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Metallo-β-lactamase inhibitory activity of 3-alkyloxy and 3-amino phthalic acid derivatives and their combination effect with carbapenem

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ABSTRACT

3-Alkyloxy and 3-amino phthalic acid derivatives were found to have metallo-β-lactamase inhibitory activity. Among them, 3-amino phthalic acid derivatives showed both potent activity against metallo-β-lactamase, IMP-1 inhibitory activity and a strong combination effect with biapenem (BIPM), carbapenem antibiotic. In particular, the 4′-hydroxy-piperidine derivative showed strong IMP-1 inhibitory activity and a combination effect with various antibiotics.

1. Introduction

Owing to their efficacy and safety, β-lactam antibiotics are widely used for the treatment of bacterial infection in the clinical field. It is well known, however, that mechanisms of resistance to β-lactam antibiotics exist. In this regard, inactivation by β-lactamase is the major mechanism of resistance against β-lactam antibiotics.

β-Lactamases are classified as four types A–D, based on their amino acid sequence homology by Amber classification.[1] Due to serine-catalysed hydrolysis of the β-lactam ring, class A, C, and D β-lactamases are called serine β-lactamases. On the other hand, class B β-lactamase is a metallo-β-lactamase (MBL) having one or two zinc ions in the active site.[2] Various types of metallo-β-lactamases, for instance, IMP, VIM, and SPM types etc., have been reported. In Japan, the IMP-1 metallo-β-lactamase has been reported so far.[3] MBLs can hydrolyze almost all β-lactam antibiotics, including penicillins, cephalosporins and carbapenems.

Carbapenems play an important role in the clinical field because they are effective against both Gram-positive and Gram-negative bacteria including *Pseudomonas aeruginosa*. Therefore, MBL-producing pathogens are a significant problem in the clinical field. In addition, multi-drug resistant *P. aeruginosa* (MDRP) strains are a significant problem because of their resistance against aminoglycosides, carbapenems and quinolones, which are normally effective against Gram-negative bacteria. Moreover, it is reported that MDRP strains produce MBLs at high frequency.[4]

As a result, we are interesting in developing a MBL inhibitor. In a previous study, we found that phthalic acids substituted with a bulky group at the 3-position had potent IMP-1 inhibitory activity.[5] In particular, compound **1**, which is substituted with a 4′-hydroxyphenyl group at the 3-position, was found to have the most potent IMP-1 inhibitory activity (IC_{50} = 1.55 μM) (Fig. 1). Furthermore, compound **1** showed a combination effect with biapenem (BIPM), a carbapenem antibiotic, against IMP-1 *P. aeruginosa* strains producing IMP-1. As a result, here we have continued to develop a more potent MBL inhibitor.

2. Results and discussion

2.1. Chemistry

Scheme 1 shows the synthesis of 3-alkyloxy phthalic acid derivatives from commercially available 3-hydroxyphthalic anhydride. Esterification of 3-hydroxyphthalic anhydride, followed by alkylation of the hydroxyl group, gave **4**. Hydrolysis of the diethylester **4** under alkaline conditions gave the phthalic acid **5** in good yield.

Scheme 2 shows the synthesis of 3-aminophthalic acid derivatives from commercially available 3-nitrophthalic acid. Two step esterification of the 3-nitrophthalic acid **6** gave the diethylester **7**. Hydrogenation of **7**, followed by alkylation of the 3-amino group of **8**, afforded **9**. Hydrolysis of the diethyl ester **9** under alkaline conditions afforded **10**.

3-Dimethylamino phthalic acid and its cyclic amine derivatives were synthesized from 3-fluorophthalic acid (Scheme 3). Esterification of the 3-fluorophthalic acid **11** afforded the diethyl

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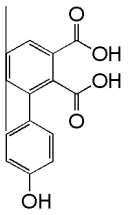

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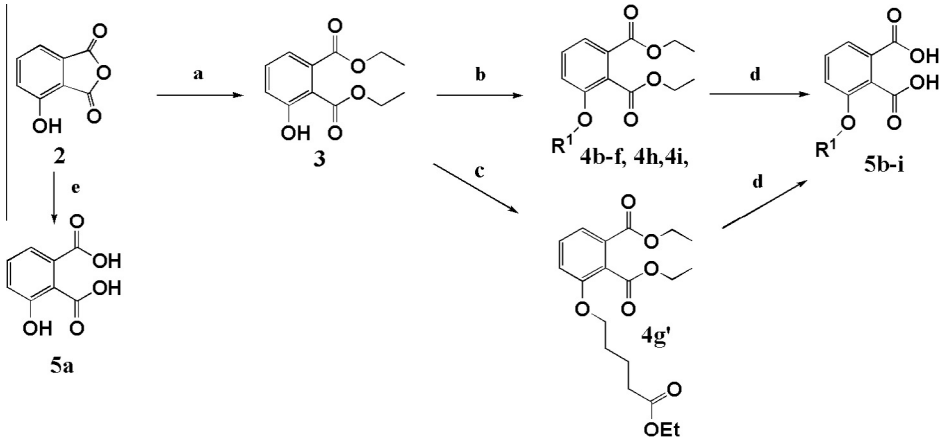
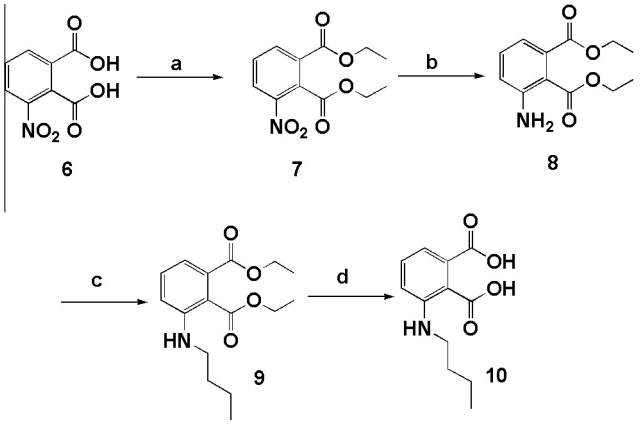
Figure 1. Lead compound of IMP-1 inhibitor



Scheme 1. Synthesis of 3-alkoxyphthalic acid derivatives. Reagents and conditions: (a) H_2SO_4, EtOH, reflux; (b) CH_3I, K_2CO_3, DMF, rt (**4b**); benzylbromide, K_2CO_3, DMF, rt (**4c**); *n*BuI, K_2CO_3, DMF, rt (**4d**); (3-bromopropyl)cyclohexane, K_2CO_3, DMF, rt (**4e**); 3-phenylpropylbromide, K_2CO_3, DMF, rt (**4f**); 2-bromoethanol, K_2CO_3, DMF, rt (**4h**); 3-bromo-1-propanol, K_2CO_3, DMF, rt (**4i**); (c) ethyl 5-bromovalerate, K_2CO_3, DMF, rt; (d) (1) NaOH, H_2O, 1,4-dioxane, 80 °C; (2) HCl, H_2O; (e) (1) NaOH, H_2O, rt; (2) HCl, H_2O.



Scheme 2. Synthesis of 3-aminophthalic acid derivatives. Reagents and conditions: (a) (1) *c*H_2SO_4, EtOH, reflux; (2) EtI, K_2CO_3, DMF; (b) H_2, 10% Pd–C, EtOH, rt; (c) *n*-butylaldehyde, acetic acid, triacetoxyborohydride, 1,2-dichloroethane, rt; (d) (1) NaOH, H_2O, 1,4-dioxane, 80 °C; (2) HCl, H_2O.

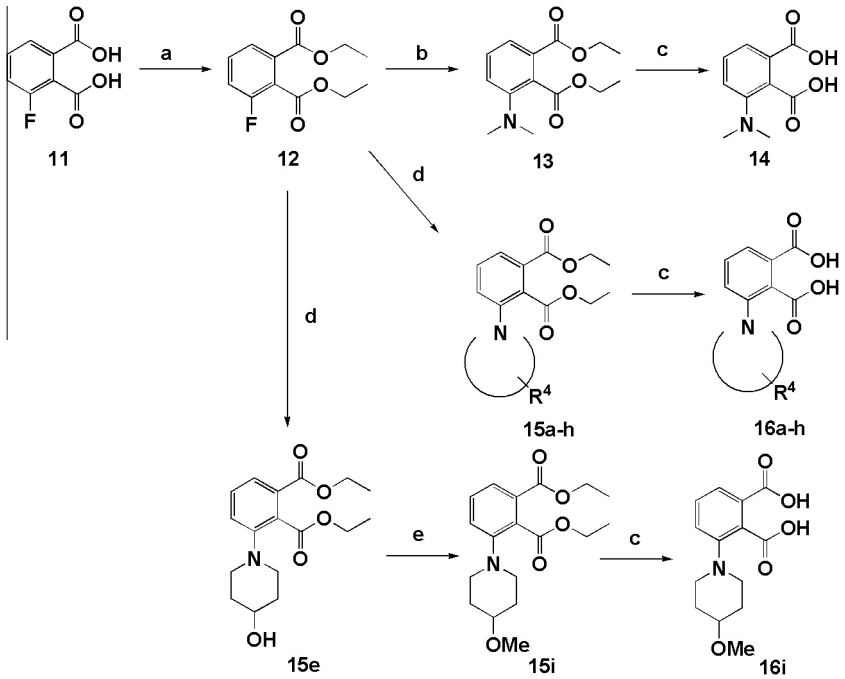
ester derivative **12**. Next, dimethyl amine or various cyclic amines were reacted with **12** to give diethyl esters of the 3-aminophthalic acid derivatives. Hydrolysis under alkaline conditions gave 3-aminophthalic acid derivatives.

2.2. Structure and activity relationship (biological activity)

The IMP-1 inhibitory activities of the 3-alkyloxy phthalic acid derivatives were shown in Table 1. Although the hydroxyl derivative **5a** showed no IMP-1 inhibitory activity, the methoxy derivative **5b** showed weak activity. The 3-benzyloxy phthalic acid **5c** showed about a 20-fold increase in activity as compared with **5b**. The derivatives **5d**, **5e** and **5f** with longer carbon chains showed potent IMP-1 inhibitory activity. In compounds **5g**, **5h** and **5i**, the hydrophilic hydroxyl or carboxyl group was introduced to the C2, C3 or C4 alkyl chain. These derivatives (**5g–i**) showed weak IMP-1 inhibitory activity (IC_{50} = 18.8–47.8 μM).

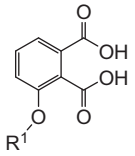
Next, we evaluated the combination effect with BIPM, a carbapenem antibiotic, against *P. aeruginosa* KG5002[6]/pMS363[7] (ΔMexAB) and PAO1/pMS363 strains that produce IMP-1. The 3-alkyloxy phthalic acids **5e** and **5f**, which were strong IMP-1 inhibitors, showed no combination effect with BIPM against *P. aeruginosa* PAO1/pMS363. Although the IMP-1 inhibitory activity of **5g**, **5h** and **5i** was weaker than that of **5e** or **5f**, the derivatives **5g–i** showed potent combination effect with BIPM against *P. aeruginosa* PAO1/pMS363. On the other hand, **5f** showed a strong combination effect with BIPM against *P. aeruginosa* KG5002/pMS363 (ΔMexAB). Interestingly, the hydroxyl derivatives **5h** and **5i** showed a combination effect against *P. aeruginosa* KG5002/pMS363 (ΔMexAB) that was equal to that against *P. aeruginosa* PAO1/pMS363. These results indicated that **5f** was affected by the efflux system of MexAB-OprM, whereas the hydroxyl derivatives **5h** and **5i** were not affected by this efflux system.

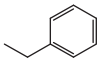
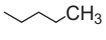
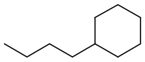
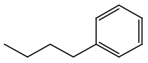
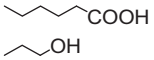
Table 2 shows the activities of the 3-aminophthalic acid derivatives. No substituted 3-aminiophthalic acid showed weak IMP-1 inhibitory activity, but the *n*-butyl (**10**) and *N*,*N*-dimethyl amino (**14**) derivatives showed improved IMP-1 inhibitory activity. Although the *N*,*N*-dimethyl amino derivative **14** had weak IMP-1 inhibitory activity, this compound showed a potent combination effect with BIPM against *P. aeruginosa* strains that produced IMP-1. On the other hand, cyclic amine compounds showed potent IMP-1 inhibitory activity and a combination effect with BIPM against *P. aeruginosa* strains producing IMP-1. In particular, the



Scheme 3. Synthesis of cyclic amine derivatives and *N*,*N*-dimethyl amino derivatives. Reagents and conditions: (a) (1) H_2SO_4, EtOH, reflux; (2) EtI, K_2CO_3, DMF, rt; (b) dimethylamine, THF, ,sealed tube, 90 °C; (c) NaOH, H_2O, 1,4-dioxane, 80 °C; (d) pyrolidine, 80 °C (**15a**); piperidine, 80 °C (**15b**); 3-hydroxypiperidine, DMSO, 80 °C (**15c**); 3-(hydroxylmethyl)piperidine, DMSO, 80 °C (**15d**); 4-hydroxypiperidine, DMSO, 80 °C (**15e**); 4-(hydroxylmethyl)piperidine, DMSO, 80 °C (**15f**); 4-(hydroxylethyl)piperidine, DMSO, 80 °C (**15g**); 4-hydroxy-4-phenylpiperidine, DMSO, 80 °C (**15h**); (c) (1) NaOH, H_2O, 1,4-dioxane, 80 °C; (2) HCl, H_2O; (e) NaH, MeI, THF, rt.

Table 1
IMP-1 inhibitory activity of 3-alkyloxy phthalic acid derivatives and their combination effect with BIPM

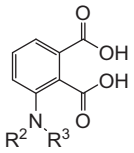


Compd	R^1	IMP-1 inhibitory activity IC_{50} (μM)	Combination effect (50 μg/mL) with BIPM MIC of BIPM (μg/mL)	
			P. aeruginosa KG5002 /pMS363 (ΔmexAB)	*P. aeruginosa* PAO1/pMS363
5a	–H	>300	NT	NT
5b	$-CH_3$	142	NT	NT
5c		7.40	NT	NT
5d		5.10	NT	NT
5e		2.00	4	64
5f		1.70	0.5	64
5g		18.8	2	8
5h		47.8	16	16
5i		21.4	16	16
		(BIPM alone)	64–128	64–128

piperidine derivative **16b** showed an excellent combination effect with BIPM.

Next, we aimed to optimize the piperidine derivatives. The IMP-1 inhibitory activity and combination effect of piperidine derivatives were shown in Table 3. As compared with **16b**, the IMP-1 inhibitory activities were mainly improved. The 4′-hydroxyethyl compound **16g** showed low potent IMP-1 inhibitory activity. In addition, this compound showed a weak effect of combination with BIPM. The 4′-hydroxy-4′-phenyl compound **16h** and the 4′-methoxy compound **16i** showed potent IMP-1 inhibitory activity and a potent combination effect against *P. aeruginosa* KG5002/pMS363 (ΔmexAB); however, these compounds showed a weak

Table 2
IMP-1 inhibitory activity of 3-amino phthalic acid derivatives and their combination effect with BIPM



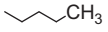
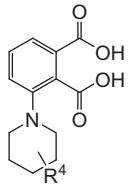
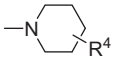
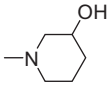
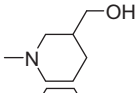
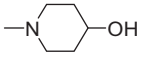
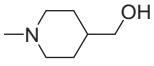
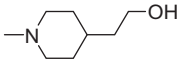
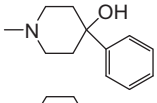
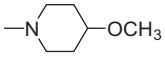
Compd	R^2	R^3	IMP-1 inhibitory activity IC_{50} (μM)	Combination effect (50 μg/mL) with BIPM MIC of BIPM (μg/mL)	
				P.aeruginosa KG5002 /pMS363 (ΔmexAB)	*P.aeruginosa* PAO1/pMS363
	–H	–H	300	NT	NT
10	–H		13.1	1	64
14	$-CH_3$	$-CH_3$	94.5	4	4
16a			2.80	0.5	4
16b			10.8	≦0.5	4
			(BIPM alone)	64–128	64–128

Table 3
IMP-1 inhibitory activity of piperidine derivatives and their combination effect with BIPM



Compd		IMP-1 inhibitory activity IC_{50} (μM)	Combination effect (50 μg/mL) with BIPM MIC of BIPM (μg/mL)	
			P. aeruginosa KG5002/pMS363 (ΔmexAB)	*P. aeruginosa* PAO1/pMS363
16b		10.8	≦0.5	4
16c		2.70	≦0.5	1
16d		2.10	0.5	2
16e		2.70	≦0.25	1
16f		2.60	1	2
16g		25.6	8	32
16h		2.30	≦0.5	16
16i		3.70	≦0.25	2
		(BIPM alone)	64–128	64–128

combination effect against *P. aeruginosa* PAO1/pMS363. From these results, it is likely that these compounds were effluxed by the MexAB system.

As compared with **16c** and **16d**, or **16e** and **16f** and **16g**, extension of the carbon chain resulted in a decreased combination effect against *P. aeruginosa* producing IMP-1. The presence of a hydroxyl group at the 3′ or 4′ position of the piperidine ring led to potent IMP-1 inhibitory activity. In particular, the 4′-hydroxy piperidine derivative **16e** showed the most potent effect of combination with BIPM.

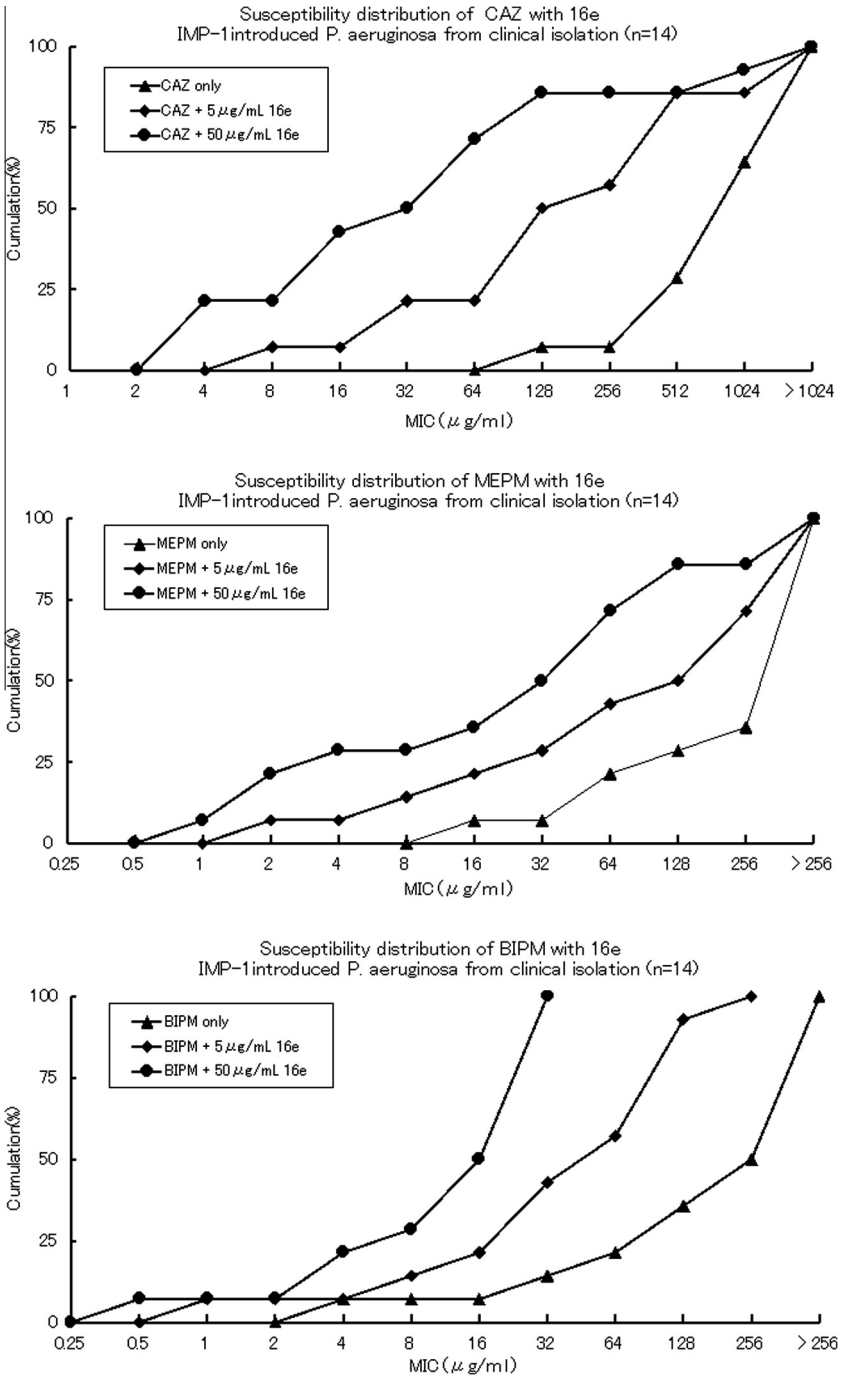


Figure 2.

From the above results, we selected the 4′-hydroxy piperidine derivative **16e**, and carried out more detailed tests. We tested the combination effect **16e** with various antibiotics, including BIPM, meropenem (MEPM) and ceftazidime (CAZ), against clinical isolates of *P. aeruginosa* producing IMP-1 (Fig. 2).

All antibiotics were shown to have a combination effect with **16e**, and this effect was dose-dependent on **16e**. These results indicate that the MBL-inhibitor **16e** is effective against clinical isolates of *P. aeruginosa* producing IMP-1. For CAZ and MEPM, however, some strains were not sensitive. It is considered that CAZ and MEPM were affected not only by IMP-1 but also by another mechanism of resistance. Thus, BIPM is the favored antibiotic for combination with the MBL-inhibitor **16e**.

3. Conclusion

In this study, we found that the 3-alkyloxy and 3-amino phthalic acid derivatives had potent IMP-1 inhibitory activity. Regarding the structure–activity relationship of 3-alkyloxy phthalic acids, although the long carbon chain compounds had potent IMP-1 inhibitory activity, their combination effect with BIPM against *P. aeruginosa* strains producing IMP-1 was not parallel. In contrast, the hydroxy derivatives **5h** and **5i** had low IMP-1 inhibitory

activity, but showed a strong combination effect with BIPM against *P. aeruginosa* without being subject to efflux pump. In the case of the 3-aminophtahalic acid derivatives, the piperidine derivatives showed strong IMP-1 inhibitory activity; in particular, polar substitution increased this activity.

The 4′-hydroxypiperidine compound **16e** showed ideal activity. This compound showed a combination effect with various antibiotics against *P. aeruginosa* strains producing IMP-1. In this study, BIPM was the most promising antibiotic for combination with the MBL inhibitor **16e** against clinical isolates of *P. aeruginosa* producing IMP-1. On the basis of these findings, further structure–activity relationship studies of MBL inhibitors are currently in progress.

4. Experimental section

^{1}H NMR spectra were recorded on JNM-LA400 spectrometers with chemical shifts reported in ppm with internal tetramethylsilane as a basis. Electron ionization (EI) mass spectra were recorded on a Hitachi M-80B instrument. Fast-atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-700 instrument. Thermospray (TSP) mass spectra were recorded on a Hewlett–Packard 5989A instrument. Electrospray ionization (ESI) mass spectra were recorded on a Hewlett–Packard 5989A instrument. Atmospheric pressure chemical ionization (APCI) mass spectra were recorded on a Hewlett–Packard 5989A instrument. High-resolution mass spectra (HRMS) were recorded under FAB conditions.

4.1. 3-Hydroxy phthalic acid (5a)

3-Hydroxyphthalic anhydride **2** (60.0 mg, 0.360 mmol) was solved in water (2.0 mL) and 1.0 mol/L NaOH (2.0 mL) solution. The solution was stirred at room temperature for 15 h. 1.0 mol/L HCl solution was added to adjust the pH until pH2, and the mixture was extracted three times with EtOAc. The organic layers were combined and dried over anhydrous $MgSO_4$. It was then concentrated under reduced pressure to give **5a** (14.0 mg, 21.4%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.07 (2H, m, phenyl), 7.44 (1H, dd, J = 7.6, 8.2 Hz, phenyl); ESIMS: m/z 181 $(M-H)^-$.

4.2. 3-Methoxyphthalic acid (5b)

conc. H_2SO_4 (3.0 mL) was added to a solution of 3-hydroxyphtalic anhydride (650 mg, 3.96 mmol) in EtOH (15 mL). The mixture was refluxed for overnight and then concentrated under reduced pressure. Water was added to the residue and the mixture was extracted three times with EtOAc. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **3** (930 mg, 98.7%). ESIMS: m/z 237 $(M-H)^-$.

To a DMF (5.0 mL) solution of **3** (100 mg, 0.420 mmol), K_2CO_3 (120 mg, 0.868 mmol) and methyliodide (0.031 mL, 0.500 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **4b** (35.0 mg, 33.1%).

ESIMS: m/z 253 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **4b** (34.0 mg, 0.135 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. The reaction mixture was extracted with EtOAc three times. 1.0 mol/L HCl solution was added to the aqueous solution to adjust the until pH3, and then extracted with EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **5b** (14.0 mg, 52.9%).

^{1}H NMR (400 MHz, CD_3OD) δ 3.08 (3H, s, methyl), 7.21 (1H, dd, J = 0.97, 8.3 Hz, phenyl), 7.51 (1H, dd, J = 8.0, 8.3 Hz, phenyl), 7.49 (1H, dd, J = 0.97, 8.0 Hz, phenyl); ESIMS: m/z 195 $(M-H)^-$.

4.3. 3-Benzyloxyphthalic acid (5c)

To a DMF (5.0 mL) solution of **3** (100 mg, 0.420 mmol), K_2CO_3 (120 mg, 0.860 mmol) and benzylbromide (0.060 mL, 0.500 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **4c** (90.0 mg, 65.3%).

ESIMS: m/z 329 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **4c** (90.0 mg, 0.274 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. The reaction mixture was extracted with EtOAc three times. 1.0 mol/L HCl solution was added to aqueous solution to adjust the pH until pH3, and then extracted with EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **5c** (64.0 mg, 85.8%).

^{1}H NMR (400 MHz, CD_3OD) δ 5.09 (2H, s, benzyl), 7.27 (7H, m, phenyl), 7.51 (1H, dd, J = 0.98, 7.6 Hz, phenyl), ESIMS: m/z 271 $(M-H)^-$.

4.4. 3-Buthoxyphthalic acid (5d)

To a DMF (5.0 mL) solution of **3** (60.0 mg, 0.252 mmol), K_2CO_3 (87.0 mg, 0.578 mmol) and 1-iodobutane (0.034 mL, 0.299 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification with preparative TLC (hexane/EtOAc = 2:1) afforded **4d** (52.0 mg, 70.2%).

ESIMS: m/z 295 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **4d** (50.0 mg, 0.170 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for 3 h. The reaction mixture was extracted with EtOAc three times. 1.0 mol/L HCl solution was added to the aqueous solution to adjust the pH until pH2, and then extracted with EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **5d** (40.0 mg, 98.9%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.95 (3H, t, J = 7.3 Hz, methyl), 1.48 (2H, m, CH_2), 1.77 (2H, m, CH_2), 4.04 (2H, t, J = 6.5 Hz, CH_2), 7.13 (1H, d, J = 8.0 Hz, phenyl), 7.38 (1H, dd, J = 8.0 Hz, phenyl), 7.60 (1H, d, J = 8.0 Hz, phenyl), EIMS: m/z 238 (M^+).

4.5. (3-Cyclohexylpropoxy)phthalic acid (5e)

To a DMF (5.0 mL) solution of **3** (290 mg, 1.22 mmol), K_2CO_3 (500 mg, 3.62 mmol) and (3-bromopropyl)cyclohexane (500 mg, 2.44 mmol) were added. The mixture was stirred at room temperature for two days. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified with silica–gel column chromatography (hexane/EtOAc = 2:1) to give **4e** (430 mg, 97.3%).

EIMS: m/z 362 (M^+).

To a 1,4-dioxane (2.0 mL) solution of **4e** (430 mg, 1.19 mmol), 5.0 mol/L NaOH solution (10 mL) was added. The mixture was stirred at 80 °C for overnight. The reaction mixture was extracted with EtOAc three times. 1.0 mol/L HCl solution was added to the aqueous solution to adjust the pH until pH2, and then extracted with EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **5e** (283 mg, 77.5%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 0.90 (2H, m), 1.09–1.37 (6H, m), 1.67 (5H, m), 1.83 (2H, m), 4.06 (2H, t, J = 6.7 Hz, CH_2), 7.19 (1H, d, J = 8.3 Hz, phenyl), 7.46 (1H, dd, J = 7.8, 8.3 Hz, phenyl), 7.65 (1H, d, J = 7.8 Hz, phenyl), ESIMS: m/z 307 $(M+H)^+$.

4.6. (3-Phenylpropoxy)phthalic acid (5f)

To a DMF (3.0 mL) solution of **3** (82.0 mg, 0.345 mmol), K_2CO_3 (120 mg, 0.868 mmol) and 3-phenylpropylbromide (81.0 mg, 0.407 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc = 1:1) to give **4f** (90.0 mg, 73.3%).

EIMS: m/z 356 (M^+).

To a 1,4-dioxane (1.0 mL) solution of **4f** (90.0 mg, 0.253 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for 3 h. The reaction mixture was extracted with EtOAc three times. 1.0 mol/L HCl solution was added to the aqueous solution to adjust the pH until pH2, and then extracted with EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **5f** (54.0 mg, 71.1%).

^{1}H NMR (400 MHz, $CDCl_3 + CD_3OD$) δ 2.11 (2H, m, CH_2), 2.81 (2H, t, J = 7.6 Hz, CH_2), 4.02 (2H, t, J = 6.2 Hz, CH_2), 7.08 (1H, d, J = 8.3 Hz, phenyl), 7.23 (5H, m, phenyl), 7.38 (1H, dd, J = 7.8, 8.3 Hz, phenyl), 7.62 (1H, d, J = 7.8 Hz, phenyl),

ESIMS: m/z 299 $(M-H)^-$.

4.7. (3-Carboxybuthoxy)phthalic acid (5g)

To a DMF (5.0 mL) solution of **3** (100 mg, 0.420 mmol), K_2CO_3 (150 mg, 1.09 mmol) and ethyl 5-bromopentanoate (100 mg, 0.478 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification with silica gel column chlomatography (hexane/EtOAc = 7:3) afforded **4g** (149 mg, 96.9%).

EIMS: m/z 366(M^+).

To a 1,4-dioxane (1.0 mL) solution of **4g** (140 mg, 0.383 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. The reaction mixture was extracted with EtOAc. 1.0 mol/L HCl solution was added to the aqueous solution to adjust the pH until pH2, and then extracted with EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **5g** (67.7 mg, 62.7%).

^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 1.48 (4H, m, CH_2), 2.39 (2H, m, CH_2), 2.51 (2H, m, CH_2), 7.13 (1H, d, J = 8.0 Hz, phenyl), 7.39 (1H, dd, J = 8.0, 8.0 Hz, phenyl), 7.62 (1H, d, J = 8.0 Hz, phenyl).

ESIMS: m/z 283 $(M+H)^+$.

4.8. 3-(2-Hydroxyethoxy)phthalic acid(5h)

To a DMF (4.0 mL) solution of **3** (100 mg, 0.420 mmol), K_2CO_3 (170 mg, 1.23 mmol) and ethyl 2-bromoethanol (100 mg, 0.800 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification with silica gel column chlomatography (hexane/EtOAc = 7:3) afforded **4h** (45.0 mg, 38.0%).

EIMS: m/z 282 (M^+).

To a 1,4-dioxane (0.6 mL) solution of **4h** (45.0 mg, 0.160 mmol), 5.0 mol/L NaOH solution (3.0 mL) was added. The mixture was stirred at 80 °C for 3 h. 1.0 mol/L HCl solution was added to the reaction mixture until pH2, and then concentrated under reduced pressure. Purification with synthetic adsorption resin SP-207 (Mitsubishi Chemical Co., Ltd,) (H_2O–CH_3CN) gave **5h** (18.2 mg, 50.3%).

^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 3.88 (2H, t, J = 4.3 Hz, CH_2), 4.16 (2H, t, J = 4.3 Hz, CH_2), 7.17 (1H, d, J = 8.5 Hz, phenyl), 7.42 (1H, dd, J = 7.8, 8.5 Hz, phenyl), 7.64 (1H, d, J = 7.8 Hz, phenyl).

ESIMS: m/z 225 $(M-H)^-$.

4.9. 3-(3-Hydroxypropoxy)phthalic acid (5i)

To a DMF (4.0 mL) solution of **3** (100 mg, 0.420 mmol), K_2CO_3 (170 mg, 1.23 mmol) and ethyl 3-bromo-1-propanol (120 mg, 0.863 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined oreganic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. Purification with preparative TLC (hexane/EtOAc = 1:1) afforded **4i** (110 mg, 88.5%).

EIMS: m/z 296 (M^+).

To a 1,4-dioxane (1.0 mL) solution of **4i** (110 mg, 0.371 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for 3 h. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. Purification with synthetic adsorption resin SP-207 (H_2O–CH_3CN) gave **5i** (35.0 mg, 39.3%).

^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 2.02 (2H, m, CH_2), 3.80 (2H, t, J = 5.7 Hz, CH_2), 4.18 (2H, t, J = 5.7 Hz, CH_2), 7.16 (1H, d, J = 8.3 Hz, phenyl), 7.41 (1H, dd, J = 7.9, 8.3 Hz, phenyl), 7.64 (1H, d, J = 7.9 Hz, phenyl).

EIMS: m/z 240 (M^+).

4.10. 3-(Butylamino)phthalic acid (10)

conc. H_2SO_4 (10 mL) was added to a solution of 3-nitrophthalic acid (5.00 g, 23.7 mmol) in EtOH (100 mL). The mixture was refluxed for 6 h. Water was added to the reaction mixture, and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated. The residue was solved in DMF (200 mL) and then K_2CO_3 (9.78 g, 70.8 mmol) and Ethyliodide (2.9 mL, 36.3 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture and the mixture was extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc) to give **7** (5.68 g, 89.9%).

ESIMS: m/z 268 $(M+H)^+$.

To a EtOH (40 mL) solution of **7** (2.68 g, 10.0 mmol), 10% Pd–C (530 mg) was added and the mixture was hydrogenated under balloon pressure of hydrogen at room temperature for 5 h. The mixture was purified with silica-gel column chromatography (hexane/EtOAc) to give **8** (2.38 g, 100%).

ESIMS: m/z 238 $(M+H)^+$.

To a 1,2-dichloroethane (10 mL) solution of **8** (237 mg, 1.00 mmol), *n*-butylaldehyde (0.13 mL, 1.44 mmol), acetic acid (0.11 mL, 1.92 mmol) and triacetoxyborohydride (420 mg, 2.22 mmol) were added. The mixture was stirred at room temperature for overnight. Saturated $NaHCO_3$ solution was added to the reaction mixture and stirred, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated. The residue was purified with preparative TLC (hexane/EtOAc) afforded **9** (190 mg, 64.8%).

ESIMS: m/z 294 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **9** (190 mg, 0.648 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. The reaction mixture was extracted with EtOAc. 1.0 mol/L HCl solution was added to the aqueous solution to adjust the pH until pH2, and then extracted with

EtOAc three times. The organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure to give **10** (112 mg, 72.9%).

^{1}H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 0.96 (3H, t, J = 7.4 Hz, CH_3), 1.45 (2H, m, CH_2), 1.64 (2H, m, CH_2), 3.16 (2H, t, J = 7.1 Hz, CH_2), 6.78 (2H, m, phenyl), 7.30 (1H, dd, J = 7.9, 7.9 Hz, phenyl).

ESIMS: m/z 236 $(M-H)^-$.

4.11. 3-Dimethylaminophthalic acid (14)

To a EtOH (200 mL) solution of **11** (9.35 g, 50.8 mmol), conc. H_2SO_4 (20 mL) was dropped, and then refluxed for 6 h. The reaction mixture was concentrated under reduced pressure. Water was added to the residue, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was solved in DMF (150 mL), K_2CO_3 (21.0 g, 152 mmol) and iodoethane (6.1 mL, 75.6 mmol) were added. The mixture was stirred at room temperature for two days. Water was added to the reaction mixture and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc) afforded **12** (9.13 g, 74.8%).

EIMS: m/z 240 (M^+).

Compound **12** (120 mg, 0.500 mmol) was added to 2.0 M dimethylamine in THF solution (3.0 mL) and then stirred at 90 °C in sealed tube for overnight. The reaction mixture was evaporated, and then purified with preparative TLC (hexane–EtOAc) to give **13** (130 mg, 98.1%).

FABMS: m/z 266 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **13** (13 0 mg, 0.49 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for 3 h. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin, Sepabeads SP-207 (H_2O–CH_3CN) to give **14** (23.0 mg, 22.5%).

^{1}H NMR (400 MHz, D_2O) δ 3.12 (6H, s, CH_3), 7.47 (1H, dd, J = 1.0, 7.6 Hz, phenyl), 7.62 (1H, dd, J = 7.6, 8.3 Hz, phenyl), 7.73 (1H, dd, J = 1.0, 8.3 Hz, phenyl).

FABMS: m/z 210 $(M+H)^+$.

4.12. 3-(Pyrolidine-1-yl) phthalic acid (16a)

Compound **12** (120 mg, 0.500 mmol) was added to pyrolidine (1.0 mL, 12.0 mmol) and then stirred at 80 °C for overnight. The reaction mixture was concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc) to give **15a** (27.0 mg, 18.6%).

FABMS: m/z 292 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **15a** (27 mg, 0.0927 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The reaction mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin (sepabeads SP-207) (H_2O–CH_3CN) to give **16a** (21.0 mg, 96.4%).

^{1}H NMR (400 MHz, D_2O) δ 2.79 (4H, m, pyrolidine), 4.24 (4H, m, pyrolidine), 8.03(1H, dd, J = 2.7, 5.8 Hz, phenyl), 8.20 (1H, d, J = 5.8 Hz, phenyl), 8.21 (1H, d, J = 2.7 Hz, phenyl).

ESIMS: m/z 236 $(M+H)^+$.

4.13. 3-(Piperidine-1-yl) phthalic acid (16b)

Compound **12** (120 mg, 0.500 mmol) was added to piperidine (0.82 mL, 8.30 mmol) and then stirred at 80 °C for overnight. Water was added to the reaction mixture, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified with preparative TLC (hexane/EtOAc) to give **15b** (166 mg, 99.7%).

EIMS: m/z 305 (M^+).

To a 1,4-dioxane (1.0 mL) solution of **15b** (150 mg, 0.492 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin (Sepabeads SP-207) (H_2O–CH_3CN) to give **16b** (70.0 mg, 57.1%).

FABMS: m/z 250 $(M+H)^+$; FAB-HMS $(M+H)^+$ calcd for $C_{13}H_{16}NO_4$: 250.1079, found: 250.1079.

4.14. 3-(3′-Hydroxypiperidine-1-yl)phthalic acid (16c)

To a DMSO (3.0 mL) solution of **12** (375 mg, 1.56 mmol), 3-hydroxypiperidine (1.26 g, 12.4 mmol) was added. The mixture was stirred at 80 °C for overnight. Water was added to the reaction mixture, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc) to give **15c** (410 mg, 81.9%).

EIMS: m/z 321 (M^+).

To a 1,4-dioxane (1.0 mL) solution of **15c** (400 mg, 1.25 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin Sepabeads SP-207 (H_2O–CH_3CN) to give **16c** (150 mg, 45.4%).

^{1}H NMR (400 MHz, D_2O) δ 1.80–2.15 (4H, m, piperidine), 3.28 (1H, dd, J = 4.2, 12.4 Hz, piperidine), 3.39 (2H, m, piperidine), 3.66 (1H, dd, J = 1.6, 12.4 Hz, piperidine), 4.21 (1H, m, piperidine), 7.46 (1H, dd, J = 1.0, 7.6 Hz, phenyl), 7.64 (1H, dd, J = 7.6, 8.1 Hz, phenyl), 7.70(1H, dd, J = 1.0, 8.1 Hz, phenyl);FABMS: m/z 266 $(M+H)^+$; FAB-HMS $(M+H)^+$ calcd for $C_{13}H_{16}NO_4$: 266.1028, found: 266.1028.

4.15. 3-(3′-Hydroxymethyl)piperidine-1-yl)phthalic acid(16d)

To a DMSO (3.0 mL) solution of **12** (200 mg, 0.833 mmol), 3-(hydroxylmethyl)piperidine (960 mg, 8.33 mmol) was added. The mixture was stirred at 80 °C for overnight. The reaction mixture was purified with silica-gel column chromatography (hexane/EtOAc) to give **15d** (220 mg, 78.8%).

EIMS: m/z 335 (M^+).

To a 1,4-dioxane (1.0 mL) solution of **15d** (220 mg, 0.657 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin Sepabeads SP-207 (H_2O–CH_3CN) to give **16d** (150 mg, 81.8%).

^{1}H NMR (400 MHz, D_2O) δ 1.36 (1H, m), 1.83 (2H, m), 2.06 (2H, m), 3.21–3.55 (6H, m), 7.44 (1H, d, J = 7.3 Hz, phenyl), 7.62–7.69 (2H, m, phenyl).

FABMS: m/z 280 $(M+H)^+$; FAB-HMS $(M+H)^+$ calcd for $C_{14}H_{18}NO_5$: 280.1185, found: 280.1185.

4.16. 3-(4′-Hydroxypiperidine-1-yl)phthalic acid (16e)

To a DMSO (10 mL) solution of **12** (1.0 g, 4.17 mmol), 4-hydroxypiperidine (4.2 g, 41.5 mmol) was added. The mixture was stirred at 80 °C for overnight. Water was added to the reaction mixture, and

then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc) to give **15e** (1.35 g, 99.4%).

EIMS: *m*/*z* 321 (M^+).

To a 1,4-dioxane (5.0 mL) solution of **15e** (1.0 g, 3.12 mmol), 5.0 mol/L NaOH solution (25.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin Sepabeads SP-207 (H_2O–CH_3CN) to give **16e** (500 mg, 60.5%).

^1H NMR (400 MHz, D_2O) δ 1.88 (2H, m, piperidiene), 2.16 (2H, m, piperidiene), 3.44 (2H, m, piperidiene), 3.58 (2H, m, piperidiene), 4.05 (1H, m, piperidiene), 7.46 (1H, dd, *J* = 0.97, 7.5 Hz, phenyl), 7.65 (1H, dd, *J* = 7.5, 8.1 Hz, phenyl), 7.70(1H, dd, *J* = 0.97, 8.1 Hz, phenyl).

FABMS: *m*/*z* 266 $(M+H)^+$; FAB-HMS $(M+H)^+$ calcd for $C_{13}H_{16}NO_5$: 266.1028, found: 266.1028.

4.17. 3-(4′-(Hydroxymethyl)piperidine-1-yl)phthalic acid (16f)

4-(Hydroxymethyl)piperidine (960 mg, 9.50 mmol) was added to **12** (200 mg, 0.833 mmol), and then stirred at 80 °C for overnight. The reaction mixture was purified with preparative TLC (hexane/EtOAc) go give **15f** (50.0 mg, 17.9%).

EIMS: *m*/*z* 335 (M^+).

To a 1,4-dioxane (1.0 mL) solution of **15f** (50.0 mg, 0.149 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with HP-20 (H_2O–CH_3CN) to give **16f** (30 mg, 72.2%).

^1H NMR (400 MHz, D_2O) δ 1.55 (2H, m, piperidiene), 1.85 (1H, m, piperidiene), 2.06 (2H, m, piperidiene), 3.48 (6H, m, piperidiene, CH_2), 7.37 (1H, dd, *J* = 2.5, 6.5 Hz, phenyl), 7.60 (1H, d, *J* = 6.5 Hz, phenyl), 7.61 (1H, d, *J* = 2.5 Hz, phenyl).

FABMS: *m*/*z* 280 $(M+H)^+$; FAB-HMS $(M+H)^+$ calcd for $C_{14}H_{18}NO_5$: 280.1185, found: 280.1185.

4.18. 3-(4′-(Hydroxyethyl)piperidine-1-yl)phthalic acid (16g)

To a DMSO (5.0 mL) solution of **12** (152 mg, 0.633 mmol), 4-(hydroxyethyl)piperidine (810 mg, 6.26 mmol) was added. The mixture was stirred at 80 °C for overnight. Water was added to the reaction mixture, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$, and then concentrated under reduced pressure. The residue was purified with preparative TLC (hexane/EtOAc) to give **15g** (52.0 mg, 23.5%).

ESIMS: *m*/*z* 350 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **15g** (50.0 mg, 0.143 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with HP-20 (H_2O/CH_3CN) to give **16g** (16.0 mg, 38.2%).

^1H NMR (400 MHz, D_2O) δ 1.55 (2H, m, piperidiene), 1.85 (1H, m, piperidiene), 2.06 (2H, m, piperidiene), 3.35 (8H, m, piperidiene, CH_2), 7.37(1H, dd, *J* = 2.5, 6.5 Hz, phenyl), 7.60 (1H, d, *J* = 6.5 Hz, phenyl), 7.61 (1H, d, *J* = 2.5 Hz, phenyl).

FABMS: *m*/*z* 292 $(M-H)^-$.

4.19. 3-(4′-Hydroxy-4′-phenylpiperidine-1-yl)phthalic acid (16h)

To a DMSO (2.0 mL) solution of **12** (120 mg, 0.500 mmol), 4-hydroxy-4-phenylpiperidine (440 mg, 2.48 mmol) was added. The mixture was stirred at 80 °C for overnight. Water was added to the reaction mixture, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$, and then concentrated under reduced pressure. The residue was purified with preparative TLC (hexane/EtOAc) to give **15h** (41.0 mg, 20.7%).

ESIMS: *m*/*z* 398 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **15h** (60.0 mg, 0.151 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with SP-207 (H_2O/CH_3CN) to give **16h** (20.0 mg, 38.8%).

^1H NMR (400 MHz, $CDCl_3$+CD_3OD) δ 2.05 (2H, m, piperidiene), 2.64 (2H, m, piperidiene), 3.14 (2H, m, piperidiene), 3.66 (2H, m, piperidiene), 7.33 (1H, d, *J* = 7.6 Hz, phenyl), 7.41 (1H, dd, *J* = 7.3,7.6 Hz, phenyl), 7.54 (1H, d, *J* = 7.3 Hz, phenyl), 7.66 (5H, br s, phenyl).

FABMS: *m*/*z* 342 $(M+H)^+$.

4.20. 3-(4′-(Methoxy)piperidine-1-yl)phthalic acid (16i)

To a THF (5.0 mL) solution of **15e** (200 mg, 0.623 mmol), sodium hydride (75.0 mg, 3.12 mmol), methyl iodide (0.15 mL, 2.40 mmol) were added. The mixture was stirred at room temperature for overnight. Water was added to the reaction mixture, and then extracted with EtOAc three times. The combined organic layers were dried over anhydrous $MgSO_4$, and then concentrated under reduced pressure. The residue was purified with silica-gel column chromatography (hexane/EtOAc) to give **15i** (58.0 mg, 27.8%).

ESIMS: *m*/*z* 336 $(M+H)^+$.

To a 1,4-dioxane (1.0 mL) solution of **15i** (58.0 mg, 0.173 mmol), 5.0 mol/L NaOH solution (5.0 mL) was added. The mixture was stirred at 80 °C for overnight. 1.0 mol/L HCl solution was added to the reaction mixture to adjust the pH until pH2, and then concentrated under reduced pressure. The residue was purified with synthetic adsorption resin Sepabeads SP-207 (H_2O/CH_3CN) to give **16i** (40.0 mg, 82.9%).

^1H NMR (400 MHz, D_2O) δ 1.95 (2H, m, piperidiene), 2.16 (2H, m, piperidiene), 3.33 (3H, s, methyl), 3.40 (2H, m, piperidiene), 3.56 (2H, m, piperidiene), 3.70 (1H, m, piperidiene), 7.41 (1H, d, *J* = 6.6 Hz, phenyl), 7.62 (2H, m, phenyl).

ESIMS: *m*/*z* 278 $(M-H)^-$; FAB-HMS $(M+H)^+$ calcd for $C_{14}H_{18}NO_5$: 280.1185, found: 280.1185.

4.21. MBL inhibitory activity

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 *Escherichia 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 incubation for 20 min 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.

4.22. Mic

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.

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