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Inhibitors of the FEZ-1 metallo-β-lactamase

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Abstract—Metallo-β-lactamases (MBLs) catalyze the hydrolysis of β-lactams including penicillins, cephalosporins and carbapenems. Starting from benzohydroxamic acid (**1**) structure-activity studies led to the identification of selective inhibitors of the FEZ-1 MBL, e.g., 2,5-substituted benzophenone hydroxamic acid **17** has a K_i of 6.1 ± 0.7 μM against the FEZ-1 MBL but does not significantly inhibit the IMP-1, BcII, CphA or L1 MBLs.

β-Lactamases can be classified into those employing a nucleophilic serine (classes A, C and D) and those binding one or two zinc ions (the metallo-β-lactamases, MBLs, class B) at their active site. To date, medical attention has focused on the serine active BLs and inhibitors of these enzymes are widely used. However, MBLs hydrolyse most β-lactam antibiotics and represent a growing clinical threat (for review, see Refs. 1,2). MBL sub-classes B1 and B3 display a broad substrate selectivity, whereas sub-class B2 are reported to efficiently hydrolyse only carbapenems.[3,4] Sub-class B1 MBLs, including BcII (*Bacillus cereus*) and IMP-1 (*Pseudomonas aeruginosa*), accept β-lactam substrates with one or two active site Zn(II) ions, whereas B3 MBLs, e.g., FEZ-1 (*Legionella gormanii*) and GOB-1 (*Chryseobacterium meningosepticum*), are only active with two Zn(II) ions. The B2 sub-class MBL CphA (*Aeromonas hydrophilia*) is only active as a monozinc protein and is non-competitively inhibited by the presence of a second Zn(II).[5] MBL inhibitors can be classified into those containing thiols (e.g., thiomandelic acid,[6] D-captopril,[7] 6-(mercaptomethyl)penicillinates[8] and homocysteinyl peptides[9]) and non-thiol-containing compounds (e.g., trifluoromethylketones,[10] hydroxamic acids,[11] biphenyl tetrazoles,[12] *N*-arylsulfonyl hydrazones[13] and 2,3-disubstituted succinic acids[14]). Notably, only thiomandelate has been reported to inhibit (non-selectively) FEZ-1 with a K_i value of 0.27 μM.[6] Despite similarities in its overall fold, crystallographic analyses indicate that the active site of the FEZ-1 MBL is significantly different to that of other sub-class B3 MBLs,[15] possibly reflecting difficulties in obtaining inhibitors for it. To demonstrate that FEZ-1 inhibitors can be prepared, and with the aim of understanding differences between FEZ-1 and other MBLs, efforts were made to identify non-thiol FEZ-1 inhibitors. We report the identification and synthesis of benzophenone hydroxamic acids as FEZ-1 selective inhibitors.

Initial studies, based on the screening of a set of small molecules bearing potential metal binding chelation functions, led to the observation that benzohydroxamic acid (**1**, Fig. 1) inhibited FEZ-1 (55% of inhibition at 100 μM under standard assay conditions), but did not inhibit IMP-1, BcII and CphA. Furthermore, **1** did not inhibit the L1 MBL, which, like FEZ-1, is from sub-class B3. The synthesis of derivatives of **1** was then carried out to identify more potent FEZ-1 inhibitors. Hydroxybenzohydroxamic acids (**2a**–**c**) were prepared from the corresponding hydroxybenzoic acids in five steps (in overall yields of 17–52%; see supporting information) and screened for inhibition against IMP-1, BcII, CphA, L1 and FEZ-1. Compounds **2a**–**c** inhibited FEZ-1 with ca. 45%, 64% and 53% inhibition observed in the standard assay. Hydroxamic acids **3** and **4** did not significantly (<10%) inhibit FEZ-1 nor the other tested MBLs, arguing against the non-specific inhibition of FEZ-1 by

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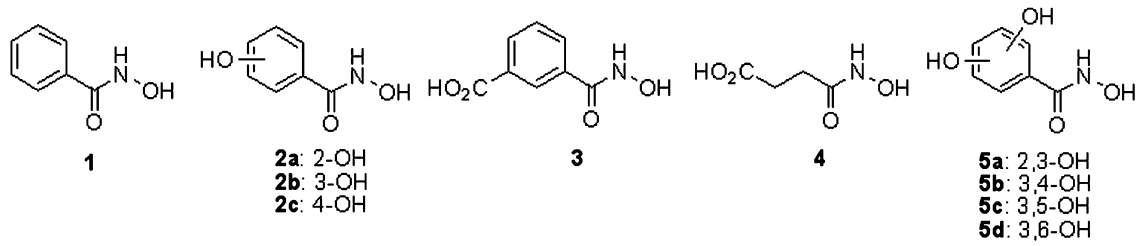


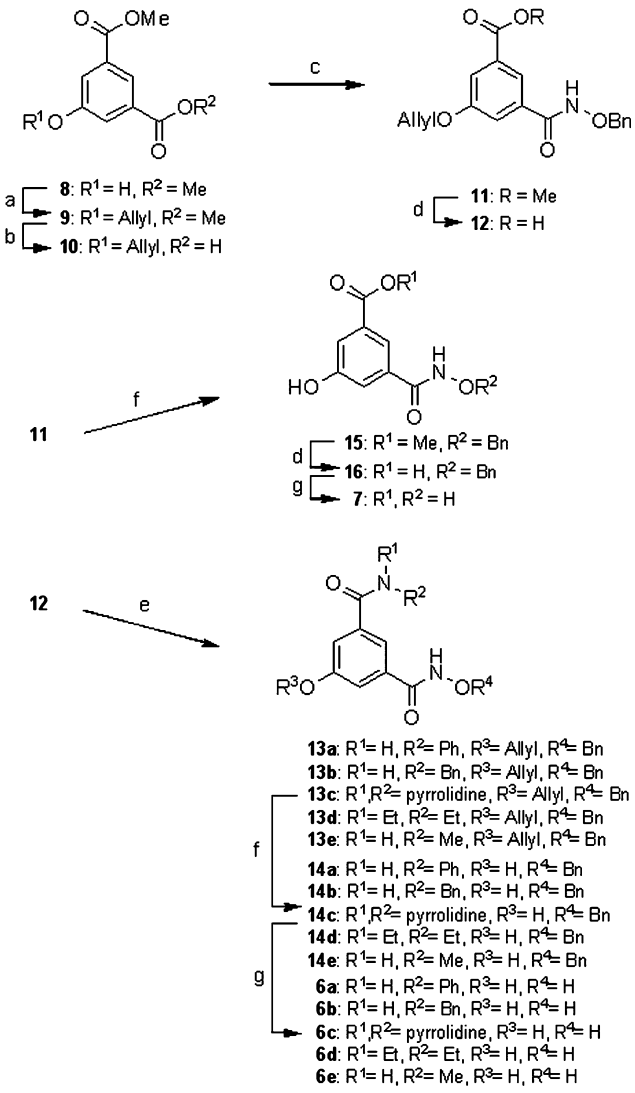
Figure 1. Derivatives of benzohydroxamic acid **1** synthesized as potential MBL inhibitors.

Zn(II) complexation in solution. Solution kinetics with FEZ-1 revealed competitive inhibition for the most potent inhibitor **2b** with a K_i of 17.9 ± 0.7 μM.

Since a *meta*-hydroxyl substituent on the phenyl ring appeared to somewhat enhance the inhibition against FEZ-1 (from 55% inhibition for **1** to 64% for **2b**) whilst retaining selectivity, dihydroxy-derivatives of **2b** were targeted. Compounds **5a**–**d** (Fig. 1) were synthesized via a similar route used for **2a**–**c** (see supporting information). Despite retaining FEZ-1 selectivity, the presence of an additional hydroxyl group at the C-2 (**5a**), C-4 (**5b**) or C-6 (**5d**) positions on the aromatic ring resulted in an apparent reduction in potency compared to **2b**. However, 3,5-dihydroxylated compound **5c** (K_i = 13.3 ± 1.0 μM) had about the same potency as **2b** (17.9 ± 0.7 μM) and was FEZ-1-selective. The apparent slight increase in the potency on derivatization at C-5 prompted the investigation of further 3-hydroxy-5-substituted benzohydroxamic acids.

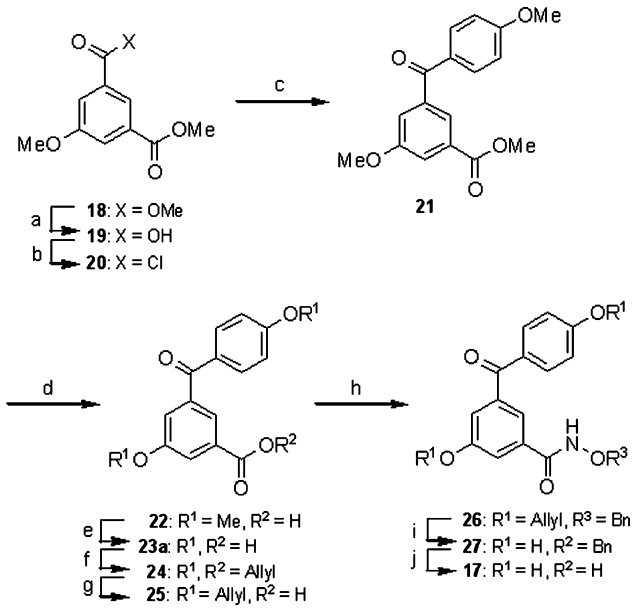
5-Amidobenzohydroxamic acids **6a**–**e** and 5-carboxybenzohydroxamic acid **7** were prepared from dimethyl ester **8**, in seven steps and in overall yields of 26–57% for **6a**–**e** and in six steps and 41% yield for **7** (Scheme 1). Thus, methyl isophthalate **8** was alkylated with allyl bromide to give ether **9**. Mono-saponification of the methyl isophthalate ester **9** to generate **10** was followed by EDCI/HOBt-mediated coupling of *O*-benzylhydroxylamine HCl to afford **11**. Hydrolysis of the methyl ester of **11** afforded carboxylic acid **12** which was then coupled with aromatic, aliphatic and cyclic amines to afford benzamides **13a**–**e**. Deprotection of allyl ethers **13a**–**e** catalyzed by tetrakis(triphenylphosphine)palladium(0) gave phenols **14a**–**e**, which were converted by palladium-catalyzed-hydrogenolysis to the target hydroxamic acids **6a**–**e**. Starting from protected benzohydroxamic acid **11**, palladium-mediated allyl deprotection afforded phenol **15**, which was followed by methyl ester hydrolysis to give **16**, and palladium-catalyzed hydrogenolysis afforded **7**.

Benzophenone hydroxamic acid **17** was prepared from dimethyl ester **18**, in 10 steps and overall yield of 8% (Scheme 2). Dimethyl ester **18** was mono-saponified using KOH in DMSO to give monomethyl ester **19**. Formation of acid chloride **20** ($SOCl_2$), followed by Friedel–Crafts acylation, afforded benzophenone **21** (*para*/*ortho* regioselectivity, 96:4). Methyl ester **21** (as an *para*/*ortho* mixture) was hydrolysed to give **22** and then demethylated using an ionic liquid solution (trimethylamine HCl and aluminium chloride[16]) to give hydroxybenzophenone **23a**.



Scheme 1. Reagents and conditions: (a) Allyl bromide, Cs_2CO_3, DMF, 1 h, 98%; (b) NaOH, MeOH/THF, 1 h, 69%; (c) $H_2NOBn{\cdot}HCl$, HOBt, EDCI, Et_3N, CH_2Cl_2, 12 h, 87%; (d) NaOH, THF, 3 h, 87%; (e) HNR_1R_2, HOBt, EDCI, Et_3N, THF, 6 h, 68–98%; (f) $Pd(PPh_3)_4$, Pyrrolidine, CH_2Cl_2, 20 min, 61–90%; (g) 10% Pd-C, H_2, MeOH, 1–16 h, 76–99%.

At this stage, the *ortho* isomer **23b** was isolated. Protection of **23a** using allyl bromide and cesium carbonate in



Scheme 2. Reagents and conditions: (a) KOH, DMSO, 130 °C, 3 h, 49%; (b) $SOCl_2$, reflux, 1 h, >95%; (c) anisole, $AlCl_3$, CH_2Cl_2, 3 h, 72% for *para* isomer, [96:4] *para*/*ortho*; (d) NaOH, MeOH, 60 °C, 1 h, 99%; (e) TMAH-Al_2Cl_7, CH_2Cl_2, reflux, 17 h, 95%; (f) Allyl bromide, Cs_2CO_3, DMF, rt, 1 h, 96%; (g) NaOH, MeOH, 60 °C, 1 h, 95%; (h) HOBt, EDCI, Et_3N, H_2NOBn, CH_2Cl_2, rt, 16 h, 67%; (i) $Pd(PPh_3)_4$, Pyrrolidine, CH_2Cl_2, rt, 1 h, 38%; (j) 10% Pd-C, MeOH, H_2, rt, 1 h, >95%.

DMF gave triallyl-protected benzophenone **24**, and saponification of the allyl ester afforded carboxylic acid **25**. Generation of benzyl-protected hydroxamic acid **26** was accomplished using the EDCI/HOBt-mediated coupling of *O*-benzylhydroxylamine HCl in CH_2Cl_2. Deprotection, first with tetrakis(triphenylphosphine)palladium(0) (to give **27**) and then 10% palladium(0) on charcoal and H_2 gave **17**.

Replacement of the 5-hydroxyl group of **5c** with a carboxylate (**7**) almost removed activity versus FEZ-1 (Table 1). However, amide derivatives of **7** (**6a**–**e**) retained some potency versus FEZ-1 with 58% (**6a**), 54% (**6b**), 36% (**6c**), 34% (**6d**) and 53% (**6e**) inhibition being observed in the standard assay. These observations prompted investigation into alternative hydrophobic more rigid aromatic derivatives such as in benzophenones **17** and **23a**.

Strikingly, when the 5-hydroxyl group of compound **5c** was replaced by a benzoyl moiety to generate benzophenone hydroxamate **17**, FEZ-1 was inhibited by 85% compared with the absence of inhibitor. Moreover, **17** remained selective for FEZ-1, with no significant inhibition detected against IMP-1, BcII, CphA or even L1. The K_i value for **17** of 6.1 ± 0.7 μM represents a three fold increase in potency compared to **2b** without reduction of selectivity. A significant drop of inhibitory activity was observed for carboxylate **23a** with ca. 33% of inhibition compared to hydroxamate **17** (85%). This result indicates that the hydroxamic acid function is important for efficient FEZ-1 inhibition (probably via interactions with the Zn(II) ion(s)) and that the benzophenone core may interact favourably with FEZ-1.

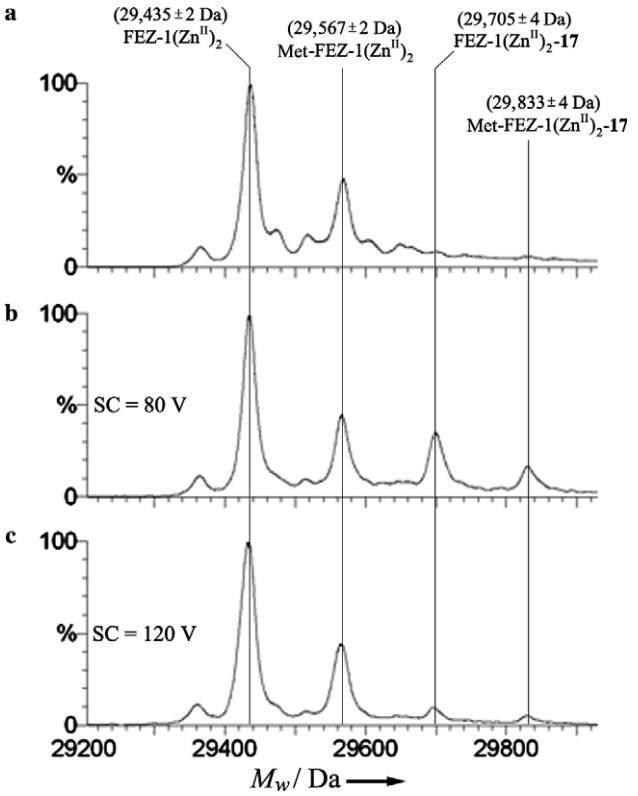


Figure 2. Deconvoluted ESI-MS spectra of (a) the FEZ-1 MBL and a 1/1 mixture of FEZ-1 and compound **17** at a sample cone voltage varied from (b) 80 V to (c) 120 V.

Table 1. Residual activity of various members of the MBL family after treatment with compounds **7**, **6a**–**e**, **23a** and **17** (100 μM)

Inhibitor	R^1	R^2	% Activity of IMP-1	% Activity of BcII	% Activity of CphA	% Activity of L1	% Activity of FEZ-1
None	—	—	100 ± 4	100 ± 2	100 ± 1	100 ± 8	100 ± 6
7	—	—	67 ± 9	93 ± 9	79 ± 9	97 ± 19	92 ± 12
6a	H	Ph	92 ± 3	82 ± 5	90 ± 18	103 ± 2	42 ± 2
6b	H	CH_2Ph	81 ± 8	103 ± 10	81 ± 5	95 ± 3	46 ± 3
6c	Pyrrolidine		85 ± 4	100 ± 5	86 ± 1	92 ± 3	64 ± 6
6d	Et	Et	85 ± 3	101 ± 10	90 ± 1	93 ± 1	66 ± 7
6e	H	CH_3	93 ± 6	103 ± 10	92 ± 5	96 ± 4	47 ± 1
23a	—	—	100 ± 7	86 ± 4	83 ± 13	107 ± 1	67 ± 7
17	—	—	83 ± 13	91 ± 11	84 ± 15	102 ± 14	15 ± 4

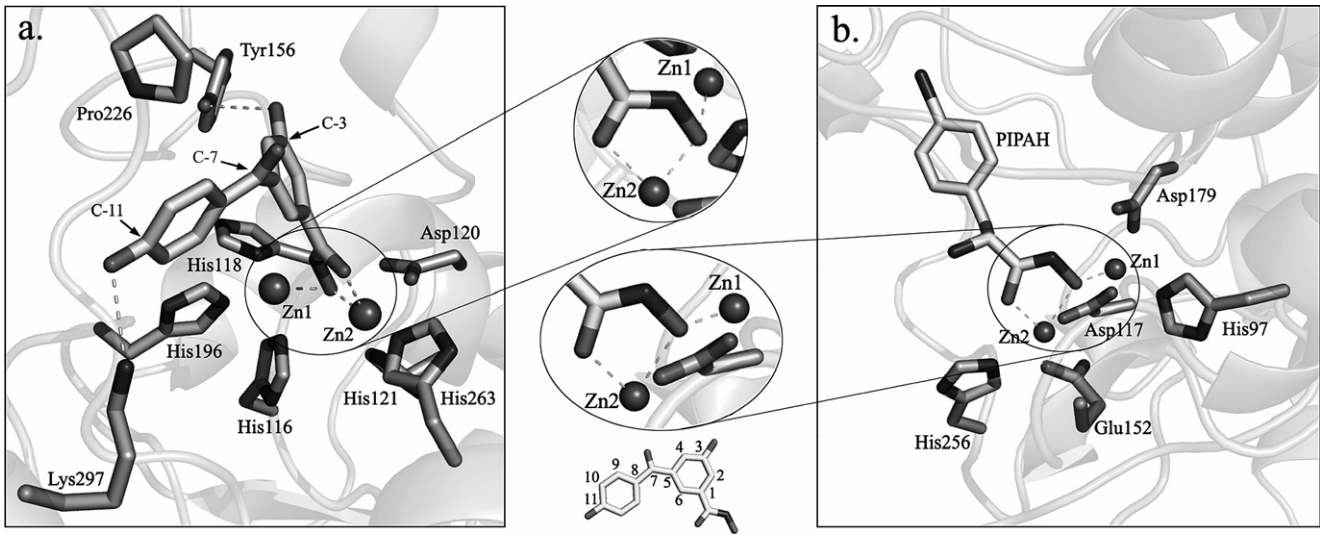


Figure 3. (a) Highest GOLD score docking conformation representing the possible binding mode of **17** to FEZ-1 (PDB code 1K07);[15] (b) view from the crystal structure of aminopeptidase(Zn^{II})$_2$-*para*-iodo-D-phenylalanine hydroxamate (PDB code 1IGB).[19] The similarity of the hydroxamate-Zn(II) interactions between the docked FEZ-1(Zn^{II})$_2$-**17** and the crystal structure of aminopeptidase(Zn^{II})$_2$-PIPAH complexes is highlighted. Factors that may contribute to the proposed binding mode of FEZ-1(Zn^{II})$_2$-**17** include electrostatic interactions between the hydroxamic acid of **17** and the Zn(II) ions, hydrogen bonding between Tyr156 and C-3 hydroxyl groups of **17**, hydrogen bonding between the Lys297 side chain and the C-11 hydroxyl group of **17** and hydrophobic interactions between Pro226 and **17** (see supporting information for additional views).

To investigate the binding of compound **17** to FEZ-1, soft ionization electrospray mass spectrometry (ESI-MS) experiments were carried out (Fig. 2a). Peaks at ca. 29,435 Da (A) and ca. 29,567 Da (B) were observed; they correspond to FEZ-1(Zn^{II})$_2$ and FEZ-1(Zn^{II})$_2$ + methionine (met-FEZ-1(Zn^{II})$_2$) complexes, respectively. The assignment of the N-terminal ± Met residue was confirmed by tryptic digests followed by MALDI-MS analyses.

Enzyme–inhibitor complexes were not observed for the less potent inhibitors **1**, **2b** and **5c**, but one molecule of **17** was observed to bind to FEZ-1 (Fig. 2b) corresponding to a mass shift of ca. 270 Da relative to the unliganded enzyme; in agreement with the MW of **17** (273 Da). The complex of **17** with FEZ-1 was not detected upon increasing the sample cone from 80 V (Fig. 2b) to 120 V (Fig. 2c), indicative of non-covalent binding.

Although there are caveats with respect to conformational changes and metal ligation, docking analyses of **17** using GOLD,[17] which has been previously used with MBLs,[18] gave 10 conformations with seven displaying identical binding modes to FEZ-1 (Fig. 3a). The predicted interactions between the carbonyl and hydroxyl oxygen atoms of the hydroxamate of **17** with the FEZ-1 Zn(II) ions are similar to the crystallographically observed interactions in the complex of a dizinc-dependent aminopeptidase (Fig. 3b) and *para*-iodo-*D*-phenylalanine hydroxamate.[19] The benzophenone core of **17** is predicted to adopt a twisted shape around Pro226, apparently maximizing the interaction with the benzophenone carbonyl and the aromatic C-4 of **17**. This interaction may explain the lack of FEZ-1 activity for **3**, **4** and **7** since the presence of a negatively charged carboxylate function at C-5 may be incompatible with Pro226. In contrast, compounds with more hydrophobic functionalities at C-5 (e.g., **6a**–**e** and **23a**) may be more suited for interaction with Pro226 leading to an increase in potency.

Comparison of the predicted binding mode for **17** with FEZ-1 MBL, with crystal structures of wildtype BcII and CphA (see supporting information), suggests that the much narrower active sites of BcII and CphA may hinder binding of the *para*-hydroxybenzoyl substituent of compound **17** possibly rationalizing the reason for the lack of inhibition of **17** against IMP-1, BcII, CphA and L1.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.11.053.

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