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# Minimum structural requirements for cell membrane leakage-mediated anti-MRSA activity of macrocyclic bis(bibenzyl)s



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#### ABSTRACT

Macrocyclic bis(bibenzyl)-type phenolic natural products, found exclusively in bryophytes, exhibit potent antibacterial activity towards methicillin-resistant Staphylococcus aureus (anti-MRSA activity). Here, in order to identify the minimum essential structure for cell membrane leakage-mediated anti-MRSA activity of these compounds, we synthesized acyclic fragment structures and evaluated their anti-MRSA activity. The activities of all of the acyclic fragments tested exhibited similar characteristics to those of the macrocycles, i.e., anti-MRSA bactericidal activity, an enhancing effect on influx and efflux of ethidium bromide (EtBr: fluorescent DNA-binder) in Staphylococcus aureus cells, and bactericidal activity towards a Staphylococcus aureus strain resistant to 2-phenoxyphenol (4). The latter result suggests that they have a different mechanism of action from 4, which is a Fabl inhibitor previously proposed to be the minimum active fragment of riccardin-type macrocycles. Thus, cyclic structure is not a necessary condition for cell membrane leakage-mediated anti-MRSA activity of macrocyclic bis(bibenzyl)s.

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We have previously reported that macrocyclic bis(bibenzyl)type phenolic natural products, such as riccardin C (RC: 1) and isoplagiochin D (2), which are found exclusively in bryophytes,<sup>1,2</sup> exhibit potent antibacterial activity towards methicillin-resistant Staphylococcus aureus (anti-MRSA activity), comparable to that of the clinically used drugs vancomycin and linezolid.<sup>3–5</sup> Structural development studies of these macrocyclic bis(bibenzyl)s, prompted us to identify some synthetic bis(bibenzyl) derivatives such as 3, which exhibited more potent anti-MRSA activity. In studies to identify the minimum essential structure of these macrocyclic bis(bibenzyl)s for anti-MRSA activity, we previously prepared three fragments of RC, i.e., 4-6 (Fig. 1). Compound 6 is identical with lunularin,<sup>6</sup> which is a precursor in the biosynthetic pathway of bis(bibenzyl)-type cyclic phenolic natural products. Among these fragments, only the northern fragment, 2-phenoxyphenol (4) exhibited substantial anti-MRSA activity, with an MIC (minimum inhibitory concentration) of 8 µg/mL, while 5 and 6 were inactive (Table 1).

However, it turned out that the antibiotic activity of **4** is distinct from that of the macrocyclic bis(bibenzyl)s. For example, survival assay indicated that an excess concentration of 1 (4  $\times$  MIC concentration) shows potent bactericidal activity towards MRSA strains (survival rate is less than 1/10,000).<sup>7</sup> On the other hand, an excess concentration of  $\mathbf{4}$  (4 × MIC concentration) did not decrease the survival of these strains.<sup>7</sup> In other words, **4** exhibited bacteriostatic activity, not bactericidal activity. 1 affected the inflow and outflow of ethidium bromide (EtBr) through Staphylococcus aureus membrane, whereas **4** did not.<sup>7</sup> Therefore **4** is not a true minimum essential structure for the activity of the macrocyclic bis(bibenzyl)s.

To confirm the difference in mode of antibacterial action, we selected an MRSA mutant resistant to 32 mg/L 4 from Staphylococcus aureus strain N315, for which the genome has been fully sequenced.<sup>7</sup> We found that the macrocyclic bis(bibenzyl) 1 still exhibited potent activity towards this strain, whereas the activity of **4** was greatly reduced, as expected.<sup>7</sup> Based on these experiments, we concluded that the northern fragment **4** is not the minimum essential structure of **1** for potent anti-MRSA activity. In addition, our genetic studies indicated that the mutation in the 4-resistant strain was located in the enoyl-acyl carrier protein reductase gene *fabI*, i.e., the molecular target of **4** is FabI.<sup>7</sup> The molecular target of the parent macrocyclic bis(bibenzyl) derivative 1 remains to be identified, but is clearly different from FabI.

In continuing studies to identify the minimum essential structure of the macrocyclic bis(bibenzyl) derivatives, aimed at developing a new lead structure for anti-MRSA agents, we further synthesized two larger acyclic fragment structures, 7 and 8 and evaluated their anti-MRSA activities. These results are presented here.

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Figure 1. Chemical structures of the macrocyclic bis(bibenzyl)s (1-3) and their fragments (4-8), including those synthesized here (7, 8).

 Table 1

 Anti-MRSA activities of macrocyclic bis(bibenzyl) derivatives and their fragments

Compd	MIC (µg/mL) S. aureus		
	N-315	OM481	Compd <b>4</b> -resistant strain
1	2	2	2
3	0.5	0.5	0.5
4	8	8	>128
5	>128	>128	>128
6	>128	>128	>128
7	8	8	8
8	16	16	16
Vancomycin	1	1	N.T.

Compounds **7** and **8** were prepared basically by Suzuki coupling to construct the biphenyl framework, and subsequent Wittig reaction to attach the side chain phenethyl group.<sup>8</sup> Wittig reaction of (2',4-dimethoxy-4'-(methoxycarbonyl)[1,1'-biphenyl]-2-yl)methyl)triphenyl-phosphonium bromide (**9**) with benzaldehyde in the presence of K<sub>2</sub>CO<sub>3</sub> and 18-crown-6 ether afforded the stilbene derivative (**10**) as a mixture of geometrical isomers. This mixture was hydrogenated to afford the phenethyl derivative (**11**). The methoxycarbonyl group of **11** was reduced with lithium aluminum hydride to obtain the hydroxymethyl derivative (**12**). The hydroxymethyl derivative was treated with triphenylphosphine HBr, followed by Wittig reaction, and finally hydrogenation to afford the bis(phenethyl) derivative (**15**). Demethylation with BBr<sub>3</sub> gave the target compound **7**.

The hydroxyl group of methyl 4-hydroxy-3-methoxybenzoate (16) was treated with trifluoromethanesulfonic anhydride, and subsequent Suzuki coupling with (4-methoxy-2-methylphenyl) boronic acid afforded methyl biphenylcarboxylate derivative 18. 18 was reduced with lithium aluminum hydride to obtain the hydroxymethyl derivative (19). 19 was treated with triphenylphosphine HBr, followed by Wittig reaction, and finally hydrogenation to afford the phenethyl derivative (22). Demethylation with BBr<sub>3</sub> gave the target compound 8 (Scheme 1).

Anti-MRSA activities were determined by the liquid microdilution method, and are expressed in terms of minimum inhibitory concentration (MIC,  $\mu$ g/mL).<sup>9</sup> The MRSA strains used for the evaluation were OM481 and N315. OM481 is a clinical isolate from Okayama University Hospital Japan, and N315 is a MRSA strain isolated in 1982 in Japan as a prototype of Japanese hospital-associated MRSA strains.<sup>10</sup> The activity of these compounds towards a compound **4**-resistant MRSA strain was also measured. The results are summarized in Table 1.

The inflow of EtBr to energized *S. aureus* N315 cells was evaluated as the increase in fluorescence intensity after addition of the test compounds (excitation 530 nm and emission 600 nm).<sup>7</sup> The outflow of EtBr from ethidium-loaded *S. aureus* N315 cells was evaluated in terms of decrease in fluorescence intensity (excitation 530 nm and emission 600 nm).<sup>7</sup>

Measurement of intracellular ion concentrations were performed with the inductively coupled plasma optical emission spectrometry (ICP-OES) analysis with a VISTA-PRO (Seiko Instruments Inc.).<sup>7</sup>

These results are summarized in Figures. 2 and 3.

All of the new acyclic fragments exhibited substantial anti-MRSA activity. Although these compounds were less potent than macrocycles such as **1**, their activity was comparable to that of **4**. However, it is very important to note that **4** exhibited decreased activity towards the **4**-resistant mutant strain compared to the parent MRSA strain, as expected, whereas **7** and **8** exhibited comparable activities towards both MRSA strains. This result suggested that the mechanism of action of **7** and **8** might be distinct from that of **4**, i.e., the activities of **7** and **8** might not be mediated through inhibition of FabI.

Encouraged by this result, we evaluated the effects of these fragments on influx and efflux of EtBr in *S. aureus* N315 cells. The results are summarized in Figure 2. When the fragments were added to energized *S. aureus* N315 cells, the fluorescence of EtBr immediately increased (Fig. 2A and C), indicating increased intracellular binding of EtBr to N315 DNA. We also investigated the effect of the acyclic fragments on outflow of EtBr from EtBr-loaded *S. aureus* cells. Addition of the fragments caused a rapid decrease in EtBr-mediated fluorescence (Fig. 2B and D). These results indicate that all the acyclic fragments tested, increase the permeability of the cytoplasmic membrane of *S. aureus*.



**Scheme 1.** Synthetic routes to compounds **7** and **8**. Reagents and conditions: (a) benzaldehyde,  $K_2CO_3$ , 18-crown-6-ether,  $CH_2Cl_2$ , reflux, 11.5 h (90%); (b) 10%-Pd/C, 0.35 MPa H<sub>2</sub>, EtOAc, rt, 0.5 h (90%); (c) LAH, THF, 0 °C to rt, 14 h (95%); (d) P(Ph)<sub>3</sub>HBr, CH<sub>3</sub>CN, reflux, 13 h (94%); (e) benzaldehyde,  $K_2CO_3$ , 18-crown-6-ether,  $CH_2Cl_2$ , reflux, 14 h (90%); (f) 10%-Pd/C, 0.35 MPa H<sub>2</sub>, EtOAc, MeOH, rt, 2 h (78%); (g) BBr<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, -78 °C to rt, 3 days (quant); (h) Tf<sub>2</sub>O, Py, CH<sub>2</sub>Cl<sub>2</sub>, 0 °C to rt, 1.5 h (94%); (i) 4-methoxy-2-methylphenyl boronic acid, Pd(PPh<sub>3</sub>), K<sub>3</sub>PO<sub>4</sub>, DMF, 100 °C, 15.5 h (92%); (j) LAH, THF, 0 °C to rt, 14 h (95%); (k) PPh<sub>3</sub>HBr, CH<sub>3</sub>CN, reflux, 4 h (quant); (l) benzaldehyde, K<sub>2</sub>CO<sub>3</sub>, 18-crown-6-ether, CH<sub>2</sub>Cl<sub>2</sub>, reflux, 21.5 h (99%); (m) 10%-Pd/C, EtOAc, 0.31 MPa H<sub>2</sub>, rt, 1 h (quant); (n) BBr<sub>3</sub>, CH<sub>2</sub>Cl<sub>2</sub>, -78 °C to rt, 6 h (69%).



Figure 2. Effects of 7, 8 on the inflow (left) and outflow (right) of EtBr in S. aureus N315 cells.

We have previously reported that macrocyclic bis(bibenzyl)s, such as **1** and **3** exhibited rapid EtBr inflow and outflow. Two possibilities were considered for the increase of fluorescence intensity observed in intracellular EtBr. One was the increase of membrane permeability, and the other was the inhibition of multidrug efflux pumps that extrude EtBr. However we would be able to omit latter possibility, for, **1** and **3** caused a rapid decrease in EtBr fluorescence

from EtBr-loaded *S. aureus* N315 cells. If **1** and **3** inhibited efflux pumps, we would not be able to detect any changes in fluorescence in the EtBr outflow assay. These results strongly suggested that **1** and **3** increased the permeability of the cytoplasmic membrane of *S. aureus*.

Thus, both the inflow and outflow of EtBr suggest that **7** and **8** may not be an inhibitor of efflux pumps.



Figure 3. Effect of the representative fragment 7 on intracellular Na<sup>+</sup> and K<sup>+</sup> concentrations (A, B), and survival of S. aureus N315 (C).



**Figure 4.** Schematic illustration of the putative molecular targets and target sites of bis(bibenzyl) derivatives and their fragments for anti-MRSA activity.

We further confirmed the effect of **7** on membrane permeability by analyzing changes in the intracellular concentrations of Na<sup>+</sup> and K<sup>+</sup> (Fig. 3A and B). When *S. aureus* cells were treated with **7**, intracellular Na<sup>+</sup> concentration was increased (Fig. 3A), while K<sup>+</sup> concentration was decreased (Fig. 3B).

Figure 3C shows the results of survival assay of *S. aureus* N315. When **7** was added at the concentration of the MIC, the number of viable cells was reduced by at least three orders of magnitude, while a concentration of 4 times the MIC rapidly killed essentially all the cells. These results are consistent with the idea that **7** and **8** exhibit bactericidal (not bacteriostatic) activity by disrupting the membrane of *S. aureus*.

In conclusion, we synthesized acyclic fragment structures of macrocyclic bis(bibenzyl) derivatives, and evaluated their antibacterial activities towards methicillin-resistant *Staphylococcus aureus* in order to identify the minimum essential structure for cell membrane leakage-mediated anti-MRSA activity. The results showed that cyclic structure is not necessary for anti-MRSA activity. In contrast to the previously reported fragment 2-phenoxyphenol (**4**), which exhibited bacteriostatic anti-MRSA activity by inhibiting the function of FabI, the present compounds **7** and **8** appear to directly permeabilize the cell membrane of methicillin-resistant *Staphylococcus aureus* in a similar manner to the macrocyclic bis (bibenzyl)s, which cause membrane leakage mediated directly or

indirectly through the formation of mesosomes, intracellular structures formed by invagination of the cell membrane (Fig. 4).<sup>7</sup>

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- Morita, D.; Sawada, H.; Ogawa, W.; Miyachi, H.; Kuroda, T. Biochim. Biophys. Acta. 2015, 1848, 2057.
- 8. Physico-chemical properties of compounds **7** and **8**: *Compound* **7**: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 7.30–7.26 (m, 2H), 7.23–7.18 (m, 5H), 7.15–7.13 (m, 1H), 7.10 (d, *J* = 8.0 Hz, 1H), 6.96–6.92 (m, 3H), 6.84–6.81 (m, 2H), 6.79–6.76 (m, 2H), 5.00–4.72 (m, 2H), 3.00–2.90 (m, 4H), 2.74–2.69 (m, 4H), <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>);  $\delta$  = 155.94, 152.88, 143.35, 143.32, 141.91, 141.66, 132.37, 130.75, 128.60, 128.50, 128.47, 128.42, 127.73, 126.09, 126.03, 124.68, 120.71, 116.77, 115.24, 113.88, 37.94, 37.87, 37.35, 35.68. MS (FAB) 395 (M+H)<sup>+</sup>. HPLC purity was estimated to be 92.0% by means of reversed phase-HPLC, using a Pegasil ODS sp100 column (4.6 mm × 250 mm, Senshu Chemical, Japan) fitted on a JASCO HPLC system, with CH<sub>3</sub>CN/H<sub>2</sub>O = 2:1 v/v as the eluent and detection at 254 nm.

Compound 8: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  = 7.32–7.28 (m, 2H), 7.23–7.19 (m, 3H), 7.10 (d, J = 8.4 Hz, 1H), 6.99 (d, J = 8.0 Hz, 1H), 6.84–6.79 (m, 3H), 6.75 (dd, J = 8.0 Hz, 2.4 Hz, 1H), 4.96 (s, 1H), 4.77 (s, 1H), 2.99–2.89 (m, 4H), 2.12 (s, 3H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>):  $\delta$  = 155.50, 152.61, 143.09, 141.81, 139.42, 131.93, 130.39, 128.43, 128.36, 127.98, 125.95, 124.86, 120.60, 117.33, 115.00, 113.35, 37.69, 37.67, 19.91. MS (FAB) 305 (M+H)<sup>+</sup>. HPLC purity was estimated to be 98.0% by means of reversed phase-HPLC, using a Pegasil ODS sp100 column (4.6 mm × 250 mm, Senshu Chemical, Japan) fitted on a JASCO HPLC system, with CH<sub>3</sub>CN/H<sub>2</sub>O = 2:1 v/v as the eluent and detection at 254 nm.

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