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Discovery of Glutathione S-Transferase Inhibitors Using Dynamic Combinatorial Chemistry

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Abstract: Protein-directed dynamic combinatorial chemistry (DCC) relies on reversible chemical reactions that can function under the near-physiological conditions required by the biological target. Few classes of reaction have so far proven effective at generating dynamic combinatorial libraries (DCLs) under such constraints. In this study, we establish the conjugate addition of thiols to enones as a reaction well-suited for the synthesis of dynamic combinatorial libraries (DCLs) directed by the active site of the enzyme glutathione S-transferase (GST). The reaction is fast, freely reversible at basic pH, and easily interfaced with the protein, which is a target for the design of inhibitors in cancer therapy and the treatment of parasitic diseases such as schistosomiasis. We have synthesized DCLs based on glutathione (GSH, **1**) and the enone ethacrynic acid, **2a**. By varying either set of components, we can choose to probe either the GSH binding region ("G site") or the adjacent hydrophobic acceptor binding region ("H site") of the GST active site. In both cases the strongest binding DCL components are identified due to molecular amplification by GST which, in the latter system, leads to the identification of two new inhibitors for the GST enzyme.

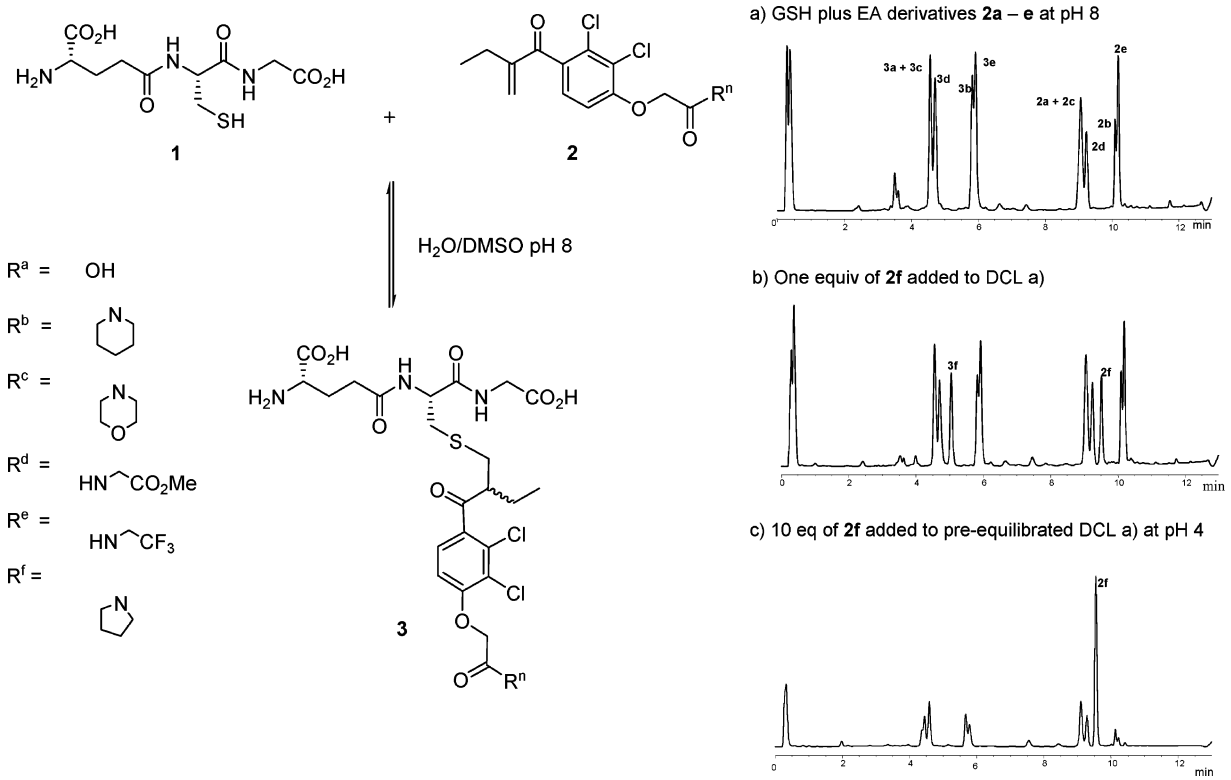
Introduction

Dynamic Combinatorial Chemistry (DCC) is a powerful approach to the discovery of small molecule ligands for large biomolecules.[1] The method uses the biomolecular target to direct the reversible, in situ assembly of a small molecule library and as such can be considered, in the wider context of target-guided synthesis (TGS), an umbrella term that covers a variety of systems that feature small-molecule synthesis orchestrated by a large biomolecule (usually a protein).[2−10] DCC contrasts with the majority of TGS methods by using reversible reactions to assemble the prospective ligands. Whereas kinetic methods use the interior of proteins to direct and accelerate an irreversible reaction, usually between isolated sets of reactants, a dynamic combinatorial library (DCL) of compounds is designed such that it can equilibrate in the presence of the target. Since the library population distribution is under thermodynamic control, stabilization of one member through selective binding to the protein is expected to amplify that species at the expense of other (nonbinding) species, generating hit structures that can be identified through analysis of the DCL population distribution. DCC thus bridges the gap between chemical synthesis of drug candidates and their biological binding assay, meshing the two operations into a single process whereby the structure of the biological target directs the assembly of its own best inhibitor in situ.

The set of chemical reactions that have been successfully applied to the construction of DCLs in the presence of a biological target is currently rather small, centering on C═N and S−S bond-forming processes.[11−14] This lack of diversity

(1) Reviews: (a) Cheeseman, J. D.; Corbett, A. D.; Gleason, J. L.; Kazlauskas, R. J. *Chem.—Eur. J.* **2005**, *11*, 1708−1716. (b) Otto, S. *Curr. Opin. Drug Discovery Dev.* **2003**, *6*, 509−520. (c) Otto, S.; Furlan, R. L. E.; Sanders, J. K. M. *Curr. Opin. Chem. Biol.* **2002**, *6*, 321−327. (d) Lehn, J.-M. *Chem.—Eur. J.* **1999**, *5*, 2455−2463.
(2) Rideout, D. *Science* **1986**, *233*, 561−563.
(3) Nguyen, R.; Huc, I. *Angew. Chem., Int. Ed.* **2001**, *40*, 1774−1776.
(4) Nicolaou, K. C.; Hughes, R.; Cho, S. Y.; Winssinger, N.; Smethurst, C.; Labischinski, H.; Endermann, R. *Angew. Chem., Int. Ed.* **2000**, *39*, 3823−3828.
(5) Nicolaou, K. C.; Hughes, R.; Cho, S. Y.; Winssinger, N.; Labischinski, H.; Endermann, R. *Chem.—Eur. J.* **2001**, *7*, 3824−3843.
(6) Erlanson, D. A.; Braisted, A. C.; Raphael, D. R.; Randal, M.; Stroud, R. M.; Gordon, E. M.; Wells, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9367−9372.
(7) Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radić, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 1053−1057.
(8) Mocharla, V. P.; Colasson, B.; Lee, L. V.; Röper, S.; Sharpless, K. B.; Wong, C.-H.; Kolb, H. C. *Angew. Chem., Int. Ed.* **2005**, *44*, 116−120.
(9) Bourne, Y.; Kolb, H. C.; Radić, Z.; Sharpless, K. B.; Taylor, P.; Marchot, P. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 1449−1454.
(10) Krasinski, A.; Radić, Z.; Manetsch, R.; Raushel, J.; Taylor, P.; Sharpless, K. B.; Kolb, H. C. *J. Am. Chem. Soc.* **2005**, *127*, 6686−6692.
(11) C═N bond formation: (a) Huc, I.; Lehn, J.-M. *Proc. Nat. Acad. Sci. U.S.A.* **1997**, *94*, 2106−2110. (b) Bunyapaiboonsri, T.; Ramström, O.; Lohmann, S.; Lehn, J.-M.; Peng, L.; Goeldner, M. *ChemBioChem* **2001**, *2*, 438−444. (c) Hochgürtel, M.; Kroth, H.; Piecha, D.; Hofmann, M. W.; Nicolau, C.; Krause, S.; Schaaf, O.; Sonnenmoser, G.; Eliseev, A. V. *Proc. Nat. Acad. Sci. U.S.A.* **2002**, *99*, 3382−3387. (d) Gerber-Lemaire, S.; Popowycz, F.; Rodriguez-Garcia, E.; Asenjo, A. T. C.; Robina, I.; Vogel, P. *ChemBioChem* **2002,** *3*, 466−470. (e) Ramström, O.; Lohmann, S.; Bunyapaiboonsri, T.; Lehn, J.-M. *Chem.—Eur. J.* **2004**, 10, 1711−1715. (f) Bugaut, A.; Toulmé, J.-J.; Rayner, B. *Angew. Chem., Int. Ed.* **2004**, *43*, 3144−3147. (g) Zameo, S.; Vauzeilles, B.; Beau, J.-M. *Angew. Chem., Int. Ed.* **2005**, *44*, 965−969. (h) Bugaut, A.; Bathany, K.; Schmitter, J.-M.; Rayner, B. *Tetrahedron Lett.* **2005**, *46*, 687−690.
(12) S−S bond formation: (a) Ramström, O.; Lehn, J.-M. *ChemBioChem* **2000**, *1*, 41−48. (b) Krishnan-Ghosh, Y.; Balasubramanian, S. *Angew. Chem., Int. Ed.* **2003**, *42*, 2171−2173. (c) Whitney, A. M.; Ladame, S.; Balasubramanian, S. *Angew. Chem., Int. Ed.* **2004**, *43*, 1143−1146. (d) Hotchkiss, T.; Kramer, H. B.; Doores, K. J.; Gamblin, D. P.; Oldham, N. J.; Davis, B. G. *Chem. Commun.* **2005**, 4264−4266. (e) Ladame, S.; Whitney, A. M.; Balasubramanian, S. *Angew. Chem., Int. Ed.* **2005**, *44*, 5736−5739.
(13) Thioester formation: Larsson, R.; Pei, Z.; Ramström, O. *Angew. Chem., Int. Ed.* **2004**, *43*, 3716−3718.

Scheme 1. DCL Composed of EA Analogues and GSH, **1**[a]



[a] Glutathione, **1**, adds to the five enones **2a**−**e** to set up a DCL that has an equitable distribution of thiol conjugates **3a**−**e** and starting enones (chromatogram a). The reversibility of the DCL is proven by adding an additional enone component, **2f**, to the pre-equilibrated library and observing the incorporation of adduct **3f** (chromatogram b). The pH control of the DCL is illustrated by pre-equilibration, acidification to pH 4, and subsequent addition of a large excess of enone **2f**, which is not incorporated into the library as the conjugate addition reaction has been switched off (chromatogram c).

is unsurprising, as the successful reaction must meet two sets of exacting criteria; it must proceed under the near physiological conditions required by the protein target as well as fulfilling the thermodynamic condition of reversibility inherent to the DCC process. An increase in the number of chemical reactions available for DCC is necessary to enable greater functional group diversity and increased applicability of DCLs as a target-guided synthesis methodology. With this in mind, we have recently introduced the conjugate addition of thiols to enones as a new method for DCL synthesis.[15] The reaction is well-suited to DCC being fast, freely reversible, responsive to pH change, and proceeding in water under mild conditions with no external reagents (Scheme 1). In addition, the choice of the tripeptide glutathione (γ-Glu-Cys-Gly, GSH, **1**) as the thiol in our preliminary studies creates DCLs that are biologically relevant and amenable to targeting by a variety of proteins, the most relevant being the enzymes responsible for mediating the conjugate addition chemistry of GSH in the cell: the glutathione S-transferases (GSTs).

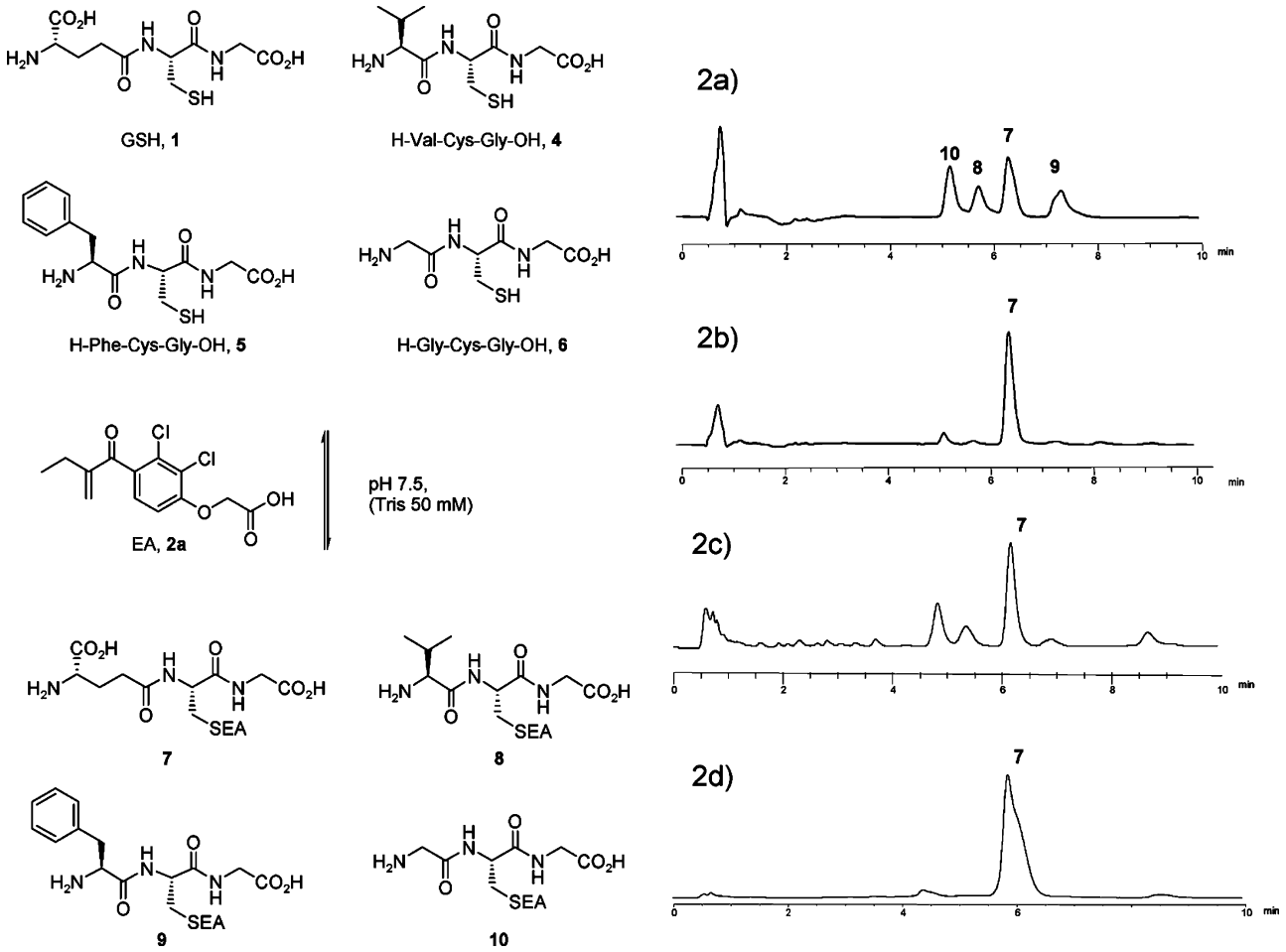
The GSTs are a large family of dimeric enzymes responsible for cell detoxification, thereby protecting the cell from cytotoxic and oxidative stress.[16,17] They catalyze the conjugation of GSH to a wide variety of xenobiotic electrophiles such as quinones, prostaglandins, and base propenals, making them more water soluble and easily eliminated from the cell. The GSTs are potential drug targets in cancer therapy, where resistance to chemotherapeutic drugs has been directly correlated with the overexpression of GSTs in tumor cells, and parastic diseases such as malaria and schistosomiasis.[18] We now describe the application of our GSH-based DCLs to the discovery of GST inhibitors.

Results and Discussion

We initially designed a biased DCL to examine the compatibility of our conjugate addition methodology with GST and to see whether the enzyme could effectively amplify the best binding component from a DCL. Since DCL synthesis requires large amounts of highly purified enzyme, we selected GST from the helminth worm *Schistosoma japonica* (SjGST, 26kDa monomer) as our target transferase, as it is well characterized and amenable to recombinant overexpression in *E. coli* as an affinity-tagged construct.[19] The DCL is shown in Scheme 2 and consists of GSH, **1**, three tripeptide GSH analogues **4**−**6**, and the enone ethacrynic acid, **2a** (EA). The tripeptide analogues were synthesized using standard solid-phase synthesis methods and differ from GSH at the γ-glutamyl residue. As a result, the three analogues are expected to be poor fits for the GSH binding region of the GST active site, as the γ-glutamyl residue is thought to be critical for binding,[20] thus biasing the DCL toward

(14) Enzymatic aldol reaction: (a) Lins, R. J.; Flitsch, S. L.; Turner, N. J.; Irving, E.; Brown, S. A. *Angew. Chem., Int. Ed.* **2002**, *41*, 3405−3407. (b) Lins, R.; Flitsch, S. L.; Turner, N. J.; Irving, E.; Brown, S. A. *Tetrahedron* **2004**, *60*, 771−780.
(15) Shi, B.; Greaney, M. F. *Chem. Commun.* **2005**, 886−888.
(16) Mannervik, B.; Danielson, U. H. *CRC Crit. Rev. Biochem.* **1988**, *23*, 283−337.
(17) Hayes, J. D.; Flanagan, J. U.; Jowsey, I. R. *Annu. Rev. Pharmacol. Toxicol.* **2005**, *45*, 51−88.
(18) Mahajan, S.; Atkins, W. M. *Cell. Mol. Life Sci.* **2005**, *62*, 1221−1233.
(19) Waugh, D. S. *Trends Biotechnol.* **2005**, *23*, 316−320.
(20) Andersson, C.; Mosialou, E.; Adang, A. E.; Mulder, G. J.; van Der Gen, A.; Morgenstern, R. *J. Biol. Chem.* **1991**, *266*, 2076−2079.

Scheme 2. DCL Composed of GSH Analogues and EA, **2a**[a]



[a] The four tripeptides **1**, **4**−**6** undergo reversible conjugate addition to EA, **2a**, at pH 7.5. Chromatogram (a) shows the blank DCL after 1 h; chromatogram (b) shows the DCL after 10 min in the presence of SjGST; chromatogram (c) shows the DCL after pre-equilibration and *then* addition of SjGST, after 2 days; chromatogram (d) shows the same system as (c) after 6 days. Although each EA adduct is formed as a mixture of diastereoisomers upon conjugate addition, giving a total of eight adducts in the DCL, the diasteromeric adducts are not separable under the LC conditions.

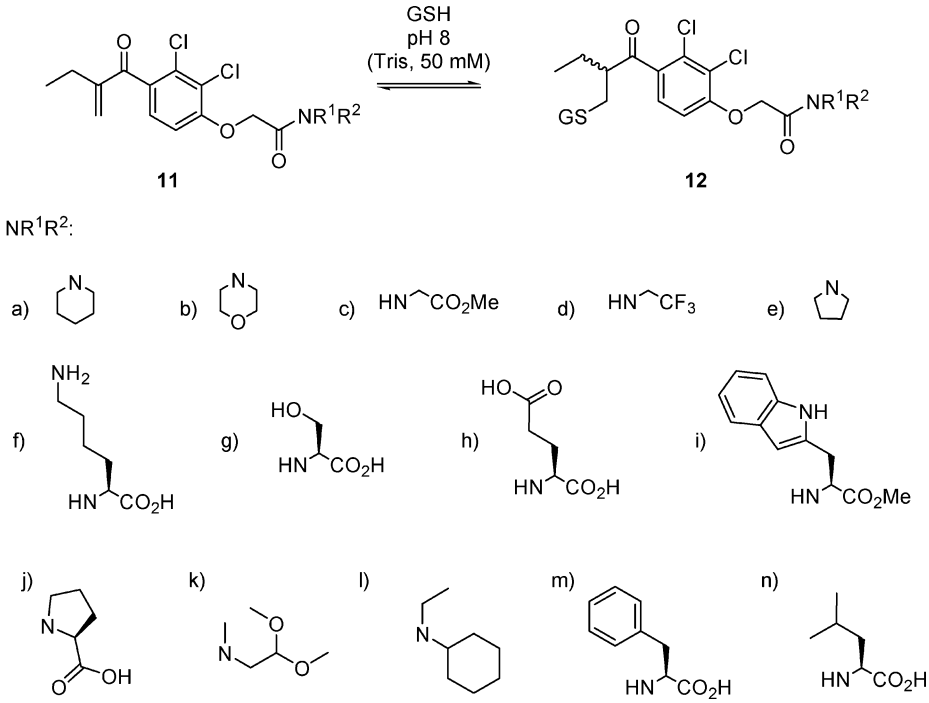
the GSH adduct **7**. The enone ethacrynic acid is a known inhibitor of GSTs and provides a motif for further structural elaboration.

The blank DCL, assembled in the absence of any enzyme, equilibrates in 1 h to give the distribution of each of the four conjugates **7**−**10** shown in chromatogram 2a (Scheme 2). Upon incubation of the same components with 0.8 equiv of SjGST for 10 min, the DCL collapsed to essentially one adduct, the expected GS-EA, **7** (chromatogram 2b), and remained unchanged thereafter. The rapid equilibration in the presence of SjGST indicates that the enzyme is accelerating the conjugate addition, as would be expected given its catalytic function as a transferase in GSH biochemistry. To verify that the composition shown in chromatogram 2b represented true equilibrium and not a metastable distribution generated by target-accelerated synthesis in the presence of the enzyme, we added SjGST to the pre-equilibrated DCL shown in chromatogram 2a. Strong amplification of adduct **7** was again observed, but at a far slower rate. After 2 days **7** was present in 55% of total adduct concentration (chromatogram 2c), and after 6 days the DCL had equilibrated to an identical distribution to that of chromatogram 2b, where adduct **7** is by far the dominant species. These results indicate that catalysis of the conjugate addition reaction by SjGST does not effect the equilibrium distribution of the DCL and that the amplified compound **7** is the thermodynamically preferred binder.

The expected adduct **7** has been amplified from 35% of total conjugate concentration to 92% at equilibrium, a large amplification that ought to reflect correspondingly large differences in binding affinity between **7** and those peptides lacking the γ-glutamyl residue. To verify that amplification and binding affinity are indeed correlated in this system, we quantified the inhibitory activity of **7** and **10** for SjGST using the standard chlorodinitrobenzene (CDNB) GST inhibition assay.[21] Measured IC_{50} values of 0.32 μM for **7** and 88 μM for **10** differ by over 2 orders of magnitude, demonstrating that the extent of molecular amplification for each component in the biased DCL can be clearly related to their binding affinity for the protein target. Similar strong amplification was also observed in slightly larger DCLs based upon seven starting tripeptides (vide infra). Control experiments using bovine serum albumin as the protein target in place of SjGST produced no changes to the blank DCL composition, indicating that the active site of SjGST is directing the amplification of the DCL. The success of these biased DCLs

(21) (a) Habig, W. H.; Pabst, M. J.; Jakoby, W. B. *J. Biol. Chem.* **1974**, *249*, 7130−7139. (b) Burg, D.; Filippov, D. V.; Hermanns, R.; van der Marel, G. A.; van Boom, J. H.; Mulder, G. J. *Bioorg. Med. Chem.* **2002**, *10*, 195−205.

Scheme 3. A DCL Made from GSH and 14 EA Analogues



encouraged us to extend the thiol addition methodology to the discovery of new GST inhibitors through the synthesis of larger, nonbiased DCLs.

All GSTs contain two recognition areas for GS−R conjugates: a highly conserved GSH binding site and a hydrophobic binding region for the electrophilic substrate, called the H-site, which varies across the different GST isoforms. The design of inhibitors which target the H-site is a promising strategy in terms of isozyme discrimination,[18] so we elected to use DCC to explore the H-site of SjGST with a series of EA analogues. We prepared a DCL with reversed stoichiometry from that in Scheme 2, whereby *n* EA analogues react with GSH to afford *n* GS-EA adducts. The EA analogues were prepared from EA via EDC-mediated amide bond formation at the carboxylic acid group, producing a series of compounds **11a**−**n** that display a variety of randomly selected functional groups (Scheme 3). The fourteen EA analogues were equilibrated with GSH in both the absence and presence of SjGST (2 equiv). Figure 1 shows the LC traces of each DCL, along with an overlay for comparison purposes. The large peak eluting at ∼5.9 min in the amplified trace is due to trace amounts of SjGST still present in the reaction mixture. We verified that all of the expected GSH conjugates were present in the DCL using ESI-MS analysis and then looked to identify only those components that have been amplified by the enzyme.

Looking at the overlay trace in the area of the GSH conjugates we can see that a single peak has been amplified, marked with a star at $R_T = 4.8$ min. This amplification is at the expense of the first peak at $R_T = 3.3$ min, which is reduced in intensity. MS analysis of the amplified peak indicated four adducts, **12a**, **d**, **m**, and **n**, while the reduced peak is due to adduct **12f**.

Deconvolution studies established that each of the adducts **12a**, **m**, and **n** were being amplified in the presence of SjGST, whereas **12d** was not.[22] Identical amplification results were obtained when SjGST was added to the pre-equilibrated DCL, indicating that the thermodynamic reversibility inherent to the thiol conjugate addition in the absence of any target is maintained in the presence of GST. To ascertain whether the hit structures generated from DCC were in fact active inhibitors of SjGST, we synthesized the amplified adducts **12a** and **12n**, a nonamplified adduct **12b**, and the depreciated adduct **12f** separately and measured their IC_{50} values (Figure 2).

Both piperidine (**12a**) and leucine (**12n**) derivatives of EA inhibit SjGST at the low micromolar/high nanomolar level, with the piperidine derivative **12a** being slightly more potent ($IC_{50} = 0.61$ μM versus $IC_{50} = 1.40$ μM). The more polar lysine amide **12f** is, as expected, the least active, with an IC_{50} value more than 10-fold lower than **12a** at 8.20 μM. The morpholine amide **12b** lies halfway between **12a** and **12f** with an IC_{50} value of 4.30 μM, approximately 7-fold lower than the piperidine amide. These figures indicate that the extent of DCL amplification not only reflects the relative binding affinities of DCL components for the protein target but is also discriminatory across an order of magnitude range of IC_{50} values. The piperidine and leucine amides **12a** and **12n** are amplified from the library at the expense of the lysine amide **12f**, which being an approximately 10-fold weaker binder is removed from the equilibrium composition of the DCL. The reversibility inherent to the thiol conjugate addition is enabling the SjGST target to amplify relatively weak-binding compounds from a nonbiased selection of small molecules, demonstrating that this TGS methodology can be applied in a medicinal chemistry lead-finding context.

As EA is known to inhibit GSTs both on its own and as a GSH conjugate,[23] we were interested in acquiring IC_{50} data for some of the enone starting materials. We assayed the five enones **2a**, **11a**, **11b**, **11f**, and **11n**, the corresponding Michael acceptors

(22) See Supporting Information.

(23) Oakley, A. J.; Rossjohn, J.; Lo Bello, M.; Caccuri, A. M.; Federici, G.; Parker, M. W. *Biochemistry* **1997**, *36*, 576−585.

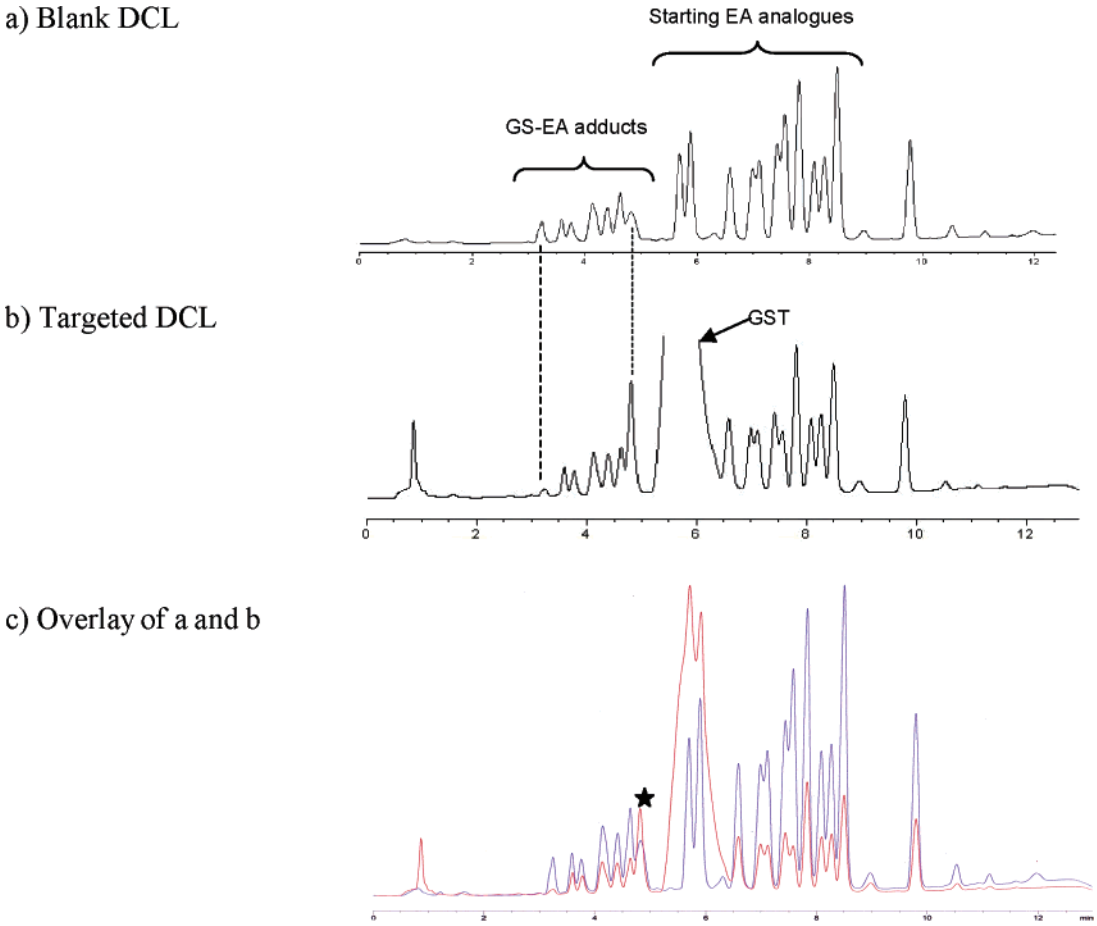


Figure 1. HPLC chromatograms for the targeted DCL of EA analogues. Chromatogram (a) shows the blank DCL distribution after 1 h, chromatogram (b) shows the DCL composition in the presence of SjGST after 30 min and chromatogram (c) shows an overlay of (a) and (b). The two dotted lines indicate the peak that is reduced in intensity on addition of the enzyme, at $R_T = 3.3$ min, along with the peak that is amplified upon enzyme addition, at $R_T = 4.8$ min.

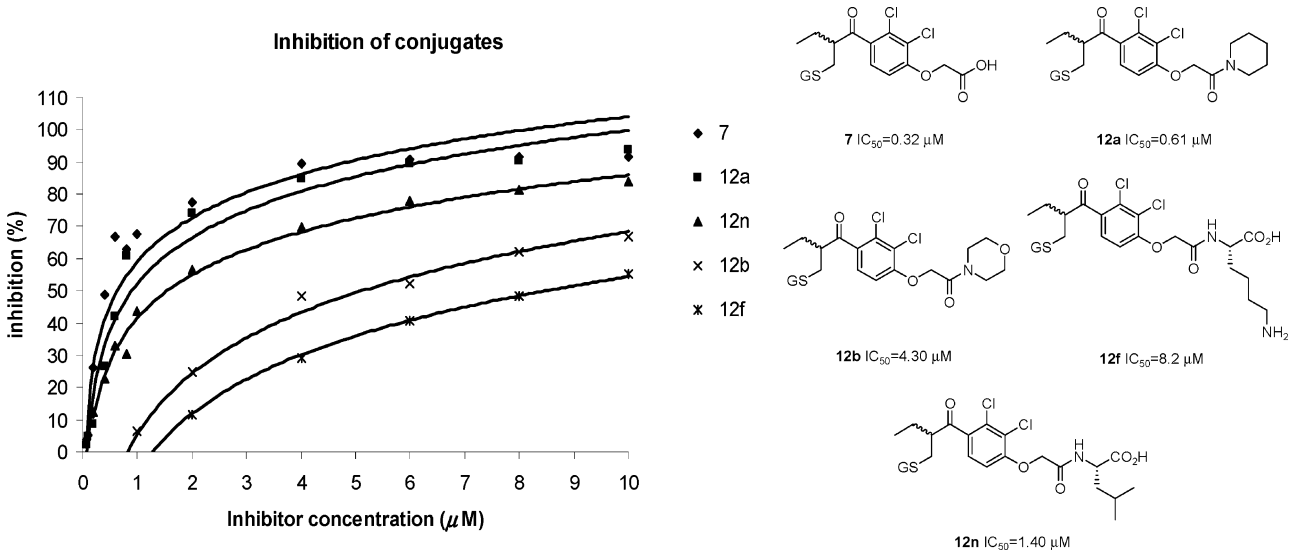


Figure 2. Inhibition profiles and IC_{50} values for amplified adducts **12a** and **12n**, nonamplified adduct **12b**, and depreciated adduct **12f** along with GS−EA, **7**, for comparison purposes.

to the five GSH conjugates we had previously investigated. The inhibition data are shown in Figure 3 and follow a similar trend to that of the conjugates, with **2a** ≈ **11a** > **11n** > **11b** > **11f**. Additionally, the quantitative IC_{50} values are similar for each enone and its corresponding GSH conjugate, a common observation across a number of GST isozymes.[23−25] The absence of any significant augmentation of binding in the case of the GS−EA conjugates relative to the EA analogues suggests that the EA moiety of the conjugate may bind in different orientations and/or positions within the H-site relative to the parent

(24) Ploemen, J. H. T. M.; van Ommen, B.; van Bladeren, P. J. *Biochem. Pharmacol.* **1990**, *40*, 1631−1635.
(25) Awasthi, S.; Srivastava, S. K.; Ahmad, F.; Ahmad, H.; Ansari, G. A. S. *Biochim. Biophys. Acta* **1993**, *1164*, 173−178.

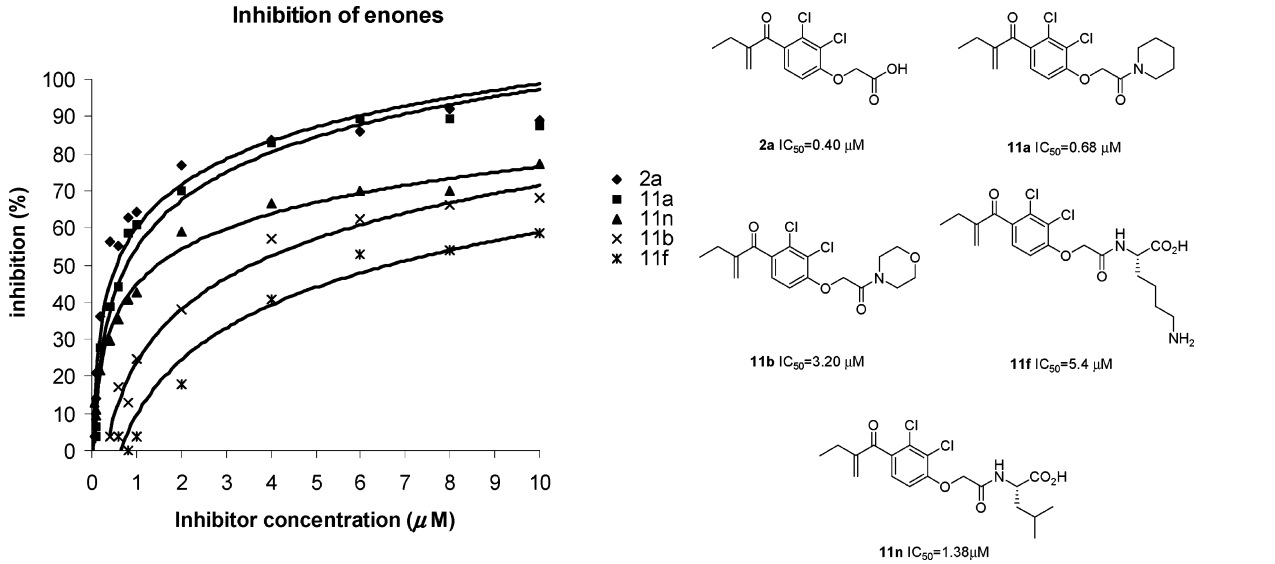


Figure 3. Inhibition profiles and IC_{50} values for enone starting materials **11a**, **b**, **f**, and **n** along with EA, **2a**.

compound. This phenomenon has been observed in the crystal structures of EA and GS−EA bound into the active site of GSTP1-1[23] and is perhaps to be expected, given that the two binding fragments of GSH and EA are linked in the conjugate by a single covalent bond which offers little flexibility for the EA group to explore analogous optimal binding modes to the individual enones.

It is important to note that GST assays use the non-natural CDNB as a substrate and the exact nature of binding of this substrate to SjGST is not known. Moreover, the details of GSH-EA binding to SjGST are also lacking since a high-resolution structure of the SjGST/GS−EA complex has not been determined. A model structure of the SjGST GSH EA Michaelis complex was recently proposed based on structures of SjGST bound to a series of glutathione derivatives (glutathione sulfonate, *S*-hexyl glutathione, and *S*-2-iodobenzyl glutathione).[26] This model identifies a series of residues (Arg103, Tyr111, Ser107, Gln 204) that could interact with the EA carboxylic acid group and proposes that the enzyme must undergo conformational changes to allow conjugate addition of the GSH thiol onto the enone of EA. Interestingly, our identification of the amplified amide compounds **12a** and **12n** from our DCLs indicates that the carboxylic acid group of EA is not essential for binding in the SjGST H site and may be successfully extended without significant loss of inhibitory activity.

Conclusions

We have established the conjugate addition reaction of thiols to enones as a new reaction for enzyme-directed DCC. Using the enzyme SjGST, we could investigate either the GSH- or the H-regions of the enzyme active site by varying DCL stoichiometry between *n* thiols/one enone and one thiol/*n* enones. DCL amplification was successful for both approaches, with the amplified compounds corresponding to the best inhibitors as measured by a competition assay. While the degree of amplification from the EA-based DCL is quantitatively far lower than that observed in the biased GSH-based DCLs, it represents a more significant application of DCC. The power of DCC as a TGS method in medicinal chemistry will likely be realized early in the drug discovery process, where libraries of weak-binding compounds can be rapidly screened to produce lead structures for further development. The ability of SjGST to amplify the low micromolar inhibitors **12a** and **12n** from a nonbiased thiol conjugate DCL, at the expense of compound **12f** which differs in IC_{50} by only a single order of magnitude, illustrates this discovery process in a microcosm and demonstrates the power of TGS in combining the chemical synthesis, discovery, and biological binding assay of enzyme inhibitors into a single operation. Future work will extend our GSH-based DCL methodology to the discovery of novel inhibitors for a range of human GST isoforms.

Experimental Section

General. All reagents were purchased from commercial suppliers and used as received. Dichloromethane (DCM) was distilled from CaH_2. TLC was performed with Merck aluminum plates silica gel 60 F_{254}. Melting points were recorded on a Gallenkamp melting point instrument and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Bruker DPX360 MHz NMR spectrometer unless otherwise specified. Chemical shifts δ are quoted in ppm relative to the $CDCl_3$ signal as reference. Coupling constants are given in Hz. Optical rotations were measured on a POLAAR 20 automatic polarimeter using a 2 mL cell. Mass spectra were obtained from the EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea.

Analytical HPLC was performed on an HP1100 instrument and analytical HPLC-MS on a Waters 2790 HPLC with a micromass Platform II quadrupole mass spectrometer. Enzyme concentrations were determined by Bradford assay on a UNICAM UV−visible spectrometer with bovine serum albumin as a standard.[27] Enzyme activity assays were conducted on a CARY 300 SCAN UV−visible spectrometer.

Synthesis of Library Components. A. General Protocol for Peptide Syntheses: Tripeptides **4**−**6**, **13**−**15** were prepared using standard Fmoc solid-phase synthesis methods. The preparation of H-Val-Cys-Gly-OH (**4**) is illustrative: Fmoc-Gly-Wang (0.6 mmol/g) was swollen in DCM at rt for 10 min. The resin was drained and treated with 20% piperidine in dry DMF. The reaction was agitated with nitrogen at rt and monitored by Kaiser test. After completion, the resin was washed with DMF, DCM, and MeOH and dried in vacuo. The

(26) Cardoso, R. M. F.; Daniels, D. S.; Bruns, C. M.; Tainer, J. A. *Proteins* **2003**, *51*, 137−146.

(27) Bradford, M. *Anal. Biochem*. **1976**, *72*, 248−254.

resulting H-Gly-Wang resin was then swollen in DCM at rt for 10 min and drained. Fmoc-Cys(Trt)-OH (5 equiv), HBTU (4.9 equiv), and HOBt·H_2O (5 equiv) were mixed in dry DMF and added to the resin. The mixture was agitated with nitrogen, while DIPEA (10 equiv) was added. The reaction was monitored by Kaiser test. After completion, the resin was washed with DMF, DCM, and MeOH and dried in vacuo to afford Fmoc-Cys(Trt)-Gly-Wang. Fmoc removal from the cysteine residue was accomplished as described for the deprotection of Gly, followed by coupling to Fmoc-Val under the same conditions as those used for coupling to cysteine with the exception that DIPEA was added to the mixture of peptide, HBTU, and HOBt·H_2O for the activation. Fmoc removal from Fmoc-Val-Cys(Trt)-Gly-Wang was carried out as before, and the resin finally dried in vacuo over KOH. Cleavage and deprotection were accomplished by adding aqueous TFA solution (94.5% TFA, 2.5% H_2O, 2.5% EDT, 1% TIS) to the resin followed by gentle filtration and washing with TFA solution (×2). All of the filtrate was collected and added dropwise to a 10-fold excess of cold ether in a centrifuge tube. After centrifugation, ether was carefully decanted, and the centrifugation was repeated twice. Water was then added to the white residue, and the aqueous solution was transferred to a separatory funnel. The aqueous phase was washed with DCM 3 times and then lyophilized to give the pure tripeptide **4** (11 mg, 35%) as a fluffy white solid: ^{1}H NMR (360 MHz, D_2O) δ = 1.06 (6H, dd, J = 6.5, 6.5 Hz, Val $(CH_3)_2$), 2.27 (1H, m, Val CH), 2.91−3.04 (2H, m, Cys CH_2), 3.91 (1H, d, J = 5.8 Hz, Val CH), 3.97−4.08 (2H, m, Gly CH_2), 4.53 (1H, t, J = 6.5 Hz, Cys CH); ^{13}C NMR (91 MHz, D_2O) δ = 17.8, 18.6, 26.2, 31.1, 42.4, 56.8, 59.4, 170.4, 172.7, 174.3; HRMS (ES+) calcd for $C_{10}H_{20}O_4N_3S$ $[M + H]^+$ 278.1169, found 278.1171.

B. Synthesis of EA derivatives. EA derivatives were synthesized according to the coupling procedure described in ref 15, followed by Boc cleavage with TFA. The preparation of **11g** is illustrative: Ethacrynic acid (151.6 mg, 0.50 mmol), HOBt·H_2O (84.2 mg, 0.55 mmol), EDCI (105.4 mg, 0.55 mmol), H-Ser(*t*Bu∼)-O*t*Bu·HCl (139.6 mg, 0.55 mmol), and DIPEA (428 μl, 2.50 mmol) were dissolved in DCM (10 mL), and the reaction was left to stir at room temperature overnight. The reaction was worked up in the usual fashion, followed by flash column chromatography (SiO_2, DCM/EtOAc 9:1) to yield the *tert*-butyl-protected amide (156 mg, 62%) as a colorless oil; R_f = 0.3 (SiO_2, DCM/EtOAc 9:1); ^{1}H NMR (360 MHz, $CDCl_3$) δ = 1.09−1.16 (12H, m, Ser ^{t}Butyl + CH_3), 1.45 (9H, s, Ser ^{t}Butyl), 2.45 (2H, q, J = 7.4, methylene CH_2), 3.54 (1H, dd, Ser CH_2, J_1 = 8.7 Hz, J_2 = 3.1 Hz), 3.85 (1H, dd, Ser CH_2, J_1 = 8.7 Hz, J_2 = 2.7 Hz), 4.54−4.61 (2H, m, OCH_2), 4.66 (1H, m, Ser CH), 5.57 (1H, s, enone H), 5.93 (1H, s, enone H), 6.85 (1H, d, J = 8.5 Hz, Ph H), 7.17 (1H, d, J = 8.5 Hz, Ph H), 7.58 (1H, d, J = 8.7 Hz, amide H); ^{13}C NMR (91 MHz, $CDCl_3$ + CD_3OD) δ = 13.4, 24.4, 28.3, 29.0, 53.8, 63.0, 69.0, 74.2, 83.1, 111.8, 124.1, 128.2, 129.8, 132.4, 135.0, 151.2, 155.6, 167.6, 169.8, 196.7; HRMS (ES+) calcd for $C_{24}H_{34}O_6NCl_2$ $[M + H]^+$ 502.1758, found 502.1748; $[\alpha]^{20}_D$ = +11° (c = 2.72, chloroform). The amide was then taken up into dry DCM (5−10 mL), and TFA (5 mL) was added. The reaction was allowed to stir under nitrogen at rt for 1 day, after which time it was partitioned between water and DCM and the organic phase was extracted (×3). Drying over $MgSO_4$ and concentration in vacuo yielded the acid **11g** (66 mg, 54%) as a white solid; mp 144−146 °C; ^{1}H NMR (360 MHz, $CDCl_3$ + CD_3OD) δ = 1.03 (3H, t, J = 7.4 Hz, CH_3), 2.34 (2H, q, J = 7.4 Hz, methylene CH_2), 3.78−3.93 (2H, m, Ser CH_2), 4.49−4.57 (3H, m, OCH_2 + Ser CH), 5.50 (1H, s, enone H), 5.87 (1H, s, enone H), 6.83 (1H, d, J = 8.5 Hz, Ph H), 7.07 (1H, d, J = 8.5 Hz, Ph H); ^{13}C NMR (91 MHz, $CDCl_3$ + CD_3OD) δ = 17.2, 28.3, 59.4, 66.9, 72.9, 116.1, 128.1, 132.1, 134.2, 136.2, 138.8, 155.0, 159.7, 172.7, 201.2; HRMS (ES+) calcd for $C_{16}H_{21}O_6N_2Cl_2$ $[M + NH_4]^+$ 407.0771, found 407.0771; $[\alpha]^{21}_D$ = +28.1° (c = 1.32, chloroform/methanol 9:1).

Protein Synthesis. A. Cloning of SjGST as a His-Tagged Fusion. The pET-6His-SjGST plasmid was designed to have SjGST fused to a 6His N-terminal tag which facilitates purification of the SjGST on nickel metal-affinity resin, rather than glutathione-affinity resins which are typically used to purify GST isoforms. This strategy thus provided large amounts of SjGST free from large amounts of glutathione (GSH) which could interfere with dynamic combinatorial library (DCL) synthesis. The *Schistosoma japonica gst* gene was amplified by PCR using pGEX6P-1 (Amersham Biosciences, Genbank Accession Number U78872) plasmid DNA as a template with primers GST-FOR (5′ GGA AAC AAG CT**T CAT GA**C CCC TAT 3′) which incorporated a *Bsp*HI site (in bold) and GST-REV (5′ GAA CTT CGG **GGA TCC** CAT GGG CCC 3′) which incorporated a *Bam*HI site (in bold). The PCR products were cloned into plasmid pUC19, sequenced to confirm the fidelity of the cloned DNA, and then cloned into plasmid pET-6His plasmid DNA digested with appropriate restriction enzymes (*Nco*I and *Bam*HI for the pET plasmid and *Bsp*HI and *Bam*HI for pUC19/SjGST). Clones containing the correct insert were isolated and sequenced to confirm the identity of the pET-6His-SjGST plasmid.

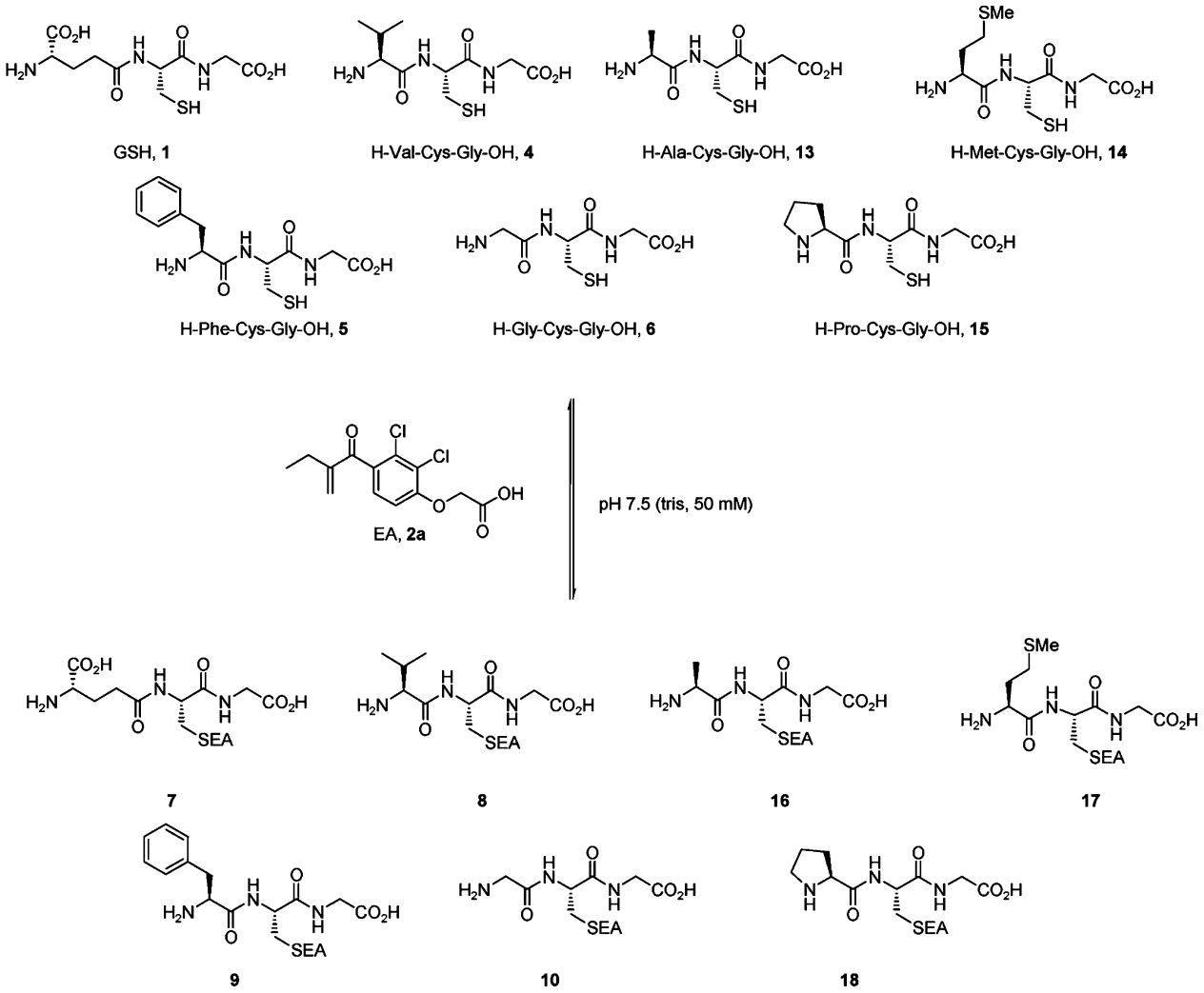
B. Expression and Purification of GST. Overexpression of *Schistosoma japonica* GST was achieved by transforming *E. coli* BL21(DE3) (Novagen) cells with the plasmid pET-6His-SjGST. A single colony was used to inoculate 50 mL of LB broth supplemented with ampicillin (100 μg/mL) and grown overnight at 37 °C with shaking. This overnight seed culture was then used to inoculate 2 L of fresh growth medium and grown at 37 °C to OD_{600} = 0.8 before induction with isopropyl thio-β-D-galactoside (IPTG) (1.0 mM final concentration). After a further 3 h agitation at 37 °C, the cells were harvested by centrifugation (6000 g for 15 min at 4 °C) and frozen at −20 °C for 16 h. The defrosted cells were resuspended in 50 mL of binding buffer (10 mM Tris/HCl, pH 7.5, 10 mM NaCl, containing one tablet of Complete Protease Inhibitor Cocktail (Roche)) and gently agitated for 1 h at 4 °C. The cells were disrupted by sonication (15 pulses of 30 s at 30-s intervals, on ice), and the cell debris was removed by centrifugation at 27 000 g for 20 min at 4 °C after which the cell lysate supernatant was filtered through a 0.45-μm membrane prior to chromatography.

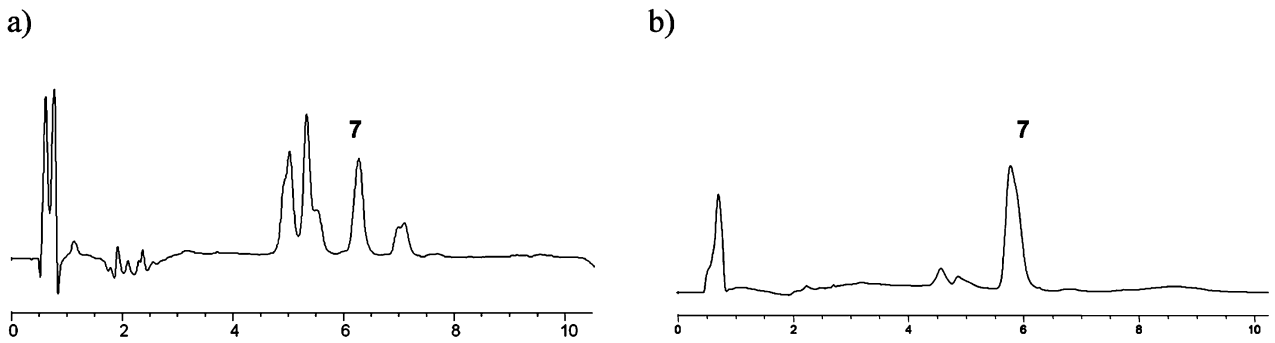
The cell lysate was applied to a HisTrap HP column (5 mL, Amersham Biosciences) pre-equilibrated with binding buffer (50 mM Tris/HCl, 0.5 M NaCl, 5 mM imidazole, pH 7.5). The column was then washed with 20 column volumes of binding buffer (100 mL) before bound material was eluted using a linear gradient of 0−100% elution buffer (50 mM Tris/HCl, 0.5 M NaCl, 0.5 M imidazole, pH 7.5, over 20 column columns, 100 mL; see Figure S5.1 in the Supporting Information). Fractions were analyzed by SDS-PAGE, and those containing GST were pooled and dialyzed overnight against 4 L of 10 mM Tris/HCl (pH 7.5) at 4 °C. SDS-PAGE analysis suggested that the protein was greater than 95% pure (Figure S5.2 in the Supporting Information). Further purification was achieved using MonoQ anion exchange (1 mL, Amersham Biosciences). The protein was loaded onto the column, washed with 50 mM Tris, pH 7.5, and then eluted with a linear 0−1 M NaCl gradient over 20 mL. Fractions were analyzed by SDS-PAGE, and the purest GST fractions were pooled, concentrated by ultrafiltration (10 kDa cutoff), and frozen. A 1 mg aliquot of the isolated 6His-SjGST was analyzed by gel filtration chromatography (Sephacryl HR-75, Amersham Bioscience) to confirm that it was dimeric in solution.

Electrospray mass spectrometry (ESI-MS) analysis of the pure enzyme gave the molecular mass of the 6His-SjGST as 28 068 Da (±5 Da), in good agreement with the predicted value of 28 057 Da for the monomer. The final yield of GST using this method was ∼20 mg per L of bacterial culture, and this protein was used for all subsequent library synthesis and inhibition assays.

Dynamic Combinatorial Chemistry. Representative procedure for GSH analogue DCL: The seven GSH tripeptides **1**, **4**−**6**, **13**−**15** (7 × 8.2 μL, 10 mM aqueous) and ethacrynic acid (0.82 μl, 0.1 M in DMSO) were added to tris buffer (200 μL, 50 mM, pH 7.5). The DCL was allowed to stand at rt with occasional gentle shaking and monitored by HPLC at regular intervals to establish the blank DCL composition

Scheme 4. HPLC Analysis of DCL Made from Seven GSH-Based Tripeptides **1**, **4**−**6**, **13**−**15**[a]





[a] Chromatogram (a) shows the blank DCL, and chromatogram (b) shows the DCL in the presence of SjGST. The GSH−EA adduct **7** is amplified from 35% of total adduct concentration to 88%.

(HPLC conditions: Column: Luna 5 μ C18(2) 30 mm × 4.60 mm, flow rate 1 mL min^{-1}, wavelength 254 nm, temperature 23 °C, gradient H_2O/MeCN (0.01% TFA) from 80% to 70% over 8 min, then to 40% over 1 min eventually to 20% over 2 min).

The DCL was then resynthesized in the presence of SjGST (200 μl, 160 μM in 50 mM tris buffer pH 7.5), and the HPLC trace was compared to that of the blank (Scheme 4). The DCL composition was identical in the case of addition of SjGST from the start and the case of SjGST addition to a pre-equilibrated library.

Representative procedure for EA analogue DCL: Reduced glutathione (20 μL, 10 mM aqueous) and the 14 ethacrynic acid derivatives **11a**−**n** (14 × 2 μL, 0.1 M in DMSO) were added to tris buffer (3.3 mL, 50 mM, pH 8). The DCL was allowed to stand with occasional gentle shakes at rt and monitored by HPLC at regular intervals. LC−MS verified that each of the expected adducts was present in the blank DCL (Figure S6.1). HPLC conditions: Column: Luna 5 μ C18(2) 30 mm × 4.60 mm, flow rate 1 mL min^{-1}, wavelength 254 nm, temperature 23 °C, gradient H_2O/MeCN (0.01% TFA) from 80% to 5% over 10 min.

The DCL was then resynthesized in the presence of SjGST (1.1 mL, 180 μM in 50 mM tris buffer pH 7.5) and added to tris buffer (2.2 mL, 50 mM, pH 8), and the HPLC traces were compared (Figure 1). The

DCL composition was identical in the case of addition of SjGST from the start and the case of SjGST addition to a pre-equilibrated library.

Inhibition Assays. To a 500 μL cuvette were added phosphate buffer (415 μL, 0.1 M pH 6.6), GST (10 μL, 0.148 mg/mL), and inhibitor (12.5 μL in DMSO), and the solution was mixed well. After incubation at ambient temperature for 5 min, CDNB (12.5 μl, 40 mM in EtOH) and GSH (50 μl, 10 mM) were added and quickly mixed well. Absorbance was measured at 340 nm at 20 °C for 5 min. Prior to each experiment, the baseline of the UV spectrometer was corrected by replacing GST solution with phosphate buffer. For each inhibitor, a group of experiments were conducted by varying inhibitor concentrations (0.02−10 μM). After each experiment absorbance against time graphs were plotted such that the initial gradients as velocities were worked out. To calculate the IC_{50} of each inhibitor, the ratio of these velocities and the velocity without any inhibitor were plotted against inhibitor concentrations.

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Supporting Information Available: Characterization data for all compounds and analyses of GST protein purity. This material is available free of charge via the Internet at http://pubs.acs.org.

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