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Assay Platform for Clinically Relevant Metallo-β-lactamases

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ABSTRACT: Metallo-β-lactamases (MBLs) are a growing threat to the use of almost all clinically used β-lactam antibiotics. The identification of broad-spectrum MBL inhibitors is hampered by the lack of a suitable screening platform, consisting of appropriate substrates and a set of clinically relevant MBLs. We report procedures for the preparation of a set of clinically relevant metallo-β-lactamases (i.e., NDM-1 (New Delhi MBL), IMP-1 (Imipenemase), SPM-1 (São Paulo MBL), and VIM-2 (Verona integron-encoded MBL)) and the identification of suitable fluorogenic substrates (umbelliferone-derived cephalosporins). The fluorogenic substrates were compared to chromogenic substrates (CENTA, nitrocefin, and imipenem), showing improved sensitivity and kinetic parameters. The efficiency of the fluorogenic substrates was exemplified by inhibitor screening, identifying 4-chloroisoquinolinols as potential pan MBL inhibitors.

INTRODUCTION

Selection pressure caused by frequent use of β-lactam antibiotics, including penicillins, cephalosporins, carbapenems, and monobactams, has resulted in the emergence and dissemination of highly efficient resistance mechanisms in clinically relevant bacterial pathogens. This rapid adaptation of bacterial strains to commonly used antibiotics is an increasing global threat to public health care. An important mechanism of resistance to β-lactam antibiotics, including clinically challenging Gram-negative organisms, involves β-lactamase catalysis. β-Lactamases can be classified into those employing a nucleophilic serinyl residue at their active site (serine-β-lactamases, SBLs, classes A, C, and D) and those employing one or two zinc ions to promote hydrolysis (metallo-β-lactamases, MBLs, class B). The combination of a penicillin antibiotic and an SBL inhibitor has substantially extended the utility of the former, as, for example, in the case of amoxicillin and clavulanic acid (Augmentin). In addition to clavulanic acid, two other class A (penicillinas) β-lactamase inhibitors are used, tazobactam and sulbactam; all three inhibitors are themselves β-lactams, though in contrast to penicillins they react with SBLs to form relatively stable acyl-enzyme complexes. These SBL inhibitors, however, appear to have no effect on MBLs. β-Lactam antibiotics have been developed that possess resistance to SBLs, or as in the case of carbapenems, they have been developed to be inhibitors or relatively poor substrates. However, since the pioneering introduction of the SBL-targeting inhibitors, progress in the development of clinically useful, β-lactamase-specific inhibitors has been limited. In the case of SBLs, at least one compound, Avibactam, is currently in clinical trials as a combined class A and class C SBL inhibitor.

MBLs, which, unlike the SBLs, are not structurally or mechanistically related to the penicillin-binding proteins (PBP), were first identified approximately 50 years ago. However, as a result of their apparently restricted distribution to the chromosomes of less pathogenic species, they were long not considered to represent a significant threat to the clinical effectiveness of β-lactams. The emergence of horizontally acquired MBLs, where horizontal gene transfer permits genetic material to be transferred between individual bacteria of the same species or between different species, has led to their extensive dissemination across geographical and species
boundaries and now threatens the effectiveness of almost all β-lactams, including carbapenems (i.e., “last resort” antibiotics), the monobactam aztreonam is a current exception. MBLs identified on transferrable plasmids (e.g., the NDM-1, IMP-1, VIM-1, GIM-1, and SPM-type enzymes) are considered a prevalent and immediate clinical problem. Various structurally diverse types of MBL inhibitors have been described, including carboxylic/succinic acids, triazoles/tetrazoles, thiols, trifluoromethyl ketones, and others. To date, no clinically useful MBL inhibitor has been reported. This, in part, may reflect the technical and scientific challenges in the development of an MBL–β-lactam-based combination therapy. There is also extensive structural diversity in the MBL family, particularly in the proposed mobile loop regions around the active site. For an MBL inhibitor to be clinically useful, it is possible that more than one MBL will need to be inhibited. In an effort to help enable work that will lead to the development of useful MBL inhibitors, we here describe an assay platform for clinically relevant MBLs, including protein production procedures and assay conditions using both chromogenic and fluorogenic substrates.

**RESULTS AND DISCUSSION**

Several screening methods for the in vitro and in vivo detection of β-lactamases have been reported. Representative substrates currently in use for β-lactamases include chromogenic cephalosporin-based substrates, such as CENTA, PADAC, and nitrocefin, cephalosporin-based fluorogenic substrates, bioluminescent probes, and fluorescence resonance energy transfer (FRET)-based substrates. These substrates have been used in research centered on SBLs and only in one case on MBLs (L1 and CccA). The large-scale application of these type of compounds in automated high-throughput screening (HTS) for MBL inhibitors is, however, hampered by their lengthy and often difficult synthesis and the high costs associated with these substrates. More importantly, these substrates suffer from poor substrate recognition by some MBLs as a result of the high diversity of this enzyme family whose members vary significantly in sequence, structure, and substrate specificity, thus making it hard to use a single substrate for broad MBL activity screening. The development of new assays for broad-range MBL activity screening, based on hydrolysis of chromogenic or fluorogenic β-lactams, would, however, significantly facilitate inhibitor identification. Not unimportantly, large-scale inhibitor screening against different MBLs needs an inexpensive substrate, thus requiring a simple, scalable, and cost-efficient synthetic route. To develop assay procedures suitable for a range of MBLs, we targeted four clinically relevant enzymes as well as the MBL from Bacillus cereus (BcII), which has been extensively used as a model enzyme. The clinically relevant enzymes chosen were NDM-1 (New Delhi MBL), IMP-1 (Imipenemase), SPM-1 (São Paulo MBL), and VIM-2 (Verona integron-encoded MBL). To identify MBL substrates useful for efficient inhibitor screening, we prepared and tested substrates on the above-mentioned panel of MBLs. These substrates included three chromogenic substrates, i.e., imipenem, nitrocefin, and CENTA, and five coumarin-linked substrates, i.e., FC1–FC5 (Figure 1). Fluorescent substrates FC1 and FC2 release 7-mercapto-4-methylcoumarin upon hydrolysis, resulting in a decrease of fluorescence. Conversely, fluorescent substrates FC3–FC5 liberate 7-hydroxycoumarin upon hydrolysis of the lactam ring, producing an increased fluorescence signal (Figure 2).
and SPM-1 sequences (i.e., with the periplasmic export sequences removed) fused to N-terminal hexahistidine tags, cleavable with 3C protease. The VIM-2 and SPM-1 genes were codon optimized for \textit{E. coli} (Genscript, Piscataway, NJ) and inserted into the pOPINF vector. All the MBLs were produced using in-house-constructed \textit{E. coli} BL21(DE3) pLyS cells, which were grown in 2TY medium. For the protein purifications standard procedures were used. The purity of the MBL proteins was determined to be >95% by SDS–PAGE analysis (see the Supporting Information, Figure SI_4).

### Table 1. Kinetic Data for Chromogenic and Fluorogenic Substrates on MBLs

| Entry | Enzyme   | Substrate   | [E] (pM) | $K_M$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (μM$^{-1}$·s$^{-1}$) | $k_{cat}/K_M$ (μM$^{-1}$·s$^{-1}$) | Reference |
|-------|----------|-------------|----------|-----------|---------------------|-----------------------------------|----------------------------------|-----------|
| 1     | NDM-1    | nitrocefin | 1000     | 8.8 ± 1.1 | 25.3                | 2.9                               | 4.1                              | 33        |
| 2     | CENTA    | nitrocefin | 1000     | 34.6 ± 6.6| 92.5                | 2.7                               | ND                               | ND        |
| 3     | imipenem | nitrocefin | 250      | 111.2 ± 11.7| 398.5            | 3.6                               | 5.8                              | 33        |
| 4     | FC3      | nitrocefin | 50       | 17.6 ± 1.7| 102.6              | 5.8                               | ND                               | ND        |
| 5     | FC4      | nitrocefin | 50       | 4.0 ± 0.8 | 125.7              | 32.0                              | ND                               | ND        |
| 6     | FC5      | nitrocefin | 50       | 2.4 ± 0.2 | 298                | 124.1                             | ND                               | ND        |
| 7     | VIM-2    | nitrocefin | 100      | 7.2 ± 0.6 | 225.6              | 31.2                              | 42.7                             | 34        |
| 8     | CENTA    | nitrocefin | 1000     | 26.1 ± 3.1| 48.2               | 1.8                               | ND                               | ND        |
| 9     | imipenem | nitrocefin | 1000     | 37.8 ± 11.5| 41.6              | 1.1                               | 3.8                              | 34        |
| 10    | FC4      | nitrocefin | 50       | 6.3 ± 0.4 | 125.5              | 20.0                              | ND                               | ND        |
| 11    | FC5      | nitrocefin | 100      | 15.2 ± 1.2| 291.2              | 19.1                              | ND                               | ND        |
| 12    | IMP-1    | nitrocefin | 50       | 55.7 ± 4.8| 2794               | 50.2                              | 2.3                              | 35        |
| 13    | CENTA    | nitrocefin | 100      | 17.1 ± 2.0| 431.9              | 25.3                              | 2.0                              | 35        |
| 14    | imipenem | nitrocefin | 50       | 42.7 ± 6.7| 1200               | 28.1                              | 1.2                              | 35        |
| 15    | FC4      | nitrocefin | 5        | 15.2 ± 0.7| 12200              | 807                               | ND                               | ND        |
| 16    | FC5      | nitrocefin | 5        | 16.8 ± 1.9| 8706               | 517                               | ND                               | ND        |
| 17    | SPM-1    | nitrocefin | 50 × 10$^3$| 16.0 ± 3.7 | 0.7               | 0.04                              | 0.12                             | 36        |
| 18    | CENTA    | nitrocefin | 10 × 10$^3$| 25.3 ± 6.1| 6.1               | 0.2                               | ND                               | ND        |
| 19    | imipenem | nitrocefin | 10 × 10$^3$| 330.9 ± 35.5| 37.2             | 0.1                              | 1.0                              | 36        |
| 20    | FC4      | nitrocefin | 500      | 2.5 ± 0.2 | 8.7                | 3.5                               | ND                               | ND        |
| 21    | FC5      | nitrocefin | 1000     | 2.7 ± 0.2 | 27.38              | 10.3                              | ND                               | ND        |
| 22    | BcII     | nitrocefin | 1000     | 8.1 ± 1.0 | 14.9               | 1.8                               | 0.64                             | 37        |
| 23    | CENTA    | nitrocefin | 1000     | 135.9 ± 16.4| 8.9             | 0.06                              | 0.15                             | 27        |
| 24    | imipenem | nitrocefin | >1000   | 151.1              | 807                               | ND                               | ND        |
| 25    | FC4      | nitrocefin | 1000     | 24.4 ± 1.7| 17.1               | 0.7                               | ND                               | ND        |
| 26    | FC5      | nitrocefin | 1000     | 40.4 ± 3.3| 151.1              | 3.7                               | ND                               | ND        |

**Reagents and conditions:** (a) MMC, DiPEA, DMF, rt, 2 h; (b) TFA/anisole (5:1), 0 °C, 30 min; (c) mCPBA (1 equiv), CH$_2$Cl$_2$, 0 °C, 1 h; (d) NaI (10 equiv), acetone, rt, 2 h; (e) 7-HC, K$_2$CO$_3$, MeCN, rt, 4 h; (f) mCPBA (2 equiv), CH$_2$Cl$_2$, 0 °C, 3 h.

**Table 1. Kinetic Data for Chromogenic and Fluorogenic Substrates on MBLs**

**The enzyme concentrations of the purified proteins were determined using a NanoDrop spectrometer; concentrations of diluted solutions used in the assay were calculated from the original concentration.**

**ND = not determined.**

**The following apparent $K_i$ value for substrate/product inhibition was determined:** 36.6 ± 15.2.
**Synthesis of Substrates.** CENTA was prepared using a modified literature procedure.\textsuperscript{27} Cleavage of \(S,S'-\text{dithiobis}(2\text{-nitrobenzoic acid})\) by dithiothreitol (DTT) yielded 3-carboxyl-4-nitrothiophenol, which was subsequently used to convert commercially available cephalothin into CENTA by heating (65 °C, 3 h). Use of chromatographically purified 3-carboxyl-4-nitrothiophenol gave CENTA in high purity and satisfactory yield after a single acid/base extraction.\textsuperscript{28}

Fluorogenic cephalosporin 1 (FC1) was prepared from commercially available p-methoxybenzyl (PMB) ester-protected chlorocephalosporin (CC) via substitution with 7-mercaptop-4-methylcoumarin (MMC), followed by acid-mediated PMB deprotection (Scheme 1A). Treatment of CC with MMC resulted in the formation of both the \(\Delta_2\)- and \(\Delta_3\)-alkene isomers of compound 1. Subjecting the \(\Delta_2\)-\(\Delta_3\) isomer mixture to acid-mediated PMB deprotection conditions led to complete deprotection within 1 h, generating FC1 (\(\Delta_2\)-\(\Delta_3\) mix). To circumvent the \(\Delta_2\)-\(\Delta_3\) isomerization issue, oxidation of the thiazine sulfur atom with \(m\)-CPBA was performed prior to alkylation (Scheme 1A).\textsuperscript{29} The \(\Delta_3\)-alkene (S)-sulfoxide 2 was obtained in good yield (72%); subsequent alkylation with MMC in the presence of \(N,N'-\text{disopropylethylamine}\) gave 3 as a single isolate isomer in reasonable yield (49%). Acid-mediated PMB deprotection yielded FC2 in good yield (88%). Notably, MMC appears to be nonfluorescent in its free form, whereas the thioether cephalosporin derivatives (i.e., 1 and 3) showed significant fluorescence. While this was not our objective, FC2 has potential as an “inverse fluorogenic” substrate (for spectroscopic data see the Supporting Information, Figure SI_1).

With the aim of developing a fluorogenic MBL substrate, we focused on 7-hydroxycoumarin (7-HC) cephalosporin derivatives, with the knowledge that umbelliferone shows strong fluorescence in its free form while being nearly nonfluorescent when ether-derivatized.\textsuperscript{30} During the course of our investigations, Rao and co-workers reported the synthesis and application of FC3 and FC4 as SBL fluorogenic substrates (specific for BclI over TEM-1).\textsuperscript{31} Their reported procedure (with minimal \(\Delta_3\)-\(\Delta_4\) isomerization) for the synthesis of FC3 and FC4 proved to be efficient and yielded the desired fluorogenic probes (FC3 and FC4) in satisfactory yields (Scheme 1B). Besides substrates FC3 (sulfide) and FC4 ((S)-sulfoxide), we also prepared substrate FC5 (sulfone). Treatment of compound 4 with 2 equiv of \(m\)-CPBA gave clean conversion to compound 6, which upon PMB deprotection yielded substrate FC5 in good yield.

**Metallo-\(\beta\)-lactamase Assays.** Initially time-dependent changes in absorbance and/or emission spectra upon hydrolysis of the potential MBL substrates were recorded to investigate the optimal wavelengths for analysis (see the Supporting Information, Figures SI_2 and SI_3). For the frequently used chromogenic substrates, i.e., imipenem (carbapenem) and nitrocefin and CENTA (cephalosporins), this resulted in preferred readout absorption wavelengths of 300, 405, and 492 nm, respectively. For fluorogenic substrates FC3–FC5 the absorption and emission spectra of umbelliferone in HEPES assay buffer (pH 7.5) were measured and their corresponding optimal wavelengths applied in the fluorescence-based assay (\(\lambda_{ex} = 380 \text{ nm}\) and \(\lambda_{em} = 460 \text{ nm}\)).\textsuperscript{32}

We then determined kinetic parameters for different MBL–substrate combinations. The data for the chromogenic substrates (Table 1) are generally in good agreement with previously reported data,\textsuperscript{27,39} with the only substantial dissimilarity being the lower \(K_M\) values obtained by us in the case of CENTA, possibly because of increased purity of the substrate. Direct quantitative comparison of the obtained data with reported values is, however, difficult as a result of variations in protein production conditions, concentration of metal ions added, etc. The improved stability of CENTA compared to imipenem renders it an attractive substrate for inhibitor identification. However, MBL inhibition by the hydrolyzed cephalosporin products (for BclI,\textsuperscript{40} CphA\textsuperscript{41} and Sfh-I\textsuperscript{42}) or the liberated thiophenol from CENTA (for IMP-1) could complicate interpretation of the results. Examination of the data in Table 1 reveals the high sensitivity of the fluorogenic MBL substrates, which enable the use of enzyme concentrations (generally \(\gg 1 \text{ nM}\)) well below those used with chromogenic substrates (generally 1–50 nM).

In the case of the combination of NDM-1 and FC3–FC5, up to 5–20 times lower enzyme concentration was used as compared to enzyme concentrations required for imipenem or nitrocefin. Further testing of NDM-1 with the three fluorogenic substrates FC3–FC5, with different sulfur oxidation states, disclosed significantly different \(k_{cat}/K_M\) values (up to 20-fold), implying catalytic efficiency is altered by the oxidation state of the thiazine sulfur atom. We observed that the stability of FC3 in aqueous solution is substantially lower than that of FC4 and FC5. Autohydrolysis studies in buffer at room temperature showed substantial hydrolysis of FC3 after 3 h, while both FC4 and FC5 were found to be stable for at least 8 h. Solutions of FC4 and FC5 could be used for 1–2 days when stored on ice. Moreover, DMSO stock solutions, stored at −20 °C, could be used several months after preparation without any detectable decomposition. Storage of compounds FC4 and FC5 as solids at −20 °C enabled use of these compounds over a prolonged period of time (i.e., months). As a result, FC4 and FC5 were tested on the other MBLs.

For nearly all the clinically important MBLs, FC4 and FC5 showed higher \(k_{cat}/K_M\) values than the chromogenic substrates (with VIM-2 being the only exception). On analysis of the VIM-2 results, we found that FC5 is preferred over FC4 and performs equally well as nitrocefin, having similar \(k_{cat}\) values though slightly different \(K_M\) values (7.2 μM vs 15.2 μM, respectively) and consequently different \(k_{cat}/K_M\) values (31.2 μM\(^{-1}\cdot s^{-1}\) vs 19.1 μM\(^{-1}\cdot s^{-1}\)).

The kinetic data of the five substrates for IMP-1 reveal that FC4 outperforms all other substrates mainly as a result of its high \(k_{cat}\) value (i.e., 807 μM\(^{-1}\cdot s^{-1}\)). As depicted in Figure 2A substrate concentrations up to 100 μM could be applied without perturbing the measurement. Although FC4 has a low \(K_M\) compared to the other substrates tested on IMP-1, its high sensitivity allows for accurate measurement as indicated by the low interexperimental error (Figure 3A, experiment performed in triplicate).\textsuperscript{43} As a result of the high sensitivity of both FC4 and FC5, a 20-fold lower IMP-1 concentration could be used (i.e., picomolar enzyme concentration range) while retaining a \(K_M\) value in the micromolar range, thus allowing for the detection of weak binding fragments or slow-binding inhibitors.\textsuperscript{44}

In the case of SPM-1, both FC4 and FC5 outperform all the tested chromogenic substrates. Interestingly, we found that SPM-1 is inhibited by nitrocefin/nitrocefin-derived species at a concentration of 36 μM (see Figure 3B and Table 1). Although \(\beta\)-lactams can act as inhibitors of MBLs, as is apparent for nitrocefin and imipenem at high concentrations (e.g., nitrocefin for SPM-1, this paper, and for CphA and Sfh-I, refs 45 and 42, 47). The relative activity of FC4 was similar to that of nitrocefin despite their differing structures (nitrocefin vs 19.1 μM\(^{-1}\cdot s^{-1}\)).

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sequence similarity between MBLs of di different subfamilies (B1, B2, B3). Most clinically relevant MBLs are from the B1 subfamily; however, SPM-1 presents a high similarity with both the B1 and B2 subfamilies and hence was used as a starting point for proof of principle inhibitor screening. To validate our screening method, we screened a series of 4-chloroisooquinolinol derivatives (7a–7g) against SPM-1 using FC4 as a reporter substrate (Table 2). This set of compounds was selected as a

![Figure 3. (Top) IMP-1 with FC4. (Bottom) SPM-1 with nitrocefin (substrate or product inhibition).](image-url)

respectively); the high sensitivity of the here reported fluorogenic substrates (FC4 and FC5) allows for the use of extremely low substrate concentrations.

**In Silico Studies.** In an attempt to investigate the different substrate binding constants of nitrocefin, imipenem, CENTA, and FC4 for the different MBLs, we performed in silico docking studies. Docking of nitrocefin, imipenem, CENTA, and FC4 in IMP-1, NDM-1, and VIM-2 (PDB IDs 1JJT, 1FKL, and 1KO3, respectively) was performed using AutoDock 4 and SPROUT software. After analysis of the obtained unhydroyzed—enzyme models (see the Supporting Information), the different docking poses, calculated by Autodock 4 and SPROUT, were scored and compared to the substrate binding constants (Table SI_1, Supporting Information). Assessment of the SPROUT docking scores generally reveals a better correlation with the kinetic parameters obtained in vitro (specifically $K_m$ values in the cases of NDM-1 and VIM-2) compared to the docking scores generated by Autodock 4. However, for all three enzymes, the highest ranking substrate in terms of the SPROUT and Autodock scores is FC4, which was also measured to have the highest binding affinity for all three enzymes. Although in-depth structural analyses are required, these modeling results suggest that the cephalosporins may bind to the active site in more than one orientation, including catalytically nonproductive ones. Overall, these results emphasize the importance of experimentally determining optimal MBL–substrate combinations and the need for more structural analyses on MBL–substrate complexes.

**Inhibitor Screening.** To achieve clinical utility, MBL inhibitors may need to act on more than one MBL subfamily. This is potentially challenging because of the generally low sequence similarity between MBLs of different subfamilies (B1, B2, B3). Most clinically relevant MBLs are from the B1 subfamily; however, SPM-1 presents a high similarity with both the B1 and B2 subfamilies and hence was used as a starting point for proof of principle inhibitor screening. To validate our screening method, we screened a series of 4-chloroisooquinolinol derivatives (7a–7g) against SPM-1 using FC4 as a reporter substrate (Table 2). This set of compounds was selected as a

| Table 2. Residual Activities (RAs) on SPM-1 at 1 mM and 200 μM. |
|------------------------|------------------|
| ![Substrate]          | RA 1 mM | RA 200 μM |
| β-Resorcylic acid     | 76%     | -        |
| Salicylic acid        | 89%     | -        |
| R = (S)-CH$_2$-4-OH-Ph (S-7a) | 33%     | -        |
| R = (R)-CH$_2$-4-OH-Ph (R-7a) | 21%     | -        |
| R = (S)-CH$_2$Ph (S-7b) | <5      | -        |
| R = (R)-CH$_2$Ph (R-7b) | 13%     | -        |
| R = (S)-iPr (S-7c)    | 16%     | -        |
| R = (R)-iPr (R-7c)    | 16%     | -        |
| R = (S)-3-methylene-1H-indole (S-7d) | <5%     | 3%       |
| R = (R)-3-methylene-1H-indole (R-7d) | <5%     | 8%       |
| R = (S)-iBu (S-7e)    | <5      | 26%      |
| R = (R)-iBu (R-7e)    | <5      | 32%      |
| R = (S)-CH$_2$CO$_2$H (S-7f) | <5      | 37%      |
| R = (R)-CH$_2$CO$_2$H (R-7f) | <5      | 41%      |
| R = (S)-(CH$_2$)$_2$CO$_2$H (S-7g) | <5      | 38%      |
| R = (R)-(CH$_2$)$_2$CO$_2$H (R-7g) | <5      | 48%      |

For the synthesis of compounds 7a–7g see the Supporting Information. The remaining activity was measured by applying a 10 min preincubation time used in previous MBL work; see refs 18a and 44.
determined. Thus, under the same assay conditions, we found that the fluorescent substrate (FC4) and the chromogenic substrate (imipenem) produced similar IC_{50} and K_{i} values. However, our values are lower than the previously reported IC_{50} and K_{i} values for 1-captopril on NDM-1 using imipenem as the reported substrate (202 μM and 39 μM, respectively) possibly reflecting differences in assay conditions, protein constructs, and enzyme production procedures.

**CONCLUSIONS**

The development of broad-screen MBL inhibitors has been hampered by the lack of an appropriate, efficient screening platform for multiple MBLs. The development of such a screening platform has been hindered by the lack of suitable detection substrates. The results of our comparative study, employing five substrates and five MBLs, reveal striking differences in the kinetic parameters for different enzyme-substrate combinations. Fluorogenic substrates FC4 and FC5 are highly sensitive and efficient substrates for the clinically relevant MBLs, with one or both of them having k_{cat}/K_{M} values of >10 μM^{-1}s^{-1}. The suitability of FC4 for inhibitor screening was demonstrated by testing a set of 4-chloroisouquinolines as MBL inhibitors. This work yielded two compounds ((S)-7d and (R)-7d) that displayed inhibitory activity against our panel of MBLs, albeit at moderate inhibition concentrations (μM); nonetheless, the results reveal the feasibility of broad-spectrum MBL inhibition.

**EXPERIMENTAL SECTION**

The Supporting Information contains a complete general experimental section, including all procedures and equipment used. In general, chemicals were purchased from commonly used suppliers (Aldrich, Acros, Alfa Aesar, and TCI) and were used without further purification. (6R,7R)-4-Methoxybenzyl 3-(chloromethyl)-8-oxo-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (PMB-protected Cep-Cl) was obtained from Activate Scientific (Prien, Germany).

(6R,7R)-4-Methoxybenzyl 8-Oxo-3-((2-oxo-2H-chromen-7-yloxy)methyl)-7-(2-phenylacetamido)-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate (4). To a suspension of Cl-Cep-OPMB ester (680 mg, 1.4 mmol) in acetonitrile (10 mL) was added NaI (2.10 g, 14.0 mmol, 1 equiv). The reaction was stirred at rt for 2 h, after which the 7H_{2}O was removed in vacuo. The crude mixture was partitioned between H_{2}O (10 mL) and EtOAc (10 mL), and the layers were separated. The H_{2}O layer was extracted with EtOAc (2 × 15 mL), after which the combined organic layers were washed with a 5% Na_{2}SO_{4} aq solution (2 × 20 mL) and brine (20 mL). The organic layer was dried over Na_{2}SO_{4} filtered, and concentrated in vacuo. The crude product was dissolved in MeCN (15 mL), and 7-hydroxycoumarin (545 mg, 2.8 mmol, 2 equiv) and K_{2}CO_{3} (42 mmol, 4 equiv) were added. The conversion of the reaction was monitored by TLC analysis (cHex/EtOAc, 1:1), and completion was reached after 4 h. The solvent was evaporated in vacuo, and the resulting crude mixture was partitioned between H_{2}O (10 mL) and EtOAc (10 mL). After separation of the layers, the H_{2}O layer was extracted with EtOAc (2 × 15 mL), after which the combined organic layers were washed with a 5% Na_{2}SO_{4} aq solution (2 × 20 mL) and brine (20 mL). The organic layer was dried over Na_{2}SO_{4} filtered, and
monitoring of the conversion by TLC analysis (CH$_2$Cl$_2$/EtOAc, 8:2). Upon completion of the reaction, cold Et$_2$O (5 mL) was added, which resulted in the formation of a precipitate. The solid material was filtered off, washed with Et$_2$O (2 × 5 mL), and subsequently dried under high vacuum to yield the desired product as an off-white solid (15 mg, 37%). $R_f = 0.10$ (CH$_2$Cl$_2$/MeOH +AcOH, 9:1). $^1$H NMR (400 MHz, DMSO-d$_6$): $\delta = 8.45$ (d, $J = 8.5$ Hz, 1H), 5.97 (dd, $J = 14.0$ Hz, 1H) (part of AB system), 3.62 (d, 3H), 3.98 (d, $J = 4.90$ Hz, 1H), 7.65 (d, $J = 8.5, 4.5$ Hz, 1H), 5.14 (d, $J = 14.0$ Hz, 1H), 4.87–4.93 (m, 1H), 3.98 (d, $J = 18.0$ Hz, 1H), 3.70 (d, $J = 14.0$ Hz, 1H) (part of AB system), 3.62 (d, $J = 18.5$ Hz, 1H), 3.54 (d, $J = 14.0$ Hz, 1H) (part of AB system) ppm. $^{13}$C NMR (101 MHz, DMSO-d$_6$): $\delta = 171.1, 164.2, 162.2, 161.1, 155.3, 143.4, 135.8, 129.6, 129.1$ (2C), 128.3 (2C), 126.6, 112.8 (2C), 101.6, 67.5, 58.3, 45.3, 41.5 ppm. LRMS: mass calcd for C$_{14}$H$_{15}$N$_2$O$_7$S (M − H) 707.0, mass found (M − H) 707.0. Retention time: 7.15 min (purity 99%).

### B Abbreviations Used

MBL, metallo-β-lactamase; SBL, serine-β-lactamase; FC, fluorogenic cephalosporin; DipeA, N,N′-disopropylethylamine; DMF, dimethylformamide; 7-HC, 7-hydroxycoumarin; MeCN, acetonitrile; mCPBA, m-chloroperoxybenzoic acid; MMC, 7-mercapto-4-methylcoumarin; PMB, p-methoxybenzyl; AcOH, acetic acid; TFA, trifluoroacetic acid

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