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Metallo-β-lactamase inhibitory activity of phthalic acid derivatives

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ABSTRACT

4-Butyl-3-methylphthalic acid was recognized as a metallo-β-lactamase inhibitor. The structure–activity relationship study of substituted phthalic acids afforded 3-phenylphthalic acid derivatives as potent IMP-1 inhibitors. On the other hand, 3-substituted with 4-hydroxyphenyl phthalic acid derivative displayed a potent combination effect with biapenem (BIPM) against *Pseudomonas aeruginosa* that produce IMP-1.

β-Lactam antibiotics are the most widely used as clinical agents for the treatment of bacterial infection. Among the β-lactam antibiotics, carbapenems, such as imipenem (IPM), meropenem (MEPM) and biapenem (BIPM), have potent broad-spectrum antibacterial activities that are widely used to combat serious infections. It is well known that β-lactams are inactivated by β-lactamases. Based on their amino acid sequence homology, β-lactamases are divided into four classes A, B, C and D according to the Amber classification.[1] Among them, class B β-lactamases are known as metallo-β-lactamases (MBLs) because their active sites contain zinc.[2] MBLs hydrolyze β-lactams, penicillins, cephalosporins and carbapenems. Multi-drug resistant *Pseudomonas aeruginosa* (MDRP) are reported to produce MBL at high frequency, which can result in significant problems in clinical practice.[3] β-Lactamase inhibitors that are effective against the class A serine β-lactamase, clavulanic acid, sulbactam or tazobactam are used with β-lactam antibiotics in the clinical field. Although some MBL inhibitors have been reported[4] none of them are clinically approved. Here, we aimed to develop MBL inhibitors that are effective against Gram-negative bacteria, in particular *P. aeruginosa*. We focused on the IMP-1[5] subclass of MBL because these enzymes are present at high frequency in clinical isolates.[6] Initially, we screened our compound library for IMP-1 inhibitory activity using nitrocefin as substrate and selected 230 candidate compounds. The 230 compounds were then screened for a combination effect with MEPM or ceftazidime (CAZ) against IMP-1 producing *Escherichia coli* and obtained 40 active compounds. Of these compounds, we selected the phthalic acid derivative **1** as the lead compound (Fig. 1).

We reasoned that the two carboxyl groups of compound **1** might interact with zinc in the active site of IMP-1. Therefore, we fixed the two carboxyl groups and investigated the effect of substitution of the phenyl ring on MBL inhibitory activity. First, we synthesized the 4-subsutituted phthalic acid derivatives (Scheme 1).

Esterification of commercially available **2** gave diester derivative **3**, and then Pd catalyzed C–C bond formation afforded the 4-substituted derivative **4**. Hydrolysis under alkali conditions gave compound **5a** and **5c**, respectively. Similarly, the hydrolysis of commercially available 4-*t*-butylphthalic anhydride **6** gave compound **5b**. The IMP-1 inhibitory activities of 4-substituted phthalic acid derivatives **5a–e** are shown in Table 1.

IMP-1 inhibitory activity (IC_{50}) of lead compound **1** was 16.0 μM. Because phthalic acid **5d** displayed no IMP-1 inhibitory activity, it was recognized that substitution of the phenyl ring was necessary for IMP-1 inhibitory activity. Substitution at the 4-position with a methyl (**5e**), *t*-butyl (**5b**) or phenyl (**5c**) moiety also resulted in a compound with no IMP-1 inhibitory activity. By contrast, 4-*n*-butylphthalic acid **5a** showed weak IMP-1 inhibitory activity (IC_{50} = 243 μM), but this activity was less than a one-tenth that of the lead compound **1**. From these results, we

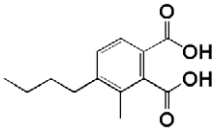

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Figure 1. Lead compound of MBL inhibitor.

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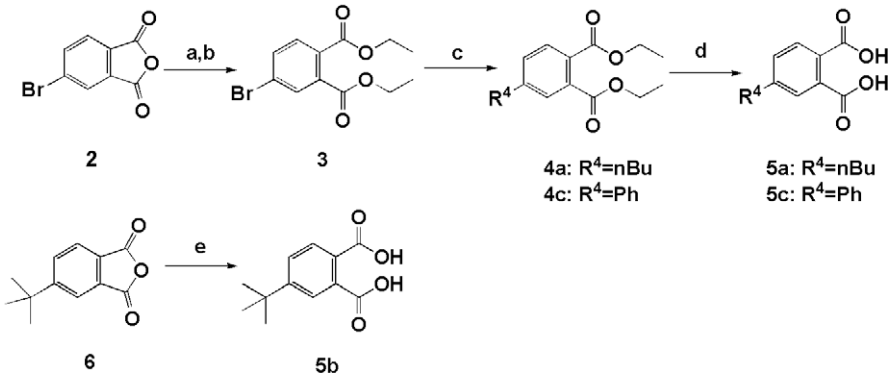
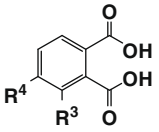


Scheme 1. Synthesis of 4-substituted phthalic acid derivatives. Reagents and conditions: a) *p*-TsOH H_2O, EtOH, 100 °C; b) Bop-reagent, iPr_2NEt, EtOH, rt (79%); c) $nBuB(OH)_2$, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C (**4a**), $PhB(OH)_2$, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C (93%, **4c**); d) (1)NaOHaq, THF, MeOH, rt (2)HClaq (4.2%, 2steps, **5a**), (88%, **5c**); e) (1)NaOHaq, THF, rt, (2)HClaq (55%).

Table 1
IMP-1 inhibitory activity of 4-subsutituted phthalic acid derivatives[12]

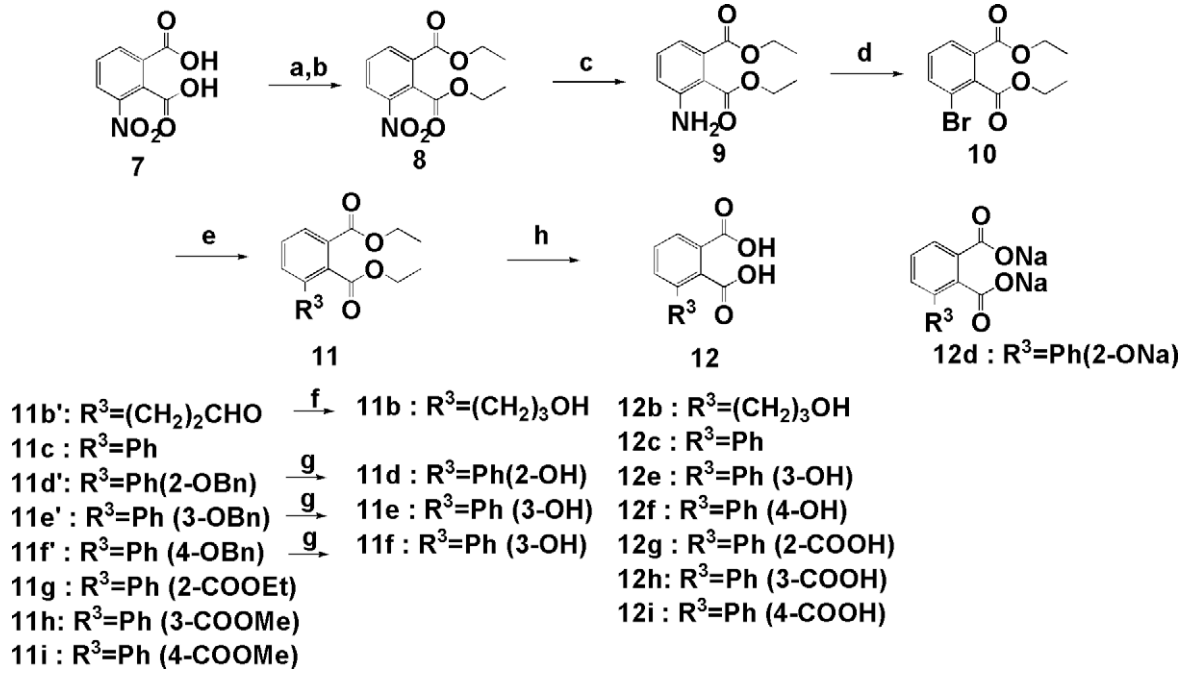


Compd	R^3	R^4	IMP-1 inhibitory activity IC_{50} (μM)
1	Me	*n*-Bu	16.0
5a	H	*n*-Bu	243
5b	H	*t*-Bu	>300
5c	H	Ph	>300
5d[a]	H	H	>100
5e[a]	H	Me	>300

[a] Compounds **5d** and **5e** were commercially available.

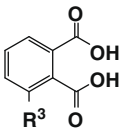
concluded that substitution of the phenyl ring, in particular at the 3-position, is important for IMP-1 inhibitory activity. We then synthesized a series of 3-substituted phthalic acid derivatives (Scheme 2).[11]

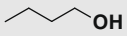
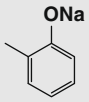
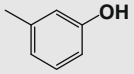
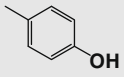
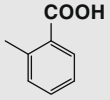
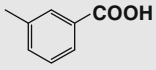
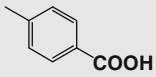
A two step esterification of commercially available **7** gave diethylester derivative **8.** Reduction of the nitro group then afforded **9** in good yield. The 3-amino derivative **9** was subjected to a Sandmeyer reaction to give the 3-bromo derivative **10**. Pd catalyzed C–C formation (and sequential reduction for **11b′**, **11d′–f′** then) gave 3-substituted phthalic acid derivatives **11b–i**.[7] Hydrolysis of each diethylester **11b–i** under alkali conditions gave the corresponding compound **12b–i**, respectively. By contrast with the 4-substituted derivatives, hydrolysis of 3-substituted derivatives was performed at elevated temperatures. In the case of **12d**, it was confirmed that the hydroxyl group on



Scheme 2. Synthesis of 3-substituted phthalic acid derivatives. Reagents and conditions: a) H_2SO_4aq, EtOH, 80 °C; b) EtI, K_2CO_3, DMF, (90%, 2steps); c) H_2/Pd-C, EtOH, rt, (100%); d) $NaNO_2$, HBr then CuBr, HBr, 70 °C (73%); e) allyl alchol, $Pd(tBu_3P)_2$, *N,N*-dicyclohexylmethylamine , K_2CO_3, $Pd(PPh_3)_4$, toluene, rt, (63%, **11b′**), phenylboronic acid, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (54%, **11c**), 2-(2-benzyloxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolan, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (23%, **11d′**), 2-(3-benzyloxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolan, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (28%, **11e′**), 2-(4-benzyloxyphenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (52%, **11f′**), ethyl 2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (11%, **11g**), methyl 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (23%, **11h**), methyl 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzoate, K_2CO_3, $Pd(PPh_3)_4$, toluene, 80 °C, (75%, **11i**); f) $NaBH_4$, EtOH, rt, (80%); g) H_2, Pd-C, rt; h) (1)NaOH aq, 1,4-dioxane, 80 °C (2)HClaq (58%, **12b**; 88%, **12c**; 53%, 2steps, **12d**; 59%, 2steps, **12e**; 17%, 2steps, **12f**; 60%, **12g**; 32%, **12h**; 27%).

Table 2
IMP-1 inhibitory activity of 3-subsutituted phthalic acid derivatives



Compd	R^3	IMP-1 inhibitory activity IC_{50} (μM)
12a	–Me	160
12b		13.2
12c		0.968
12d[a]		2.44
12e		1.75
12f		1.55
12g		207
12h		17.9
12i		2.25

[a] Bis-sodiumbenzoate.

the phenyl moiety at the 3-position and the carboxyl group at the 2-position lactonized under acidic conditions. As a result, **12d** was treated as the sodium salt. By contrast, 3-methylphthalic acid **12a** was obtained from the hydrolysis of 3-methylphthalic anhydride under the same conditions used for the conversion of **6**–**5b**. The IMP-1 inhibitory activities of 3-substituted phthalic acid derivatives **12a–i** are shown in Table 2.

3-Methylphthalic acid **12a** showed weak IMP-1 inhibitory activity whereas compound **12b,** with a 3-hydroxypropyl group at the 3-position, displayed a 10-fold increase in IMP-1 inhibitory activity. These results suggested that a bulky group at the 3-position of the phenyl ring increases IMP-1 inhibitory activity. Therefore, we decided to introduce the phenyl group, as a more bulky substitution, at the 3-position. Substitution with the phenyl ring at the 3-position **12c** increased IMP-1 inhibitory activity 100-fold compared with the methyl derivative **12a**. Next we investigated the influence of substitutions of the phenyl ring at the 3-position of phthalic acid. Hydroxyl derivatives **12d**–**12f** all showed strong IMP-1 inhibitory activity. Unlike the hydroxyl group derivatives, IMP-1 inhibitory activity of carboxyl group derivatives **12g**–**12i** are strongly influenced by their substitution position. Although the *ortho*-substituted derivative **12g** displayed only weak IMP-1 inhibitory activity, *para*-substituted derivative **12i** increased the inhibitory activity about 100-fold.

Table 3
Combination effect of 3-substituted phthalic acid derivatives with BIPM

Compd	Combination effect (50 μg/mL) with BIPM MIC of BIPM (μg/mL)	
	P. aeruginosa KG5002[9]/ pMS363[10] (ΔmexAB)	*P. aeruginosa* PAO1/ pMS363[10]
12a	4	16
12b	1	4
12c	≦0.5	2
12d	2	8
12e	0.5	4
12f	≦0.25	1
12g	64	64
12h	32	64
12i	0.5	2
BIPM only	64–128	64–128

In this study, we found that 3-substituted phthalic acid derivatives had potent IMP-1 inhibitory activity. Next, we investigated the combination effect of 3-substituted phthalic acid derivatives with the carbapenem antibiotic, Biapenem against *P. aeruginosa.* The combination effect of 3-substituted phthalic acid derivatives **12a**–**12i** with BIPM is shown in Table 3.[13]

The phenyl derivative **12c**, *m*-hydroxyphenyl derivative **12e**, *p*-hydroxyphenyl derivative **12f** and *p*-carboxyphenyl derivative **12i** showed potent combination effects with BIPM against *P. aeruginosa* KG5002/pMS363(ΔmexAB).

Some interesting approaches to the development of MBLs inhibitors have previously been reported.[4] However, to our knowledge, there are very few examples of MBL inhibitors displaying a combination effect with carbapenem antibiotics against *P. aeruginosa*. In this study, we discovered that the 3-substituted phthalic acid showed potent MBL inhibitory activity. Moreover these compounds displayed a combination effect with BIPM against *P. aeruginosa* that produces IMP-1.

The efflux systems of *P. aeruginosa* make an important contribution to antibiotic resistance. The resistance nodulation division (RND) efflux system of MexAB-OprM is such examples. It has been reported that carbapenems are effluxed by MexAB-OprM of the RND efflux system. Therefore, we tested the combination effect with BIPM against *P. aeruginosa* expressing efflux system MexAB-OprM.[8]

The *p*-hydroxyphenyl derivative **12f** showed the most potent combination effect. It is interesting that the MIC of *P. aeruginosa* KG5002/pMS363(ΔMexAB) is smaller than that of *P. aeruginosa* PAO1/pMS363. Thus, the influence of the efflux system of MexAB-OprM on the MIC is about fourfold. In conclusion, we found that the 3-substituted phthalic acid showed potent MBL inhibitory activity. Moreover these compounds displayed a combination effect with BIPM against *P. aeruginosa* that produces IMP-1. This combination effect was also shown against *P. aeruginosa* with efflux system MexAB-OprM. Based on these findings, further structure–activity relationship studies of this class of compound are currently in progress.

Acknowledgements

We thank Professor Naomasa Gotoh from Kyoto Pharmaceutical University for kindly providing us with the *P. aeruginosa* PAO1*ΔmexAB-oprM* strain. We thank Dr. Shizuko Iyobe for kindly providing us with the IMP-1 producing plasmid. We thank Mizuyo

Ida and Erumi Murase for helpful discussion and technical assistance. We also thank Shigeko Miki for NMR spectroscopic and mass spectrometric analyses, respectively.

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11. Selected spectral data. Compound **12b**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 1.69 (2H, m), 2.64 (2H, t, J = 8.0 Hz), 3.39 (2H, t, J = 6.4 Hz), 7.42 (1H, dd, J = 7.6, 7.6 Hz), 7.49 (1H, d, J = 7.6 Hz), 7.72 (1H, d, J = 7.6 Hz), FABMS: m/z 225 $[M+H]^+$. Compound **12c**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.40 (5H, m), 7.54–7.61 (2H, m), 8.05 (1H, dd, J = 1.5, 7.6 Hz), FABMS: m/z 243 $[M+H]^+$. Compound **12f**: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.81 (2H, d, J = 8.3 Hz), 7.17 (2H, d, J = 8.3 Hz), 7.47 (2H, m), 7.87 (1H, dd, J = 2.7, 6.3 Hz), FABMS: m/z 259 $[M+H]^+$. Compound **12i**: ^{1}H NMR (DMSO-d_6, 400 MHz) δ 7.50 (2H, d, J = 8.2 Hz), 7.59 (2H, m), 7.92 (1H, dd, J = 2.5, 6.6 Hz), 7.98 (2H, d, J = 8.2 Hz), ESIMS: m/z 287 $[M+H]^+$.
12. MBL inhibitory activity was determined spectrophotometrically using nitrocefin (Oxoid, Basingstoke, England) as the substrate. IMP-1 was PCR amplified from plasmid DNA prepared from a carbapenems-resistant *P. aeruginosa* MSC15369. The PCR product was cloned into pTrcHis2 TOPO vector (Invitrogen, Carlsbad, CA) and expressed in *E. coli* DH5α (Toyobo, Osaka, Japan) after induction with 0.5 mM isopropyl-β-$_D$(−)-thiogalactopyranoside (Wako, Osaka, Japan) for 3 h at room temperature. Soluble IMP-1 was purified from cell extracts by Ni-NTA slurry (Qiagen, Valencia, CA). The IC_{50} of inhibitors were determined following 20 min incubation at room temperature with IMP-1 (1.0 nM in 50 mM HEPES, pH7.5) in the presence of 100 μM $ZnSO_4$ and 20 μg/ml BSA (Sigma–Aldrich, St. Louis, MO). Using initial velocity as a measure of activity, inhibition was monitored spectrophotometrically at 490 nm in a Wallac ARVOsx 96-well plate reader (Perkin Elmer, Waltham, MA) employing nitrocefin as the reporter substrate at 100 μM.
13. The in vitro activities were determined by the microbroth dilution method in accordance with CLSI. Mueller-Hinton II broth (Becton, Dickinson and Company, Sparks, MD) was used for testing procedure. MICs were determined for BIPM alone and in combination with the inhibitor at a constant 50 μg/ml. The bacterium inoculum size was approximately 5×10^4 CFU/well.