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Probing the stereochemistry of the active site of gamma-glutamyl transpeptidase using sulfur derivatives of L-glutamic acid

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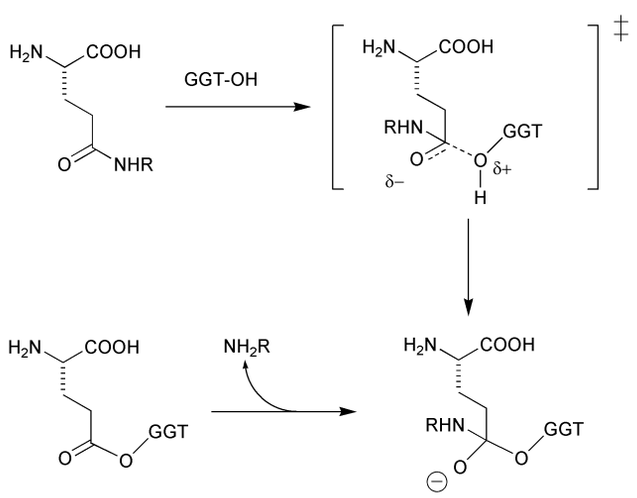
Gamma-glutamyl transpeptidase (GGT) catalyses the transfer of a γ-glutamyl moiety from a donor substrate to different acceptors, such as amino acids and water. GGT is known to display relatively low stereospecificity with respect to the α-stereocentre of its donor substrates. In this study we have studied its stereospecificity with respect to the stereocentre at the δ-position of different analogues of L-glutamic acid. Notably, L-methionine sulfoxide is well-recognised whereas L-methionine sulfone and L-methionine sulfoximine are not. Furthermore, when the synthetic γ-diastereoisomers of L-methionine sulfoxide were separated and tested, it was discovered that GGT shows remarkable stereospecificity at the γ-position, binding the S_CS_S diastereoisomer with a K_i of 3.5 mM, whereas the S_CR_S diastereoisomer is not recognised. Finally, using a sulfoxide as a new pharmacophore for GGT, we have synthesized and tested an analogue of glutathione to obtain a very promising competitive inhibitor with a K_i of (53 ± 3) μM.

Introduction

γ-Glutamyl transpeptidase (GGT; E.C. 2.3.2.2) is a highly glycosylated heterodimeric enzyme found mainly in the kidney, but also in the brain and pancreas.[1] It catalyses the formation of leukotriene D_4, is implicated in the inflammatory response,[2] and is involved in cellular detoxification[3] owing to the critical role that glutathione (γ-glutamylcysteinylglycine), its *in vivo* substrate, plays in this process. It also plays numerous roles in physiological disorders, namely inhibition of apoptosis,[4,5] Parkinson's disease,[6] diabetes[7] and asthma.[8,9] However, its precise pathological function is not well known, due to lack of information about its mechanism and its structure.

GGT catalyses the transfer of the γ-glutamyl moiety of glutathione (donor substrate) to acceptor substrates, usually amino acids or dipeptides, or to a water molecule, forming either a γ-glutamyl–acceptor compound containing an isopeptide bond or glutamate, respectively, through a modified ping-pong mechanism.[10] The enzyme binds glutathione and releases cysteinylglycine as its first product to form an acyl–enzyme intermediate in the acylation step.[11] This transient intermediate then binds the acceptor substrate and transfers its acyl moiety to form the second product (γ-glutamyl–acceptor) and regenerate the free enzyme in the deacylation step. α-Amino acids serve as good acceptor substrates, and the resulting transamidation reaction has been suggested to represent the principal physiological function of the enzyme, particularly in the γ-glutamyl cycle.[12] However, a water molecule can also act as an acceptor 'substrate', leading to hydrolysis of the acyl–enzyme and formation of glutamate. The nature of the amino acids implicated in substrate binding and the catalysis of these transamidation and hydrolysis reactions are not well known.

The active-site nucleophile has not been identified for all types of GGT. *E. coli* GGT has been inhibited with a fluorophosphonate analogue of glutamic acid and subsequent mass spectrometric sequencing revealed the *N*-terminal Thr-391 of the light subunit, conserved at that position in all mammalian GGTs, to be covalently modified, leading the authors to classify this enzyme as an *N*-terminal nucleophile (Ntn) hydrolase.[13] However, other work based on site-directed mutagenesis of human GGT has suggested Ser-405 or Ser-406 may be the active site nucleophile.[1] For the widely studied rat GGT, no nucleophile has been unambiguously identified. We have previously proposed that the nucleophile (probably a Thr hydroxyl group) attacks the carbonyl group of the γ-glutamyl amide bond to form a tetrahedral intermediate (Scheme 1). The rate-limiting step of the acylation process is the decomposition of this intermediate, namely the cleavage of the C–N bond with concomitant protonation of the departing amine, probably by a nearby His residue.[14]



Scheme 1 Reaction catalysed by rat kidney GGT.

The binding site for the donor substrate has been studied in detail, showing that GGT is intolerant of certain modifications of the γ-glutamyl moiety. For example, the free ammonium group and the carbonyl at the α-position are necessary for good recognition of the substrate.[15] However, donor substrates of either L- or D-configuration are recognised without significant selectivity.[10] The side chain cannot bear any alkyl substituents, except at the α-position.[16] Upon substitution of a methylene unit by heteroatoms such as oxygen or nitrogen, the resulting compounds no longer act as donor substrates.[17] However, due to the lack of a crystal structure suitable for analysis of any type of GGT, it is difficult to specify the precise nature of the amino acids involved in the mechanism or affecting the stereochemistry of the active site near the nucleophile.

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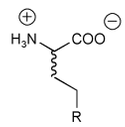
Table 1 Inhibition constant (K_i) and IC_{50} values for compounds **1**–**9** as inhibitors of the transpeptidation reaction between L-γ-glutamic acid *p*-nitroanilide and glycylglycine catalysed by rat kidney GGT at pH 8.0 and 37 °C



α-Isomer, R group (compound no.)	K_i/mM	Type of inhibition	α-Isomer, R group (compound no.)	K_i/mM	Type of inhibition
L, CO_2^- **(1)**	14.1 [a]	Competitive	L, SO_2CH_3 **(6)**	N/I [c]	—
L, CN^- **(2)**	20.6 [b]	—	DL, SO_3^- **(7)**	11.1	Uncompetitive
5-Hydroxy-DL-lysine **(3)**	N/I [c]	—	L, $SOCH_3$ [d] **(8)**	5.9	Competitive
L, SCH_3 **(4)**	26.9 [a]	Competitive	DL, PO_3^{2-} **(9)**	35.8	Competitive
L, $SO(NH)CH_3$ **(5)**	N/I [c]	—			

[a] See reference 19. [b] IC_{50} value determined in the presence of 400 μM L-γ-glutamic acid *p*-nitroanilide and 20 mM glycylglycine. [c] N/I: negligible inhibition at concentrations up to 20 mM. [d] 50 : 50 mixture of diastereoisomers at the sulfur atom.

In order to gain insight into the steric environment near the catalytic nucleophile, active site mapping was carried out with various L-glutamic acid analogues bearing different functionalities at the γ-position on the lateral chain. As shown in Fig. 1, the carbonyl group of the glutamyl moiety was replaced by various functional groups, thereby altering the geometry of the donor substrate near the nucleophile. Analysis of the relative affinities of these compounds as competitive inhibitors at the donor substrate binding site has permitted an examination of the stereospecificity of the active site and the design and synthesis of a glutathione analogue that is one of the most potent inhibitors of GGT. These results are discussed herein.

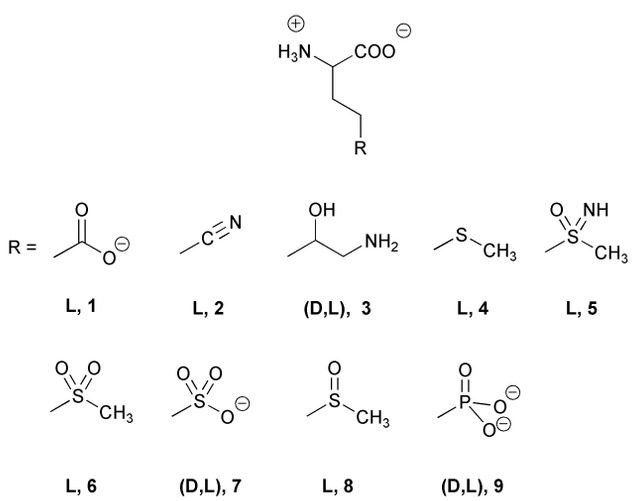


Fig. 1 Different compounds tested with GGT.

Results and discussion

Previous studies from our laboratories [15] and others [18] have shown that an inhibitor must incorporate certain features to display good affinity for the active site of GGT. For example, it appears that a free α-ammonium group and an α-carbonyl group are essential for recognition. A series of compounds were designed to contain these functional groups while varying the geometry of the atom at the δ-position of the corresponding glutamine model compound. Compounds **1**–**9**, as shown in Fig. 1, were tested for their ability to act as inhibitors of the GGT-mediated transpeptidation reaction between L-γ-glutamic acid *p*-nitroanilide as the donor substrate and glycylglycine as the acceptor substrate. Glycylglycine was chosen as an acceptor substrate both for its efficiency and its known inability to act as an inhibitor at the donor substrate binding site,[19] such that our inhibition results are relevant to the compounds tested and not contaminated by background inhibition inherent in the test reaction. For each compound tested, a K_i (or an IC_{50}) value was determined and is reported in Table 1. Almost all of the compounds were found to be competitive inhibitors, as was obvious from the Lineweaver–Burk plots of the inhibition data (not shown).

Compound **1**, L-glutamic acid, is a reasonable analogue of L-glutamine, which serves as a simple donor substrate for GGT,[19] and functions as a modest competitive inhibitor (Table 1). Compound **2**, the nitrile of L-glutamine, was shown to be a weaker inhibitor of GGT, perhaps due to its linear geometry at the δ-position, even though inhibitors of this type have been effective for other enzymes such as chymotrypsin.[20]

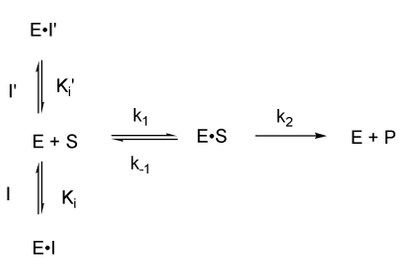
Certain compounds have been reported that are excellent inhibitors of GGT, purportedly due to their similarity to the transition state of GGT acylation.[21] For example, a complex of L-serine and borate has been used to inhibit GGT with a K_i of 20 μM.[22] The L-glutamic acid boronate analogue (*ABBA*) has an *in vitro* K_i of 17 nM and is one of the best competitive inhibitors for GGT, although it also inhibits several other glutamate-dependant enzymes, such as glutamic pyruvic transaminase. Its efficacy as an inhibitor of cell growth has been demonstrated, although *in vivo* concentrations of 1 mM are required. Hoping to capitalise on the general efficiency of transition-state analogues and to improve upon the specificity of known GGT inhibitors, a series of compounds were tested that bear functional groups that resemble the geometric and/or electrostatic character of the transition state of the acylation step of GGT catalytic cycle.

5-hydroxy-DL-lysine (**3**) has also been tested with other enzymes with some success [23] due to its resemblance to glutamine. However, when we tested **3** with GGT, no inhibition was observed. L-Methionine, thioether **4**, has previously been tested as an inhibitor of the donor substrate and found to have low affinity for the enzyme.[19] The absence of the double bonded oxygen may explain the lack of recognition of this compound. In order to test for the necessity of this double bond, we tested the sulfoximine **5**, which is known to be a good inhibitor for enzymes recognizing a γ-glutamyl moiety such as glutamine synthetase.[25] However, in light of the high IC_{50} obtained for GGT, compound **5** is not well recognised by GGT. Thinking that the NH group could affect its recognition by the enzyme, we prepared and tested sulfone **6**. This compound also proved incapable of inhibiting the enzyme, even though compound **6** has been used as a L-glutamic acid analogue for inhibition of enzymes such as glutamate synthase [24] and glutamine synthetase.[25] To probe the effect of replacing the methyl group with a heteroatom, sulfonic acid **7**, DL-homocysteic acid, was tested. For this compound, weak inhibition was observed (K_i = 11.1 mM) that appeared to be uncompetitive with respect to the donor substrate. This signifies that while this substitution did not improve affinity for the donor substrate binding site, it

apparently led to a modest increase in affinity for a second site on the enzyme. Further tests showed that it is not a good competitive inhibitor of glycyclycine, however, so it does not appear to be bound in the acceptor substrate binding site. In partial summary of these results, from comparison of the inhibition results obtained for compounds **4**–**7**, it would appear that the presence of two double bonds at the δ-position adversely affects the affinity of the compound for the donor substrate binding site, the simple thioether **4** being the best of this subset and a weak inhibitor at that.

Conversely, when the commercial sulfoxide analogue **8** was tested it was found to act as a surprisingly efficient competitive inhibitor of the donor substrate of the transpeptidation reaction. Its K_i value was found to be less than half of that of compound **1**, L-glutamic acid. The sulfoxide functionality has interesting geometric and electrostatic properties and has been widely used as L-glutamic acid analogue inhibitors with different enzymes.[26,27] The sulfoxide group can be represented as a sulfur atom double bonded with an oxygen, or forming a dative bond with an oxygen, or bearing a partial positive charge bonded to an oxygen atom with a partial negative charge.[28] In all cases, the sulfur atom at the δ-position of compound **8** represents a stereocentre, the fourth corner of the asymmetric tetrahedron occupied by a lone pair of electrons. For the K_i value reported in Table 1, a mixture of the two δ-diastereoisomers of **8** was used. Stereoselectivity for chiral sulfoxide derivatives has been observed in the inhibition of peptidyl transferase by the antibiotic sparsomycin,[29] so we were intrigued to see if GGT would display greater affinity for one diastereomer of **8** compared to the other. In order to measure the stereoselectivity of GGT for the δ-position of the molecule, we synthesised and separated the two diastereoisomers according to a literature procedure[30–32] as described in the Experimental section. The diastereomers **8a** (S_CS_S) and **8b** (S_CR_S) were thus obtained, but only **8a** was obtained as a relatively pure stereoisomer, having a diastereoisomeric excess (de) of > 95%, as determined by ^{13}C NMR.[30] The S_CS_S molecule gave a K_i value of 3.4 mM, nearly half the value of the diastereoisomeric mixture. This suggested the enzyme has a lower affinity for the S_CR_S diastereomer, compound **8b**. Although purification of **8b** to a de of 95% has been reported elsewhere,[31] in our hands it was always obtained as a mixture of the two δ-diastereomers. Our highest enrichment gave a mixture of 76% **8b** and 24% **8a**. Since it was impossible for us to obtain diastereoisomer **8b** in pure form, we used an indirect kinetic method to determine its K_i.

Scheme 2 signifies the inhibition of an enzyme by two different competitive inhibitors (I and I′), each having different affinities for the same donor substrate binding site, represented by the inhibition constants (K_i and K_i'). As is shown in detail in the Experimental section, one may derive from this competitive inhibition scheme an equation for the variation of the apparent inhibition constant as a function of the mole fraction of inhibitor, namely $\frac{1}{K_{i\,app}} = f \cdot \left(\frac{1}{K_i} - \frac{1}{K_i'} \right) + \frac{1}{K_i'}$. This equation provides the basis for the graphical analysis of the inhibition data obtained for a series of mixtures of diastereomers **8a** (S_CS_S) and **8b** (S_CR_S), displaying satisfactory linear correlation ($r^2 = 0.987$)



Scheme 2 Inhibition by a mixture of two competitive inhibitors.

as shown in Fig. 2. Extrapolation of the data indicated clearly that the value of K_i' (for **8b**) is very high, giving an intercept of around zero in Fig. 2. In this way, the inhibition constant of diastereomer **8b** was determined indirectly, since the compound could not be isolated as a pure diastereomer. From the slope of Fig. 2 the inhibition constant of **8a** was determined to be K_i = 3.4 mM. This lack of recognition by GGT of the S_CR_S isomer **8b**, in contrast to its relatively high affinity for the S_CS_S diastereoisomer **8a** clearly illustrates the stereospecificity of its interactions with the δ-position of γ-glutamyl analogues. These interactions may include an electrostatic interaction of some positively charged amino acid residue with the (partially) anionic oxygen of the sulfoxide, and/or a dipole–dipole interaction between the active site nucleophile (possibly a threonine hydroxyl group) and the partially positively charged sulfur, as represented in Fig. 3. The stereospecificity of these interactions presents an interesting feature for the design of future high-affinity inhibitors of GGT.

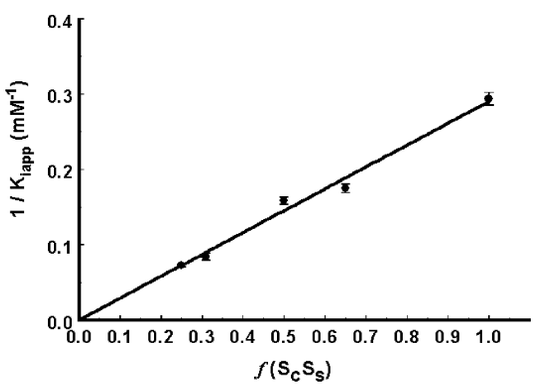


Fig. 2 Plot of $\frac{1}{K_{i\,app}}$ *vs.* f where f represents the fraction of L-methionine sulfoxide S_CS_S (**8a**) in the mixture of the δ-diastereoisomers. The error bars shown for each point arise from the determination of K_i from the secondary plots. (See Experimental section for equations and kinetic analysis.)

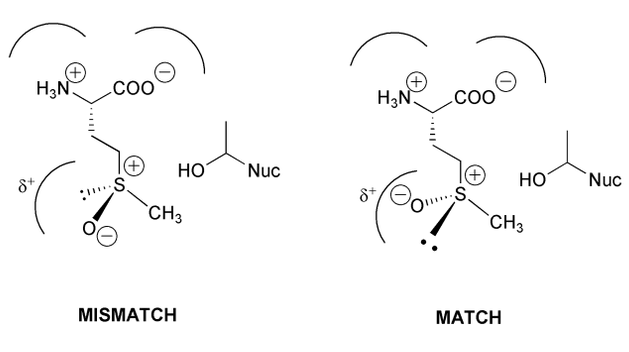
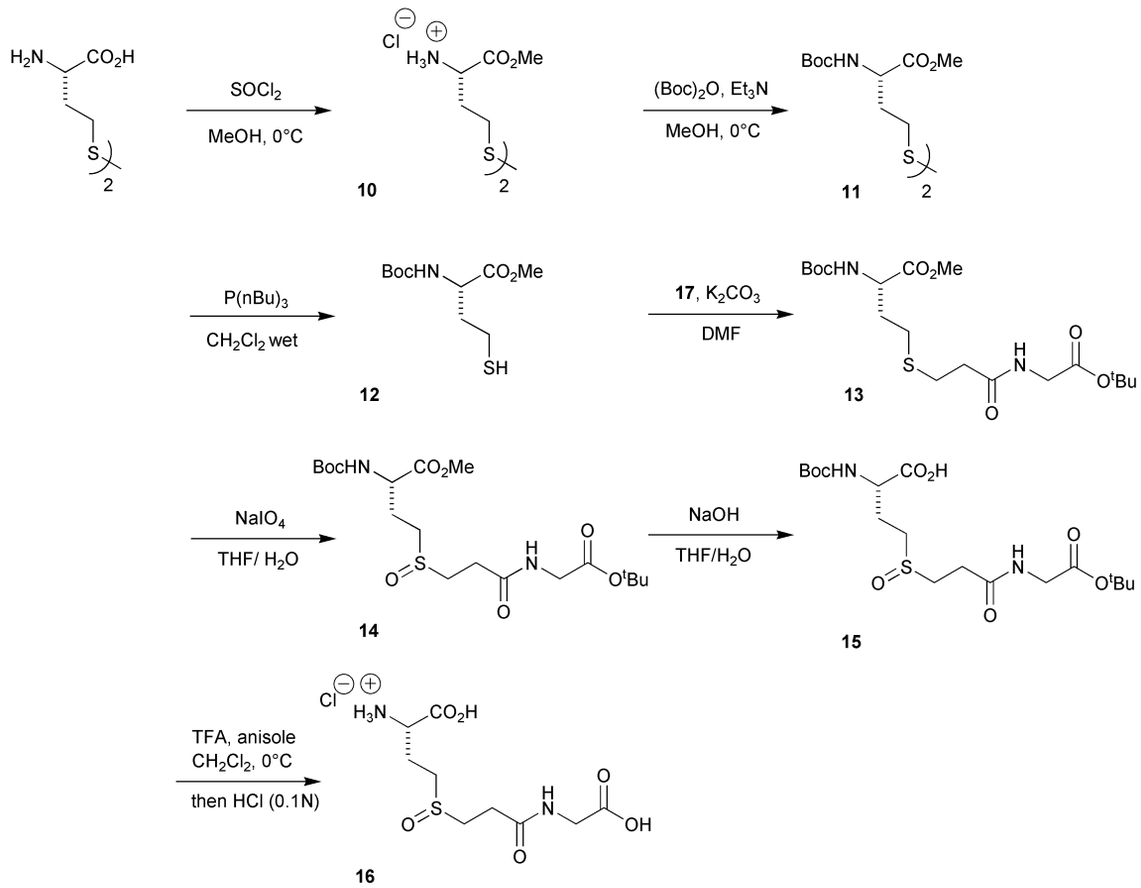


Fig. 3 Figurative representation of the stereochemical interaction of L-methionine sulfoxide in the active site of GGT (Nuc represents the active site nucleophile).

The affinity displayed for the sulfoxides tested herein suggested to us that the distribution of partial charges in this functional group may also be important for favourable interaction with the donor substrate binding site. Phosphorus-containing compounds have been used widely as transition-state analogues of enzymatic reactions.[33] For example, with GGT, a fluorophosphoric acid derivative of glutamic acid has been used as an (irreversible) inhibitor to identify the nucleophile for bacterial GGT.[13] When we tested the commercially available compound **9**, DL-2-amino-4-phosphonobutyric acid, a relatively high K_i of 35.8 mM was determined at pH 8.0. This weak affinity for the donor substrate binding site may be due to charges on the side chain phosphonate present at this pH. The pK_a of the



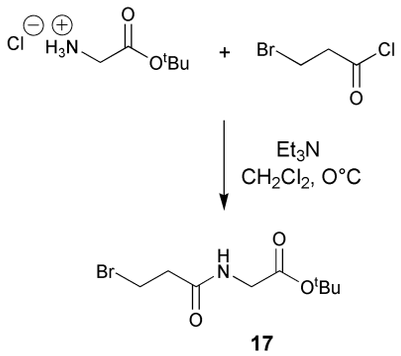
Scheme 3 Synthesis of the glutathione analogue sulfoxide **16**.

α-ammonium and α-carboxylate groups do not vary greatly according to the nature of the side chain, such that the compounds tested all exist predominantly as α-ammonium carboxylates around neutrality. Likewise, one of the two hydroxyl groups bound to the phosphorus atom would have a pK_a value around 2.4[34] and would always be ionised at the pH range of the kinetic analyses. However, the pK_a of the second hydroxyl group varies with the length of the side chain and the nature of adjacent substituents. Compound **9** was therefore titrated to determine that the second pK_a value of the phosphonic acid group is 7.022 ± 0.002. Thus, during the inhibition tests at pH 8.0, both oxygens were negatively charged. One could hypothesize that the low affinity of compound **9** for the donor substrate binding site may be due to electrostatic repulsion. On repeating the same competitive inhibition experiment at successively lower pHs of 7.0 and 6.0, we found increasingly lower K_i values of 5.91 mM and 0.6 mM, respectively. It is noteworthy that the K_i determined for compound **9** at pH 6.0, where the second oxygen would be predominantly protonated, is 60 times lower than at pH 8.0, where the oxygen would be predominantly ionised. Of course, these results may also be due to the ionisation of an amino acid residue in or near the substrate binding site of GGT. We[11,14] and others[35] have shown that GGT-mediated transpeptidation shows a bell-shaped pH-rate profile, with a kinetic pK_a on the acidic limb of around 7.8. Although this pK_a has been attributed to the α-amino group of the acceptor substrate, it may overlap with an ionisation of a residue on the enzyme itself. In any case, it is clear that at lower pH the affinity between compound **9** and the donor substrate binding site is dramatically improved. This suggests that electrostatic interactions at this position of the γ-glutamyl sub-site probably play an important role in inhibitor affinity. This may provide another reason, in addition to its geometry, why sulfoxide **8**, bearing one (partial) negative charge, shows relatively better affinity for GGT.

Methyl sulfoxide **8** is a new type of competitive inhibitor for GGT, but does not contain any recognition element on the sulfoxide moiety. The natural substrate of GGT is glutathione and the presence of the Cys–Gly moiety on the other side of the scissile γ-glutamyl bond is also important in the recognition of this donor substrate by the enzyme. For example, L-glutamine functions as a donor substrate of GGT, but with a K_M value around 10 times greater than that of glutathione. In order to extend the potential binding interactions and consequent inhibitory ability of the parent sulfoxide **8**, an analogue of glutathione was designed, namely sulfoxide derivative **16**. The pendant propionylglycine moiety of **16** was added with the notion that it could be bound in the sub-site usually occupied by the cysteinylglycine moiety of glutathione.

The general procedure used to synthesize compound **16** was based on a published protocol[36] and is shown in Scheme 3. Initially, the α-carboxylate group of L-homocystine was protected as a methyl ester (**10**). Ester **10** was then efficiently converted to the Boc-protected compound **11**. This derivative was reduced by tri-*n*-butylphosphine in the presence of CH_2Cl_2 saturated with water to obtain **12**. The key step of this synthesis is the subsequent condensation of L-homocysteine derivative **12** with bromide **17**. This bromomethylene analogue of glycylglycine was synthesized in one step through the simple condensation of glycine *tert*-butyl ester with 3-bromopropionyl chloride (Scheme 4). Bromide **17** and thiol **12** were then condensed to provide a sulfide **13** which was oxidized directly with $NaIO_4$ to give sulfoxide **14** in good yield. The remaining steps represent the straightforward deprotection of the molecule. The cleavage of the α-methyl ester group was carried out successfully with NaOH to give **15** and the Boc group and *tert*-butyl ester were finally removed with TFA to give compound **16** in good yield.

Compound **16** was then tested in the same way as the other compounds shown in Table 1. It gave a surprisingly low K_i value



17

Scheme 4 Synthesis of the glycylglycine analogue moiety **17**.

of (53 ± 3) μM, well below all the other inhibitors tested in this work, and 100-fold lower than the other sulfoxide, compound **8**. Obviously, the presence of some recognition element *N*-terminal to the analogous γ-glutamyl group contributed greatly to the overall binding affinity of this compound, giving an inhibitor that compares well with other known GGT inhibitors. It is important to note that the K_i value for **16** was measured on a 50 : 50 mixture of the two *S*-diastereoisomers, since we were unable to separate them by the same techniques used for compound **8**. Although one may speculate that the stereospecificity that GGT manifested for compound **8** would suggest that the S_S diastereomer of **16** would give a K_i value even lower than 53 μM, we were unable to test this hypothesis.

Other analogues of glutathione have been synthesized containing heteroatoms in the γ-position on the lateral chain of the γ-glutamyl moiety,[37,38] or modifications on the sulfur of the cysteinyl moiety. These compounds are good inhibitors, probably due to their affinity for the binding site of the cysteinyl moiety. The synthesis of a γ-sulfonamide analogue of glutathione has been reported, but no kinetic data is available yet to indicate if this (possibly irreversible) inhibitor shows good affinity for GGT.[39]

In conclusion, we have shown that γ-glutamyl derivatives containing a sulfoxide moiety at the δ-position are a promising new class of GGT inhibitors. GGT displays stereospecific affinity for one diastereoisomer (S_CS_S) of methionine sulfoxide, as determined by an indirect kinetic analysis method that should prove useful for the analysis of other known mixtures of competitive inhibitors. We have also synthesized a sulfoxide analogue of glutathione which is a potent inhibitor of GGT and demonstrates the importance of interactions with the putative cysteinylglycine sub-site of the glutathione binding site to the overall affinity with the inhibitor. Studies are currently in progress in our laboratory to design and prepare better glutathione analogue inhibitors and to glean more structural information regarding the active site of GGT.

Experimental

Material

All compounds (except **2**) were purchased from Sigma-Aldrich and were used without further purification. Compound **2** is a generous gift from Dr B. Badet[40] (CRNS, Gif–sur–Yvette, France). Compounds **8a** and **8b** were synthesized as described below. The buffers MES, MOPS and Tris and glycylglycine were purchased at Sigma. The donor substrate L-γ-glutamic acid *p*-nitroanilide was synthesized as described previously.[41] Rat kidney GGT was purified as previously described in an established protocol.[14] For the kinetic studies, absorbance values at 410 nm were recorded on a Cary 100 Bio spectrophotometer or on a Pharmacia Biotech Ultrospec 2000 spectrophotometer. The pK_a of compound **9** was determined by using a Mettler Toledo DL53 Titrator.

All chemical products used in synthesis were purchased from Sigma-Aldrich. ^{1}H and ^{13}C spectra were recorded on Brukers AMX300 or ARX300 (300 MHz) or Brukers AMX400 or ARX400 (400 MHz). All solvents were dried using a drying column on a GlassContour system (Irvine, CA). Low- and high-resolution mass spectra were obtained from the Regional Centre of Mass Spectrometry at the Université de Montréal. Melting points were determined with a capillary tube with an Uni-melt Thomas-Hoover melting point apparatus and are reported as corrected values. IR spectra were recorded on a FT-IR Paragon 1000 spectrophotometer (Perkin Elmer). Specific optical rotations were measured at 20 °C in units of 10^{-1} deg cm^2 g^{-1}.

Synthesis

Synthesis of sulfoxides 8a and 8b. Sulfoxides **8a** and **8b** were prepared according to a previously described procedure,[30,31] with some slight modifications. The hydrogen peroxide oxidation of *N*-phthaloyl L-methionine was done at room temperature over 2 days and not at −20 °C. The S_CR_S and S_CS_S stereoisomers were initially isolated as a 50 : 50 mixture, as determined by comparison of the S–CH_3 and the C_α peaks of the ^{13}C NMR.[31] The S_CS_S diastereoisomer was crystallised in MeOH several times to obtain a purity > 95% by ^{1}H NMR. Different attempts to obtain the other diastereoisomer S_CR_S were unsuccessful. In our hands, separation by simple chromatography (gradient 100% AcOEt to 20% MeOH in AcOEt) gave a maximum ratio of 76 : 24 (S_CR_S : S_CS_S). To obtain mixtures containing different proportions of both diastereoisomers, we varied the number of subsequent recrystallisations in MeOH and determined the diastereomeric ratio of the recrystallised product by NMR ^{13}C according to the published procedure.[30] In this way we generated and tested diastereomeric mixtures of which the mole fraction of diastereomer **8a**, *f* was 0.24, 0.31, 0.5, 0.65, and 1.0.

L-Homocystine methyl ester hydrochloride (10). To a solution of L-homocystine (2.262 mmol, 0.607 g) in methanol (15 mL) at 0 °C, was added thionyl chloride (13.6 mmol, 0.988 mL). The solution was kept overnight at room temperature and then removed under reduced pressure. To remove the traces of $SOCl_2$, the resulting oil was diluted in $CHCl_3$ and concentrated again to give a hygroscopic gummy solid (0.833 g, 100%). This compound **10** was used without further purification. $[\alpha]_D$ +52.6 (*c* 0.63, MeOH); IR (NaCl, cm^{-1}) 3410, 2956, 1746, 1620, 1516, 1442, 1234; ^{1}H NMR (MeOD, 400 MHz) 2.32 (2H, m), 2.85 (2H, m), 3.82 (3H, s), 4.18 (1H, t, J = 5.4 Hz); ^{13}C NMR (MeOD, 100 MHz) 30.6, 33.4, 52.4, 53.7, 170.2. HRMS (MAB, M^+) 296.0876. $C_{10}H_{20}N_2O_4S_2$ calculated 296.0865.

***tert*-Butoxycarbonyl L-homocystine methyl ester (11).** To a solution of **10** (0.815 mmol, 0.300 g) in MeOH (10 mL) at 0 °C, was added Boc_2O (2.445 mmol, 0.534 g) and triethylamine (3.261 mmol, 0.454 mL). The solution was stirred overnight at room temperature. The MeOH was removed under reduced pressure and the residue was diluted in $CHCl_3$ and washed with aqueous HCl (0.1 M). The organic layer was dried over $MgSO_4$ and removed under reduced pressure, giving a white solid which was purified by flash chromatography (30% EtOAc in hexane) (0.396 g, 98%). R_f 0.5 (50% EtOAc in hexane). $[\alpha]_D$ +30.4 (*c* 0.53, $CHCl_3$); IR (NaCl, cm^{-1}) 3363, 2978, 1714, 1515, 1439, 1367, 1250, 1219, 1165, 1051, 1025; ^{1}H NMR ($CDCl_3$, 300 MHz) 1.38 (18H, s), 1.95 (2H, m), 2.25 (2H, m), 2.66 (4H, t, J = 7.4 Hz), 3.74 (6H, s), 4.34 (2H, m), 5.22 (2H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) 28.4, 32.6, 34.6, 52.6, 80.2, 155.5, 172.8; HRMS (MAB, M^+) 496.1910. $C_{20}H_{36}N_2O_8S_2$ calculated 496.1913.

***tert*-Butoxy-L-homocysteine methyl ester (12).** To a solution of **11** (0.786 mmol, 0.390 g) in wet CH_2Cl_2 (6 mL), was slowly

added tri-(*n*-butyl)phosphine (0.786 mmol, 0.197 mL) under a nitrogen atmosphere. The mixture was stirred for 30 minutes and the solvent removed under reduced pressure. The residue was purified by flash chromatography (75% hexane in EtOAc) to give **12** (0.392 g, 100%) as an oil. R_f 0.5 (33% EtOAc in hexane). $[\alpha]_D$ +13 (*c* 0.60, $CHCl_3$); IR (NaCl, cm^{-1}) 3352, 2978, 2580, 1710, 1513, 1436, 1366, 1164, 1050; ($CDCl_3$, 300 MHz) 1.42 (9H, s), 1.56 (1H, t, *J* = 8.1 Hz), 1.87–2.09 (2H, m), 2.55 (2H, m), 3.73 (3H, s), 4.43 (1H, m), 5.09 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) 20.8, 28.4, 37.3, 52.3, 52.6, 80.2, 155.5, 172.9; HRMS (MAB, M^+) 249.1035. $C_{10}H_{19}NO_4S$ calculated 249.1035.

2-*tert*-Butoxycarbonylamino-4-[2-(*tert*-butoxycarbonylmethyl-carbamoyl)ethylsulfanyl]-butyric acid methyl ester (13). To a solution of **12** (1.570 mmol, 0.390 g) in DMF (7 mL) at 0 °C, was added **17** (1.883 mmol, 0.5 g) and then K_2CO_3 (1.727 mmol, 0.239 g) under a nitrogen atmosphere. The solution was warmed to room temperature. After stirring overnight, the solution was diluted with water and the desired product was extracted 5 times with EtOAc. The organic layer was concentrated under reduced pressure and the residue was purified by flash chromatography (35% EtOAc in hexane) to obtain a colourless oil (0.489 g, 72%). R_f 0.27 (35% EtOAc in hexane). $[\alpha]_D$ +6.6 (*c* 0.43, $CHCl_3$); IR (NaCl, cm^{-1}) 3330, 2979, 1744, 1708, 1663, 1529, 1439, 1368, 1225, 1161; ^1H NMR ($CDCl_3$, 300 MHz) 1.42 (9H, s), 1.45 (9H, s), 1.87–2.09 (2H, m), 2.50 (4H, m), 2.75 (2H, t, *J* = 7.1 Hz), 3.72 (3H, s), 3.93 (2H, d, *J* = 5.1 Hz), 4.38 (1H, m), 5.28 (1H, d, *J* = 8.1 Hz), 6.55 (1H, t, *J* = 4.7 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz); 27.4, 28.1, 28.4, 32.5, 36.5, 42.1, 52.5, 52.7, 80.1, 82.4, 155.5, 169.1, 171.2, 172.8; HRMS (FAB, MH^+) 435.2161. $C_{19}H_{35}N_2O_7S$ calculated 435.2165.

2-*tert*-Butoxycarbonylamino-4-[2-(*tert*-butoxycarbonylmethyl-carbamoyl)ethylsulfinyl]-butyric acid methyl ester (14). To a solution of **13** (0.375 mmol, 0.163 g) in THF : H_2O (7 : 2) (6 mL) at 0 °C, was added 2 mL H_2O containing 0.073 g of $NaIO_4$ (0.342 mmol). After 1 day of reaction, the THF was evaporated under reduced pressure. The remaining aqueous solution was diluted with water and the desired product was extracted with $CHCl_3$ (3 × 10 mL). The organic layer was dried over $MgSO_4$ and the resulting oil was purified by flash chromatography (gradient 100% EtOAc to 80% EtOAc in MeOH) to give the desired product as an oil (0.154 g, 91%); R_f 0.2 (100% EtOAc); $[\alpha]_D$ +12.7 (*c* 0.29, $CHCl_3$); IR (NaCl, cm^{-1}) 3294, 2979, 2928, 1741, 1716, 1656, 1530, 1448, 1368, 1226, 1159, 1021; ^1H NMR ($CDCl_3$, 300 MHz) 1.40 (9H, s), 1.42 (9H, s), 2.01–2.37 (2H, m), 2.75 (4H, m), 2.84–3.12 (2H, m), 3.71 (3H, s), 3.88 (2H, dd, *J* = 5.1 Hz, *J* = 2.3 Hz), 4.36 (1H, m), 5.58 (1H, m), 7.00 (1H, m); ^{13}C NMR ($CDCl_3$, 75 MHz) 26.0 (d, 50 : 50 mixture of diastereoisomers), 28.1, 28.3, 28.5, 42.2, 47.0 (d), 48.4 (d), 52.6, 52.7, 80.2, 82.2, 155.6, 169.0, 170.3, 172.2. HRMS (FAB, MH^+) 451.2127. $C_{19}H_{35}N_2O_8$ calculated 451.2114.

2-*tert*-Butoxycarbonylamino-4-[2-(*tert*-butoxycarbonylmethyl-carbamoyl)ethylsulfinyl]-butyric acid (15). A solution of **14** (0.361 mmol, 0.163 g) with NaOH (0.543 mmol, 0.022 g) in THF–H_2O (7 : 2) (5 mL) at 0 °C was stirred for 2 h. The reaction was concentrated under reduced pressure, diluted in $CHCl_3$ and extracted with H_2O. The aqueous layer was acidified to pH 2–3 and EtOAc (3 × 20 mL) was added to extract compound **15**. The organic layer was dried with $MgSO_4$ and concentrated to obtain an oil (0.112 g, 71%). R_f 0.13 (75% EtOAc in MeOH); $[\alpha]_D$ +21.0 (*c* 0.56, $CHCl_3$); IR (NaCl, cm^{-1}) 3320, 2979, 2930, 1710 (br), 1531, 1368, 1230, 1160, 1023; ^1H NMR ($CDCl_3$, 400 MHz) 1.42 (9H, s), 1.44 (9H, s), 2.26 (m, 2H), 2.78–2.90 (4H, m), 3.00–3.18 (2H, m),3.92 (2H, br s), 4.38 (1H, m), 5.80 (1H, dd, *J* = 6.4 Hz, *J* = 30 Hz), 7.19 (1H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) 25.9 (d, 50 : 50 mixture of diastereoisomers), 28.2, 28.5, 30.4, 42.3 (d), 47.2, 52.5, 77.2, 80.3, 82.5, 155.9, 169.2 (d), 170.8, 174.5 (d). HRMS (FAB, MH^+) 437.1953. $C_{18}H_{33}N_2O_8S$ calculated 437.1958.

2-Amino-4-[2-(carboxymethylcarbamoyl)ethylsulfinyl]-butyric acid (16). To a solution of **15** (0.248 mmol, 0.108 g) in CH_2Cl_2 (5 mL) at 0 °C, was added anisole (0.248 mmol, 0.027 mL) and TFA (7.428 mmol, 0.572 mL). The solution was stirred overnight at room temperature. A supplementary volume of 0.191 mL (2.476 mmol) of TFA was added and the mixture was stirred again for 5 h. The solvent was removed under reduced pressure. Cyclohexane was added to the residue and the solution was concentrated under reduced pressure 3 times. The resulting oil was dissolved in $CHCl_3$ and the desired product was extracted with aqueous HCl (0.1 M). The aqueous layer was concentrated under reduced pressure to obtain a pale yellow very hygroscopic gum (0.074 g, 95%). $[\alpha]_D$ +13.1 (*c* 0.300, MeOH); IR (NaCl, cm^{-1}) 3372, 2933, 1735, 1654, 1550, 1414, 1230, 1002; ^1H NMR (D_2O, 300 MHz) 2.32 (2H, m), 2.73 (2H, t, *J* = 7.2 Hz), 2.80–3.22 (4H, m), 3.90 (2H, s), 4.07 (1H, td, *J* = 3.0 Hz, *J* = 6.6 Hz); ^{13}C NMR (D_2O, 75 MHz); 23.6 (d, 50 : 50 mixture of diastereoisomers), 28.3, 41.4, 46.5 (d), 46.6 (d), 51.8 (d), 171.0, 173.6; HRMS (FAB, MH^+) 281.0817. $C_9H_{17}N_2O_6S$ calculated 281.0807.

(3-Bromopropionylamino)acetic acid *tert*-butyl ester (17). To a solution of glycine *tert*-butyl ester hydrochloride (0.978 g, 5.833 mmol) and Et_3N (1.626 mL, 1.167 mmol) in CH_2Cl_2 (10 mL) at 0 °C, was added a solution of 3-bromo-propionyl chloride (0.588 mL, 5.833 mmol) in CH_2Cl_2 (5 mL) under a nitrogen atmosphere. The mixture was allowed to stir overnight. The residue was purified by flash chromatography (20% EtOAc in hexane) to give **17** (1.026 g, 66%) as a white solid. R_f 0.20 (35% EtOAc in hexane). mp 104–106 °C; IR (NaCl, cm^{-1}) 3288, 3091, 2977, 1741, 1651, 1567, 1413, 1367, 1227, 1154, ^1H NMR ($CDCl_3$, 400 MHz) 1.43 (9H, s), 2.81 (2H, t, *J* = 6.7 Hz), 3.60 (2H, t, *J* = 6.7 Hz), 3.92 (2H, d, *J* = 5.0 Hz), 6.49 (1H, br s); ^{13}C NMR ($CDCl_3$, 100 MHz) 27.2, 28.1, 39.3, 42.2, 82.5, 169.1, 170.0; HRMS (MAB, MH^+) 266.0404. $C_9H_{17}NO_3Br$ calculated 266.0392.

pK_a determination

A solution of **9** (final concentration 1.5 mM) in 55 mM KCl plus 4 mM HCl was titrated with a solution of 0.1 M NaOH from pH 2.0 to pH 12.0 in triplicate. The pK_a was calculated using an internal routine.

Enzyme inhibition studies

Stock solutions for each compound tested were prepared as follows: L-γ-glutamic acid *p*-nitroanilide (5 mM), glycylglycine (0.1 M), L-glutamine nitrile **2** (50 mM), 5-hydroxy-DL-lysine **3** (200 mM), L-methionine sulfoximine **5** (200 mM), L-methionine sulfone **6** (200 mM), DL-homocysteic acid **7** (200 mM), L-methionine sulfoxide **8** (200 mM), DL-2-amino-4-phosphono-butyric acid **9** (200 mM), and sulfone **16** (10 mM) were all prepared in 0.1 M Tris-HCl at pH 8.0. Compound **9** was also prepared in 0.1 M MOPS at pH 7.0 and in 0.1 M MES at pH 6.0.

Calibration curves for the extinction coefficient of *p*-nitroaniline were constructed at pH 6.0 and pH 7.0; the exctinction coefficient at pH 8.0 was previously determined[14] to be 8800 M^{-1} cm^{-1}. A stock solution of 5 mM *p*-nitroaniline was made in 0.1 M MES pH 6.0 buffer. Concentrations in the range 0–100 μM were used and their absorbance at 410 nm was measured to make a standard absorbance *vs.* concentration curve in duplicate, giving 8440 M^{-1} cm^{-1} as the molar extinction coefficient. When the same experiment was done at pH 7.0 with 0.1 M MOPS buffer, an extinction coefficient of 8110 M^{-1} cm^{-1} was obtained.

All inhibition kinetic experiments were performed using a reaction mixture consisting of L-γ-glutamic acid *p*-nitroanilide (67.2 μM to 1400 μM), glycylglycine (20 mM) and fixed concentrations of inhibitors (0 mM to 20 mM, except for compound **16**, 0 mM to 1 mM) and completed to 1 mL by 0.1 M Tris–HCl pH 8.0. Reactions were initiated by the addition of approximately 7.8 mU of GGT. After 15 min, the slope of the graph of absorbance *versus* time was measured and converted to velocity by dividing by the extinction coefficient ($\varepsilon = 8800\ M^{-1}\ cm^{-1}$ [10] at pH 8.0, 8110 $M^{-1}\ cm^{-1}$ at pH 7.0 and 8440 $M^{-1}\ cm^{-1}$ at pH 6.0 (*vide supra*)). A Lineweaver–Burk plot was prepared for each inhibitor concentration, using a broad range of substrate concentrations ($0.1 \times K_M - 2 \times K_M$) judiciously chosen so as to be evenly distributed over the reciprocal *x*-axis. The linear slopes ($K_{M\ app}/V_{max}$) of these plots, determined using the curve-fitting software Axum 5.0, were re-plotted against the inhibitor concentration to obtain K_i as the intercept on the abscissa of these secondary plots. All plots displayed excellent linearity ($r^2 > 0.99$).

Kinetic analysis of diastereomeric mixture

Eqn. (1) represents a typical double reciprocal equation (*cf*. Lineweaver–Burk plot) of an enzyme-catalysed reaction in the presence of a competitive inhibitor, where *v* is the reaction rate, K_M is the Michaelis constant of the donor substrate, [*S*] is the concentration of the substrate, V_{max} is the maximal rate of the enzymatic reaction, [*I*] is the concentration of the competitive inhibitor and K_i is the inhibition constant. The observed apparent Michaelis constant $K_{M\ app}$ varies according to the inhibitor concentration and the inhibition constant as shown in eqn. (2).

$$\frac{1}{v} = \frac{K_M\left(1 + {[I]}/{K_i}\right)}{V_{max}[S]} + \frac{1}{V_{max}} \quad (1)$$

$$K_{M\ app} = K_M\left(1 + {[I]}/{K_i}\right) \quad (2)$$

When a second competitive inhibitor is added to the inhibition scheme as shown in Scheme 2, the double reciprocal equation can be shown to include an additional term, as indicated in eqn. (3), after application of the steady state approximation (k_1[E][S] = (k_{-1} + k_2)[E·S]) and the mass balance equation ($[E]_0 = [E] + [E{\cdot}S] + [E{\cdot}I] + [E{\cdot}I']$) and defining $K_i = \frac{[E][I]}{[E \bullet I]}$ and $K_i' = \frac{[E][I']}{[E \bullet I']}$

$$\frac{1}{v} = \frac{K_M\left(1 + {[I]}/{K_i} + {[I']}/{K_i'}\right)}{V_{max}[S]} + \frac{1}{V_{max}} \quad (3)$$

In eqn. (3), K_i and K_i' represent the inhibition constants for inhibitor *I* (compound **8a** (S_CS_S) in this case) and *I*′ (compound **8b** (S_CR_S) in this case), respectively. With this model, we can modify eqn. (2) to give eqn. (4).

$$K_{M\ app} = K_M\left(1 + {[I]}/{K_i} + {[I']}/{K_i'}\right) \quad (4)$$

From eqn. (4) we note that the combined effect of both competitive inhibitors to increase $K_{M\ app}$ can be roughly defined as a function of total inhibitor concentration $[I]_{tot}$ as follows:

$${[I]_{tot}}/{K_{i\ app}} = \left({[I]}/{K_i} + {[I']}/{K_i'}\right) \quad (5)$$

where $K_{i\ app}$ is the apparent inhibition constant. The fraction of the total inhibitor concentration corresponding to one diastereoisomer **8a** can be written as follows:

$$f = \frac{[I]}{[I] + [I']} \quad (6)$$

and eqn. (6) can be used to re-write eqn. (5) in the following linear equation:

$$\frac{1}{K_{i\ app}} = f \cdot \left(\frac{1}{K_i} - \frac{1}{K_i'}\right) + \frac{1}{K_i'} \quad (7)$$

From eqn. (7) it can be seen that a plot of the reciprocal of the (observed) apparent competitive inhibition constant as a function of the fraction of **8a** in the mixture of diastereomer inhibitors will give a slope of $\left(\frac{1}{K_i} - \frac{1}{K_i'}\right)$ and an intercept of $\frac{1}{K_i'}$, allowing the apparent inhibition constants for both diastereomers to be determined.

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