(12) **STANDARD PATENT**   (11) Application No. **AU 2017348434 B2**
(19) **AUSTRALIAN PATENT OFFICE**

(54)   Title
Peripheral modifications on pocket-redesigned vancomycin analogs synergistically improve antimicrobial potency and durability

(51)   International Patent Classification(s)
C07K 7/54 (2006.01)   *A61K 38/00* (2006.01)

(21)   Application No: 2017348434   (22)   Date of Filing: **2017.10.31**

(87)   WIPO No: **WO18/081797**

(30)   Priority Data

(31)   Number   (32)   Date   (33)   Country
         62/415,191   **2016.10.31**   **US**

(43)   Publication Date: **2018.05.03**
(44)   Accepted Journal Date: **2021.10.07**

(71)   Applicant(s)
The Scripps Research Institute

(72)   Inventor(s)
Boger, Dale L.

(74)   Agent / Attorney
FPA Patent Attorneys Pty Ltd, Level 46 101 Collins Street, Melbourne, VIC, 3000, AU
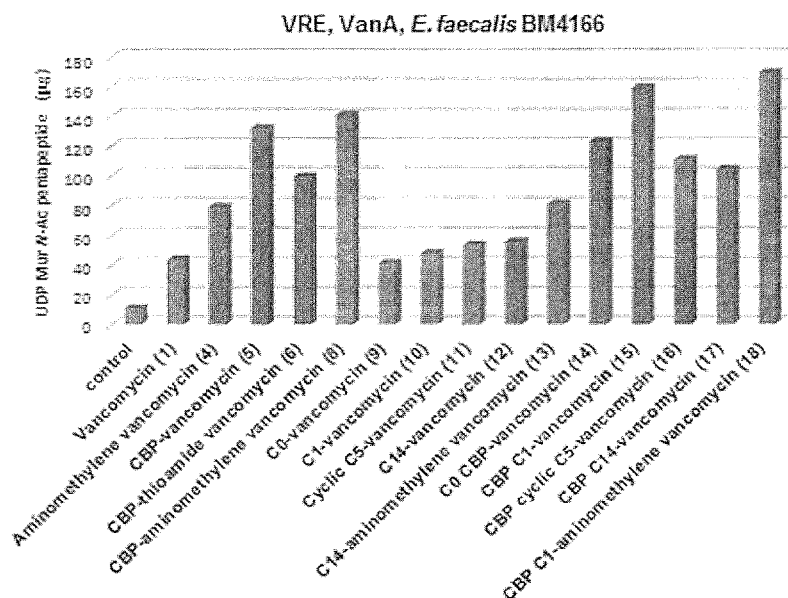
(56)   Related Art
WO 1998000153 A1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification:
 C07K 7/54 (2006.01)   *A61K 38/00* (2006.01)

(21) International Application Number:
PCT/US2017/059289

(22) International Filing Date:
31 October 2017 (31.10.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/415,191   31 October 2016 (31.10.2016)   US

(71) Applicant: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10550 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventor: BOGER, Dale, L.; 8212 Caminito Maritimo, La Jolla, CA 92037 (US).

(74) Agent: GAMSON, Edward, P. et al.; Husch Blackwell, LLP, 120 South Riverside Plaza, Suite 2200, Chicago, IL 60606 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(54) Title: PERIPHERAL MODIFICATIONS ON POCKET-REDESIGNED VANCOMYCIN ANALOGS SYNERGISTICALLY IMPROVE ANTIMICROBIAL POTENCY AND DURABILITY

Fig. 1

VRE, VanA, *E. faecalis* BM4166

(57) Abstract: A C-terminus modification to a binding pocket-modified vancomycin introduces a quaternary ammonium salt that provides a binding pocket-modified vancomycin analog with a second mechanism of action that is independent of D-Ala-D-Ala/D-Ala-D-Lac binding. The modification disrupts cell wall integrity and induces cell wall permeability complementary to the glycopeptide inhibition of cell wall synthesis, and provides synergistic improvements in antimicrobial potency (200-fold) against vancomycin-resistant bacteria. Combining the C- terminus and binding pocket modifications with an orthogonal (4-chlorobiphenyl) methyl addition to the vancomycin disaccharide provides even more potent antimicrobial agents whose activity can be attributed to three independent and synergistic mechanisms of action, only one of which requires D-Ala-D-Ala/D-Ala- D-Lac binding. The resulting modified vancomycins display little propensity for acquired resistance through serial exposure of vancomycin-resistant Enterococci and their durability against

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

---

such challenges as well as their antimicrobial potency follow predicable trends. Methods of treatment with and compositions containing the modified vancomycins are disclosed.

PERIPHERAL MODIFICATIONS ON POCKET-REDESIGNED VANCOMYCIN ANALOGS SYNERGISTICALLY IMPROVE ANTIMICROBIAL POTENCY AND DURABILITY

Description

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to US application Serial No. 62/415,191, filed on October 31, 2016, whose disclosures are incorporated herein by reference.

GOVERNMENTAL SUPPORT

The present invention was made with governmental support pursuant to grant CA041101 and F32 GM114948 from the National Institutes of Health/National Cancer Institute. The government has certain rights in the invention.

TECHNICAL FIELD

The present invention relates to a vancomycin analog molecule that is modified in three ways in three different portions of the molecule. The resulting modified vancomycin compounds are many times more potent than vancomycin itself in both vancomycin-sensitive and vancomycin-resistant bacteria. Also contemplated are a pharmaceutical composition containing a modified vancomycin and a method of treating a bacterially-infected mammal using a modified vancomycin.

BACKGROUND ART

Recent years have seen a welcomed refocus on the need for new antibiotics to address the

persistent and serious threat of bacterial resistance. [WHO, *Antimicrobial Resistance. Global Report on Surveillance 2014;* Geneva, Switzerland, 2014; Centers for Disease Control and Prevention. *Antibiotic Resistance Threats in the United States;* Atlanta, GA, 2013, p 144]. A number of actions have been advanced to address the challenges posed by bacterial resistance now emerging faster than new treatment options. These actions include providing new financial incentives for countering the declining economic interests in developing new antibiotics [Laxminarayan, In *Extending the Cure, Policy Responses to the Growing Treat of Antibiotic Resistance,* Laxminarayan et al. Eds., Chapter 1, pp 25-37], revamping regulatory criteria for new drug approvals, improving the rate of diagnostic characterization of infecting organisms, enhancing nationwide resistance surveillance, encouraging a scientific focus on targeting mechanisms of resistance, identifying new therapeutic targets and approaches for antibiotic development, and championing antibiotic stewardship [Mullard, *Nat. Rev. Drug Discovery* **2014,** *13:*711-713; Sinha et al., *Bioorg Med Chem* **2016,** *24(24):*6446-6451; Brown et al., *Nature* **2016,** *529:*336-343].

Although sounding attractive, the latter efforts to restrict antibiotic use seems counter to their importance, introduces guilt into even their most legitimate of uses, challenges the prevailing practices of empirical best guess initial therapy and prophylaxis deployment, and unfortunately produces further disincentives to antibiotic development and their practical uses [Mullard, *Nat. Rev. Drug Discovery* **2014,** *13:*711-713]. Although such

initiatives highlight the pressing need for renewed
antibiotic discovery and the fundamental importance
of antibiotics in modern medicine [Wright, *ACS
Infect. Dis.* **2015**, *1*:80-84], they have done little to
define new approaches or design concepts that
directly address the underlying problem of
evolutionarily-driven and acquired resistance [Sinha
et al., *Bioorg Med Chem* **2016**, *24(24)*:6446-6451;
Laxminarayan, *Science* **2014**, *345*, 1299-1301].

The mechanisms of resistance are ancient
and increasingly accumulating in pathogenic bacteria
that have now acquired and assimilated large elements
of this bacterial resistome [Hamad, *Nat. Rev. Drug
Discovery* **2010**, *9*:675-6; Wright, et al., *Trends
Microbiol.* **2012**, *20*:157-159]. An additional and
perhaps even more important question to ask is can
one now design antibiotics that overcome the forces
of evolution and selection responsible for bacterial
resistance, that are less prone or even impervious to
resistance development, that avoid many of the common
mechanisms of resistance, and that are more durable
to widespread use than ever before. As an
alternative to championing the restricted use of
antibiotics or conceding that bacteria will always
outsmart us, can develop more durable antibiotics
capable of continued or even more widespread use.

Herein durable antibiotics are created by
deliberate design that may directly address such
evolutionary forces. The glycopeptide antibiotics
were identified as an antibiotic class already
endowed with features that avoid many mechanisms of
resistance. Following introduction of designed
structural changes that directly overcome the
molecular basis of their only prevalent mechanism of

resistance, peripheral structural changes were
created and examined in the molecules that provide
them with additional and now multiple synergistic
mechanisms of action, thereby not only increasing
their potency but also creating durable antibiotics.

Recent disclosures have discussed
attributes of the glycopeptide antibiotics
[*Glycopeptide Antibiotics*; Nagarajan, Ed.; Marcel
Dekker: New York, 1994; Kahne et al., *Chem. Rev.*
2005, *105*:425-448] that have contributed to their
sustained effectiveness in the clinic [James et al.,
ACS Chem. Biol. **2012,** 7:797-804]. Vancomycin
[McCormick et al., *Antibiot. Annu.* **1955-1956,** 606-
611], teicoplanin [Parenti et al., *J. Antibiot.* **1978,**
31, 276-283], and three recently approved
semisynthetic derivatives, oritavancin (August 2014)
[Markham, *Drugs* **2014,** *74(15):*1823-1828], dalbavancin
(May 2014) [Anderson et al., *Drugs* **2008,** *68*:639-648],
and telavancin (September 2009) [Corey et al., *Nat.
Rev. Drug Discovery* **2009,** *8*:929-930], are widely used
to treat refractory bacterial infections, including
methicillin-resistant *Staphylococcus aureus* (MRSA)
[Zhanel et al., *Drugs* **2010,** *70*:859-886].

Vancomycin (**1**, below)[Harris et al., *J. Am.
Chem. Soc.* **1983,** *105*:6915-6922] was disclosed in 1956
and introduced into the clinic in 1958 [McCormick et
al., *Antibiot. Annu.* **1955-1956,** 606-611]. Even after
nearly 60 years of clinical use and even with the
past widespread use of glycopeptide antibiotics for
agricultural livestock (avoparcin), vancomycin-
resistant pathogens have only slowly emerged and
vancomycin remains an integral and increasingly
important antibiotic today.

Clinical resistance was initially observed with vancomycin-resistant Enterococci (VRE, 1987) detected only after 30 years of clinical use [Leclercq et al., *N. Engl. J. Med.* **1988**, *319*:157-161], but now also includes vancomycin-resistant *Staphylococcus aureus* (VRSA, 2002) [Weigel et al., *Science* **2003**, *302*:1569-1571].
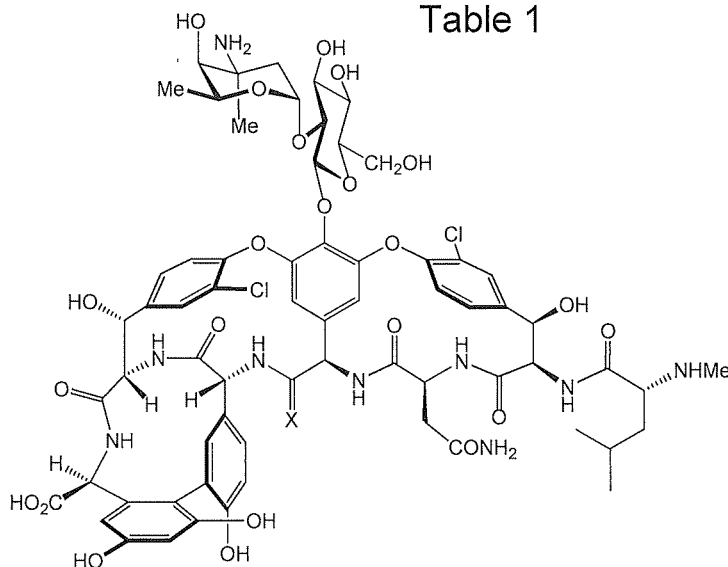
Treatment options for VRSA are limited and, beyond the newer generation glycopeptide antibiotics, these presently include antibiotics known to rapidly evoke resistance (e.g. linezolide, daptomycin) [Brickner et al., *J. Med. Chem.* **2008**, *51(7)*:1981-1990; Baltz et al., *Nat. Prod. Rep.* **2005**, *22(6)*:717-741]. As a result, these latter antibiotics have been designated as reserve antibiotics to be deployed sparingly in order to preserve their effectiveness as drugs of last resort against intractable infections. Just as significantly, some VRE organisms, like MRSA, have also reached a stage where they are now resistant to most all other classes of common antibiotics [Arias et al., *Nat Rev Microbiol* **2012** *10(4)*:266-278]. As a result, and especially because they are already vancomycin-resistant, the U.S. Centers for Disease Control (CDC) has now placed VRE on its serious threat list [cdc.gov/drugresistance/biggest threats]. Most recently, WHO has released for the first time a list of drug-resistant bacteria that pose the greatest threat to human health for which new antibiotics are desperately needed. Both VRE (4th) and VRSA (5th) appear on this ranked list [Willyard, *Nature*, 543(7643):15 **2017** Feb 28].

The structural formula and the analogs set out in Table 1, below, illustrate vancomycin (**1**) and

binding pocket-modified analogs designed and
previously explored to overcome bacterial resistance.

Table 1

Antimicrobial Activity, MIC[a] (μg/mL)

	sensitive	MRSA	VanA		VanB
	S. aureus[b]	S. aureus[c]	E. faecalis[d]	E. faecium[e]	E. faecalis[f]
1, X = O	0.5	0.5	250	250	8
2, X = S	>32	>32	>32	>32	>32
3, X = NH	nd[g]	nd[g]	0.5	0.5	nd[g]
4, X = H$_2$	nd[g]	nd[g]	31	31	nd[g]

[a]MIC = Minimum inhibitory concentration. [b]ATCC 25923. [c]ATCC 43300.
[d]BM 4166. [e]ATCC BAA-2317. [f]ATCC 51299. [g]not determined.

The primary biological target for
vancomycin and the glycopeptide antibiotics is
bacterial cell wall precursors containing the
dipeptide D-Ala-D-Ala, binding to which results in
inhibition of cell wall maturation [Perkins,
Pharmacol. Ther. **1982**, *16(2)*:181-197]. This target
is unique to bacteria and contributes to the
selectivity of the antibiotic class for bacteria

versus their mammalian hosts. It is also an atypical
biological target, being a substrate for an enzymatic
reaction and a precursor to a structural component of
the bacterial cell wall. It is not a protein or
nucleic acid biological target subject to changes by
a single genetic mutation to the target that can
result in resistance.

The primary mechanism of action of
vancomycin involves sequestration of this substrate
(D-Ala-D-Ala) for a late-stage enzyme-catalyzed (aPBP
or bPBP transpeptidase) reaction used for cell wall
cross-linking [Kahne, *Chem. Rev.* **2005**, *105(2)*:425-
448]. Thus, the nature of the target (D-Ala-D-Ala)
and the antibiotic mechanism of action (sequestration
of an enzyme substrate) are difficult for the
organism to genetically alter or overcome by a single
genetic alteration.

Vancomycin is also thought to inhibit the
preceding step in the cell wall biosynthesis, the
aPBP transglycosylase-catalyzed incorporation of
lipid intermediate II into the repeating
polysaccharide backbone of the bacterial cell wall.
In the case of vancomycin, this also requires D-Ala-
D-Ala binding [Allen et al., *Antimicrob. Agents
Chemother.* **1996**, *40(10)*:2356-2362; Goldman et al.,
FEMS Microbiol. Lett. **2000**, *183(2)*:209-214; Ge et
al., *Science* **1999**, *284(5413)*:507-511; Chen et al.,
Proc. Natl. Acad. Sci. USA **2003**, *100(10)*:5658-5663].
However, it is not yet clear whether this occurs
through direct binding of the vancomycin-appended
disaccharide to the enzyme active site and is
observed effectively only with cell wall binding
sites contributing to its localization, or whether
this occurs by indirect enzyme (aPBP) inhibition. It

may be that the impact of direct, but weak, active
site transglycosylase inhibition by the vancomycin
disaccharide is magnified by its cell wall binding
localization.

Because there may be two or more mechanisms
of action, including those yet unknown or whose role
is not yet fully appreciated like the recently
disclosed SEDS-bPBP transglycosylase-transpeptidases
[Meeske et al., *Nature* **2016**, *537(7622)*:634-638], full
bacterial resistance requires the unlikely
simultaneous changes impacting each mechanism.

Further contributing to the durability of
vancomycin is the site of action at the bacterial
cell wall surface. Cell wall penetration or import
is not needed and this permits vancomycin to avoid
the common resistance mechanisms mediated by
expression levels of proteins involved in transport,
efflux, and metabolic deactivation by cytosolic
enzymes [Wright, *Chem. Commun.* **2011**, *47(14)*:4055-
4061].

Finally, it has been suggested that there
are genetic features that presently make the
glycopeptide antibiotics less susceptible to vertical
versus horizontal gene transfer of resistance to
bacterial progeny [Hegstad et al., *Clin. Microbil.
Infect.* **2010**, *16(6)*:541-554]. Regardless of the
origins, it is most revealing that the primary
mechanism of clinical resistance to vancomycin (VanA
and VanB phenotypes) was transferred to pathogenic
bacteria from non-pathogenic organisms that produce
vancomycin and use this inducible resistance
mechanism to protect themselves during vancomycin
production [Marshall et al., *Antimicrob. Agents
Chemother.* **1998**, *42(9)*:2215-2220]. Thus, pathogenic

bacteria themselves have not yet evolved effective
resistance mechanisms to the glycopeptide antibiotics
even after nearly 60 years of widespread use
[Courvalin, *Clin. Infect. Dis.* **2006**, *42*:S25-S34].
[Identified mechanisms of resistance: VanA, VanB and
VanD (inducible D-Ala-D-Ala to D-Ala-D-Lac, 1000-fold
resistant), VanC, VanE and Van G (D-Ala-D-Ser, 10-20-
fold resistant), and thickened cell wall (increased
number of target sites, <10-fold resistant)]. This
has suggested that solutions to VanA and VanB
resistance alone may provide antibiotics with durable
clinical lifetimes.

It is an intricate mechanism of resistance
in which synthesis of the bacterial cell wall
precursors continue with installation of the pendant
N-terminus D-Ala-D-Ala. Resistant bacteria, like the
producing organisms, sense the presence of the
antibiotic [Hong et al., *Adv. Exp. Med. Biol.* **2008**,
631:200-213]. Through use of a two-component cell
surface receptor sensing and subsequent intracellular
signaling system [Bugg et al., *Biochemistry* **1991**,
30(43):10408-10415], producing and resistant
organisms initiate a late stage remodeling of their
peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-Lac
to avoid the action of the antibiotic.

The binding affinity of vancomycin for the
altered ligand is reduced 1000-fold [Walsh, *Science*
1993, *261(5119)*:308-309; McComas et al., *J. Am. Chem.
Soc.* **2003**, *125(31)*:9314-9315] resulting in a
corresponding 1000-fold loss in antimicrobial
activity. In a series of studies, the first
vancomycin analogs were reported that contain changes
at a key single atom site in its target binding
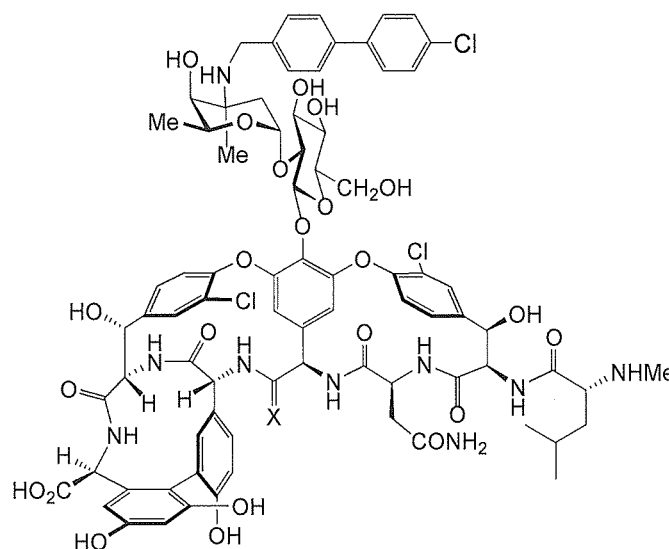pocket (residue 4 carbonyl O $\rightarrow$ S, NH, H_2), the latter

two of which were designed to directly address this underlying molecular basis of resistance to vancomycin (Table 1) [Crowley et al., *J. Am. Chem. Soc.* **2006**, *128(9)*:2885-2892; Xie et al., *J. Am. Chem. Soc.* **2011**, *133(35)*:13946-13949; Xie et al., *J. Am. Chem. Soc.* **2012**, *134(2)*:1284-1297; Okano et al., *J. Am. Chem. Soc.* **2012**, *134(33)*:8790-8793; Okano et al., *J. Am. Chem. Soc.* **2014**, *136(39)*:13522-13525; Okano et al., *J. Am. Chem. Soc.* **2015**, *137(10)*:3693-3704; Boger, *Med Res Rev 2001*, 21(5):356-381].

These two rationally designed binding pocket modifications reinstated binding to the altered target D-Ala-D-Lac and maintained binding affinity for the unaltered target D-Ala-D-Ala. Such dual target binding compounds were found to reinstate antimicrobial activity against vancomycin-resistant organisms that inducibly (D-Ala-D-Ala → D-Ala-D-Lac) or constitutively employ D-Ala-D-Lac peptidoglycan precursors, and remain active against vancomycin-sensitive bacteria that employ only D-Ala-D-Ala precursors [Okano et al., *J. Am. Chem. Soc.* **2015**, *137*:3693-3704]. Moreover, the *in vitro* antimicrobial potencies of such compounds correlated directly with the absolute trends in dual binding affinities of the pocket-modified vancomycin analogs for model target ligands.

It was subsequently found that peripheral functionalization of the binding pocket-modified vancomycin analogs, introducing the oritavancin (4-chlorobiphenyl)methyl (CBP) group to the pendant disaccharide known to enhance antimicrobial potency [Markham, *Drugs* **2014**, 74(15):1823-1828] did indeed enhance antimicrobial potency of the compounds so prepared (Table 2) [Okano et al., *J. Am. Chem. Soc.*

2014, *136(39)*:13522-13525; Okano et al., *J. Am. Chem. Soc.* **2015**, *137(10)*:3693-3704]. Data for those previously reported pocket-modified vancomycins that contain an additional peripheral (4-chlorobiphenyl)methyl (CBP) modification to the pendant disaccharide are shown below.

Table 2

Antimicrobial Activity, MIC[a] (μg/mL)

	sensitive	MRSA	VanA		VanB
	S. aureus[b]	*S. aureus*[c]	*E. faecalis*[d]	*E. faecium*[e]	*E. faecalis*[f]
5, X = O	0.03	0.03	2.5	2.5	0.03
6, X = S	2	2	4	4	2
7, X = NH	0.03	0.06	0.005	0.005	0.06
8, X = H$_2$	0.5	0.25	0.13	0.06	0.5

[a]MIC = Minimum inhibitory concentration. [b]ATCC 25923.
[c]ATCC 43300. [d]BM 4166. [e]ATCC BAA-2317. [f]ATCC 51299.

As is seen, these compounds exhibited a remarkable spectrum of antimicrobial activity (VSSA, MRSA, VanA and VanB VRE) with further improved (about 100-fold) and impressive potencies against both vancomycin-sensitive and vancomycin-resistant bacteria (MICs = 0.06-0.005 μg/mL and 0.5-0.06 μg/mL

for Compounds **7** and **8**, respectively). Moreover and with the benefit of the examination of the residue 4 thioamide **6**, which is incapable of effectively binding either D-Ala-Ala or D-Ala-D-Lac (MICs = 2-4 µg/mL), the activity of such CBP-modified analogs was recognized to be derived from two synergistic mechanisms of action, only one of which is dependent on D-Ala-D-Ala/D-Ala-D-Lac binding [Okano et al., *J. Am. Chem. Soc.* **2015**, *137(10)*:3693-3704].

The disclosures that follows provide studies that clarify this second mechanism of action, as well as detail an alternative peripheral modification that endows the pocket-modified vancomycin analogs with another, different second mechanism of action that is also independent of D-Ala-D-Ala/D-Ala-D-Lac binding. This modification also provides synergistic and similarly impressive improvements in antimicrobial potencies against vancomycin-resistant bacteria (VRE).

Further, the two such peripheral modifications are shown to be combinable with the pocket-modified vancomycins to provide even more potent antimicrobial agents whose activity can be attributed to three independent and synergistic mechanisms of action, only one of which requires D-Ala-D-Ala/D-Ala-D-Lac binding.

The following disclosure also demonstrates that such peripherally- and binding pocket-modified vancomycins display little propensity for acquired resistance through serial exposure of vancomycin-resistant Enterococci (VRE) and that these compounds' durability against such challenges as well as their potency follow predicable trends (3 > 2 > 1 mechanisms of action). Such antibiotics display
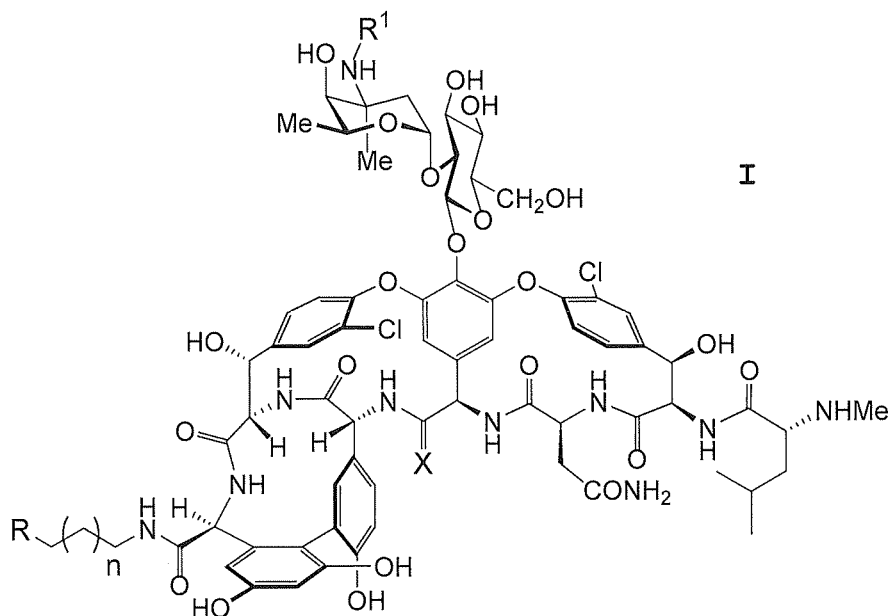
durable antimicrobial activity that is not prone to
rapidly acquired clinical resistance.

BRIEF SUMMARY OF THE INVENTION

In a quest for antibiotics that can display
durable clinical lifetimes, analogs of the
glycopeptide antibiotics including vancomycin have
been designed that not only directly overcome the
molecular basis of existing vancomycin resistance,
but that contain two added peripheral modifications
that endow them with two additional independent
mechanisms of actions not found in the parent
antibiotics. It is shown hereinafter that such
peripherally- and binding pocket-modified vancomycin
analogs described herein display little propensity
for acquired resistance by vancomycin-resistant
Enterococci (VRE), and that both their antimicrobial
potency and durability against such challenges follow
trends (3 > 2 > 1 mechanisms of action) that are now
predictable.

The present invention contemplates a
compound, a pharmaceutical composition containing the
compound and a method of treatment using the
compound. A contemplated compound corresponds in
structure to that shown in Formula **I** or its
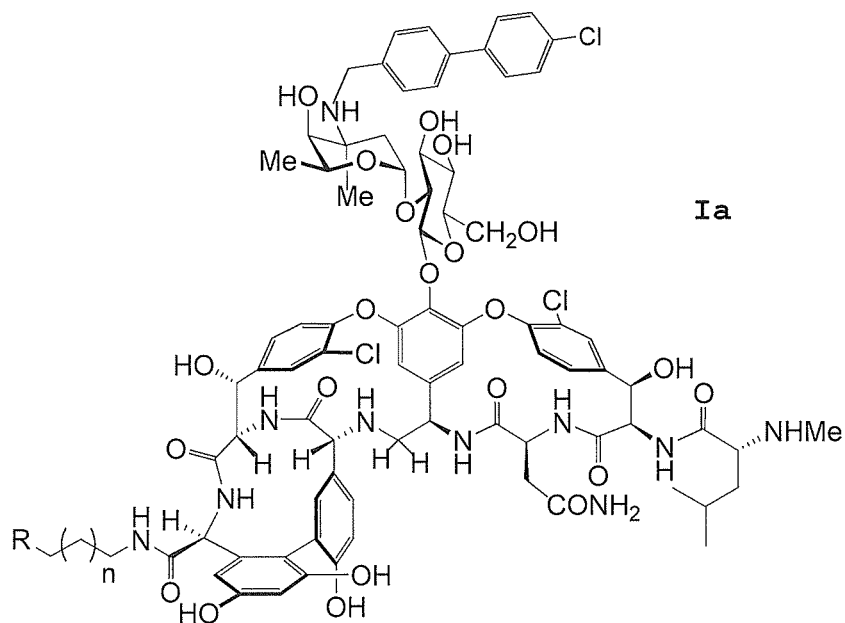pharmaceutically acceptable salt

In a contemplated compound, X is H_2, O or NH; and n is 0, 1 or 2. R is a substituent group that is selected from the group consisting of N,N-(di-C_1-C_6-hydrocarbyl)amino, N,N,N-(tri-C_1-C_6-hydrocarbyl)-ammonium, N-(C_{10}-C_{18}-hydrocarbyl)-N,N-(di-C_1-C_6-hydrocarbyl)ammonium, and N-(C_1-C_6-hydrocarbyl)-N-(C_5-C_7-cyclohydrocarbyl)ammonium; and R^1 is H (hydrido) or halo(C_1-C_{12})-hydrocarbyldiyl. When R is an ammonium group, as is preferred, an appropriate pharmaceutically acceptable anion is also present.

In a compound of Formula **I**, X is preferably H_2 or NH, and n is individually, preferably 1. Preferably, R^1 is hydrido or a 4-(4'-chlorophenyl)-phenylmethyldiyl (CBP) group, with the latter substituent being particularly preferred. Additionally, R^1 is other than hydrido when X is O.

-14-

Of the contemplated compounds, in one
embodiment, a compound corresponds in structure to
Formula **Ia**, in which R^1 is CBP, and n and R are as
defined above.

In another embodiment, a contemplated compound
corresponds in structure to Formula **Ib**, in which R^1
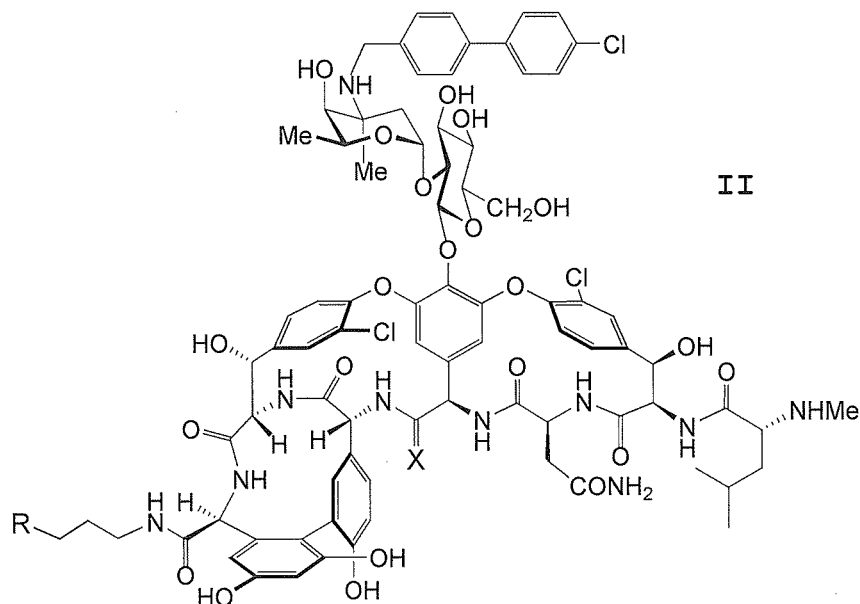is CBP, and n and R are as defined above

Ib

In a still further embodiment, a contemplated compound corresponds in structure to Formula **Ic**, in which R^1 is CBP, and n and R are as defined above

Ic

It is also particularly preferred that "n" be 1, so that the linker between the amido nitrogen

and R groups is a propan-1,3-diyl ($-CH_2CH_2CH_2-$) group.

A particularly preferred compound corresponds in structure to Formula **II**

in which X = H_2, O or NH; and R is selected from the group consisting of N,N-(di-C_1-C_6-hydrocarbyl)amino, N,N,N-(tri-C_1-C_6-hydrocarbyl)ammonium, N-(C_{10}-C_{18}-hydrocarbyl)-N,N-(di-C_1-C_6-hydrocarbyl)ammonium, and N-(C_1-C_6-hydrocarbyl)-N-(C_5-C_7-cyclohydrocarbyl)-ammonium. Again, when R is an ammonium compound, an appropriate anion is also present. Preferably, that anion is a pharmaceutically acceptable anion such as a halide like chloride, bromide, or iodide, a carbonate, bicarbonate, sulfate, bisulfate, benzene sulfonate, or a methane sulfonate.

Also contemplated is a pharmaceutical composition that comprises a pharmaceutically acceptable diluent (carrier) in which is dispersed or

dissolved an anti-microbial amount of a compound of
Formula **I** or **II** as discussed above.

Definitions

In the context of the present invention and
the associated claims, the following terms have the
following meanings:

The articles "a" and "an" are used herein
to refer to one or to more than one (i.e., to at
least one) of the grammatical object of the article.
By way of example, "an element" means one element or
more than one element.

The word "hydrocarbyl" is used herein as a
short hand term for a non-aromatic group that
includes straight and branched chain aliphatic as
well as alicyclic groups or radicals that contain
only carbon and hydrogen. Thus, alkyl, alkenyl and
alkynyl groups are contemplated, whereas aromatic
hydrocarbons such as phenyl.

Where a specific aliphatic hydrocarbyl
substituent group is intended, that group is recited;
i.e., C_1-C_4 alkyl, methyl or tert-butyl. Exemplary
hydrocarbyl groups contain a chain of 1 to 4 carbon
atoms, and preferably 1 or 2 carbon atoms.

A particularly preferred hydrocarbyl group
is an alkyl group. As a consequence, a generalized,
but more preferred substituent can be recited by
replacing the descriptor "hydrocarbyl" with "alkyl"
in any of the substituent groups enumerated herein.

Examples of alkyl radicals include methyl,
ethyl, n-propyl, isopropyl, n-butyl, isobutyl,
sec-butyl, tert-butyl. Examples of suitable alkenyl
radicals include ethenyl (vinyl), 2-propenyl, 3-
propenyl, 1,4-butadienyl, 1-butenyl, 2-butenyl, and

3-butenyl. Examples of alkynyl radicals include ethynyl, 2-propynyl, 1-propynyl, 1-butynyl, 2-butynyl, 3-butynyl, and 1-methyl-2-propynyl.

As a skilled worker will understand, a substituent that cannot exist such as a C_1 alkenyl group is not intended to be encompassed by the word "hydrocarbyl", although such substituents with two or more carbon atoms are intended.

Usual chemical suffix nomenclature is followed when using the word "hydrocarbyl" except that the usual practice of removing the terminal "yl" and adding an appropriate suffix is not always followed because of the possible similarity of a resulting name to one or more substituents. Thus, a hydrocarbyl ether is referred to as a "hydrocarbyloxy" group rather than a "hydrocarboxy" group as may possibly be more proper when following the usual rules of chemical nomenclature. Illustrative hydrocarbyloxy groups include methoxy, ethoxy, n-propoxy, isopropoxy, allyloxy, n-butoxy, iso-butoxy, sec-butoxy, and tert-butoxy groups.

The present invention has several benefits and advantages.

One salient benefit of the invention is the enhanced potency of the contemplated C-terminus-modified vancomycin compounds against both vancomycin-resistant bacteria (VRE) and those bacteria that are not vancomycin-resistant.

A salient advantage of the invention is that the C-terminal-modification provides synergistic potency enhancements to those provided by the incorporation of a (4-chlorobiphenyl)methyl (CBP) addition to the vancomycin disaccharide.

A further salient benefit of the invention is that the contemplated C-terminus-modified vancomycin compounds function by three independent, synergistic mechanisms, only one of which requires D-Ala-D-Ala/D-Ala-D-Lac binding.

A further salient advantage of the invention is a contemplated modified vancomycin displays little propensity for acquired resistance through serial exposure of vancomycin-resistant Enterococci (VRE) and that their durability against such challenges as well as their antimicrobial potency follow predicable trends (3 > 2 > 1 mechanisms of action). Such antibiotics display durable antimicrobial activity not prone to rapidly acquired clinical resistance.

Still further benefits and advantages will be apparent to the skilled worker from the detailed description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

In the drawings forming a portion of this disclosure,

Fig. 1 is a graph whose bar heights illustrate inhibition of bacterial cell synthesis in VRE, VanA, *E. faecalis* ATCC BM4166 as quantitated by accumulation of the peptidoglycan precursor UDPMurNAc-pp (**19**) in cytosol by treatment with an enumerated vancomycin analog (35 μg/mL);

Fig. 2 is a graph whose bar heights illustrate inhibition of bacterial cell synthesis in VRE, VanA, ATCC BAA-2317 as quantitated by accumulation of the peptidoglycan precursor UDPMurNAc-pp (**19**) in cytosol by treatment with a vancomycin analog (35 μg/mL);

Fig. 3 is a graph whose plots illustrate
cell wall permeability induced by compounds **1-8** (10
µM added at 5 minutes) in VanA VRE (*E. faecium* ATCC
BAA-2317);

Fig. 4 is a graph whose plots illustrate
cell wall permeability induced by compounds **9-13** (10
µM added at 5 minutes) in VanA VRE (*E. faecium* ATCC
BAA-2317);

Fig. 5 is a graph whose plots illustrate
cell wall permeability induced by compounds **14-18** (10
µM added at 5 minutes) in VanA VRE (*E. faecium* ATCC
BAA-2317);

Fig 6 is a table that provides a summary of
distinct mechanisms of action of preferred modified
vancomycin analogs and their individual and
cumulative effect on antimicrobial activity against
VanA VRE;

Fig 7 is a table that provides a summary of
the minimum inhibitory concentrations (MICs) of
various of the modified vancomycins disclosed herein
that illustrate the varying potencies provided by the
modifications;

Fig. 8 and Fig. 9 are graphs showing
increases in MIC with increasing passaging of
modified vancomycins disclosed herein and commercial
anti-bacterials daptomycin, tigecycline and linezolid
against VRE, VanA *E. faecium* (ATCC BAA 2317) (Fig. 8)
and VRE, VanA *E. faececalis* (BM 4166) (Fig. 9);

Fig. 10 is a table showing a summary of
resistance development study against VRE, VanA *E.
faecium* (ATCC BAA 2317) resulting from serial
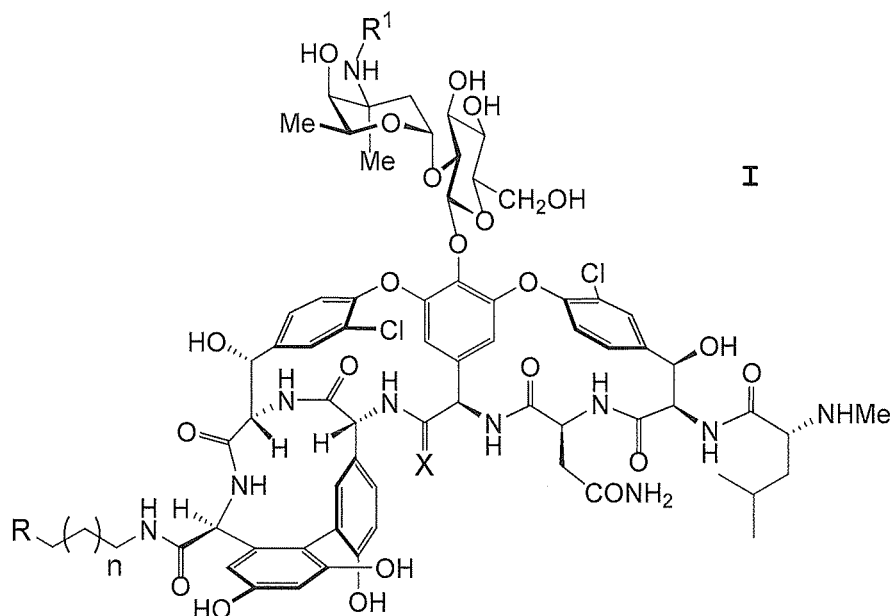passaging (up to 50 passages) in the presence of 0.5

x MIC levels of vancomycin analogs. One of two
replicate studies is presented; and

Fig. 11 and Fig. 12 are graphs illustrating
cell wall permeability similar to those of Figs. 4
and 5, but induced by Compounds **1, 12, 15 and 18** (10
mM added at 5 minutes) in VanA VRE (*E. faecalis* BM
4166; Fig. 11) and induced by those same Compounds
(10 mM added at 5 minutes) in VanA VRE (*E. faecium*
ATCC BAA-2317; Fig. 12).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

A compound, a pharmaceutical composition
containing the compound and a method of treatment
using the compound are contemplated herein. A
contemplated compound corresponds in structure to
that shown in Formula **I** or its pharmaceutically
acceptable salt

In a compound of Formula **I**, X is H_2, O or NH. As can be seen from the data in Tables 1 and 2, above, similar compounds in which X is H_2 or NH are more potent than are those in which X is O. X is therefore preferred to be H_2 or NH.

R is a substituent group that contains a tertiary amine or a quaternary amine group. An R substituent is selected from the group consisting of N,N-(di-C_1-C_6-hydrocarbyl)amino, N,N,N-(tri-C_1-C_6-hydrocarbyl)ammonium, N-(C_{10}-C_{18}-hydrocarbyl)-N,N-(di-C_1-C_6-hydrocarbyl)ammonium, and N-(C_1-C_6-hydrocarbyl)-N-(C_5-C_7-cyclohydrocarbyl)ammonium. A preferred C_1-C_6-hydrocarbyl group is a C_1 (methyl) group, so that a preferred N,N-(di-C_1-C_6-hydrocarbyl)amino group is a N,N-dimethylamino group and a preferred N,N,N-(tri-C_1-C_6-hydrocarbyl)ammonium group is a N,N,N-(tri-methylammonium) substituent. A C_{10}-C_{18}-hydrocarbyl group is preferably a straight chain alkyl or alkenyl group such as a lauryl, myristyl, palmityl, stearyl, oleyl or palmitoleyl group. When R is an ammonium group, as is preferred, an appropriate, preferably a pharmaceutically acceptable, anion is also present.

The linker chain between the amido nitrogen atom and the nitrogen atom of the R group can contain 2, 3, or 4 carbon atoms such that n is 0, 1 or 2. It is preferred that n be 1 so that three carbons are in the chain of the linker.

A R^1 substituent is H (hydrido) or halo(C_1-C_{12})-hydrocarbyldiyl. R^1 is other than hydrido when X is O. A preferred halo(C_1-C_{12})-hydrocarbyldiyl

substituent is a 4-(4'-chlorophenyl)-phenylmethyldiyl
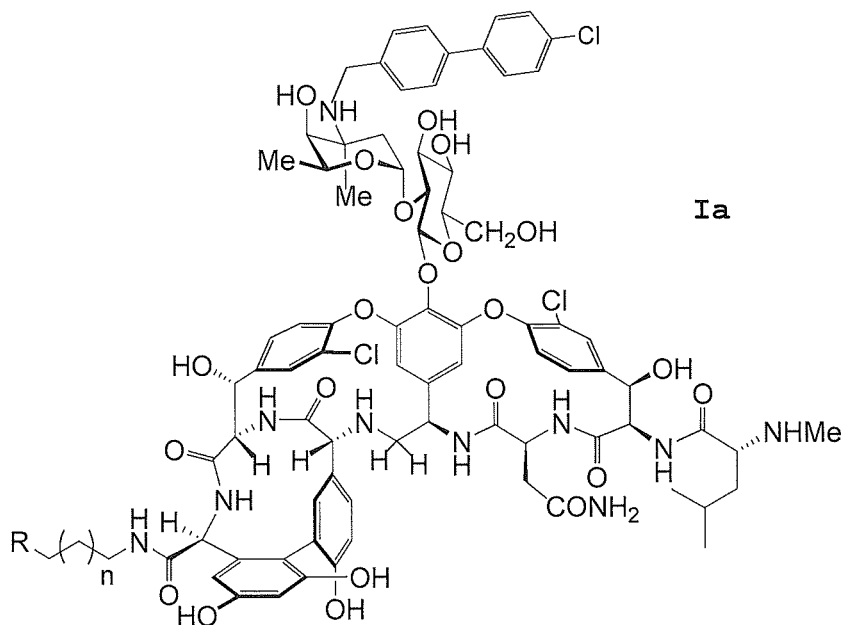group, below, that can also be named

a 4-(4'-chlorobiphenyl)methyl group (CBP), or a 4-
(4'-chlorophenyl)benzyl group, and is abbreviated
herein as "4-CBP" or just "CBP".

In a compound of Formula **I**, X is preferably
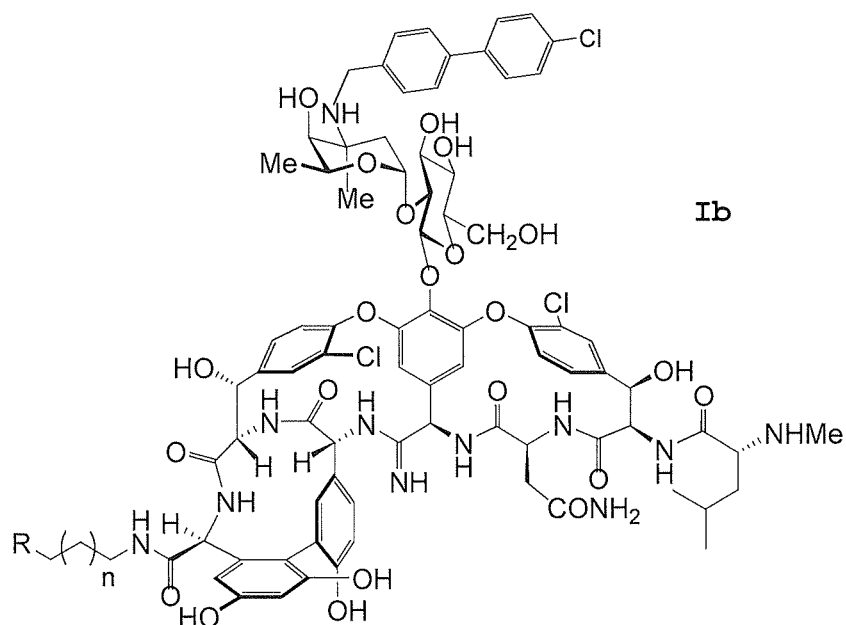H_2 or NH, and n is individually, preferably 1.

Preferably, R^1 is hydrido or a 4-(4'-chlorophenyl)-
phenylmethyldiyl (CBP) group, with the latter
substituent being particularly preferred.
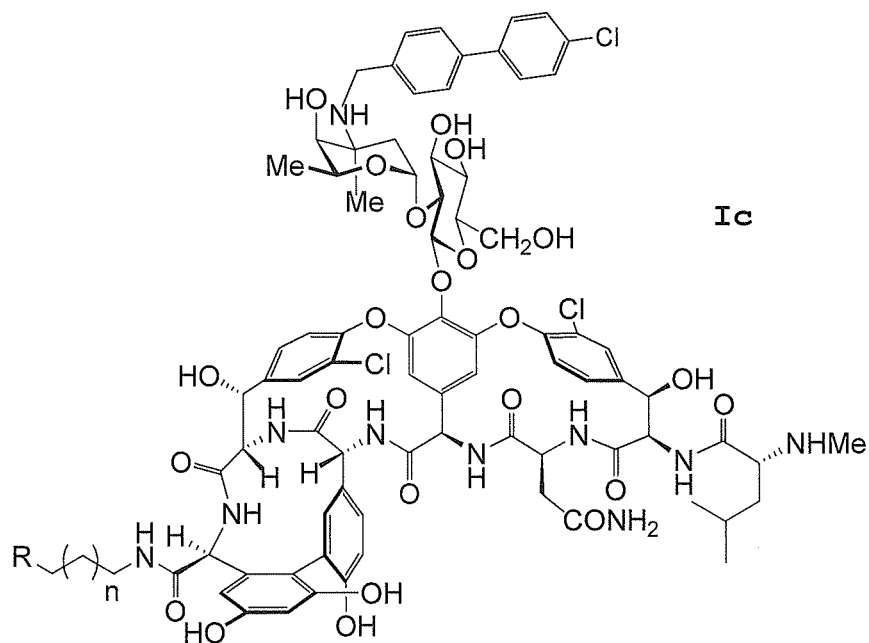Additionally, R^1 is other than hydrido when X is O.

Of the contemplated compounds, in one
embodiment, a compound corresponds in structure to
Formula **Ia**, in which R^1 is CBP, and n and R are as
defined above.

In another embodiment, a contemplated compound corresponds in structure to Formula **Ib**, in which R^1 is CBP, and n and R are as defined above
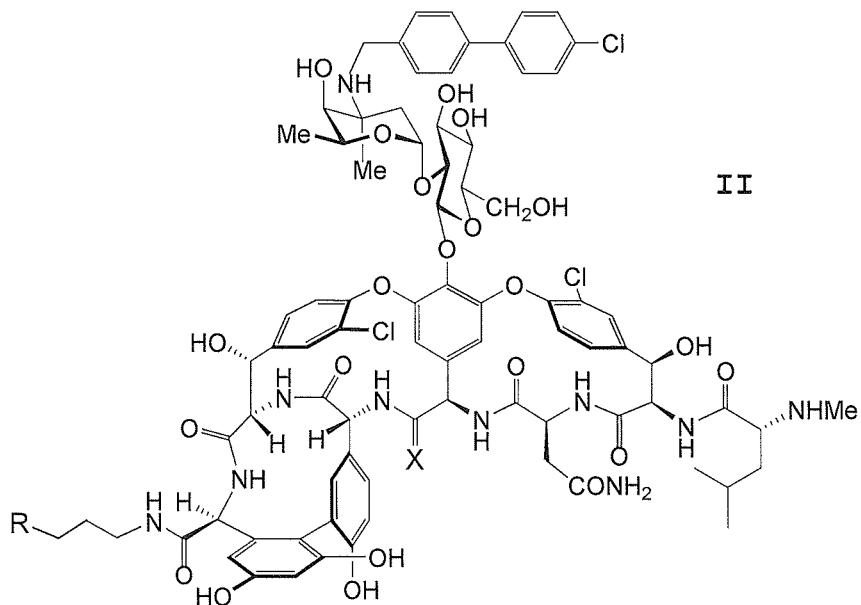

Ib

In a still further embodiment, a contemplated compound corresponds in structure to Formula **Ic**, in which R^1 is CBP, and n and R are as defined above

Ic

It is also particularly preferred that "n" be 1, so that the linker between the amido nitrogen and R groups contains a chain of 3 carbon atoms, and is therefore a propan-1,3-diyl (-CH2CH2CH2-) group.

A particularly preferred compound corresponds in structure to Formula **II**

II

in which X = H_2, O or NH; and R is selected from the group consisting of N,N-(di-C_1-C_6-hydrocarbyl)amino, N,N,N-(tri-C_1-C_6-hydrocarbyl)ammonium, N-(C_{10}-C_{18}-hydrocarbyl)-N,N-(di-C_1-C_6-hydrocarbyl)ammonium, and N-(C_1-C_6-hydrocarbyl)-N-(C_5-C_7-cyclohydrocarbyl)-ammonium. Again, when R is an ammonium compound, an appropriate anion is also present. Preferably, that anion is a pharmaceutically acceptable anion such as a halide like chloride, bromide, or iodide, a carbonate, bicarbonate, sulfate, bisulfate, benzene sulfonate, or a methane sulfonate. Appropriate anions are discussed further hereinafter.

Also contemplated is a pharmaceutical composition that comprises a pharmaceutically acceptable diluent (carrier) in which is dispersed or dissolved an anti-microbial amount of a compound of Formula **I** or **II** as discussed above.

COMPOSITION AND TREATMENT METHOD

A further aspect of the invention is a method of treating a mammal infected with a microbial infection such as a bacterial infection, typically a Gram-positive infection; i.e., an infection caused by Gram-positive bacteria, and in need of antimicrobial (antibacterial) treatment. In accordance with a contemplated method, an antibacterial-effective amount of one or more compounds of Formula **I** or **II** or a pharmaceutically acceptable salt of such a compound is administered to an infected mammal in need.

The compound can be administered as a solid or as a liquid formulation, and is preferably administered via a pharmaceutical composition

discussed hereinafter. That administration can also
be oral or parenteral, as are also discussed further
hereinafter.

It is to be understood that viable mammals
are infected with bacteria and other microbes. The
present invention's method of treatment is intended
for use against infections of pathogenic microbes
that cause illness in the mammal to be treated.
Illustrative pathogenic microbes include *S. aureus*,
methicilin-resistant *S. aureus* (MRSA), VanA strains
of *E. faecalis* and *E. feacium*, as well as VanB
strains of *E. faecalis*. Evidence of the presence of
infection by pathogenic microbes is typically
understood by physicians and other skilled medical
workers.

A mammal in need of treatment (a subject)
and to which a pharmaceutical composition containing
a Compound of Formula **I** or **II**, or its
pharmaceutically acceptable salt can be administered
can be a primate such as a human, an ape such as a
chimpanzee or gorilla, a monkey such as a cynomolgus
monkey or a macaque, a laboratory animal such as a
rat, mouse or rabbit, a companion animal such as a
dog, cat, horse, or a food animal such as a cow or
steer, sheep, lamb, pig, goat, llama or the like.

As is seen from the data that follow, a
contemplated compound is active in *in vitro* assay
studies at less than 1 μg/mL amounts, which
corresponds to a molar concentration of about 1 to
about 100 nanomolar (nM), using the molecular weight
of Compound **18**. When used in an assay such as an *in
vitro* assay, a contemplated compound is typically
present in the composition in an amount that is

sufficient to provide a concentration of about 0.1 nM to about 1 μM to contact microbes to be assayed.

The amount of a compound of Formula **I** or a pharmaceutically acceptable salt of such a compound that is administered to a mammal in a before-discussed method or that is present in a pharmaceutical composition discussed below, which can be used for that administration, is an antibiotic (or antibacterial or antimicrobial) effective amount. It is to be understood that that amount is not an amount that is effective to kill all of the pathogenic bacteria or other microbes present in an infected mammal in one administration. Rather, that amount is effective to kill some of the pathogenic organisms present without also killing the mammal to which it is administered, or otherwise harming the recipient mammal as is well known in the art. As a consequence, the compound usually has to be administered a plurality of times, as is discussed in more detail hereinafter.

A contemplated pharmaceutical composition contains an effective antibiotic (or antimicrobial) amount of a Compound of Formula **I** or **II** or a pharmaceutically acceptable salt thereof dissolved or dispersed in a physiologically (pharmaceutically) acceptable diluent or carrier. An effective antibiotic amount depends on several factors as is well known in the art. However, based upon the relative potency of a contemplated compound relative to that of vancomycin itself for a susceptible strain of *S. aureus* shown hereinafter, and the relative potencies of vancomycin and a contemplated compound against the VanA *E. faecalis* and *E. faecium* strains, a skilled worker can readily determine an appropriate dosage amount.

Exemplary salts useful for a contemplated compound include but are not limited to the following: sulfate, hydrochloride, hydro bromides, acetate, adipate, alginate, citrate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, camphorate, camphorsulfonate, digluconate, cyclopentanepropionate, dodecylsulfate, ethanesulfonate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, fumarate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxy-ethanesulfonate, lactate, maleate, methanesulfonate, nicotinate, 2-naphthalenesulfonate, oxalate, palmoate, pectinate, persulfate, 3-phenyl-propionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, mesylate and undecanoate.

The reader is directed to Berge, *J. Pharm. Sci.* 1977 **68(1)**:1-19 for lists of commonly used pharmaceutically acceptable acids and bases that form pharmaceutically acceptable salts with pharmaceutical compounds.

In some cases, the salts can also be used as an aid in the isolation, purification or resolution of the compounds of this invention. In such uses, the salt prepared need not be pharmaceutically acceptable.

A contemplated composition is typically administered repeatedly *in vivo* to a mammal in need thereof until the infection is diminished to a desired extent, such as cannot be detected. Thus, the administration to a mammal in need can occur a plurality of times within one day, daily, weekly, monthly or over a period of several months to several years as directed by the treating physician. More usually, a contemplated composition is administered a plurality of times over a course of treatment until a desired effect is achieved,

typically until the bacterial infection to be treated has ceased to be evident.

A contemplated pharmaceutical composition can be administered orally (perorally) or parenterally, in a formulation containing conventional nontoxic pharmaceutically acceptable carriers or diluents, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection, or infusion techniques. Formulation of drugs is discussed in, for example, Hoover, John E., *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, Pennsylvania; 1975 and Liberman, H.A. and Lachman, L., Eds., *Pharmaceutical Dosage Forms*, Marcel Decker, New York, N.Y., 1980.

In some embodiments, a contemplated pharmaceutical composition is preferably adapted for parenteral administration. Thus, a pharmaceutical composition is preferably in liquid form when administered, and most preferably, the liquid is an aqueous liquid, although other liquids are contemplated as discussed below, and a presently most preferred composition is an injectable preparation.

Thus, injectable preparations, for example, sterile injectable aqueous or oleaginous solutions or suspensions can be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation can also be a sterile injectable solution or suspension in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution, and isotonic sodium chloride solution, phosphate-buffered saline.

Other liquid pharmaceutical compositions include, for example, solutions suitable for parenteral

administration. Sterile water solutions of a Compound of
Formula **I** or **II** or its salt or sterile solution of a
Compound of Formula **I** or **II** in a solvent comprising water,
ethanol, or propylene glycol are examples of liquid
compositions suitable for parenteral administration. In
some aspects, a contemplated Compound of Formula **I** or **II**
is provided as a dry powder that is to be dissolved in an
appropriate liquid medium such as sodium chloride for
injection prior to use.

In addition, sterile, fixed oils are
conventionally employed as a solvent or suspending medium.
For this purpose any bland fixed oil can be employed
including synthetic mono- or diglycerides. In addition,
fatty acids such as oleic acid find use in the preparation
of an injectable composition. Dimethyl acetamide,
surfactants including ionic and non-ionic detergents,
polyethylene glycols can be used. Mixtures of solvents
and wetting agents such as those discussed above are also
useful.

A sterile solution can be prepared by
dissolving the active component in the desired solvent
system, and then passing the resulting solution through a
membrane filter to sterilize it or, alternatively, by
dissolving the sterile compound in a previously sterilized
solvent under sterile conditions.

Solid dosage forms for oral administration
can include capsules, tablets, pills, powders, and
granules. The amount of a contemplated Compound or
salt of Formula **I** or **II** such as Compound **18** in a
solid dosage form is as discussed previously, an
amount sufficient to provide an effective antibiotic
(or antimicrobial) amount. A solid dosage form can
also be administered a plurality of times during a
one week time period.

In such solid dosage forms, a compound of
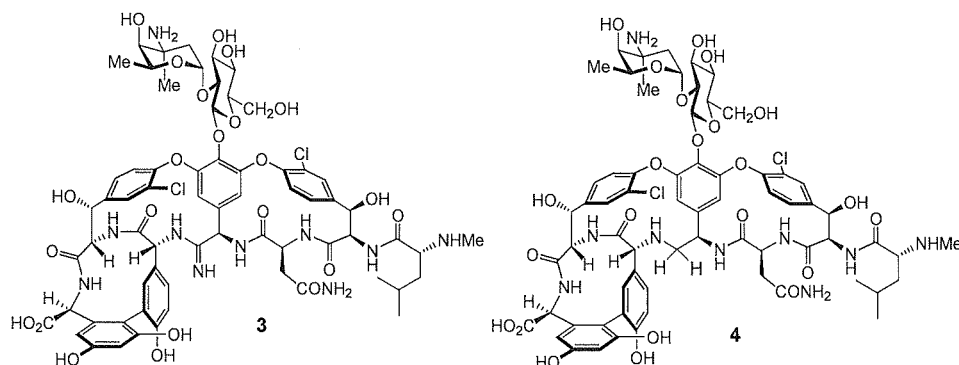this invention is ordinarily admixed as a solution or

suspension in one or more diluents appropriate to the indicated route of administration. If administered *per os*, the compounds can be admixed with lactose, sucrose, starch powder, cellulose esters of alkanoic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate, polyvinylpyrrolidone, and/or polyvinyl alcohol, and then tableted or encapsulated for convenient administration. Such capsules or tablets can contain a controlled-release formulation as can be provided in a dispersion of active compound in hydroxypropylmethyl cellulose. In the case of capsules, tablets, and pills, the dosage forms can also comprise buffering agents such as sodium citrate, magnesium or calcium carbonate or bicarbonate. Tablets and pills can additionally be prepared with enteric coatings.

Where an *in vitro* assay is contemplated, a sample to be assayed such as cells and tissue can be used. These *in vitro* compositions typically contain water, sodium or potassium chloride, and one or more buffer salts such as and acetate and phosphate salts, Hepes or the like, a metal ion chelator such as EDTA that are buffered to a desired pH value such as pH 4.0 -8.5, preferably about pH 7.2-7.4, depending on the assay to be performed, as is well known.

Preferably, the pharmaceutical composition is in unit dosage form. In such form, the composition is divided into unit doses containing appropriate quantities of the active compound. The unit dosage form can be a packaged preparation, the package containing discrete quantities of the preparation, for example, in vials or ampules.

RESULTS AND DISCUSSION

  The studies described herein are described
with the methylene pocket-modified vancomycin analog
Compound **4** ([Ψ[CH₂NH]Tpg⁴]vancomycin; below), [Okano
et al., *J. Am. Chem. Soc.* **2015**, *137(10)*:3693-3704]
prepared by total synthesis. [Okano et al., *J. Am.
Chem. Soc.* **2015**, *137(10)*:3693-3704; Boger, *Med. Res.
Rev.* **2001**, *21*:356-381; Nicolaou et al., *Angew. Chem.,
Int. Ed.* **1999**, *38*:2096-2152; Wright et al., *Angew.
Chem. Int. Ed.* **2014**, *53*:8840-8869]. Because it also
exhibits the more modest dual D-Ala-D-Ala/D-Ala-D-Lac
binding affinity Crowley et al., *J. Am. Chem. Soc.*
2006, *128(9)*:2885-2892] and antimicrobial activity
against vancomycin-resistant organisms of the pocket-
modified vancomycin analogs (**4** vs **3**, Table 1), the
impact of an alternative as well as multiple
peripheral modifications was anticipated to be most
easily observed.

  The second alternative, peripheral
modification examined was C-terminus amide
functionalization with incorporation of either a
basic amine capable of protonation or a quaternary
ammonium salt bearing a cationic charge [Xie et al.,
J Am Chem Soc **2012**, *134(2)*:1284-1297]. Several
classes of commercial quaternary ammonium salts are

used as antimicrobial agents and act by disrupting
the bacterial cell membrane [Jennings et al., *ACS
Infect. Dis.* **2015,** *1*:288-303]. Recently, such
modifications have been introduced into vancomycin,
resulting in improved antimicrobial activity against
vancomycin-resistant organisms where analogs
containing the modifications were shown to disrupt
bacterial cell wall membrane integrity, increasing
cell permeability and inducing bacterial membrane
depolarization [Yarlagadda et al., *J. Med. Chem.*
2014, *57(11)*:4558-4568].

Although inspired by the nonselective
membrane disruption induced by quaternary ammonium
salts, the observed results illustrate a modification
that exhibits only a subset of such effects (membrane
permeability, and acts by a more specific mechanism
not resulting in cell lysis. The above-described
behavior and mechanism are not observed with the
naturally occurring glycopeptide antibiotics or their
more typical analogs, but the mechanism is one that
may contribute to the improved spectrum of activity
of the semisynthetic clinical drugs dalbavancin and
telavancin [Higgins et al., *Antimicrob Agents
Chemother* **2005,** 49(3):1127-1134]. In vancomycin-
resistant organisms, such modifications do not
directly contribute to inhibition of bacterial cell
wall biosynthesis, do not improve D-Ala-D-Lac binding
needed to express such effects, and act independent
of mechanisms resulting from inhibition of the action
of aPBP transpeptidases or transglycosylases.

A select series of such vancomycin analogs
were prepared, including Compounds **10** and **11** not
previously examined. For simplicity, they are
referred to as C0 (**9**) [Yarlagadda et al., *J. Med.*

Chem. **2014**, *57(11)*:4558-4568], C1 (**10**), cyclic C5
(**11**) and C14 (**12**) (*Ibid.*), denoting the terminal
tertiary dimethylamine (**9**, C0) or the quaternary
ammonium salts bearing a methyl (**10**, C1), C5 cyclic
(**11**, C5), or tetradecyl (**12**, C14) substituent. These
compounds are illustrated hereinafter in Table 3.

Based on their assessment in antimicrobial
assays against VanA VRE, the most potent C-terminus
modification found in **12** (C14) was incorporated into
the analogous C14 derivative **13** of the pocket-
modified vancomycin analog **4**. In each case, this was
accomplished in a single step from the fully
functionalized vancomycins **1** or **4** without need for
intermediate protection by coupling the C-terminus
carboxylic acid with the corresponding functionalized
amines (N,N,N',N'-tetramethyl-O-(1H-benzotriazol-1-
yluronium hexafluorophosphate (HBTU), N-methyl-
morpholine (NMM) under conditions modified from those
previously described, (e.g., 1:1 DMF:DMSO, 0.01 M, 25
°C, 5 minutes)[Sundram et al., *J Org Chem* **1995**,
60(5):1102-1103].

These C-terminus changes had a
progressively pronounced impact on activity against
the VanA vancomycin-resistant organisms (VanA VRE)
where the more hydrophobic quaternary ammonium salts
incrementally increased activity up to as much as
100-fold (**12** $\geq$ **11** > **10** > **9**, **1**; Table 3). Notably,
all the quaternary ammonium salts (C1, cyclic C5 and
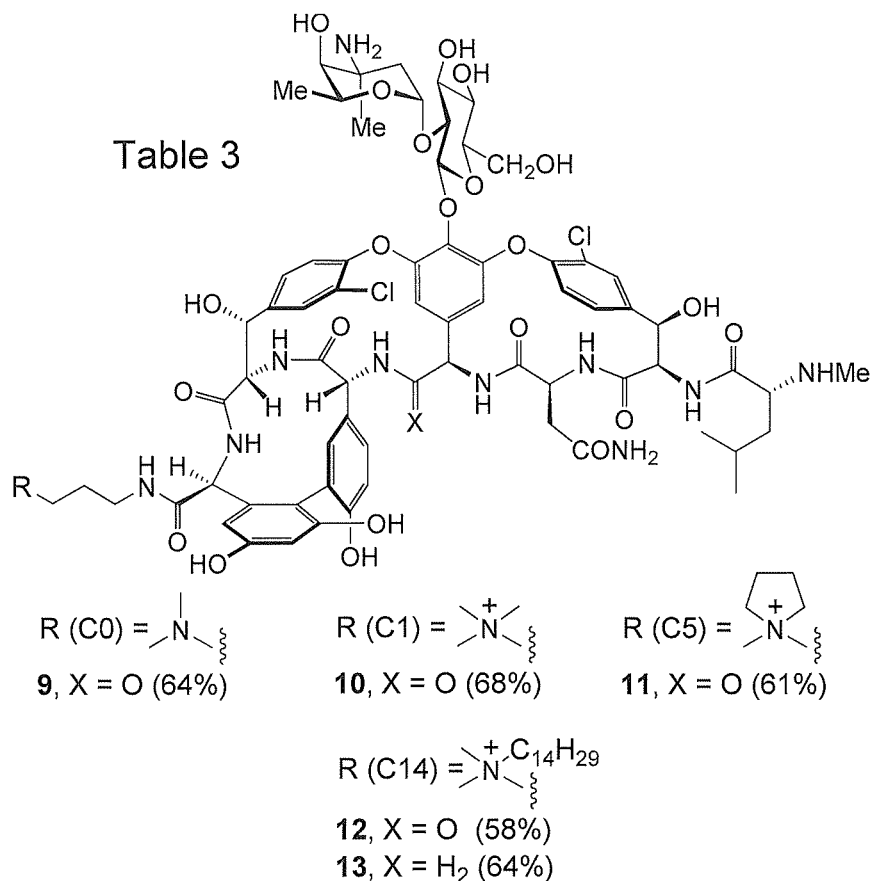C14) improved activity.

For the pocket modified vancomycin analog,
the most potent of these modifications produced a
200-fold increase in potency against VanA VRE,
reducing the MIC value from the modest activity of 31
µg/mL for **4** to 0.16 µg/mL for **13**. This vancomycin

analog is >10-fold more potent than its comparison
vancomycin derivative **12** and >1000-fold more potent
than vancomycin itself.

Thus, an additional and now second
peripheral modification of a pocket-modified
vancomycin analog synergistically increased
antimicrobial activity against the most stringent of
the vancomycin-resistant phenotypes (VanA VRE). As
shown below, this result arises through an
independent second mechanism of action, involving
disruption of bacterial cell membrane integrity,
increasing membrane permeability. This second
synergistic mechanism of action incorporated into **4**
is different from that observed with the peripheral
CBP modification found in **8**.

Table 3

R (C0) = $\overset{|}{N}$
9, X = O (64%)

R (C1) = $\overset{+}{N}$
10, X = O (68%)

R (C5) = $\overset{+}{N}$
11, X = O (61%)

R (C14) = $\overset{+}{N}$—C$_{14}$H$_{29}$
12, X = O (58%)
13, X = H$_2$ (64%)

Antimicrobial Activity, MIC[a] (μg/mL)

	VanA	
	E. faecalis[b]	*E. faecium*[c]
1, vancomycin	250	250
9, X = O (C0)	500	500
10, X = O (C1)	63	126
11, X = O (C5)	4	2
12, X = O (C14)	2	2
4, X = H$_2$	31	31
13, X = H$_2$ (C14)	0.16	0.16

[a]MIC = Minimum inhibitory concentration. [b]BM 4166. [c]ATCC BAA-2317.

More significantly, the impact of combining the two different peripheral modifications was examined. This was explored first with CBP-vancomycin (**5**), coupling its C-terminus carboxylic acid with the same functionalized amines bearing the terminal tertiary dimethylamine (**14**, C0) or C1 (**15**),

cyclic C5 (**16**) and C14 (**17**) quaternary ammonium salts. Based on their assessment in VanA VRE antimicrobial assays, the most effective C-terminus modification found in **15** (C1) was incorporated into the analogous C1 derivative **18** of the pocket-modified CBP-vancomycin analog **8**.

A particularly preferred C-terminus carboxamide derivative of a CBP-vancomycin (X =O), aminomethylene CBP-vancomycin (X = H_2), or amidino CBP-vancomycin (X = NH) can be prepared in one step as shown in Scheme 1, below and in the syntheses hereinafter. The three starting materials (X = O, X = H_2 and X= NH) are prepared as described in Okano et al., *J. Am. Chem. Soc.* **2014**, *136*:13522-13525; and in Okano et al., *J. Am. Chem. Soc.* **2015**, *137*:3693-3704.
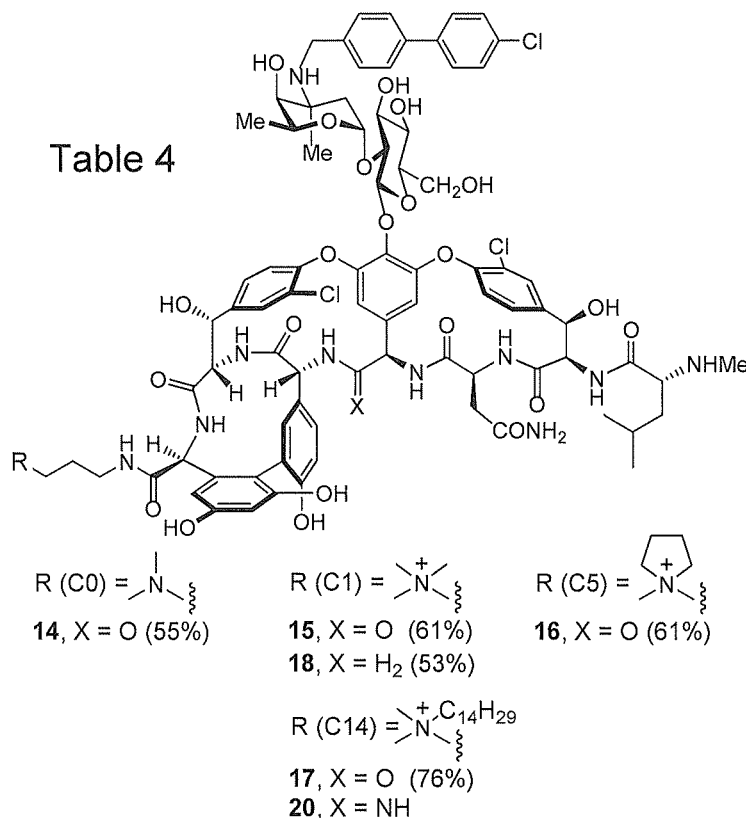
Scheme 1

X= O, NH, or H_2

Notably, the preparation requires a single amide bond coupling reaction and can be conducted without the need for protected intermediates. This second peripheral modification of CBP-vancomycin did not display the same trends observed with vancomycin itself with most couplings not resulting in an

altering of the antimicrobial activity of CBP-
vancomycin against VanA VRE (Table 4, below).

Table 4

R (C0) = $\searrow$N$\swarrow$

14, X = O (55%)

R (C1) = $\searrow$N$^+$$\swarrow$

15, X = O (61%)
18, X = H₂ (53%)

R (C5) = [pyrrolidinium]

16, X = O (61%)

R (C14) = $\searrow$N$^+$–C₁₄H₂₉

17, X = O (76%)
20, X = NH

Antimicrobial Activity, MIC^a (μg/mL)

	VanA	
	E. faecalis^b	*E. faecium*^{c}
1, vancomycin	250	250
5, X = O (CBP)	2.5	2.5
14, X = O (CBP/C0)	5	5
15, X = O (CBP/C1)	0.25	0.5
16, X = O (CBP/C5)	2	2
17, X = O (CBP/C14)	2	2
4, X = H₂	31	31
8, X = H₂ (CBP)	0.13	0.06
18, X = H₂ (CBP/C1)	0.01	0.005

^{a}MIC = Minimum inhibitory concentration. ^{b}BM 4166. ^{c}ATCC BAA-2317.

The exception was Compound **15**, containing
the C1 quaternary ammonium salt, which alone
displayed a 5-10-fold increase in activity against
VanA VRE, exhibiting exceptional activity for a

compound incapable of binding D-Ala-D-Lac. In fact,
it represents a compound likely devoid of the
original glycopeptide antibiotic mechanism of action
(D-Ala-D-Ala binding-dependent cell wall
biosynthesis) in the resistant organisms, but
possesses two alternative effective mechanisms of
action independent of D-Ala-D-Ala/D-Ala-D-Lac
binding.

        The impact of these modifications is more
than additive, improving the activity beyond what
either does alone and, as shown below, results from
inhibition of bacterial cell wall biosynthesis by
direct transglycosylase inhibition due to the CBP
modification and through induced membrane
permeability by the C1 quaternary ammonium salt.
Although unanticipated, the most effective C-terminus
modifications is now C1 for the CBP derivatives.
Subsequent studies show clearly that that that
modification alone imparts membrane permeability not
found with derivatives lacking this particular
C-terminus modification.

        For the pocket modified CBP-vancomycin
analog capable of dual D-Ala-D-Ala/D-Ala-D-Lac
binding, this second additional peripheral
modification with Compound **18** produced a >10-fold
increase in potency against VanA VRE relative to
Compound **8**, lowering the MIC value for **18** to 0.01-
0.005 µg/mL. This vancomycin analog is >10-fold more
potent than the CBP derivative **8**, >1,000-fold more
potent than the pocket analog **4**, and a stunning
>10,000-fold more potent than vancomycin itself. It
is also >25-100-fold more potent than its comparison
C1/CBP-vancomycin derivative Compound **15** and >250-

fold more active than either CBP-vancomycin (**5**) or
C14-vancomycin (**12**).

To clarify the contributing mechanisms
responsible for the antimicrobial activity in
vancomycin-resistant organisms (VanA VRE), the key
analogs were examined in a range of assays, two of
which defined the origin of their effects. One assay
was used to establish inhibition of bacterial cell
wall synthesis and the second measured induced
membrane permeability.

The inhibition of bacterial cell wall
synthesis was established in an assay that
quantitates the accumulation of the peptidoglycan
precursor UDP-*N*-acetyl-muramyl-depsipentapeptide
(Compound **19**, UDPMurNAc-pp), resulting from inhibited
cell wall incorporation following antibiotic
treatment. This was accomplished by following a
protocol that employs vancomycin-resistant *E. faecium*
with tetracycline pretreatment (30 minutes, 37 °C)
[Allen et al., *Antimicrob Agents Chemother* **1996**
40(10):2356-2362]. This procedure was reduced in
scale and modified to enlist reverse-phase HPLC
separation, UV detection, and calibration curve
determination of the amount of isolated **19**,
permitting use with limited sample sizes. A larger
scale isolation and full characterization of **19**
provided the material needed to establish a linear
calibration curve.

The test compounds were incubated with
tetracycline pretreated VanA VRE for 30 minutes (37
°C) before the cultured bacteria was collected by
centrifugation, washed, and resuspended in pH 7.2
buffer (5 mM HEPES and 5 mM glucose, 1:1). The
bacterial suspension was heated at 100 °C for 15

minutes to release cytosolic **19**. The entire
supernatant was analyzed by semi-preparative reverse-
phase HPLC with a single injection and the amount of
19 was quantitated with use of the calibration curve.
The results of the evaluation of Compounds **5-18** in
VanA vancomycin-resistant *E. faecalis* (BM 4166) and
E. faecium (ATCC BAA-2317) are presented in Fig. 1
and Fig. 2, respectively, and represent the same
strains employed in the antimicrobial assays.

The effect of the compounds on cell
membrane permeability was examined by measuring
cytoplasmic membrane uptake of the fluorescent probe
propidium iodide in the same VanA vancomycin-
resistant *E. faecalis* (BM 4166) and *E. faecium* (ATCC
BAA-2317) strains. This fluorescent probe only
enters cells with permeabilized cell membranes and is
detected by the emission of fluorescence upon
intracellular nucleic acid binding [Boulos et al., *J
Microbiol Methods* **1999**, *37(1)*:77-86].

Fresh, mid-log phase VanA VRE in pH 7.2
buffer at 25 °C were preincubated with propidium
iodide (10 µM) for 5 minutes before the test
compounds were added, monitoring the fluorescence
emission at 617 nm (excitation at 535 nm) over time,
both pre (5 minutes) and post (15 minutes) compound
addition. Rapid and sustained increases in
fluorescence intensity are observed immediately upon
addition of test compounds that induce bacterial cell
membrane permeability.

Assessments of the compounds, identifying
their contributing mechanisms of action in
vancomycin-resistant VRE, were conducted with the two
assays. The results are discussed below in sets
first defining the role of the pocket modification

found in **4**, and the subsequent impact of the
peripheral CBP modification to the vancomycin
disaccharide found in **5-8**. This is followed by the
discussion of the results from the examination of the
peripheral quaternary ammonium salt modifications
found in **9-13**, including their effects on both
unmodified and pocket modified vancomycin analogs.
Finally, the effects of the two combined peripheral
modifications in **14-18** are detailed.

        Consistent with their inactivity,
vancomycin (**1**) and the thioamide pocket modified
vancomycin analog **2** (not shown) do not effectively
inhibit bacterial cell wall synthesis in VanA VRE and
do not result in the significant accumulation of **19**
in the assay (Figs. 1 and 2). In contrast, the
pocket-modified analog Compound **4** designed for dual
D-Ala-D-Ala/D-Ala-D-Lac binding inhibits bacterial
cell wall biosynthesis, resulting in the buildup of
the precursor **19** in the assay at levels consistent
with its relative model ligand binding affinities and
antimicrobial activity.

        As anticipated, none of these compounds
significantly impact membrane integrity and none
result in cytoplasmic membrane permeability as
measured by propidium uptake. Thus, the
antimicrobial activity of Compound **4** correlates
directly with its expected impact on bacterial cell
wall biosynthesis, binding D-Ala-D-Lac and inhibiting
cell wall maturation. Incorporation of the
peripheral CBP modification in Compound **5**
(ineffective binding to D-Ala-D-Lac) and Compound **6**
(ineffective binding to either D-Ala-D-Ala or D-Ala-
D-Lac) produced analogs with good activity against
VanA VRE that was found to correlate with their

ability to inhibit cell wall synthesis of VanA VRE, resulting in the accumulation of **19** in the assay (Figs. 1 and 2). Neither compound significantly impacts membrane permeability.

Compounds **5** and **6** exhibit nearly equivalent antimicrobial activity against VanA VRE and both inhibit cell wall biosynthesis to a similar extent, yet **6** is incapable of binding either D-Ala-D-Ala or D-Ala-D-Lac. Thus, this inhibition of bacterial cell wall synthesis is not derived from inhibition of transpeptidase-catalyzed cross-linking derived from D-Ala-D-Ala/D-Ala-D-Lac binding, but rather likely arises from direct inhibition of transglycosylase by the peripherally modified disaccharide. Previous studies of Kahne and others have demonstrated such direct inhibition of transglycosylase by Compound **5** and related CBP-bearing analogs [Allen et al., *Antimicrob Agents Chemother* **1996**, *40(10)*:2356-2362; Goldman et al., *FEMS Microbiol Lett* **2000**, *183(2)*:209-214; Ge et al., *Science* **1999**, *284(5413)*:507-511; Chen et al., *Proc Natl Acad Sci. USA* **2003**, *100(10)*:5658-5663; Allen et al., *FEMS Microbiol Rev* **2003**, *26(5)*:511-532].

Finally, the potent pocket-modified vancomycin analog Compound **8**, containing the peripheral CBP modification, inhibits cell wall synthesis more effectively than **4**, lacking the CBP modification, and more potently than either Compounds **5** or **6**, lacking a productive pocket modification. This is the result of the combined effects of the two independent mechanisms of action, both of which impact cell wall biosynthesis but only one of which depends on D-Ala-D-Ala/D-Ala-D-Lac binding. These observations are interpreted to represent inhibition

of both transpeptidase-catalyzed cross-linking, requiring D-Ala-D-Ala/D-Ala-D-Lac binding, and inhibition of the transglycosylase-catalyzed cell wall incorporation of Lipid II, presumably by a direct enzyme interaction that does not require D-Ala-D-Ala/D-Ala-D-Lac binding.

The examination of the analogs that contain the peripheral C-terminus amides with quaternary ammonium salt modifications (Compounds **9-13**) was similarly revealing and clear. Despite the progressive increase in antimicrobial activity observed against VanA VRE with Compounds **9-12,** little or no change in their ability to inhibit bacterial cell wall synthesis was observed and they remained, like vancomycin itself, essentially inactive in this assay (Figs. 1 and 2).

By contrast, the two vancomycin derivative Compounds **11** and **12** that were active against VanA VRE produced pronounced, rapid cell membrane permeability immediately upon their addition, whereas the inactive (**9**) and less active (**10**) variants did not do so when examined at 10 μM (Fig. 11 and Fig. 12). The less active compound **10** exhibited this induced permeability when examined at a higher concentration (100 μM, not shown).

Here, the antimicrobial activity against VanA VRE can be attributed to a mechanism independent of cell wall biosynthesis and independent of D-Ala-D-Ala/D-Ala-D-Lac binding. The antimicrobial activity correlates with disruption of the cell wall integrity, as measured by its increased permeability. The incorporation of the most potent of these peripheral C-terminus modifications into the pocket modified analog **4** with **13** (C14) further enhanced

antimicrobial activity against VanA VRE 200-fold
(Table 3). This modification did not improve,
diminish, or alter the inhibition of cell wall
biosynthesis, where **4** and **13** were found to be equally
active (Figs. 1 and 2). However, it did provide an
analog that, unlike **4,** produced pronounced cell
membrane permeability immediately upon addition (Fig.
4).

Thus, compound **13** represents a pocket
modified vancomycin analog that displays potent and
further improved activity against VanA VRE derived
from two independent and synergic mechanisms of
action. One mechanism relies on the dual D-Ala-D-
Ala/D-Ala-D-Lac binding like **4** and results in
effective cell wall synthesis inhibition. The second
mechanism is independent of this ligand binding
property and is derived from induced cell wall
permeability.

The two combined vancomycin modifications
and the accompanying two synergistic mechanisms of
action provide a vancomycin analog >1000-fold more
active than vancomycin against the most stringent
vancomycin-resistant organisms, VanA VRE, displaying
superb *in vitro* MICs (0.16 μg/mL). It represents now
the second such example, complementing the
observations made with Compound **8,** but now with a
different second mechanism of action introduced by a
second alternative peripheral modification and
generalizes the opportunities provided by such design
principles.

The results of the examination of the
analogs that incorporate the two peripheral
modifications (Compounds **14-17**) and their combination
with the pocket modified vancomycin analog in

Compound **18** were even more revealing. In addition to
demonstrating that this can be successfully achieved,
they highlight that it is not necessarily each of the
most effective variants of the two peripheral
modifications that combine to produce the desired
effects, but rather a combination that allows
expression of the two independent mechanisms. Likely
this represents an interplay of molecular properties
and perhaps the impact of the modifications on
membrane localization sites.

As expected based on the CBP modification,
Compounds **14-17** inhibit VanA VRE bacterial cell wall
synthesis and their relative activities are reflected
in their functional activity in the antimicrobial
assays (Fig. 1 and Fig. 2). The C14 and cyclic C5
quaternary ammonium salts diminish the inhibition of
cell wall synthesis relative to CBP-vancomycin itself
and C0 was equally active, whereas C1 may have
improved activity slightly (activity: Compound **15** >
14 = **5** > **16** and **17**).

Even more revealing, their examination in
the cell wall permeability assay indicates that only
C1 combined with the CBP modification induced a
pronounced, rapid, and potent cell membrane
permeability (Fig. 5). By contrast, the combination
of the peripheral CBP modification with the C14 and
C5 quaternary ammonium salts was not productive,
providing compounds that fail to express the enhanced
activity.

Compound **18,** which incorporates the
redesigned pocket modification for dual D-Ala-D-
Ala/D-Ala-D-Lac binding (blocks cell wall synthesis
by ligand binding including inhibition of
transpeptidase-catalyzed cross-linking), the CBP

peripheral disaccharide modification (blocks cell
wall synthesis by direct transglycosylase inhibition
without D-Ala-D-Ala/D-Ala-D-Lac binding), and the C1
quaternary ammonium salt C-terminus peripheral
modification (induces membrane permeability),
exhibited the most potent inhibition of cell wall
synthesis in the assay of all compounds assessed
(Fig. 1 and Fig. 2), as well as the most pronounced
and potent cell membrane permeability in the assay of
membrane integrity (Fig. 5).

This result indicates that all three
mechanisms of action are productively contributing to
the expression of the antimicrobial activity of
Compound **18** against VanA VRE, resulting in its potent
VanA VRE antimicrobial activity (MIC = 0.01-0.005
µg/mL). To the inventor's knowledge, this represents
the first analog of vancomycin deliberately designed
to address vancomycin resistance that incorporates
three designed modifications that impart three
distinct and independent mechanisms of action, only
one of which depends on reengineered dual D-Ala-D-
Ala/D-Ala-D-Lac ligand binding and each of which
contributes to the expression of the antimicrobial
activity.

Notably, these latter comparisons provided
a direct correlation of the results of the
permeability assay with the functional expression of
antimicrobial activity and follow unanticipated
trends with only C1 effectively expressing the
functional behaviors. This result provides
compelling evidence that the assay is both an
accurate readout of the correlated functional
behaviors and that the underlying mechanistic
interpretation is similarly accurate. It is

remarkable that this effect is so specific for C1
when combined with the CBP modification, suggesting
the mechanism responsible for induction of membrane
permeability may involve specific interaction(s)
within the bacterial cell wall.

Finally, the results are summarized in Fig.
6 for the key analogs alongside their antimicrobial
activity. Within this series, CBP-vancomycin
(Compound **5**) is representative of the potency and
characteristics of the clinically approved
semisynthetic vancomycin analogs.

For VanA VRE, the progression through the
series of Compounds **1** to **4**, then to Compound **8** or
Compound **13**, and finally to Compound **18** represent
vancomycin analogs with zero (**1**), one (**4**), two (**8** and
13), and three (**18**) distinct and synergistic
mechanisms of action that progressively provide
increasingly potent antibacterial activity. This
progression culminates in Compound **18** (MICs = 0.005–
0.01 µg/mL) with activity 25,000–50,000-fold more
potent than vancomycin against VanA VRE. Notably,
Compound **18** is also 250–500-fold more potent than
CBP-vancomycin (Compound **5**), which is representative
of the semisynthetic vancomycin analogs presently
used in the clinic. Of special note, each structural
modification and mechanism of action independently
expresses its functional activity at the level of 2–
30 µg/mL (1–15 µM) in both the antimicrobial and
mechanistic assays, but each provides synergistic
improvements in the functional antimicrobial activity
when combined.

The discussion above focused on the
identification of the contributing mechanisms of
action and the demonstration that each independently

improve antimicrobial activity potency. However, an
additional and even more important feature of the
expression of multiple independent mechanisms of
action is a mechanism's impact on the rate at which
bacterial resistance may emerge. As a result, CBP-
vancomycin (**5**, 1 mechanism of action), the
peripherally C14-modified vancomycin analog (**12**, 1
mechanism of action), the peripherally CBP-modified
pocket analog Compound **8** (2 mechanisms of action),
the peripherally C14-modified pocket analog Compound
13 (2 mechanisms of action), and the pocket analog
Compound **18** that contains the two complementary
peripheral C1 and CBP-modifications (3 mechanisms of
action) were examined for their susceptibility to
acquired resistance upon sub-lethal (0.5 x MIC)
serial exposure to the same two VRE bacterial
strains, monitoring MICs daily (Fig. 8 and Fig. 9).

Distinct from most related studies
enlisting MRSA or other vancomycin-sensitive
bacterial strains, this study was conducted with the
most stringent (VanA vs VanB) vancomycin-resistant
strains for which the mechanism of action associated
with D-Ala-D-Ala binding is no longer effective.
Consequently, D-Ala-D-Ala binding is not counted
among the number of effective mechanisms of action
imbedded in the compound structure. Notably, the
antibiotic susceptibility profiles of the VanA VREs
used herein indicate they are resistant to a number
of additional classes of antibiotics and are on the
verge of being classified as multi-drug resistant
VRE, indicating they have already assimilated a
number of common resistance mechanisms.

Because the changes for Compound **18** were so
small throughout a typical 25 day study, the

examination was extended to 50 days. These studies revealed that resistance to Compounds **5** and **12** emerged most rapidly and was pronounced (1 mechanism). Changes in the potency of both of Compound **8** and **13** were much slower and more muted (2 mechanisms, only 2-4-fold after 25 passages, Compound **8** slower than Compound **13**). No change in susceptibility to Compound **18** was observed after 25 days (3 mechanisms), and little change in susceptibility to Compound **18** was observed even after 50 daily passages (only 2-4-fold change in the MICs of 0.01 and 0.005 µg/mL in the two strains).

Moreover, the magnitude of the changes in the MICs for the compounds acting by two or more mechanisms is sufficiently small to indicate none experience a full loss of one of the contributing mechanisms. As such, each mechanism is rendered more robust when combined with structural modifications that provide one or two additional mechanisms of action. Thus, the durability of the antimicrobial activity in such challenges and the effectiveness of each individual mechanism of action, as well as the compound potency, were found to follow now predictable trends (3 > 2 > 1 mechanisms of action).

Most striking, resistance to daptomycin, linezolid and tigecycline, each of which is now a frontline single target antibiotic, arises much faster and is much more pronounced, highlighting the exceptional durability of the antimicrobial activity detailed for Compounds **8**, **13** and especially Compound **18**. Within the series examined, CBP-vancomycin (**5**) is representative of the expected behavior of the clinically approved semisynthetic vancomycin analogs.

The key compounds in the series were
examined for *in vitro* toxicity that might result from
the combined mechanisms of action, especially the
introduction of structural modifications (quaternary
ammonium salt) that might impact host as well as
bacterial cell wall integrity. The compounds were
examined for red blood cell hemolytic activity,
resulting from membrane lysis. No compound in the
series, including Compound **18**, exhibits any hemolytic
activity even at concentrations >1000-fold above
their MICs.

In addition, the mammalian cell toxicity of
Compounds **5, 12, 13, 15** and **18** was established in
cell growth inhibition assays against two mammalian
cell lines, NIH/3T3 (ATCC CRL-1658, mouse embryonic
fibroblast) and HepG2 (ATCC HB-8065, human liver
cancer cell line). No growth inhibition (cytotoxic
activity) was observed up to 100 µM, the highest dose
tested. They were also found to be inactive (>100
µM, highest dose tested) against HCT116 (human colon
cancer cell line).

No correlation in activity with cLogP was
found for the series of compounds studied, and none
of the compounds (**5, 12, 13, 15, 17** and **18**) exhibited
aggregation, higher order complex formation, or
micelle formation at concentrations up to 100 µM in
PBS buffer (25 °C), indicating such effects are not
playing a role in the expression of the activity of
the compounds. Compound **18** also failed to produce
bacterial cell membrane depolarization in the same
VanA vancomycin-resistant *E. faecium* (ATCC BAA-2317)
as measured by fluorescence of a released membrane
imbedded dye (DiSC$_3$5, 3,3′-dipropylthiadicarbocyanine

iodide) [TeWinkel et al., *Front Cell Develop Biol*
2016, 4:article 29, 1-10].

Because the C1 introduction is such a small
and seemingly benign structural modification and
because it induces membrane permeability without
membrane depolarization or cell wall lysis, it
suggests a more specific mechanism of action. The
mechanism by which Compounds **15** and **18** exert their
effects on membrane permeability is currently under
investigation.

Conclusions

Several programs have disclosed the
development of antibiotic products that act by two
mechanisms of action. These programs have included
the optimization of a single pharmacophore to
independently bind two related targets (e.g.
fluoroquinolones targeting both bacterial DNA gyrase
and topoisomerase IV), the design of hybrids of two
antimicrobial pharmacophores including the covalent
linkage of two antibiotics, the use of combinations
of single target antimicrobials to overcome or avoid
resistance (e.g. combination drug treatment of
multidrug-resistant TB), and the design of
antibiotics that display additional drug-target
binding contacts to enhance the robustness of target
engagement and decrease resistance susceptibility
[Silver, *Nat Rev* **2007**, *6(1)*:41-55(57).

A complementary approach is described
herein that is perhaps a subset of one of these
approaches to design durable antibiotics endowed with
multiple synergistic mechanisms of action. To the
inventor's knowledge, this work has provided the
first prototype antibiotics with *three* independent

mechanisms of action, targeting VRE for which
vancomycin is ineffective.

Because VRE are already vancomycin
resistant and because many have already reached a
point where they are no longer susceptible to most
other antibiotic classes, the CDC recently placed VRE
on its serious threat list and the WHO placed it
fourth on its list of drug-resistant bacteria that
pose the greatest threat to human health. The
glycopeptide antibiotics constitute an antibiotic
class already endowed with features that avoid many
mechanisms of resistance [James et al., *ACS Chem Biol*
2012, *7(5)*:797-804].

With an understanding of the molecular
basis of bacterial resistance to the glycopeptide
antibiotics, binding pocket modifications designed
for dual ligand binding reinstated binding to the
altered target D-Ala-D-Lac and maintained binding
affinity for the unaltered target D-Ala-D-Ala were
developed. These modifications were found to
reinstate antimicrobial activity against vancomycin-
resistant organisms that employ the altered D-Ala-D-
Lac peptidoglycan precursor targets and remain active
against vancomycin-sensitive bacteria that employ
only D-Ala-D-Ala precursors.

There is reason to expect that these
solutions to VanA and VanB VRE resistance alone may
provide antibiotics with durable clinical lifetimes,
perhaps approaching those of vancomycin itself (>50
years). Subsequent to these studies, the peripheral
structural changes in the molecules that provide them
have been explored with additional and now multiple
synergistic mechanisms of action.

Complementary to the inventor and co-workers' initial disclosure with a carbohydrate CBP modification that produced a 100-fold increase in antimicrobial activity [Okano et al., *J Am Chem Soc* **2015**, *137(10)*:3693-3704], a second peripheral modification at the C-terminus of the pocket-modified analogs is detailed herein that enhances antimicrobial activity (200-fold) against VanA VRE by another additional mechanism of action (induced membrane permeability). These two peripheral modifications and their synergistic mechanisms of action were then combined with a pocket modification to provide a vancomycin analog endowed with three independent mechanisms of action, only one of which is dependent on D-Ala-D-Ala/D-Ala-D-Lac binding.

This synthetic strategy not only further increased the antimicrobial potency against VanA VRE (>6000-fold), but also reduced the susceptibility to resistance. Thus, the durability of the antimicrobial activity in a resistance challenge and the robustness of each individual mechanism of action, as well as the compound potency, were shown to follow now predictable trends (3 > 2 > 1 mechanisms of action).

Most striking, resistance to the frontline antibiotics daptomycin, linezolid and tigecycline, some of which are regarded as durable by today's standards, was found to arise much faster and was more pronounced in the same resistance challenge, highlighting the exceptional durability of the antimicrobial activity detailed for Compounds **8, 13** and especially **18** (Fig. 8 and Fig. 9).

An important question these results raise is presently what to do with conventional

semisynthetic vancomycin analogs active against VanA
VRE that incorporate a single peripheral modification
and act by a single mechanism of action that is
independent of D-Ala-D-Lac binding [e.g., CBP-
vancomycin (**5**), oritavancin and C14-vancomycin (**12**)].
Should their use be encouraged for VRE but at the
risk of raising resistance to this otherwise
effective approach for other challenging bacterial
infections (e.g. MRSA)? The answer would seem to be
to encourage their use for challenging vancomycin-
sensitive bacterial infections (e.g., VSSA, MRSA)
where they are not only more potent than vancomycin,
but also benefit from two independent mechanisms of
action.

Clinical resistance or loss in sensitivity
to either mechanism would likely be slow to emerge
and slower than for vancomycin itself. However,
their use against vancomycin-resistant bacteria
(e.g., VRE, VRSA), where they are less potent and
where only a single and less durable mechanism of
action remains operative, likely would more rapidly
raise resistance, compromising not only its future
use but also potentially transfer that resistance to
other organisms (e.g. MRSA).

The approach employed herein, is suggested
to represent a case of durable antibiotic discovery
by design, relied on the total synthesis of the
candidate antibiotics [Nakayama et al., *Org Lett*
2014, *16(13)*:3572-3575; Boger et al., *J Am Chem Soc*
1999, *121(43)*:10004-10011; Walker et al., *Chem Rev*
2005, *105(2)*:449-476] to obtain the previously
inaccessible compounds. Although not highlighted in
the preceding discussion, the total synthesis of the
starting pocket modified aglycon(s) (26 steps) [Okano

et al., *J Am Chem Soc* **2015**, *137(10):*3693-3704],
enzymatic installation of the disaccharide (2 steps)
[Nakayama et al., *Org Lett* **2014**, *16(13):*3572-3575],
and subsequent addition of the two peripheral
modifications (2 steps) represent remarkable
accomplishments in their own right.

Finally, most of the work herein was
conducted with the aminomethylene analog of
vancomycin in which the residue 4 amide carbonyl was
removed. A more potent pocket modified vancomycin
analog is the residue 4 amidine (Compound **3** vs **4**),
which exhibits antimicrobial activity against both
vancomycin-resistant and vancomycin-sensitive
bacteria equipotent with the activity vancomycin
displays against vancomycin-sensitive bacteria.
Incorporation of such peripheral changes on Compound
3 or **7**, providing all three independent mechanisms of
action, can further improve on the already stunning
potency of Compound **18** (about 30-fold), while
displaying the outstanding durability of Compound **18**.

Materials and Methods

Compound C14:

A solution of **S1** (300 mg, 1.5 mmol) in
anhydrous EtOH (4 mL) was treated with $C_{14}H_{29}Br$ (0.74
g, 3.0 mmol) at 25 °C and the reaction mixture was
stirred at 70 °C for 2 hours. The reaction mixture
was cooled to 25 °C and the solvent was removed under
a stream of N_2. The residue was purified by flash

chromatography (SiO$_2$, 5-15% MeOH-CH$_2$Cl$_2$) to afford the corresponding ammonium salt as a yellow oil. This oil was treated with conc. HCl/MeOH = 1/5 (2 mL) at 25 °C and the mixture was stirred at 25 °C for 3 hours. The solvent and HCl were removed under a stream of N$_2$ to afford **C14** (271 mg, 61%, 2 steps) as a white solid identical in all respects with authentic material (^{1}H NMR, D$_2$O) [Boulos et al., *J. Microbiol. Methods* **1999**, *37*:77-86].
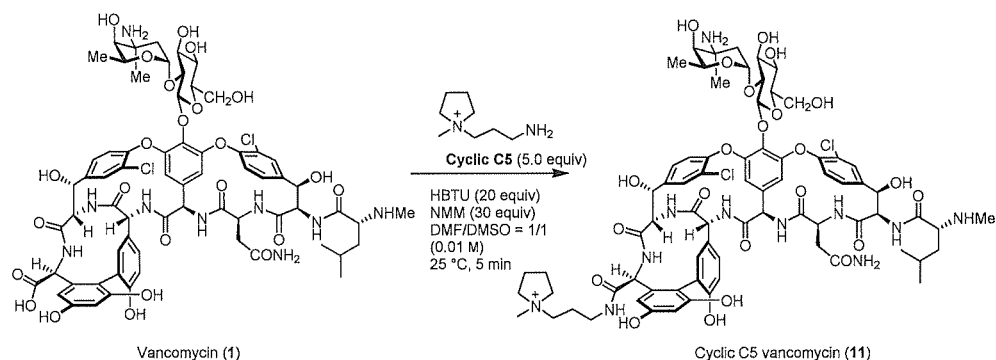

Compound Cyclic C5:

i) Boc$_2$O, THF
25 °C, 5 min

ii) MeI, DCM, 60 °C
10 min

iii) TFA/CH$_2$Cl$_2$ = 1/1
25 °C, 1 h

S2   Cyclic C5

A solution of **S2** (300 mg, 2.4 mmol) in anhydrous THF (3 mL) was treated with Boc$_2$O (510 mg, 2.3 mmol) at 25 °C and the reaction mixture was stirred at 25 °C for 5 minutes. The solvent was removed under a stream of N$_2$ to afford the crude Boc protected amine as a colorless oil. This oil was dissolved in anhydrous CH$_2$Cl$_2$ (2 mL) and treated with MeI (3.3 g, 23.4 mmol) at 25 °C and the reaction mixture was stirred at 60 °C for 10 minutes. The reaction mixture was cooled to 25 °C and the solvent and MeI were removed under a stream of N$_2$. The residue was purified by flash chromatography (SiO$_2$, 5-20% MeOH-CH$_2$Cl$_2$) to afford the corresponding ammonium salt as a yellow oil. This oil was treated with TFA/CH$_2$Cl$_2$ (1/1, 2 mL) at 25 °C and the reaction mixture was stirred at 25 °C for 1 hour. The solvent and TFA were removed under a stream of N$_2$ to afford **Cyclic C5** (154 mg, 46%, 3 steps) as a yellow oil: ^{1}H NMR (D$_2$O, 600 MHz, 298 K) δ 3.50-3.39 (m, 4H), 3.38-3.34 (m, 2H), 3.18 (s, 1H), 2.97 (s, 3H), 2.94 (t, 2H, *J* =

7.2 Hz), 2.15-2.05 (m, 6H); ESI-TOF HRMS m/z 143.1547 (M + H$^+$, $C_8H_{19}N_2$ requires 143.1548).

Compound 9:

Vancomycin (1)   CO vancomycin (9)

A solution of **1** (2.0 mg, 1.4 μmol) in DMF/DMSO (1/1, 60 μL) was treated with **CO** (1 M in DMF/DMSO = 1/1, 7.0 μL, 7.0 μmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 41.2 μL, 41.2 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 27.6 μL, 27.6 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 minutes and quenched with the addition of 50% MeOH in H$_2$O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 1-40% MeCN/H$_2$O-0.07% TFA gradient over 40 minutes, 3 mL/minute, t_R = 20.8 minutes) to afford **9** (1.4 mg, 64%) as a white amorphous solid identical in all respects with authentic material (^{1}H NMR, D$_2$O) [Boulos et al., *J. Microbiol. Methods* **1999**, *37*:77-86].

Compound 10:

Vancomycin (1)   C1 vancomycin (10)

A solution of **1** (1.5 mg, 1.0 µmol) in DMF/DMSO (1/1, 100 µL) was treated with **10** (1 M in DMF/DMSO = 1/1, 5.2 µL, 5.2 µmol), N-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 31.2 µL, 31.2 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 20.8 µL, 20.8 µmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 minutes and quenched with the addition of 50% MeOH in H_2O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10 × 150 mm, 1-40% MeCN/H_2O-0.07% TFA gradient over 40 minutes, 3 mL/minute, t_R = 20.6 min) to afford **10** (1.1 mg, 68%) as a white film: ^{1}H NMR (DMSO-d_6, 600 MHz, 298 K) δ 9.43 (br s, 1H), 9.02 (s, 1H), 8.69 (s, 1H), 8.56 (s, 1H), 8.20 (s, 1H), 7.82 (s, 1H), 7.76 (s, 1H), 7.73 (s, 1H), 7.58 (d, 2H, J = 8.4 Hz), 7.47 (d, 2H, J = 9.0 Hz), 7.37 (d, 1H, J = 8.4 Hz), 7.31 (s, 1H), 7.19 (d, 1H, J = 8.4 Hz), 7.02 (br s, 1H), 6.88 (d, 1H, J = 10.8 Hz), 6.78 (d, 1H, J = 8.4 Hz), 6.71 (d, 1H, J = 8.4 Hz), 6.39 (s, 1H), 6.22 (s, 1H), 5.76 (s, 1H), 5.57 (s, 1H), 5.36 (s, 1H), 5.26 (d, 1H, J = 7.8 Hz), 5.23 (s, 1H), 5.20 (s, 1H), 4.96 (s, 1H), 4.69 (d, 1H, J = 7.8 Hz), 4.48 (s, 1H), 4.28 (s, 1H), 4.25 (d, 1H, J = 5.4

Hz), 3.96 (s, 2H), 3.69 (s, 1H), 3.67 (s, 1H), 3.60-
3.40 (m, 5H), 3.27 (s, 1H), 3.25-3.10 (m, 4H), 3.09-
3.05 (m, 2H), 3.00 (s, 9H), 2.69 (s, 6H), 2.66 (s,
1H), 2.25-2.10 (m, 1H), 1.91 (d, 1H, J = 11.4 Hz),
1.85 (s, 2H), 1.74 (d, 1H, J = 13.2 Hz), 1.69-1.64
(m, 1H), 1.59-1.51 (m, 2H), 1.30 (s, 3H), 1.07 (d,
3H, J = 6.0 Hz), 0.90 (d, 3H, J = 6.0 Hz), 0.85 (d,
3H, J = 6.0 Hz); ESI-TOF HRMS m/z 774.2867 ([M +
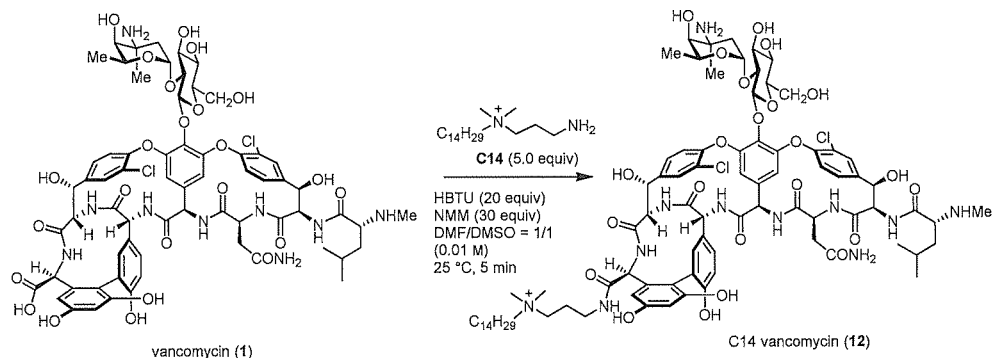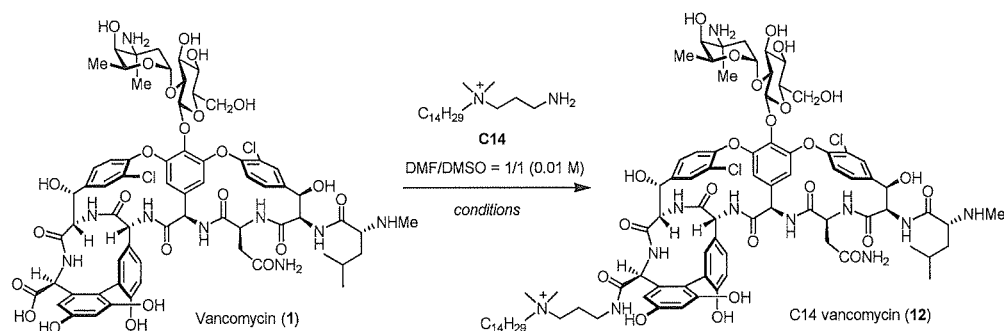2H]$^{+2}$, $C_{72}H_{90}Cl_2N_{11}O_{23}$ requires 774.2861).

Compound 11:

Vancomycin (1)   Cyclic C5 vancomycin (11)

A solution of **1** (1.8 mg, 1.1 µmol) in
DMF/DMSO (1/1, 50 µL) was treated with **Cyclic C5** (1 M
in DMF/DMSO = 1/1, 5.5 µL, 5.5 mol), N-methyl-
morpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1,
32.7 µL, 32.7 µmol), and HBTU (Chem-Impex
International, Inc., 1 M in DMF/DMSO = 1/1, 21.8 µL,
21.8 µmol) at 25 °C. The reaction mixture was
stirred at 25 °C for 5 minutes and quenched with the
addition of 50% MeOH in H$_2$O (0.5 mL) at 25 °C. The
mixture was purified by semi-preparative reverse-
phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10
× 150 mm, 1-40% MeCN/H$_2$O-0.07% TFA gradient over 40
minutes, 3 mL/minute, t_R = 20.4 minutes) to afford **11**
(1.2 mg, 61%) as a white film: ^{1}H NMR (DMSO-d_6, 600
MHz, 298 K) δ 9.14 (br s, 1H), 8.99 (s, 1H), 8.80-8.65

-62-

(m, 1H), 8.56 (s, 1H), 8.18 (s, 1H), 7.85–7.81 (m, 1H), 7.67 (s, 1H), 7.60–7.45 (m, 3H), 7.35 (dd, 1H, J = 6.6, 3.0 Hz), 7.30 (s, 1H), 7.25 (d, 1H, J = 8.4 Hz), 7.20 (d, 1H, J = 7.8 Hz), 7.08 (br s, 1H), 6.85 (d, 1H, J = 11.4 Hz), 6.78 (d, 1H, J = 8.4 Hz), 6.70 (d, 1H, J = 8.4 Hz), 6.38 (d, 1H, J = 2.4 Hz), 6.22 (d, 1H, J = 2.4 Hz), 5.77 (d, 1H, J = 7.8 Hz), 5.58 (d, 1H, J = 13.2 Hz), 5.35–5.15 (m, 5H), 4.93 (br s, 1H), 4.69 (dd, 1H, J = 5.4, 5.4 Hz), 4.49 (d, 1H, J = 5.4 Hz), 4.27 (d, 1H, J = 5.4 Hz), 4.00–3.90 (m, 1H), 3.35–3.25 (m, 2H), 3.24–3.15 (m, 2H), 3.11–3.02 (m, 1H), 2.93 (s, 3H), 2.67–2.60 (m, 3H), 2.54 (s, 1H), 2.20–2.00 (m, 5H), 1.95–1.83 (m, 3H), 1.80–1.50 (m, 4H), 1.29 (d, 3H, J = 13.8 Hz), 1.07 (d, 3H, J = 6.0 Hz), 0.91 (d, 3H, J = 6.6 Hz), 0.86 (d, 3H, J = 6.6 Hz); ESI-TOF HRMS m/z 787.2922 ([M + 2H]$^{+2}$, $C_{74}H_{92}Cl_2N_{11}O_{23}$ requires 787.2948).

Compound 12:

A solution of **1** (4.0 mg, 2.8 µmol) in DMF/DMSO (1/1, 150 µL) was treated with **C14** (1 M in DMF/DMSO = 1/1, 13.8 µL, 13.8 µmol), *N*-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 82.8 µL, 82.8 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 55.2 µL, 55.2 µmol) at 25 °C. The reaction mixture was

stirred at 25 °C for 5 minutes and quenched with the addition of 50% MeOH in H$_2$O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 20-80% MeCN/H$_2$O-0.07% TFA gradient over 30 minutes, 3 mL/minute, t_R = 21.2 minutes) to afford **12** (2.8 mg, 58%) as a white amorphous solid identical in all respects with authentic material (^{1}H NMR, DMSO-d_6) [Boulos et al., *J. Microbiol. Methods* **1999**, *37*:77-86].

Note: This reaction was run on scales of 0.5-10 mg (51-63%) during the optimization of conditions (Table 5, below). Compounds **13** and **18** were synthesized on 0.7 mg and 0.25 mg scales with respect to their starting material.

Table 5

entry	reaction scale	C14	HBTU	base	temp.	time	isolated yield
1[a]	10-2 mg	2 equiv	2 equiv	DIEA (5 equiv)	0 °C	12 h	52-58%
2	500 mg	2 equiv	2 equiv	DIEA (5 equiv)	0 °C	12 h	45%
3	500 mg	5 equiv	10 equiv	DIEA (15 equiv)	0 °C	2 h	51%
4	500 mg	5 equiv	20 equiv	DIEA (30 equiv)	0 °C	2 h	58%
5	500 mg				25 °C		
6	500 mg	5 equiv	20 equiv	DIEA (30 equiv)	25 °C	5 min	55%
		5 equiv	20 equiv	NMM (30 equiv)		5 min	63%

[a] Adapted procedure from: Haldar, J. *et al. J. Med. Chem.* **2014**, *57*, 4558.

Compound 4

Experimental for the total synthesis of aminomethylene vancomycin **4** [Crowley et al., *J. Am. Chem. Soc.* **2006**, *128(9)*:2885-2892; Okano et al., *J. Am. Chem. Soc.* **2015**, *137(10)*:3693-3704] has been previously disclosed.
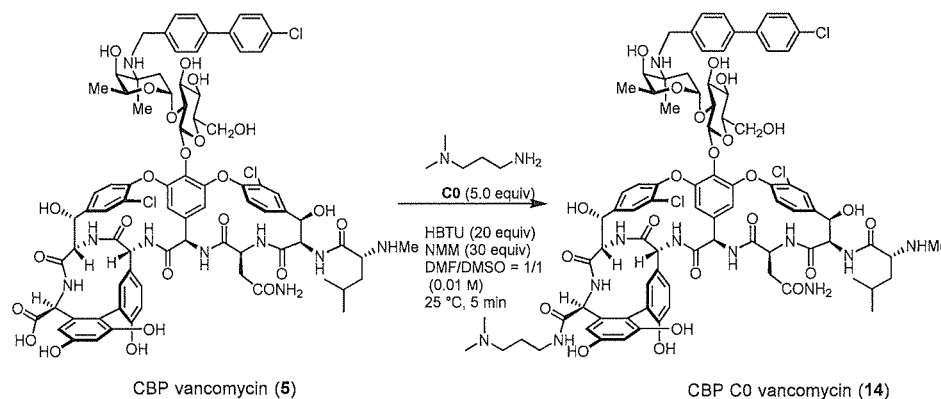
Compound 13:

aminomethylene vancomycin (**4**)   C14 aminomethylene vancomycin (**13**)

A solution of **4** (0.69 mg, 0.48 µmol) in DMF/DMSO (1/1, 30 µL) was treated with **C14** (1 M in DMF/DMSO = 1/1, 2.4 µL, 2.4 µmol), *N*-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 14.4 µL, 14.4 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 9.6 µL, 9.6 µmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 minutes and quenched with the addition of 50% MeOH in H_2O (0.2 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10 × 150 mm, 20-80% MeCN/H_2O-0.07% TFA gradient over 30 minutes, 3 mL/minute, t_R = 18.4 minutes) to afford **13** (0.53 mg, 64%, typically 61-67%) as a white film: ^{1}H NMR (CD$_3$OD, 600 MHz, 298 K) δ 8.85-8.80 (m, 1H), 8.50-8.45 (m, 1H), 8.35-8.30 (m, 1H), 7.98 (s, 1H), 7.82 (d, 1H, *J* = 8.7 Hz), 7.74 (dd, 1H, *J* = 8.4, 1.8 Hz), 7.59 (d, 1H, *J* = 2.4 Hz), 7.41 (d, 1H, *J* = 9.0 Hz), 7.31 (d, 1H, *J* = 2.4 Hz),

7.25-7.17 (m, 2H), 7.15 (dd, 1H, J = 8.4, 2.4 Hz),
6.93 (d, 1H, J = 8.4 Hz), 6.46 (d, 1H, J = 2.4 Hz),
6.33 (d, 1H, J = 2.4 Hz), 5.54 (d, 1H, J = 3.0 Hz),
5.44 (d, 1H, J = 7.8 Hz), 5.41 (d, 1H, J = 2.4 Hz),
5.37 (d, 1H, J = 5.4 Hz), 4.57□4.54 (m, 1H),
4.43-4.31 (m, 2H), 4.29-4.19 (m, 1H), 4.18-3.98 (m,
1H), 3.88-3.70 (m, 3H), 3.69-3.59 (m, 1H), 3.58-3.50
(m, 2H), 3.40-3.32 (m, 4H), 3.29-3.24 (m, 4H), 3.05
(d, 1H, J = 3.6 Hz), 3.00 (s, 3H), 2.86 (s, 3H), 2.79
(s, 3H), 2.69-2.66 (m, 5H), 2.64-2.56 (m, 1H),
2.33-2.25 (m, 1H), 2.10-1.92 (m, 5H), 1.83-1.70 (m,
3H), 1.67-1.57 (m, 2H), 1.54 (s, 3H), 1.45-1.25 (m,
23H), 1.20 (d, 3H, J = 6.6 Hz), 0.95 (d, 3H, J = 7.2
Hz), 0.93-0.87 (m, 6H); ESI-TOF HRMS m/z 857.8965 ([M
+ 2H]$^{+2}$, $C_{85}H_{117}Cl_2N_{11}O_{22}$ requires 857.8948).

Compound 5

Experimental data for the synthesis of CBP
vancomycin **5** [Okano et al., *J. Am. Chem. Soc.* **2015**,
137(10):3693-3704] has been previously disclosed.

Compound 14:

A solution of **5** (2.0 mg, 1.2 μmol) in
DMF/DMSO (1/1, 100 μL) was treated with **C0** (1 M in
DMF/DMSO = 1/1, 6.1 μL, 6.1 μmol), *N*-methylmorpholine

(Acros, distilled, 1 M in DMF/DMSO = 1/1, 36.4 µL,
36.4 µmol), and HBTU (Chem-Impex International, Inc.,
1 M in DMF/DMSO = 1/1, 24.3 µL, 24.3 µmol) at 25 °C.
The reaction mixture was stirred at 25 °C for 5
minutes and quenched with the addition of 50% MeOH in
H_2O (0.5 mL) at 25 °C. The mixture was purified by
semi-preparative reverse-phase HPLC (Nacalai Tesque,
Inc., ARII-C18, 5 µm, 10 × 150 mm, 20-80% MeCN/H_2O-
0.07% TFA gradient over 30 minutes, 3 mL/minute, t_R =
13.1 minutes) to afford **14** (1.2 mg, 55%) as a white
film: ^1H NMR (CD$_3$OD, 600 MHz, 298 K) δ 9.00 (d, 1H, J =
6.6 Hz), 8.73 (s, 1H), 7.73–7.60 (m, 7H), 7.58–7.54
(m, 2H), 7.48–7.45 (m, 2H), 7.32 (d, 1H, J = 9.0 Hz),
7.27 (d, 1H, J = 9.0 Hz), 7.10 (br s, 1H), 7.02 (d,
1H, J = 9.0 Hz), 6.82 (d, 1H, J = 9.0 Hz), 6.46–6.45
(m, 1H), 6.37–6.35 (m, 1H), 5.80 (s, 1H), 5.77 (s,
1H), 5.51 (d, 1H, J = 8.4 Hz), 5.46 (s, 1H),
5.38–5.30 (m, 3H), 4.25 (s, 1H), 4.18 (d, 1H, J =
13.2 Hz), 4.10–4.06 (m, 3H), 3.90–3.82 (m, 2H),
3.77–3.72 (m, 1H), 3.67–3.60 (m, 3H), 3.20–3.18 (m,
1H), 3.15–3.10 (m, 2H), 2.91–2.87 (m, 8H), 2.76 (s,
3H), 2.33–2.24 (m, 1H), 2.21–2.16 (m, 2H), 2.07 (s,
1H), 2.04 (s, 1H), 2.03–1.95 (m, 3H), 1.89–1.83 (m,
1H), 1.80–1.75 (m, 1H), 1.70–1.64 (m, 5H), 1.30–1.29
(m, 2H), 1.26 (d, 3H, J = 7.2 Hz), 1.02 (d, 3H, J =
6.6 Hz), 0.98 (d, 3H, J = 6.0 Hz); ESI-TOF HRMS m/z
866.7940 ([M + 2H]$^{+2}$, C$_{74}$H$_{96}$Cl$_3$N$_{11}$O$_{23}$ requires 866.7952).

Compound 15:

CBP vancomycin (**5**)   CBP C1 vancomycin (**15**)

A solution of **5** (1.4 mg, 0.85 μmol) in DMF/DMSO (1/1, 100 μL) was treated with **C1** (1 M in DMF/DMSO = 1/1, 4.3 μL, 4.3 μmol), *N*-methylmorpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 25.5 μL, 25.5 μmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 17.0 μL, 17.0 μmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 minutes and quenched with the addition of 50% MeOH in H_2O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 μm, 10 × 150 mm, 20-80% MeCN/H_2O□0.07% TFA gradient over 30 minutes, 3 mL/minute, t_R = 13.7 min) to afford **15** (0.91 mg, 61%) as a white film: ^{1}H NMR (CD$_3$OD, 600 MHz, 298 K) δ 9.01 (d, 1H, J = 4.2 Hz), 8.75 (s, 1H), 8.37 (d, 1H, J = 7.2 Hz), 7.73-7.55 (m, 8H), 7.46 (d, 2H, J = 8.4 Hz), 7.32 (d, 1H, J = 9.0 Hz), 7.28 (dd, 1H, J = 9.0, 3.6 Hz), 7.11 (br s, 1H), 7.01 (dd, 1H, J = 9.0, 2.4 Hz), 6.82 (d, 1H, J = 9.0 Hz), 6.46 (s, 1H), 6.37 (d, 1H, J = 2.4 Hz), 5.80 (s, 1H), 5.76 (s, 1H), 5.52-5.46 (m, 2H), 5.40-5.31 (m, 4H), 4.35-4.24 (m, 1H), 4.17 (d, 1H, J = 12.6 Hz), 4.14-4.05 (m, 3H), 3.89-3.82 (m, 2H), 3.74 (dd, 1H, J = 12.6, 5.4 Hz), 3.67-3.60 (m,

2H), 3.14–3.09 (m, 11H), 3.00 (d, 1H, J = 9.0 Hz),

2.78 (s, 3H), 2.66 (s, 1H), 2.34–2.23 (m, 1H), 2.20

(dd, 1H, J = 12.0, 4.8 Hz), 2.09–2.01 (m, 5H),

1.87□1.82 (m, 1H), 1.79–1.75 (m, 1H), 1.65 (s, 3H),

1.27 (d, 3H, J = 6.0 Hz), 1.02 (d, 3H, J = 6.6 Hz),

0.99 (d, 3H, J = 6.6 Hz); ESI-TOF HRMS m/z 873.8042

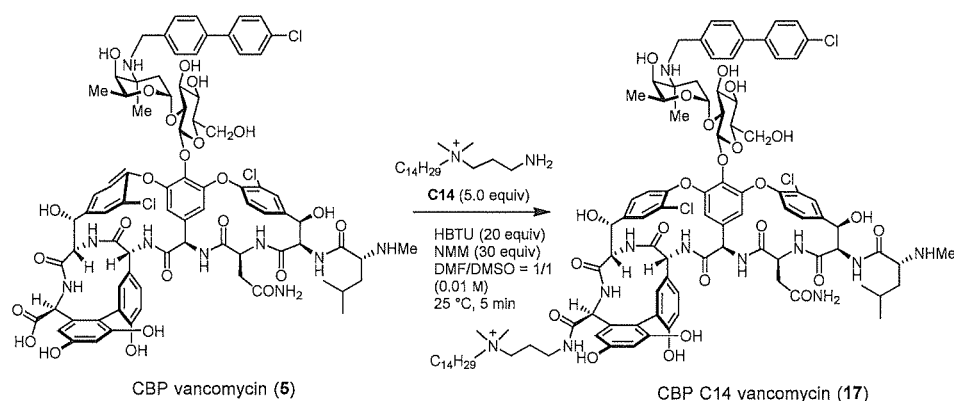($[M + 2H]^{+2}$, $C_{85}H_{98}Cl_3N_{11}O_{23}$ requires 873.8027).

Compound 16:

CBP vancomycin (5)　　　　　　　CBP cyclic C5 vancomycin (16)

A solution of **5** (1.8 mg, 1.1 µmol) in

DMF/DMSO (1/1, 100 µL) was treated with **Cyclic C5** (1

M in DMF/DMSO = 1/1, 5.5 µL, 5.5 µmol), N-methyl-

morpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1,

21.8 µL, 21.8 µmol), and HBTU (Chem-Impex

International, Inc., 1 M in DMF/DMSO = 1/1, 32.7 µL,

32.7 µmol) at 25 °C. The reaction mixture was

stirred at 25 °C for 5 minutes and quenched with the

addition of 50% MeOH in H_2O (0.5 mL) at 25 °C. The

mixture was purified by semi-preparative reverse-

phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10

× 150 mm, 20-80% $MeCN/H_2O$-0.07% TFA gradient over 30

minutes, 3 mL/minute, t_R = 12.7 minutes) to afford **16**

(1.2 mg, 61%) as a white film: ^1H NMR (CD$_3$OD, 600 MHz,

298 K, rotamers (4:1)) δ 9.04 (s, 0.25H), 8.74 (s,

1H), 8.35 (s, 0.25H), 8.00 (s, 1H), 7.76–7.56 (m,

18H), 7.50–7.48 (m, 4H), 7.35 (d, 0.25H, J = 9.6 Hz),
7.30 (d, 1H, J = 9.0 Hz), 7.13–7.09 (m, 1.25H), 7.11
(s, 1H), 6.83 (d, 1H, J = 9.0 Hz), 6.48–6.46 (m,
1.25H), 6.40–6.39 (m, 1.25H), 5.84 (br s, 1H), 5.54
(d, 1H, J = 7.8 Hz), 5.49 (d, 1H, J = 4.8 Hz), 5.42
(s, 1H), 5.37–5.34 (m, 2.5H), 4.59 (dd, 0.25H, J =
6.0, 6.0 Hz), 4.37–4.31 (m, 1H), 4.27–4.17 (m, 5H),
4.15–4.04 (m, 4.25H), 3.93–3.86 (m, 1.75H), 3.82–3.75
(m, 1.25H), 3.72 (s, 1H), 3.71–3.64 (m, 3H),
3.59–3.47 (m, 9H), 3.45–3.42 (m, 2.5H), 3.40–3.35 (m,
2H), 3.25–3.21 (m, 1H), 3.18 (d, 1H, J = 2.4 Hz),
3.16–3.12 (m, 1H), 3.09–3.06 (m, 5.5H), 3.04–3.02 (m,
4.25H), 2.97–2.93 (m, 3.5H), 2.89 (s, 3H), 2.82–2.77
(m, 5H), 2.68 (s, 6H), 2.30–2.18 (m, 8.5H), 2.12–2.04
(m, 4.25H), 1.91–1.85 (m, 1.5H), 1.83–1.76 (m, 2H),
1.74–1.65 (m, 5H), 1.42–1.27 (m, 8H), 1.09–0.95 (m,
11H); ESI-TOF HRMS m/z 886.8121 ([M + 2H]$^{+2}$,
$C_{87}H_{101}Cl_3N_{11}O_{23}$ requires 886.8103).

Compound 17:

CBP vancomycin (**5**) CBP C14 vancomycin (**17**)

A solution of **5** (2.1 mg, 1.3 µmol) in
DMF/DMSO (1/1, 100 µL) was treated with **C14** (1 M in
DMF/DMSO = 1/1, 6.4 µL, 6.4 µmol), N-methylmorpholine
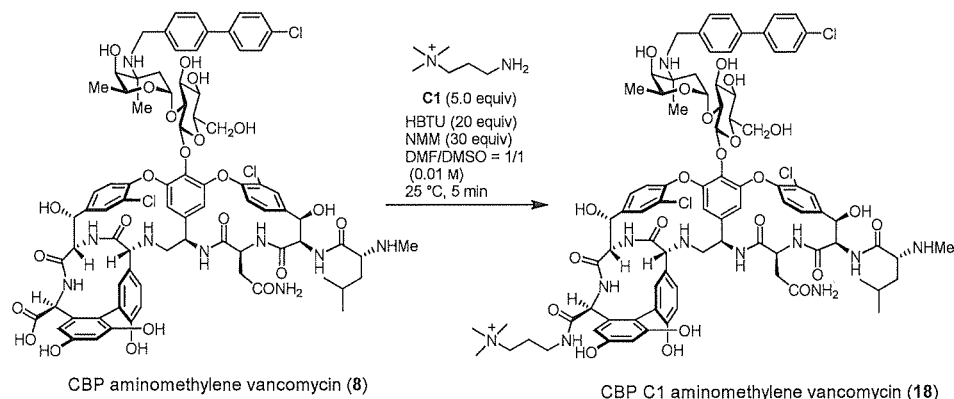(Acros, distilled, 1 M in DMF/DMSO = 1/1, 38.3 µL,

-70-

38.3 μmol), and HBTU (Chem-Impex International, Inc.,
1 M in DMF/DMSO = 1/1, 25.5 μL, 25.5 μmol) at 25 °C.
The reaction mixture was stirred at 25 °C for 5
minutes and quenched with the addition of 50% MeOH in
H_2O (0.2 mL) at 25 °C. The mixture was purified by
semi-preparative reverse-phase HPLC (Nacalai Tesque,
Inc., ARII-C18, 5 μm, 10 × 150 mm, 20-80% $MeCN/H_2O$-
0.07% TFA gradient over 30 minutes, 3 mL/minute, t_R =
18.4 minutes) to afford **17** (1.9 mg, 76%) as a white
film: ^1H NMR (CD_3OD, 600 MHz, 298 K, rotamers (2:1)) δ
9.16 (s, 0.5H), 9.09 (s, 0.5H), 8.74 (s, 1H), 8.40
(s, 1H), 7.98 (s, 0.5H), 7.89 (s, 0.5H), 7.80-7.53
(m, 11H), 7.51 (d, 1H, J = 7.8 Hz), 7.48□7.42 (m,
3H), 7.38-7.25 (m, 3H), 7.20 (d, 0.5H, J = 8.4 Hz),
7.17-7.12 (m, 2H), 7.04 (d, 0.5H, J = 2.4 Hz), 6.91
(s, 0.5H), 6.83 (d, 1H, J = 8.4 Hz), 6.51 (s, 0.5H),
6.47 (s, 1H), 6.41 (s, 0.5H), 6.37 (s, 1H), 6.29 (d,
0.5H, J = 7.8 Hz), 5.81 (br s, 1H), 5.65 (s, 0.5H),
5.58-5.51 (m, 1H), 5.48-5.38 (m, 2H), 5.35 (s, 1H),
5.22 (d, 0.5H, J = 2.4 Hz), 5.01 (s, 0.5H), 4.70 (s,
1H), 4.62 (s, 0.5H), 4.60-4.52 (m, 2H), 4.36 (s,
0.5H), 4.28 (s, 1H), 4.19-4.06 (m, 5H), 3.98 (d,
0.5H, J = 10.8 Hz), 3.92-3.80 (m, 1.5H), 3.78-3.71
(m, 2H), 3.63-3.59 (m, 2H), 3.57-3.51 (m, 3H), 3.47
(s, 1H), 3.43-3.38 (m, 1.5H), 3.12-3.06 (m, 11H),
3.02-3.00 (m, 8H), 2.92 (d, 1H, J = 2.4 Hz), 2.89 (s,
1H), 2.87 (s, 0.5H), 2.83-2.79 (m, 4H), 2.66 (s, 6H),
2.22-2.18 (m, 2H), 2.16-2.10 (m, 0.5H), 2.07-1.99 (m,
4H), 1.95-1.83 (m, 2H), 1.81-1.62 (m, 12H), 1.44-1.19
(m, 54H), 1.09-0.96 (m, 3.5H), 0.93-0.88 (m, 7H),
0.81 (d, 1H, J = 4.2 Hz), 0.71 (d, 1H, J = 3.6 Hz);

ESI-TOF HRMS m/z 1928.7987 (M^+, $C_{98}H_{125}Cl_3N_{11}O_{23}$ requires 1928.8010).

Compound 8

Experimental procedures for the total synthesis of **8** [Okano et al., *J. Am. Chem. Soc.* **2015**, *137(10)*:3693-3704] has been previously disclosed.

Compound 18:

CBP aminomethylene vancomycin (**8**)

CBP C1 aminomethylene vancomycin (**18**)

A solution of **8** (0.24 mg, 0.15 µmol) in DMF/DMSO (1/1, 20 µL) was treated with **C1** (0.1 M in DMF/DMSO = 1/1, 7.4 µL, 0.74 µmol), *N*-methyl-morpholine (Acros, distilled, 1 M in DMF/DMSO = 1/1, 4.5 µL, 4.5 µmol), and HBTU (Chem-Impex International, Inc., 1 M in DMF/DMSO = 1/1, 3.0 µL, 3.0 µmol) at 25 °C. The reaction mixture was stirred at 25 °C for 5 minutes and quenched with the addition of 50% MeOH in H_2O (0.5 mL) at 25 °C. The mixture was purified by semi-preparative reverse-phase HPLC (Nacalai Tesque, Inc., ARII-C18, 5 µm, 10 × 150 mm, 20-80% MeCN/H_2O-0.07% TFA gradient over 30 minutes, 3 mL/minute, t_R = 12.9 minutes) to afford **18** (0.14 mg, 53%) as a white film: ^{1}H NMR (CD$_3$OD, 600 MHz, 298 K) δ 9.20-9.05 (m, 1H), 9.01 (s, 1H), 8.61-8.54 (m, 1H), 7.98 (s, 1H), 7.80-7.55 (m, 10H), 7.47 (d, 1H, J =

10.2 Hz), 7.29 (d, 1H, $J = 9.0$ Hz), 7.25 (s, 1H),
7.09 (d, 1H, $J = 9.6$ Hz), 6.86 (d, 1H, $J = 9.0$ Hz),
6.84 (s, 1H), 6.73 (d, 1H, $J = 2.4$ Hz), 6.47 (s, 1H),
6.34 (s, 1H), 5.85–5.75 (m, 2H), 5.55–5.40 (m, 3H),
5.35–5.25 (m, 1H), 4.26 (br s, 1H), 4.20–4.05 (m,
4H), 3.95–3.80 (m, 2H), 3.79–3.70 (m, 2H), 3.68–3.55
(m, 3H), 3.54–3.40 (m, 7H), 3.19 (s, 1H), 3.11 (s,
1H), 3.05 (s, 3H), 3.03–2.95 (m, 7H), 3.00 (s, 3H),
2.92 (s, 1H), 2.87 (s, 3H), 2.76 (s, 3H), 2.66 (s,
3H), 2.30–2.15 (m, 6H), 2.14–2.01 (m, 4H), 1.87–1.73
(m, 2H), 1.68 (s, 3H), 1.26 (d, 3H, $J = 7.2$ Hz), 1.03
(s, 3H, $J = 7.2$ Hz), 0.99 (s, 3H, $J = 7.2$ Hz); ESI-
TOF HRMS m/z 866.8129 ([M + 2H]$^{+2}$, $C_{85}H_{102}Cl_3N_{11}O_{22}$
requires 866.8130).

In vitro antimicrobial assays[*]

One day before a study was run, fresh
cultures of vancomycin-sensitive *Staphlococcus aureus*
(VSSA strain ATCC 25923), methicillin and oxacillin-
resistant *Staphlococcus aureus subsp. aureus* (MRSA
strain ATCC 43300), vancomycin-resistant *Enterococcus
faecalis* (VanA VRE, BM4166), *Enterococcus faecium*
(VanA VRE, ATCC BAA-2317), vancomycin-resistant
Enterococcus faecalis (VanB VRE, strain ATCC 51299),
Escherichia coli (ATCC 25922), *Acinetobacter
baumannii* (ATCC BAA-1710), *Pseudomonas aeruginosa*
(ATCC 15442), *Klebsiella pneumoniae* (ATCC 700603)
were inoculated and grown in an orbital shaker at 37
°C in 100% Mueller-Hinton broth (VSSA, MRSA and VanB
VRE), 100% brain-heart infusion broth (VanA VRE, *A.
baumannii* and *K. pneumoniae*) or 100% Luria broth (*E.
coli* and *P. aeruginosa*). After 24 hours, the
bacterial stock solutions were serially diluted with

the culture medium (10% Mueller-Hinton broth for
VSSA, MRSA and VanB VRE or 10% brain-heart infusion
broth for VanA VRE *A. baumannii* and *K. pneumoniae* or
10% Luria Broth for *E. coli* and *P. aeruginosa*) to
achieve a turbidity equivalent to a 1:100 dilution of
a 0.5 M McFarland solution. This diluted bacterial
stock solution was then inoculated in a 96-well
V-shaped glass coated microtiter plate, supplemented
with serial diluted aliquots of the antibiotic
solution in DMSO (4 μL), to achieve a total assay
volume of 0.1 mL. The plate was then incubated at 37
°C for 18 hours, after which minimal inhibitory
concentrations (MICs) were determined by monitoring
the cell growth (observed as a pellet) in the wells.

     The lowest concentration of antibiotic (in
μg/mL) capable of eliminating cell growth in the
wells is the reported MIC value. The reported MIC
values for the vancomycin analogues were determined
against vancomycin as a standard in the first well.*

     For VanA *E. faecalis* (VanA VRE, BM 4166):
resistant to erythromycin, gentamicin,
chloramphenicol, and ciprofloxacin as well as
vancomycin and teicoplanin; sensitive to daptomycin.
For VanA *E. faecium* (VanA VRE, ATCC BAA-2317):
resistant to ampicillin, benzylpenicillin,
ciprofloxacin, erythromycin, levofloxacin,
nitrofurantoin, and tetracycline as well as
vancomycin and teicoplanin, insensitive to linezolid;
sensitive to tigecycline and dalfopristine.
[*Clinical and Laboratory Standards Institute.
*Methods for Dilution Antimicrobial Susceptibility
Tests for Bacteria That Grow Aerobically;* Approved
Standard, 7th ed.; CLSI document M07-A8; Clinical and
Laboratory Standards Institute: Wayne, PA, 2009.]

For VanA *E. faecalis* (VanA VRE, BM 4166):
resistant to erythromycin, gentamicin,
chloramphenicol, and ciprofloxacin as well as
vancomycin and teicoplanin; sensitive to daptomycin.
For VanA *E. faecium* (VanA VRE, ATCC BAA-2317):
resistant to ampicillin, benzylpenicillin,
ciprofloxacin, erythromycin, levofloxacin,
nitrofurantoin, and tetracycline as well as
vancomycin and teicoplanin, insensitive to linezolid;
sensitive to tigecycline and dalfopristine.

Cell wall permeability assay[**]

One day before a study was run, cultures of
vancomycin-resistant *Enterococcus faecalis* (VanA VRE,
BM4166) and *Enterococcus faecium* (VanA VRE, ATCC BAA-
2317), were inoculated and grown in an orbital shaker
at 37 °C in 100% brain-heart infusion broth for 12
hours. The above bacterial solution was subjected to
a subculture to obtain fresh mid-log phase bacterial
cells (total volume of bacterial suspension = 7 mL,
incubation time = 6 hours, OD_{600} = 0.6). After the
cultured bacteria were harvested (3000 rpm, 4 °C, 20
minutes), the white bacterial precipitate was washed
and resuspended in 5 mM glucose and 5 mM HEPES buffer
(1:1, 500 μL, pH = 7.2).

This bacterial suspension (130 μL) was
charged into a 96-well black plate with a clear
bottom (Corning 3650). The propidium iodide dye (10
μL, 150 μM DMSO solution) was added to the above
suspension and the fluorescence was monitored at 25
°C for 5 minutes at 30 second intervals using a
microplate reader (Molecular Devices®, Max Gemini EX)
at an excitation wavelength of 535 nm and an emission
wavelength of 617 nm. The test compound (150 μM, 10

µL) was added to the cell suspension and the
fluorescence was monitored at 25 °C for an additional
15 minutes.

The impact of the structural modifications
on cell wall permeability against both VanA VRE
examined herein was also examined (Fig. 11).
Vancomycin (1), C14-vancomycin (12), CBP C1-
vancomycin (15), and CBP C1-aminomethylene vancomycin
(18) displayed similar induced permeabilities against
both vancomycin-resistant *Enterococcus faecalis* (VanA
VRE, BM4166) and *Enterococcus faecium* (VanA VRE, ATCC
BAA-2317). [**Sahal *et al*. *J. Med. Chem.* **2010**,
53:6079-6088; Boulos *et al*. *J. Microbiol. Methods*
1999, *37*:77-86.]

Cell wall depolarization assay ***

One day before a study was run, cultures of
vancomycin-resistant *Enterococcus faecium* (VanA VRE,
ATCC BAA-2317), were inoculated and grown in an
orbital shaker at 37 °C in 100% Brain-Heart Infusion
for 12 hours. The above bacterial solution was
subjected to subculture to obtain fresh mid-log phase
bacterial cells (total volume of bacterial suspension
= 7 mL, incubation time = 6 hours, OD_{600} = 0.6).
After cultured bacteria media was harvested (3000
rpm, 4 °C, 20 minutes), the bacterial precipitate was
washed and resuspended in a mixture of 5 mM glucose,
5 mM HEPES, and 5 mM KCl buffer (1:1:1, pH = 7.2).
This bacterial suspension (130 µL) was charged in a
96-well black plate (Corning 3650).

The dye (DiSC3(5): 3,3'-Dipropylthiadi-
carbocyanine iodide, 150 µM DMSO solution, 2.5 µL)
was added to the above suspension and the
fluorescence was monitored for 10 minutes at 1 minute

intervals using a microplate reader (Molecular
devices, Max Gemini EX) at an excitation wave length
of 622 nm and an emission wave length of 670 nm. The
test compound (150 µM, 10 µL) was added to the cell
suspension and the fluorescence was monitored for a
further 30 minutes. [*** Yarlagadda et al., *J. Med.
Chem.* **2014**, *57(11)*:4558-4568; Uppu et al., *Chem.
Commun.* **2013**, *49*:9389-9391; Zhang et al., *Antimicrob.
Agents Chemother.* **2000**, *44*:3317-3321.]

Resistance development study[****]

        The MICs of the vancomycin analogues
against vancomycin-resistant *Enterococcus faecalis*
(VanA VRE, BM4166) and *Enterococcus faecium* (VanA
VRE, ATCC BAA-2317) were determined. The bacterial
suspension (40 µL) in the 96-well plate at sub-MIC
concentration (MICs/2) was inoculated with 100%
brain-heart infusion broth and the bacteria were
grown in an orbital shaker at 37 °C for 6 hours until
the value of OD_{600} became 0.6. A new MIC assay was
performed with the same protocol. This process was
repeated for 50 passages, and the fold increase in
MIC was determined at each passage. [****Pollard *et
al.*, *J. Antimicrob. Chemother.* **2012**, *67*:2665-2672.]

Cell wall biosynthesis inhibition assay ***

        Cultures of vancomycin-resistant
Enterococcus faecalis (VanA VRE, BM4166) and
Enterococcus faecium (VanA VRE, ATCC BAA-2317) were
inoculated and grown in an orbital shaker at 37 °C in
100% brain-heart infusion broth for 12 hours. The
above bacterial solution was subjected to a
subculture to obtain fresh mid-log phase bacterial
cells (total volume of bacterial suspension = 5 mL,

incubation time = 6 hours, OD_{600} = 0.6). Tetracycline
(5 mg/mL, 130 µL) was added to the above bacterial
suspension to ensure complete inhibition of protein
synthesis and incubated at 37 °C for 30 minutes.
Vancomycin analogues were added and the mixture was
incubated at 37 °C for a further 30 minutes.

After the bacteria were harvested (3000
rpm, 4 °C, 20 minutes), this bacterial precipitate
was washed and resuspended in 5 mM glucose and 5 mM
HEPES buffer (500 µL, 1:1, pH = 7.2). This bacterial
suspension was heated at 100 °C for 15 minutes and
centrifuged (13000 rpm, 25 °C, 10 minutes). The
entire volume of supernatant was directly purified by
semi-preparative reverse-phase HPLC without further
manipulation (Agilent Technologies, Zorbax® SB-C18, 5
µm, 9.4 × 150 mm, 1-40% MeCN/H_2O-0.07% TFA gradient
over 40 minutes, 3 mL/minute, t_R = 11.9 minutes) to
afford UDP Mur *N*-Ac depsipentapeptide (**19**) as a white
film identical in all respects with authentic
material (^{1}H NMR, D_2O). [*****Allen et al.,
Antimicrob. Agents Chemother. **1996**, *40(10)*:2356-
2362]; Liu et al., *J. Am. Chem. Soc.* **2001**, *123*:9916-
9917.] The amount of UDP Mur *N*-Ac pentapeptide (**19**)
was quantified by use of calibration curves for *E.
faecalis* BM4166, and for *E. faecium* ATCC BAA-2317
based on the area under the curve (AUC).

Hemolysis assay[#]

The blood cells in pig whole blood (2 mL,
Pel-Free Biologicals, non-sterile, sodium citrate)
were harvested (3000 rpm, 4 °C, 20 minutes), and the
red blood precipitate was washed and resuspended in
phosphate buffered saline (pH 7.4). This diluted red
blood cell stock solution (384 µL) was incubated with

the antibiotic solution in DMSO (16 µL) in a 1 mL microtube to achieve the final concentration of the test compounds. The mixture was then incubated at 37 °C for 1 hour.

The solution was diluted with phosphate buffer saline (pH 7.4, 200 µL) at 25 °C and centrifuged (3000 rpm, 4 °C, 20 minutes). The supernatant (200 µL) was transferred to a microtiter plate. A positive control (0.2 % vol% Triton® X-100, 100% total hemolysis) and the negative control (no antibiotic, 0% hemolysis) were prepared. A_{350} was measured using a microplate reader (Molecular Devices®, Max Gemini EX). The % hemolysis was determined by calculating the following equation shown below (eq. 1).

$$\text{Hemolysis (\%)} = \frac{(A_{test} - A_{zero})}{(A_{total} - A_{zero})} \times 100 \quad \text{(eq. 1)}$$

A_{test} : Absorbance with test compound
A_{total} : Absorbance of 100% hemolysis
A_{zero} : Absorbance of 0% hemolysis

[#Patch et al., *J. Am. Chem. Soc.* **2003**, *125*:12092-12093.]

The key compounds in the series were examined for *in vitro* toxicity that might result from the combined mechanisms of action, especially the introduction of structural modifications (quaternary ammonium salt) that might impact host as well as bacterial cell wall integrity. The compounds were examined for red blood cell hemolytic activity, resulting from membrane lysis.

Although the differences in mammalian and bacterial cell wall composition are extensive, including the more highly anionic composition of the bacterial cell wall responsible for a preferential and differential cation binding, lysis of mammalian

cell membranes (red blood cells) are potential off-
target consequences of cationic compounds that impact
bacterial cell membrane integrity. The standard red
blood cell hemolysis assay was conducted and measures
the extent of red blood cell lysis after 1 hour
exposure to candidate compounds (pH 7.4, PBS, 37 °C, 1
hour). No compound in the series, including Compound
18, exhibited any hemolytic activity even at
concentrations >1000-fold above their MICs.

Because this set of observations did not
distinguish between any of the derivatives (no
hemolytic activity with any derivative), the time of
the assay out was extended to 24 hours. However, red
blood cells deteriorate under the conditions of the
assay as time progresses and such extended time
assays are not recommended or utilized by any in the
field.

The results should therefore not be taken
as reflective of potential toxicity. However, it is
notable that Compound **18** was the best compound in the
series even with an extended exposure, displaying
little hemolytic activity and behaving no differently
from the control linezolid, which does not act on the
bacterial cell membrane. Compound **18** was also
substantially better than vancomycin itself, which
was no different from the control tigecycline that
also does not act on the bacterial cell wall
membrane, and it was much better and readily
distinguishable from control daptomycin that acts by
permeabilizing (not lysing) the bacterial cell
membrane.

The only compound in the series examined
that performed worse than the control daptomycin was
the C-terminus C14 quaternary ammonium salt of

vancomycin (**12**). Importantly this combined set of studies indicate compound **18,** as well as **15,** have less of an impact on mammalian red blood cell membranes than even vancomycin itself. Finally, the extraordinary potency of the key analog Compound **18** would also be expected to minimize any nonselective toxicity because the amounts required for observation of antimicrobial activity are so low.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e., to at least one) of the grammatical object of the article. Each of the patents, patent applications and articles cited herein is incorporated by reference.

The foregoing description and the examples are intended as illustrative and are not to be taken as limiting. Still other variations within the spirit and scope of this invention are possible and will readily present themselves to those skilled in the art.

Reference to any prior art in the specification is not an acknowledgement or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be combined with any other piece of prior art by a skilled person in the art.

By way of clarification and for avoidance of doubt, as used herein and except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additions, components, integers or steps.

CLAIMS:

1.   A compound that corresponds in structure to that shown in Formula **I** or its pharmaceutically acceptable salt,
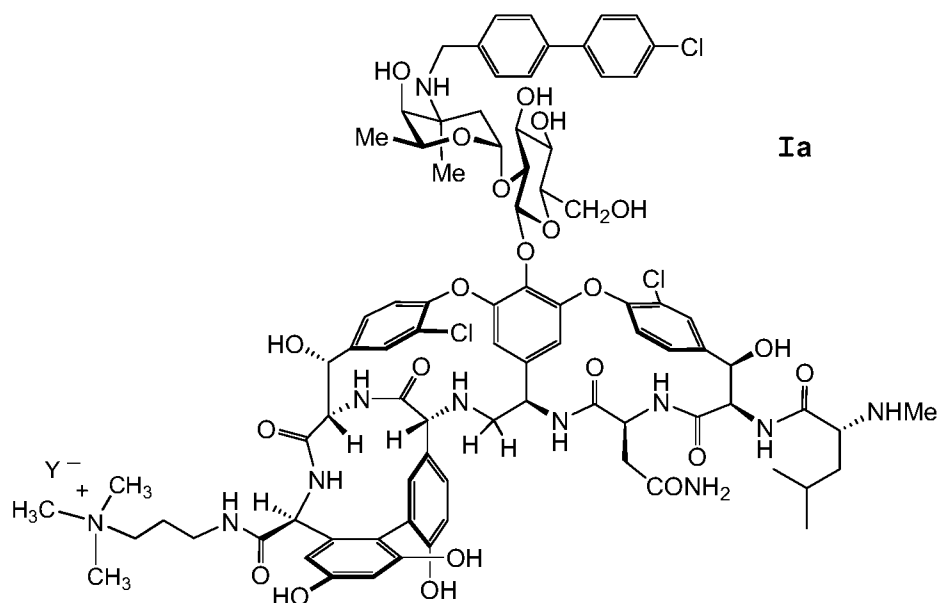

I

wherein

X = O, NH or 2 H atoms attached to the carbon that X is attached to; and
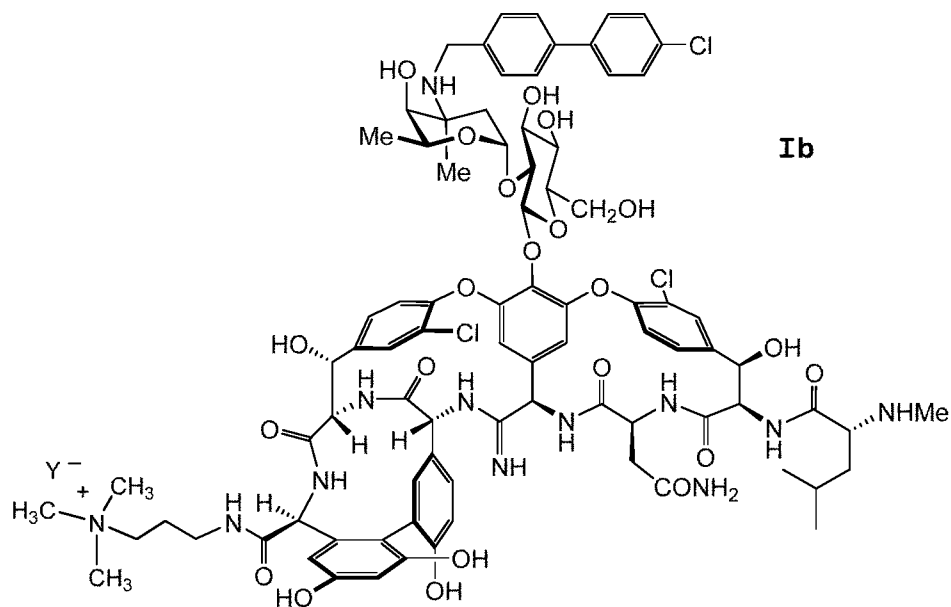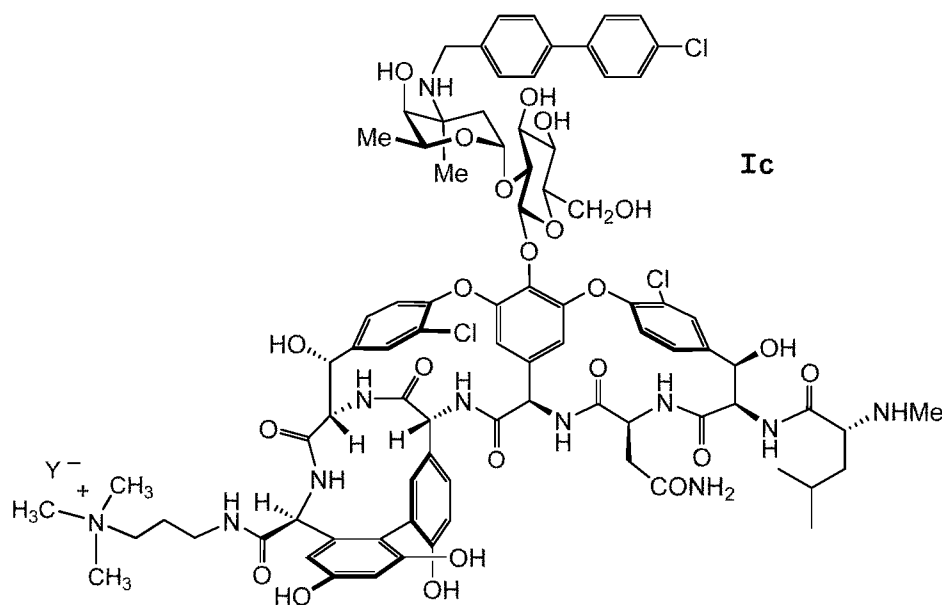
Y⁻ is a pharmaceutically acceptable anion.

2.   The compound or its pharmaceutically acceptable salt according to claim 1 that corresponds in structure to Formula **Ia,**

Ia

3.   The compound or its pharmaceutically acceptable salt according to claim 1 that corresponds in structure to Formula **Ib**,

Ib

4.   The compound or its pharmaceutically acceptable salt according to claim 1 that corresponds

-83-

in structure to Formula **Ic**,

Ic

5.   A pharmaceutical composition that comprises an antimicrobial amount of a compound of any one of claims 1-4 or a pharmaceutically acceptable salt thereof dissolved or dispersed in a physiologically acceptable diluent.

6.   A method of treating a mammal that is infected with bacteria that are vancomycin-sensitive and/or have become vancomycin-resistant and is in need of antibacterial treatment that comprises administering an antibacterial-effective amount of a compound according to any one of claims 1-4 or a pharmaceutically acceptable salt of such a compound, or the composition of claim 5, to said infected mammal in need.

7.   The method according to claim 6, wherein the bacteria that infect said bacterially-infected mammal are Gram-positive bacteria.

1003633879

8.   The method according to claim 7, wherein said Gram-positive bacteria are selected from the group consisting of one or more of *S. aureus*, methicillin-resistant *S. aureus* (MRSA), VanA *E. faecalis*, VanA *E. faecium*, and VanB *E. faecalis*.
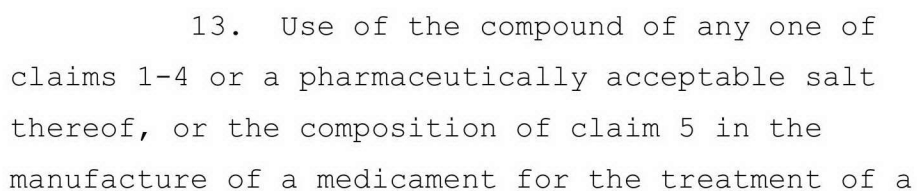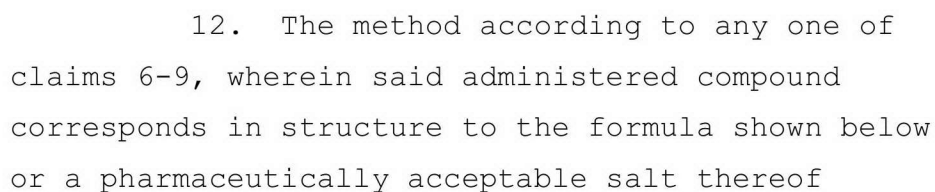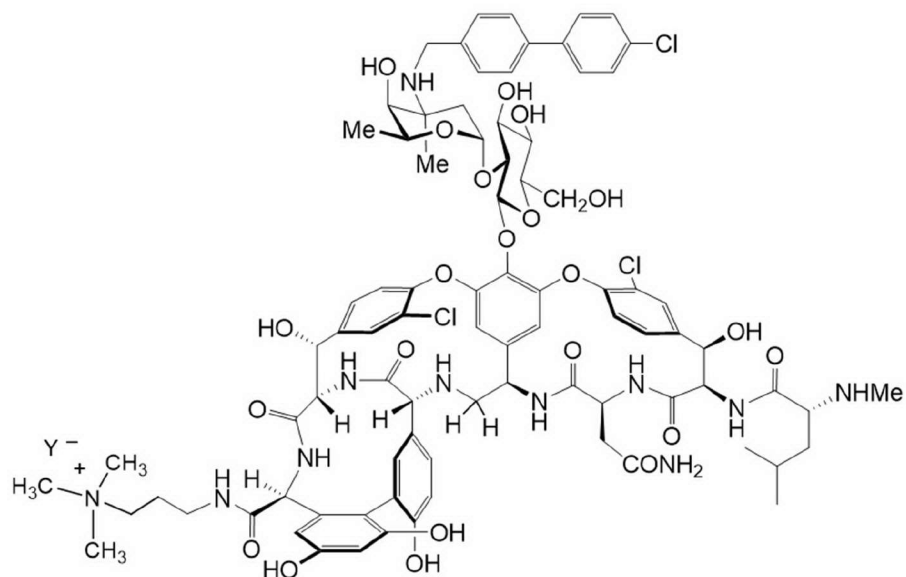
9.   The method according to any one of claims 6-8, wherein said administration is repeated a plurality of times.

10.   The method according to any one of claims 6-9, wherein said administered compound corresponds in structure to the formula shown below or a pharmaceutically acceptable salt thereof

.

11.   The method according to any one of claims 6-9, wherein said administered compound corresponds in structure to the formula shown below or a pharmaceutically acceptable salt thereof

12.   The method according to any one of claims 6-9, wherein said administered compound corresponds in structure to the formula shown below or a pharmaceutically acceptable salt thereof

13.   Use of the compound of any one of claims 1-4 or a pharmaceutically acceptable salt thereof, or the composition of claim 5 in the manufacture of a medicament for the treatment of a

-86-

bacterial infection in a mammalian subject in need thereof, wherein the mammalian subject is infected with bacteria that are vancomycin-sensitive and/or have become vancomycin-resistant.

14. The use according to claim 13, wherein the bacteria that infect said bacterially-infected mammal are Gram-positive bacteria.

15. The use according to claim 14, wherein said Gram-positive bacteria are selected from the group consisting of one or more of *S. aureus*, methicillin-resistant *S. aureus* (MRSA), VanA *E. faecalis*, VanA *E. faecium*, and VanB *E. faecalis*.

16. The use according to any one of claims 13-15, wherein the medicament is formulated for administration a plurality of times.

17. The use according to according to any one of claims 13-16, wherein the medicament comprises a compound that corresponds in structure to the formula shown below or a pharmaceutically acceptable salt thereof

1003633879

18. The use according to according to any one of claims 13-16, wherein the medicament comprises a compound that corresponds in structure to the formula shown below or a pharmaceutically acceptable salt thereof

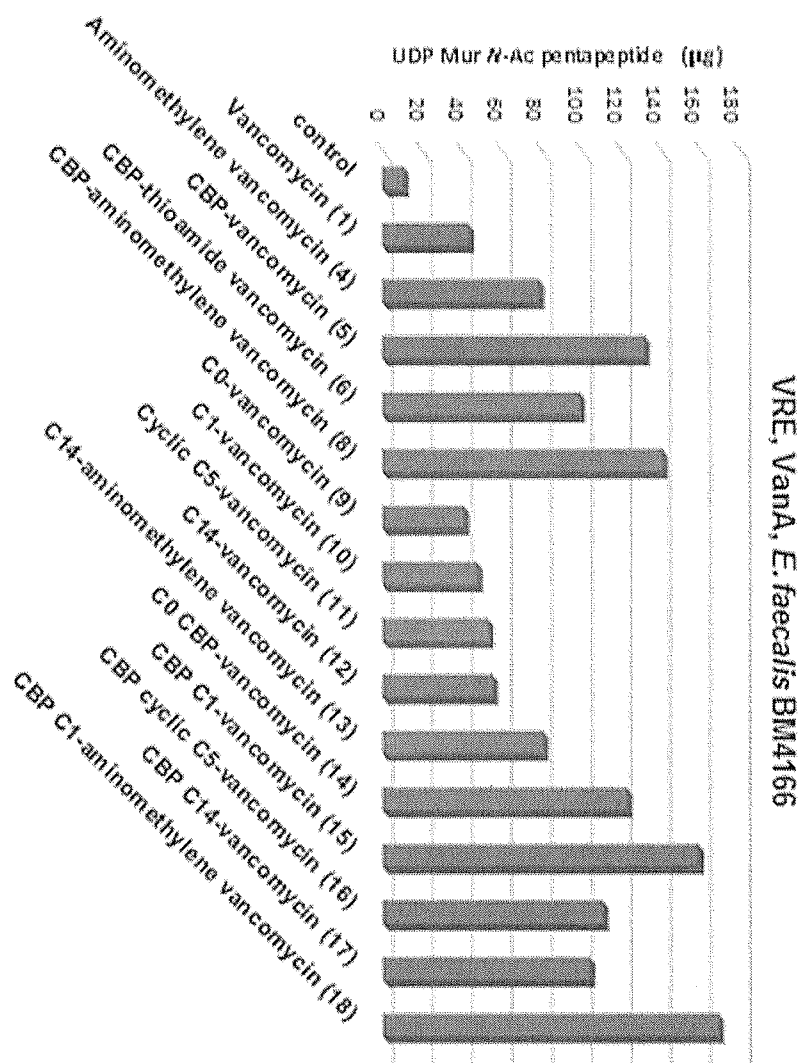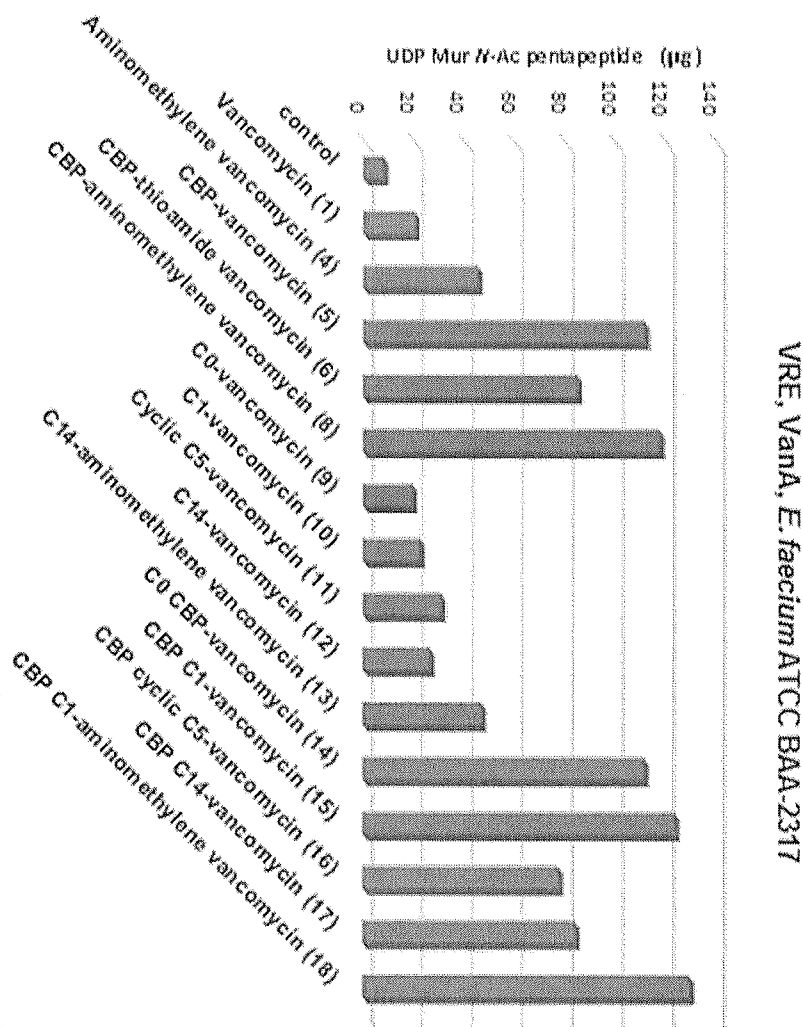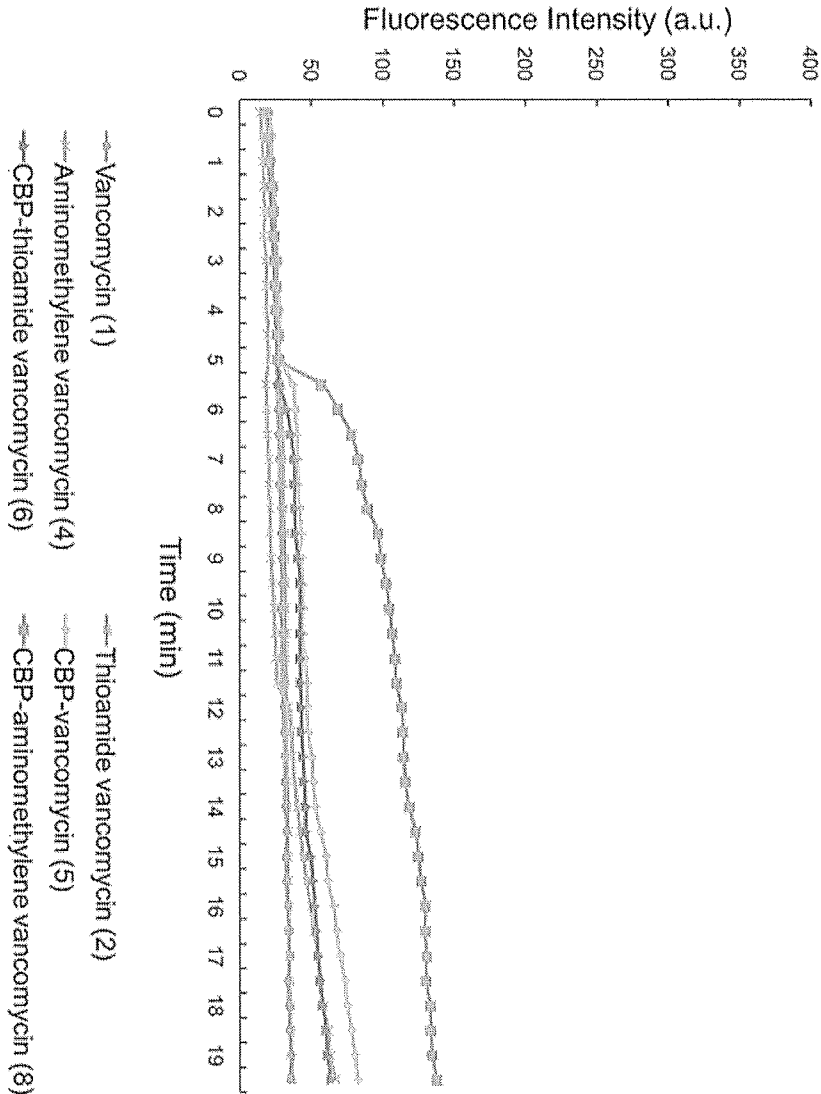19. The use according to according to any one of claims 13-16, wherein the medicament comprises

-88-

a compound that corresponds in structure to the formula shown below or a pharmaceutically acceptable salt thereof
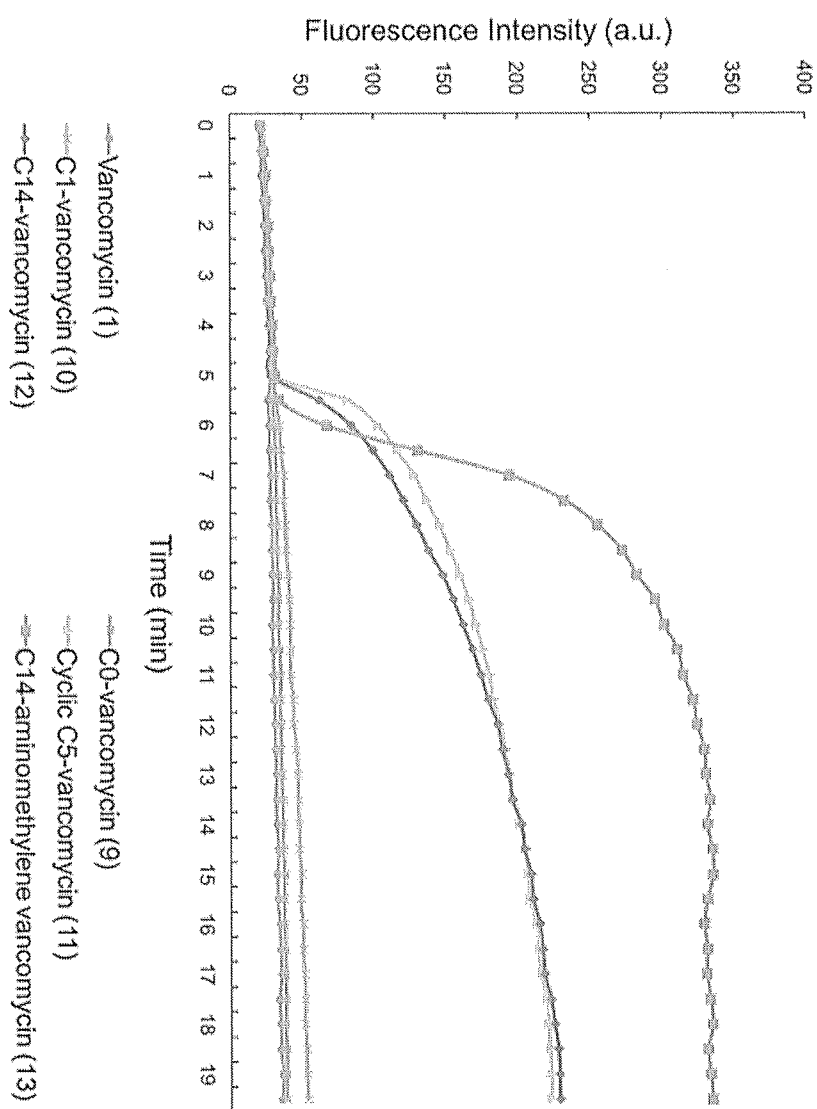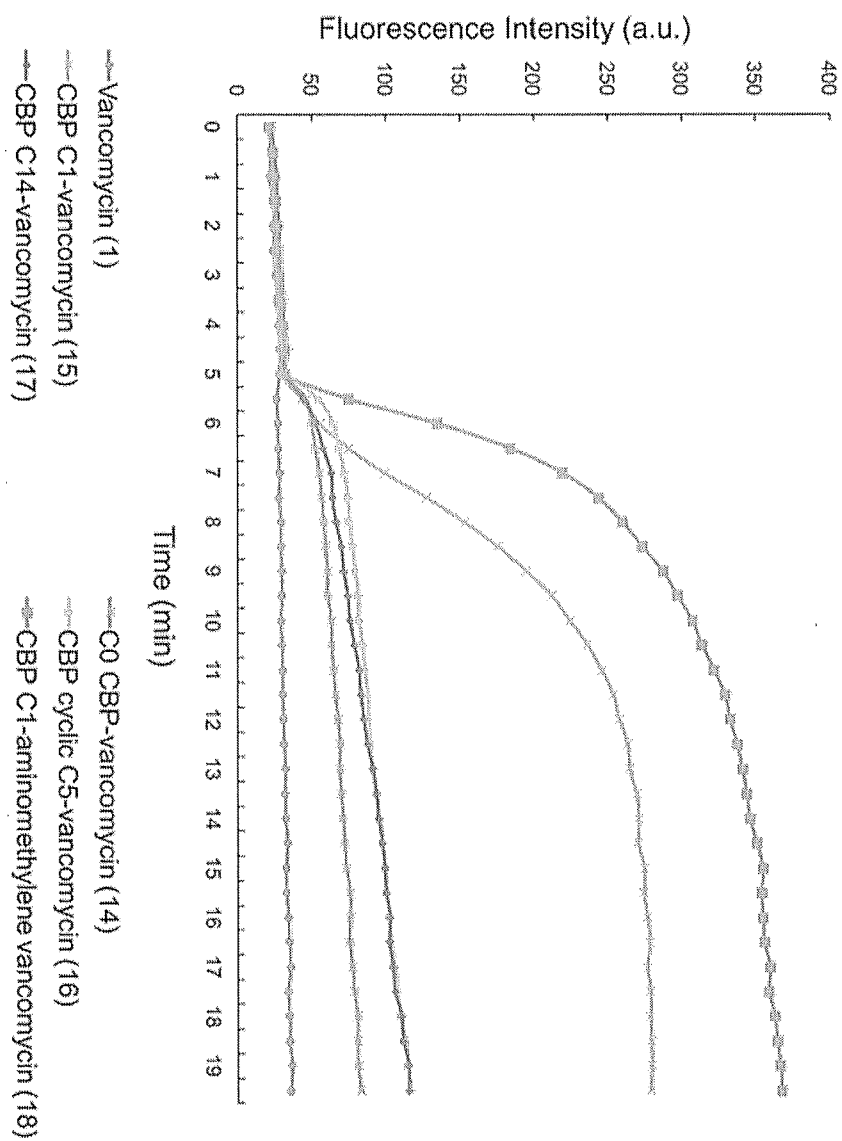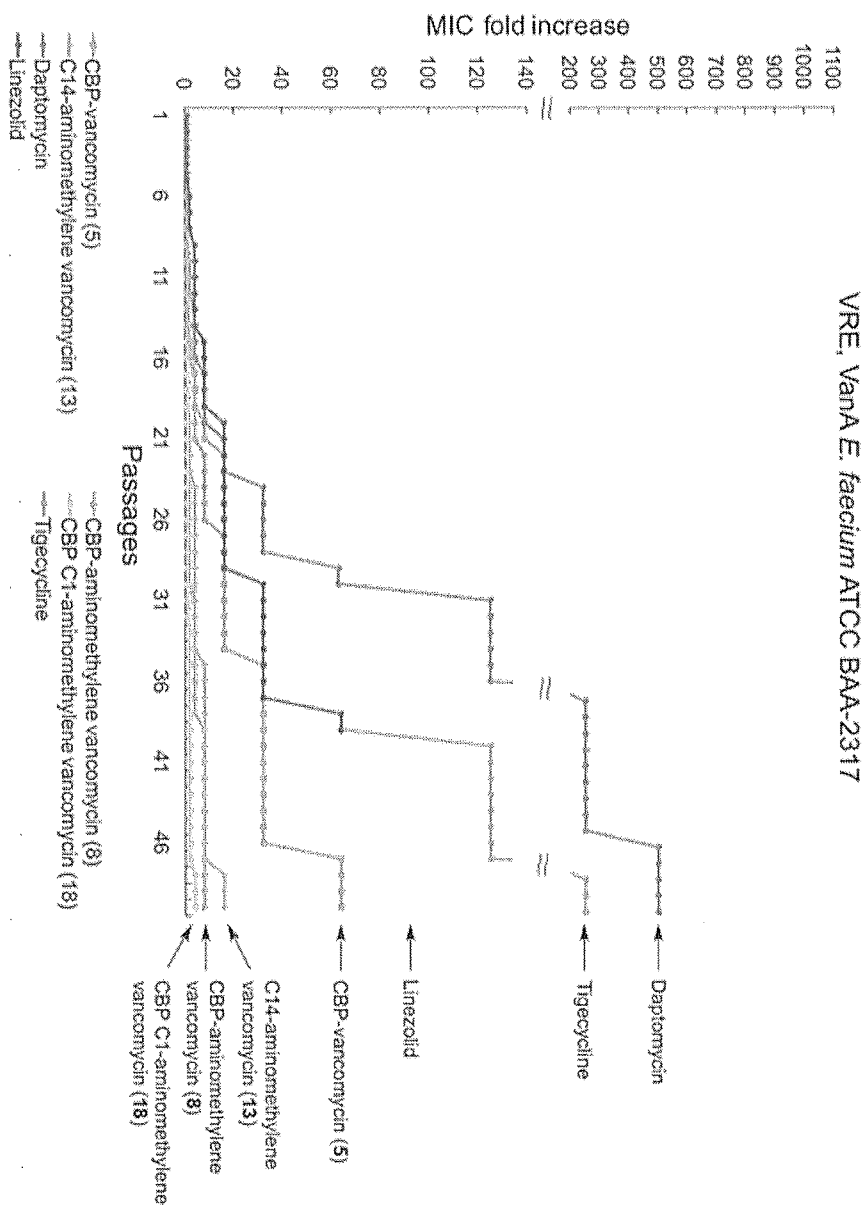

.

1/12

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

select vancomycin analogs	D-Ala-D-Ala binding	D-Ala-D-Lac binding	cell wall synthesis inhibition with ligand binding	cell wall synthesis inhibition without ligand binding	disrupts cell wall integrity
Thioamide vancomycin (2)	✗	✗	✗	✗	✗
CBP-thioamide vancomycin (6)	✗	✓	✓	✓	✗
Vancomycin (1)	✓	✗	✗	✗	✗
CBP-vancomycin (5)	✓	✗	✗	✓	✗
C14-vancomycin (12)	✓	✗	✗	✗	✓
CBP C1-vancomycin (15)	✓	✗	✗	✓	✓
Aminomethylene vancomycin (4)	✓	✓	✓	✗	✗
CBP-aminomethylene vancomycin (8)	✓	✓	✓	✓	✗
C14-aminomethylene vancomycin (13)	✓	✓	✓	✗	✓
CBP C1-aminomethylene vancomycin (18)	✓	✓	✓	✓	✓

Fig. 7

select vancomycin analogs	MIC (μg/mL)	
	VanA E. faecium BM4166	VanA E. faecalis ATCC BAA-2317
Thioamide vancomycin (2)	>32	>32
CBP-thioamide vancomycin (6)	4	4
Vancomycin (1)	250	250
CBP-vancomycin (5)	2.5	2.5
C14-vancomycin (12)	2	2
CBP C1-vancomycin (15)	0.5	0.25
Aminomethylene vancomycin (4)	31	31
CBP-aminomethylene vancomycin (8)	0.06	0.13
C14-aminomethylene vancomycin (13)	0.16	0.16
CBP C1-aminomethylene vancomycin (18)	0.005	0.01

Fig. 8

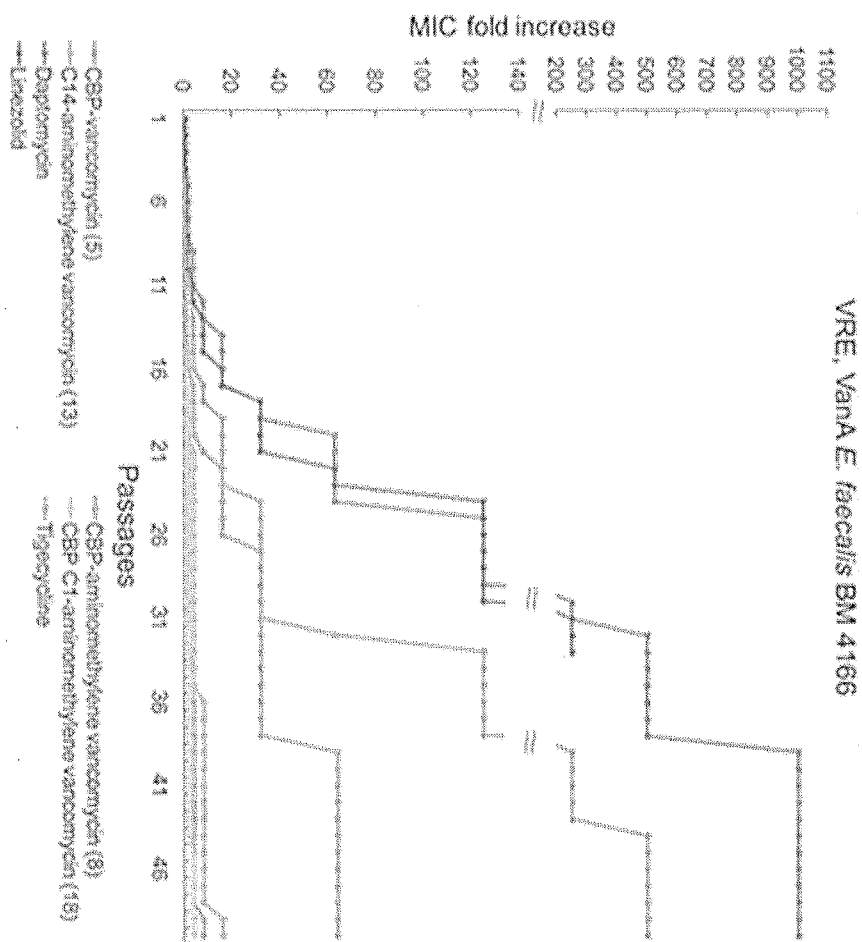VRE, VanA E. faecium ATCC BAA-2317

Fig. 9

Fig. 10

Summary of resistance development study against VRE, VanA E. faecium (ATCC BAA 2317)

	initial MIC	MIC[a] after 25 passages	fold-increase in MIC @ 25 passages	MIC[a] after 50 passages	fold-increase in MIC @ 50 passages	number of distinct effective mechanisms[b]
CBP-vancomycin (5)	2.5	40	16	160	64	1
C14-aminomethylene vancomycin (13)	0.16	0.64	4	2.5	16	2
CBP-aminomethylene vancomycin (8)	0.06	0.12	2	0.5	8	2
CBP C1-aminomethylene vancomycin (18)	0.005	0.005	0	0.02	4	3

[a]VanA E. faecium (ATCC BAA 2317), µg/mL. [b]Number excludes mechanism of D-Ala-D-Ala binding ineffective against VanA VRE.
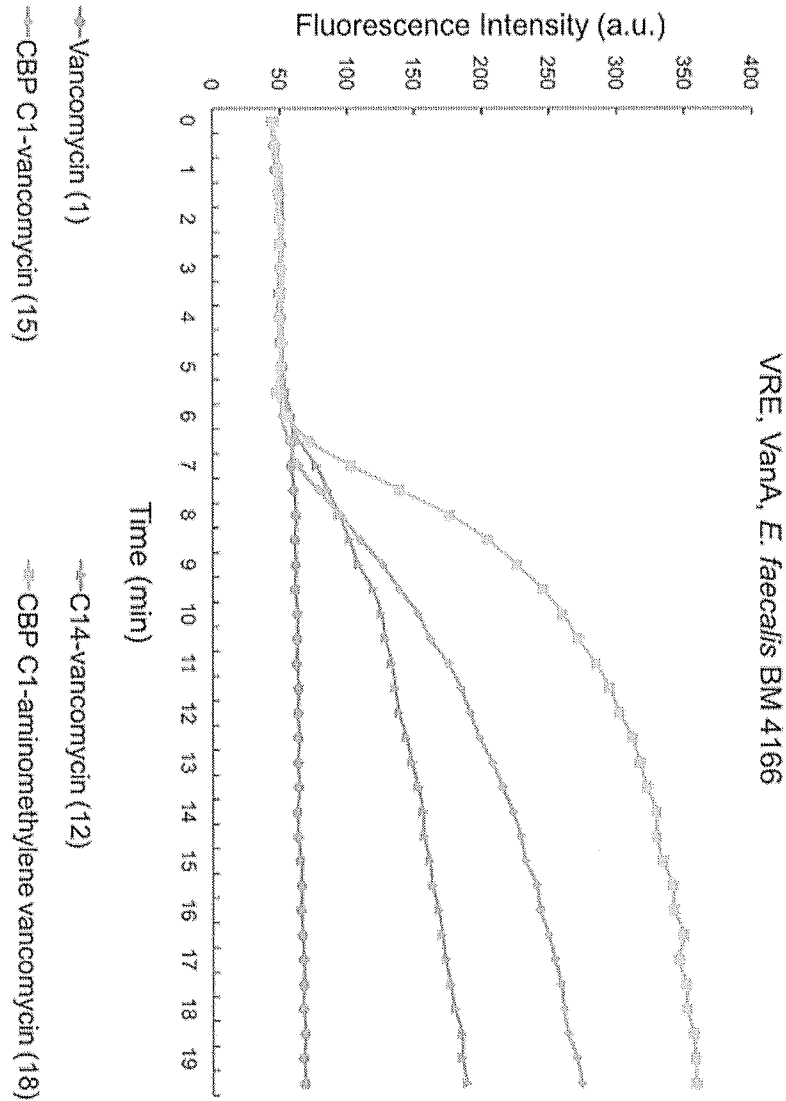
Fig. 11

Fig. 12

VRE, VanA, E. faecium BAA-2317

Fluorescence Intensity (a.u.)

Time (min)

—◦— Vancomycin (1)
—■— C14-vancomycin (12)
—△— CBP C1-vancomycin (15)
—▲— CBP C1-aminomethylene vancomycin (18)