Supporting Information

Discovery of Benzodiazepine Sulfonamide Based Bombesin Receptor Subtype 3 Agonists and Their Unusual Chirality

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Contents of Supporting Information:

General Information. All reagents were purchased from Aldrich and used without further purification unless otherwise stated. Column chromatography was carried out on flash silica gel (Merck 230-400 mesh). TLC analysis was conducted on ANALTECH silica gel plates. The LC/MS analyses were performed using a MICROMASS ZMD mass spectrometer coupled to an AGILENT 1100 Series HPLC utilizing a YMC ODS-A 4.6 x 50 mm column eluting at 4.5 mL/min with a solvent gradient of 10 to 95% B over 2.5 min, followed by 0.5 min at 95% B: solvent A = 0.06% TFA in water; solvent B = 0.05% TFA in acetonitrile. ^{1}H-NMR spectra were obtained on a 500 MHz VARIAN Spectrometer in $CDCl_3$ or CD_3OD as indicated and chemical shifts are reported as δ using the solvent peak as reference and coupling constants are reported in hertz (Hz).

For selectivity counter-screen, 168 radioligand binding or enzymatic assays were carried at MDS Pharma Services as a contract service to Merck. A summary of each assay protocol and the reference for each assay are listed in the MDS Pharma catalog.

(*S*)-6-[(4-*tert*-Butylphenyl)sulfonyl]-7,8-dimethyl-2-(trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine (8a) and (*R*)-6-[(4-*tert*-Butylphenyl)sulfonyl]-7,8-dimethyl-2-(trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine (8b)

Step A: A mixture of 3,4-dimethylbenzene-1,2-diamine (6.8 g, 50 mmol) and 2-chloro-6-(trifluoromethyl)nicotinic acid (11 g, 50 mmol) in 2-butoxyethanol (150 mL) was heated at 150 °C for 6 h. The reaction mixture was poured into water and was made basic (pH=8) with 50% aqueous NaOH solution. The precipitate was collected by filtration, washed with water, and dried in a vacuum oven at 45 °C to give the diazepinone, which was used in Step B without further purification.

Step B: To the diazepinone of Step A in 100 mL of THF at 0 °C was added 1.0 M BH_3 in THF (150 mL, 150 mmol). After stirring at rt overnight, the reaction was carefully quenched with MeOH at 0 °C. The resulting mixture was concentrated, and the residue was purified on silica gel eluting with 10-50% EtOAc in hexanes to give 7,8-Dimethyl-2-(trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine.

Step C: To the solution of Step B product 7,8-Dimethyl-2-(trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine (0.44 g, 1.5 mmol) in DCM (4 mL) at rt was added sequentially (4-*tert*-butylphenyl)sulfonyl chloride (0.39 g, 1.5 mmol), pyridine (1 mL), and DMAP (18 mg, 0.15 mmol). The mixture was stirred at room temperature for 24 h and quenched with water. The product was extracted with EtOAc (3x), and the combined extracts were washed with water and brine, dried over $MgSO_4$ and concentrated. After silica gel column purification eluting with 10-50% EtOAc in hexanes, the racemic mixture was resolved by HPLC using a Chiralcel OD column (10% IPA in heptane) to afford two enantiomers **8a** and **8b**. The faster eluting enantiomer (**8a**) has a retention time of 10.6 min

and rotation of $[\alpha]_D$ -157°, while the slower eluting enantiomer (**8b**) has a retention time of 12.6 min and rotation of $[\alpha]_D$ +155°. ^{1}H NMR (500 MHz, CD_3OD): δ 7.58 (1H, d), 7.19 (2H,d), 7.08 (2H, d), 7.07 (1H), 6.99 (1H, d), 6.79 (1H, d), 5.19 (1H, d), 4.42 (1H, d), 2.43 (3H, s), 2.28 (3H, s), 1.24 (9H, s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.9, 139.7, 138.9, 137.2, 136.6, 132.3, 130.8, 128.7, 127.4, 125.2, 119.2, 116.8, 116.7, 110.4, 54.4, 35.4, 31.2, 20.4, 16.5; LCMS: *m/e* 490.2 $(M+H)^+$.

(*S*)-6-[(4-*tert*-Butylphenyl)sulfonyl]-7-chloro-2-(trifluoromethyl)-6,11-dihydro-5-pyrido[2,3-*b*][1,5]benzodiazepine (8i)

Step A: A solution of 3-chloro-2-nitroanailine (8.6 g, 50 mmol) in 150 mL of EtOH was stirred with Pt(IV) oxide (0.9 g) under 1 atm hydrogen (balloon) for 4 h. The reaction mixture was filtered through Celite and the filtrate was concentrated to afford 3-chlorobenzene-1,2-diamine.

Step B: A mixture of 3-chlorobenzene-1,2-diamine and 2-chloro-6-(trifluoromethyl)nicotinic acid (11 g, 50 mmol) in 2-butoxyethanol (150 mL) was heated at 150 °C for 22 h. The reaction mixture was concentrated to give the diazepinone, which was used without further purification.

Step C: To the diazepinone of Step B in 100 mL of THF at 0 °C was added 1.0 M BH_3 in THF (150 mL, 150 mmol). After stirring at rt overnight, and reaction was carefully quenched with MeOH at 0 °C. The resulting mixture was concentrated, and the residue was purified on silica gel eluting with 10-50% EtOAc in hexanes to give the title compound. LCMS: *m/e* 300.0 $(M+H)^+$.

Step D: To a solution of 7-chloro-2-(trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine (from Step C, 2.60 g, 8.8 mmol) in CH_2Cl_2 (40 mL) at 0 °C was added (4-*tert*-butylphenyl)sulfonyl chloride (6.2 g, 26.0 mmol), pyridine (3.7 mL) and DMAP (500 mg). After stirring at rt for 12 h, the reaction mixture was concentrated and the residue was purified by silica gel chromatography (40-80% CH_2Cl_2:hexanes) to give the title compound as a racemic mixture. The racemic mixture was resolved by HPLC using a Chiralcel OD column (10% IPA in heptane) to afford **8i** as a faster eluting enantiomer. ^{1}H NMR (500 MHz, $(CD_3)_2CO$): δ 8.69 (s, 1H), 8.47 (s, 1H), 7.70 (d, 1H), 7.33 (m, 3H), 7.20 (dd, 1H), 7.15 (d, 2H), 5.19 (d, 1H), 4.59 (d, 1H), 1.22 (s, 9H). LCMS: *m/z* 496.0 $(M+H)^+$.

6-[(4-*tert*-Butylphenyl)sulfonyl]-2-(trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine (8k)

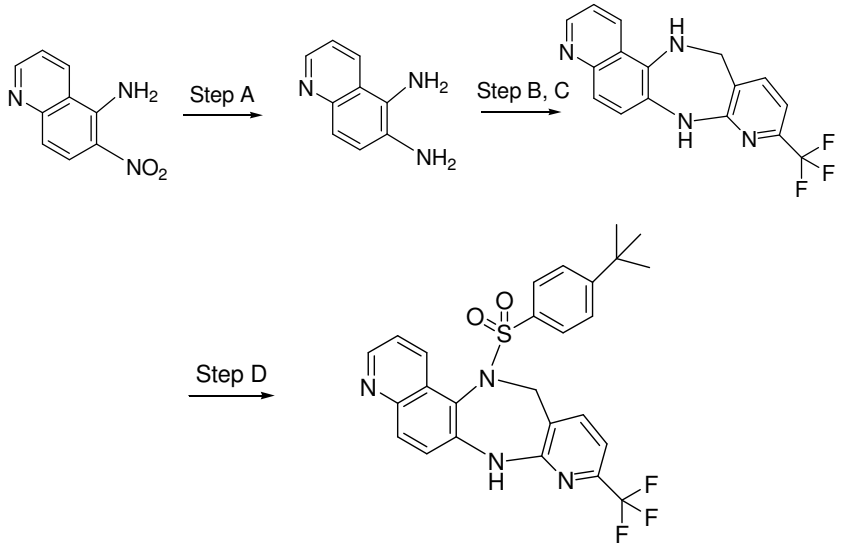
Step A: A mixture of 1,2-diaminobenzene (5.4 g, 50 mmol), 2-chloro-6-(trifluoromethyl)nicotinic acid (11.25g, 50 mmol) in 2-butoxyethanol (150 mL) was heated at 150 °C for 6 h. The reaction mixture was poured into water and made basic (pH=8) with 50% aqueous NaOH solution. The precipitate was

collected by filtration, washed with water, and dried in a vacuum oven at 45 °C to give the diazepinone product, which was used without further purification. LCMS: *m/e* 280.1 $(M+H)^+$.

Step B: To the diazepinone of Step A in 100 mL of THF was added 1.0 M BH_3 in THF (150 mL, 150 mmol) at 0 °C. After stirring at rt overnight, the reaction was carefully quenched with MeOH at 0 °C. The resulting mixture was concentrated, and the residue was purified on silica gel eluting with 10-50% EtOAc in hexanes to give 2-(Trifluoromethyl)-6,11-dihydro-5*H*-pyrido[2,3-*b*][1,5]benzodiazepine. LCMS: *m/e* 266.1 $(M+H)^+$.

Step C: To a solution of Step B product (3.98 g, 15 mmol) in DCM (75 mL) at rt was added sequentially (4-*tert*-butylphenyl)sulfonyl chloride (9.1 g, 37.5 mmol), pyridine (7.11 g, 90 mmol), and DMAP (0.366 g, 3 mmol). The mixture was stirred at room temperature for 24 h and quenched with water. The mixture was extracted with EtOAc (3x), and the combined extracts were washed with water and brine, dried over $MgSO_4$ and concentrated. The crude product was purified by silica chromatography eluting with 10-50% EtOAc in hexanes to afford the title compound. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.63 (1H, d), 7.57 (1H, d), 7.28 (1H, t), 7.11 (2H, d), 7.07 (1H, d), 7.06 (1H, t), 6.94 (2H, d), 6.76 (1H, d), 6.72 (1H, broad s), 4.80 (2H, s), 1.23 (s, 9H). LC/MS: *m/e* 462.1 $(M+H)^+$.

(*S*)-13-[(4-*tert*-Butylphenyl)sulfonyl]-9-(trifluoromethyl)-12,13-dihydro-7*H*-pyrido[2',3':5,6][1,4]diazepino[2,3-*f*]quinoline (10)



Step A: A mixture of 5-Amino-6-nitroquinoline (650 mg, 3.44 mmol), Pd/C (10 wt %, 70 mg) and 15 mL of EtOH was stirred under H_2 (balloon) overnight. The reaction mixture was filtered through Celite and the filtrate was concentrated to give 5,6-diaminoquinoline.

Steps B and C: 5,6-Diaminoquinoline was converted to 9-(trifluoromethyl)-12,13-dihydro-7*H*-pyrido[2',3':5,6][1,4]diazepino[2,3-*f*]quinoline following the procedure of Steps A and B described for compound **8k** above.

Step D: 9-(Trifluoromethyl)-12,13-dihydro-7*H*-pyrido[2',3':5,6][1,4]diazepino[2,3-*f*]quinoline was converted to the title compound as a racemic mixture following the procedure described for compound **8k** (Step C) above. The racemic mixture was resolved by HPLC on a chiralcel OD column (30% EtOH in hexanes) to afford compound **10** as a faster eluting enantiomer with retention time of 7.6 min. ^{1}H NMR (500 MHz, CD_3OD): δ 8.83 (1H, d), 8.75 (1H, d), 7.96 (1H, d), 7.72 (1H, d), 7.59 (1H, m), 7.53 (1H, d), 7.19 (2H,d), 7.17 (d, 1H), 6.97 (2H, d), 5.38 (1H, d), 4.53 (1H, d), 1.23 (9H, s). LCMS: *m/e* 513.2 $(M+H)^+$.

Bombesin Receptor Subtype 3 (BRS-3) Binding Assays. For human BRS-3 binding assays, 1 to 4 μg of membrane protein obtained from NFAT-CHO cells expressing the receptor were incubated with 0.3 pM [^{125}I]-[D-Tyr^6,β-Ala^{11},Phe^{13},Nle^{14}]-Bombesin (6-14) (^{125}I-dY-peptide) and various concentrations of test compounds in 200 μL of binding buffer (50 mM Tris, pH 7.2, 5 mM $MgCl_2$, 0.1% BSA). After 2 h incubation at room temperature, the binding reaction was terminated by filtering through a GF/c filter and washing the filter with PBS using a Packard 96-well Harvester. The amount of radioligand bound to the receptor was measured by liquid scintillation counting of the radioactivity on the filter. The nonspecific binding was defined as the binding in the presence of 100 nM unlabeled dY-bombesin. The data, as % inhibition of binding, was plotted vs. the log molar concentration of receptor ligand (compound). The IC_{50} was reported as the inflection point of the resulting sigmoidal curve.

BRS-3 Functional Assays. The functional assay is an aequorin bioluminescence assay. It was performed in a 96-well format using a Wallac Microbeta luminometer equipped with microinjector module. Compounds in DMSO (0.5% final concentration) were titrated in the plates at 2x concentration in a volume of 0.1 mL ECB buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 1 mM CaCl, 5 mM glucose, 0.1 mg/ml BSA). The HEK293AEQ cells from lines expressing either human, rat or mouse BRS3 (20,000 per well) were charged with coelenterazine (Molecular Probes) and then injected in 0.1 mL ECB buffer into the compound containing wells. The bioluminescence was monitored for 30 seconds, or alternatively, total bioluminescence was determined over 10 minutes. The bioluminescent readings were plotted vs. the log molar concentration of receptor ligands (compounds). The EC_{50} for activation was reported as the inflection point of the resulting sigmoidal curve. The percentages of activation are the maxim activations of tested compounds relative to that of dY-peptide.

Pharmacokinetic study. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY, USA) were dosed intravenously at 1 mg/kg and orally at 4 mg/kg for pharmacokinetic (PK) evaluations. The blood samples were collected at various time points into lithium heparin tubes and centrifuged. The plasma

samples were kept at -70 °C until analysis. The plasma samples were extracted by protein precipitation and analyzed by LC/MS/MS.

***In vivo* overnight food intake and body weight in C57 Obese Male Mice.** Male C57 mice were made obese by being maintained on a high fat diet (45-60% kcal from fat), such as Research Diets RD12492, starting at 6 weeks of age. Obese mice, approximately 20-52 weeks old and weighing approximately 45-62 g, were individually housed and acclimated for several days prior to testing. On the day of study, mice were orally dosed (n=6-8/ group) with either vehicle only (10% Tween- water) or BRS-3 agonists (various doses). A known CB1R inverse agonist, AM251 (3 mg/kg), was used as the positive control for inter- and intra-experimental control. BRS-3 agonists were dosed approximately 60 minutes prior to the onset of the dark cycle. Overnight food intake and body weight were measured and analyzed. All data are presented as mean ± SEM. Statistical significance was calculated using Student's *t-test* with differences considered significant when 2-tailed $p<0.05$.

***In vivo* sub-chronic administration on body weight in C57 Obese Male Mice.** The same obese male mice were used. During the study, mice were orally dosed (n=7-9/ group) with either vehicle only (10% Tween- water) or BRS-3 agonists (various doses). AM251 (3 mg/kg) was used as the positive control. Two doses (PO) of BRS-3 agonist were administered each day for 4 days. The first dose was given approximately 60 minutes prior to the onset of the dark cycle and the second, 5 hours after the first dose. A single dose of AM251 was given approximately 60 minutes prior to the onset of the dark cycle and vehicle was dosed for the second dose, 5 hours after the first dose. Daily food intake and body weight were measured and analyzed. All data are presented as mean ± SEM. Statistical significance was calculated using Student's *t-test* with differences considered significant when 2-tailed $p<0.05$.

X-ray crystallography analysis. X-ray diffraction analysis of single-crystals of **8a** and **8b** showed an interesting geometry at the sulfonamide nitrogen. The bond distances from this N to the connected atoms are all typical for single-bond lengths. The geometry around the N is almost planar (distance from N to the plane formed from the S and two C atoms is only 0.14 Å) and the position for the lone-pair orbital is approximately perpendicular to the plane of the aromatic ring. Comparison of structures **8a** and **8b** shows that the origin of the chirality arises from the puckered, conformationally-constrained diazepine ring (planar chirality), where the ring flip is restricted by the van der Waals interaction between the sulfonyl group and the 7-Me substituent on the phenyl rather than either a restricted rotation about the N-S bond or an inversion of the sulfonamide nitrogen.

Diffraction data for compounds **8a** and **8b** were collected on a Bruker CCD diffractometer. The

structures were solved by direct methods (SHELXS-97, Sheldrick, G.M. *Acta Crystallogr.*, 2008, A64, 112-122) and refined using full-matrix least-squares on F^2 (SHELXL-97, Sheldrick, G.M. *Acta Crystallogr.*, 2008, A64, 112-122). The absolute stereochemistry has been determined using anomalous dispersion calculations. All non-hydrogen atoms were refined with anisotropic thermal displacements. CCDC contains the supplementary crystallographic data for this paper. This data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Compound ID	liup0105a (**8a**)	liup0105b (**8b**)
CCDC deposition#	841543	841544
Formula	$C_{25}H_{26}F_3N_3O_2S$	$C_{25}H_{26}F_3N_3O_2S$
Fw	489.560	489.560
Crystal dimen. (mm)	0.02 x 0.06 x 0.06	0.06 x 0.10 x 0.26
Lattice symmetry	monoclinic	monoclinic
Space group	$P2_1$	$P2_1$
a , (Å	8.686(8)	8.657(3)
b	7.752(8)	7.745(2)
c	17.511(17)	17.510(5)
〈	90.00	90.00
®	99.619(17)	99.233(4)
©	90.00	90.00
V (Å^3)	1163(2)	1158.9(6)
Z	2	2
D_{calc} (Mg m^{-3})	1.398	1.403
Radiation (*K*〈)	Mo	Mo
Wavelength (Å)	0.71073	0.71073
Temperature (K)	100	100
⌠ (mm^{-1})	0.192	0.192
Diffractometer	Bruker SMART1K CCD	Bruker SMART1K CCD
Reflections measured	10312	13975
Resolution (Å)	0.75	0.75
Unique reflections	5230	5476
R_{int}	0.052	0.035
Absorp. corr.	none	multi-scan
Trans. (max, min)	0.9960, 0.9890	0.9890, 0.9520
Reflections used	5230	5476
Refl. obsd. criterion	> 2⌠(*I*)	> 2⌠(*I*)
Variables	312	312
Refined on	F^2	F^2
R	0.047	0.032
R_w	0.111	0.080
S	0.98	1.02
Flack, x	0.00(8)	0.01(5)
residual peak (eÅ^{-3})	0.26(6)	0.25(5)
Computer programs:		
Solution	SHELXS-97	SHELXS-97
Refinement	SHELXL-97	SHELXL-97
Absorption	--	SADABS

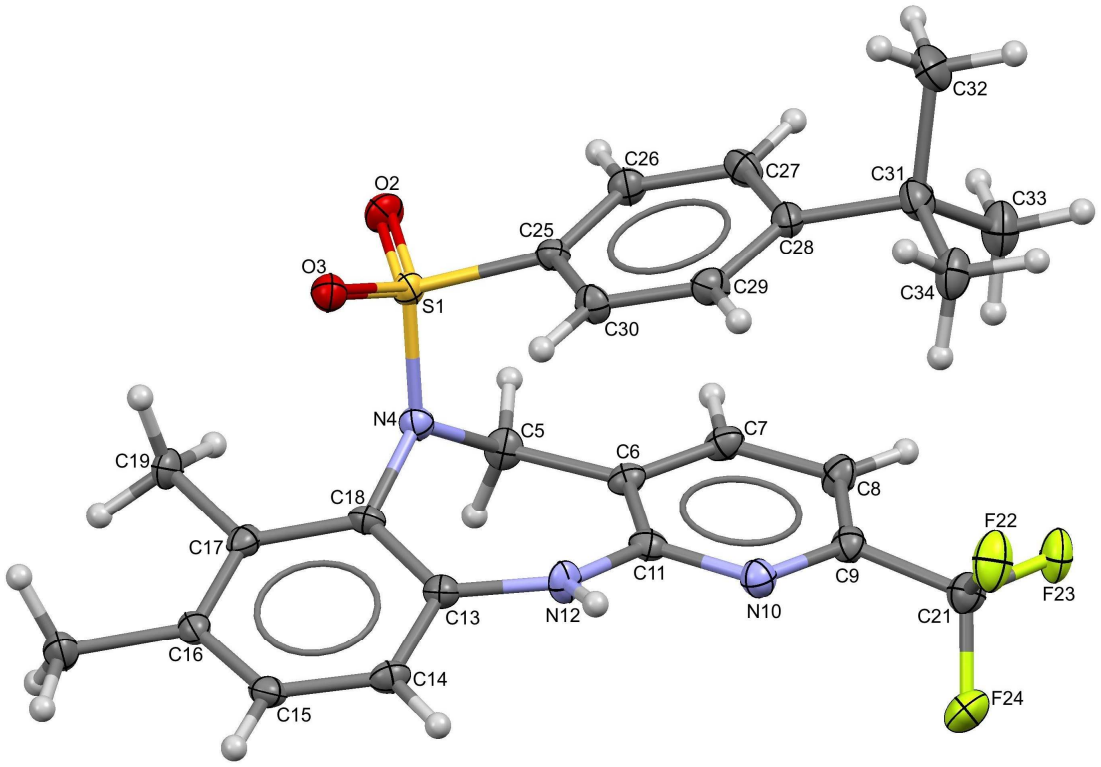


Figure 1. X-ray structure of **8a**

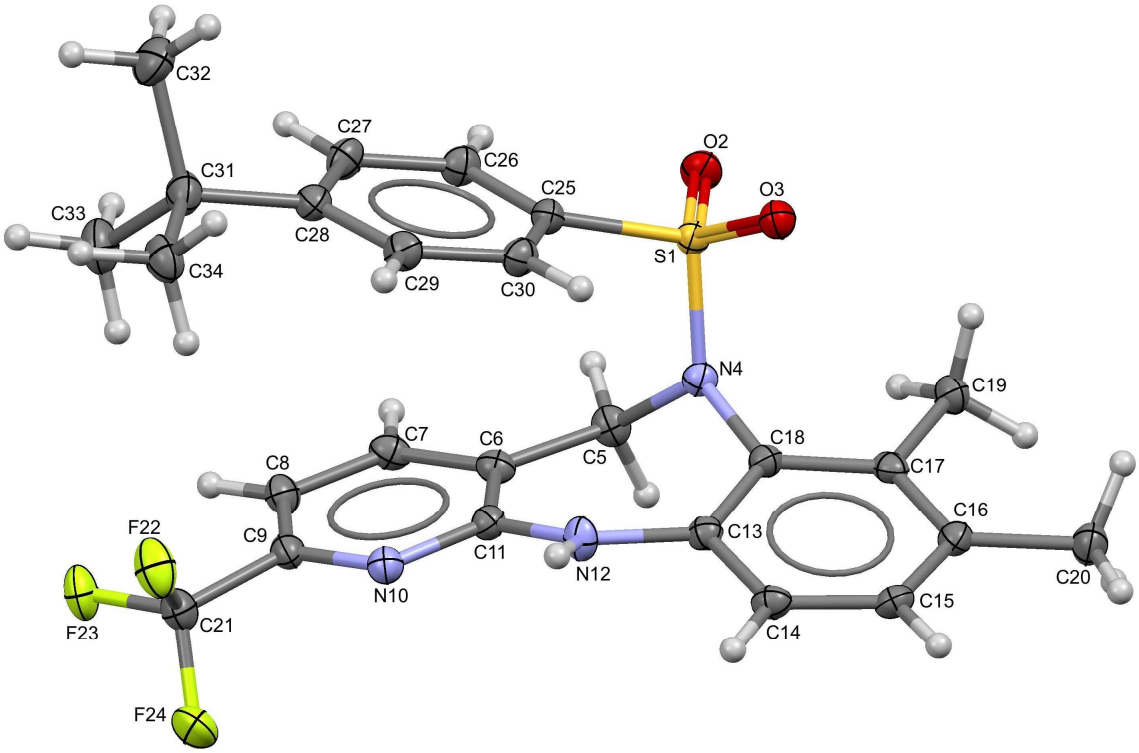


Figure 2. X-ray structure of **8b**

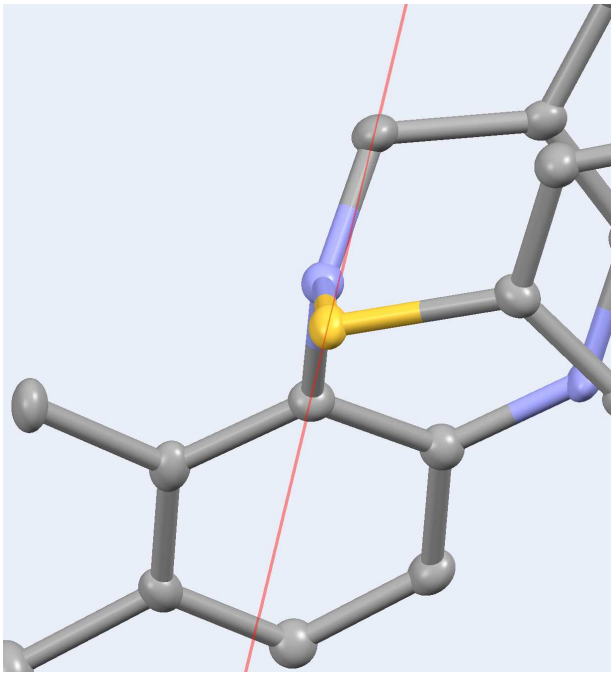


Figure 3.

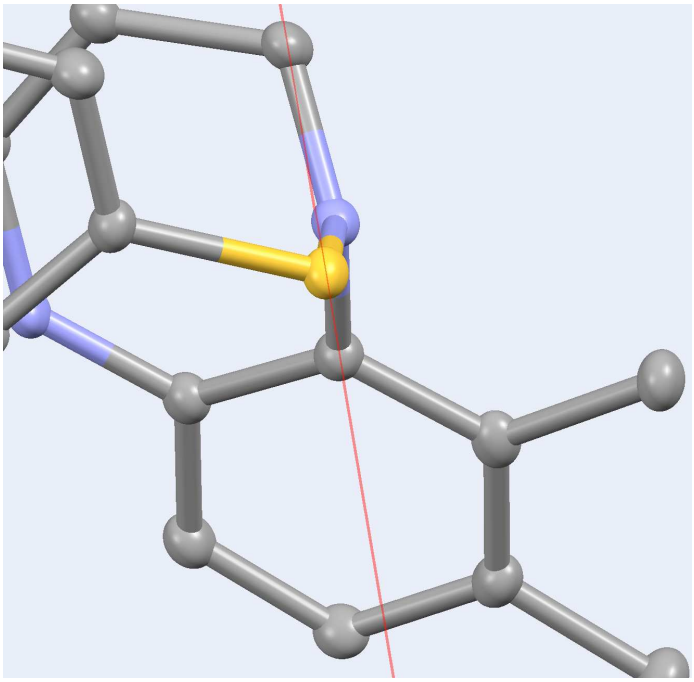


Figure 4.

Figures 3 and 4 show the line for the plane (S1-C5-C18) and the slight displacement of N4 from that plane (0.142 Å) in **8a** and **8b**, respectively. The plane barely intersects the ellipsoid drawn at the 50% probability envelope. Based on the electron density there is no evidence that N4 is inverting in the crystal structure.

Chiral liquid chromatography and conversion kinetics study. Chiral LC was developed to monitor conversion between **8a** and **8b**. Instrument: Agilent LC1100 with DAD and temperature control of sample compartment was used for experiment at 25°C. Waters ZQ LC/MS was used for experiment at 40°C. Both sample compartment and column were maintained at same temperature. Auto sampler was covered by aluminum foil to protect the sample from the light. Lux Cellulose-4 150x4.6 3u, 30% 0.1% H_3PO_4 70% MeCN isocratic, 1 mL/min at 210nm detection. Sample preparation: 0.298 mg/ml sample in acetonitrile (25°C) and 0.633 mg/ml sample in acetonitrile (40°C) were prepared immediately before analysis in amber HPLC vials and injected at different time points.

The conversion was slow but measurable at 25 °C. A total of 35 data points were collected in 22 days (Figure 5) and the data were integrated and regression-analyzed (Figure 6).

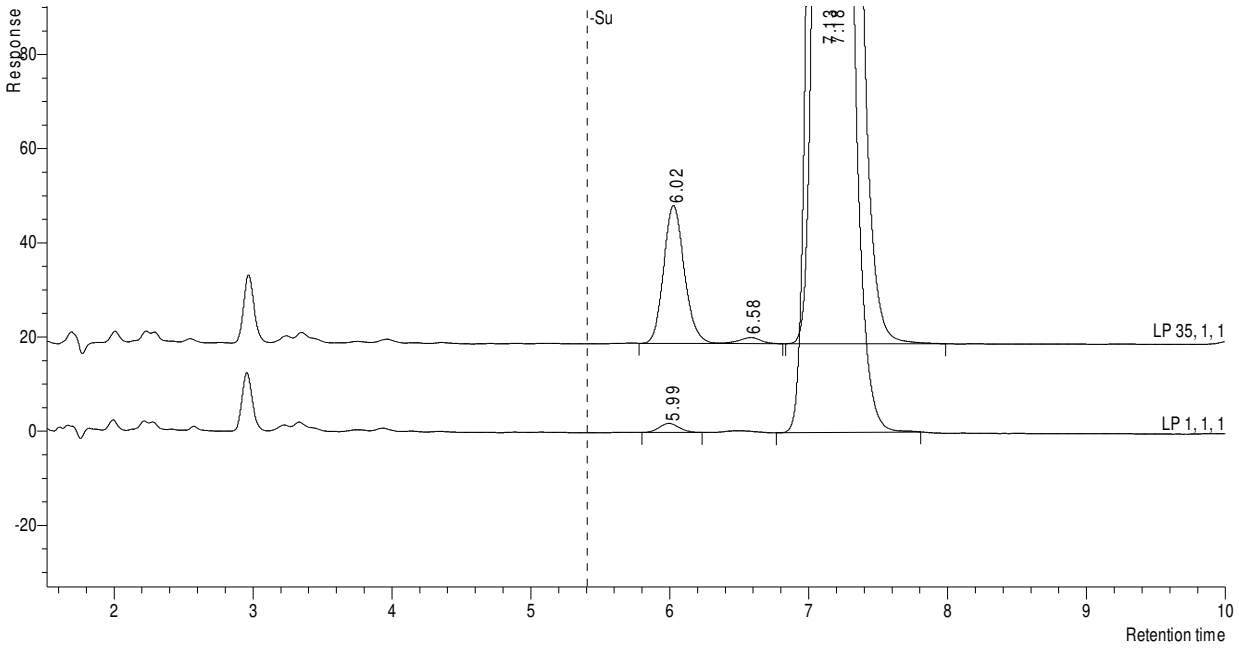


Figure 5. Chiral chromatogram overlay bottom: 0.2% at 0 hour; top: 2.7% after 22 days at 25°C

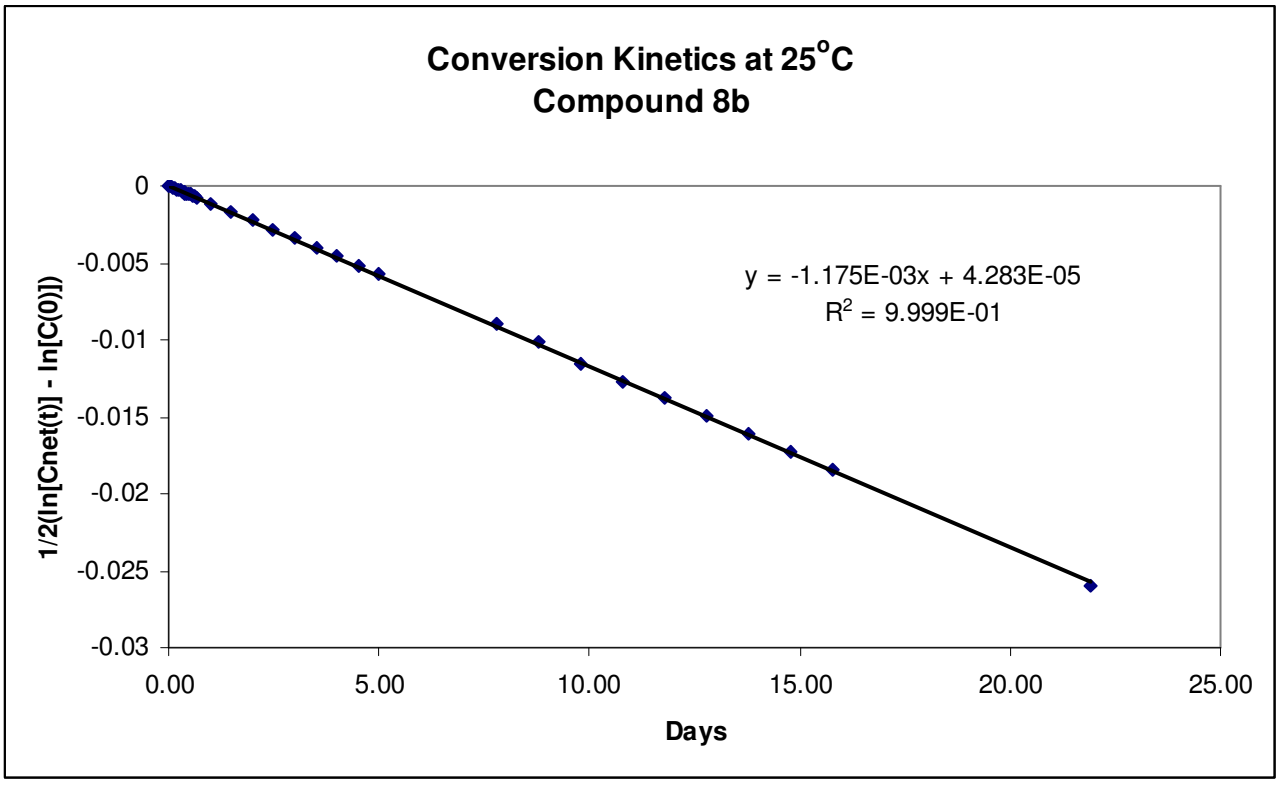


Figure 6. Conversion kinetics plot at 25°C by chiral LC data

The conversion between **8a** and **8b** was also measured at slightly raised temperature of 40°C and a total 20 points were collected in 3 days by Chiral HPLC (Figure 7).

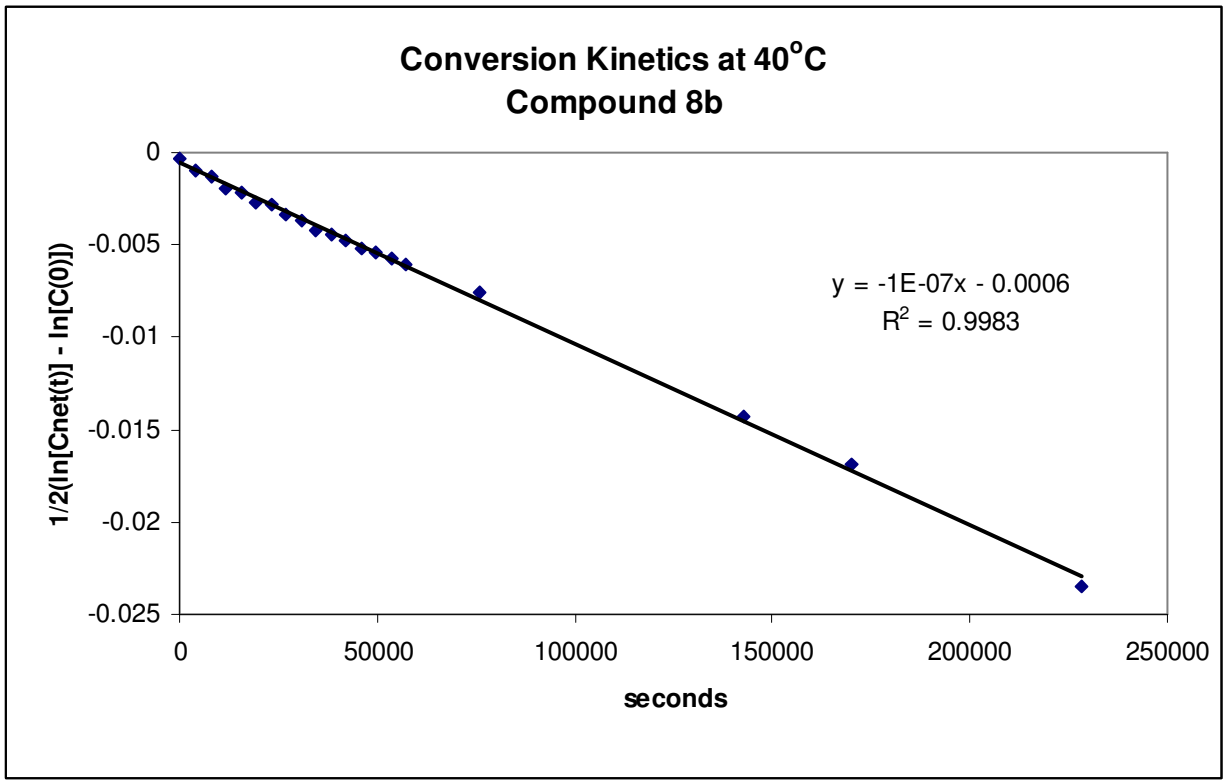


Figure 7. Conversion kinetics plot at 40°C by chiral LC data

The rate constant K and half time at 25 °C and 40 °C from regression analysis are listed in the following table.

	25 °C	40 °C
K	-1.35 x 10^{-8} sec^{-1}	-1.03 x 10^{-7} sec^{-1}
$T_{1/2}$	296.0 days	38.9 days

At 25 °C: $T_1 = 298$; $k_1 = A \times \exp(-Ea/RT_1)$; $k_1 = 1.35 \times 10^{-8}$ s^{-1}

At 40 °C: $T_2 = 313$; $k_2 = A \times \exp(-Ea/RT_2)$; $k_2 = 1.03 \times 10^{-7}$ s^{-1}

From these two equations, the activation energy Ea was calculated as 25.2 kcal/mol.

Molecular modeling. Molecular modeling hypothesized that the conversion between **8a** and **8b** is due to the pucker change in the diazepine ring system, which is mediated by a co-planar conformation. In order to understand the transition energy barrier, simple calculations at the *ab initio* HF/6-31G** level were carried out along the torsion angle χ (C_{13}-C_{19}-N_{11}-S_{20}) of the diazipine ring and estimated an energy barrier at about 33 Kcal/mol at room temperature. Experimentally measured rate constants by Chiral LC studies led to an estimated energy barrier of 25 Kcal/mol, which explains the very slow conversion of these two isomers at room temperature and is in line with the calculated energy barrier caused by the pucker change.