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Membrane-active antimicrobial poly(amino-modified alkyl) β-cyclodextrins synthesized *via* click reactions†

Hatsuo Yamamura, *[ab] Miho Nonaka,[b] Shingo Okuno,[b] Ryogo Mitsuhashi,[b] Hisato Kato, [c] Takashi Katsu,[c] Kazufumi Masuda,[d] Koichi Tanimoto,[e] Haruyoshi Tomita[f] and Atsushi Miyagawa[ab]

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The emergence of drug-resistant bacteria has led to the high demand for new antibiotics. In this report, we investigated membrane-active antimicrobial β-cyclodextrins. These contain seven amino-modified alkyl groups on a molecule, which act as functional moieties to permeabilize bacterial cell membranes. The polyfunctionalization of cyclodextrins was achieved through a click reaction assisted by microwave irradiation. A survey using derivatives with systematically varied functionalities clarified the unique correlation of the antimicrobial activity of these compounds with their molecular structure and hydrophobicity/hydrophilicity balances. The optimum hydrophobicity for the compounds being membrane-active was specific to bacterial strains and animal cells; this led to specific compounds having selective toxicity against bacteria including multidrug-resistant pathogens. The results demonstrate that cyclodextrin is a versatile molecular scaffold for rationally designed structures and can be used for the development of new antibiotics.

Introduction

Antimicrobial resistance is a clear and present danger facing humanity. It is estimated that at least 700 000 people die every year from antibiotic-resistant infections.[1] In Europe, 25 000 deaths each year are caused by drug-resistant bacteria.[2] To combat antimicrobial resistance, the World Health Organization and national governments have encouraged the development of new antibiotics that utilize alternative mechanisms of action; many trials have been performed worldwide on these new antibiotics. It is within this context that antimicrobial peptides have become attractive as novel agents against drug-resistant bacteria.[3–6] The multiple positive charges of the peptides interact with the negatively charged bacterial cell membranes, and their multiple hydrophobic groups interact with the apolar lipid acyl chains in the membranes. This leads to membrane disruption and a broad-spectrum antimicrobial activity.[7,8] Resistance to membrane-active antimicrobial peptides develops more slowly than that to conventional drugs, as cell membrane alteration can be metabolically expensive.[5,9,10] Despite these advantages, applications of the peptides are limited by their high cytotoxicity and hemolytic activity as well as high production costs. Furthermore, their synthetic complexity means that it is difficult to introduce functional moieties into them that would allow their antimicrobial activity to be maximized and their toxicity to be minimized.

To solve the problems related to antimicrobial peptides and mimic their favorable biological activity, we have designed antimicrobial oligosaccharides.[11–14] A molecular scaffold that we found to be useful was a cyclic oligosaccharide called cyclodextrin (CD). The CD has a cone structure (about 1 nm in diameter) whose size is comparable to that of cyclic antimicrobial peptides gramicidin S and polymyxin B. The molecule is rimmed by hydroxyl groups which can be chemically modified. In order to mimic the polycationic regions of peptides, we synthesized γ-CD derivatives containing polyamino groups on the C6 positions (eight groups on the molecule) which strongly disrupted the bacterial membranes.[11] By adding benzyl groups as hydrophobic moieties to the amino groups, we were able to enhance membrane permeabilization and inhibit bacterial proliferation in a manner similar to that by natural peptides. These suggest that

[a] *Life and Applied Chemistry, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan.*
E-mail: cyamamura.hatsuo@nitech.ac.jp
[b] *Materials Science and Engineering, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan*
[c] *School of Pharmacy, Shujitsu University, 1-6-1 Nishigawara, Naka-ku, Okayama-shi, Okayama 703-8516, Japan*
[d] *Graduate School of Clinical Pharmacy, Shujitsu University, 1-6-1 Nishigawara, Naka-ku, Okayama-shi, Okayama 703-8516, Japan*
[e] *Laboratory of Bacterial Drug Resistance, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*
[f] *Department of Bacteriology and Laboratory of Bacterial Drug Resistance, Graduate School of Medicine, Gunma University, 3-39-22 Showa-machi, Maebashi, Gunma 371-8511, Japan*

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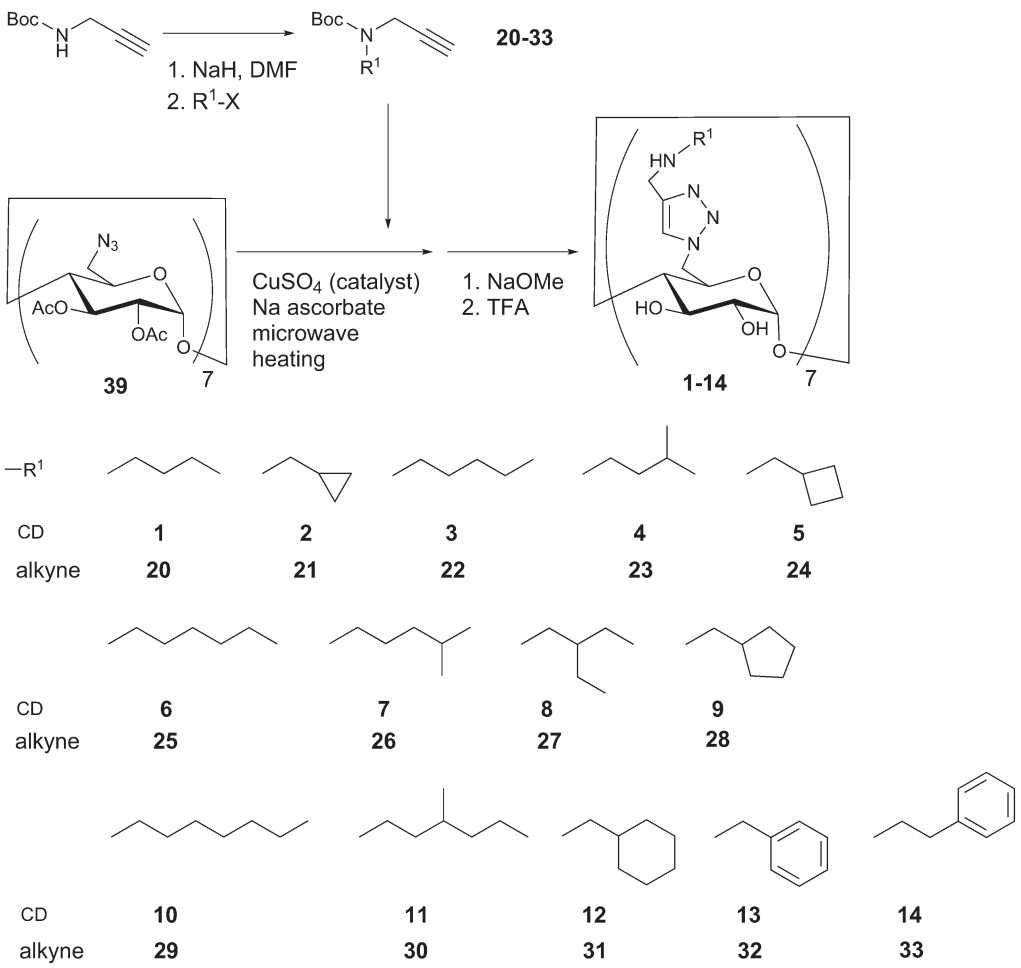
the amino groups and the hydrophobic benzyl moieties on the CD cooperatively work to be antimicrobial. Furthermore, we developed a microwave (MW)-assisted Huisgen 1,3-dipolar cycloaddition method that could be used for the poly-functionalization of CDs[15] and prepared γ-CD derivatives that contained alkylamino groups that could act as membrane-active functionalities.[12,13] Additional research conducted using glucose, maltose, maltooctaose and amylose found that the antimicrobial activity of these molecules depends on the number of functional groups on the molecular scaffold.[14] We therefore present in this paper a systematic survey of β-CD derivatives that possess a series of amino-modified alkyl groups (seven groups per CD molecule) as antimicrobial functional groups. This study is expected to provide greater insight into the correlation of the structure–antimicrobial activity of CD derivatives for the sake of developing novel antibiotics to combat pathogens.

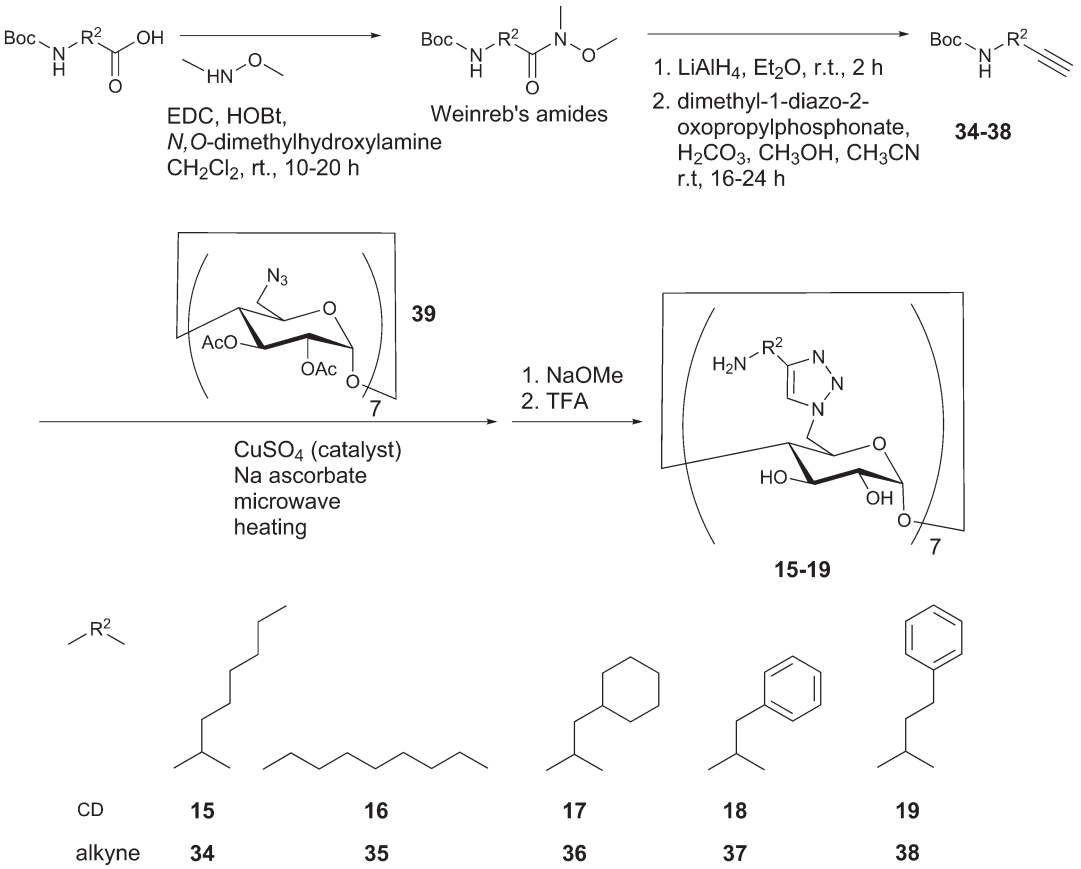
Results and discussion

Chemistry

We prepared β-CD derivatives **1–19**, which were hepta-modified with 19 types of amino-modified alkyl groups (Schemes 1 and 2). Compounds **1–14** had R^1–NH–CH_2 moieties linked to the triazole rings (Scheme 1); in other words, they were secondary amines possessing alkylamino groups. The alkyl groups (R^1) varied from butyl to heptyl groups and included isomeric linear, branched, and ring structures. Compounds **13** and **14** incorporated aromatic benzene moieties. The CD secondary amines **1–14** were prepared by an MW-assisted Huisgen 1,3-dipolar cycloaddition of per-2,3-acetylated β-CD heptaazide (**39**)[16] using the corresponding Boc-protected propargyl-alkylamines **20–33** (Scheme 1). The alkynes were obtained by alkylation of the Boc-protected propargylamines with the corresponding haloalkanes. Click reactions using the alkynes were completed in 10 min by MW heating (120 °C), which attached the seven amino-modified alkyl groups onto the β-CD molecules. Deprotection of the acetyl groups and the Boc groups produced the desired CD secondary amines **1–14** as trifluoroacetic acid (TFA) salts. Compounds **15** and **17–19** had 1-aminoalkyl moieties on the triazoles, which are, more specifically, 1-aminoheptyl (**15**), 1-amino-2-cyclohexylethyl (**17**), 1-amino-2-phenylethyl (**18**), and 1-amino-3-phenylpropyl (**19**) moieties, respectively (Scheme 2). Whereas the amino groups of **15** and **17–19** existed on the α carbon linked to the triazole ring, the



Scheme 1 Preparation of β-CD secondary amines **1–14** *via* MW-assisted click reactions using alkynes **20–33**.



Scheme 2 Preparation of β-CD primary amines **15–19** using alkynes **34–38** to introduce primary amine-modified alkyl groups into them using an MW-assisted click reaction.

7-aminoheptyl CD (**16**) had terminal amino groups. **15–19** were primary amines and isomers of the corresponding secondary amines shown in Scheme 1. The primary aminoalkyl groups were introduced onto the CDs through the click reaction with the corresponding alkynes **34–38** (Scheme 2). The alkynes were prepared from the corresponding *N*-Boc amino acids according to chemistry with the Ohira–Bestmann modification of the Gilbert–Seyferth reaction.[17–19] Each of the amino acids reacted with *N*,*O*-dimethylhydroxylamine to produce the corresponding Weinreb amides. The reduction of the amides with LAH produced aldehydes that were subsequently converted into the alkynes **34–38** *via* reaction with dimethyl-1-diazo-2-oxopropylphosphonate through Horner–Wadsworth–Emmons-type reactions, loss of nitrogen, and rearrangement of the resulting alkenylidenecarbenes. The MW-assisted Huisgen reaction of the azide **39** with the alkynes **34–38**, followed by acetyl and Boc group deprotection promoted the per-functionalization of the β-CD molecules to efficiently produce **15–19**. These demonstrated that the click reaction is applicable to both the poly-primary and secondary amino modification of CD molecules. The good results of the syntheses of the per-functionalized β-CD derivatives as well as those of γ-CD[12,13] and other sugar derivatives[14] confirm that MW-assisted click reactions are advantageous for sugar molecule polyfunctionalization.

Antimicrobial activity

The inhibition of bacterial proliferation by the CDs was evaluated using minimum inhibitory concentration (MIC) values against Gram-positive *Staphylococcus aureus* and *Bacillus subtilis* strains and Gram-negative *Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* strains (Table 1). Unmodified native β-CD was not antimicrobial (>113 μM). In contrast, most of the CD secondary amines **1–14** were found to effectively inhibit the growth of the two Gram-positive bacteria (MIC values = 1–11 μM). The only two exceptions were cyclopropylmethyl-modified derivative **2** (MIC value >45 μM against *S. aureus* and *B. subtilis*) and cyclopentylmethyl derivative **9** (MIC value 21 μM against *B. subtilis*). The benzene-containing CD derivatives **13** and **14** were also found to be antimicrobial as well as the aliphatic group-containing CDs. The CD primary amines (**15–19**) were also found to inhibit the growth of Gram-positive bacteria. Their MIC values were almost comparable to those of their corresponding secondary amine isomers (more specifically, **15** and **16** corresponded to **6**, **17** corresponded to **12**, **18** corresponded to **13**, and **19** corresponded to **14**). The derivative **15**, whose *n*-heptyl moiety was internally aminated, and the terminally aminated **16** showed similarly good antimicrobial activity against the two Gram-positive bacteria. Against the Gram-negative bacteria,

Table 1 Minimum inhibitory concentration (MIC, μM) of CD derivatives against both Gram-positive *S. aureus* and *B. subtilis* strains and Gram-negative *E. coli*, *S. typhimurium*, and *P. aeruginosa* strains[a]

Compounds	Carbon number[b]	log *P*[c]	Gram-positive strains		Gram-negative strains		
			S. aureus	*B. subtilis*	*E. coli*	*S. typhimurium*	*P. aeruginosa*
Secondary amines							
1	5	−0.90	11	11	>44	>44	>44
2		−1.40	>45	>45	>45	>45	>45
3	6	−0.48	3	1	43	>43	>43
4		−0.57	3	1	>43	>43	>43
5		−1.00	5	3	>43	>43	>43
6	7	−0.06	3	3	5	10	10
7		−0.15	3	3	10	10	5
8		−0.08	3	3	10	42	42
9		−0.58	1	21	21	>42	>42
10	8	0.36	5	5	20	20	40
11		0.27	3	10	5	10	10
12		−0.16	3	3	5	10	20
13		−0.26	5	5	21	21	21
14	9	−0.13	3	3	10	10	10
Primary amines							
15	7	−0.18	3	5	10	10	21
16		−0.43	3	5	>42	>42	>42
17	8	−0.35	10	3	10	10	20
18		−0.67	21	5	21	>41	>41
19	9	−0.25	20	5	20	>40	>40
Polymyxin B			23	6	1	1	1
Gramicidin S[d]			7		28		56

[a] MICs were determined by the liquid microdilution method. [b] Number of carbons in the substituent on a triazole ring. [c] Values of the corresponding substituted glucose. [d] See ref. 22.

we were able to observe unique antimicrobial activities. Among the secondary amines, **6**, **7**, **11**, **12**, and **14** exhibited good activity. More specifically, the 4-methylpentyl CD **7** had a MIC value against *P. aeruginosa*, which is comparable to that of antimicrobial peptide polymyxin B. Increasing the size of the cycloalkyl moiety led to increased antimicrobial activity [order of the MIC values for such compounds: cyclopropyl (**2**) ≈ cyclobutyl (**5**) > cyclopentyl (**9**) > cyclohexyl (**12**)]. Among the linear alkyl groups (**1**, **3**, **6**, and **10**), the medium-size hexyl (**6**) group demonstrated the best activity. The benzene-containing CDs **13** and **14** showed good antimicrobial activity, and their MIC values were slightly larger than that of the cyclohexane-containing derivative (**12**). The primary amines **15** and **17** exhibited good antimicrobial activity, and their MIC values were almost the same as those of their secondary amine isomers **6** and **12**, respectively. However, the activities of the phenyl-modified primary amines **18** and **19** were slightly less than those of the secondary amine isomers **13** and **14**, respectively. Interestingly, the terminally aminated *n*-heptyl compound (**16**) showed much less activity than its internally aminated isomer (**15**) against the three Gram-negative bacteria. From our results, it appears that the activity of the CDs against the Gram-positive bacteria was stronger than that against the Gram-negative bacteria; the results are comparable to those found for the membrane-active antimicrobial peptide gramicidin S.[20]

Hemolytic activity

We examined the hemolysis of red blood cells from rabbits with the CDs to determine whether the CDs disrupt the membranes of animal cells (Table 2). The *n*-heptyl-aminomethyl CD (**10**), which was found to have strong antimicrobial activity (MIC value against *S. aureus*, 5 μM), exhibited significant hemolysis (116% at 20 μM), whereas the less antimicrobial cyclopropylmethyl-aminomethyl CD (**2**) (MIC value against *S. aureus*, >45 μM) was a lot less hemolytically active (≃0% at 20 μM). Because membrane-active peptides such as gramicidin S are often known to disrupt both bacterial and red blood cell membranes,[21,22] it is reasonable for the antimicrobial and hemolytic activities of individual CD compounds to correlate. As such, the MIC values should be inversely proportional to the observed hemolysis values, as was found to be the case for CDs **2**, **6**, **10**–**12**, **15**, and **17**–**19** (Fig. 1). However, we did find that some of the CD derivatives deviated from such correlations, in that they exhibited strong antimicrobial activity but weak hemolytic activity, which means that they exhibited selective toxicity against bacteria: **3**–**5**, **8**, **9**, **13**, and **16** were derivatives that exhibited such traits. It is noteworthy that a ratio of the concentration producing 50% hemolysis divided by MIC as a therapeutic index for the β-CDs **3**–**5**, **8**, **9**, **13**, and **16** may be >10 for Gram-positive bacteria.

Antimicrobial activity against drug-resistant Gram positive bacteria

The antimicrobial activity of the CDs against clinically isolated multidrug-resistant Gram-positive bacteria was examined (Table 3). Against methicillin-resistant *Staphylococcus aureus* (MRSA), the CD derivatives, *n*-pentyl- (**3**), 3-methylbutyl- (**4**), 2-ethylbutyl- (**8**), and cyclopentylmethyl- (**9**)

Table 2 Hemolysis induced by the CD derivatives[a]

Compounds	Carbon number[b]	log P[c]	Hemolysis (%) Concentration of CD (μM) 50	20	10	5	2	1
Primary amines								
1	5	−0.90	≃0	≃0	≃0	≃0	≃0	≃0
2		−1.40	≃0	≃0	≃0	≃0	≃0	≃0
3	6	−0.48	1	≃0	≃0	1	≃0	≃0
4		−0.57	≃0	≃0	≃0	≃0	≃0	≃0
5		−1.00	≃0	≃0	≃0	≃0	≃0	≃0
6	7	−0.06	110	64	25	7	4	≃0
7		−0.15	56	23	15	7	5	5
8		−0.08	12	8	8	3	1	5
9		−0.58	8	2	1	1	1	2
10	8	0.36	137	116	110	85	39	9
11		0.27	119	108	99	73	30	10
12		−0.16	107	78	38	13	≃0	≃0
13		−0.26	11	3	2	4	4	2
14	9	−0.13	40	23	12	7	3	3
Secondary amines								
15	7	−0.18	118	113	109	101	37	10
16		−0.43	3	4	3	4	1	3
17	8	−0.35	118	117	107	89	31	13
18		−0.67	4	4	6	3	4	5
19	9	−0.25	113	94	47	17	6	4

[a] Rabbit erythrocytes were incubated with a CD derivative at 37 °C for 20 min, and hemolysis was estimated by measuring the absorbance at 540 nm. Lysolecithin (50 μM) was used to determine the 100% level of hemolysis. [b] Number of carbon in the substituent on a triazole ring. [c] Values of the corresponding substituted glucose.

aminomethyl CDs which were less hemolytic, exhibited MIC values of around 20 μM. Furthermore, there were CD derivatives that were good at inhibiting the growth of clindamycin (ClDM)-resistant *Streptococcus agalactiae*. While the MIC value of ClDM against the bacterium was *ca.* 300 μM and that of erythromycin (EM) was >174 μM, the MIC values of **3** and **8** were notably in single digits (3 μM) and those of **4** and **9** were around 10 μM. The MIC values for *Enterococcus faecium* [MICs of EM, >174 μM; gentamicin (GM), >536 μM; kanamycin (KM), >528 μM; and streptomycin (SM), 440 μM] were almost similar to those for MRSA. In contrast, the antimicrobial activities of the CDs against vancomycin-resistant *Enterococcus* (VRE) [MIC values of the following antibiotics against this bacterium are vancomycin (VCM), >174 μM; ampicillin (ABPC), 366 μM; EM, >174 μM; ciprofloxacin (CPFX), >386 μM; GM, >536 μM; and KM, >528 μM] were a lot less. Only the 2-ethylbutyl-modified CD (**8**) exhibited MIC values at around 20 μM. None of the CDs was found to result in MIC values of less than 40 μM against *E. faecalis* [the MIC values of the antibiotics minocycline (MINO) and SM were 65 and 110 μM, respectively]. The evaluation against the drug-resistant clinical isolates exhibited that the β-CDs **3**, **4**, **8**, and **9** maintained activity against resistant pathogens. These are interesting results and may be a foundation for further drug development.

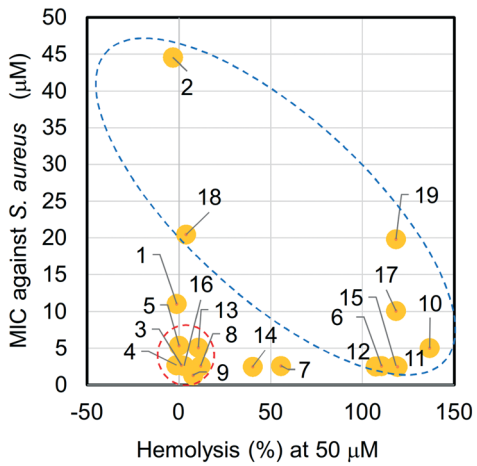


Fig. 1 Relationship between the antimicrobial activity of β-CD derivatives against *S. aureus* and their hemolytic activity. The antimicrobial activities of the derivatives in the area demarcated by the blue-dashed line correlated with their hemolytic activities, whereas those contained within the red-dashed circle had high antimicrobial activities but low hemolytic activities.

Bacterial membrane disruption

The above observed antimicrobial activity of β-CDs can be attributed to membrane disruption. Bacteria contain large concentrations of K^+ (100–500 mM) within their cells, and permeabilization of their membranes causes efflux of the ion.[24,25] To examine the cytoplasmic membrane disruption abilities of β-CDs, we treated Gram-positive *S. aureus* with the *n*-alkyl-aminomethyl β-CD **1**, **3**, **6** and **10** and monitored the K^+ efflux from the bacterial cells using a K^+ electrode, where

Table 3 Minimum inhibitory concentration (MIC, μM) of CD derivatives against Gram-positive clinically isolated MRSA, *E. faecium*, *E. faecalis*, VRE, and *S. agalactiae* strains[a]

	Drug-sensitive strain	Drug-resistant strains				
Compounds	Methicillin-sensitive *S. aureus*	Methicillin-resistant *S. aureus*	*S. agalactiae*	*E. faecium*	*E. faecalis*	Vancomycin-resistant *Enterococcus*
Primary amines						
1	22	>44	>44	>44	>44	>44
3	5	21	3	21	>43	>43
4	5	21	11	21	>43	>43
5	11	>43	>43	>43	>43	>43
6	10	21	10	10	>42	42
7	10	21	5	10	>42	42
8	5	21	3	10	>42	21
9	5	21	10	21	>42	>42
10	40	>40	>40	>40	>40	>40
11	20	40	20	20	>40	40
12	10	40	40	20	>40	40
13	5	41	21	41	>41	41
14	5	20	10	20	40	40
Secondary amines						
15	10	10	10	21	42	21
16	21	42	42	21	>42	>42
17	10	20	20	20	40	20
18	21	41	21	21	>41	>41
19	20	20	20	20	40	40

[a] The MICs were determined by the agar dilution method according to CLSI recommendations.[23]

changes to cell viability were simultaneously measured.[11,12] The *n*-pentyl-modified derivative (**3**) that exhibited good activity against resistant pathogens showed significant K^+ efflux (Fig. 2), while the *n*-butyl CD **1** showed very less efflux. Native β-CD showed no efflux. Also, it was found that increases in the K^+ efflux were proportional to decreases in the cell viability. These results provided direct evidence that **3** disrupted the cytoplasmic membrane of *S. aureus* and subsequently killed the bacteria. The CD concentrations producing 50% K^+ efflux (ED_{50}) and those producing a 50% decrease in bacterial cell survival (LC_{50}) for β-CDs **1**, **3**, **6**, and **10** are summarized in Table 4. All these derivatives showed good correlation between K^+ efflux and cell viability. We therefore concluded that the point of action of the CDs was the cytoplasmic membranes of the Gram-positive bacteria, and these CDs were bactericidal.

Relationship between hydrophobicity and antimicrobial and hemolytic activity

In this study, we observed a unique correlation between the hydrophobicity of the CD molecules and their antimicrobial activity and hemolytic activities. The partition coefficient logarithm ($\log P$) is a measure of the hydrophobicity of a compound; $\log P$ is proportionally related to a compound's hydrophobicity. We determined the presence of an optimum region of $\log P$ values of the corresponding substituted glucose of β-CDs that were antimicrobial against resistant pathogens (Fig. 3). Against MRSA, ClDM-resistant *S. agalactiae* and *E. faecium*, the CD derivatives (including **3**, **4**, **8**, and **9**) with $\log P$ values between −0.6 and −0.06, exhibited MIC values of around 20 μM or less. In contrast, against VRE, the CDs including **8**, whose $\log P$ values were from −0.35 to −0.08, exhibited MIC values at around 20 μM. The hydrophobicity of CD molecules was also found to affect their abilities to disrupt animal cell membranes. The correlation between red blood cell hemolysis of the CDs and their $\log P$ values is shown in Fig. 3. The membrane-permeabilizing activity against red blood cells drastically changed at $\log P$ values of around −0.2, and the more hydrophilic CDs showed almost no hemolytic activity. This correlation is different from that observed above for the antimicrobial activity of the CDs. Therefore, the difference enables CDs with selective toxicity against Gram-positive bacteria to be identified in such as **3**, **4**, **8**, and **9**.

The observed correlation between the membrane activity and antimicrobial activity with the hydrophobicity of the β-CDs suggests that β-CD derivatives aminated with suitable hydrophobic moieties can be membrane active and therefore antimicrobial. A CD that is too hydrophilic and has small negative values for $\log P$ can interact with a bacterial cell membrane's anionic surface, but it will not be able to penetrate into the apolar hydrocarbon core of the lipid bilayer membrane. If a CD is too hydrophobic and has a large positive $\log P$ value, then it may disturb the polar surface interaction. The unique activities of the CDs against the bacteria may be due to the differences between the bacterial cell membranes and the lipid molecules. The cytoplasmic membrane of Gram-positive bacteria such as *S. aureus* is rich in anionic phosphatidylglycerol and cardiolipin, respectively, whereas that of Gram-negative *E. coli* is rich in neutrally charged phosphatidylethanolamine.[28] Therefore, the CDs

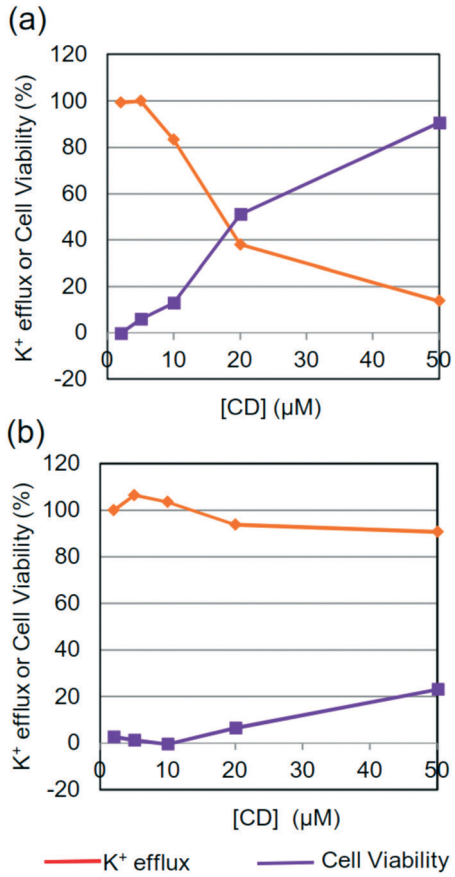


Fig. 2 Dose-dependence curves for the K^+ efflux and the cell viability of *S. aureus* on the addition of CDs ((a) **3** and (b) **1**). The purple lines indicate K^+ efflux, and the orange lines indicate cell viability. The cells were incubated with the CD derivatives for 30 min at 37 °C. To determine the level of 100% K^+ efflux, melittin (10 μM for *S. aureus*) was used.

Table 4 ED_{50} (CD concentration giving 50% K^+ efflux, μM) and LC_{50} (CD concentration giving 50% bacterial cell viability, μM) for CDs **1**, **3**, **6**, and **10**[a]

	S. aureus	
	K^+ efflux	Cell viability
Compounds	ED_{50}/μM	LC_{50}/μM
1	>50	>50
3	20	20
6	15	15
10	30	15
β-CD	>50	>50
Gramicidin S[b]	2–5	2–5
Melittin[c]	0.2–0.5	0.2–0.5

[a] Cells were incubated with a CD or a peptide for 30 min at 37 °C. [b] See ref. 26. [c] See ref. 27.

possessing cationic amino moieties can be more active against Gram positive bacteria than Gram negative pathogens. The outer membrane of Gram-negative bacteria may be associated with the less antimicrobial activity of the CDs. Moreover, the unique bacterial selectivity was observed in Gram-positive resistant pathogens (*S. agalactiae* > *E. faecium* ≥ MRSA > VRE > *E. faecalis*). The CDs may discriminate between the different bacterial cell membranes. The red blood cell membrane permeation of the CDs may also be related to the lipids that the cells contain, such as phosphatidylcholine and sphingomyelin.[28] The neutral lipids seem to prefer the relatively hydrophobic CDs. Thus, the lowest log*P* value (≈−0.2) being hemolytically active may lead to the selective toxicity of the CDs **3**, **4**, **8** and **9** against the Gram-positive pathogens as described above. The antimicrobial and hemolytic activities observed for the CDs in this study showed that the membrane activity of the CDs depended on their hydrophobicity (or a balance of their hydrophobicity and hydrophilicity). Aminoglycoside-derived amphiphiles have shown characteristic relationships between hydrophobicity and antimicrobial activity, and this has led to them having excellent activity against Gram-negative bacteria such as *P. aeruginosa*.[29,30] The relationship for the CDs in this study is different from those found for the antibiotics and is unique.

Conclusion

In this study, we developed membrane-active and antimicrobial β-CD derivatives containing a variety of amino-modified alkyl substituents that included primary and secondary amino groups. The β-CDs exhibited good antimicrobial activities similar to those found for natural peptides. The activity of the CD derivatives observed in this study may reflect the characteristic cell membranes of bacteria, which could lead to selective activity against bacteria. CD derivatives may therefore be promising agents against Gram-positive bacteria. In particular, the *n*-pentyl- (**3**), 3-methylbutyl- (**4**), 2-ethylbutyl- (**8**), and cyclopentyl- (**9**) aminomethyl CDs were found to have strong antimicrobial activity against drug-resistant Gram-positive bacteria such as clindamycin-resistant group B *Streptococcus* and far less toxicity against red blood cells. Considering the results of the γ-CD analogs,[12,13] glucose, maltose, maltooctaose and amylose derivatives,[14] the seven and eight alkylamino substituents on the molecule are qualified for expressing antimicrobial activity, which suggests that β-CD molecules could be good molecular scaffolds for antibacterial derivatives. Moreover, the molecular hydrophobicity may be related to the membrane activity and therefore the antimicrobial activity of the CD derivatives. The antimicrobial activity of the CDs described in this paper is comparable to that found for antimicrobial peptides such as gramicidin S and polymyxin B; at present it is not superior to daptomycin. However, the versatile derivatization of CDs may allow rationally designed structures with desired activities to be developed for new antibiotic compounds. The MW-assisted poly click reaction is a powerful tool for synthesizing these derivatives. The total yield of the antimicrobial derivatives from native CDs here can reach 65% (with five steps), which suggests that CDs are practical compounds for the development of novel antibiotics. CD derivatives such as 1-aminoheptyl- (**15**) and 1-amino-2-cyclohexylethyl CDs (**17**)

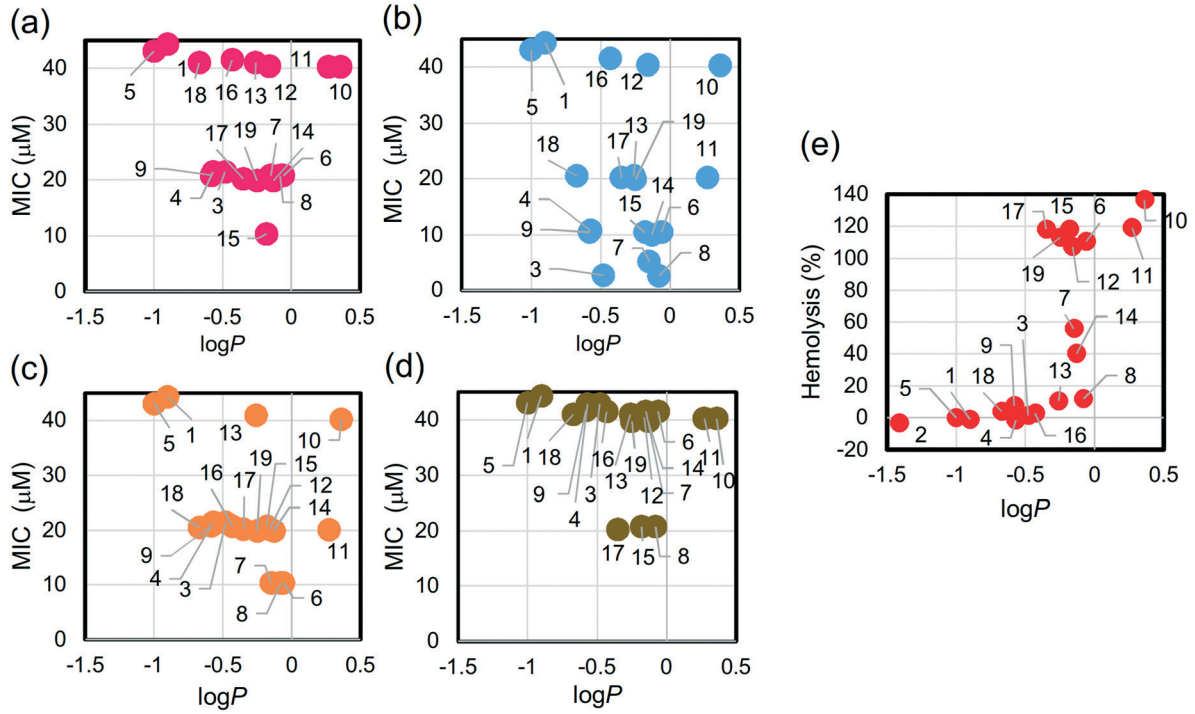


Fig. 3 Relationship between the antimicrobial and hemolytic activity of β-CD derivatives **1–19** and their hydrophobicities (the log *P* values of the corresponding substituted glucoses). Plots are shown for (a) MRSA, (b) *S. agalactiae*, (c) *E. faecium*, (d) VRE and (e) hemolytic activity (CD concentration, 50 μM).

were found to have strong activity against bacteria including drug-resistant pathogens but significant hemolytic activity; they could therefore be well-suited for topical use in treating infectious skin diseases as the case of the peptide gramicidin S. In addition, the preliminary experiment of resistance development showed that the resistance of *S. aureus* to the *n*-hexylaminomethyl CD (**6**) did not develop by multipassage treatment (see the ESI†). These suggest that the antimicrobial CDs are promising and further studies including resistance development experiment of **3**, **4**, **8**, and **9** found here are underway.

Experimental section

General chemistry

^{1}H spectra were recorded at 30 °C on a Bruker AVANCE400Plus Nanobay and AVANCE500US CryoProbe. MALDI-TOF mass measurements were carried out with a JEOL JMS-S3000 spectrometer. The microwave reactor used was a Biotage Initiator EXP used for the Huisgen reaction. The log *P* values of the corresponding substituted glucose of β-CDs were obtained using CambridgeSoft ChemDraw Professional 15.1.

General procedure for synthesis of *N*-alkyl-Boc-propargylamines 20–33 for preparation of CD secondary amines

N-2-Ethylbutyl-Boc-propargylamine **27** was prepared as follows. Boc-propargylamine (1.0 g, 6.45 × 10^{-3} mol) was stirred with 55% NaH (570 mg, 1.30 × 10^{-2} mol) in DMF (30 cm^3) for 30 min under argon. After addition of 1-bromo-2-ethylbutane (2.14 g, 1.94 × 10^{-2} mol) the reaction mixture was stirred for 12.5 h. After addition of a small amount of methanol and then EtOAc the reaction mixture was washed with water and saturated aq. NaCl. It was dried with anhydrous Na_2SO_4 and concentrated *in vacuo*. Silica gel chromatography (hexane/AcOEt) yielded the corresponding alkyne **27** as oil (1.22 g, 79.2%). The other derivatives were prepared similarly. Spectral details of **20–33** are given in the ESI.†

General procedure for synthesis of Boc-protected amino-alkyne 34–38 for preparation of CD primary amines

1-Cyclohexylmethyl-Boc-propargylamine **36** was prepared as follows. Boc-L-cyclohexylalanine (1.05 g, 3.88 × 10^{-3} mol), EDC·HCl (876 mg, 4.56 × 10^{-3} mol), and HOBt (562 mg, 4.16 × 10^{-3} mol) were dissolved in CH_2Cl_2 (15.7 cm^3). The solution was stirred at rt for 30 min under argon. *N*,*O*-Dimethylhydroxylamine·HCl (423 mg, 4.34 × 10^{-3} mol) and *N*-methylmorpholine (460 mg, 4.50 × 10^{-3} mol) were added and the solution was stirred for 16 h. The solution was evaporated *in vacuo*, diluted with EtOAc, and then washed with 1 M dHCl, saturated aq. $NaHCO_3$, and saturated aq. NaCl. It was dried with anhydrous Na_2SO_4 and concentrated *in vacuo*. Silica gel chromatography (hexane/AcOEt) yielded the corresponding Weinreb amide as oil (976 mg); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.87–2.05 (22H, cyclohexylmethyl, Boc), 3.20 (s, 3H, CH_3–N–), 3.78 (s, 3H, CH_3–O–), 4.70–4.80 (1H, Boc-NH–C<u>H</u>–), 5.02 (d, J = 9.6, 1H, Boc-N<u>H</u>–). The amide (717 mg) was

dissolved in Et_2O (12.2 cm^3) and cooled to 0 °C under argon. $LiAlH_4$ (95 mg, 1.85×10^{-3} mol) in Et_2O (3.5 dm^3) was added and the mixture was stirred for 2 h at 0 °C. 5% aq. $NaHSO_4$ was added and diluted with Et_2O. The organic layer was washed with water and saturated aq. NaCl. It was dried with anhydrous Na_2SO_4, concentrated *in vacuo*, and dissolved in EtOH. Dimethyl(1-diazo-2-oxopropyl)phosphonate (495 mg, 2.58×10^{-3} mol) and K_2CO_3 (888 mg, 4.69×10^{-3} mol) were added and the reaction mixture was stirred at rt under argon for 20 h. The solution was concentrated *in vacuo*, diluted with Et_2O, and washed with water and saturated aq. NaCl. The organic layer was dried over Na_2SO_4 and concentrated *in vacuo*. Silica gel chromatography (hexane/AcOEt) yielded the corresponding alkyne **36** (361 mg, 53.8%, 2 steps). The other derivatives were prepared similarly. Spectral details of **34–38** are given in the ESI.†

General procedure for synthesis of CD amines 1–19

Preparation of 1-amino-2-phenylethyl-modified **18** was performed as follows. A reaction solution was prepared by dissolving per-2,3-acetylated β-CD octaazide **39** (19.0 mg, 1.00×10^{-5} mol) in DMSO–H_2O (10 : 1) (1.1 cm^3) containing the alkyne **37** (21.4 mg, 1.25 mol eq. of an azide group), $CuSO_4 \cdot 5H_2O$ (1.75 mg, 0.1 mol eq.), and sodium ascorbate (17.3 mg, 1.25 mol eq.). After MW heating (120 °C, 10 min), ethyl acetate was added followed by washing with 5% aq. EDTA. Silica gel column chromatography (CH_2Cl_2/methanol) gave the click reaction product (33.0 mg). Deprotection of the acetyl groups with NaOMe–MeOH followed by that of the Boc group with TFA gave the desired product **18** (76.3%, overall yield). Other derivatives were prepared similarly. Spectral details of **1–19** are given in the ESI.†

Bacteria

The microorganisms *Bacillus subtilis* 168, *Staphylococcus aureus* FDA 209P, *Escherichia coli* K12 W3110, *S. typhimurium* LT2, and *Pseudomonas aeruginosa* PAO1 were used as drug-sensitive bacteria. Drug-resistant bacteria MRSA (methicillin-resistant *Staphylococcus aureus*) MS29202, *Enterococcus faecium* MS29120, *Enterococcus faecalis* MS29030, VRE (vancomycin resistant *Enterococcus*) MS29016, and *Streptococcus agalactiae* Str.B-1 were clinical isolates. Their MIC values against conventional antibiotics are shown in the ESI.†

Minimum inhibitory concentration (MIC)

MICs against *B. subtilis*, *S. aureus*, *E. coli*, *S. typhimurium*, and *P. aeruginosa* were determined by the liquid microdilution method, using serially diluted (two-fold) CDs. Cells (1×10^4) were cultured at 37 °C for 20 h in Mueller–Hinton broth (0.1 cm^3) containing CDs in a 96-well microtiter plate. The MIC was determined as the lowest concentration of CD at which cells were unable to grow. The MICs against MRSA, *S. agalactiae*, *E. faecium*, *E. faecalis*, and VRE were determined by the agar dilution method according to CLSI recommendations.[23] Overnight cultures of the strains grown in broth were diluted 100 times with fresh broth. One loopful (5 μl, about 10^5 to 10^4 CFUs (Colony Forming Units) of bacterial cells) of each dilution was plated using a Microplanter (Sakuma: Tokyo, Japan) on agar plates containing the drug. The growth of the bacteria was determined after incubation of the plates at 37 °C for 18–20 hours.

Hemolytic activity

Rabbit erythrocytes obtained from rabbit blood (NIPPON BIO-TEST LABORATORIES INC.) were suspended in buffer (150 mM NaCl/10 mM Hepes-NaOH, pH 7.4) at a final concentration of 0.5% hematocrit. After incubation with CDs at 37 °C for 30 min, hemolysis was estimated by measuring the absorbance at 540 nm. Lysolethicin (50 μM) was used to determine the 100% level of hemolysis.

K^+ efflux and cell viability

Bacterial cells were washed twice with a buffer (100 mM choline chloride/50 mM Mops-Tris, pH 7.2) and suspended in this buffer at 2×10^9 cells per cm^3. The final volume of the cell suspension was 1 cm^3. The cells were incubated with the CD at 37 °C for 30 min. After incubation, 0.1 cm^3 of the cell suspension was taken, diluted with physiological saline, and dispersed on an agar plate prepared with 1% polypeptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar (pH was adjusted by adding 1 M NaOH). The colonies were counted after being left to stand overnight at 37 °C and the viability of the cells was determined. The remaining cell suspension was centrifuged, and the amount of K^+ in the supernatant was measured using a K^+-selective electrode. Melittin (10 μM) was used to determine the 100% level of K^+ efflux from *S. aureus*.

Conflicts of interest

There are no conflicts of interest to declare.

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