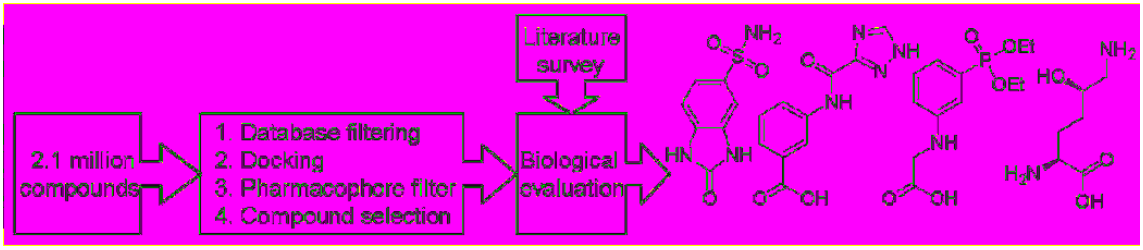
Graphical abstract



Evaluation of the amino acid binding site of *Mycobacterium tuberculosis* glutamine synthetase for drug discovery

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Abstract

A combination of a literature survey, structure-based virtual screening and synthesis of a small library was performed to identify hits to the potential antimycobacterial drug target, glutamine synthetase. The best inhibitor identified from the literature survey was (*2S,5R*)-2,6-diamino-5-hydroxyhexanoic acid (**4**, IC_{50} of 610 ± 15 μM). In the virtual screening 46,400 compounds were docked and subjected to a pharmacophore search. Of these compounds, 29 were purchased and tested in a biological assay, allowing three novel inhibitors containing an aromatic scaffold to be identified. Based on one of the hits from the virtual screening a small library of 15 analogues was synthesized producing four compounds that inhibited glutamine synthetase.

Keywords

Virtual screening; Glutamine synthetase; γ-glutamyl:ammonia ligase.

1 Introduction

Tuberculosis (TB) is one of the most serious infectious diseases. In 2005 the World Health Organization (WHO) estimated that 1.6 million deaths were caused by TB worldwide (www.who.int). Although the recommended Directly Observed Treatment Short-course therapy (DOTS) provides a cure for TB in many cases, there is a great need for new more effective TB drugs to combat multidrug-resistant tuberculosis and to shorten the lengthy treatment time.[1, 2] Special effort is needed to find and develop lead compounds acting against already validated TB drug targets.[2]

Glutamine synthetase (GS, EC 6.3.1.2) catalyzes the synthesis of glutamine from glutamate and ammonia with concurrent hydrolysis of adenosine triphosphate (ATP). The reaction passes through a phosphorylated tetrahedral intermediate.[3] GS is important in bacterial nitrogen metabolism and the synthesized L-glutamine is also a major component of the cell

wall of pathogenic mycobacteria.[4] The potential of *Mycobacterium tuberculosis* GS as a drug target was established in a transposon mutagenesis study of strain H37Rv, classifying MtGS as essential for optimal growth.[5] Furthermore, it has been shown that an antisense oligodeoxyribonucleotide directed at the *glnA1* gene, encoding MtGS, reduced bacterial replication and the amount of poly-L-glutamate/glutamine in the cell wall.[6] Finally, *glnA1* has been found to be essential for *M. tuberculosis* virulence.[7] The two most active known GS inhibitors described in the literature, L-methionine-(*S*)-sulfoximine (MSO, **1**) and phosphinothricin (PPT, **2**) have a profound effect on the growth of *M. tuberculosis*.[8] These are also the only known inhibitors of MtGS. MSO inhibits growth of *M. tuberculosis* in culture and within human mononuclear phagocytes[8] and the L-(*SR*)-diastereomer has also been demonstrated to have *in vivo* activity in a guinea pig model.[9]

Please insert Chart 1, chemical structures **1**-**5**

Amino acid analogues of MSO, PPT and mimics of the phosphorylated intermediate have been synthesized and evaluated on GS from different species in a number of studies.[10-29] Much effort has been invested in developing GS inhibitors not only for antibacterial research, but also for weed control,[20, 25, 30-32] since GS is a key enzyme in ammonia assimilation in plants. However, no inhibitor has shown improved activity compared to MSO or PPT. One of the most active PPT analogues (**3**) had a K_i in the same range as PPT when tested against GS isolated from *Escherichia coli*, spinach leaves and tobacco cultured cells.[19, 20] Other unnatural α-amino acids have also been shown to exert inhibitory effects on GS.[31, 33-36] Two such examples are (*2S,5R*)-2,6-diamino-5-hydroxyhexanoic acid (**4**, K_i 40 μM for *Salmonella typhimurium* GS,[29] and also inhibiting sheep brain and soybean GS[35]) and L-2-amino-4-hydroxyaminobutyric acid (**5**, K_i 7 μM and 21 μM for sheep brain and soybean GS,[35] respectively). Additionally, a set of aminomethylene-bisphosphonic acid derivatives inhibit

rice GS.[32] It should be noted that prokaryotic GSs are distantly related to eukaryotic ones, and that inhibition patterns may not be directly comparable.

Hit identification, i.e. the process of finding small-molecule inhibitors of a target enzyme, is a key step in drug discovery. Hit identification strategies[37, 38] include literature surveys, identification of endogenous ligands or active natural products, high-throughput screening, virtual screening, chemogenomics, ligand design and combinatorial chemistry. This study aims to find new hits against the antimycobacterial drug target MtGS utilizing three of the above mentioned hit identification strategies. This work is focused on the amino acid site, where all of the presently known inhibitors bind. First the literature was surveyed to identify small molecules known to be active on GS in other species that could be purchased or easily synthesized. In parallel, a structure-based virtual screening of the amino acid binding site was performed to uncover possible novel hits. Based on the virtual screening results, a small library of analogues was synthesized. Herein we present the results of our efforts.

2 Results and discussion

2.1 Biological assay

A GS assay was developed based on the biosynthetic reaction, considered to be the most biologically relevant.[29] The sensitivity of the present assay for inhibitors binding to the amino acid site was optimized by using relatively high concentrations of ATP and magnesium ions, and choosing a glutamate concentration similar to the K_m for that substrate. DMSO, used for dissolving some inhibitors, was found to have an activating effect on MtGS. Approximately 10% activation was observed at a DMSO concentration of 2% (v/v). However, the increase in activity could be corrected for by including DMSO in the compound-free reaction as well as in the background reaction. That the correction for the effect of DMSO did not interfere with the inhibition analysis was demonstrated by tests with compound **4,** which was dissolved in both MilliQ water and DMSO, and found to have a % inhibition of 58 ± 16 and 56 ± 10 %, respectively.

2.2 Literature survey

Since the residues in the amino acid binding site are highly conserved,[29] we anticipated that known inhibitors of GS from other species, including distantly related GS types, could also be active on MtGS. In fact a number of inhibitors, including MSO,[8] or its diastereomeric mixture L-(*SR*),[8, 11, 13, 23] PPT,[8, 19, 20, 22-25, 28] **3**,[19, 20] and **4**[29, 35] have shown inhibitory activity when tested on both bacterial and eukaryotic GSs. Therefore a literature search was performed to identify known GS inhibitors for both eukaryotic and prokaryotic enzymes. The majority of the inhibitors reported previously were amino acid based and had activities ranging from μM to mM. Compounds that we could identify as commercially available (**1**-**2**, **4** and **6**-**8**, Table 1) were purchased. Furthermore, the diastereomer of **4** (compound **11**) was synthesized. Another compound (**5**) described in the literature, which inhibits both sheep brain GS and soybean GS with similar potency to that of MSO,[35] was also synthesized, as well as the analogue having a methyl group in the same position as MSO and PPT (**12**). Finally, three close analogues (**9**, **10** and **13**) to these were purchased.

The compounds above were screened for inhibitory activity against MtGS at a single concentration (in general 1.0 mM) using a biosynthetic assay. Inhibition of MtGS was determined by comparison to that of uninhibited enzyme. The stereochemistry of the tested compounds was mostly the pure L-form (**1**, **5**-**7**, **9**-**10** and **12**-**13**). PPT (**2**) was tested as a racemic mixture and **8** was tested as L-(*SR*)-sulfoxide. Compounds **4** and **11** were synthesized with defined stereochemistry at both stereocenters as described in the experimental section. Furthermore the compounds were docked to the active site of GS in an attempt to rationalize the structure-activity relationship.

The most active inhibitor of *S. typhimurium* GS,[29] compound (**4**), was also found to inhibit MtGS. According to the proposed binding mode from the docking calculation, **4** retains all the important interactions to Arg329, His276, Gly272 and Glu135 in the amino acid backbone

that are found in the complex structure of MSO with MtGS (Figure 1). The 5-aminomethyl group approaches the proposed ammonium ion binding site[39] (residues Glu219, Tyr186 and Asp54´and Ser57', Figure 1). Combined, these interactions could explain the relatively good inhibition. The stereochemistry of the hydroxyl group of **4** is required to be *R*, since the *S*-isomer (**11**) was inactive. We therefore suggest that the interaction of the hydroxyl group with Arg368 is important, since the inactive compound (**11**) lacks this hydrogen bond (Figure 1). An alternative explanation to the inactivity of **11** could be that this position must be occupied by an oxygen atom. The importance of the stereochemistry at the same position in the active site has also previously been reported for the sulfoximine group, with the L-(*S*)-enantiomer of MSO being a more potent inhibitor of sheep brain GS[40] and *E. coli* GS.[41]

Because of the strong inhibitory activity of **5** with regard to GS from other species (*vide supra*) we resynthesized this compound and evaluated its inhibitory potency against MtGS. Unfortunately, compound **5** was only weakly potent. The analogue having a terminal methyl group corresponding to the methyl group in the sulfoximine and phosphinyl moiety of MSO and PPT, respectively, was also synthesized (**12**). However, this compound lacked inhibitory activity. Exchanging the hydroxylamine nitrogen of **5** with a carbon generates the inactive compound **9**. Thus an oxygen atom at the 5-position of an amino acid is not the only characteristic of an active compound, although this has been identified as a common structural feature in many known inhibitors.[35] L-homoserine (**10**), with a carbon chain that is one methylene unit shorter than **9**, was also tested and found to be inactive.

Compound **6** has previously been reported to inhibit *E. coli* GS (K_{is} 54 μM)[17] but was found to be inactive against MtGS. This finding was verified using a slightly different biosynthetic assay (for experimental details, see Lagerlund et al.[42]) where the activity of **6** was evaluated on GS from both species. Compound **6** was fairly potent on *E. coli* GS (61% inhibition at 1.1 mM), but lacked inhibitory potency on MtGS. Thus, despite the conserved residues in the

amino acid binding site, there are differences in the catalytic properties of *E. coli* GS and MtGS that are reflected in the different inhibition pattern seen for this compound.

The sulfonic acid (**13**) did not show any inhibition. However, the corresponding sulfone (**7**) inhibited MtGS, with 33% inhibition at a concentration of 1.0 mM. The same pattern was observed when comparing results with PPT and **6**. Additionally, the (*SR*)-sulfoxide (**8**) did not show any inhibition. Thus, the structural features including a methyl group together with two anchor points (imine nitrogen and/or double bonded oxygen(s)) seem to be very important for inhibition of the MtGS enzyme.

The IC_{50} values were determined for the most potent compound (**4**) and for the two previously known inhibitors (**1** and **2**). MSO and PPT were the best inhibitors, with IC_{50} values of 51 ± 6 μM and 1.9 ± 0.4 μM, respectively (Figure 2). Inhibitor **4** had an IC_{50} value of 610 ± 15 μM which is 12 times higher than that of MSO. In total the literature survey identified three new inhibitors of MtGS among the tested compounds.

2.3 Virtual screening

Starting from a database of 2.1 million commercially available compounds, the database was reduced to 46,400 compounds with a molecular weight <300 g/mol containing carboxylic acid bioisosters[43] (substrate), amides (product) or functional groups that have previously been used as substitutes for the sulfoximine group of MSO[29] (Figure 3). A docking protocol with FlexX was used that successfully reproduced the binding of phosphorylated MSO (MSO-P) observed in the crystallographic structure of MtGS[44] as the top scoring pose. When non-phosphorylated MSO was docked, the top scoring pose overlaid well with the equivalent portions of MSO-P. PPT was also used to evaluate the docking protocol. Its top scoring pose was generally in agreement with the complex structure of *S. typhimurium* with PPT,[45] with the only difference being that the methyl group is rotated by ~180°. This is in accordance with the binding mode of PPT suggested by Krajewski et al. on the basis of high resolution data.[44]

The 46,400 compounds were then subjected to ligand pre-treatment and docked in the amino acid binding site. The top scoring poses were filtered using a rigid pharmacophore search (Figure 3), which yielded 3511 compounds that were visually inspected. Among these, 29 compounds were selected, purchased and screened for activity against MtGS using the biosynthetic assay at a single concentration, in general 1.0 mM, The most interesting of these are presented in Table 2.

The virtual screening approach identified three active hits **14**, **15** and **23** with 24%, 26% and 48% inhibition, respectively (Table 2). Interestingly these are not related to any previously known inhibitors of GS. The FlexX docking results suggested that compound **14** fits in the amino acid binding site (Figure 4A) with the urea functionality facing Arg329 and Gly272. It places the tetrahedral sulphonamide group at the position of the sulfoximine of MSO-P, fulfilling one criterion possibly important for an active compound (*vide supra*). The sulphonamide functionality has also been used in an amino acid analogue that inhibited *E. coli* GS.[17] Both compound **15** (Figure 4B) and **23** (Figure 4C) interact with the carboxyl group in the same manner as MSO, forming an ionic interaction and hydrogen bonds to Arg329 and His276. Compound **23** lacks the backbone amino group, however the secondary aromatic amino group instead forms hydrogen bonds to Glu135. Compound **15** extends somewhat further than MSO and can find additional interactions with its heterocyclic ring higher up in the amino acid binding site. All three compounds form a hydrogen bond between the key residue Arg368 (*vide supra*) and the double bonded oxygen of the sulphonamide, amide or sulfonic acid group, respectively.

Glutamate analogues with a longer carbon chain have been reported as substrates of sheep brain GS[46, 47] and substituted glutamate analogues have been successfully converted to active PPT analogues.[23-27] However, none of the 2-amino-phosphono-carboxylic acids (**16-18**) identified in the virtual screening showed any inhibitory activity, despite the fact that the

phosphono group of docked 2-amino-6-phosphonohexanoic acid (**17**) overlaps well with the phosphono group of MSO-P. This is in accordance with results for DL-homophosphinothricin, which did not show any inhibition of sheep brain GS.[23] Thus the patterns of inhibition observed here agree well between the prokaryotic and eukaryotic GSs.

In addition to the 29 compounds that were selected, the *ortho*-substituted phenylalanine derivative **20** was identified as a promising compound, for which the amino acid backbone and the phosphono group overlaid well with the corresponding functional groups of MSO-P. However, it could not be purchased and was therefore synthesized based on the method of Dorville et al. (Scheme 1).[48] It was also realized that compound (**21**) carrying the *ortho*-substituted aminomethyl group could be obtained using this route. Starting from 2-cyanobenzylbromide, the benzyliodide **42** was prepared in four steps. Phosphorylation of **42** with triethylphosphite gave the diethyl protected phosphate **43**, which was treated with 9 M HCl to afford the desired compound **20**. In addition, the corresponding methyl ester **22** was also isolated. It is believed to have been formed *via* a transesterification with MeOH in the first step of the synthesis of **20**.

Compound **20** was also inactive even though it can adopt a conformation that overlaps well with MSO-P. Considering the results with **20** and the 2-amino-phosphono-carboxylic acids (**16**-**18**), the tetrahedral sulfoximine group or a similar moiety four bonds distant from the carbonyl carbon seems to be important for inhibitory activity. The *ortho*-substituted aminomethyl compound (**21**), the methyl ester (**22**) obtained from the synthesis of **20**, and the purchased *para*-substituted analogue (**19**) did not inhibit MtGS.

2.4 Synthesis of a small library

Compound **23**, identified in the virtual screening, also served as the starting point for a small synthetic library (Table 3). Several functional groups, including a carboxylic acid, a thioether,

a sulfoxide, a sulfonamide and a phosphonate ester, were investigated as R^2 substituents. The compounds were synthesized from glycine (**24**-**28**) or from L- (**29**-**33**) or D-serine (**34**-**38**), as described in the experimental section. The desired products were prepared via a CuI catalyzed *N*-arylation of glycine, D- and L-serine, using K_2CO_3 as a base, with an appropriately substituted arylbromide (Scheme 2).[49] After work-up the products were purified by preparative HPLC. Chiral HPLC analysis was used to determine the chiral purity (>95%) of the products.

The compounds were screened for activity against MtGS at a single concentration, in general 1.0 mM, using the biosynthetic assay. Four weak inhibitors were identified, with **28**, a phosphonate ester being the most potent. The majority of the compounds displayed an apparent activation of the enzyme. Additional studies will be required to establish the structural basis for such activation, an effect that has been reported previously in the literature for plant GSs.[30] However, to the best of our knowledge, activation of a bacterial GS has not been described.

3 Conclusions

By using a literature survey, virtual screening and synthesis of analogues, ten new compounds inhibiting MtGS have been identified. Hits were identified by all three approaches. The best compound identified (**4**) had an IC_{50} of 610 ± 15 μM, and has previously been reported as an inhibitor of *S. typhimurium* GS,[29] and to a lesser extent, sheep brain and soybean GS.[35] Three novel inhibitors with aromatic scaffolds were found among the virtual screening compounds. These compounds (**14**, **15** and **23**) showed 24-48% inhibition at 1.0 mM, respectively. Among the synthesized *N*-arylated amino acids, one inhibitor (**28**) was identified with 42% inhibition at 1.0 mM. However, we note that none of the inhibitors were particularly potent against MtGS, suggesting that the amino acid binding site may not be the best focus for future efforts. Recent structural information[50] indicates that the less-conserved nucleotide-binding site could

instead be explored as a means of attaining the desired specificity for the mycobacterial as compared to the mammalian enzyme.

4 Experimental

4.1 General information

^{1}H NMR and ^{13}C NMR spectra were obtained on a Varian Mercury 400 spectrometer (^{1}H 400 MHz, ^{13}C 100.6 MHz). Chemical shifts are reported as δ values (ppm) referenced to TMS via the solvent signal. GC-MS analyses were performed with a CP-SIL 8 CB Low Bleed (30 m×0.25 mm) or a CP-SIL 5 CB Low Bleed (30 m×0.25 mm) capillary column using a 40-300^{o}C temperature gradient and EI ionization. HPLC-MS was performed on a Gilson HPLC system with a Finnigan AQA quadropole mass spectrometer, using an Onyx Monolithic C18 column (50×4.6 mm) at a flow rate of 4 mL/min and an H_2O/CH_3CN/0.05% HCOOH gradient; detection was by UV (DAD) and MS (ESI+). Preparative RP-HPLC was performed by UV-triggered fraction collection with a similar Gilson-Finnigan AQA system and a Zorbax SB C8 column (150×21.2 mm) at a flow rate of 10-12 mL/min and an H_2O/CH_3CN/0.05% HCOOH gradient. Chiral HPLC analysis was performed using an AGP 150.4 column (150×4.0 mm) using a phosphate buffer (pH=5.5) and 2-3% 2-propanol as solvent (flow rate 1 mL/min).

Column chromatography was performed using silica gel 60 (particle size 0.040-0.063 mm, E. Merck). For reversed phase flash chromatography, silica gel RP-18 (0.040-0.063 mm, E. Merck) was used. Thin-layer chromatography was performed with aluminium sheets coated with silica gel 60 F_{254} (0.2 mm E. Merck), using UV-light or iodine vapour for visualisation. Optical rotations were obtained at room temperature on a polarimeter; specific rotations ($[\alpha]_D$) are reported in deg/dm and the concentration (*c*) is given in g/100 mL in the specified solvent. THF was freshly distilled over Na/benzophenone. Acetone was dried over 3 Å molecular sieves. All products were >95% pure according to either GC-MS or LC-MS (TIC).

All other reactants and reagents were commercially available and used without further purification unless otherwise stated. L-methionine-(*S*)-sulfoximine (**1**) and (*2S,5R*)-2,6-diamino-5-hydroxyhexanoic acid (**4**) were purchased from Fluka. DL-phosphinothricin (**2**) was purchased from Duchefa Biochemie, Haarlem, The Netherlands. L-methionine sulfone (**7**), L-methionine-(*SR*)-sulfoxide (**8**), L-2-amino-4-sulfobutyric acid (**13**) and (**23**) were purchased from Sigma Chemical Co., St Louis, MO USA. L–homoserine (**10**) was purchased from Novabiochem, Merck KGaA, Darmstadt, Germany. Compounds (**6**, **16**-**19**) were purchased from InterBio Screen.[51] Compounds **6**, **16**-**18** were purchased as L-amino acids and **19** was supplied as a racemic mixture. Compound **14** was purchased from Asinex.[52] Compound **15** was purchased from Chemdiv.[53] The structure of the purchased virtual screening compounds was confirmed by ^{1}H NMR and HPLC-MS. L-2-amino-4-hydroxyaminobutyric acid (**5**),[35] L-5-hydroxynorvaline (**9**)[54] (2*S*,5*S*)-2,6-diamino-5-hydroxyhexanoic acid (**11**),[55] L-2-amino-4-(hydroxymethylamino)butyric acid (**12**)[34] and 2-Amino-3-(2-phosphonomethylphenyl)propanoic acid (**20)**[48] are known compounds. Compound **24** is a known structure, where additional data are given. Compounds **21**, **22** and **25**-**38** are new compounds. *N*-arylated amino acids gradually decomposed at room temperature, prohibiting accurate elemental analysis.

Stocks of ATP (100 mM) were prepared, neutralized by sodium hydroxide and stored at -20°C. Phosphate detection reagents (PiColorlock Gold) were purchased from Innova Bioscience (Cambridge, UK. url: http://www.innovabiosciences.com). Sodium L-glutamate was purchased from Sigma Aldrich, St Louis, MO USA. A stock solution was prepared using MilliQ water (Millipore) and the pH was adjusted to 7.5; aliquots were stored at -20 °C.

4.2 Biological testing

4.2.1 GS samples

MtGS was heterologously expressed in a GlnE-deficient *E. coli* strain (a generous gift of AstraZeneca India, Limited), which yields a completely unadenylylated enzyme. The protein was purified and stored as previously described.[44]

4.2.2 Assay development

Quantification of released phosphate in the assay was performed using a commercial inorganic phosphate detection reagent that produces a green colour with an absorption maximum at 635 nm. The reaction (100 μL) was stopped by addition of PiLock Gold reagent (25 μL, PiLock reagent mixed 1:100 with accelerator reagent). After 5 min incubation at room temperature, stabilizer (10 μL) was added to the solution, and an additional 30 min incubation at room temperature was performed before measuring the absorbance at 635 nm using a microtiter plate reader. A linear response was observed from 1.6 μM to 50 μM, in agreement with the manufacturer's specifications. The highest P_i concentration tested, 100 μM, deviated from linearity, due to the precipitation of the developed coloured compound.

For enzyme assays, GS was diluted to 3.6 ng/μL (approximate subunit concentration of 70 nM) into HEPES-HCl (100 mM), pH 7.5, $MgCl_2$ (50 mM) and equilibrated at room temperature for at least 30 minutes. The assay was subsequently performed in HEPES-HCl (50 mM), pH 7.5, $MgCl_2$ (25 mM), ATP (1 mM), NH_4Cl (30 mM) and monosodium L-glutamate (10 mM). The reaction was initiated by the addition of glutamate (50 μL), resulting in a final volume of 100 μL and an enzyme subunit concentration of 7 nM. The assay was performed in 96-well microtiter plate format and incubated at room temperature.

A linear increase in the amount of phosphate detected was observed for 2 hours. Running the assay reaction for a longer time would be expected to generate phosphate concentrations outside the linear range of the detection system. Therefore, under standard conditions during the inhibition study the assay was run for 60 min. To assess the quality of the results, Z' was calculated as described previously by Zhang et al.[56] A Z'-factor greater than 0.5 is regarded as an excellent outcome; Z'-factors of 0.73 ± 0.15 (H_2O) and 0.69 ± 0.19 (2.3 % (v/v) DMSO) were obtained here.

4.2.3 Initial inhibition studies

Compounds were dissolved in MilliQ water or in DMSO to prepare a stock solution (50 mM) that was stored frozen at -20°C. Compound (2 µl) was added to a microtiter plate and mixed with enzyme solution (50 µl) containing all components except L-glutamate. The enzyme-compound-substrate mixture was then pre-incubated for 20 min at room temperature prior to addition of L-glutamate to start the reaction (50 µl, 20 mM). The final compound concentration after addition of glutamate was 1 mM. For reactions with compounds dissolved in DMSO the final concentration of solvent was 2% (v/v).

Each compound was assayed in triplicate and the results reported as means together with a standard deviation. The activity is presented as the percentage of inhibition. The student's 2-sided T-test was used to determine if the observed activity was significantly different from the uninhibited reaction. The p-value ($p < 0.1$) was used as the threshold for scoring a compound as active.

To correct for an increased signal due to contaminating phosphate in the compound sample, and for possible intrinsic absorbance of the compound itself, a control plate was used.[57] Each compound (2 µL) was treated as in the inhibition experiment except that GS and ATP were excluded from the reaction. The signal resulting from the phosphate contamination was

subtracted from that obtained in the inhibitory assay ($OD_{corr} = OD_{assay} - OD_{CBG}$, where OD_{assay} is defined as the optical density reading of the inhibition assay, and OD_{CBG} as the optical density reading of the compound background).

4.2.4 IC_{50} determination

To determine the IC_{50} values, initial stock solutions of compound were prepared in MilliQ water and serially diluted to an appropriate concentration range. The final compound concentrations ranged from 1.2 mM with a 2-fold serial dilution to a concentration of 4.7 μM (MSO **1**), 1 mM to 1.5 μM (PPT **2**, 3-fold serial dilution) and 10 mM to 39 μM (**4**, 2-fold serial dilution). The assay was performed as previously described for the inhibition assay. Only compounds that led to more than 50% inhibition at 1mM were evaluated. Three or more independent experiments were carried out for each compound. IC_{50} values were separately determined from each dilution series by nonlinear regression, fitting a three-parameter equation (Eq. 1) to the data from the microtiter plate readings at 635 nm, i.e.

$$Y = Lo + \frac{Hi - Lo}{1 + \frac{X}{IC_{50}}} \qquad \text{(Eq. 1)}$$

where *Hi* is the estimated highest absorbance at zero inhibitor concentration, *Lo* the estimated lowest absorbance at infinite inhibitor concentration, *X* the concentration of inhibitor and *Y* the measured absorbance. Regression analysis was performed using the SOLVER function in the Microsoft Excel spreadsheet software[58-60] with the goal of maximizing the square of the correlation coefficient (R^2), and including the constraint that $IC_{50} \geq 0$. The reported IC_{50} values are an average of those obtained from three (**1** and **2**) or five (**4**) separate experiments together with the standard deviation.

4.3 Virtual screening

A database containing approximately 2.1 million compounds was assembled.[51-53, 61-68] A 2D UNITY substructure search[69] was performed to identify compounds containing carboxylic

acid bioisosters[43] (substrate analogues), primary amides (product analogues) or functional groups that have previously been used to substitute for the sulfoximine group of the MtGS inhibitor MSO[29] (Figure 3). The search was limited to compounds with a molecular weight less than 300 g/mol, due to the small size of the amino acid binding pocket. The ligprep module implemented in Maestro[70] was used for ligand pretreatment steps. Salts were removed and hydrogens were added to all ligands. Stereoisomers, tautomers and two ring conformations were automatically generated and the ligands were ionized and minimized with the force field OPLS2003. The UNITY search yielded 46,400 compounds, which was expanded to 102,379 structures to be used in the docking calculations. Babel[71] was used for file conversion between sdf and mol2 format. The protein[44] (PDB code 2BVC) was prepared with the protein preparation and refinement feature implemented in Maestro. ADP and MSO-P were removed, as well as all water molecules.

FlexX[72, 73] was used as a docking tool with default settings. The active site was defined by the residues Glu133, Glu135, Tyr186, Glu219, Glu227, Asn271, Gly272, His276, Arg329, Glu335, Arg347, Arg352, Glu366, Arg368, Tyr406 from one subunit, as well as Asp54 and Ser57 from a neighbouring molecule, and the three Mg^{2+} ions. Only the highest ranked pose in the docking was retained and subjected to a rigid pharmacophore search (Figure 3) in UNITY. The pharmacophore model that was built based on MSO consisted of two hydrogen bond acceptors and one hydrogen bond donor, with a tolerance criterion of 2 Å. A partial match constraint to fit at least two out of three pharmacophore points was employed. The remaining 3511 compounds that fulfilled the pharmacophore criterion were visually inspected and 29 compounds were selected for purchase, based on how well they fulfilled the following criteria: (a) a realistic conformation in the active site, (b) a tetrahedral atom in close proximity to the sulphur atom of MSO, (c) a negatively charged functional group in place of the

phosphate from MSO-P and (d) an overall similarity to an amino acid. Compounds with large hydrophobic substituents were not selected.

4.4 Chemistry

4.4.1 Diethyl (2-cyanobenzyl)acetamidomalonate (**39**)[48]

To a 21% solution of NaOEt in EtOH (10.5 mL, 28.1 mmol), diethyl acetamidomalonate (5.00 g, 23 mmol) and 2-cyanobenzylbromide (5.01 g, 25.5 mmol) were added successively. The reaction was stirred at room temperature under nitrogen for 36 h. The precipitate was dissolved in MeOH, and dichloromethane (DCM) and Celite were added before the solvent was removed under reduced pressure. Flash chromatography of the crude product with DCM-acetone (19:1) gave **39** as a white solid (4.7 g, 62% yield) with ethyl methyl (2-cyanobenzyl)acetamidomalonate, approximately 12:1 as determined from ^{1}H NMR, resulting from transesterification, as a sideproduct. Experimental data were in accordance with literature data.[48]

4.4.2 Diethyl [2-(aminomethyl)benzyl]acetamidomalonate (**40**)[48]

To a suspension of 10% Pd on charcoal (1.22 g) in EtOH (1.2 mL) was added a solution of **39** (3.7 g, 11 mmol) in $CHCl_3$ (45 mL), EtOH (23 mL) and concentrated HCl (3.5 mL). The reaction was stirred under H_2 at 1 atm, 40°C for 24 h. After filtration and evaporation, the residue was taken up in water (20 mL) and extracted with DCM (2×20 mL). From the organic phase unreacted **39** was recovered after evaporation. The water phase was freeze dried to obtain **40** (0.48 g, 12% yield) as a light yellow powder with ethyl methyl [2-(aminomethyl)benzyl]acetamidomalonate as a sideproduct. Experimental data were in accordance with literature data.[48]

4.4.3 Diethyl [2-(hydroxymethyl)benzyl]acetamidomalonate (**41**)[48]

Compound **40** (1.5 g, 4.02 mmol) was dissolved in water (25 mL) and the pH was adjusted to 6 with 1 M NaOH. $NaNO_2$ (0.405 g, 5.87 mmol) was added and the reaction mixture was refluxed for 5 h, cooled and extracted with EtOAc (4×25 mL). The combined organic phases were washed with 1 M HCl (10 mL), water (10 mL), 5% $NaHCO_3$ (10 mL), water (10 mL) and brine (10 mL) and dried over Na_2SO_4. Flash chromatography with EtOAc-isohexane (6:4) gave **41** as a yellow oil (0.47 g, 34% yield) with ethyl methyl [2-(hydroxymethyl)benzyl]acetamidomalonate as a sideproduct. Experimental data were in accordance with literature data.[48]

4.4.4 Diethyl [2-(iodomethyl)benzyl]acetamidomalonate (**42**)

To a solution of **41** (0.50 g, 1.48 mmol) in dry THF (8 mL) at 0°C was added NEt_3 (0.35 mL, 2.51 mmol) and MsCl (0.2 mL, 2.58 mmol).[74] The resulting mixture was stirred for 1 h and quenched with 10% (w/w) citric acid (10 mL), extracted with EtOAc (3×25 mL) and washed with brine (10 mL). The organic phase was dried over $MgSO_4$, filtered and evaporated. Crude diethyl [2-methanesulfonyloxymethyl)benzyl]acetamidomalonate was directly dissolved in dry acetone (10 mL). NaI (0.907 g, 6.05 mmol) was added and the reaction mixture was stirred at room temperature for 2 h.[74] The reaction solvent was removed and the residue taken up in EtOAc (40 mL). The organic layer was washed sequentially with 10% (w/w) citric acid (30 mL), 10% (w/w) $Na_2S_2O_3$ (30 mL) and dried over $MgSO_4$. Evaporation of the solvent gave **42** as a yellow oil (0.50 g, 75% yield) with ethyl methyl [2-(iodomethyl)benzyl]acetamidomalonate as a sideproduct. The product was used directly in the next step without further purification.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.37-7.34 (m, 1H), 7.20-7.12 (m, 2H), 6.94-6.91 (m, 1H), 4.43 (s, 2H), 4.30 (dq, J = 10.7, 7.2 Hz, 2H), 4.23 (dq, J = 10.7, 7.2 Hz, 2H), 3.75 (s, 2H), 2.00 (s,

3H), 1.28 (t, *J* = 7.2 Hz, 6H) ^{13}C NMR ($CDCl_3$, 100.5 MHz): δ 169.5, 167.6, 138.9, 133.2, 130.9, 130.8, 128.0, 127.9, 66.6, 62.8, 34.4, 29.7, 23.1, 13.9.

4.4.5 Diethyl [2-((diethoxyphospinyl)methyl)benzyl]acetamidomalonate (**43**)[48]

42 (0.50 g, 1.12 mmol) was dissolved in 5 mL $P(OEt)_3$, refluxed overnight and concentrated *in vacuo*. Flash chromatography with isohexane-EtOAc-MeOH (6:4:0.5) gave **43** as a yellow oil (0.25 g, 49% yield) with ethyl methyl [2-((diethoxyphospinyl)methyl)benzyl]acetamidomalonate as a sideproduct. Experimental data were in accordance with literature data.[48]

4.4.6 2-Amino-3-(2-phosphonomethylphenyl)propanoic acid (**20**)[48]

Compound **43** (0.186 g, 0.41 mmol) was refluxed overnight in 9 M HCl (6.6 mL) and concentrated to dryness. The crude product was purified using preparative HPLC (45 min 0-50% CH_3CN) and **20** was obtained as white powder (11 mg, 10% yield). Experimental data were in accordance with literature reports.[48]

4.4.7 2-Amino-3-(2-aminomethylphenyl)propanoic acid (**21**)

Compound **40** (100 mg, 260 μM) was refluxed over night in 9 M HCl (5 mL) and concentrated to dryness. The crude product was purified using preparative HPLC (45 min 0-30% CH_3CN) and **21** was obtained as green crystals (55 mg, 86% yield).

^{1}H NMR (D_2O, 400 MHz): δ 7.42 (m, 4H), 4.25 (s, 2H), 4.13 (dd, *J* =8.0, 6.8 Hz, 1H), 3.40 (dd, *J* =14.9, 8.0 Hz, 1H), 3.22 (dd, *J* =14.9, 6.8 Hz, 1H) ^{13}C NMR (D_2O-CD_3OD, 2:1, 100.5 MHz): δ 174.3, 136.0, 132.6, 131.9, 131.4, 130.9, 129.4, 56.8, 41.0, 34.3. Anal. calcd. for $C_{10}H_{14}N_2O_2$+HCl+0.5 H_2O: C, 50.11; H, 6.73; N, 11.69; Cl, 14.79. Found: C, 49.79; H, 6.57; N, 11.52; Cl, 14.50.

4.4.8 Methyl 2-amino-3-(2-phosphonomethylphenyl)propanoate (**22**)

Compound **22** was obtained in the deprotection of **20** and was easily separated using preparative HPLC (45 min 0-50% CH_3CN), which produced **22** as a white powder (53 mg, 46% yield).

^{1}H NMR (D_2O, 400 MHz): δ 7.32 (m, 4H), 4.49 (m, 1H), 3.86 (s, 3H), 3.50 (dd, *J* = 14.8, 9.0 Hz, 1H), 3.30 (dd, *J* = 14.8, 5.8 Hz, 1H), 3.10 (m, 2H) ^{13}C NMR (D_2O-CD_3OD, 2:1, 100.5 MHz): δ 171.5, 135.7 (d, *J* = 8.7 Hz), 133.4 (d, *J* = 5.9 Hz), 132.9 (d, *J* = 5.1 Hz), 130.6 (d, *J* = 3.1 Hz), 128.9 (d, *J* = 3.2 Hz), 128.0 (d, *J* = 3.6 Hz), 54.5, 54.4, 34.2 (d, *J* = 128 Hz), 33.2. Anal. calcd. for $C_{11}H_{16}NO_5P$+0.5 H_2O: C, 46.81; H, 6.07; N, 4.96. Found: C, 46.97; H, 5.90; N, 4.84.

4.4.9 (*SR*)-1-Bromo-3-methanesulfinylbenzene (**44**)[75]

Phenol (5.2 g, 55.3 mmol) was melted at 45°C and mixed with 1-bromo-3-methylsulfanyl-benzene (0.4 mL, 2.98 mmol). Afterwards 30% (w/w) aq H_2O_2 was added (0.56 mL) and the reaction mixture was stirred for 30 s. Excess H_2O_2 was quenched with saturated Na_2SO_3 solution (20 mL) and the pH was adjusted to 10 with 1 M NaOH. The water phase was extracted with EtOAc (3×30 mL) and the combined organic phases were washed with water (30 mL), brine (30 mL) and dried over Na_2SO_4. The solvent was removed and **44** was obtained after flash chromatography with petrol ether-EtOAc (6:1 → 1:2) as white crystals (627 mg, 96% yield).

$[\alpha]_D$: 0.00 R_f (petrol ether-EtOAc, 2:1, v/v) 0.52. Experimental data were in accordance with literature data.[76]

4.4.10 (3-Bromophenyl)phosphonic acid diethyl ester (**45**)[77]

To a mixture of palladium acetate (354 mg, 1.56 mmol) and 3-bromoiodobenzene (2.54 mL, 19.9 mmol) was added $P(OEt)_3$ (3.6 mL, 21 mmol). The reaction mixture was stirred at 90°C for 20 h. After cooling, diethylether (20 mL) was added and the mixture was filtered. The

solvent was removed under reduced pressure and flash chromatography with DCM-CH_3OH (200:1 → 100:1) gave **45** as a colourless oil (4.4 mg, 75%).

R_f (DCM-CH_3OH, 19:1, v/v) 0.51. Experimental data were in accordance with literature data.[78]

4.4.11 General procedure for the *N*-arylation of amino acids. Synthesis of **24-38**.

Aryl bromide (1 mmol), CuI (19.5 mg, 0.1 mmol) and K_2CO_3 (166 mg, 1.2 mmol) were dissolved in a CH_3CN–H_2O mixture ([1:4, v/v], 2.5 mL). The amino acid (2 mmol) was added and the reaction mixture was stirred for 24-50 h at 90°C. The reaction was cooled (0°C), diluted with DCM (20 mL) and H_2O (20 mL) and the pH was adjusted to 2-3 with conc. HCl. The water phase was extracted with DCM (2×30 mL) and the combined organic phases were washed with brine (20 mL). Either the product was found in the water phase, which was concentrated and the crude product was isolated after freeze drying, or the product was found the organic phase, which was dried over $MgSO_4$ and evaporation of the solvent yielded the crude product. The crude product was purified by with preparative RP-HPLC (30 min 10-60% or 80% CH_3CN).

4.4.12 3-(Carboxymethylamino)benzoic acid (**24**)

Following the general procedure, 3-bromobenzoic acid (0.1 mmol) was *N*-arylated with glycine. **24** (6.2 mg, 31.8%) was obtained after preparative HPLC.

^{1}H NMR (D_2O, 400 MHz): δ 7.60-7.40 (m, 3H), 7.15 (m, 1H), 4.06 (s, 2H). HRMS (M+1): Calcd: 196.0610 Found: 196.0614. Experimental data were in accordance with literature data.[79]

4.4.13 (3-Methylsulfanylphenylamino)acetic acid (**25**)

Following the general procedure, 3-bromthioanisole (0.05 mmol) was *N*-arylated with glycine to obtain 2.8 mg (28.4%) of the **25** after preparative HPLC.

^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 7.05 (t, *J* = 7.8 Hz, 1H), 6.59 (s, 1H), 6.56 (d, *J* = 7.8 Hz, 1H), 6.46 (dd, *J* = 1.4, 8.2 Hz, 1H), 3.92 (s, 2H), 3.92 (s, 1H), 2.42 (s, 3H). ^{13}C NMR ($(CD_3)_2CO$, 100.5 MHz): δ 206.4, 172.4, 140.1, 130.2, 116.0, 111.2, 110.5, 45.6, 15.4. HRMS (M+1): Calcd: 198.0589 Found: 198.0595.

4.4.14 (3-Methanesulfinylphenylamino)acetic acid (**26**)

Following the general procedure, **44** (0.1 mmol) was *N*-arylated with glycine, and **26** (5.0 mg, 23.5%) was obtained after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.09 (t, *J* = 7.8 Hz, 1H), 6.97 (t, *J* = 7.8 Hz, 1H), 6.68 (t, *J* = 7.4 Hz, 1H), 6.51 (dd, *J* = 2.0, 8.1 Hz, 1H), 3.74 (s, 2H) 2.97 (s, 1H), 2.51 (s, 3H). HRMS (M+1): Calcd: 214.0538 Found: 214.0533.

4.4.15 (3-Sulfamoylphenylamino)acetic acid (**27**)

Following the general procedure, 3-bromo-benzenesulfonamide (0.1 mmol) was *N*-arylated with glycine to produce 12 mg (52.2%) of **27** after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.14 (t, *J* = 7.0 Hz, 1H), 6.99 (s, 1H), 6.70 (s, 1H), 6.42 (s, 1H), 5.80 (s, 1H), 3.85 (s, 2H), 1.92 (s, 2H). HRMS (M+1): Calcd: 231.0440 Found: 231.0435.

4.4.16 [3-(Diethoxyphosphoryl)phenylamino]acetic acid (**28**)

Following the general procedure, **45** (0.1 mmol) was *N*-arylated with glycine to give 5.8 mg (20.2%) of **28** after preparative HPLC.

^{1}H NMR (CD_3CN, 400 MHz): δ 7.10-7.04 (m, 1H), 6.97 (dt, *J* = 1.6, 14.9 Hz, 1H), 6.85 (dd, *J* = 7.4, 12.9 Hz, 1H), 6.76 (d, *J* = 8.2 Hz, 1H), 4.0-3.84 (m, 6H), 3.22 (s, 1H), 1.15 (t, *J* = 7.0 Hz, 6H). HRMS (M+1): Calcd: 288.1001 Found: .288.1006.

4.4.17 3-((*S*)-1-Carboxy-2-hydroxyethylamino)benzoic acid (**29**)

Following the general procedure, 3-bromobenzoic acid (0.1 mmol) was *N*-arylated with L-serine and **29** (5.1 mg, 22.7%) was obtained after preparative HPLC.

^{1}H NMR (D_2O, 400 MHz): δ 7.60-6.90 (m, 4H), 4.58-3.79 (m, 4H). HRMS (M+1): Calcd: 226.0715 Found: 226.0721. $[\alpha]_D^{20}$ = 45.6 (c 1.2, H_2O)

4.4.18 (*S*)-3-Hydroxy-2-(3-methylsulfanylphenylamino)propionic acid (**30**)

Following the general procedure, 3-bromthioanisole (0.05 mmol) was *N*-arylated with L-serine to obtain 2.0 mg (17.6%) of **30** after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.62 (t, *J* = 7.4 Hz, 3H), 6.54 (d, *J* = 8.1 Hz, 1H), 4.88 (s, 1H), 3.88-3.52 (m, 3H), 3.15 (s, 1H), 2.18 (s, 3H). HRMS (M+1): Calcd: 228.0694 Found: 228.0690. $[\alpha]_D^{20}$ = -67 (c 0.1, $CHCl_3$)

4.4.19 (*S*)-3-Hydroxy-2-(3-methanesulfinylphenylamino)propionic acid (**31**)

Following the general procedure, **44** (0.1 mmol) was *N*-arylated with L-serine. Compound **31** (4.8 mg, 19.8%) was obtained after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.92 (t, *J* = 7.1 Hz, 1H), 6.55 (s, 1H), 6.53 (t, *J* = 6.0 Hz, 1H), 6.39 (d, *J* = 7.4 Hz, 1H), 4.73 (s, 1H), 3.79 (s, 1H), 3.60 (s, 2H), 3.20 (s, 1H), 2.36 (s, 1H). HRMS (M+1): Calcd: 244.0644 Found: 244.0652.

4.4.20 (*S*)-3-Hydroxy-2-(3-sulfamoylphenylamino)propionic acid (**32**)

Following the general procedure, 3-bromo-benzenesulfonamide (0.1 mmol) was *N*-arylated with L-serine to obtain 14 mg (53.8%) of **32** after preparative HPLC.

^{1}H NMR ($(CD_3)_2CO$, 400 MHz): δ 6.82 (s, 1H), 6.72-6.40 (m, 2H), 6.30-6.20 (m, 1H), 3.48 (s, 2H), 2.94 (s, 1H), 1.58 (s, 2H). Calcd: 261.0545 Found: 261.0550. $[\alpha]_D^{20}$ = 14 (c 0.5, $CHCl_3$).

4.4.21 (*S*)-2-[3-(Diethoxyphosphoryl)phenylamino]-3-hydroxypropionic acid (**33**)

Following the general procedure, **45** (0.1 mmol) was *N*-arylated with L-serine to obtain 20.4 mg (64.4%) of **33** after preparative HPLC.

^{1}H NMR (CD_3CN, 400 MHz): δ 7.30-7.22 (m, 1H), 7.12 (d, *J* = 14.6 Hz, 1H), 7.03 (dd, *J* = 6.4, 12.9 Hz, 1H), 6.92 (d, *J* = 7.8 Hz, 1H), 5.34 (s, 1H), 4.23 (t, *J* = 4.4 Hz, 1H), 4.08-3.96 (m, 6H), 3.55 (s, 1H), 1.26 (t, *J* = 7.0 Hz, 6H). HRMS (M+1): Calcd: 318.1107 Found: 318.1101. $[\alpha]_D^{20}$ = 106 (c 0.4, $CHCl_3$).

4.4.22 3-((*R*)-1-Carboxy-2-hydroxyethylamino)benzoic acid (**34**)

Following the general procedure, 3-bromobenzoic acid (0.1 mmol) was *N*-arylated with D-serine to obtain 5.5 mg (24.4%) of **34** after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.92-6.75 (m, 3H), 6.38 (s, 1H), 4.80 (s, 2H), 3.64-3.44 (m, 2H), 2.16 (m, 1H). HRMS (M+1): Calcd: 226.0715 Found: 226.0708. $[\alpha]_D^{20}$ = -50.6 (c 1.1, H_2O).

4.4.23 (*R*)-3-Hydroxy-2-(3-methylsulfanylphenylamino)propionic acid (**35**)

Following the general procedure, 3-bromthioanisole (0.05 mmol) was *N*-arylated with D-serine to obtain 1.2 mg (10.6%) of **35** after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.62 (t, *J* = 7.4 Hz, 3H), 6.54 (d, *J* = 8.1 Hz, 1H), 4.88 (s, 1H), 3.88-3.52 (m, 3H), 3.15 (s, 1H), 2.18 (s, 3H). M= 227.28 g/mol MS m/z (70 eV) 228 ($M+H^+$). $[\alpha]_D^{20}$ = - 60 (c 0.1, $CHCl_3$).

4.4.24 (*R*)-3-Hydroxy-2-(3-methanesulfinylphenylamino)propionic acid (**36**)

Following the general procedure, **44** (0.1 mmol) was *N*-arylated with D-serine. Compund **36** (5.3 mg, 21.8%) was obtained after preparative HPLC.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.93 (t, *J* = 7.2 Hz, 1H), 6.57 (s, 1H), 6.52 (t, *J* = 6.1 Hz, 1H), 6.39 (d, *J* = 7.4 Hz, 1H), 5.14 (s, 1H), 3.79 (s, 1H), 3.60 (s, 2H), 3.12 (s, 1H), 2.36 (s, 1H). HRMS (M+1): Calcd: 244.0644 Found: 244.0649.

4.4.25 (*R*)-3-Hydroxy-2-(3-sulfamoylphenylamino)propionic acid (**37**)

Following the general procedure, 3-bromo-benzenesulfonamide (0.1 mmol) was *N*-arylated with D-serine to obtain 9.5 mg (36.5%) of the **37** after preparative HPLC.

^{1}H NMR ($(CD_3)_2CO$-$CDCl_3$, 1:1, 400 MHz): δ 6.87 (s, 1H), 6.73 (s, 1H), 6.42 (s, 1H), 6.18 (s, 1H), 4.0-3.40 (m, 3H), 1.69 (s, 2H). HRMS (M+1): Calcd: 261.0545 Found: 261.0541. $[\alpha]_D^{20}$ = - 5 (c 0.4, $CHCl_3$).

4.4.26 (*R*)-2-[3-(Diethoxyphosphoryl)phenylamino]-3-hydroxypropionic acid (**38**)

Following the general procedure, **45** (0.1 mmol) was *N*-arylated with D-serine to obtain 10.2 mg (32.2%) of **38** after preparative HPLC.

^{1}H NMR (CD_3CN, 400 MHz): δ 7.32-7.20 (m, 1H), 7.12 (d, *J* = 13.8 Hz, 1H), 7.02 (dd, *J* = 6.3, 12.8 Hz, 1H), 6.95 (d, *J* = 7.8 Hz, 1H), 4.27 (t, *J* = 4.4 Hz, 1H), 4.10-3.96 (m, 6H), 3.50 (s, 1H), 1.25 (t, *J* = 7.0 Hz, 6H). HRMS (M+1): Calcd: 318.1107 Found: 318.1111. $[\alpha]_D^{20}$ = - 95 (c 1, $CHCl_3$).

Acknowledgements

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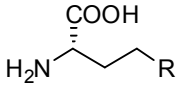
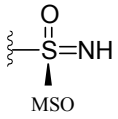
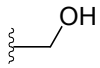
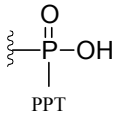
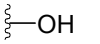
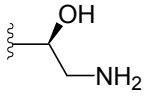
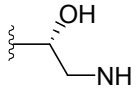
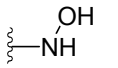
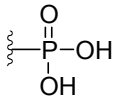
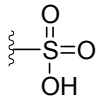
Table 1

Compounds suggested by literature surveys. Compounds **1** (MSO) and **2** (PPT) are known MtGS inhibitors, while **4**-**8** are known inhibitors of GSs of other species. Compounds **9**-**13** are analogues of previously known inhibitors. Results for MtGS are reported as percent inhibition in the biosynthetic assay in the presence of 1 mM compound. Literature values for GSs from other species were based on the transferase assay and are reported either as percent inhibition (where the concentration of compound is given in parenthesis), or where available, as K_i. In the transferase assay, glutamine and hydroxylamine are converted into γ-glutamyl hydroxamate and ammonia, in the presence of ADP, manganese and arsenate.



	R=	*M. tuberculosis*[a]	*E. coli*	*S. typhimurium*	Sheep brain	Soybean		R=	*M. tuberculosis*[a]
1	 MSO	81 ± 3[b]					**9**		NI[b]
2	 PPT	76 ± 7[b,c]					**10**		NI[b]
4		58 ± 16[b] 56 ± 10[d]		K_i=40 μM[29]	22 (0.5 mM)[35]	21 (0.5 mM)[35]	**11**		NI[b]
5		26 ± 14[b]			K_i=7 μM[35]	K_i=21 μM[35]	**12**		NI[b]
6		NI[b]	K_{is}=54 μM[17]				**13**		NI[d]
7		33 ± 13[b]			67 (25 mM)[11]				
8		NI[b,c]			11-14 (25 mM)[11]				

[a] Reported values for MtGS is an average from three independent experiments, reported together with the standard deviation. NI indicates no inhibition. Substrate concentrations in the assay were 1 mM ATP, 10 mM L-glutamate and 30 mM ammonium chloride.

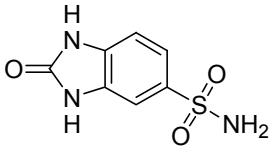
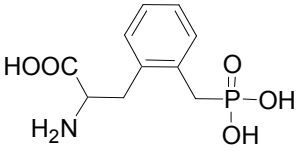
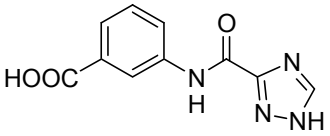
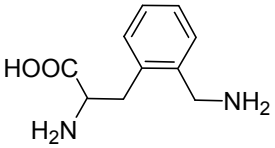
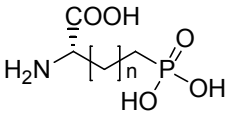
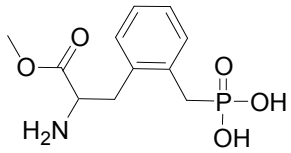
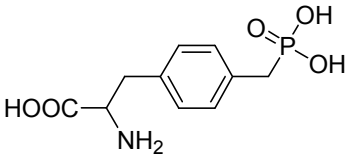
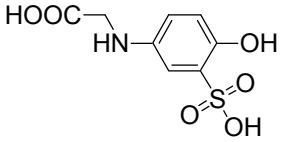
[b] Dissolved in H_2O.

[c] Tested as a racemic mixture or a mixture of two diastereomers.

[d] Dissolved in DMSO.

Table 2

Compounds derived from the virtual screening. Inhibition of MtGS is reported as percent at a compound concentration of 1.0 mM, as compared to uninhibited enzyme. Substrate concentrations in the assay were 1 mM ATP, 10 mM, L-glutamate and 30 mM ammonium chloride. NI indicates no inhibition.

	Compound[a]	% Inhibition[b]		Compound[a]	% Inhibition[b]
14		24 ± 3[c]	**20**		NI[d,e]
15		26 ± 13[c]	**21**		NI[d]
6 **16** **17** **18**	 n=1-4	n=1, NI[d] n=2, NI[c,f] n=3, NI[d] n=4, NI[d]	**22**		NI[d]
19		NI[d,g]	**23**		48 ± 16[d]

[a] Compounds **6**, **16-18** were tested as L-amino acids and **19-22** as racemic mixtures.

[b] The value is an average from three independent experiments, reported together with the standard deviation.

[c] Dissolved in DMSO

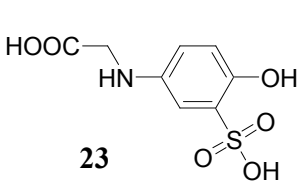
[d] Dissolved in H_2O

[e] Concentration in assay 0.20 mM

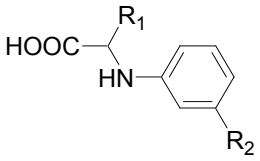
[f] Concentration in assay 0.75 mM

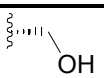
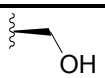
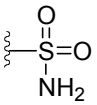
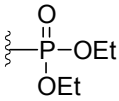
[g] Concentration in assay 0.93 mM

Table 3
Synthetic library based on **23** identified in the virtual screening. Inhibition is reported as percent inhibition at a concentration of 1.0 mM, as compared to uninhibited enzyme. Substrate concentrations in the assay were 1 mM ATP, 10 mM L-glutamate and 30 mM ammonium chloride. NI indicates no inhibition and AA indicates apparent activation.



23



	R_1= 		R_1= 		R_1= 	
R_2=	Compound	% Inhibition[a]	Compound	% Inhibition[a]	Compound	% Inhibition[a]
	**24**	AA[b]	**29**	AA[b]	**34**	AA[b]
	**25**	NI[b,c]	**30**	AA[b,d]	**35**	NI[b,d]
	**26**	30 ± 18[b]	**31**	13 ± 15[b]	**36**	NI[b]
	**27**	NI[b]	**32**	AA[b]	**37**	33 ± 10[b]
	**28**	42 ± 5[e]	**33**	AA[b]	**38**	AA[b]

[a] The value is an average from three independent experiments, reported together with the standard deviation.
[b] Dissolved in H_2O
[c] Concentration in assay 0.90 mM
[d] Concentration in assay 0.50 mM
[e] Dissolved in DMSO

Figure 1
Active site of MtGS. Compound **4** (black carbon atoms) and **11** (magenta) docked in the amino acid binding site of MtGS, compared to the crystal structure of phosphorylated L-methionine-(*S*)-sulfoximine (green). Hydrogen bonds between **4** and the enzyme are indicated with dotted lines.

Figure 2
IC_{50} determination. Experimental data are shown for MSO (**1**, triangles), PPT (**2**, squares) and (2*S*,5*R*)-2,6-diamino-5-hydroxycaproic acid (**4**, circles). Data points were normalized to ease comparison, using the experimentally determined *Hi* and *Lo* values, such that normalized fractional activity = (Y-Lo) / (Hi-Lo). The accompanying curves are those calculated from equation 1 using the determined IC_{50} values (**1**, 51 μM, n=3; **2**, 1.9 μM, n=3; **4**, 610 μM, n=5) together with *Hi* = 1 and *Lo* = 0. Substrate concentrations used in the assay were 1 mM ATP, 10 mM L-glutamate and 30 mM ammonium chloride.

Figure 3
Virtual screening flowchart. The 2D substructures were used in primary filtering of the database. The pharmacophore with one hydrogen bond donor (DON) and two hydrogen bond acceptors (ACC) was used to select only the compounds where the first docking solution was placed deep inside the amino acid binding site with a hydrogen bonding pattern similar to L-methionine-(*S*)-sulfoximine (**1**).

Figure 4
Hits from the virtual screening. The three inhibitors identified in the virtual screening and the docked solution of these in the amino acid binding site. Compounds **14** (A) and **15** (B) and **23** (C) show key interactions with residues in the binding site.

Scheme 1
Synthesis of **20**, identified in the virtual screening as a potential GS inhibitor, and two analogues (**21**-**22**). (a) $AcNHCH(CO_2Et)_2$, NaOEt, EtOH; (b) H_2, Pd/C, 1 atm, HCl, EtOH, $CHCl_3$; (c) $NaNO_2$, H_2O, reflux; (d) i–NEt_3, MsCl, THF; ii–NaI, acetone; (e) $P(OEt)_3$; (f) 9 M HCl.

Scheme 2
Procedure for the synthesis of a library based on **23** originating from the virtual screening. (a) ArBr, amino acid, 10 mol% CuI in water-acetonitrile, 90°C.

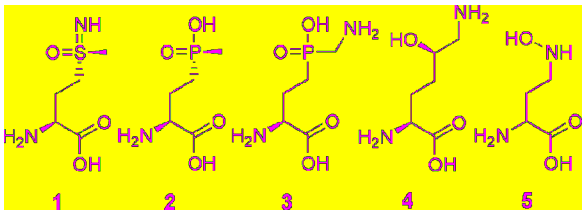
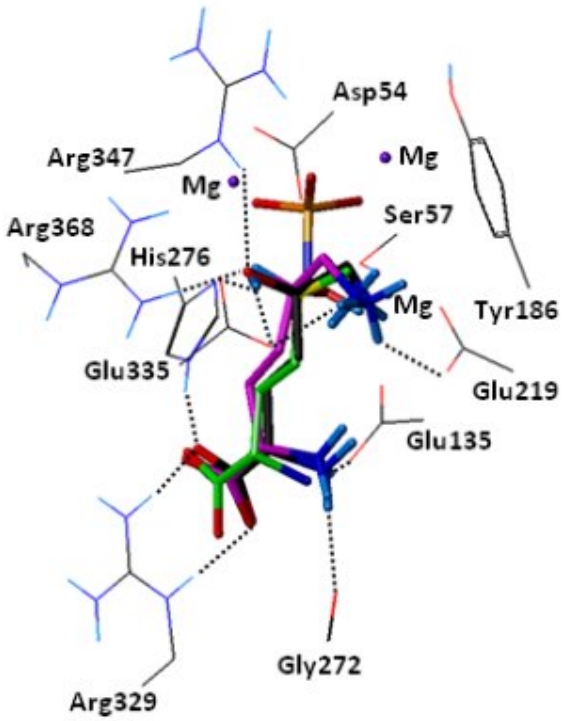

1
2
3
4
5

Figure 1


Asp54
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Mg
Ser57
Arg368
His276
Mg
Tyr186
Glu335
Glu219
Glu135
Gly272
Arg329

Figure 2

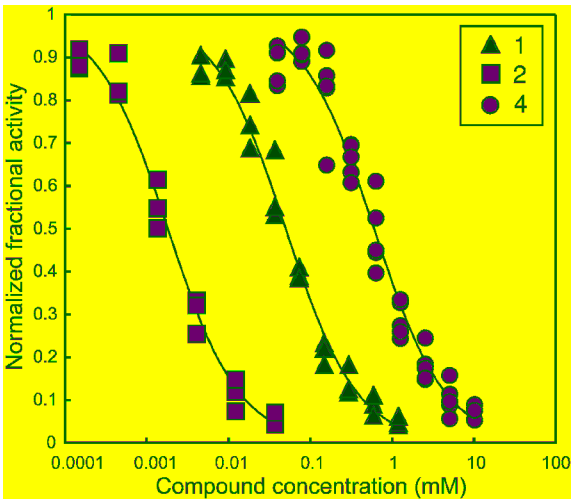

Normalized fractional activity
Compound concentration (mM)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
0.0001
0.001
0.01
0.1
1
10
100
1
2
4

Figure 3

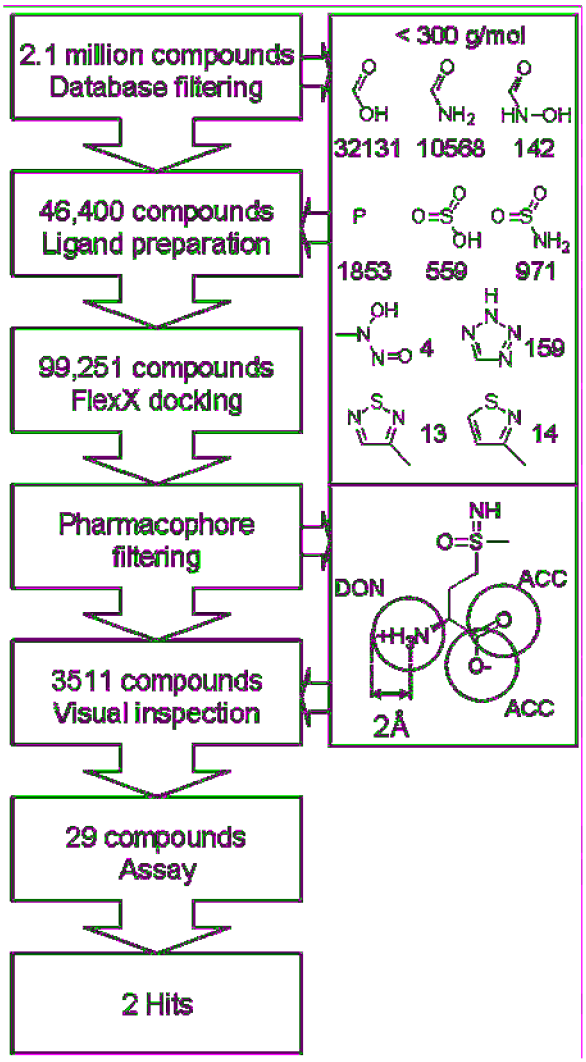
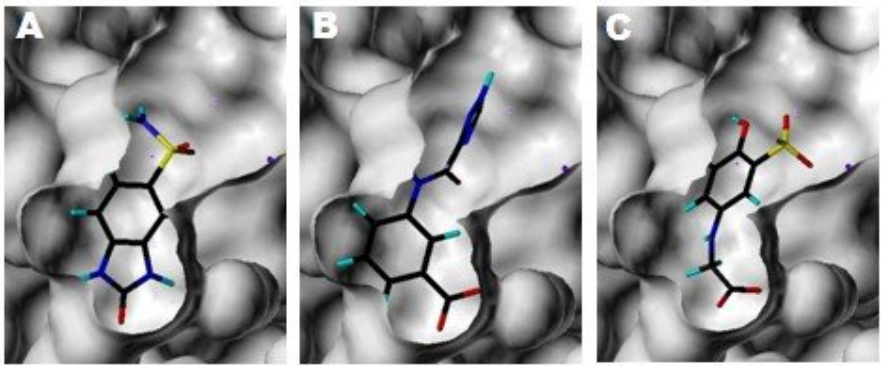
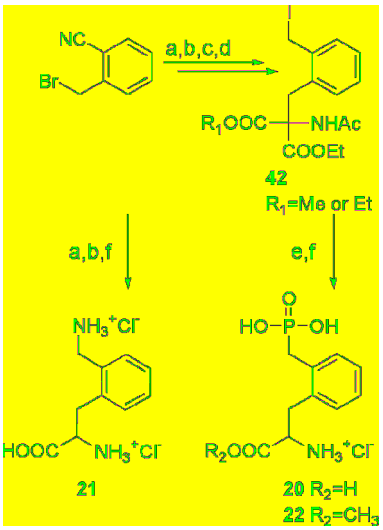

2.1 million compounds
Database filtering
< 300 g/mol
32131
10568
142
46,400 compounds
Ligand preparation
1853
559
971
99,251 compounds
FlexX docking
4
159
13
14
Pharmacophore
filtering
DON
ACC
ACC
2Å
3511 compounds
Visual inspection
29 compounds
Assay
2 Hits

Figure 4



Scheme 1



Scheme 2

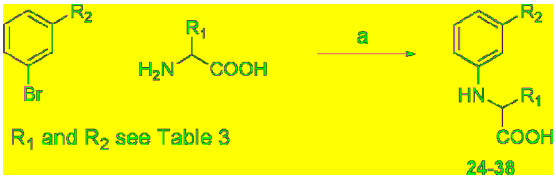


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Figure1

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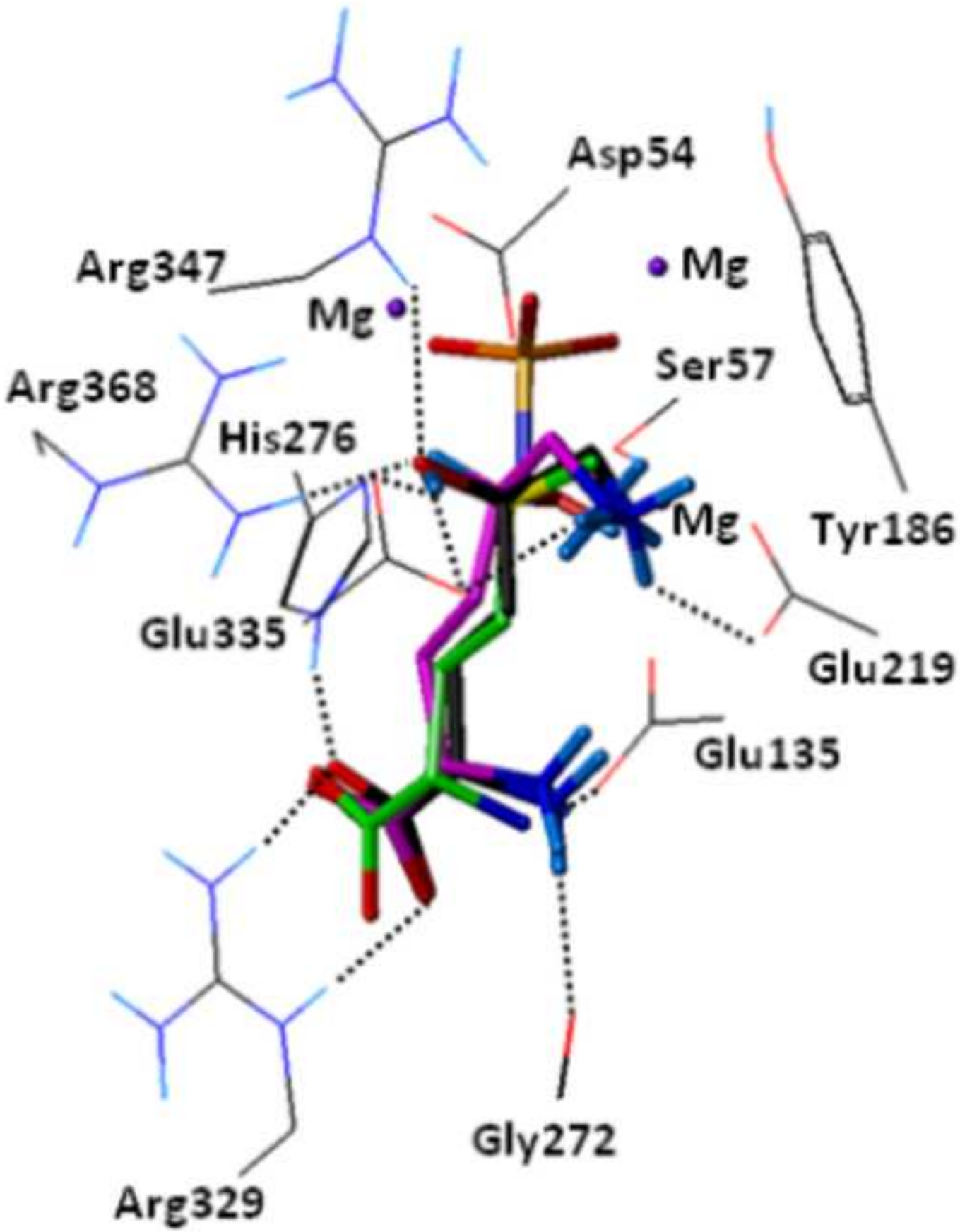

Figure2

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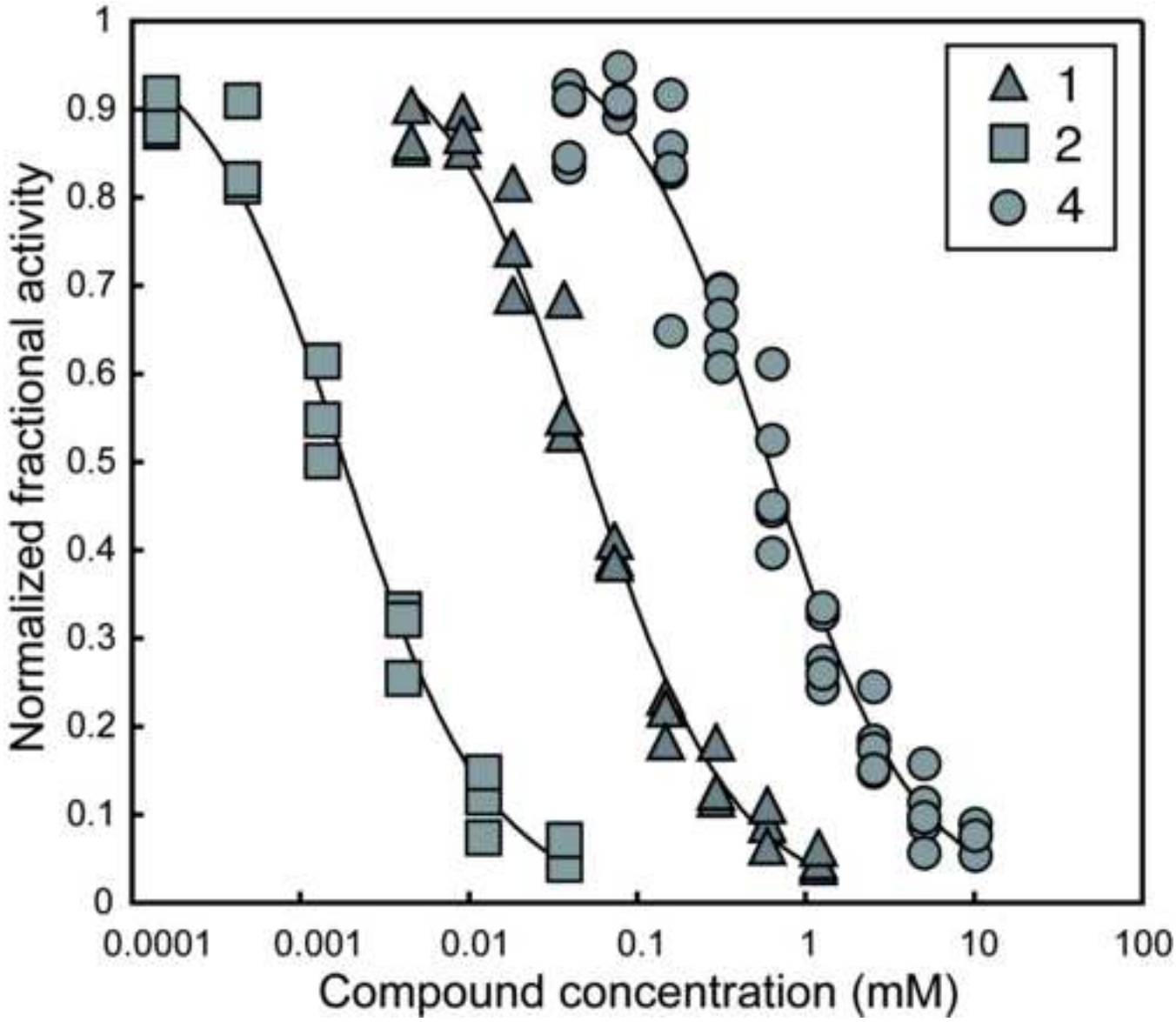

Figure3

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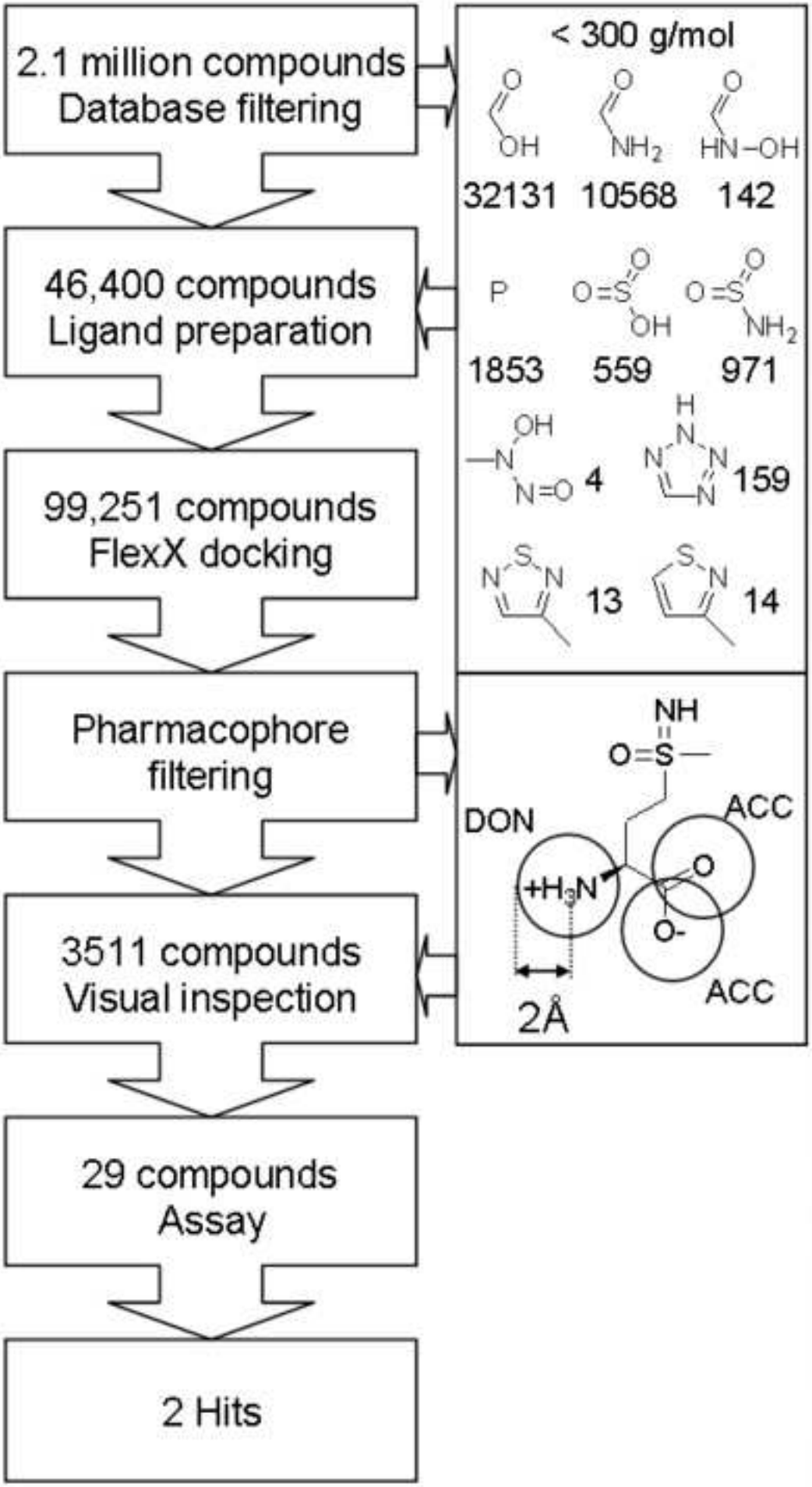

Figure4

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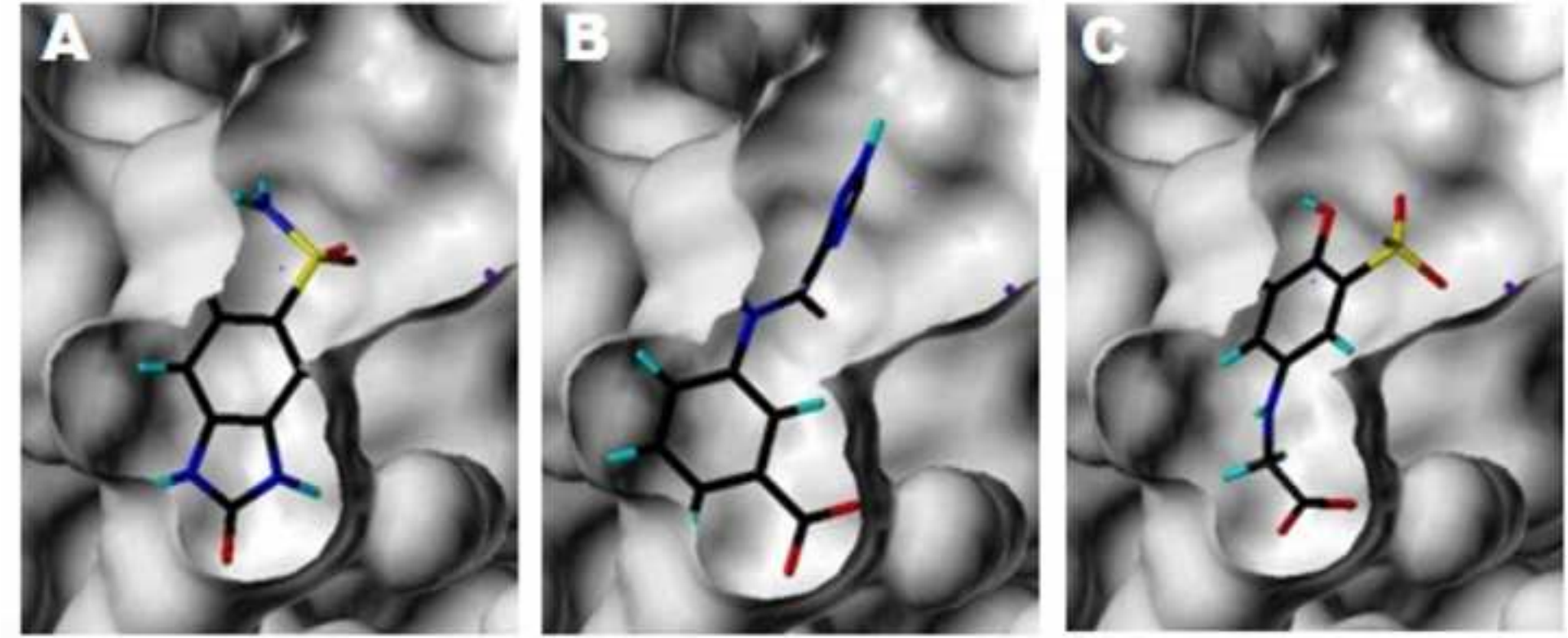

Scheme1

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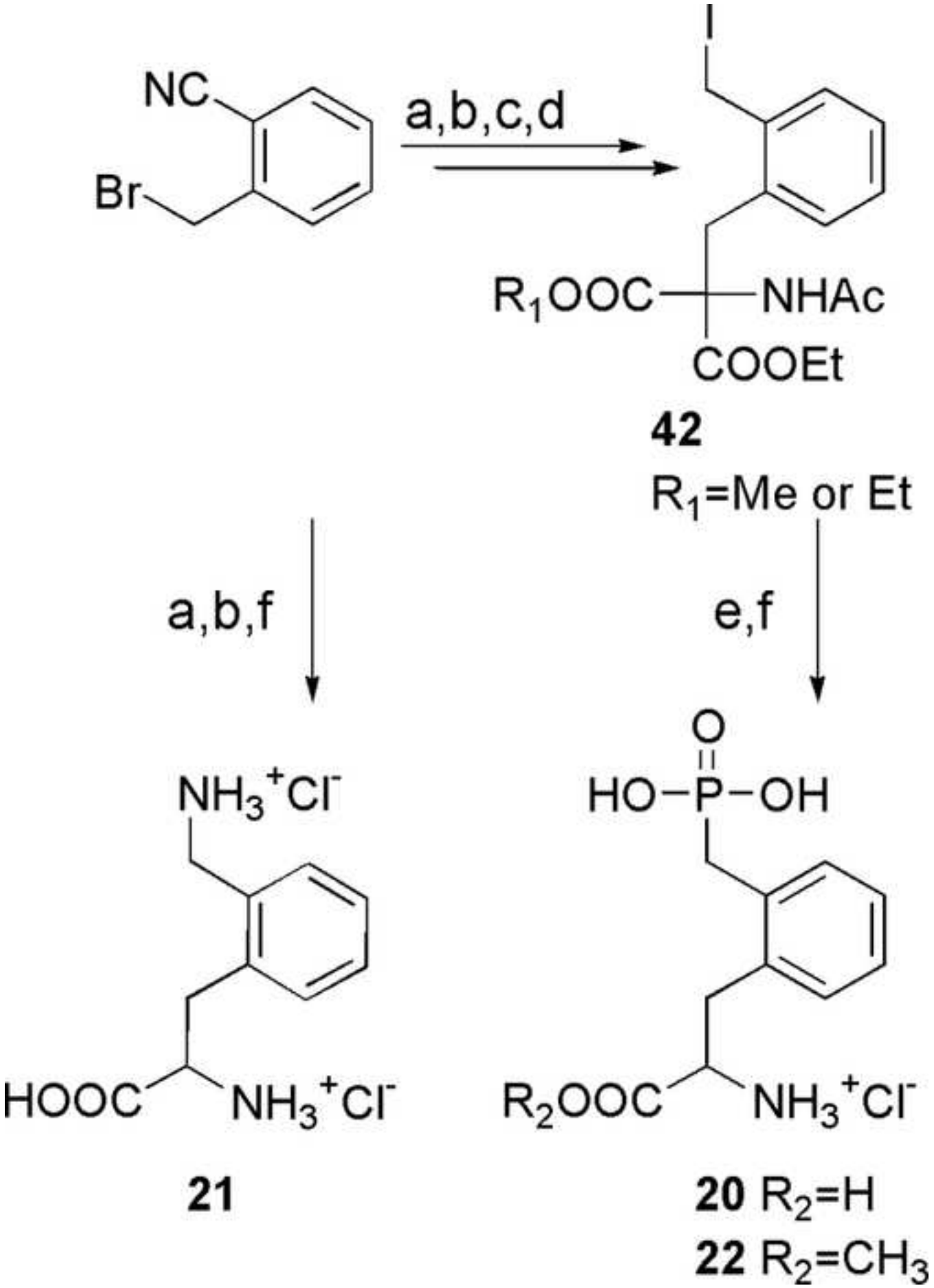

Scheme2

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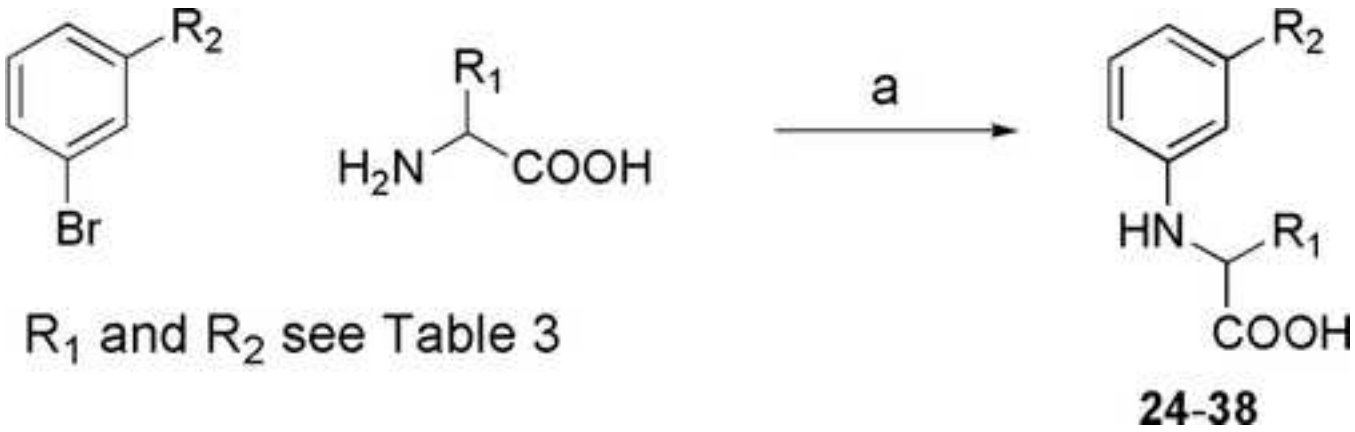

R_1 and R_2 see Table 3

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Chart1

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