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Chapter 5

Cephalosporin conjugates of the thiol inhibitors of metallo-β-lactamases are potent inhibitors of IMP enzymes

Parts of this chapter have been published in:

(1) 2019 Dutch Patent application filing; Title: "Prodrug metallo-beta-lactamase inhibitors"; Inventors: Martin, N. I., van Haren, M. J., Tehrani, K. H. M. E. Priority date: April 1, 2019.

(2) Tehrani, K. H. M. E., Wade, N., Mashayekhi, V., Brüchle, N. C., Voskuil, K., Jespers, W., Pesce, D., van Haren, M. J., van Westen, G. J. P., and Martin, N. I. (2020) Novel cephalosporins selectively inhibit the IMP type metallo-β-lactamases. *Manuscript in preparation*

1. Introduction

Despite the growing threat of β-lactam resistance caused by metallo-β-lactamases (MBLs), there are no approved drugs in the market that target this class of enzymes. Unlike serine-β-lactamases, MBLs (figure 1) are metalloenzymes containing one or two zinc ions in their active site and an activated water molecule coordinated by the zinc ions hydrolyzes all classes of β-lactams (except monobactams).[1] The MBLs of particular clinical significance are the New Delhi metallo-β-lactamase (NDM), Verona integron-encoded metallo-β-lactamase (VIM) and imipenemase (IMP) families all of which possess broad β-lactamase activity.[2] The previously reported inhibitors of MBLs have been the subject of several comprehensive review articles.[3–6] Indeed, a wide range of compounds have been reported as MBL inhibitors with the majority acting by either sequestering zinc and/or by forming a ternary complex with the metalloenzyme.[7,8] In chapter 4, we described the *in vitro* ability of a selected group of thiols (**1**-**3**, figure 2A) to inhibit MBLs and in doing so resensitize a panel of MBL-producing clinical isolates to meropenem, a potent carbapenem antibiotic.[9] The binding experiments described in chapter 4 employed isothermal titration calorimetry (ITC) to demonstrate that thiols **1** and **2** bind zinc with K_d values of 10 μM and 20 μM respectively. However, as we also demonstrated, these thiol-containing compounds are prone to rapid oxidation to the corresponding disulfides, leading to the loss of zinc-binding affinity, MBL inhibition, and synergistic activity.[9] As a means of limiting this unwanted oxidation, we were drawn to consider the hydrolysis mechanism of the cephalosporin class of β-lactam antibiotics (figure 2B). Specifically, we hypothesized that if conjugated to the 3-position of the cephalosporin core, these thiols could be delivered as MBL-inhibitor prodrugs which would enhance their selectivity and stability. Only upon MBL-mediated hydrolysis of the cephalosporin moiety would the active thiol-based inhibitor be released, providing both spacial and temporal control of inhibitor delivery and activation. This chapter describes the preparation of cephalosporin-thiol conjugates based on thiols **1**-**3** and evaluation of

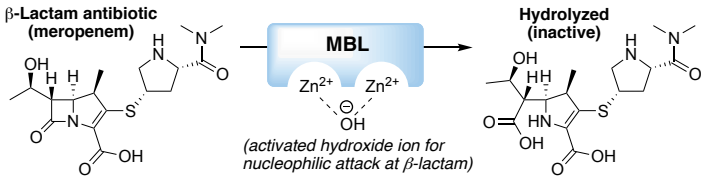


Figure 1. Metallo-β-lactamases as zinc metallo-enzymes

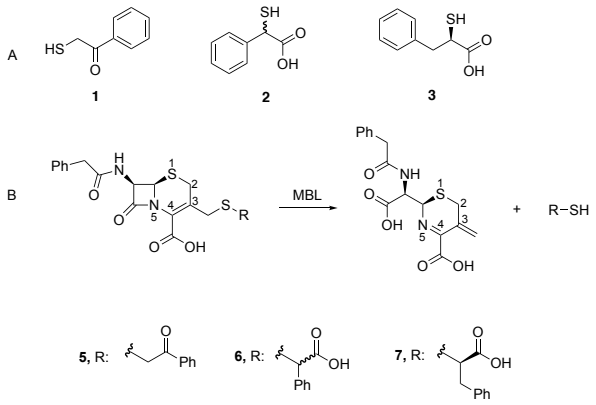
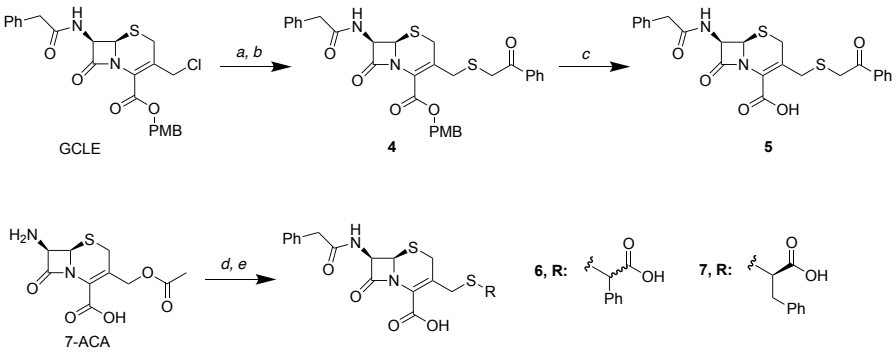


Figure 2. **A**. The previously reported thiols as MBL-inhibitors. **B**. Cephalosporin prodrugs of the thiols **1**-**3**.

their performance as MBL-inhibitor prodrugs capable of resensitizing MBL-expressing strains to β-lactam antibiotics.

2. Results and discussion

The cephalosporin-thiol conjugates were synthesized using two different routes (scheme 1). Thioalkylation of mercaptoacetophenone with the chloromethyl cephalosporin "GCLE", a common intermediate used in the industrial synthesis of cephalosporin antibiotics, yielded intermediate **4** followed by deprotection with TFA to yield compound **5**. Alternatively, compounds **6** and **7** were prepared via the BF_3-promoted substitution of 7-aminocephalosporanic acid (7-ACA) with thiomandelic acid or 2-mercapto-3-phenylpropionic acid, followed by acylation of the 7-amino group (see experimental section for detailed procedures). To assess the zinc-binding properties of the MBL-inhibitor prodrugs **5**-**7**, ITC binding studies were performed which revealed no binding interaction with zinc. This was in contrast with the starting thiols which were found to be relatively strong zinc-binders with low-μM K_d values[9]. In addition, stability analyses were performed to test whether inhibitor release occurred spontaneously. Following overnight incubation in Mueller-Hinton broth, HPLC analysis of the conjugates **5**-**7** showed very good stability (>95% after 15 h, table 1).



Scheme 1. Chemical route to the cephalosporin conjugates. Reagents and conditions: a. NaI, DMF, r.t., 30 min.; b. $NaHCO_3$, **1**, r.t., 20 h; c. TFA, anisole, 0 °C, 1 h; d. $BF_3.OEt_2$, **2**/**3**, ACN, 45 °C, 2 h; e. phenylacetyl chloride, saturated $NaHCO_3$ solution, acetone, r.t., 20 h.

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Table 1. Stability of compounds **5**-**7** in Mueller-Hinton broth

Compound	$(A_{T15}/A_{T0})\%$ [a]
5	95 ± 2
6	98 ± 1
7	98 ± 2

[a] A_{T15}: peak area after 15 h incubation, A_{T0}: peak area at time 0.

The compounds were next tested for their ability to restore the activity of meropenem against a panel of MBL-producing clinical isolates. The results showed that compound **6** and **7** were the most potent resensitizers, lowering the MIC of meropenem against IMP-producing isolates most effectively (figure 3).

Encouraged by the promising results against the MBL-producing clinical isolates, we tested the ability of the conjugates to inhibit purified IMP-1, IMP-28, VIM-2, and NDM-1 enzymes. The biochemical assay used for these studies employed the chromogenic cephalosporin nitrocefin as substrate. The IC_{50} data obtained (table 2) are consistent with the trend observed in bacterial growth inhibition synergy assays with IMP enzymes most strongly inhibited by the conjugates with **6** and **7** demonstrating the most potent inhibitory activity.

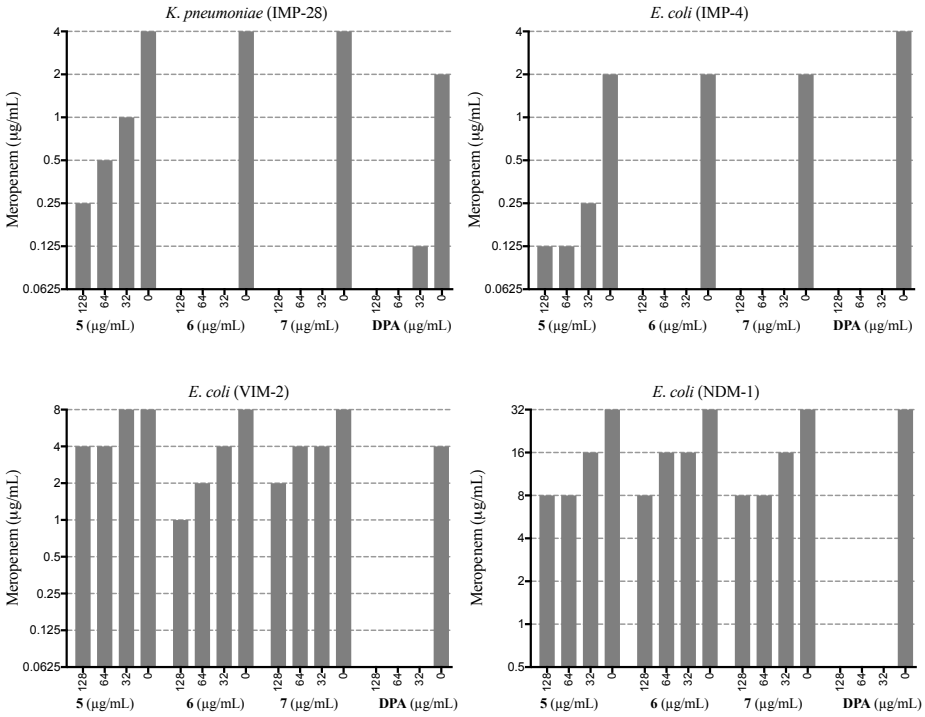

Figure 3. MIC of meropenem in combination with different concentrations of compounds **5**-**7** and dipicolinic acid (DPA).

Table 2. IC_{50} (μM) of thiol conjugates reported as mean ± SD

Compound	IMP-1	IMP-28	NDM-1	VIM-2
5	3.3 ± 0.2	14 ± 1	77 ± 12	76 ± 11
6	0.47 ± 0.08	0.46 ± 0.04	123 ± 8	10 ± 0.5
7	4.7 ± 0.4	1.1 ± 0.2	94 ± 0.2	16 ± 1
DPA	29 ± 0.5	29 ± 5	10 ± 0.1	10 ± 0.8

To assess the release of the thiol inhibitors, the cephalosporin conjugates were incubated with IMP-28 and analyzed using ^{1}H-NMR and LC-MS techniques. It has been shown previously that the molecular mechanism of cephalosporin hydrolysis can be probed *in situ* using NMR techniques.[10,11] For our studies, we used the 7-phenylacetylamide derivative of 7-ACA (compound **8**, figure 4) as a positive control. After incubating this compound with IMP-28, we detected the vinylic hydrogens of the corresponding elimination product as two singlets

resonating *ca*. 5.50 ppm (figure 4). Notably, however, when **6** and **7** were subjected to the same experiment, the vinylic hydrogens were not detected (figure 5 and 6). The results of these ^{1}H-NMR studies were further corroborated by LC-MS analyses of the hydrolysis products which revealed hydrolyzed β-lactam compounds **6H** and **7H** as the only detectable products (figure 7).

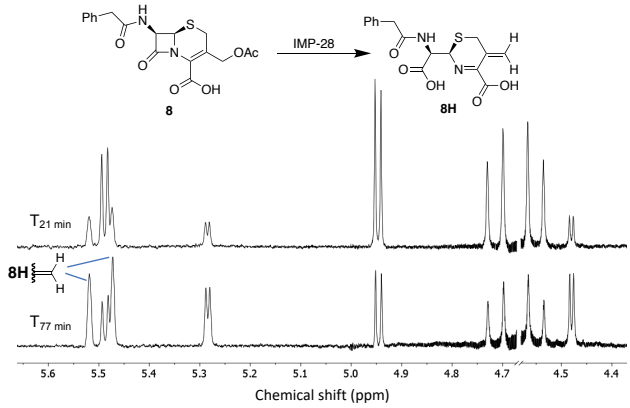

Figure 4. Hydrolysis of **8** monitored by ^{1}H-NMR

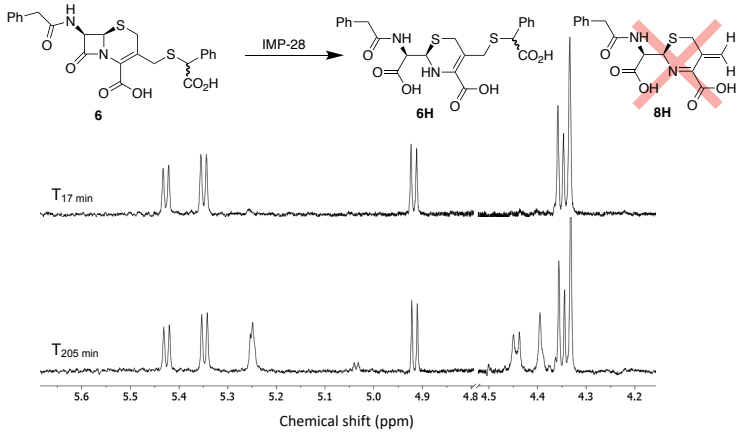

Figure 5. Hydrolysis of **6** monitored by ^{1}H-NMR

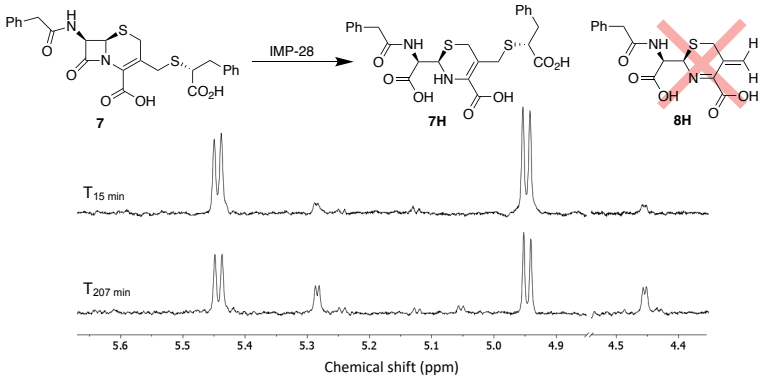

Figure 6. Hydrolysis of **7** monitored by ^{1}H-NMR

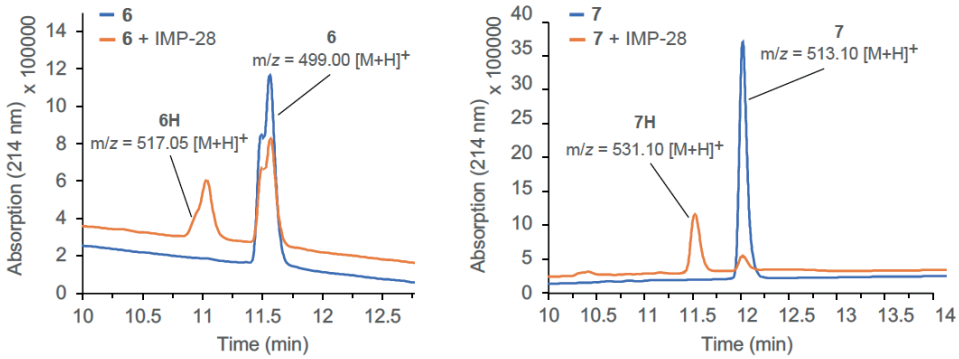

Figure 7. IMP-28-mediated hydrolysis of **6** and **7** monitored by HPLC-MS (procedure described in the experimental section).

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The finding that compounds **6** and **7** demonstrate potent inhibition of IMP-28 despite not releasing the corresponding zinc-binding thiol inhibitors upon MBL-mediated β-lactam hydrolysis was surprising. To better understand the mechanism of inhibition of these cephalosporin conjugates, a series of structural variants of compound **6** were prepared to establish which structural features are most important for the inhibitory activity observed (figure 8, synthesis described in detail in the experimental section). Specifically, we designed compounds **9**, **10**, and **11** to systematically evaluate the contribution made by the aromatic group, the carboxylate moiety, or both.

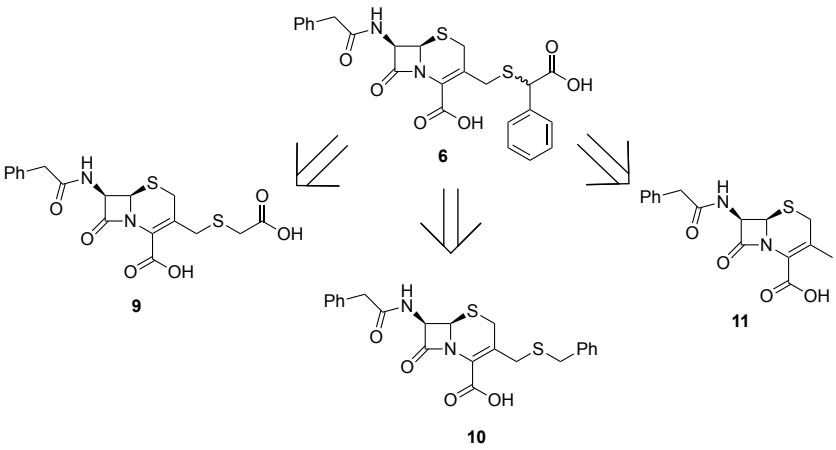

Figure 8. New derivatives of **6** synthesized for structure-activity relationship (SAR) clarification.

The IC_{50} data obtained (table 3) shows that upon the elimination of the phenyl group (**9**), carboxylic acid (**10**) or the entire thiomandelic acid fragment (**11**), the activity against IMP-1 and IMP-28 is decreased at least by ~100 times, suggesting that the thiomandelic acid fragment introduces productive biding interactions with the IMP active site.

The same trend was observed when the cephalosporin conjugates were tested for their synergy with meropenem against MBL-producing clinical isolates. To compare the potency of the cephalosporins, their ability to lower the MIC of meropenem by 4-fold were determined (table 4). The synergy data show the IMP selectivity of the cephalosporins among which **5** and **6** can reduce the MIC of meropenem by 4-fold when added at 1 μg/mL or lower.

Table 3. IC_{50} (μM) of cephalosporins **9**-**11** reported as mean ± SD

Compound	IMP-1	IMP-28	NDM-1	VIM-2
9	>200	101 ± 4	72 ± 2	53 ± 12
10	43 ± 3	45 ± 5	131 ± 11	73 ± 0.1
11	>200	>200	>200	57 ± 9

Table 4. $C_{MIC/4}$ values defined as the lowest concentration of the inhibitors leading to 4-fold reduction in the MIC of meropenem

Isolate	MBL	$C_{MIC/4}$ (μg/mL)					
		5	**6**	**7**	**9**	**10**	**11**
E. coli	IMP-4	16	1	≤0.5	4	4	32
K. pneumoniae	IMP-28	32	0.5	0.25	8	32	64
E. coli	VIM-2	>128	64	128	128	128	128
E. coli	NDM-1	64	128	64	128	>128	>128

The kinetic analysis of the hydrolysis of the cephalosporins by IMP-28, NDM-1 and VIM-2 provided additional insights on the observed IMP-28 selectivity for the inhibitors and the greater potency of **6** and **7**. These analyses showed that IMP-28 has the lowest catalytic efficiency for **6** and **7** among the tested cephalosporins (see table 5 for relative k_{cat}/K_M data). Comparison with the other major MBL families also revealed that **6** and **7** were hydrolyzed more efficiently by NDM-1 and VIM-2 than by IMP-28. These findings indicate that conjugates **6** and **7** inhibit IMP-28 either by acting as slowly turned-over substrates or that the hydrolyzed products **6H** and **7H** are more tightly bound within the IMP active site than either the NDM or VIM active sites.

To evaluate the inhibitory activity of their corresponding hydrolysis products **6H** and **7H**, the intact conjugates **6** and **7** were fully hydrolyzed by incubation with NDM-1 as described in the experimental section. Following hydrolysis, the NDM-1 enzyme was completely removed via spin-filtration as confirmed by the lack of nitrocefin activity by the filtrate. The partially hydrolyzed **6H** and **7H** (see figure 5 and 6 for chemical structures) were then tested for their inhibition of MBLs. Interestingly both hydrolysis products were found to possess potent activity against IMP-1 and IMP-28 with sub-μM IC_{50} values (table 6). In addition, the hydrolysis products **6H** and **7H** were evaluated for their zinc-binding affinity using ITC. When zinc was titrated into the solution of **6** and **7** preincubated with NDM-1, a binding interaction with K_d values of 11.8 μM and 2.9 μM were observed respectively (figure 9), while the intact cephalosporins showed no zinc-binding affinity. This may suggest that the acquired affinity to zinc could play a partial role in the inhibitory activity of **6H** and **7H**.

Table 5. The Michaelis-Menten parameters determined for the cephalosporin conjugates as substrates of IMP-28, VIM-2 and NDM-1.

Enzyme	Substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}.s^{-1}$)	Relative k_{cat}/K_M
IMP-28	**6**	130 ± 14	0.386	0.003	0.3
	7	250 ± 8	2.84	0.011	1.2
	9	219 ± 24	37.1	0.169	18
	10	21 ± 4	10.8	0.529	57
	11	393 ± 74	23.3	0.059	6.3
VIM-2	**6**	8.29 ± 2.3	4.06	0.490	52
	7	4.31 ± 1.2	2.35	0.546	58
NDM-1	**6**	14 ± 3	13.13	0.936	100
	7	21 ± 4	17.5	0.821	88

Table 6. IC_{50} (μM) of the hydrolysis products **6H** and **7H** reported as mean ± SD

Compound	IMP-1	IMP-28	NDM-1	VIM-2
6H	0.54 ± 0.02	0.75 ± 0.06	135.8 ± 32.6	65.6 ± 8.9
7H	0.59 ± 0.06	0.64 ± 0.05	118.4 ± 24.5	69 ± 15

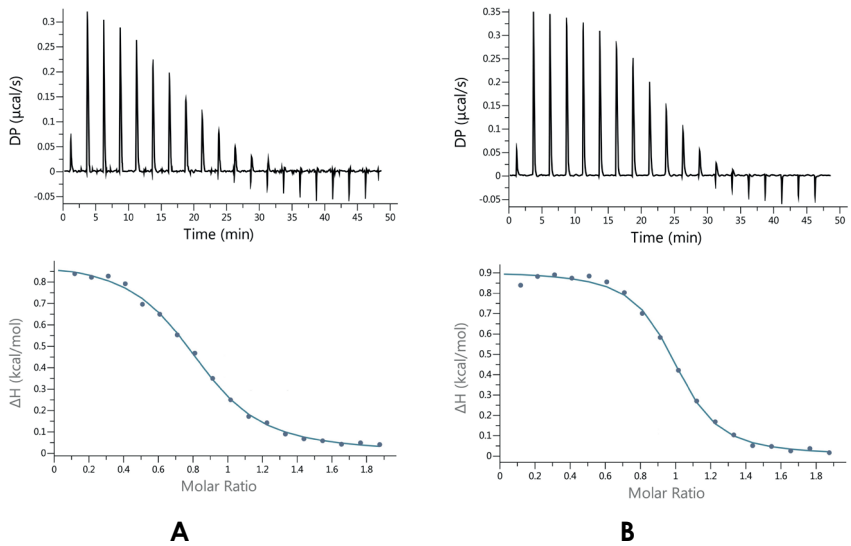

Figure 9. Thermograms of zinc sulphate (2 mM) titrated in a 0.2 mM solution of **6** (A) and **7** (B) incubated with NDM-1 (187 nM) for 2 h at room temperature.

3. Conclusion

We here describe a series of cephalosporin-based MBL inhibitor prodrugs designed to release zinc-chelating small molecule thiols upon MBL-mediated hydrolysis. Notably, these conjugates did not function as expected. While MBL-mediated hydrolysis was observed, the release of the small molecule thiol fragment did not spontaneously occur for the conjugates included in this study. This lack of release is presumably due to the pK_a of the corresponding thiols not being low enough, *i. e.* they do not possess sufficient "leaving group character". It was therefore surprising to find that the cephalosporin conjugates (**5**-**7**) selectively inhibit IMP enzymes despite the fact that β-lactam hydrolysis does not result in release of thiols **1**-**3**. Based

on kinetic analyses, the most potent conjugates **6** and **7** were shown to be slowly turned over substrates of IMP-28. In addition, the hydrolysis products **6H** and **7H** were found to be IMP-selective inhibitors. Taken together these findings suggest that the inhibitory activity observed for the conjugates is due to combination of effects. Future investigations will probe the interaction of these cephalosporin conjugates with the IMP active site as compared with the other MBLs as a means of better understanding the observed selectivity of inhibition.

4. Experimental Section

General

The chlorocephalosporin GCLE, 7-ACA, and 7-ADCA were purchased from Combi-Blocks (US) and nitrocefin from Cayman chemical. The preparation of thiols **1-3** has been described in chapter 4. Compound **8** was synthesized via the acylation of 7-ACA following a previously reported procedure.[12] Proton and carbon nuclear magnetic resonance spectra were recorded on an AV400 NMR spectrometer (Bruker) and samples were dissolved in $CDCl_3$ or DMSO-d_6. HRMS analyses were performed on a Thermo Scientific Dionex UltiMate 3000 HPLC system with a Phenomenex Kinetex C18 column (2.1 x 150 mm, 2.6 µm) at 35 °C and equipped with a diode array detector. The samples were eluted over a gradient of solution A (0.1 % formic acid in water) vs. solution B (0.1% formic acid in acetonitrile). This system was connected to a Bruker micrOTOF-Q II mass spectrometer (ESI ionization) calibrated internally with sodium formate.

Synthesis

*Compound **5***. GCLE (1.0 g, 2.1 mmol) and NaI (314 mg, 2.1 mmol) were stirred in DMF (10 mL) for 30 min at room temperature. Then mercaptoacetophenone (479 mg, 3.15 mmol) and sodium bicarbonate (200 mg, 2.38 mmol) were added successively and the mixture was stirred overnight. The reaction mixture was then partitioned between water and DCM followed by washing the organic layer with brine (3×20 mL). Concentration of the organic layer and purification of the residue on silica using ethyl acetate and DCM mixture as eluent furnished the intermediate **4** as a pale yellow solid (854 mg, 68%). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.89 (d, J = 8.3 Hz, aromatic H, 1H), 7.58 (t, J = 8.0 Hz, aromatic H, 1H), 7.45 (t, J = 8.0 Hz, aromatic H, 2H), 7.37-7.25 (m, aromatic H, 7H), 6.85 (dd, J = 8.6 Hz, J = 1.8 Hz, aromatic H, 2H), 5.99 (d, J = 9.2 Hz, 1H), 5.77 (m, β-lactam C-H, 1H), 5.14 (s, benzyloxy CH_2, 2H), 4.90 (d, J = 4.9 Hz, 1H), 3.99-3.45 (m, aliphatic H, 11H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ 194.41, 171.14, 164.50, 161.52, 159.83, 135.37, 133.72, 133.46, 130.67, 129.40, 129.10, 128.69, 128.53,

128.49, 127.64, 126.78, 124.58, 113.91, 67.93, 59.03, 57.74, 55.23, 43.26, 37.81, 33.81, 27.72. HRMS (ESI): $[M+H]^+$ calculated: 603.1624, found: 603.1620. To **4** (600 mg, 1.0 mmol) was added TFA/anisole (15 mL/3 mL) and the mixture was stirred at 0 °C for 1 h. It was then concentration under vacuum and the residue was precipitated by 1:1 mixture of diethyl ether and petroleum ether. The solid was isolated by centrifugation and purified by reversed-phase prep-HPLC using C18 and an optimal gradient of buffer A (H_2O 95%, ACN 5%, TFA 0.1%) vs. buffer B (ACN 95%, H_2O 5%, TFA 0.1%) to afford **5** (51 mg, 35%, based on the purification of ~100 mg of the crude product by prep-HPLC). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.85 (d, J = 7.3 Hz, aromatic H, 1H), 7.53-7.22 (m, aromatic H, 8H), 6.50 (d, J = 8.8 Hz, 1H), 5.72 (dd, J = 8.9 Hz, J = 4.7 Hz, β-lactam C-H, 1H), 4.90 (d, J = 4.7 Hz, β-lactam C-H, 1H), 3.97-3.44 (m, aliphatic H, 8H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ 195.06, 171.36, 165.01, 163.45, 136.24, 135.91, 133.78, 129.44, 129.15, 128.79, 128.63, 127.36, 126.90, 125.49, 59.37, 58.22, 42.03, 38.20, 33.70, 27.45. HRMS (ESI): $[M-H]^-$ calculated: 481.0897, found: 481.0863.

*General procedure for the synthesis of compounds **6**, **7**, **9**, and **10***. To a solution of $BF_3.OEt_2$ (2.6 mL, 21.3 mmol, 3.0 eq.) in acetonitrile (10 mL) were added the corresponding thiols (10.7 mmol, 1.5 eq.) and 7-ACA (1.9 g, 7.1 mmol, 1.0 eq.) successively. The mixture was stirred at 45-50 °C for 2 h after which it was diluted with water and pH was adjusted to 4 by adding 28% ammonium hydroxide solution. The precipitate was filtered off and washed with cold water and acetone respectively. The crude product (1.0 g) was added to a mixture of saturated bicarbonate solution (6 mL) and acetone (9 mL). Then phenylacetyl chloride (2.0 eq.) was added dropwise and the mixture was stirred overnight at room temperature. Diluting the mixture with water followed by acidification to pH 2.0 using 1.0 M HCl resulted in a white solid which was filtered off and washed with minimum water and ether respectively. The crude material was purified by reversed-phase prep-HPLC using C18 and an optimal gradient of buffer A (H_2O 95%, ACN 5%, TFA 0.1%) vs. buffer B (ACN 95%, H_2O 5%, TFA 0.1%). The quantities and yields below are reported based on the purification of ~100 mg of the crude product by prep-HPLC.

*Compound **6***. 40 mg (26%, over two steps). ^{1}H-NMR (400 MHz, DMSO-d_6): diastereomeric mixture δ 9.07 (apparent t, 1.8 H), 7.44-7.21 (m, aromatic H, 9H), 5.61 (m, β-lactam C-H, 1.8H), 5.04 (d, J = 4.8 Hz, β-lactam C-H, 0.8H), 4.88 (d, J = 4.7 Hz, β-lactam C-H, 1H), 4.65 (apparent d, aliphatic C-H, 1.8H), 3.69-3.32 (m, aliphatic CH_2, 10.8H), HRMS (ESI): $[M-H]^-$ calculated: 497.0847, found: 497.0842.

Compound ***7***. 69 mg (47%, over two steps). ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.41-7.14 (m, aromatic H, 10H), 6.16 (br s, 1H), 5.81 (dd, J = 8.9 Hz, J = 4.7 Hz, β-lactam C-H, 1H), 4.92 (d, J = 4.8 Hz, β-lactam C-H, 1H), 4.19 (d, J = 13.7 Hz, aliphatic C-H, 1H), 3.71-2.92 (m, aliphatic C-H, 8H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ 172.61, 170.99, 164.66, 163.08, 138.14, 135.84, 129.03, 129.01, 128.27, 128.23, 127.45, 126.55, 126.50, 124.95, 58.96, 57.75, 48.23, 41.58, 38.16, 33.38, 26.97, HRMS (ESI): $[M-H]^-$ calculated: 511.1003, found: 511.1000.

Compound ***9***. 88 mg (27%, over two steps). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.14 (d, J = 8.3 Hz, N-H, 1H), 7.34-7.21 (m, aromatic H, 5H), 5.65 (dd, J = 8.3 Hz, J = 4.7 Hz, β-lactam C-H, 1H), 5.11 (d, J = 4.8 Hz, β-lactam C-H, 1H), 3.73-3.20 (m, aliphatic H, 8H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ 170.55, 170.44, 164.10, 162.47, 135.31, 128.51, 127.72, 126.48, 125.99, 124.48, 58.43, 57.31, 41.10, 32.94, 32. 74, 26.42. HRMS (ESI): $[M+H]^+$ calculated: 423.0685, found: 423.0702.

Compound ***10***. 82 mg (74%, over two steps). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.13 (d, J = 8.3 Hz, NH, 1H), 7.35-7.22 (m, aromatic H, 5H), 5.65 (dd, J = 8.3 Hz, J = 4.7 Hz, β-lactam C-H, 1H), 5.06 (d, J = 4.7 Hz, β-lactam C-H, 1H), 3.79-3.47 (m, aliphatic H, 8H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ 171.41, 165.11, 163.62, 138.81, 136.29, 129.49, 129.34, 128.89, 128.69, 128.22, 127.37, 126.96, 125.25, 59.40, 58.36, 42.06, 35.81, 33.78, 27.44. HRMS (ESI): $[M+H]^+$ calculated 455.1099, found: 455.1098.

Compound ***11***. 7-ADCA (2.14 g, 10 mmol) was dissolved in saturated bicarbonate solution (20 mL) to which phenylacetyl chloride (1.5 mL, 11.3 mmol) dissolved in acetone (10 mL) was added in several portions. The mixture was stirred overnight at room temperature, then acidified to pH 2.0 using 1 M HCl. The precipitate was filtered off and washed with minimum amount of cold water. The crude was purified by reversed-phase prep-HPLC using C18 and an optimal gradient of buffer A (H_2O 95%, ACN 5%, TFA 0.1%) vs. buffer B (ACN 95%, H_2O 5%, TFA 0.1%). (85 mg, 75%, based on the purification of ~100 mg of the crude product by prep-HPLC). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.09 (d, J = 8.2 Hz, NH, 1H), 7.33-7.21 (m, aromatic H, 5H), 5.60 (dd, J = 8.2 Hz, J = 4.6 Hz, β-lactam C-H, 1H), 5.03 (d, J = 4.7 Hz, β-lactam C-H, 1H), 3.61-3.35 (m, aliphatic H, 4H), 2.03 (s, methyl, 3H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ 171.44, 164.82, 163.98, 136.33, 130.21, 129.48, 128.68, 126.93, 123.21, 59.33, 57.56, 42.03, 29.40, 19.87. HRMS (ESI): $[M+H]^+$ calculated: 333.0909, found: 333.0917.

Enzyme production and purification

NDM-1, VIM-2, and IMP-28 were expressed and purified as described in chapter 2. The procedure for the production of IMP-1 has been reported in previous literature.[13]

Enzymatic preparation of 6H and 7H

Compound **6** or **7** (2.0 mM each) was incubated with NDM-1 (187 nM) at room temperature in 50 mM HEPES-NaOH, pH 7.2 supplemented with 1 μm $ZnSO_4$ and 0.01% triton X-100. The progress of hydrolysis was monitored by LC-MS (figure 10). After 2 h the conversion was complete, and compounds **6H** and **7H** were separated from the enzyme by spin-filtration (3K filter cutoff, Amicon) at 12000 rpm for 5 min.

Enzyme inhibition assay

The cephalosporin derivatives were tested for their inhibitory activity against NDM-1, VIM-2 and IMP-28 using the chromogenic substrate nitrocefin. The assay buffer was 50 mM HEPES pH 7.2, supplemented with 1 μM $ZnSO_4$ and 0.01% triton X-100. Briefly, on a flat-bottom polystyrene 96-well microplate NDM-1 (6 nM), VIM-2 (8 nM) IMP-1 (2 nM) or IMP-28 (1 nM) were incubated with various concentrations of the test compounds for 15 min at 25 °C. Nitrocefin (10 μM for NDM-1, VIM-2, and IMP-28, 13 μM for IMP-1) was added to the wells and absorption at 492 nm was immediately monitored on a TECAN Spark microplate reader over 30 scan cycles. The initial velocity data were used for IC_{50} curve-fitting using GraphPad Prism 7.

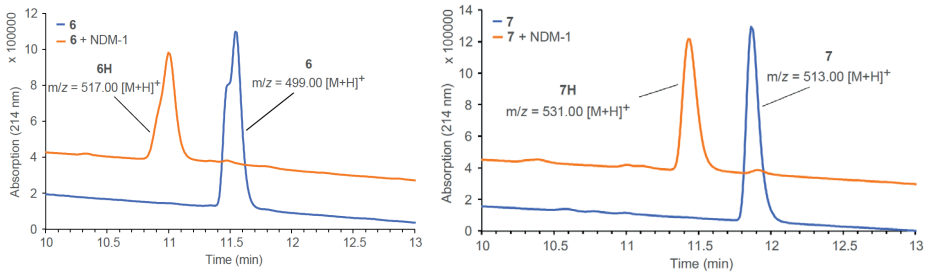

Figure 10. LCMS trace of the enzymatic preparation of **6H** and **7H**.

Determination of the kinetic parameters of cephalosporin conjugates

Hydrolysis of the cephalosporin conjugates was monitored on a Tecan Spark microplate reader using UV-transparent 96-well plates (UV-Star®, Greiner). Various concentrations of the test compounds were dissolved in 50 mM HEPES-NaOH, pH 7.2 supplemented with 1 μm $ZnSO_4$ and 0.01% triton X-100. Followed by the addition of MBLs dissolved in the same buffer, absorption at 260 nm was measured immediately over 30-40 scan cycles at 25 °C. The obtained initial velocity data were plotted against substrate concentration, and K_M and V_{max} were determined using Michaelis-Menten fitting model on GraphPad Prism 7.

MIC determination and synergy assays

Minimum inhibitory concentration (MIC) of the test compounds were determined following the guidelines published by clinical and laboratory standards institute (CLSI) and as described earlier.[9] Synergy between the cephalosporin derivatives and β-lactam antibiotics were evaluated by the following protocol: β-lactam antibiotics dissolved in Mueller-Hinton broth (MHB) with the concentration corresponding to 4×MIC was added to polypropylene 96-well microplates and serially diluted (25 μL/well). Then each 3 columns received a fixed concentration of the test compounds dissolved in MHB (25 μL/well). Multiple concentrations of the test compounds were evaluated this way. Finally, bacterial suspensions grown to the OD_{600} of 0.5 were diluted 100x in MHB before adding to the plate (50 μL/well). The microplates were then covered with breathable seals and incubated overnight with shaking at 37 °C for 15-20 h. Dipicolinic acid was used as positive control.

Stability analysis in MHB

The solutions of the test compounds (1.0 mM) in MHB were incubated at 37 °C for 15 h. Then, 100 μL of the MHB solution was precipitated by adding to acetonitrile (200 μL) supplemented with 2 mM benzocaine, vortexed and centrifuged (12000 rpm, 5 min). The supernatant was analyzed by reversed-phase analytical HPLC using a C_{18} column and an optimal gradient of buffer A (H_2O 95%, ACN 5%, TFA 0.1%) vs. buffer B (ACN 95%, H_2O 5%, TFA 0.1%). The detector wavelength was set at 254 nm.

Isothermal titration calorimetry

The ITC titrations were performed on a PEAQ-ITC calorimeter (Malvern). All the test compounds and zinc sulfate were dissolved in 20 mM Tris-HCl buffer (pH 7.0). The experiments consisted of titrating 2 mM zinc sulfate through 19×2.0 µL aliquots (except the first aliquot which was 0.4 µL) into 200 µM solutions of the cephalosporin conjugates incubated with NDM-1 (187 nM) for 2 h at room temperature. Experiments were performed at 25 °C with 150 s interval between titrations and reference power was set at 10.0 µcal/s. Data was analyzed using Microcal PEAQ-ITC analysis software. In separate experiments, upon the titration of zinc sulfate into the solutions of cephalosporin conjugates or NDM-1, no binding interaction was observed (figure 11).

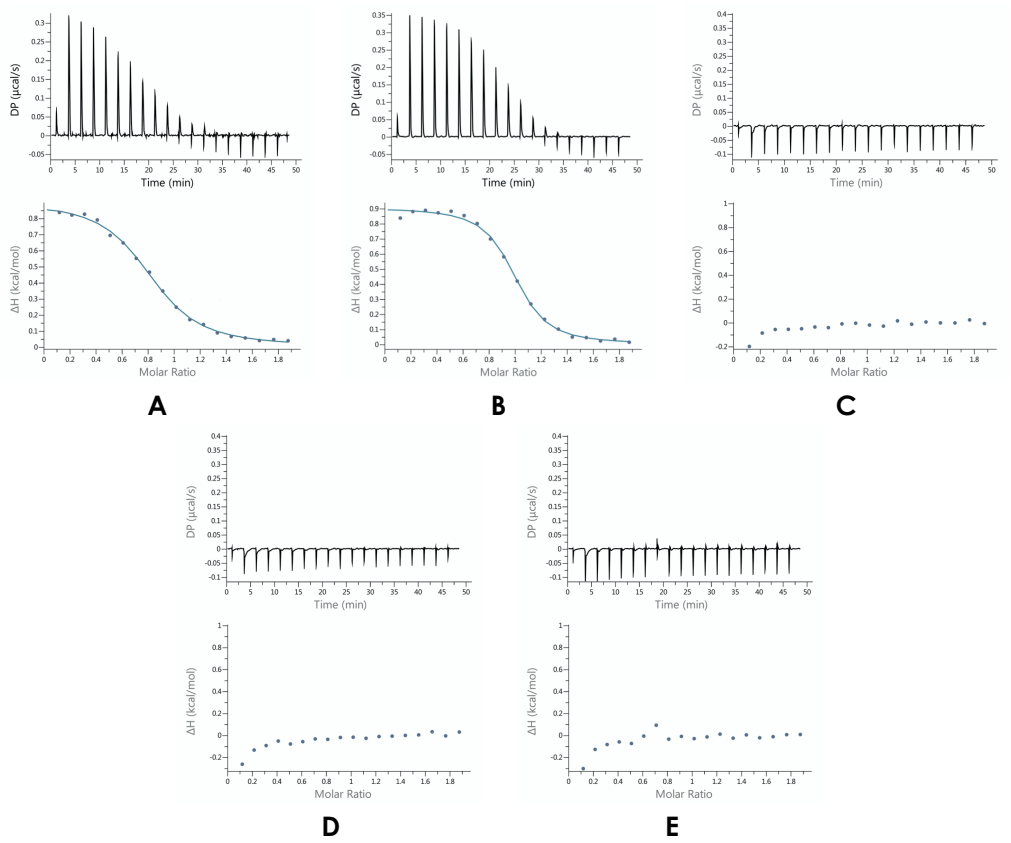

Figure 11. Thermograms of zinc sulphate (2 mM) titrated in a 0.2 mM solution of **6** (**A**) and **7** (**B**) incubated with NDM-1 (187 nM) for 2 h at room temperature. Control experiments include: **C**. zinc sulphate (2 mM) titrated to **6** (0.2 mM), **D**. zinc sulphate (2 mM) titrated to **7** (0.2 mM), and **E**. zinc sulphate (2 mM) titrated to NDM-1 (187 nM).

NMR-based monitoring of the enzymatic hydrolysis

The cephalosporin conjugates dissolved in DMSO-d_6 were diluted in deuterated PBS (pH 7.4) or deuterated 20 mM HEPES (pH 7.4) each supplemented with 1 μM $ZnSO_4$. IMP-28 was added to the solution and the final concentration of the enzyme, test compounds and DMSO were 320 nM, 1 mg/mL and 1% respectively. Following incubation at 25 °C, the ^{1}H-NMR spectra were measured on a Bruker 400 MHz spectrometer in various time points.

LCMS-based monitoring of the enzymatic hydrolysis

The cephalosporin conjugates were dissolved in 20 mM HEPES buffer (pH 7.2) supplemented with 1 μM $ZnSO_4$ and 0.01% triton X-100. IMP-28 was added to the solution and the final concentration of the enzyme, test compounds and DMSO were 320 nM, 1 mg/mL and 1% respectively. Following incubation at 25 °C and in different time points, the solution was diluted in ACN (1:2 v/v) and centrifuged at 12000 rpm for 5 min. The supernatant was analyzed on an LCMS-8040 triple quadrupole liquid chromatograph mass spectrometer (LC-MS/MS, Shimadzu) using a C18 column (3 μm, 3.0×150 mm, Shimadzu) and a gradient of 5-100% pure acetonitrile against 0.5% formic acid.

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References

1 S. M. Drawz and R. A. Bonomo, *Clin. Microbiol. Rev.*, 2010, **23**, 160–201.

2 R. A. Bonomo, E. M. Burd, J. Conly, B. M. Limbago, L. Poirel, J. A. Segre and L. F. Westblade, *Clin. Infect. Dis.*, 2017, **66**, 1290–1297.

3 R. P. McGeary, D. T. Tan and G. Schenk, *Future Med. Chem.*, 2017, **9**, 673–691.

4 P. W. Groundwater, S. Xu, F. Lai, L. Váradi, J. Tan, J. D. Perry and D. E. Hibbs, *Future Med. Chem.*, 2016, **8**, 993–1012.

5 K. H. M. E. Tehrani and N. I. Martin, *Medchemcomm*, 2018, **9**, 1439–1456.

6 W. Fast and L. D. Sutton, *Biochim. Biophys. Acta - Proteins Proteomics*, 2013, **1834**, 1648–1659.

7 L. C. Ju, Z. Cheng, W. Fast, R. A. Bonomo and M. W. Crowder, *Trends Pharmacol. Sci.*, 2018, **39**, 635–647.

8 C. M. Rotondo and G. D. Wright, *Curr. Opin. Microbiol.*, 2017, **39**, 96–105.

9 K. H. M. E. Tehrani and N. I. Martin, *ACS Infect. Dis.*, 2017, **3**, 711–717.

10 S. Hanessian and J. Wang, *Can. J. Chem.*, 1993, **71**, 896–906.

11 H. Feng, J. Ding, D. Zhu, X. Liu, X. Xu, Y. Zhang, S. Zang, D.-C. Wang and W. Liu, *J. Am. Chem. Soc.*, 2014, **136**, 14694–14697.

12 R. Keltjens, S. K. Vadivel, E. De Vroom, A. J. H. Klunder and B. Zwanenburg, *European J. Org. Chem.*, 2001, **2001**, 2529–2534.

13 S. S. van Berkel, J. Brem, A. M. Rydzik, R. Salimraj, R. Cain, A. Verma, R. J. Owens, C. W. G. Fishwick, J. Spencer and C. J. Schofield, *J. Med. Chem.*, 2013, **56**, 6945–6953.