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A New Class of Multi-Armed Antibiotics with Low Risk of Resistance

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Antibiotic resistance of pathogenic bacteria is a serious threat to public health. New antibacterial agents with novel structures or targets are urgently needed. Here we discover a new class of multi-armed antibiotics (MAAs) structurally distinct from known antibiotics. MAAs possess a multi-armed structure composed of a nucleus like ethylene, carbon, benzene, nitrogen or triazine, and three or four symmetrical arms like phenylbenzoic acid or 4-methynylbenzoic acid. The independence from a fixed functional moiety greatly increases their molecular diversity and avoids the rapid emergence of resistance. MAAs have excellent antibacterial activities against Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus*. The action mechanism involves selective internalization into Gram-positive bacteria, inhibition of cell wall assembly and influence on cell membrane. Our study not only identifies seven potential antibiotics but also

provides a completely new chemical library for future antibiotic designs. Their unique structure and multiple action mechanism allow us to develop a large family of antibiotics with low risk of resistance.

The wide spread of multi-drug resistant (MDR) Gram-positive bacteria is a serious risk to human health. β -lactams initially provided effective cures for bacterial infections, but the rapid emergence of resistance poses an imminent threat to their clinical therapies.^{1, 2} Methicillin-resistant *S. aureus* (MRSA) which is resistant to β -lactams mediated by the expression of β -lactamases or penicillin-binding protein 2a, is widespread leading to more than 80,000 infections per year in the United States.³⁻⁵ Even worse, currently available drugs like vancomycin, daptomycin and linezolid are increasingly losing efficacy, making a mild infection incurable and fatal.^{6, 7} Other pathogens including *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus pneumonia*, and *Enterococcus faecium* (*E. faecium*) are also facing serious drug resistance.^{8, 9} MRSA and vancomycin-resistant *E. faecium* (VR *E. faecium*) have been classified as high priority pathogens by WHO.¹⁰ Thus, there is an urgent demand for new antibiotics to combat the MDR gram-positive bacteria.

Antibiotics with novel structures or targets are particularly needed. Traditional antibiotics mainly come from the isolations of natural products.¹¹ While the discovery of natural antibiotics is becoming increasingly difficult as the same molecules are repeatedly screened. Scientists often resort to alternatives from semisynthetic tailoring of natural products.^{12, 13} However, semisynthetic antibiotics often suffer from cross resistance with existing drugs and increasingly limited chemical spaces. Even though people have access to the large synthetic chemical library, only few synthetic scaffolds, like sulfonamides, furans, quinolones, and oxazolidone, have been made into clinical uses.¹⁴⁻¹⁶ So we need much more effort to search for new synthetic antibiotics.

Here we propose a new chemical library, multi-armed molecules (MAMs), for antibiotic screening. These molecules are commonly used in the synthesis of metal organic frameworks, but their biological properties have not been explored.¹⁷ We started this work based on an accidental discovery of an excellent antimicrobial molecule E-4PBA when we developed detecting methods for bacteria using small molecules with tetraphenyl ethylene structures.¹⁸ After a structure-activity relationship (SAR) study, we find that the multi-armed structure, rather than the tetraphenyl ethylene structure, is particularly important for their antibacterial actions. By screening the nucleus and arms, we discovered a new class of multi-armed antibiotics (MAAs) structurally distinct from existing antibiotics. Seven MAAs with a nucleus like ethylene (E), carbon (C), benzene (B), nitrogen (N) and triazine (T), and three or four symmetrical arms like phenylbenzoic acid (PBA) and 4-methynylbenzoic acid (MBA) show excellent efficiencies for Gram-positive bacteria *in vitro* and *in vivo*. We investigate the action mechanism and find that MAAs can be selectively internalized into Gram-positive bacteria, inhibit cell wall assembly and influence cell membrane. MAMs can be a new chemical library for antibiotic screening. Their independence from a fixed structural moiety and the multiple action mechanism can greatly avoid the rapid emergence of resistance. Our findings provide a new class of antibiotics and a new direction for future antibiotic designs.

Results

SAR Study Yields MAAs

This work starts with the discovery of a new antimicrobial molecule E-4PBA, while we were looking for small molecule-based dyes to identify different types of bacteria (Figure 1).¹⁸ We found that it is a multi-armed molecule (MAM) composed of an ethylene (E) nucleus and four 4-

phenylbenzoic acid (PBA) arms, neither of which is active as antibiotics for pathogens (Table S2).

The unique multi-armed structure may contribute to its antimicrobial activities. To screen the practical structure of E-4PBA, we carried out a SAR study based on an orthogonal screening of the nucleus and arms. When the nucleus is replaced by a carbon atom (**2**), the molecule shows equivalent activities to E-4PBA. Another molecule with benzene nucleus (**3**) is also active toward *S. aureus*. By contrast, when the nucleus changes to porphyrin (**4**), benzidine (BZ, **18**) or terophenyl (TP, **19**), the molecules lose antibiotic activity (Figure S1A). Besides four-armed molecules, MAMs with three PBA arms and a nucleus like nitrogen atom (**5**), B (**6**), and triazine (**7**) are also active for bacteria. So, E, C, B, N and T nucleus are effective for MAMs to exert antibacterial actions.

Figure 1 SAR study of MAMs with different nucleus. MAMs with nucleus including ethylene (**1**) carbon atom (**2**), benzene (**3**), porphyrin (**4**), nitrogen atom (**5**), benzene (**6**) and triazine (**7**), and phenylbenzoic acid (PBA) arms. MAMs in boxes are active MAMs for *S. aureus*.

79 We evaluate the effects of arms on antibacterial activities based on MAMs with B nucleus (Figure
80 2). Compared with the three-armed molecule (**6**, MIC is 0.06 $\mu\text{g/mL}$), MAM with four PBA arms (**3**,
81 MIC is 0.5 $\mu\text{g/mL}$) shows decreased efficacy (Figure 2A). When the number of arms increases to six,
82 the molecule (**8**) fails to inhibit bacteria. The length of arms also affects antibacterial activities
83 (Figure 2B). MAMs with benzoic acid (BA, **10**) or carboxylic acid (CA, **9**) arms, which lack one or
84 two benzene groups compared with PBA, are inactive toward bacteria. When the arms increase to
85 terphenylbenzoic acid (TBA, **11**), the molecule is inactive. Structure modifications of arms lead to
86 diverse effects (Figure 2C, S1B). Replacements of the carboxyl groups on PBA arms by amine (**12**),
87 hydroxyl (**13**) or nitril (**14**) groups lead to inactive compounds. Changing the inner benzene group to
88 oxygen (**15**) or secondary amine (**16**) disables the MAMs. While when the inner benzene group
89 changes to alkynyl, the MAM (**17**, B-3MBA) retains efficacy. MAMs with other arms do not show
90 antibacterial activity. So PBA and MBA are optional for antibacterial MAMs.

91 We have discovered a new chemical library for antibiotic screening and identified seven multi-
92 armed antibiotics (MAAs) from thirty-three synthetic compounds based on the SAR study. The
93 multi-armed structure is pivotal in their antibacterial actions. Increases of the sizes of nucleus
94 influence their antibacterial activities. Changes of numbers, lengths or structures of the arms also
95 affect their efficacy. The antimicrobial activities of small molecules are always dictated by their
96 physicochemical properties especially polarity and molecular weight.^{19, 20} MAAs have ClogD_{7.4} (the
97 predicted octanol/water distribution coefficient at pH 7.4) in the range of -1.77 to 1.48, and their
98 MWs range from 510.49 to 862.92 (Table S1). MAMs outside the efficient ClogD_{7.4} or MW ranges
99 are inactive for bacteria. So polarity and molecular weight simultaneously restrict the antibacterial
100 actions of MAMs. The effective range where $-1.77 \leq \text{ClogD}_{7.4} \leq 1.48$ and $510.49 \leq \text{MW} \leq 862.92$

101 can be a new guideline for antibiotic screening among MAMs.

102

103 **Figure 2** SAR study of MAMs with B nucleus and different arms. **A** MAMs with B nucleus and
 104 different number of arms including three PBA (6), four PBA (3) and six PBA (8). **B** MAMs with B
 105 nucleus and different length of arms including carboxylic acid (CA, 9), benzoic acid (BA, 10), PBA
 106 (6) and terphenylbenzoic acid (TBA, 11). **C** MAMs with B nucleus and different arms including 4-

aminobiphenyl (ABP, **12**), 4-phenylphenol (PP, **13**), 4-nitrobiphenyl (NBP, **14**), oxybenzoic acid (OBA, **15**), amino-benzoic acid (ABA, **16**) and 4-methynylbenzoic acid (MBA, **17**). MAMs in boxes are active MAMs for *S. aureus*.

Antibacterial and Bactericidal Activities of MAAs

MAAs show a broad antibacterial spectrum against Gram-positive bacteria including *S. aureus*, *S. epidermidis*, *Staphylococcus haemolyticus* (*S. haemolyticus*), *Bacillus subtilis* (*B. subtilis*), *Streptococcus pneumoniae* and *E. faecium*, as well as clinical MDR isolates including MRSA, MRSE, MDR *S. haemolyticus* and VRE (Table 1).²¹ The potency against most species is below 1 µg/mL, which is much lower than traditional antibiotics. N-3PBA is most efficient and its MICs can be as low as 0.008 µg/mL, which is 32, 1024 and 256 fold lower than ampicillin, oxacillin and vancomycin respectively. MAAs are exceptionally active against *S. aureus* and its MDR counterparts with MICs from 0.008 µg/mL to 2 µg/mL. The MDR strains are difficult to treat and resistant to many antibiotics like ampicillin, oxacillin and even vancomycin (Table S3). Thus our findings are meaningful for the treatment of MDR bacterial infections.

We evaluate the antibacterial and bactericidal activities of MAAs. MAAs and vancomycin can completely inhibit the growth of *S. aureus* and MRSA, and the turbidities and OD₆₀₀ values of bacterial suspensions do not increase after 24-h culturing (Figure S2, S3). While ampicillin or oxacillin cannot inhibit MRSA even at 32 µg/mL due to the drug resistance. MAAs also show inhibiting effects on other strains including *S. epidermidis*, *E. faecium*, MRSE and VR *E. faecium*. MAAs have excellent bactericidal activities and most of them can kill more than 99.99 % MRSA after 24 h (Figure 3A, Figure S4).^{22, 23} Particularly, 0.5 µg/mL E-4PBA, C-4PBA, B-3PBA and T-

128 3PBA can kill more than 99.99 % MRSA after 12-h treatment. Even after 8 h, 2 µg/mL MAAs can
129 eliminate 99.99 %, 99.99 %, 98.47 %, 99.91 %, 99.99 %, 99.99 % and 99.63 % MRSA respectively.
130 By contrast, the cell number of MRSA treated with 32 µg/mL vancomycin decreases slightly with
131 killing rates of 53.40 % after 8 h and 96.10 % after 12 h. The bactericidal effect is not observed for
132 oxacillin (32 µg/mL). MAAs are also superior to vancomycin in killing late exponential phase
133 populations of MRSA (10⁹ CFU/mL. Figure 3B, Figure S5). Failure to eliminate MDR bacteria is
134 probably the reason for clinical failures of antibiotics in patient treatments. MAAs can be promising
135 candidates for the treatment of MDR infections when traditional antibiotics, such as vancomycin,
136 fail.

137 **Table 1 Antibacterial activities of MAAs against pathogenic bacteria.**

Organism	MAAs MIC (µg/mL)						
	E-4PBA	C-4BPA	B-4PBA	N-3PBA	B-3PBA	T-3PBA	B-3MBA
<i>S. aureus</i>	0.06	0.125	0.5	0.008	0.06	0.25	0.06
<i>S. aureus</i> (MRSA) ¹	0.06	0.125	2	0.008	0.03	0.06	0.125
<i>S. aureus</i> (MRSA) ²	0.06	0.125	2	0.016	0.03	0.25	0.125
<i>S. epidermidis</i>	0.5	0.5	8	0.016	0.125	2	0.125
<i>S. epidermidis</i> (MRSE)	1	0.25	8	0.016	0.125	2	0.25
<i>S. haemolyticus</i>	1	0.5	0.125	0.06	1	4	0.125
<i>S. haemolyticus</i> (MDR)	2	0.5	16	0.125	1	4	0.25
<i>B. subtilis</i>	0.5	0.5	8	0.03	0.06	0.06	0.25
<i>Streptococcus pneumoniae</i>	1	1	16	0.25	0.25	1	2
<i>E. faecium</i>	16	2	32	1	2	1	0.5
<i>E. faecium</i> (MDR)	16	2	32	0.5	0.5	0.25	0.5
<i>E. faecium</i> (VRE)	8	1	16	1	2	2	0.5

S. aureus, *Staphylococcus aureus*, MRSA, methicillin-resistant *S. aureus*, MRSA¹ and MRSA² are two MRSA strains, MRSE, methicillin-resistant *S. epidermidis*, MDR, multi-drug resistant, VRE, vancomycin-resistant *Enterococcus*.

Figure 3 Bactericidal activities of MAAs. **A** Time-dependent killing of MRSA at early exponential phase (10⁶ CFU/mL) by Van (32 µg/mL) and MAAs (0.5, 1 and 2 µg/mL). **B** Time-dependent killing of MRSA at late exponential phase (10⁹ CFU/mL) by Van (32 µg/mL) and MAAs (1, 2 and 4 µg/mL).

Antibacterial Mechanisms of MAAs

We investigate the interactions of MAAs with bacterial cells based on the fluorescent property of N-3PBA (Figure S6). All Gram-positive bacteria show intense fluorescence after the treatment of N-

146 3PBA (Figure 4A). Gram-positive bacteria lack the outer membrane, and MAAs can be internalized
147 into bacterial cells, which explains the selective susceptibilities of Gram-positive bacteria to
148 MAAs.^{19, 24} We further observe the cellular localization of N-3PBA by structured illumination
149 microscopy (SIM).²⁵ Strong fluorescent signals are observed at the septa and peripheries where
150 should be the cell envelop (cell membrane or cell wall, Figure 4B). We use two fluorescent dyes,
151 FM4-64 and BODIPY-FL-labeled vancomycin (Van-FL) to stain cell membrane and cell wall. They
152 show co-localizations with N-3PBA, further demonstrating the cell envelop localization.²⁶
153 Membrane integrity has been verified by propidium iodide (PI), a fluorescent dye that can cross
154 compromised membranes and bind to the DNA or RNA to cause increased fluorescence. Few cells of
155 N-3PBA-treated bacteria can be stained by PI, indicating the bacteria are not killed via membrane
156 lysis (Figure 4C).²¹ Time-dependent observation shows that N-3PBA gradually accumulates to the
157 septa (Figure 4D). Meanwhile, N-3PBA-treated *S. aureus* shows evident stagnation of division
158 (Figure 4E, Figure S8). During division, bacteria build a septum by synthesizing new cell walls.^{25, 27}
159 So MAAs inhibit bacteria mainly by affecting cell walls.

160 We visualize the morphological changes of *S. aureus* by scanning electron microscope (SEM) and
161 transmission electron microscope (TEM). In SEM images, some of the N-3PBA-treated cells are
162 bigger than those in the control group due to the inhibition of cell division (Figure 4F, G, S9). There
163 are pits in the middle of some cells, but we do not observe the collapse of cell wall. To investigate
164 intercellular changes, we make super-thin slices of *S. aureus* and visualize them by TEM (Figure
165 4H). In the control group, the normal bacterial cells possess a thin and clear boundary, and a flat
166 septum can be observed in dividing cells. While in the N-3PBA-treated group, the bacterial cell walls
167 appear thickened accompanied with concave membranes. Statistical results show a 5.5-fold increase

Figure 4 Antibacterial mechanism of MAAs. **A** Fluorescent images of Gram-positive bacteria (G+) and Gram-negative bacteria (G-) treated with N-3PBA. **B** Co-localizations of N-3PBA with FM4-64 and Van-FL imaged by SIM. **C** Fluorescent images of *S. aureus* treated with N-3PBA and stained by PI/Syto9. **D** Time-dependent monitoring of *S. aureus* treated with N-3PBA by SIM. **E** Time-dependent monitoring of *S. aureus* division. **F** SEM images of *S. aureus* and MAAs-treated *S. aureus*. **G** Bacterial size and septa thickness. **H** TEM images of *S. aureus* and *S. aureus* treated with MAAs and **I** antibiotics including

175 ampicillin (Amp, 32 $\mu\text{g/mL}$) and vancomycin (Van, 32 $\mu\text{g/mL}$). **G** Statistical results of bacterial sizes
176 from SEM images and septa thickness from TEM images (*** $P < 0.01$). The scale bars are 5 μm in
177 **A, C and E**, 500 nm in **B, D, H and I**, 1 μm in **F**.

178 of the thickness of bacterial septa from 71.1 ± 18.5 nm to 393.0 ± 142.8 nm (Figure 4G, S10). The
179 cell wall thickening may result from the inhibition of cell wall biosynthesis which lead to
180 accumulations of cell wall precursor or their complexes with MAAs. Same phenomena are observed
181 in *S. aureus* treated with antibiotics targeting cell wall biosynthesis like ampicillin and vancomycin
182 (Figure 4I).²⁸ Thus, disturbance on cell wall biosynthesis should be the major impact of MAAs on *S.*
183 *aureus*.

184 The primary component of the cell wall is peptidoglycan (PG), whose synthesis involves a series
185 of enzymatic reactions classified as cytoplasmic steps, lipid-linked steps, and extracellular steps
186 (Figure 5A).^{29, 30} As the cell wall thickening occurs outside membrane, MAAs should mainly
187 influence the extracellular steps including transglycosylation (TG) and transpeptidation (TP).
188 Blocking of the late steps of cell wall biosynthesis commonly result in intracellular accumulations of
189 the soluble cell wall precursor undecaprenyl-N-acetylmuramic acid-pentapeptide (UDP-MurNAc-
190 pentapeptide). Consistent with vancomycin, high accumulations of UDP-MurNAc-pentapeptide
191 (HPLC, $[M + H]^+ = 1150.36$) are observed after the treatment of N-3PBA, which confirms that one
192 of the subsequent steps of cell wall biosynthesis is blocked (Figure 5B).^{31, 32} Resistance study based
193 on passages of *S. aureus* in the presence of sub-MIC levels of N-3PBA do not yield resistant mutant
194 like ampicillin, suggesting that the target of MAAs is not a protein (Figure 5E).²³ We further carry
195 out an antagonization assay in which the MICs are tested in the presence of putative binding sites of

196 the possible targets including Lipid II, C₅₅-P and C₅₅-PP. Ac-Lys(Ac)-D-Ala-D-Ala (Ac₂Kaa), the
197 Lipid II binding site of vancomycin, can ablate the activity of vancomycin (Figure 5C).³³ Since
198 Ac₂Kaa does not antagonize the activity of N-3PBA, MAAs must affect bacteria in a different way to
199 vancomycin. N-3PBA can be antagonized by the lipid carriers C₅₅-P or C₅₅-PP, demonstrating that
200 MAAs inhibit cell wall biosynthesis by targeting the lipid carriers (Figure 5D). C₅₅-P and C₅₅-PP are
201 the central carriers for the transport of sugar intermediates in PG and wall teichoic acids (WTA)
202 biosynthesis in Gram-positive bacteria.³⁴ MAAs should cause a dual effect on precursor cycling and
203 cell wall assembly in Gram-positive bacteria. In addition, accumulations of MAAs outside the
204 membrane should influence the function of cell membrane. As expected, disturbance of membrane
205 potential is observed which further influence the bacterial physiology like cell division and ATP
206 synthesis (Figure 5F, G). Thus, MAAs play a multiple role on bacterial cells including inhibition of
207 the assembly of functional cell wall and influence on cell membrane. The multiple antibacterial
208 mechanism contributes to the high efficacy of MAAs and would avoid the rapid emergence of
209 resistance.

Figure 5 Antibacterial mechanism of MAAs. **A** Model for the antibacterial mechanism of MAAs. MAAs inhibit bacteria by inhibiting PG and WTA biosynthesis and affecting cell membrane. **B** Intracellular accumulations of the UDP-MurNac-pentapeptide of *S. aureus* treated with ampicillin (Amp), vancomycin (Van) and N-3PBA. Antagonism of the antibacterial activities of **C** Van and **D** N-3PBA by Ac-Lys(Ac)-D-Ala-D-Ala (Ac₂Kaa), Gly₅, N-acetylglucosamine (GlcNAc), N-acetylmuramic acid (MurNAc), C₅₅-P and C₅₅-PP. **E** Resistance study in the presence of sub-MIC levels of antimicrobials. **F** Membrane potential and **G** intracellular ATP level of *S. aureus* treated with N-3PBA.

220 ***In vivo* efficacy of MAAs**

221 We test the wound healing capabilities of MAAs by an MRSA wound infection model (Figure
222 6A). Full-thickness wounds with diameters of 2 cm are prepared on the dorsal side of Wistar rats.
223 The wounds are infected by MRSA for 30 min and treated with MAAs wound dressings (E-4PBA,
224 C-4PBA, B-4PBA, N-3PBA, B-3PBA, T-3PBA and B-3MBA). On day 6, all wound sizes decrease
225 slightly (Figure 6B). Histological graphs show that inflammatory cells such as neutrophils (Ne) and
226 lymphocytes (Ly) emerge in all groups due to the inflammatory response of the host. After 12 days,
227 the wound sizes in MAAs groups are much smaller than those in the control group. Elongated
228 fibroblasts (Ef) and epithelial cells (Ec), which are the signals of reepithelization are observed
229 accompanied with the emergence of horny layers. While there still exist many inflammatory cells in
230 the control group. We also record the local bacteria counts at the wound area after MRSA infections
231 for 1, 6 and 12 days. After 6 and 12 days, the bacterial levels at MAAs-treated wounds decrease
232 obviously (Figure 6C). These results indicate that MAAs can reduce the local bacterial levels of
233 infected wounds and possess outstanding wound-healing effects on MRSA infections.

234 We evaluate the therapeutic effects of MAAs in a mouse septicemia model (Figure 6A, D). Mice
235 are infected with MRSA via intraperitoneal injections. E-4PBA and N-3PBA at single doses of 0.13,
236 0.25, 0.5 and 1 mg/kg are introduced intravenously at 0.5 h post-infection. Physiological saline,
237 oxacillin (4 mg/kg) and vancomycin (0.5 and 1 mg/kg) are also applied as negative and positive
238 controls. The treatment of 0.5 mg/kg E-4PBA, 1 mg/kg N-3PBA and 1 mg/kg vancomycin can
239 rescue all mice with septicemia. By contrast, none of the mice survive with the treatment of
240 physiological saline or oxacillin. The PD₅₀ (protective dose at which half of the animals survive) of
241 E-4PBA and N-3PBA are 0.15 and 0.45 mg/kg, which compare favorably to that of vancomycin

(0.68 mg/kg). MAAs show outstanding therapeutic efficacy for MRSA infection *in vivo*, revealing that they are potential antibiotics for serious MDR bacterial infections.

Figure 6 MAAs are efficacious in MRSA infections. **A** Scheme of the experimental protocol of MRSA wound infections and septicemia. **B** Histological graphs of skin tissue after H&E staining (Ly, lymphocyte; Ne, neutrophil; Ec, epithelial cells; Ef, elongated fibroblasts) and photographs of the infected wounds treated with gauze (control) and MAAs (E-4PBA, C-4PBA, N-3PBA, B-3PBA, T-3PBA and B-3MBA) for 6 and 12 days. **C** Local bacteria counts in the wound area on day 1, 6, and 12 post-infections. P is the significant statistical differences (**P < 0.05, ***P < 0.001). **D** *In vivo* efficacy of E-4PBA, N-3PBA, Van and Oxa in a mouse septicemia model. The white and black scale bars are 1 cm and 100 μ m.

Discussion

In this study, we discover a new class of antibiotics structurally and functionally distinct from known antibiotics. The rapid emergence of MDR bacteria seriously compromises the clinical

therapies of currently available antibiotics. In contrast, the development of new antibiotics is increasingly difficult. New classes of antibiotics with novel structures or targets are particularly needed because they kill bacteria in a different way to existing antibiotics, which protect them from the resistance of pathogens. MAAs possess a completely new multi-armed structure with a nucleus and three or four symmetrical arms, which has not been reported in other studies. The nucleus can be ethylene (E), carbon (C), nitrogen (N), benzene (B), and triazine (T), and the arms can be phenylbenzoic acid (PBA) and 4-methynylbenzoic acid (MBA). Different from traditional antibiotics like β -lactams, the activities of MAAs do not rely on a fixed structural moiety. This independence from a fixed functional moiety greatly increases the molecular diversity and avoids the rapid emergence of resistance. The MAMs in this study are commonly applied as ligands in the synthesis of metal organic frameworks, but their biological properties have not been explored. So this is a new chemical library for antibiotic screening, and more MAMs with different permutations of nucleus and arms can be involved in our future study. The SAR study shows that the antibacterial activities of MAMs are related to their physicochemical properties like polarity and molecular weight. The efficient range where $-1.77 \leq \text{ClogD}_{7.4} \leq 1.48$ and $510.49 \leq \text{MW} \leq 862.92$ can be a guideline for future antibiotic screening among MAMs.

MAAs show excellent antibacterial activities against Gram-positive bacteria and MDR strains including MRSA and VRE which have been classified as high priority pathogens by WHO. N-3PBA is most efficient and its MICs can be as low as 0.008 $\mu\text{g/mL}$ that are much lower than traditional antibiotics and other reported compounds. For *S. aureus*, the MIC of N-3PBA is 32, 1024 and 256 fold lower than ampicillin, oxacillin and vancomycin respectively. MAAs are also superior to traditional antibiotics in killing MDR bacterial strains achieving more than 99.99 % eliminations for

278 MRSA. MAAs at concentration 64 fold lower than vancomycin, show much higher bactericidal
279 efficacy than vancomycin. *In vivo* evaluations show that they can effectively treat MRSA wound
280 infections and septicemia.

281 We carry out a comprehensive study on the antibacterial mechanism of MAAs based on a series of
282 chemical and biological technologies including fluorescent imaging, electron microscopy and cell-
283 based assays. Fluorescent imaging show that MAAs can be selectively internalized into Gram-
284 positive bacteria leading to increased fluorescence of bacterial cells, which due to the lack of outer
285 membrane of Gram-positive bacteria and contributes to their selective activities to Gram-positive
286 bacteria. TEM and SEM imaging indicate the influence of MAAs on bacterial cell wall. Further cell-
287 based assays confirm the inhibition of the assembly of functional cell walls including PG and WTA
288 biosynthesis and membrane associated reactions. The multiple action mechanism contributes to the
289 high efficacy of MAAs and would avoid the rapid emergency of resistance developed by a single
290 mutation of bacteria.

291 Our study not only identifies seven potential antibiotics for serious MDR bacterial infection but
292 also provides a new chemical library for new antibiotic designs. We hope that our results will inspire
293 chemists to develop a large family of new antibiotics with low risk of drug resistance.

294 **Methods**

295 **Materials.** All synthetic molecules for antibiotic screening are from Chemsoon and Alfachem.
296 Dimethyl sulfoxide (DMSO) and other chemical reagents are from Sigma. CCK-8 kits are from
297 Beyotime Institute of Biotechnology, China. Syto9, PI (propidium iodide), diSC3(5) (3,3-
298 dipropylthiadicarbocyanine iodide), FM4-64, and Van-FL are from Invitrogen Molecular Probes, Inc.

299 C₅₅-P and C₅₅-PP are from Larodan Fine Chemicals, Sweden. Human umbilical vein endothelial cells
300 (HUVECs) are from Guangzhou Xiangbo Biotechnology Co. Ltd., China. Balb/c female mice,
301 Sprague-Dawley (SD) female rats and rabbit come from Beijing Vital River Laboratory Animal
302 Technology Co., Ltd. Lysogeny broth (LB) medium, LB agar medium are from Solarbio life science,
303 China. Bacteria strains are grown in LB and LB agar plates at 37 °C.

304 **Bacteria strains.** *Staphylococcus aureus* ATCC 6538P (*S. aureus*), *Staphylococcus epidermidis*
305 ATCC 12228 (*S. epidermidis*), *Bacillus subtilis* ATCC 6633 (*B. subtilis*), *Streptococcus pneumonia*
306 ATCC 6303 and *Enterococcus faecium* (*E. faecium*), *Escherichia coli* (*E. coli*) are from China
307 General Microbiological Culture Collection Center. Clinical isolates, methicillin-resistant *S. aureus*
308 (MRSA), methicillin-resistant *S. epidermidis* (MRSE), MDR *Staphylococcus. haemolyticus* (MDR .
309 *haemolyticus*), MDR *E. faecium* and *E. faecium* (VRE) are from local hospitals.

310 **Minimum inhibitory concentration (MIC).** All molecules are dissolved in DMSO and then diluted
311 with deionized water or LB medium. We determine the MICs using a microbroth dilution method.^{23,}
312 ³⁵ Bacteria strains are cultured to logarithmic-phase ($OD_{600} \approx 0.1$) and diluted for a thousand folds
313 with LB medium. MAAs are diluted exponentially in a 96-well plate (100 μ L), and we add 10 μ L of
314 bacteria suspensions in each well. MIC is the minimal concentration at which no turbidity is
315 observable in all three parallels after 24 h incubation. We also compare the inhibition effects of
316 MAAs and ampicillin, Oxacillin and vancomycin by testing the optical density at 600 nm (OD_{600}) of
317 bacterial suspensions by microplate readers (Tecan infinite M200), and recording the turbidities of
318 bacterial suspensions by Nikon D90 camera.³⁶

319 **Time-dependent killing assay.** We incubate MRSA ($\approx 10^6$ CFU/mL) with different concentration of

MAAs (0.5, 1 and 2 µg/mL), oxacillin (32 µg/mL) and vancomycin (32 µg/mL) in LB medium for 4, 8, 12, 16 and 24 h.^{23, 37, 38} The suspensions are diluted for 10, 1000, and 100000 folds, and 100 µL of the dilutions are placed on LB plates and incubated at 37 °C for 24 h. Colonies are counted and CFU/ml is calculated. The group without MAAs is the control. We also test the time-dependent killing of MAAs against late exponential phase populations of MRSA (10⁹ CFU/mL) based on the same method.

Characterization of MAAs. MAAs are dissolved in DMSO with a concentration of 4096 µg/mL and diluted by deionized water to 256 µg/mL. We characterize the excitation and emission spectra of MAAs in an aqueous solution by fluorescence spectrophotometry. Physiochemical properties like MWs and ClogD_{7.4} values are calculated by the online compound property calculation software FAFdrugs⁴ (<http://fafdrugs4.mti.univ-paris-diderot.fr/index.html>).¹⁹

Fluorescent imaging by confocal microscopy. We investigate the interactions of MAAs with bacterial cells based on their fluorescent properties.¹⁸ We incubate *S. aureus* and *E. coli* with MAAs (2 µg/mL) in LB medium for 15 min and image them by confocal microscopy (Zeiss LSM710) using a 405 nm excitation light. Other Gram-positive bacteria including *S. epidermidis*, *B. subtilis*, *S. haemolyticus*, *E. faecium* and Gram-negative strains like *P. aeruginosa*, *K. pneumoniae*, *A. baumannii* and *Salmonella* are incubated with N-3PBA for 15 min and imaged by confocal microscopy. We assess the influence of MAAs on the integrity of cell membranes using Syto9/PI staining. Bacteria are incubated with MAAs (4 µg/mL) for 4 h, and stained with PI (200 µM) and Syto9 (30 µM) for 30 min, and then imaged by confocal microscopy. We monitor the cell division of *S. aureus* and N-3PBA-treated *S. aureus* for 60 min by confocal microscopy.

341 **Fluorescent imaging by structured illumination microscopy (SIM).** We study the localization of
342 N-3PBA by structured illumination microscopy (SIM, Nikon N-C2-SIM).^{25, 39} *S. aureus* are
343 incubated with N-3PBA for 15 min and imaged by SIM using a 405 nm excitation light. We use
344 FM4-64 or Van-FL to stain the cell membrane or cell wall of bacteria. The N-3PBA treated *S. aureus*
345 are incubated with FM4-64 (10 µg/mL) or Van-FL (10 µg/mL) for 2 min, and imaged by SIM. We
346 also monitor the interactions of N-3PBA with *S. aureus*. *S. aureus* is incubated with N-3PBA for 15
347 min, 1h, 2h, 3h and 4h, and imaged by SIM.

348 **SEM imaging of bacteria.** We observe the morphological changes of *S. aureus* after the treatment of
349 MAAs by SEM.²⁵ *S. aureus* are incubated with MAAs (4 µg/mL) in LB medium at 37 °C for 4 h. We
350 fix the samples with 2.5% glutaraldehyde for 3 h, and wash them with PBS twice. The samples are
351 dehydrated through graded ethanol solutions with 30, 50, 70, 90, 95, 100 % (v/v, in water) at room
352 temperature. We use Hitachi S4800 SEM to record the images.

353 **TEM imaging of bacteria.** We investigate the influence of MAAs on *S. aureus* by TEM.^{16, 31} We fix
354 MAAs-treated bacteria for 3 h with 2.5 % glutaraldehyde and wash them with PBS twice. We further
355 fix the bacteria with 0.1 % osmic acid for 30 min and wash them with PBS for three times. We
356 dehydrate the samples through graded ethanol solutions with 30, 50, 70, 90, 95, 100 % (v/v, in
357 water), 50 % ethanol in acetone for 10 min each time finally with pure acetone twice for 15 min. The
358 dehydrated samples are embedded in Epon812 and polymerized at 60 °C for 24 h. We cut the
359 samples into super thin slices and place them on Formvar-coated grids. The slices are stained with
360 2 % uranyl acetate and 0.2 % lead citrate. We use Tecnai G2 20 ST TEM to take the pictures.

361 **Accumulation of UDP-N-acetyl-muramic acid-pentapeptide.** *S. aureus* is cultured to logarithmic-

362 phase ($OD_{600} \approx 0.5$) and incubated with chloramphenicol (130 $\mu\text{g/mL}$) for 15 min.^{23, 31} We add N-
 363 3PBA (4 $\mu\text{g/mL}$), ampicillin (20 $\mu\text{g/mL}$) and vancomycin (20 $\mu\text{g/mL}$) to the bacteria suspensions and
 364 incubate them for 60 min. Bacteria are collected by centrifugation and extracted by boiling water.
 365 The extracts are centrifuged, and the supernatant is lyophilized. We use HPLC-MS to analyze the
 366 UDP-N-acetyl-muramic acid-pentapeptide. Experiments are performed with biological replicates.

367 **Resistance study.** We assess the resistance development of *S. aureus* to ampicillin, vancomycin and
 368 N-3PBA by sequential passaging.⁴⁰ *S. aureus* is cultured to logarithmic-phase and diluted to OD_{600} of
 369 0.01 in LB medium with ampicillin, vancomycin or N-3PBA at 0.5 MIC. Bacteria are incubated at 37
 370 °C with agitation and passaged at 12 h intervals. We transfer the bacteria to fresh LB medium and
 371 culture them to OD_{600} of 0.5. MICs are test based on the microbroth dilution method. Experiments
 372 are performed with biological replicates.

373 **Antagonization assay.** Antagonization of the antibiotic activity of vancomycin and N-3PBA by
 374 potential target molecules is performed via a MIC based method.^{32, 41} Vancomycin or N-3PBA are
 375 mixed with putative binding sites of Lipid II {Ac-Lys(Ac)-D-Ala-D-Ala (Ac₂Kaa), Gly5, MurNAc,
 376 and GlcNAc}, C₅₅-P and C₅₅-PP in 96-well plates, and then incubated for 3 h. We add 10 μL bacteria
 377 in every well and incubate the plate at 37 °C for 24 h. MICs in the presence of different molar ratios
 378 of target molecules with vancomycin or N-3PBA are recorded. Experiments are performed with
 379 biological replicates.

380 **Cytotoxicity of MAAs.** We detect the cytotoxicity of MAAs to human umbilical vein endothelial
 381 cells (HUVECs) via a commercial CCK-8 assay kit. We culture HUVECs (10^4 cells per well) in 96-
 382 well plates for 24 h.^{21, 42} 10 μL MAAs at different concentrations (final concentration of 0-64 $\mu\text{g/mL}$)

are added and incubated for another 24 h. We add 10 μ L CCK-8 solution to each well and incubate the samples at 37 °C for 2 h. The optical density at 450 nm (OD₄₅₀), which represents cell viability, is detected by microplate readers.

Hemolysis assay. We perform the hemolysis assay using fresh rabbit blood.^{43, 44} We centrifuge 1 mL aliquot of whole blood at 12, 000 r/min for 5 min and discard the supernatant. The red blood cells (RBCs) pellet are collected and washed for three times by PBS. For hemolysis assays, we incubate MAAs (final concentration of 0-64 μ g/mL) with RBCs (final concentration of 4 %) for 3 h. Pure water is set as a positive control, and physiological saline is negative control. After incubation, we centrifuge the samples at 12, 000 r/min for 15 min, take pictures and analyze the supernatant based on the optical density at 540 nm (OD₅₄₀).

***In vivo* toxicity.** We also carry out a dose tolerance study by challenging female balb/c mice with MAAs. Mice (n = 5 per group) are administered with a single intravenous dose of MAAs molecule at 0, 0.5, 1, and 2 mg/kg and clinically monitored for 7 days. We record the survival rates of each group after 7 days.

MRSA wound infection model. We build a wound infection model using female rats aged 8 weeks with an average weight of 250 g.^{22, 45} Full-thickness wounds with diameters of 2 cm are prepared by removing dorsal flank skin from the rats. We fabricate electrospun nanofibers with PLGA and MAAs (PLGA-MAAs) as wound dressings. The wounds are infected by 0.2 mL MRSA (5×10^9 CFU/mL) for 30 min and covered with gauze and PLGA-MAAs, respectively. We take photos of the infected wounds on day 6 and day 12 after the operation to study the wound-healing progress. For histological analysis, we gather wound tissues and obtain isometric continuum cut sections using a

404 microtome in vertical planes of each fixed tissue and stain them with hematoxylin and eosin (HE
405 staining). We also record the local bacterial counts at the infected wounds on day 1, day 6, and day
406 12 by grinding the wound tissues and culturing the suspensions on LB plates.

407 **Mouse septicemia model.** We make a mouse septicemia model to evaluate the *in vivo* bioavailability
408 of MAAs.^{23, 33} Female balb/c mice aged 6-8 weeks are infected with 0.5 mL of MRSA suspension
409 (10^9 CFU/mL) via intraperitoneal injection. After 30 min, the mice (five mice per group) are treated
410 with different concentrations of E-4PBA and N-3PBA (0.13, 0.25, 0.5, and 1 mg/kg), oxacillin (4
411 mg/kg), vancomycin (1 and 0.5 mg/kg) and saline by intravenous injection. Saline is set as the
412 negative control, and vancomycin is a positive control. Mice are clinically monitored for 48 h, and
413 survival rates are recorded. The animal experiments are conducted in Southern University of Science
414 and Technology and Chinese Academy of Sciences. We have complied with all relevant ethical
415 regulations in the *in vivo* experiments.

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426 in the experiments of antibiotic screening, antibacterial assay, and fluorescent imaging.

427 **Author contributions**

428 The study was designed by Y.X. J., W.W.C. and R.B.T, and supervised by X.Y.J. *In vitro*
429 screening, antibacterial and bacterial experiments were performed by Y.X. J. and W.W.C. The
430 animal experiments were performed by Y. X. J., R.B.T. and R. H. D. The fluorescent and electronic
431 imaging were conducted by Y.X. J., W.W.C., R.B.T. and X.Y.L. X.Y.J., Y.X. J., W.W.C. analyze
432 the data and wrote the paper.

433 **Competing interest declaration**

434 There are no conflicts of interest to declare.

435 **Data availability**

436 The data that support the findings of this study are available from the corresponding authors
437 upon request.

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Figures

Figure 1

SAR study of MAMs with different nucleus.

Figure 2

SAR study of MAMs with B nucleus and different arms.

Figure 3

Bactericidal activities of MAAs.

Figure 4

Antibacterial mechanism of MAAs.

Figure 5

Antibacterial mechanism of MAAs.

Figure 6

MAAs are efficacious in MRSA infections.

Supplementary Files

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