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Combined Mass Spectrometry and Dynamic Chemistry Approach to Identify Metalloenzyme Inhibitors**

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Dynamic combinatorial chemistry (DCC) has the potential to enable the rapid definition of structure–activity relationships (for review see [1,2]). However, cases where this approach has been applied to medicinally important protein targets are limited,[3,4] probably in part due to the lack of methodology for identifying members of dynamic combinatorial libraries (DCLs) that preferentially bind to a templating macromolecule. As noncovalent protein–ligand interactions can be observed by electrospray ionization mass spectrometry (ESI–MS)[5–7] and MS has been used to screen for both reversible and irreversible enzyme inhibitors, direct protein MS analyses may be suited for the screening of DCLs.[8] Recently, Poulsen reported the use of FTICR–MS (Fourier transform ion cyclotron resonance mass spectrometry) to identify carbonic anhydrase binders from a DCL of equilibrating hydrazones/aldehydes.[9] Thiol-disulfide interchange chemistry binding (mutant) cysteinyl residues has been used to investigate active-site topography with the "tethering" approach.[10]

Herein, we report studies aimed at combining a thiol-disulfide based (see for example [11,12]) DCL and ESI–MS for investigation of structure–activity relationships (SAR) in mild conditions for the clinically relevant *Bacillus cereus* metallo-β-lactamase (BcII). By recruitment of one or two zinc ions, BcII catalyzes the hydrolysis of a range of clinically used β-lactam antibiotics.[13] BcII complexed with zinc and thiol inhibitors can be analyzed by ESI–MS.[14] We envisaged that it may be possible to obtain SAR data with a combined MS–DCL method using reversibly bound dithiols, with one thiol acting as a tether for a DCL approach and the other binding to the active site zincs (Figure 1). The latter requirement meant that it was necessary to carry out the analyses without oxidation of both thiols of the support (tethering) ligands to disulfides. Herein, we report that this approach is viable and led to the identification of potent monothiol BcII inhibitors.

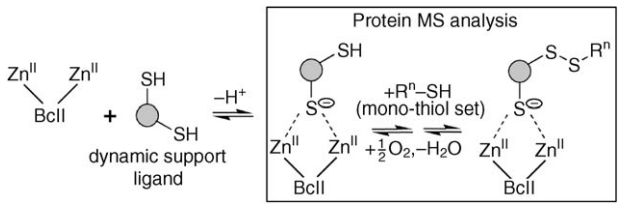

Figure 1. Scheme showing how reversible binding of a dithiol support ligand to the target protein BcII$(Zn^{II})_2$, coupled to dynamic disulfide formation could lead to selected enzyme-bound disulfide complexes. (Not all possible reactions are shown, for example, the support ligand could oligomerize before binding to the protein and disulfide formation could occur before binding to the enzyme).

Initially thiophenols **1**–**4** were analyzed for binding to BcII by ESI–MS (Figure 2a–d). The observation of a peak at 25246 Da provided evidence for the formation of BcII$(Zn^{II})_2$ complexes with isomeric mercaptobenzoic acids **2**–**4**, but not thiophenol **1**.

The importance of the thiol and carboxylate groups in binding was shown by the relative lack of binding observed for mercaptoanilines **5**–**7** (Figure 2e–g) and 2-mercaptopyridines **8** and **9** (Figure 2h and i). Of these compounds, only mercaptoaniline **6** exhibited weak binding affinity, as illustrated by a peak at 25217 Da corresponding to the BcII$(Zn^{II})_2$–**6** complex (calcd 25215 ± 2 Da) (Figure 2f). Mercaptopyridines **8** and **9** probably exist predominantly as thiopyridones diminishing their binding potential for BcII$(Zn^{II})_2$. The phenol analogues of **2**–**4** did not lead to significant BcII$(Zn^{II})_2$–ligand complex formation (data not shown). As the K_i values in solution of **2**, **4**,[15] and **3** for BcII are 346, 29, and 185 μM respectively, the results support the proposal[15] that potency is related to the spatial proximity of the thiol and carboxylate groups.

As the thiols of **2**–**4** likely chelate the BcII zinc ions, dithiol derivatives were then targeted with a view to their use as dynamic support ligands for a DCL enabled by a non zinc-interacting thiol. Dithiols **10a**–**e** (Figure 3), prepared from the corresponding commercially available diols in three steps (overall yields 23–87%), were incubated with BcII$(Zn^{II})_2$ and analyzed by ESI–MS (see Supporting Information).

10a–**e** were mixed with BcII$(Zn^{II})_2$ under anaerobic conditions because of their instability with respect to poly/oligomerization in the presence of oxygen; data acquisition was started approximately 1 min after exposure to atmospheric oxygen. The progressive disappearance of all BcII$(Zn^{II})_2$–ligand complex signals was usually apparent after a few hours, thus time-course analyses were important.

Whereas all the dithiols **10a**–**e** were observed to form BcII$(Zn^{II})_2$–sulfide complexes, one, **10c**, was clearly observed to form a significant BcII$(Zn^{II})_2$–homodimer (**10c**–**10c**) complex.

A set of thiols **1**–**9** and **11**–**20** (Figure 4) were chosen as potential coupling partners for support ligands **10a**–**e**. Exposure of BcII$(Zn^{II})_2$ to an equimolar mixture of **1**–**9** and **11**–**20** in the absence of **10a**–**e** produced a single peak at 25239 ± 1 Da, assigned as a quaternary complex of BcII$(Zn^{II})_2$ with any or all of isomers **2**, **3**, or **4** (Figure 5a and b). Strikingly, when **10c** was added to the set **1**–**9** and **11**–**20**, a significant increase in the

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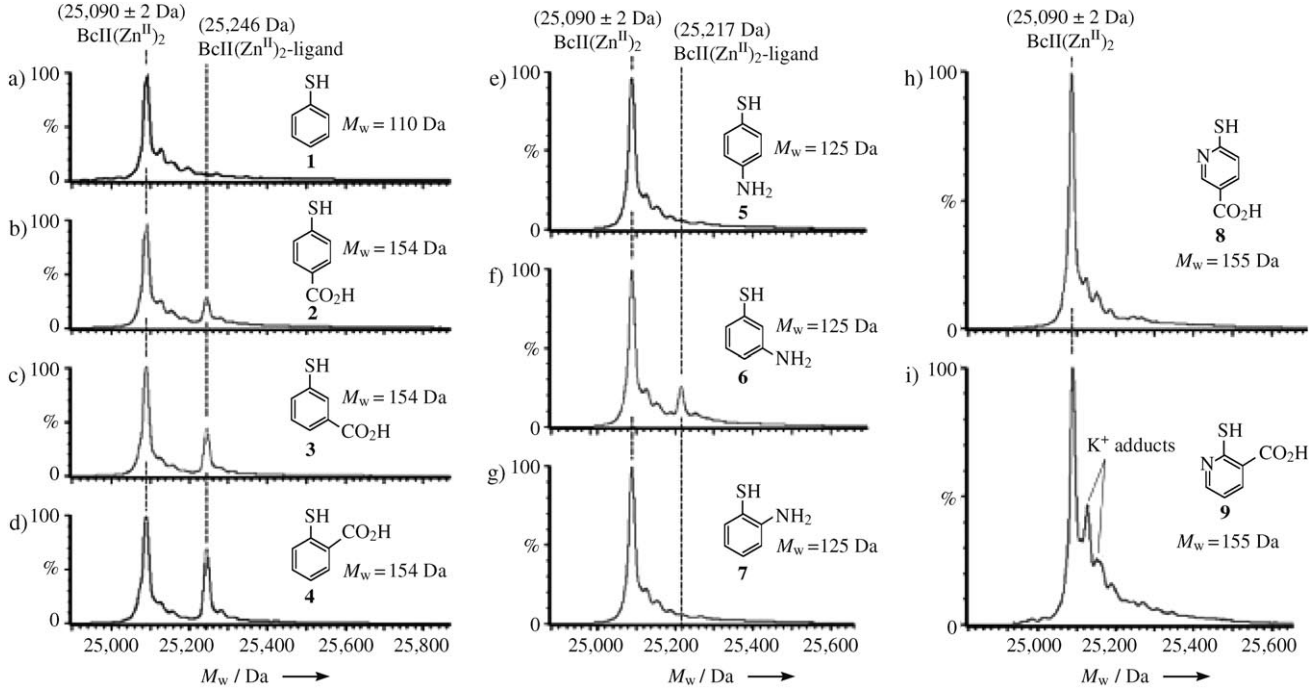


Figure 2. Deconvoluted ESI mass spectra from BcII(Zn^{II})$_2$ in the presence of thiol **1**–**9** (a–i). Analyses were carried out after a 10 min incubation at 23 °C.

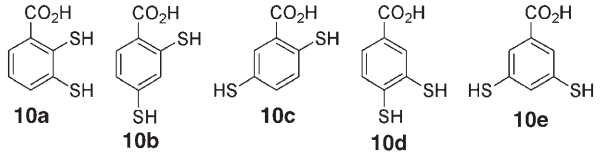


Figure 3. Dynamic support ligands **10a**–**e** (M_w = 186 Da).

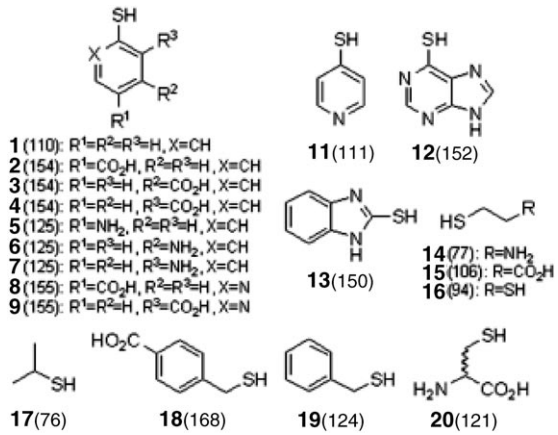


Figure 4. The thiol set with molecular weights (Da) in parentheses.

number of stable BcII(Zn^{II})$_2$–ligand complexes was observed (Figure 5c and d). After 20 h, heterodisulfides preliminarily assigned as resulting from oxidative coupling of **10c** with thiol isomers **2**/**3**/**4** and of **10c** with thiol isomers **5**/**6**/**7** were observed to form BcII(Zn^{II})$_2$–(**10c**–**2**/**3**/**4**) (Figure 5d, obsd 25424 ± 2 Da, calcd 25423 ± 2 Da) and BcII(Zn^{II})$_2$–(**10c**–**5**/**6**/**7**) complexes (Figure 5d, obsd 25391 ± 2 Da, calcd 25394 ± 2 Da). In contrast, dithiol **10b** did not appear to support the apparent formation of any heterodisulfide complexes (Figure 5e and f). Notably, only the heterodisulfide resulting from oxidative coupling of support ligand **10e** and thiol **18** was observed to form a BcII(Zn^{II})$_2$–(**10e**–**18**) complex (Figure 5h, obsd 25435 ± 1 Da, calcd 25437 ± 1 Da). Experiments using *ortho*-dithiols **10a** and **10d** as support ligands led to relatively complex data (see Supporting Information), possibly due to partial in situ formation of apo-BcII and BcII(Zn^{II}), leading to some covalent adducts involving members of the thiol library and the Zn^{II}-binding cysteine (Cys168). The importance of carrying out kinetic analyses was again emphasized by the fact that when BcII(Zn^{II})$_2$ was added to a mixture of a template and the thiol set, which had been allowed to equilibrate for 24 h under the same conditions as those of the time-course analyses, no binding of thiols was observed even after prolonged incubation. This observation is consistent with the reported relatively rapid oligomerization of dithiols, such as **10c**.[16] Using knock-out experiments (removal of specific components from the thiol set **1**–**9** and **11**–**20**, see Supporting Information), it was established that isomeric thiols **5**–**7** likely all contributed to the formation of the complex BcII(Zn^{II})$_2$–(**10c**–**5**/**6**/**7**) (Figure 5d). In contrast, the results implied that only the *para*-isomer of mercaptocarboxylic acid (**2**) formed a significant complex with **10c**, that is, BcII(Zn^{II})$_2$–(**10c**–**2**) (Figure 5d). To test the predicted structures of the complexes between dithiols **10c**, **10e**, and thiols **2** and **18** upon binding to BcII(Zn^{II})$_2$, mixtures of one support dithiol and one thiol were then analyzed. Only heterodisulfides resulting from oxidative coupling of **10c** and **2** (Figure 6a) and **10e** and **18** (Figure 6d) were observed to form BcII(Zn^{II})$_2$–(**10c**–**2**) and BcII(Zn^{II})$_2$–(**10e**–**18**) complexes, respectively. Together

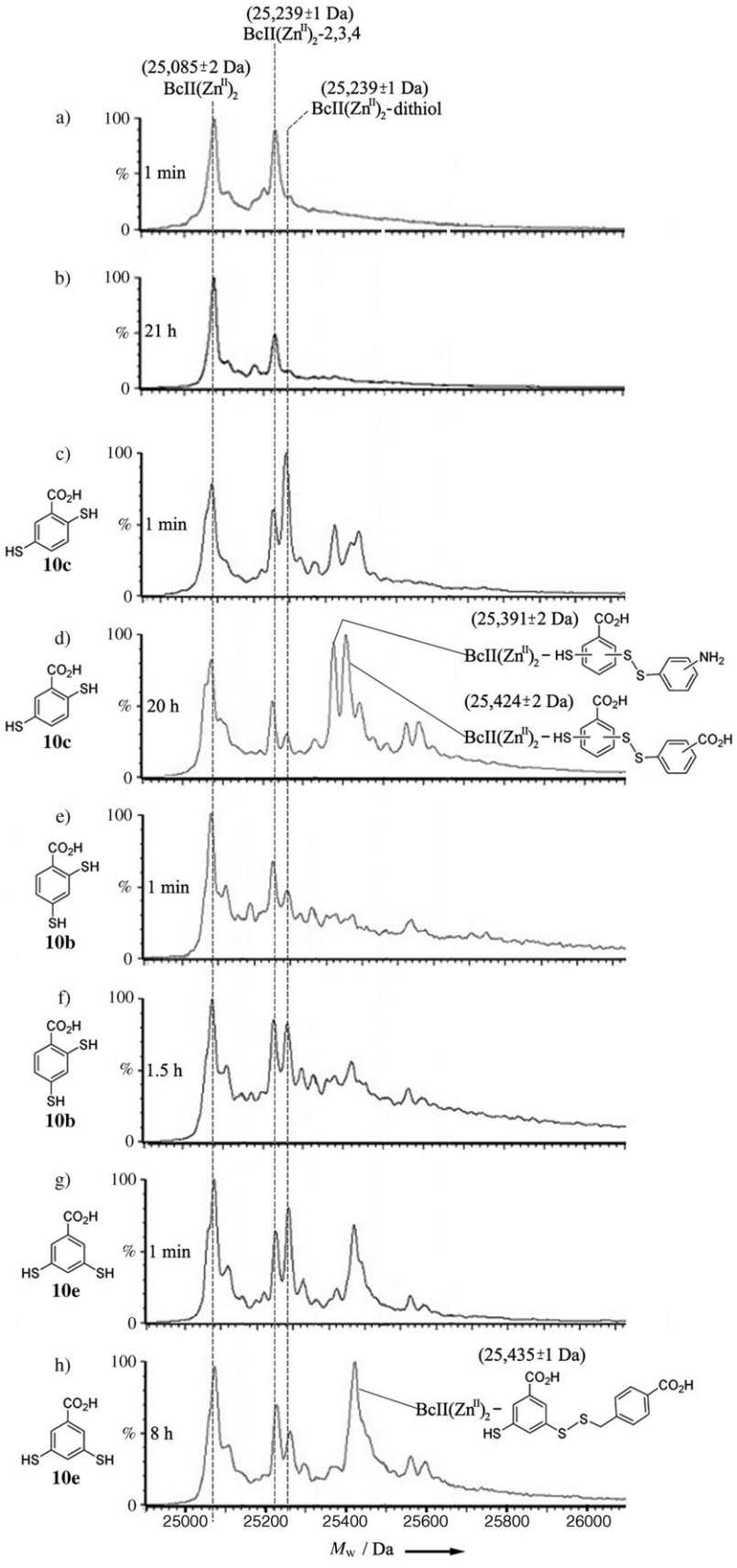


Figure 5. Deconvoluted ESI mass spectra from an equimolar mixture of 19 thiols (19×10 μM) (shown in Figure 3) and a) BcII(Zn^{II})$_2$ (15 μM) after 1 min, b) 21 h of aerial exposure; c) BcII(Zn^{II})$_2$ (15 μM) + **10c** (30 μM) after 1 min, d) 20 h of aerial exposure; e) BcII(Zn^{II})$_2$ (15 μM) + **10b** (30 μM) after 1 min, f) 1.5 h of aerial exposure; g) BcII(Zn^{II})$_2$ (15 μM) + **10e** (30 μM) after 1 min, h) 8 h of aerial exposure.

these observations imply specific disulfide coupling products resulting from the thiol set can be preferentially recognized by BcII-(Zn^{II})$_2$. In particular, the (**10c**–**2**) and (**10e**–**18**) disulfides form complexes with the MBL, whereas disulfides (**10c**–**18**) or (**10e**–**2**) do not.

Thus, two candidates, disulfides (**10c**–**2**) and (**10e**–**18**) were identified as having the potential to improve the potency of lead inhibitor **3**. As the disulfides (for example (**10e**–**18**)) cannot be readily isolated, the syntheses of stable carba-analogues of the disulfide (**10e**–**18**) were investigated (Figure 7). The intrinsic symmetry of the dithiol core (**10e**) made synthesis of the possible derivatives reasonably accessible. As the disulfide bond is long and can adopt conformations that may be difficult to mimic we investigated the effect of different length methylene linkers. Thiols **21a**–**e** were synthesized from diol **22** (Scheme 1 and Supporting Information).

Analysis of monothiols **21a**–**e** for BcII(Zn^{II})$_2$ binding indicated that they possess significant affinity (see Supporting Information). The activities of thiols **21a**–**e** against BcII in solution indicated that the optimum chain length comprises two methylene groups, as demonstrated by a K_i value of 6 μM for compound **21b**, which is approximately 30 times more potent than the lead thiol **3** (Figure 7).

Interestingly, compound **21a** (one CH_2) displayed the poorest inhibition value, with a K_i of 102 μM, approximately 17 times less potent than compound **21b**. This result correlates with the observation that **10e** was shown not to dimerize with any thiophenols (**1**–**7**), but preferentially dimerized with benzylic thiol **18** to form a stable (**10e**–**18**) complex with BcII(Zn^{II})$_2$ (Figure 4 h). The sensitivity of inhibitory potency to chain length

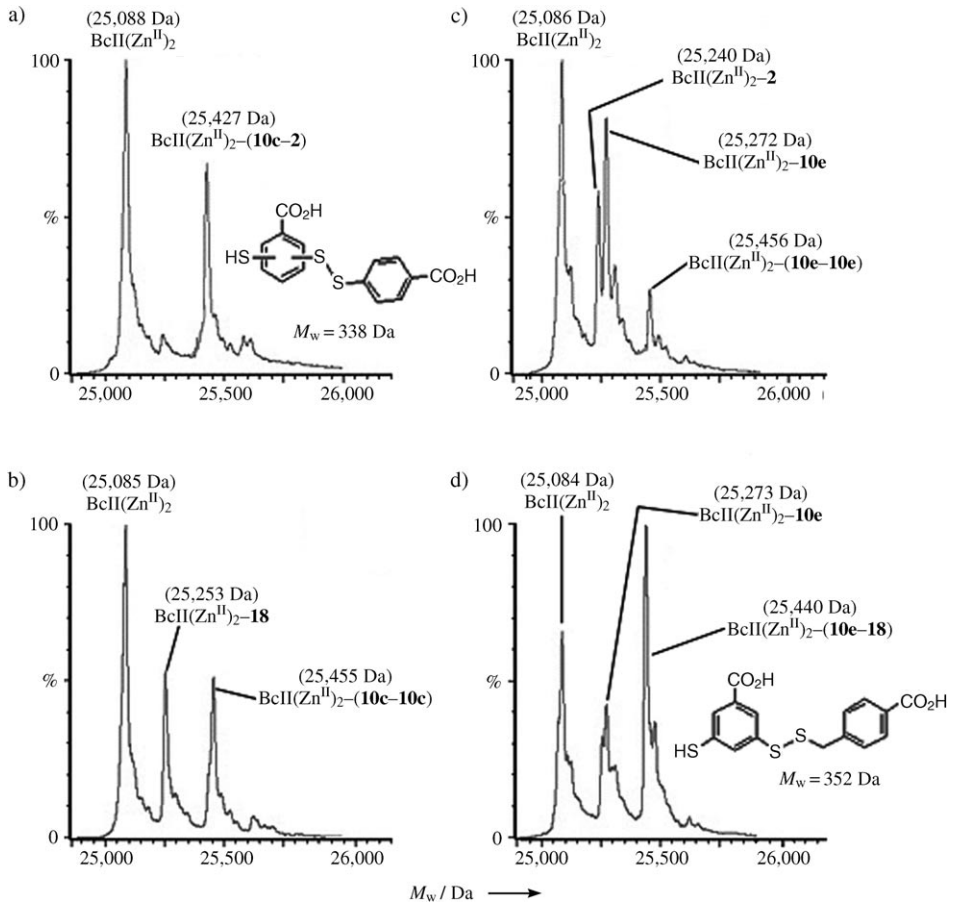


Figure 6. Deconvoluted ESI mass spectra from an equimolar mixture (15 μM) of a) **10c** and **2**, b) **10c** and **18**, c) **10e** and **2** and d) **10e** and **18** incubated with BcII(Zn^{II})$_2$ (15 μM) after 8 h of aerial exposure.

may be related to the conformation of a flexible loop (see Supporting Information) involved in BcII substrate recognition and catalysis.[17]

Overall, the results reveal that dynamic chemistry coupled to ESI–MS protein analyses can be productively combined to identify oligomers under sensitive enzyme-suitable conditions that act as a useful template for inhibitor discovery. It should be emphasized that the useful data were not acquired under equilibrium conditions (that is, after prolonged incubation) but were obtained from time-course analyses. There are limitations to the methodology, including the lack of suitability of all proteins for analysis by MS under mild conditions and technical difficulties in the case of thiol/disulfide chemistry. However, given that the latter may be avoided by other types of reversible reactions and that BcII is a relatively challenging case in that it binds two zinc ions and that (different)

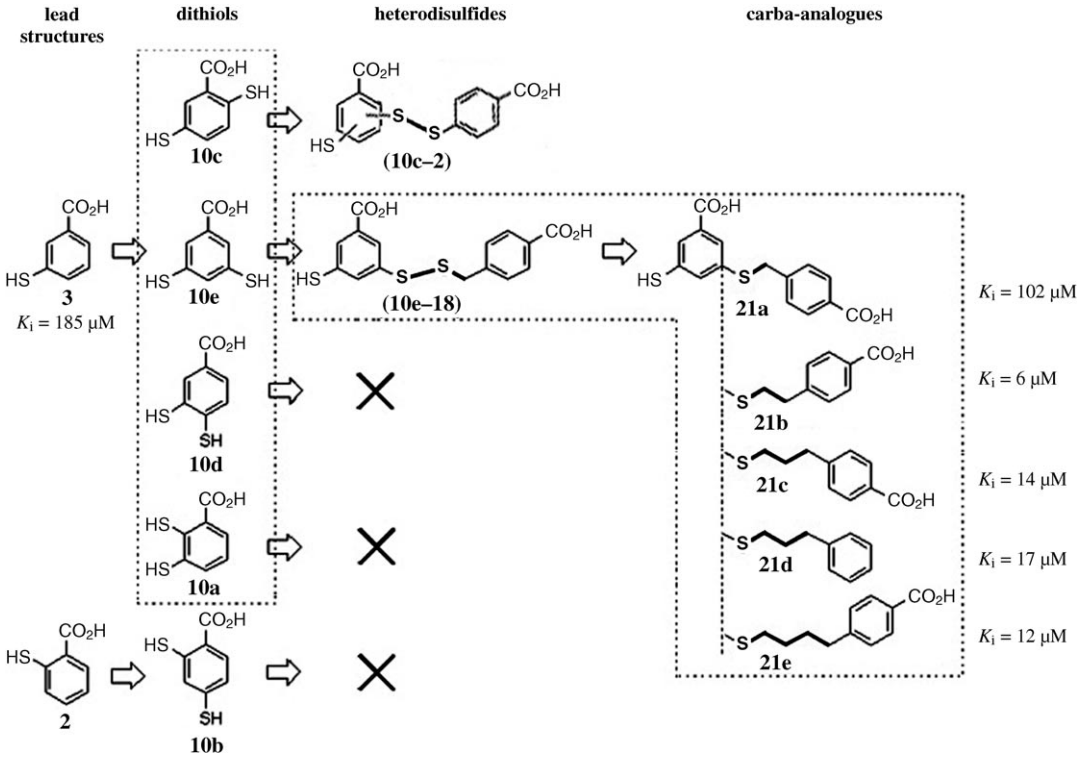
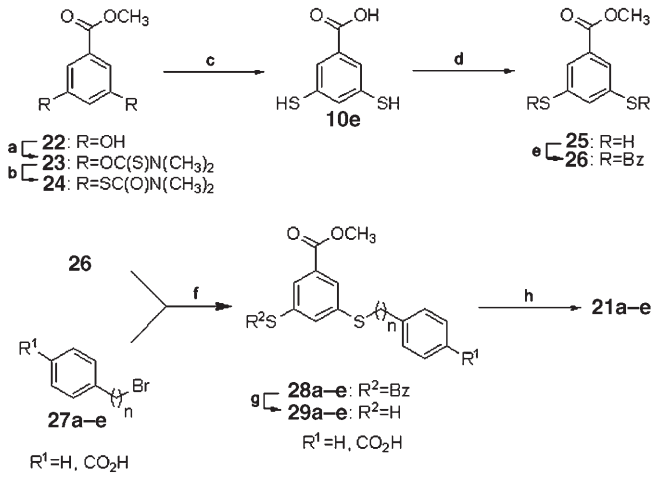


Figure 7. Carba-analogues of (**10e**–**18**), their K_i values versus BcII(Zn^{II})$_2$, and relationship with heterodisulfides observed to bind to BcII.



Scheme 1. Synthesis of compounds **21a–e**. a) DABCO, $(CH_3)_2NC(S)Cl$, DMF, RT, 1 h, 93%; b) Ph_2O, 230 °C, 3 h, 97%; c) NaOH (1 N), 70 °C, 8 h, 96%; d) MeOH, H_2SO_4, reflux, 12 h, 98%; e) BzCl, Et_3N, THF, RT, 14 h, 91%; f) **26**, MeOH, MeONa, 0 °C, 1 h then **27a–e**, K_2CO_3, CH_2Cl_2, reflux, 31–61%; g) MeOH, MeONa, 0 °C, 1 h, 68–95%; h) NaOH (1 N), 70 °C, 83–99%.

thiols were used both for anchorage and dynamic modification, the methodology may be applicable to other metalloenzymes, and by the use of different dynamic support ligands to other macromolecular targets.

Experimental Section

Prior to each run, under hypoxic atmosphere (< 1 ppm O_2), individual thiols were freshly dissolved in DMSO at a final concentration of 100 mM. Each monothiol was then diluted to 75 μM into the same mixture in ammonium acetate buffer (15 mM, pH 7.5). The pH of the resulting mixture was then adjusted to the required value with a 2.8% aqueous solution of NH_4OH. Each dithiol solution was diluted to a concentration of 100 μM in 15 mM ammonium acetate buffer at pH 7.5. The experimental samples were prepared by mixing the appropriate volumes of the monothiols, dithiol, and the enzyme stock solution in 15 mM ammonium acetate at pH 7.5. An aliquot of this mixture was placed in a 96-well plate sealed with adhesive aluminium foil and was subsequently taken out of the oxygen-free environment to be analyzed. ESI–MS analyses used a Q-TOF mass spectrometer (Q-TOFmicro Micromass, Altrincham, UK) interfaced with a NanoMate™ chip-based nano-ESI source (Advion Biosciences, Ithaca, NY, USA). Time-courses were started when the nanomate tip pierced the aluminium seal covering the 96-well plate and introduced O_2 into the system. Samples were then infused into the Q-TOF through the ESI chip (estimated flow rate approximately 100 nL min^{-1}). Typically a spraying voltage of 1.70 kV ± 0.1 kV depending on the "sprayability" of the sample and a sample pressure of 0.25 psi was applied. The instrument was equipped with a standard Z-spray source block. Clusters of $Cs_{(n+1)}I_n$ (1 mg mL^{-1} CsI in 100% methanol) were used for calibration. Calibration and sample acquisitions were performed in the positive ion mode in the range of *m/z* 500–5000. Operating conditions for the mass spectrometer were: sample cone voltage (varied) between 20 and 200 V (only the data acquired at sample cone voltage 50 V are shown in the figures), source temperature 20 °C. Acquisition and scan time were 30 s and 1 s, respectively. The pressure at the interface between the atmospheric source and the high vacuum region was fixed at 6.6 mbar (measured with the roughing pump Pirani gauge) by throttling the pumping line using an Edwards Speedivalve to provide collisional cooling.

Keywords: disulfide exchange · dynamic chemistry · mass spectrometry · metallo-β-lactamase · structure–activity relationships

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