Methyl 3-(benzyloxycarbonylamino)-4-hydroxycyclohexanecarboxylate (10). To a solution of **9** (0.91 g, 3.1 mmol) in anhydrous methanol (30 mL) was added concentrated sulfuric acid (0.1 mL), and the solution was heated at reflux for 24 h. The solvent was evaporated at reduced pressure, and the residue was redissolved in ethyl acetate (60 mL), washed with saturated sodium carbonate (20 mL) and brine (20 mL), dried over sodium sulfate, evaporated, and washed with 1:7 of ethyl acetate and hexanes to afford a white solid (0.83 g, 87%); mp 115.0–116.5 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.57–1.60 (m, 1H, ring CH_2), 1.70–1.77 (m, 3H, ring CH_2), 1.88–1.97 (m, 2H, ring CH_2), 2.23 (br s, 1H, OH), 2.41 (m, 1H, H-1), 3.66 (br s, 4H, $COOCH_3$ and H-3), 3.98 (s, 1H, H-4), 5.08 (s, 2H, $PhCH_2O$), 5.43–5.45 (d, 1H, J = 7.5 Hz, NH), 7.34 (m,

5H, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.77 (C-6), 28.94 (C-5), 31.32 (C-2), 41.74 (C-1), 51.95 (C-3), 52.11 ($COOCH_3$), 66.92 (C-4), 67.48 ($PhCH_2O$), 128.31 (Ar), 128.70 (Ar), 136.58 (Ar), 156.06 (OCONH), 175.41 (COOMe). HRMS (EI) calcd for $C_{16}H_{21}NO_5$ (M^+) 307.1414, found 307.1421.

3.2.4. (*cis*)-Methyl 5-(benzyloxycarbonylamino)cyclohex-3-enecarboxylate (11) and (±)-(1*S*,3*S*,4*S*)-methyl 3-(benzyloxycarbonylamino)-4-fluorocyclohexanecarboxylate (12). (Diethylamino)sulfur trifluoride (0.53 mL, 4.0 mmol) was added dropwise over 10 min to a solution of **10** (0.61 g, 2.0 mmol) in anhydrous methylene chloride (50 mL) cooled in a dry ice-acetone bath. The solution was stirred further at −78 °C for 2 h before it was allowed to rise to room temperature and stirred for an additional 18 h. Water (10 mL) was then added, and the organics were washed with saturated sodium carbonate (2 × 10 mL) and brine (2 × 10 mL). Drying with sodium sulfate and evaporation of solvent afforded a yellow oil, which was chromatographed (ethyl acetate/hexanes, 1:5) to give **11** (91 mg, 16%) and **12** (0.1 g, 16%) both as white solids.

For **11**: R_f = 0.30 (ethyl acetate/hexanes, 1:3); mp 67.0–69.0 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.50–1.59 (q, 1H, J = 11.1 Hz, H-2), 2.27 (m, 2H, H-6), 2.36–2.39 (d, 1H, J = 11.6 Hz, H-2), 2.71–2.73 (m, 1H, H-1), 3.67 (s, 3H, $COOCH_3$), 4.36 (m, 1H, H-3), 4.92–4.94 (d, 1H, J = 8.8 Hz, NH), 5.10 (s, 2H, $PhCH_2O$), 5.58–5.60 (d, 1H, J = 9.2 Hz, H-4), 5.78–5.80 (m, 1H, H-5), 7.28–7.41 (m, 5H, ArH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.35 (C-2), 32.30 (C-6), 38.42 (C-1), 47.45 (C-3), 52.00 (CH_3), 66.82 ($PhCH_2O$), 128.07 (Ar), 128.21 (Ar), 128.57 (Ar), 128.61 (C-4 and C-5), 136.56 (Ar), 155.87 (OCONH), 175.25 (COOMe). HRMS (EI) calcd for $C_{16}H_{19}NO_4$ (M^+) 289.1309, found 289.1308.

For **12**: white needle crystallized from ethyl acetate/hexanes; R_f = 0.25 (ethyl acetate/hexanes, 1:3); mp 92.0–93.3 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.42–1.56 (m, 2H, ring CH_2), 1.59–1.65 (t, 1H, J = 11.8 Hz, ring CH_2), 2.05–2.08 (m, 1H, ring CH_2), 2.18–2.24 (m, 1H, ring CH_2), 2.37 (m, 1H, ring CH_2), 2.44–2.50 (t, 1H, J = 11.6 Hz, H-1), 3.63–3.73 (m, 1H, H-3), 3.68 (s, 3H, $COOCH_3$), 4.20–4.32 (dm, 1H, J = 50.0 Hz, H-4), 4.99 (br s, 1H, NH), 5.11 (s, 2H, $PhCH_2O$), 7.31–7.37 (m, 5H, ArH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 26.18–26.28 (d, J = 11.0 Hz, C-6), 30.08–30.27 (d, J = 19.2 Hz, C-5), 33.30 (C-2), 41.12 (C-1), 52.16 ($COOCH_3$), 53.64–53.85 (d, J = 20.2 Hz, C-3), 67.08 ($PhCH_2O$), 91.81–93.60 (d, J = 180.3 Hz, C-4), 128.31 (Ar), 128.68 (Ar), 136.47 (Ar), 156.05 (OCONH), 174.59 (COOMe). ^{19}F NMR ($CDCl_3$, 376 MHz) δ −182.06 to −181.92 (d, 1F, J = 48.9 Hz). HRMS (EI) calcd for $C_{16}H_{20}NO_4F$ (M^+) 309.1371, found 309.1372. Irradiation of H-4 resulted in a NOE of 1.6% and 0.1% for Cbz benzyl and H-3, respectively. Thus, H-4 should be *trans* to H-3.

3.2.5. (±)-(1*S*,3*S*,4*S*)-3-Amino-4-fluorocyclohexanecarboxylic acid (3). To a mixture of **12** (38 mg, 0.12 mmol) and sodium iodide (99 mg, 0.66 mmol) in anhydrous acetonitrile (2 mL) was added dropwise chlorotrimethylsilane (0.1 mL, 0.8 mmol), and the mixture was heated at reflux for 9 h. Water (20 mL) was then added, and the solution was extracted with dichloromethane (3 × 10 mL) to give a colorless aqueous layer, which was evaporated at reduced pressure to give a white solid. The crude material was then loaded onto a short column containing Dowex 50WX8-200 ion-exchange resin (hydrogen form, 2 g), which had been pre-eluted with 1 M pyridine (80 mL) and water (100 mL). The column was washed with water (100 mL) and then the product was eluted with 1 M pyridine (100 mL) to give a white powder (21 mg, 86%); mp 240.0–243.5 °C. ^{1}H NMR (D_2O, 500 MHz) δ 1.56–1.63 (q, 1H, J = 13.2 Hz, ring CH_2), 1.71–1.79 (q, 2H, J = 12.3 Hz, ring CH_2), 2.18–2.21 (dm, 1H, J = 13.0 Hz, ring CH_2), 2.35–2.45 (m, 2H, ring CH_2), 2.66–2.71 (t, 1H, J = 12.2 Hz, H-1), 3.51–3.55 (m, 1H, H-3), 4.64–4.67 (dt, 0.5H, J = 10.1 Hz, 4.8 Hz, H-4; the other half of the peaks was obscured by the water peak). ^{13}C NMR (D_2O, 125 MHz) δ 28.42 (C-6), 31.88 (C-5), 32.81 (C-2), 43.02 (C-1), 55.74–55.89 (d, J = 18.4 Hz, C-3), 94.02–95.40 (d, J = 173.3 Hz, C-4), 180.71 (COOH). ^{19}F NMR (D_2O, 376 MHz) δ −182.85 to −182.72 (d, 1F, J = 50.4 Hz). HRMS (ESI) calcd for $C_7H_{13}NO_2F$ $(M+H)^+$ 162.0930, found 162.0925. Anal. Calcd for $C_7H_{12}NO_2F{\cdot}0.4H_2O$: C, 49.93; H, 7.66; N, 8.32. Found: C, 49.81; H, 7.48; N, 8.21.

3.2.6. 3-Amino-5-hydroxybenzoic acid hydrochloride (14). The procedures by Becker and Rickards[22] were followed. A mixture of **13** (12.0 g, 0.078 mol), ammonium chloride (10.2 g, 0.191 mol), and aqueous ammonium hydroxide (14.8 N, 36.0 mL, 0.533 mol) was heated in a steel bomb at 180 °C for 65 h. The mixture was evaporated to dryness, and the residue was taken up in hydrochloric acid (6 N, 120 mL) and refluxed for 16 h. The reaction mixture was then cooled to room temperature and filtered, and the filtrate was extracted with ethyl acetate (3 × 40 mL), from which was obtained, after evaporation of solvent, a gray solid as the unreacted starting material (4.7 g). The separated aqueous phase was evaporated to dryness, taken up in hot methanol (100 mL), filtered, and evaporated to give a red solid. Water (20 mL) was added, and the solution was loaded onto an ion-exchange column comprised of a Dowex AG 50WX8-200 hydrogen form resin (20 g). The column was washed with water (200 mL), then it was eluted with hydrochloric acid (0.3 N) to afford a white solid (5.6 g, 62% based on converted starting material); mp 269–272 °C (dec). ^{1}H NMR (CD_3OD, 400 MHz) δ 6.98 (m, 1H, H-4), 7.41–7.44 (m, 2H, H-2 and H-6). ^{13}C NMR (CD_3OD, 100 MHz) δ 115.44 (C-6), 115.77 (C-4), 118.14 (C-2), 133.23 (C-3), 135.27 (C-1), 160.51 (C-5), 168.00 (COOH).

3.2.7. (*cis*,*cis*)-3-(Benzyloxycarbonylamino)-5-hydroxycyclohexanecarboxylic acid (15). A mixture of **14** (1.89 g, 10.0 mmol) and 5 wt% rhodium on alumina (0.2 g) in water (70 mL) was hydrogenated in a Parr apparatus for 40 h at 90 °C and 100 atm. The mixture was cooled to room temperature and filtered to give a clear solution, which was directly used without isolation of the saturated amino acid. The aqueous solution was

then adjusted to pH 9 with saturated sodium carbonate and cooled in an ice bath before benzyl chloroformate (2.14 mL, 15.0 mmol) was added dropwise over 30 min. The mixture was allowed to rise to room temperature over 30 min, and it was stirred further at room temperature for 4 h. The solution was then extracted with ethyl acetate (2 × 30 mL), and the organic phase was discarded. The separated aqueous phase was then adjusted to pH 1 with concentrated hydrochloric acid, extracted with ethyl acetate (3 × 30 mL), washed with brine (30 mL), dried with sodium sulfate, and evaporated to give a semisolid, which was washed with a 1:2 mixture of ethyl acetate and hexanes, and filtered to afford a white powder (1.10 g, 38% for two steps); mp 137.0–140.0 °C. ^{1}H NMR (CD_3OD, 500 MHz) δ 1.10–1.26 (m, 3H, ring CH_2), 2.08–2.14 (m, 3H, ring CH_2), 2.36–2.41 (tm, J = 12.5 Hz, 1H, H-1), 3.46–3.50 (m, 1H, H-3), 3.59–3.63 (m, 1H, H-5), 5.04 (s, 2H, $PhCH_2O$), 7.14–7.32 (m, 5H, ArH). ^{13}C NMR (CD_3OD, 125 MHz) δ 35.78 (C-2), 38.40 (C-6), 40.73 (C-4), 42.39 (C-1), 67.47 ($PhCH_2O$), 69.04 (C-5), 128.92 (Ar), 129.10 (Ar), 129.60 (Ar), 138.53 (Ar), 158.20 (OCONH), 178.14 (COOH). HRMS (CI) calcd for $C_{15}H_{20}NO_5$ $(M+H)^+$ 294.1336, found 294.1329.

3.2.8. (*cis*,*cis*)-Methyl 3-(benzyloxycarbonylamino)-5-hydroxycyclohexanecarboxylate (16). The title compound was prepared as a white solid (0.24 g, quantitative) from **15** (0.21 g, 0.7 mmol) using the same conditions as described for **10**; mp 96.5–97.8 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.11–1.37 (3 q, J = 11.5 Hz, 3H, ring CH_2), 2.17–2.19 (m, 3H, ring CH_2), 2.39–2.40 (m, 1H, H-1), 2.93 (br s, 1H, OH), 3.57–3.70 (m, 2H, H-3 and H-5), 3.65 (s, 3H, $COOCH_3$), 5.06 (s, 2H, $PhCH_2O$), 5.14–5.15 (d, J = 7.5 Hz, 1H, NH), 7.32 (s, 5H, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 34.53 (C-2), 36.82 (C-6), 39.23 (C-4), 41.45 (C-1), 47.66 (C-3), 53.00 ($COOCH_3$), 66.82 (C-5), 67.96 ($PhCH_2O$), 128.25 (Ar), 128.28 (Ar), 128.66 (Ar), 131.51 (Ar), 155.76 (OCONH), 174.89 (COOMe). HRMS (EI) calcd for $C_{16}H_{21}NO_5$ (M^+) 307.1414, found 307.1420.

3.2.9. (*cis*)-Methyl 3-(benzyloxycarbonylamino)-5-oxocyclohexanecarboxylate (17). A mixture of **16** (0.15 g, 0.5 mmol) and Dess–Martin periodinane (0.29 g, 0.7 mmol) in methylene chloride (15 mL) was stirred at room temperature for 16 h. The solvent was removed under reduced pressure, and the residue was redissolved in ethyl acetate (50 mL). A mixed solution of sodium bicarbonate (5%, 5 mL) and sodium thiosulfate (1 M, 5 mL) was added, and the mixture was stirred at room temperature for 10 min. The two phases were separated, and the organics were washed with sodium hydroxide (1 N, 2 × 10 mL) and brine (2 × 10 mL), dried with sodium sulfate, and evaporated to afford white flakes (0.14 g, 93%); mp 113.0–114.5 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.71–1.78 (q, 1H, J = 11.5 Hz, ring CH_2), 2.21–2.26 (t, 1H, J = 12.5 Hz, ring CH_2), 2.45–2.47 (d, 2H, J = 13.5 Hz, ring CH_2), 2.56–2.59 (d, 1H, J = 13.5 Hz, H-1), 2.70–2.72 (d, 2H, J = 11.5 Hz, ring CH_2), 3.69 (s, 3H, $COOCH_3$), 3.89 (br s, 1H, H-3), 5.08 (s, 2H, $PhCH_2O$), 5.12 (s, 1H, NH), 7.33 (s, 5H, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 34.38 (C-2), 39.22 (C-6), 42.48 (C-1), 47.43 (C-4), 48.68 (C-3), 52.47 ($COOCH_3$), 67.03 ($PhCH_2O$), 128.34 (Ar), 128.44 (Ar), 128.73 (Ar), 136.29 (Ar), 155.38 (OCONH), 173.75 (COOMe), 205.94 (C-5). HRMS (EI) calcd for $C_{16}H_{19}NO_5$ (M^+) 305.1258, found 305.1256.

3.2.10. (*cis*)-Methyl 3-(benzyloxycarbonylamino)-5,5-difluorocylcohexanecarboxylate (18). (Diethylamino)sulfur trifluoride (0.15 mL, 1.1 mmol) was added dropwise to a solution of **17** (0.115 g, 0.38 mmol) in anhydrous benzene (15 mL), and the solution was heated at reflux for 18 h. The reaction mixture was cooled to room temperature, diluted in ethyl acetate (40 mL), and washed with saturated sodium carbonate (15 mL) and brine (2× 15 mL). The organics were dried with sodium sulfate and evaporated to give a yellow oil, which was chromatographed (ethyl acetate/hexanes, 1:7 to 1:5) to afford a white solid (87 mg, 71%); mp 90.9–92.0 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.26–1.38 (m, 1H, ring CH_2), 1.54–1.68 (m, 1H, ring CH_2), 1.75–1.87 (m, 1H, ring CH_2), 2.36–2.46 (m, 3H, ring CH_2), 2.68–2.73 (t, 1H, J = 12.0 Hz, H-1), 3.71 (s, 3H, $COOCH_3$), 3.84–3.85 (m, 1H, H-3), 4.83–4.84 (d, 1H, J = 6.5 Hz, NH), 5.10 (s, 2H, $PhCH_2O$), 7.33–7.39 (m, 5H, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 34.27 (C-2), 35.36–35.76 (t, J = 25.2 Hz, C-6), 37.84–37.93 (d, J = 10.7 Hz, C-1), 39.80–40.18 (t, J = 23.4 Hz, C-4), 46.62–46.71 (d, J = 11.4 Hz, C-3), 52.40 ($COOCH_3$), 67.12 ($PhCH_2O$), 120.37–124.22 (dd, J = 239.6 Hz, 244.1 Hz, C-5), 128.41 (Ar), 128.48 (Ar), 128.79 (Ar), 136.35 (Ar), 155.48 (OCONH), 173.58 (COOMe). ^{19}F NMR ($CDCl_3$, 376 MHz) δ −98.83 to −98.00 (dtt, 1F, J = 247.4 Hz, 33.2 Hz, 9.0 Hz), −90.67 to −90.01 (d, 1F, J = 245.8 Hz). HRMS (EI) calcd for $C_{16}H_{19}NO_4F_2$ (M^+) 327.1277, found 327.1276.

3.2.11. (*cis*)-3-(Benzyloxycarbonylamino)-5,5-difluorocylcohexanecarboxylic acid (19). A solution of **18** (81 mg, 0.2 mmol) in a mixture of THF (5 mL) and lithium hydroxide (1 N, 5 mL) was stirred at room temperature overnight. The reaction mixture was diluted in water (15 mL) and extracted with ethyl acetate (2 × 20 mL), which was discarded. The separated aqueous phase was adjusted to pH 1 with hydrochloric acid (3 N), and the suspension was extracted with ethyl acetate (3 × 20 mL). The combined organics were washed with brine (20 mL), dried with sodium sulfate, evaporated, and chromatographed (5% methanol with 0.5% acetic acid in dichloromethane) to afford a white solid (40 mg, 52%); mp 150.0–151.8 °C. ^{1}H NMR (CD_3OD, 500 MHz) δ 1.30–1.37 (q, 1H, J = 12.5 Hz, ring CH_2), 1.59–1.83 (m, 2H, ring CH_2), 2.20–2.26 (m, 3H, ring CH_2), 2.56–2.58 (t, 1H, J = 11.5 Hz, H-1), 3.68 (br s, 1H, H-3), 5.04 (s, 2H, $PhCH_2O$), 7.22–7.31 (m, 5H, ArH). ^{13}C NMR (CD_3OD, 125 MHz) δ 35.12 (C-2), 36.46–36.86 (t, J = 25.2 Hz, C-6), 39.34 (d, J = 12.3 Hz, C-1), 40.38–40.78 (t, J = 24.8 Hz, C-4), 47.91–48.01 (d, J = 12.3 Hz, C-3), 67.64 ($PhCH_2O$), 122.16–125.99 (dd, J = 242.7 Hz, 238.8 Hz, C-5), 128.99 (Ar), 129.17 (Ar), 129.63 (Ar), 138.38 (Ar), 158.09 (OCONH), 176.61 (COOH). ^{19}F NMR (CD_3OD, 376 MHz) δ −100.04 to −99.15 (dtt, 1F, J = 245.5 Hz,

34.6 Hz, 9.7 Hz), −90.79 to −90.14 (d, 1F, J = 244.0 Hz). HRMS (CI) calcd for $C_{15}H_{18}NO_4F_2$ $(M+H)^+$ 314.1198, found 314.1204; calcd for $C_{15}H_{17}NO_4F$ $(M-F)^+$ 294.1136, found 294.1134.

3.2.12. (*cis*)-3-Amino-5,5-difluorocylcohexanecarboxylic acid (4). A mixture of **19** (31 mg, 0.1 mmol) and 10 wt% palladium on activated carbon (10 mg) in methanol (10 mL) was hydrogenated at near atmospheric pressure overnight. Filtration over Celite and evaporation of solvent afforded a white powder (17.5 mg, 99%); mp 236.1–238.5 °C. ^{1}H NMR (D_2O, 500 MHz) δ 1.60–1.68 (q, 1H, J = 12.5 Hz, ring CH_2), 1.90–2.05 (m, 2H, ring CH_2), 2.39–2.55 (m, 3H, ring CH_2), 2.80–2.85 (t, 1H, J = 12.7 Hz, H-1), 3.51–3.56 (t, 1H, J = 12.2 Hz, H-3). ^{13}C NMR (D_2O, 125 MHz) δ 33.25 (C-2), 36.89–37.28 (t, J = 24.8 Hz, C-6), 39.22–39.63 (t, J = 25.2 Hz, C-4), 39.91–40.00 (d, J = 10.7 Hz, C-1), 49.00–49.09 (d, J = 11.4, C-3), 123.08–126.91 (dd, J = 238.5 Hz, 243.0 Hz, C-5), 179.35 (COOH). ^{19}F NMR (D_2O, 376 MHz) δ −98.61 to −97.73 (dtt, 1F, J = 245.5 Hz, 33.9 Hz, 10.1 Hz), −91.13 to −90.48 (d, 1F, J = 245.8 Hz). HRMS (ESI) calcd for $C_7H_{12}NO_2F_2$ $(M+H)^+$ 180.0836, found 180.0823. Anal. Calcd for $C_7H_{11}NO_2F_2 \cdot 0.4H_2O$: C, 45.11; H, 6.38; N, 7.52. Found C, 45.08; H, 6.40; N, 7.29.

3.2.13. (*cis*)-Methyl 4-aminocyclopent-2-enecarboxylate hydrochloride (21). To a solution of **20** (1.1 g, 10.0 mmol) in methanol (25 mL) was added concentrated hydrochloric acid (2 mL), and the solution was heated at reflux for 22 h. The solvent was evaporated, and the residual oil was crystallized from dichloromethane/diethyl ether to afford a white solid (1.6 g, 89%); mp 89.0–92.0 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.39–2.42 (m, 1H, H-5, *trans* to H-1), 2.50–2.53 (m, 1H, H-5, *cis* to H-1), 3.62–3.63 (m, 1H, H-1), 3.74 (s, 3H, $COOCH_3$), 4.50 (s, 1H, H-4), 6.16 (s, 1H, H-2), 6.27 (s, 1H, H-3), 8.50 (br s, 2H, NH_2). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 32.63 (C-5), 49.88 (C-1), 53.16 ($COOCH_3$), 56.46 (C-4), 131.66 (C-2), 136.62 (C-3), 175.05 (COOMe).

3.2.14. (*cis*)-Methyl 4-phthalimidocyclopent-2-enecarboxylate (*cis*-22). A solution of **21** (0.51 g, 2.9 mmol) in dichloromethane (50 mL) was washed once with saturated sodium carbonate (20 mL), dried with sodium sulfate, and evaporated to give a clear oil as the corresponding free amine. Phthalic anhydride (0.45 g, 3.0 mmol) was added, followed by anhydrous toluene (30 mL), and the solution was heated at reflux for 8 h. The solvent was evaporated under reduced pressure, chromatographed (ethyl acetate/hexanes, 1:5), and crystallized with isopropyl ether to afford fine needles (0.45 g, 58%); mp 83.9–85.8 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.50–2.56 (dt, 1H, J = 14.0 Hz, 6.6 Hz, H-5, *trans* to H-1), 2.65–2.73 (dt, 1H, J = 13.2 Hz, 9.2 Hz, H-5, *cis* to H-1), 3.64–3.68 (tt, 1H, J = 9.2 Hz, 2.4 Hz, H-1), 3.80 (s, 3H, $COOCH_3$), 5.34–5.38 (tt, 1H, J = 8.8 Hz, 2.2 Hz, H-4), 5.80–5.82 (dt, 1H, J = 5.6 Hz, 2.4 Hz, H-3), 6.16–6.18 (dt, 1H, J = 5.6 Hz, 2.4 Hz, H-2), 7.70–7.72 (dd, 2H, J = 3.0 Hz, 5.4 Hz, ArH), 7.81–7.83 (dd, 2H, J = 3.0 Hz, 5.4 Hz, ArH). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 31.36 (C-5), 49.80 (C-1), 52.36 (C-4), 55.01 ($COOCH_3$), 123.36 (Ar), 130.87 (C-3), 132.06 (Ar), 132.32 (Ar), 134.09 (C-2), 168.00 (phthalyl CO), 173.57 (COOMe). HRMS (CI) calcd for $C_{15}H_{14}NO_4$ $(M+H)^+$ 272.0917, found 272.0912.

3.2.15. (*cis*)- and (*trans*)-Methyl 4-phthalimidocyclopent-2-enecarboxylate (*cis*- and *trans*-22). A solution of **21** (3.0 g, 17 mmol) in hydrochloric acid (1 N, 20 mL) was stirred at room temperature for 24 h to give a mixture of the corresponding *cis* and *trans* amino acids. The solvent was evaporated under reduced pressure, and the residue was redissolved in methanol (60 mL). Concentrated hydrochloric acid (4 mL) was added, and the solution was heated at reflux for 24 h. The solvent was evaporated, and the residual oil was redissolved in ethyl acetate (60 mL) and washed with saturated sodium carbonate (20 mL). Drying with sodium sulfate and evaporation of solvent afforded a clear oil as a mixture of *cis* and *trans*-methyl 4-aminocyclopent-2-ene-1-carboxylate. Phthalic anhydride (3.6 g, 24 mmol) was then added, followed by anhydrous pyridine (2 mL), and the mixture was heated at 120 °C for 22 h. The resulting dark solution was diluted in ethyl acetate (70 mL), washed with hydrochloric acid (1 N, 20 mL), saturated sodium carbonate (20 mL) and brine (20 mL), dried with sodium sulfate, and evaporated to give a yellow oil. Chromatography (ethyl acetate/hexanes, 1:7) afforded *trans*-**22** (1.08 g, 24% overall yield) followed by *cis*-**22** (0.98 g, 21% overall yield), both as white solids.

For *cis*-**22**: R_f = 0.24 (ethyl acetate/hexanes, 1:3). See analytical data above.

For *trans*-**22**: R_f = 0.33 (ethyl acetate/hexanes, 1:3); mp 75.0–77.0 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.30–2.35 (m, 1H, H-5, *cis* to H-1), 2.61–2.66 (m, 1H, H-5, *trans* to H-1), 3.70 (s, 3H, $COOCH_3$), 4.10–4.13 (m, 1H, H-1), 5.48–5.50 (dt, 1H, J = 7.5 Hz, 2.1 Hz, H-4), 5.71–5.73 (m, 1H, H-3), 6.07–6.08 (m, 1H, H-2), 7.67–7.69 (dd, 2H, J = 3.0 Hz, 5.5 Hz, ArH), 7.77–7.79 (dd, 2H, J = 3.0 Hz, 5.5 Hz, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 31.55 (C-5), 50.90 (C-1), 52.21 ($COOCH_3$), 55.46 (C-4), 123.26 (Ar), 130.56 (C-3), 132.07 (Ar), 133.80 (C-2), 134.10 (Ar), 167.99 (phthalyl CO), 174.28 (COOMe). *m*/*z* 271, 239, 212, 130, 104.

3.2.16. (±)-(1*S*,2*R*,4*S*,5*S*)-Methyl 6,6-difluoro-4-phthalimidobicyclo[3.1.0]hexane-2-carboxylate (23). A 10 mL round-bottomed flask equipped with a magnetic stirrer and water condenser was charged with *cis*-**22** (0.20 g, 0.74 mmol), sodium fluoride (11 mg, 0.26 mmol), and anhydrous toluene (0.1 mL). The mixture was heated in a 120 °C oil bath for 30 min before trimethylsilyl-2,2-difluoro-2-(fluorosulfonyl)acetate[25] (TFDA, 0.3 mL, 1.5 mmol) was added via a syringe pump over 1 h. The reaction was allowed to proceed for 5 h, and a second portion of TFDA (0.3 mL) was added via a syringe pump over 1 h. After 16 h of heating, the reaction mixture was cooled and diluted in ethyl acetate (50 mL), washed with saturated sodium carbonate (20 mL) and brine (20 mL), dried with sodium sulfate, and evaporated to give a yellow semisolid. NMR analysis showed a 1:0.35 ratio of the desired product to the starting alkene.

The crude product was then dissolved in 1,4-dioxane (5 mL) and added dropwise to a potassium permanganate solution (0.1 M, 10 mL). The mixture was heated at 50 °C for 1 h, and it was cooled and extracted with ethyl acetate (2 × 30 mL). The organics were washed with saturated sodium carbonate (20 mL), sodium thiosulfate (1 M, 10 mL), and brine (20 mL), dried with sodium sulfate, evaporated under reduced pressure, and chromatographed (ethyl acetate/hexanes, 1:5) to afford a white powder (0.15 g, 63%; 81% based on converted starting material); mp 173.0–176.0 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.19–2.24 (dd, 1H, J = 8.7 Hz, 13.7 Hz, H-5), 2.48–2.55 (m, 1H, H-3, *cis* to H-2), 2.63–2.66 (dm, 1H, J = 14.2 Hz, H-3, *trans* to H-2), 3.02–3.06 (dd, 1H, J = 9.5 Hz, 14.2 Hz, H-1), 3.18–3.20 (d, 1H, J = 10.0 Hz, H-2), 3.83 (s, 3H, $COOCH_3$), 4.96–4.98 (d, 1H, J = 8.5 Hz, H-4), 7.72–7.74 (dd, 2H, J = 3.2 Hz, 5.2 Hz, ArH), 7.82–7.84 (dd, 2H, J = 3.0 Hz, 5.0 Hz, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 32.10–32.38 (t, J = 10.7 Hz, C-5), 32.36–32.53 (t, J = 10.7 Hz, C-1), 36.93 (C-3), 44.31 (C-2), 49.94(C-4), 52.82 (COO CH_3), 110.82–115.41 (dd, J = 281.6 Hz, 294.5 Hz, C-6), 123.66 (Ar), 131.93 (Ar), 134.40 (Ar), 166.77 (phthalyl CO), 173.18 (COOMe). ^{19}F NMR ($CDCl_3$, 376 MHz) δ −149.69 to −149.25 (d, 1F, J = 164.9 Hz), −127.73 to −127.22 (dt, 1F, J = 166.4 Hz, 14.0 Hz). HRMS (EI) calcd for $C_{16}H_{13}NO_4F_2$ (M^+) 321.0807, found 321.0805. Irradiation of H-2 resulted in NOE of 1.9%, 0.7%, and 0.4% for H-3 (*cis* to H-2), H-3 (*trans* to H-2), and H-1, respectively. Irradiation of H-4 resulted in NOE of 2.1%, 0.7%, and 0.8% for H-3 (*cis* to H-2), H-3 (*trans* to H-2), and H-5, respectively. Thus, the *gem*-difluorocyclopropane moiety is *trans* to both the ester and phthalimide groups.

3.2.17. (±)-(1*S*,2*S*,4*S*,5*S*)-Methyl 6,6-difluoro-4-phthalimidobicyclo[3.1.0]hexane-2-carboxylate (24). Starting from *trans*-**22** (0.21 g, 0.8 mmol), the above procedures for the preparation of **23** before oxidation were followed. NMR analysis indicated a 1:1 ratio of the desired product to the starting alkene. So the same procedures were repeated twice on the mixed product, and the same ratio increased to 1:0.36 after the second round and to 1:0.13 after the third round of reaction. The mixture was then treated with potassium permanganate as described above to give a white solid (0.16 g, 62%); mp 92.0–95.5 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.08–2.12 (dd, 1H, J = 8.0 Hz, 14.0 Hz, H-5), 2.21–2.26 (t, 1H, J = 12.0 Hz, H-3, *cis* to H-2), 2.47–2.55 (m, 1H, H-3, *trans* to H-2), 2.63–2.68 (m, 1H, H-1), 3.75 (s, 3H, $COOCH_3$), 4.11–4.17 (m, 1H, H-2), 5.05–5.07 (d, 1H, J = 9.0 Hz, H-4), 7.74–7.76 (dd, 2H, J = 3.0 Hz, 5.5 Hz, ArH), 7.84–7.86 (dd, 2 H, J = 3.0 Hz, 5.5 Hz, ArH). ^{13}C NMR ($CDCl_3$, 125 MHz) δ 31.72–31.89 (t, J = 10.7 Hz, C-5), 32.71–32.89 (t, J = 10.8 Hz, C-1), 34.85–34.90 (d, J = 6.0 Hz, C-3), 44.98 (C-2), 50.56 (C-4), 52.38 ($COOCH_3$), 111.14–115.73 (dd, J = 279.3 Hz, 296.8 Hz, C-6), 123.63 (Ar), 131.87 (Ar), 134.54 (Ar), 167.86 (phthalyl CO), 173.26 (COOMe). ^{19}F NMR ($CDCl_3$, 376 MHz) δ −147.39 to −146.92 (dt, 1F, J = 169.4 Hz, 4.5 Hz), −126.37 to −125.86 (dt, 1F, J = 167.9 Hz, 13.7 Hz). HRMS (EI) calcd for $C_{16}H_{13}NO_4F_2$ (M^+) 321.0807, found 321.0824. Irradiation of H-2 resulted in NOE of 1.7%, 0.2%, and 1.6% for H-3 (*cis* to H-2), H-3 (*trans* to H-2), and H-1, respectively. Irradiation of H-4 resulted in NOE of 0.5%, 2.4%, and 0.7% for H-3 (*cis* to H-2), H-3 (*trans* to H-2), and H-5, respectively. Thus, the *gem*-difluorocyclopropane moiety is *trans* to the C-4 phthalimide, while *cis* to the C-2 methyl ester.

3.2.18. (±)-(1*S*,2*R*,4*S*,5*S*)-6,6-Difluoro-4-phthalimidobicyclo[3.1.0]hexane-2-carboxylic acid (25). The title compound was prepared as a white solid (0.11 g, 92%) from **23** (0.13 g, 0.4 mmol) using the same conditions as described for **19**; mp 195.0–197.0 °C. ^{1}H NMR (CD_3OD, 500 MHz) δ 2.12–2.19 (m, 1H, H-3, *cis* to H-2), 2.22–2.24 (dm, 1H, J = 14.5 Hz, H-3, *trans* to H-2), 2.33–2.38 (dd, 1H, J = 8.0 Hz, 15.0 Hz, H-5), 2.42–2.46 (dd, 1H, J = 8.0 Hz, 14.5 Hz, H-1), 3.10–3.11 (d, 1 H, J = 8.5 Hz, H-2), 4.55–4.56 (d, 1H, J = 7.0 Hz, H-4), 7.38–7.39 (d, 1H, J = 7.5 Hz, ArH), 7.45–7.48 (t, 1H, J = 7.2 Hz, ArH), 7.52–7.55 (t, 1H, J = 7.0 Hz, ArH), 7.89–7.90 (d, 1H, J = 7.0 Hz, ArH). ^{13}C NMR (CD_3OD, 125 MHz) δ 32.51–32.70 (t, J = 11.8 Hz, C-5), 34.06–34.24 (t, J = 11.9 Hz, C-1), 36.16 (C-3), 44.23 (C-2), 52.64 (C-4), 113.26–117.80 (dd, J = 295.2 Hz, 275.5 Hz, C-6), 128.88 (Ar), 130.87 (Ar), 131.55 (Ar), 133.23 (Ar), 139.83 (Ar), 169.18 (phthalyl CO), 171.98 (phthalyl CO), 177.90 (COOH). ^{19}F NMR (CD_3OD, 376 MHz) δ −148.95 to −148.51 (d, 1F, J = 167.9 Hz), −126.29 to −125.77 (dt, 1F, J = 167.9 Hz, 14.9 Hz). HRMS (CI) calcd for $C_{15}H_{12}NO_4F_2$ $(M+H)^+$ 308.0729, found 308.0725.

3.2.19. (±)-(1*S*,2*S*,4*S*,5*S*)-6,6-Difluoro-4-phthalimidobicyclo[3.1.0]hexane-2-carboxylic acid (26). The title compound was prepared as a white solid (0.28 g, 99%) from **24** (0.29 g, 0.9 mmol) using the same conditions as described for **19**; mp 174.0–176.3 °C. ^{1}H NMR (CD_3OD, 500 MHz) δ 1.98–2.08 (m, 2H, H-3), 2.17–2.28 (m, 2H, H-1 and H-5), 3.44–3.50 (m, 1H, H-2), 4.50–4.52 (d, 1H, J = 6.5 Hz, H-4), 7.32–7.33 (d, 1H, J = 7.5 Hz, ArH), 7.41–7.44 (t, 1H, J = 7.5 Hz, ArH), 7.49–7.52 (t, 1H, J = 7.5 Hz, ArH), 7.87–7.89 (d, 1H, J = 8.0 Hz, ArH). ^{13}C NMR (CD_3OD, 125 MHz) δ 30.56–30.74 (t, J = 11.4 Hz, C-5), 33.87–34.06 (t, J = 11.8 Hz, C-1), 34.30–34.37 (d, J = 8.4 Hz, C-3), 44.60 (C-2), 52.66 (C-4), 114.11–118.66 (dd, J = 273.9 Hz, 297.5 Hz, C-6), 128.98 (Ar), 130.69 (Ar), 131.57 (Ar), 133.39 (Ar), 140.10 (Ar), 169.09 (phthalyl CO), 172.51 (phthalyl CO), 176.40 (COOH). ^{19}F NMR (CD_3OD, 376 MHz) δ −146.64 to −146.19 (d, 1F, J = 169.4 Hz), −125.15 to −124.63 (dt, 1F, J = 169.4 Hz, 13.6 Hz). HRMS (EI) calcd for $C_{15}H_{11}NO_4F_2$ (M^+) 307.0656, found 307.0652.

3.2.20. (±)-(1*S*,2*R*,4*S*,5*S*)-4-Amino-6,6-difluorobicyclo[3.1.0]hexane-2-carboxylic acid (5). Sodium borohydride (19 mg, 0.5 mmol) was added to a mixture of **25** (15.6 mg, 0.05 mmol) in 2-propanol (3.0 mL) and water (0.5 mL), and the solution was stirred at room temperature for 24 h. Acetic acid (0.2 mL) was then carefully added dropwise, and the solution was heated at 80 °C for 2 h after the foam had subsided. The crude reaction

mixture was then directly loaded onto Dowex 50WX8-200 resin (5 g) and washed with water (100 mL). Elution with ammonium hydroxide (0.4 N, 100 mL) afforded a white solid (7.0 mg, 77%); mp 249.4–251.3 °C (dec). ^{1}H NMR (CD_3OD, 500 MHz) δ 2.00–2.01 (m, 2H, H-3), 2.28–2.32 (dd, 1H, J = 7.2 Hz, 14.7 Hz, H-5), 2.40–2.44 (dd, 1H, J = 8.0 Hz, 14.0 Hz, H-1), 2.98–2.99 (m, 1H, H-2), 3.85 (s, 1H, H-4). ^{13}C NMR (CD_3OD, 125 MHz) δ 33.16–33.35 (t, J = 12.2 Hz, C-3), 34.54–34.88 (2 t, J = 11.5 Hz, C-1 and C-5), 48.23 (C-2), 53.90 (C-4), 113.25–117.79 (dd, J = 274.7 Hz, 296.0 Hz, C-6), 181.79 (COOH). ^{19}F NMR (CD_3OD, 376 MHz) δ −148.33 to −147.88 (d, 1 F, J = 169.4 Hz), −125.44 to −124.91 (dt, 1F, J = 169.4 Hz, 14.9 Hz). HRMS (ESI) calcd for $C_{15}H_{12}NO_4F_2$ $(M+H)^+$ 178.0680, found 178.0662. Anal. Calcd for $C_7H_9NO_2F_2 \cdot 0.5H_2O$: C, 45.16; H, 5.41; N, 7.52. Found: C, 45.34; H, 5.31; N, 7.33.

3.2.21. (±)-(1*S*,2*S*,4*S*,5*S*)-4-Amino-6,6-difluorobicyclo[3.1.0]hexane-2-carboxylic acid (6). A mixture of **26** (62 mg, 0.2 mmol) and hydrazine hydrate (0.3 mL, 4.9 mmol) in methanol (5 mL) was heated at reflux overnight. The solvent was evaporated under reduced pressure, and the residue was redissolved in hydrochloric acid (3 N, 5 mL) and refluxed for 3 h. The solution was evaporated under an oil-pump vacuum to dryness, and the residue was dissolved in water (1 mL) and loaded onto 2 g of Dowex 50WX8-200 ion-exchange resin, which had been pre-eluted with 50 mL of 1 N pyridine in water and 150 mL water. The column was washed with water (100 mL), then it was eluted with 50 mL of 1 N pyridine, evaporated, and recrystallized from water/ethanol to give a white powder (27 mg, 76%); mp 244.0–246.3 °C (dec). ^{1}H NMR (D_2O, 500 MHz) δ 2.14–2.16 (m, 2H, H-3), 2.29–2.32 (dd, 1H, J = 7.7 Hz, 15.2 Hz, H-5), 2.51–2.54 (m, 1H, H-1), 3.57–3.61 (m, 1H, H-2), 4.04 (s, 1H, H-4). ^{13}C NMR (D_2O, 125 MHz) δ 32.77–32.94 (t, J = 11.1 Hz, C-5), 33.53–33.73 (t, J = 12.6 Hz, C-1), 34.95 (C-3), 47.98 (C-2), 55.18 (C-4), 114.65–119.20 (dd, J = 275.9 Hz, 302.0 Hz, C-6), 182.93 (COOH). ^{19}F NMR (CD_3OD, 376 MHz) δ −146.54 to −146.09 (d, 1F, J = 169.0 Hz), −124.90 to −124.38 (dt, 1F, J = 167.9 Hz, 13.7 Hz). Anal. Calcd for $C_7H_9NO_2F_2 \cdot 1.2H_2O$: C, 42.30; H, 5.78; N, 7.05; F, 19.12. Found: C, 42.24; H, 5.62; N, 6.99; F, 18.92.

3.3. Enzyme and assays

3.3.1. General. GABA aminotransferase was isolated from pig brain by the published procedure.[31] Succinic semialdehyde dehydrogenase (SSDH) was isolated from GABAse, a commercially available mixture of SSDH and GABA-AT, using the method of Jeffery et al.[32] GABA-AT activity was assayed using a modification of the coupled assay of Scott and Jakoby.[33] The assay solution has final concentrations of 11 mM GABA, 1.1 mM $NADP^+$, 5.3 mM α-ketoglutarate, 2 mM β-mercaptoethanol, and excess SSDH in 50 mM potassium pyrophosphate buffer at pH 8.5. With this assay, the change in absorbance at 340 nm, corresponding to the formation of NADPH from $NADP^+$ at 25 °C, is proportional to the GABA-AT activity.

3.3.2. Time-dependent inactivation test of GABA-AT by 3–6. GABA aminotransferase (16.7 μM, 20 μL) was added to solutions of **3**–**6** (100 μL final volume, 10 mM) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 15 mM α-ketoglutarate and 1 mM β-mercaptoethanol at 25 °C. At timed intervals, aliquots (20 μL) were withdrawn and added to the assay solution (575 μL) followed by the addition of SSDH (5 μL, excess amount), and reaction rates were measured spectrophotometrically at 340 nm.

3.3.3. Competitive inhibition test of GABA-AT by 3–6 and 3-aminocyclohexanecarboxylic acid. The activity of GABA-AT (16.7 μM, 4 μL) at 25 °C in 600 μL of 50 mM potassium pyrophosphate buffer (pH 8.5) containing excess SSDH, 5 mM α-ketoglutarate, and 1 mM $NADP^+$ was determined upon the introduction of varying concentrations of potential inhibitors at different GABA concentrations. The percentage of inhibition was obtained by comparison to an untreated enzyme control.

3.3.4. Substrate activity test of 3–6 and 3-aminocyclohexanecarboxylic acid. Each compound (2.5 mM) was individually incubated in a 0.5 mL amber microcentrifuge tube (Fisher Scientific) for 48 h with GABA-AT (16.7 μM, 3 μL) in 50 mM potassium pyrophosphate buffer, pH 8.5, containing 2.0 mM β-mercaptoethanol in a total volume of 100 μL in the presence of 2.8 mM [^{14}C]α-ketoglutarate (specific activity = 181.8 μCi/mmol) at 25 °C. A control with everything but the test compound was run in parallel. Aqueous trichloroacetic acid (20% solution, 20 μL) was added, and the mixture was loaded onto a disposable glass pipette containing 1.0 g of Dowex 50WX8-200 ion-exchange resin. The tube was washed with water (2 × 120 μL), and the rinses were also loaded onto the column. The column was eluted with water (3 × 2 mL) followed by ammonium hydroxide solution (2.0 N, 3 × 2 mL) into six 20 mL scintillation vials. To each of the vials was added Packard Ultima Gold XR cocktail (15 mL), and the radioactivity was counted on a Packard 2100TR Tri-Carb liquid scintillation analyzer.

Acknowledgment

The authors are grateful to the National Institutes of Health (Grant GM66132) for financial support of this research.

Supplementary data

Supplementary data (^{1}H, ^{13}C, and ^{19}F NMR spectra of all synthesized compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.11.010.

References and notes

1. Cooper, A. J. *Methods Enzymol.* **1985**, *113*, 80.
2. Krnjevic, K. *Physiol. Rev.* **1974**, *54*, 418.

3. Gale, K. *Epilepsia* **1989**, *30*, 1.
4. Hornykiewicz, O.; Lloyd, K. B.; Davidson, L. In *GABA in Nervous System Function*, Roberts, E., Chase, T. N., Tower, D. B., Eds.; Raven Press: New York, 1976; pp 479–485.
5. Perry, T. L.; Hansen, S.; Kloster, M. *New Eng. J. Med.* **1973**, *288*, 337.
6. Aoyage, T.; Wada, T.; Nagai, M.; Kojima, F.; Harada, S.; Takeuchi, T.; Takahashi, H.; Kirokawa, K.; Tsumita, T. *Chem. Pharm. Bull.* **1990**, *38*, 1748.
7. (a) Kushner, S. A.; Dewey, S. L.; Kornetsky, C. *J. Pharmacol. Exp. Ther.* **1999**, *290*, 797; (b) Dewey, S. L.; Morgan, A. E.; Ashby, C. R., Jr.; Horan, B.; Kushner, S. A.; Logan, J.; Volkow, N. D.; Fowler, J. S.; Gardner, E. L.; Brodie, J. D. *Synapse* **1998**, *30*, 119.
8. Nanavati, S. M.; Silverman, R. B. *J. Med. Chem.* **1989**, *32*, 2413.
9. (a) Silverman, R. B.; Levy, M. A. *J. Org. Chem.* **1980**, *45*, 815; (b) Silverman, R. B.; Levy, M. A. *Biochemistry* **1981**, *20*, 1197; (c) Silverman, R. B.; Invergo, B. J. *Biochemistry* **1986**, *25*, 6817.
10. Sugase, K.; Horikawa, M.; Sugiyama, M.; Ishiguro, M. *J. Med. Chem.* **2004**, *47*, 489.
11. Edmonds, M. K.; Abell, A. D. *J. Org. Chem.* **2001**, *66*, 3747.
12. Fernandez, M. M.; Diez, A.; Rubiralta, M.; Montenegro, E.; Casamitjana, N.; Kogan, M. J.; Giralt, E. *J. Org. Chem.* **2002**, *67*, 7587.
13. Freidinger, R. M.; Veber, D. F.; Perlow, D. S. *Science* **1980**, *210*, 656.
14. (a) Qiu, J.; Silverman, R. B. *J. Med. Chem.* **2000**, *43*, 706; (b) Storici, P.; Qiu, J.; Schirmer, T.; Silverman, R. B. *Biochemistry* **2004**, *43*, 14057.
15. (a) Rando, R. R. *Biochemistry* **1977**, *16*, 4604; (b) Fu, M.; Silverman, R. B. *Bioorg. Med. Chem.* **1999**, *7*, 1581.
16. Qiu, J.; Pingsterhaus, J. M.; Silverman, R. B. *J. Med. Chem.* **1999**, *42*, 4725.
17. Ye, Q.; Komarov, I. V.; Kirby, A. J.; Jones, M., Jr. *J. Org. Chem.* **2002**, *67*, 9288.
18. Middleton, W. *J. Org. Chem.* **1975**, *40*, 574.
19. Anet, F. A. L.; Bourn, A. J. R. *J. Am. Chem. Soc.* **1965**, *87*, 5250.
20. Katagiri, N.; Matsuhashi, Y.; Kokufuda, H.; Takebayashi, M.; Kaneko, C. *Tetrahedron Lett.* **1997**, *38*, 1961.
21. Olah, G. A.; Narang, S. C.; Gupta, B. G. B.; Malhotra, R. *J. Org. Chem.* **1979**, *44*, 1247.
22. Becker, A. M.; Rickards, R. W. *Tetrahedron* **1983**, *39*, 4189.
23. Dess, D. B.; Martin, J. C. *J. Org. Chem.* **1983**, *48*, 4155.
24. Allan, R. D.; Fong, J. *Aust. J. Chem.* **1986**, *39*, 855.
25. Tian, F.; Kruger, V.; Bautista, O.; Duan, J.-X.; Li, A.-R.; Dolbier, W. R., Jr.; Chen, Q.-Y. *Org. Lett.* **2000**, *2*, 563.
26. Sasaki, T.; Minamoto, K.; Itoh, H. *J. Org. Chem.* **1978**, *43*, 2320.
27. Osby, J. O.; Martin, M. G.; Ganem, B. *Tetrahedron Lett.* **1984**, *25*, 2093.
28. Neal, M. J.; Bowery, N. G. *Brain Res.* **1977**, *138*, 169.
29. Storici, P.; Capitani, G.; De Biase, D.; Moser, M.; John, R. A.; Jansonius, J. N.; Schirmer, T. *Biochemistry* **1999**, *38*, 8628.
30. Murahashi, S.; Taniguchi, Y.; Imada, Y.; Tanigawa, Y. *J. Org. Chem.* **1989**, *54*, 3292.
31. Churchich, J. E.; Moses, U. *J. Biol. Chem.* **1981**, *256*, 1101.
32. Jeffery, D.; Weitzman, P. D. J.; Lunt, G. G. *Insect Biochem.* **1988**, *28*, 347.
33. Scott, E. M.; Jakoby, W. B. *J. Biol. Chem.* **1958**, *234*, 932.