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Rediscovery of Tetronomycin as a Broad-Spectrum and Potent Antibiotic against Drug-Resistant Gram-Positive Bacteria

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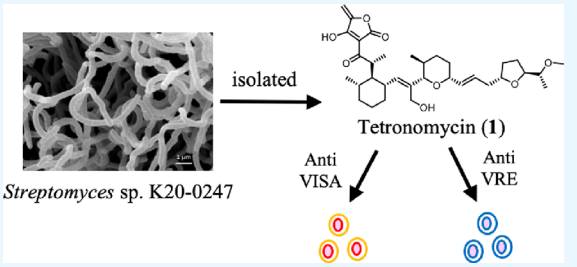
ABSTRACT: Tetronomycin (**1**), first isolated from a cultured broth of *Streptomyces* sp. by Juslen *et al.* in 1974, is a polycyclic polyether compound. However, the biological activity of **1** has not been thoroughly examined. In this study, we found that **1** exhibits more potent antibacterial activity than two well-known antibacterial drugs (vancomycin and linezolid) and is effective against several drug-resistant clinical isolates including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. Furthermore, we reassigned the ^{13}C NMR spectra of **1** and performed a preliminary structure−activity relationship study of **1** to synthesize a chemical probe for target identification, which implied different targets based on its ionophore activity.



INTRODUCTION

Antimicrobial resistance (AMR) is one of the major health threats to humankind, animals, and our shared environment. The number of AMR-related deaths in 2019 was estimated as 5 million annually.[1] Vancomycin, which was first isolated in 1957 and approved by the Food and Drug Administration in 1958, is one of the oldest antibiotics in clinical use for ∼60 years and has long been considered a drug of the last resort, owing to its efficacy against Gram-positive bacteria as a broad-spectrum antibiotic.[2] However, the increasing emergence of vancomycin-resistant bacteria such as vancomycin-intermediate *Staphylococcus aureus* (*S. aureus*) (VISA), which was initially discovered in methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *Enterococci* (VRE) has been a global concern.[3] The US Centers for Disease Control and Prevention projected that VRE caused 54,500 cases of infections and 5400 deaths in 2017.[4] As alternatives to vancomycin, the development of new broad-spectrum antibiotics against Gram-positive bacteria is urgently required.

This study describes the identification of tetronomycin (**1**) as a broad-spectrum and potent antibiotic against Gram-positive bacteria including clinical isolates of VISA and VRE. Further, this study presents the reassignment of the NMR spectra of **1** and its preliminary structure−activity relationship (SAR).

RESULTS AND DISCUSSION

Isolation of 1 and Reassignment of Its NMR Spectra.

Compound **1** was isolated as a light-yellow oil from the culture broth of the *Streptomyces* sp. K20-0247 strain, which was isolated from the soil collected at Izu Ōshima, Tokyo, Japan (Figure 1). Originally, **1** was isolated by Juslen *et al.* in 1974,

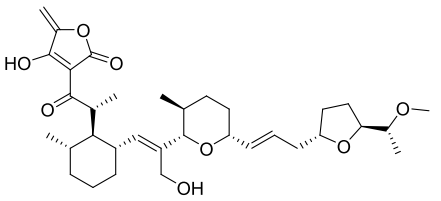


Figure 1. Structure of tetronomycin (**1**).

and its absolute structure was determined via the X-ray analysis of the mono-*O*-acetyltetronomycin silver salt.[5] To assign the peaks in the ^{1}H and ^{13}C NMR spectra of **1**, they derivatized **1** to its sodium salt **2**, probably owing to the acidic nature of the tetronic acid moiety. Therefore, we also obtained **2**; its ^{1}H and ^{13}C NMR spectra and optical rotation values are in good agreement with those described in the original report.[5] However, as a result of our full reassignment of **2** by correlated spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation

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Table 1. NMR Spectroscopy Data for Tetronomycin Sodium Salt (2)[a]

position	original δ_C, type	reassigned δ_C, type	original δ_H	reassigned δ_H (J in Hz)
1	172.59, C	172.32, C		
2	96.82, C	96.74, C		
3	180.45, C	180.40, C		
4	154.22, C	153.87, C		
5	88.21, CH_2	88.48, CH_2	5.22	5.20, d (1.6)
			4.74	4.73, d (1.6)
6	201.95, C	202.00, C		
7	43.31, CH	43.26, CH	3.93	3.92, dq (4.0, 6.8)
8	8.76, CH_3	8.72, CH_3	0.99	0.98[c], d (6.8)
9	47.32, CH	47.08, CH	1.89	1.87[g], ddd (4.0, 10.8, 10.8)
10	32.64[b], CH	33.92, CH	1.32–1.42	1.37[e], m
11	19.84, CH_3	19.76, CH_3	1.21	1.20[d], d (6.8)
12	35.40[a], CH_2	35.22, CH_2	1.52–1.67	1.61[f], m
			1.1–1.3	1.14[d], m
13	25.76, CH_2	25.62, CH_2	1.52–1.67	1.57[f], m
			1.1–1.3	1.22[d], m
14	32.20[a], CH_2	35.10, CH_2	1.32–1.42	1.34[e], m
			1	1.01[c], m
15	36.18, CH	36.05, CH	2.49	2.48, dddd (4.4, 10.2, 10.4, 10.4)
16	141.94, CH	141.68, CH	5.15	5.14, d (10.2)
17	130.72, C	130.51, C		
18	56.29, CH_2	56.15, CH_2	4.29	4.27, d (11.0)
			3.78	3.79[h], d (11.0)
19	90.86, CH	90.62, CH	3.26	3.24, d (10.0)
20	34.07[b], CH	32.47, CH	1.32–1.42	1.39[e], m
21	18.16, CH_3	18.15, CH_3	0.52	0.52, d (6.8)
22	35.27[a], CH_2	32.01, CH_2	1.79	1.78, dddd (3.6, 3.6, 3.6, 13.4)
			1.1–1.3	1.17[d], m
23	31.58[a], CH_2	31.46, CH_2	1.52–1.67	1.58[f], m
			1.45	1.43[e], dddd (3.7, 12.1, 12.1, 15.4)
24	79.45, CH	79.36, CH	3.82	3.80[h], ddd (1.8, 8.8, 8.8)
25	132.62, CH	132.92, CH	5.48	5.47, ddd (1.6, 8.8, 15.2)
26	135.23, CH	135.10, CH	6.16	6.13, ddd (3.8, 11.2, 15.2)
27	39.87, CH_2	39.77, CH_2	2.33	2.34, dddd (1.6, 1.8, 3.8, 13.2)
			1.96	1.97[g], ddd (10.8, 11.2, 13.2)
28	78.05, CH	77.97, CH	4.1–4.2	4.13[i], dddd (1.8, 6.2, 8.0, 10.8)
29	31.93[a], CH_2	31.90, CH_2	2.11	2.09, m
			1.52–1.67	1.56[f], m
30	27.73, CH_2	27.74, CH_2	1.85–1.95	1.90[g], m
			1.52–1.67	1.55[f], m
31	80.17, CH	80.08, CH	4.1–4.2	4.16[i], ddd (2.4, 6.0, 8.4)
32	78.46, CH	78.32, CH	3.33	3.31, dq (2.4, 6.4)
33	10.43, CH3	10.19, CH_3	0.92	0.92, d (6.4)
34	57.42, CH_3	57.35, CH_3	3.4	3.37, s
18			4.36	4.41, br, s

[a]Original data were collected at 360 MHz for ^{1}H NMR spectra and 90.5 MHz for ^{13}C NMR spectra. Reassigned data were collected at 400 MHz for ^{1}H NMR spectra and 100 MHz for ^{13}C NMR spectra. [a,b]Exchangable. [c,d,e,f,g,h,i]Overlapped.

(HMBC) analyses (Figures S1–S7), we found that the chemical shifts of C-14 and C-22 in ^{13}C NMR were found to be inconsistent with the original data; thus, we reassigned these shifts (Table 1 and Figure 2). The HSQC cross-peak from H_2-14 (δ_H 1.34 and 1.01) to C-14 (δ_C 35.10), COSY cross-peaks of H-16 (δ_H 5.14)/H-15 (δ_H 2.49)/H_2-14, and the HMBC cross-peak from H-16 to C-14 supported the reassignment of C-14 to δ_C 35.10. The HSQC cross-peaks from H_2-22 (δ_H 1.78 and 1.17) to C-22 (δ_C 32.01), COSY cross-peaks of H_3-21 (δ_H 0.52)/H-20 (δ_H 1.35)/H_2-22, and the HMBC cross-peak from H-21 to C-22 supported the reassignment of C-22 to δ_C 32.01 (Table 1 and Figure 2). Therefore, based on the spectral data, the ^{13}C NMR chemical shift values of C-4 and C-22 in **2** were reassigned.

Preliminary SAR Study of 1. As a first approach to identify the target molecules of **1** with broad antibacterial activity against Gram-positive bacteria, a preliminary SAR study was investigated (Scheme 1). Because the broad antibacterial activity spectrum of **1** is similar to that of related polyether tetronic compounds[6,7] as well as based on the carrier-mediated transport studies of **1**,[8,9] it is considered as an ionophore compound. Furthermore, some studies have confirmed that polyether tetronic acid antibiotics accelerate the transport of ions across a model membrane.[10,11] However, owing to insufficient evidence from these studies, a causal relationship between the ionophore and antibacterial activity could not be established. In addition, tetrodecamycin, one of the tetronate natural products, possesses an *exo*-methylene group on the tetronic acid moiety identical to **1**, and the *exo*-methylene group is crucial for antibacterial activity.[12,13] Therefore, we decided to chemically modify the *exo*-methylene group to determine its importance for antibiotic activity. Considering the electrophilicity of the *exo*-methylene group, thiophenol was used as a soft nucleophile, and a diastereomixture of the thioacetal derivative **3** was produced. However, as measured by the paper-disc method, the biological activity of **3** against *S. aureus* was slightly less than that of **1**. We believed that the retro Michael reaction of **3** was possible to produce **1**; therefore, we attempted the selective reduction of the *exo*-methylene group to prevent the retro Michael reaction. The use of H_2 and 10% Pd/C afforded a diastereomixture of the target product **4**. As anticipated, **4** lost its antibiological activity, indicating that the *exo*-methylene group on the tetronic acid moiety is crucial for antibacterial activity and that **1** may covalently bind to its target binding sites at the *exo*-methylene group by nucleophilic amino acid residues. This finding suggests that **1** has other targets of antibacterial activity in addition to ionophore activity. Subsequently, we derivatized the C-18 OH group using acid anhydrides to elucidate the role of the hydroxyl group in the antibiotic activity and provide insight into the chemical probe synthesis. The C-18 OH group barely tolerated the introduction of acyl groups, such as acetyl, propynoic, and pivaloyl, with reduced antibacterial activity slightly [the

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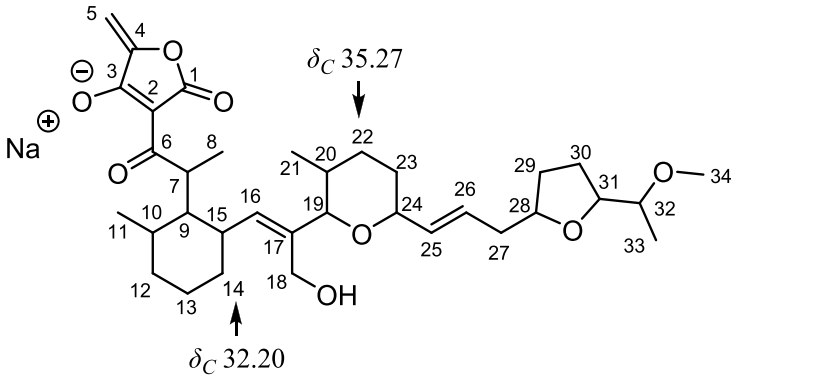
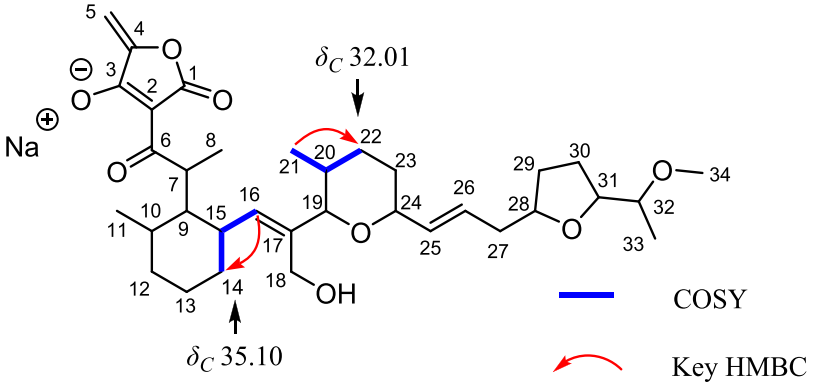


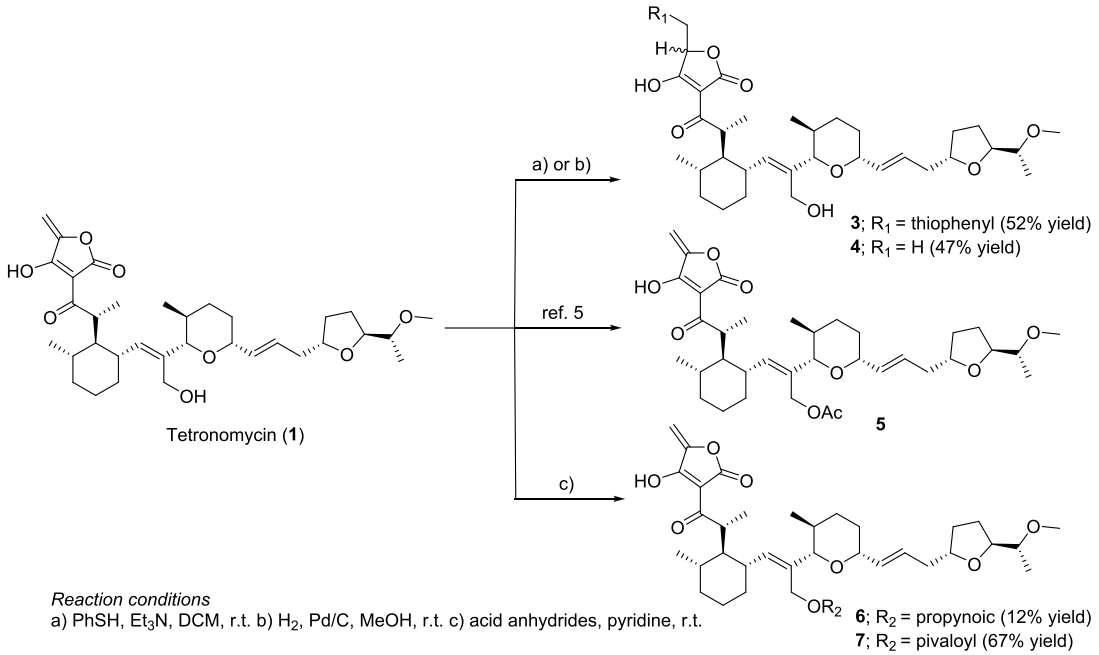
Originally assigned carbons in ^{13}C NMR spectrum.



Reassigned carbons in ^{13}C NMR spectrum.

Figure 2. Reassignment of ^{13}C NMR chemical shift values of **2**.

Scheme 1. Derivatization of 1



minimal inhibitory concentration (MIC) values of acyl derivatives (compounds **5**−**7**) are shown in Table 2]. This result allowed us to use compound **6** as a chemical probe for target identification using the click-chemistry strategy.[14]

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Table 2. Antibacterial Activity of 1 and Its Derivatives as well as the Known Drugs[a]

strains		MIC (μg/mL)					
		1	5	6	7	VCM	LZD
Staphylococcus aureus	ATCC6538P	0.125	0.5	1	2	1	1
Staphylococcus aureus	ATCC 19636	0.063	0.25	1	2	1	1
Staphylococcus aureus	ISP217	0.063	0.125	0.5	0.5	1	1
Staphylococcus aureus	KUB854	0.125	0.25	1	2	1	2
Staphylococcus aureus	KUB855	0.125	0.25	0.5	1	0.5	1
Staphylococcus aureus	KUB856	0.125	0.5	1	2	1	2
Staphylococcus aureus	ATCC700699	0.063	0.125	0.5	1	8	1
Staphylococcus aureus	KUB877	0.125	0.25	1	2	1	16
Staphylococcus epidermidis	KUB795	0.25	0.5	2	2	1	1
Kocuria rhizophila	ATCC9341	0.063	0.125	0.5	0.125	0.5	1
Enterococcus faecalis	ATCC29212	0.063	0.063	0.25	0.25	4	1
Enterococcus faecalis	NCTC12201	0.063	0.125	0.25	0.25	>64	1
Enterococcus faecalis	KUB7012	0.016	0.063	0.25	0.016	>64	1
Enterococcus faecium	NCTC12204	0.063	0.125	0.5	0.25	>64	1
Enterococcus faecium	KUB7013	0.031	0.125	0.5	0.125	>64	2
Enterococcus gallinarum	KUB7014	0.063	0.031	0.25	0.25	8	1
Escherichia coli	NIHJ JC-2	>8	>8	>8	>8	>64	>64
Citrobacter freundii	ATCC8090	>8	>8	>8	>8	>64	>64
Klebsiella pneumoniae	NCTC9632	>8	>8	>8	>8	>64	>64
Klebsiella aerogenes	NCTC10006	>8	>8	>8	>8	>64	>64
Proteus vulgaris	ATCC8427	>8	>8	>8	>8	>64	16
Morganella morganii	IID 602	>8	>8	>8	>8	>64	>64
Serratia marcescens	IFO12648	>8	>8	>8	>8	>64	>64
Enterobacter cloacae	IFO13535	>8	>8	>8	>8	>64	>64
Pseudomonas aeruginosa	46001	>8	>8	>8	>8	>64	>64
Pseudomonas aeruginosa	E-2	>8	>8	>8	>8	>64	>64
Acinetobacter calcoaceticus	IFO12552	>8	>8	>8	>8	>64	>64

[a]Abbreviations: VCM, vancomycin; LZD, linezolid.

Evaluation of the Antibacterial Activity of 1 and Its Derivatives. According to the original study,[5] the antibacterial activity evaluation of **1** against drug-resistant clinical isolates has not been performed yet. Therefore, using the paper-disc method and agar dilution method, we evaluated the antibacterial activity of **1** and its derivatives that maintained antibacterial activity against Gram-positive and Gram-negative bacteria (Table 2 and Table S2). The disc method is widely used in natural product chemistry to identify active compounds. Further, we compared the antibacterial potency of **1** to those of two well-known drugs (vancomycin and linezolid) (Table 2). Compound **1** showed a selective and more potent antibacterial activity against Gram-positive bacteria than the two known drugs. Compared with the MIC values of the known drugs toward *S. aureus*, *Staphylococcus epidermidis*, and *Kocuria rhizophila*, **1** exhibited antibacterial activity at a lower MIC. Furthermore, **1** was effective against *S. aureus* strains resistant to linezolid and macrolide. Notably, **1** exhibits remarkable antibacterial activity against VISA and other types of VRE. Thus, **1** is expected to be a new broad-spectrum antibiotic against Gram-positive bacteria and an effective alternative to vancomycin.

The antibacterial activity of acyl derivatives (compounds **5**–**7**) slightly decreased but remained at a high level compared with the known drugs (Table 2). Strain information is provided in Table S3.

CONCLUSIONS

We reported the reassignment of the ^{13}C NMR chemical shifts and showed that **1** is a broad-spectrum, potent antibiotic against Gram-positive bacteria and exhibits antibacterial activity against drug-resistant clinical isolates including VISA and VRE. As a preliminary attempt to identify the target molecules of **1** with broad antibacterial activity against Gram-positive bacteria, a preliminary SAR study was conducted. The *exo*-methylene group on the tetronic acid moiety is crucial for antibacterial activity, suggesting that in addition to its ionophore activity, **1** may covalently bind to its target binding sites. Overall, we expect that **1** will become an effective antibiotic against Gram-positive bacteria. While the MIC values of tetronomycin-nonproducing strains such as *Streptomyces lividans* TK24 and *Staphylococcus albus* J1074 were below 3 μg/mL, *Streptomyces* sp. K20-0247 was resistant to **1** (MIC >10 μg/mL). Thus, we speculated that *Streptomyces* sp. K20-0247 possesses resistance genes against **1** in the biosynthetic gene cluster of **1**. The genome sequence of *Streptomyces* sp. K20-0247 was analyzed, and the biosynthetic gene cluster of **1** was discovered (Figure S18 and Table S1). This cluster was extremely similar to the previously reported tetronomycin biosynthetic gene cluster (Figure S18 and Table S1). In these clusters, resistance gene candidates such as genes encoding the DedA family and phosphatase were identified (Table S1). Thus, we hypothesized that these genes were responsible for *Streptomyces* sp. K20-0247 showing resistance against **1**. Our next objective is to analyze the function of candidate resistance genes and the mode of action and the resistance mechanism using the propynoic derivative of **1**.

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EXPERIMENTAL SECTION

General Experimental Procedures. High- and low-resolution mass spectra were obtained using an AB Sciex QSTAR Hybrid LC/MS/MS system (AB Sciex, Framingham, MA, USA) and a JEOL JMS-T100LP (JEOL, Tokyo, Japan). NMR spectra were measured using a Varian XL-400 spectrometer (Agilent Technologies, CA, USA), with ^{1}H NMR and ^{13}C NMR at 400 and 100 MHz, respectively, and a JEOL JNM-ECA-500 (JEOL, Tokyo, Japan), with ^{1}H NMR obtained at 500 Hz in $CDCl_3$. The chemical shifts are reported in ppm and referenced to $CDCl_3$ (7.26 ppm) in the ^{1}H NMR spectra and $CDCl_3$ (77.16 ppm) in the ^{13}C NMR spectra. Optical rotation was measured using a JASCO P-2200 polarimeter (JASCO Corporation, Tokyo, Japan).

Isolation of Strain K20-0247. Strain K20-0247 was isolated from the soil sample collected from Izu Ōshima, Tokyo, Japan via incubation at 27 °C for two weeks on water-proline agar (proline 1%, agar 1.5%, and tap water) with 25 μg/mL benlate (DuPont de Nemours, Inc., Delaware, USA), 25 μg/mL nalidixic acid (TCI, Tokyo, Japan), and 1 μg/mL vancomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). After incubation, colonies were isolated and maintained on an agar medium (starch 1.0%, *N*-*Z*-amine 0.3%, yeast extract 0.1%, meat extract 0.1%, $CaCO_3$ 0.3%, agar 1.2%, and distilled water, with pH 7.0).

Genome Analysis. The genomic DNA of strain K20-0247 was obtained using the cetyltrimethylammonium bromide method.[15] The genomes were sequenced using the Pacbio RSII technology (Macrogen, Inc., Seoul, Republic of Korea). The genome of *Streptomyces* sp. K20-0247 comprised a linear 8,931,955 bp genome and a circular 102,312 bp plasmid. The obtained genomic sequence was annotated using DFAST and analyzed via antiSMASH.[16,17]

The comparison of tetronomycin biosynthetic gene clusters generated from *Streptomyces* sp. K20-0247 and *Streptomyces* sp. NRRL 11266 was visualized using Clinker.[18] BLAST search of the 16S rRNA gene sequence was performed using the EzBioCloud server (http://eztaxon-e.ezbiocloud.net) to determine the taxonomic position of the 16S rRNA gene sequence.

Fermentation. Strain K20-0247 was maintained on agar medium (glucose 1.0%, peptone 0.5%, 35% Ehrlich bonito extract 0.5%, NaCl 0.3%, and agar 1.0%). For seed culture, a loop of strain K20-0247 spores was inoculated into each 100 mL of seed medium (starch 2.4%, glucose 0.1%, peptone 0.3%, 35% Ehrlich bonito extract 0.3%, yeast extract 0.5%, and $CaCO_3$ 0.4%) (adjusted to pH 7.0 before sterilization) in a 500 mL Erlenmeyer flask (total = 2 flasks). The flask was incubated on a rotary shaker (210 rpm) at 27 °C for four days. A 1 mL portion of the seed culture was inoculated into each 100 mL of production medium (starch 2.0%, glycerol 0.5%, defatted wheat germ 1.0%, 35% Ehrlich bonito extract 0.3%, dry yeast 0.3%, and $CaCO_3$ 0.3%) in 500 mL Erlenmeyer flasks (total = 120 flasks). The flasks were incubated on a rotary shaker (210 rpm) at 27 °C for six days.

Isolation of Tetronomycin (1). MeOH (12 L) was added to the culture, and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was collected and evaporated under reduced pressure to remove MeOH. The residual aqueous solution (12 L) was loaded onto an ODS column and eluted stepwise using a mixture of MeCN:H_2O (0:100, 30:70, 60:40, and 100:0) and MeOH (with 0.1% TFA). The MeOH (0.1% TFA) fraction was concentrated *in vacuo* to remove MeOH. The residue was dissolved in 800 mL of EtOAc and rinsed three times using an equal volume of water. The organic layer was concentrated to dryness to afford a crude extract (477.3 mg). Finally, the crude extract was purified via preparative TLC (plate size = 200 × 200 mm and thickness = 1 mm) using two plates eluted with *n*-hexane:EtOAc (1:1, R_f = 0.35) to produce 129.1 mg of **1** as a light-yellow oil. **1** was isolated for its antimicrobial activity against *S. aureus* ATCC43300.

Preparation Procedure of the Tetronomycin Sodium Salt 2. To a solution of **1** (10.0 mg, 17.1 μmol) in dichloromethane (2.0 mL, 8.5 mM), 1.0 M $NaHCO_3$ aq. (200.0 μL, 200.0 μmol) was added at room temperature. After stirring for 3 h, the reaction mixture was quenched using sat. NH_4Cl aq., and the organic layer was separated. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated under vacuum to afford **2** (7.0 mg, 11.4 μmol, 66.8%). $[\alpha]^{22}_{D}$ +80.5 (*c* 0.28, MeOH); ^{1}H and ^{13}C NMR are presented in Table 1; ESI-MS *m*/*z* 609.3401 $[M + Na]^+$ (calcd. for $C_{34}H_{50}O_8Na$, *m*/*z* 609.3403 $[M + Na]^+$).

Preparation Procedure of the Tetronomycin Thioacetal Derivative 3. To a solution of **1** (10.0 mg, 17.1 μmol) in dichloromethane (0.3 mL, 56.8 mM), benzenethiol (8.0 μL, 78.4 μmol) and triethylamine (4.0 μL, 28.7 μmol) were added and stirred at room temperature for 5 h. The reaction mixture was purified via preparative TLC to afford **3** as a diastereomixture (6.2 mg, 8.9 μmol, 52.2%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.54−7.51 (2H, overlapped), 7.44−7.37 (2H, overlapped), 7.28−7.20 (4H, overlapped), 7.18−7.09 (2H, overlapped), 6.13 (2H, overlapped), 5.55−5.34 (2H, overlapped), 5.32 (1H, d, *J* = 10.3 Hz), 5.11 (1H, d, *J* = 10.3 Hz), 4.61 (1H, t, *J* = 3.7 Hz), 4.53 (1H, dd, *J* = 8.2, 2.9 Hz), 4.26 (1H, d, *J* = 11.1 Hz), 4.21 (1H, d, *J* = 11.1 Hz), 4.18−3.92 (4H, overlapped), 3.92−3.70 (6H, overlapped), 3.60−3.56 (4H, overlapped), 3.37−3.28 (6H, overlapped), 2.53−2.27 (4H, overlapped), 2.12−2.04 (2H, overlapped), 2.04−1.70 (8H, overlapped), 1.70−1.40 (8H, overlapped), 1.40−1.28 (6H, overlapped), 1.28−1.00 (4H, overlapped), 1.16 (6H, d, *J* = 6.4 Hz), 0.99−0.90 (12H, overlapped), 0.52 (3H, d, *J* = 6.5 Hz), 0.42 (3H, d, *J* = 6.5 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 201.97, 201.57, 193.52, 193.23, 175.99, 175.80, 142.36, 141.60, 137.53, 136.36, 135.06, 134.98, 132.76, 132.67, 130.81, 130.00, 128.99, 128.93, 128.86, 126.15, 125.64, 90.71, 90.21, 82.11, 80.31, 80.23, 79.46, 79.13, 78.97, 78.60, 78.51, 78.47, 78.31, 78.21, 78.13, 57.48, 57.17, 56.33, 56.25, 47.62, 46.65, 43.34, 43.25, 39.92, 39.90, 38.74, 37.02, 36.36, 36.33, 36.10, 35.52, 35.35, 35.33, 35.00, 34.10, 33.96, 32.64, 32.52, 32.22, 32.08, 32.05, 31.99, 31.61, 31.57, 29.83, 27.84, 25.79, 25.74, 22.82, 19.98, 19.81, 18.32, 18.29, 15.72, 15.66, 14.25, 10.50, 10.48, 8.86, 8.80; ESI-MS *m*/*z* 714.4043 $[M + NH_4]^+$ (calcd. for $C_{40}H_{60}NO_8S$, *m*/*z* 714.4040 $[M + NH_4]^+$).

Preparation Procedure of Reduced Tetronomycin 4. To a solution of **1** (11.7 mg, 20.0 μmol) in MeOH (0.5 mL, 40.0 mM), 10% Pd/C (1.5 mg, 1.4 mmol) was added and stirred at room temperature under a H_2 atmosphere for 15 min. The reaction mixture was filtered and purified via preparative TLC to afford **4** as a diastereomixture (5.5 mg, 9.4 μmol, 46.9%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.15 (2H, overlapped), 5.48 (2H, overlapped), 5.14 (1H, d, *J* = 10.6 Hz), 5.08 (1H, d, *J* = 10.6 Hz), 4.58 (1H, br), 4.51 (1H, br), 4.44 (1H, q, *J* = 6.9 Hz), 4.34 (1H, q, *J* = 6.9 Hz), 4.27 (2H, d, *J* = 11.0 Hz), 4.20−4.09 (4H, overlapped), 3.91 (2H, dq, *J* = 7.0, 3.5 Hz), 3.89−3.76 (4H, overlapped), 3.36 (6H, s), 3.34−3.29

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(2H, overlapped), 3.28 (1H, d, J = 9.9 Hz), 3.25 (1H, d, J = 9.9 Hz), 2.50 (2H, dddd, J = 10.8, 10.8, 10.6, 4.6 Hz), 2.32 (2H, m), 2.12−2.04 (2H, m), 2.01−1.72 (8H, overlapped), 1.67−1.50 (10H, overlapped), 1.48 (3H, d, J = 6.9 Hz), 1.43 (3H, d, J = 6.9 Hz), 1.50−1.27 (6H, overlapped), 1.27−1.06 (12H, overlapped), 1.05−0.95 (8H, overlapped), 0.95−0.91 (6H, overlapped), 0.57−0.49 (6H, overlapped); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 201.96, 196.47, 196.21, 176.14, 141.66, 135.06, 134,89, 132.79, 132.68, 130.73, 95.61, 95.55, 90.87, 90.68, 80.23, 79.38, 78.37, 78.06, 57.44, 56.30, 47.52, 43.08, 41.05, 39.87, 36.27, 35.49, 35.47, 35.33, 34.08, 32.61, 32.17, 31.97, 31.94, 31.58, 31.56, 27.81, 27.74, 25.79, 19.94, 19.83, 18.31, 17.90, 17.78, 10.40, 8.89, 8.79; ESI-MS m/z 606.4006 $[M + NH_4]^+$ (calcd. for $C_{34}H_{56}NO_8$, m/z 606.4006 $[M + NH_4]^+$).

Preparation Procedure of Acetyl Tetronomycin 5. According to ref 5, the acetyl tetronomycin Ag salt (15.8 mg, 26.0 μmol) was synthesized and purified via preparative TLC to afford **5** (6.2 mg, 9.9 μmol, 38.0%). $[\alpha]^{25}_D$ +94.0 (c 0.59, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.12 (1H, ddd, J = 15.1, 10.9, 4.1 Hz), 5.41 (1H, ddd, J = 15.1, 9.1, 1.4 Hz), 5.30 (1H, d, J = 10.2 Hz), 5.19 (1H, d, J = 1.7 Hz), 4.71 (1H, d, J = 1.7 Hz), 4.68 (1H, d, J = 12.1 Hz), 4.56 (1H, d, J = 12.1 Hz), 4.22−4.13 (1H, m), 4.13 (1H, ddd, J = 14.3, 7.3, 7.2 Hz), 4.10 (1H, ddd, J = 9.3, 6.5, 2.8 Hz), 3.86 (1H, ddd, J = 11.4, 9.1, 2.1 Hz), 3.78 (1H, dq, J = 6.7, 3.5 Hz), 3.44 (1H, s), 3.42 (1H, dq, J = 6.7, 2.8 Hz), 3.36 (1H, q, 3.78 J = 1.8 Hz), 3.35 (1H, s), 3.25 (1H, d, J = 10.1 Hz), 2.31 (2H, dddd, J = 10.9, 10.7, 10.7, 4.1 Hz), 2.39−2.25 (1H, m), 2.21−1.70 (8H, overlapped), 1.65−1.52 (5H, overlapped), 1.49−1.28 (3H, overlapped), 1.20 (3H, d, J = 6.4 Hz), 1.28−1.00 (4H, overlapped), 0.98 (3H, d, J = 6.7 Hz), 0.92 (3H, d, J = 6.7 Hz), 0.62 (3H, d, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 200.94, 180.04, 173.52, 171.56, 154.27, 144.50, 134.61, 133.02, 127.15, 96.72, 90.28, 88.38, 79.75, 79.14, 77.93, 59.62, 57.40, 57.20, 47.35, 43.92, 39.49, 37.16, 35.56, 35.00, 34.19, 32.84, 32.17, 31.67, 31.48, 29.85, 27.24, 25.93, 21.30, 19.85, 17.99, 10.41, 8.88; ESI-MS m/z 646.3956 $[M + NH_4]^+$ (calcd. for $C_{36}H_{56}NO_9$, m/z 646.3955 $[M + NH_4]^+$, molecular formula).

Preparation Procedure of Propynoic Tetronomycin 6. To a solution of **1** (5.9 mg, 10.0 μmol) in propiolic acid (100.0 μL, 0.1 M), triethylamine (150.0 μL, 1.1 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) (19.0 mg, 99.1 μmol) were added at 0 °C. After stirring for 50 min, the reaction mixture was quenched using 1.0 M HCl aq. The organic layer was separated and washed with 1.0 M HCl aq., dried over Na_2SO_4, and concentrated under vacuum. The residue was purified via preparative TLC to afford **6** (0.8 mg, 1.2 μmol, 11.7%). $[\alpha]^{24}_D$ +95.9 (c 0.10, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.11 (1H, ddd, J = 15.3, 10.8, 4.3 Hz), 5.44 (1H, ddd, J = 15.3, 9.1, 1.7 Hz), 5.33 (1H, d, J = 10.3 Hz), 5.20 (1H, d, J = 1.9 Hz), 4.81 (1H, d, J = 12.1 Hz), 4.71 (1H, d, J = 1.9 Hz), 4.70 (1H, d, J = 12.1 Hz), 4.17 (1H, m), 4.11 (1H, m), 3.86 (1H, m), 3.78 (1H, m), 3.42 (3H, s), 3.27 (1H, d, J = 10.2 Hz), 2.87 (1H, s), 2.39−2.25 (2H, overlapped), 2.14−2.08 (1H, m), 2.06−1.72 (4H, overlapped), 1.68−1.54 (5H, overlapped), 1.20 (3H, d, J = 6.5 Hz), 1.48−0.98 (8H, overlapped), 0.97 (3H, d, J = 6.3 Hz), 0.92 (3H, d, J = 7.1 Hz), 0.64 (3H, d, J = 6.6 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 201.00, 180.25, 173.48, 154.20, 153.32, 145.03, 134.59, 132.91, 126.28, 96.68, 90.00, 88.47, 79.75, 79.34, 77.93, 61.28, 57.43, 47.25, 43.91, 39.46, 37.26, 35.53, 34.80, 34.15, 32.86, 32.15, 32.06, 31.61, 31.50, 29.84, 27.26, 25.87, 22.83, 19.81, 17.97, 10.42, 8.86; ESI-MS m/z 656.3796 $[M + NH_4]^+$ (calcd. for $C_{37}H_{54}NO_9$, m/z 656.3799 $[M + NH_4]^+$).

Preparation Procedure of Pivaloyl Tetronomycin 7. To a solution of **2** (4.1 mg, 6.7 μmol) in pivalic anhydride (0.1 mL, 0.1 mM), pyridine (0.1 mL, 1.2 mmol) was added and stirred at 80 °C for 8 h. The reaction mixture was quenched using sat. $NaHCO_3$ aq. The organic layer was separated and washed with sat. $NaHCO_3$ aq., dried over Na_2SO_4, and concentrated under vacuum. The residue was freeze-dried and purified via preparative TLC to afford **7** (3.0 mg, 4.5 μmol, 67.2%). $[\alpha]^{25}_D$ +125.4 (c 0.30, MeOH); ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.11 (1H, ddd, J = 15.3, 11.0, 4.1 Hz), 5.43 (1H, dd, J = 15.3, 9.1 Hz), 5.35 (1H, d, J = 10.2 Hz), 5.20 (1H, d, J = 1.7 Hz), 4.71 (1H, d, J = 1.7 Hz), 4.61 (1H, d, J = 11.6 Hz), 4.44 (1H, d, J = 11.6 Hz), 4.19 (1H, m), 4.06 (1H, ddd, J = 8.9, 6.2, 2.4 Hz), 3.88 (1H, ddd, J = 11.4, 9.1, 2.4 Hz), 3.76 (1H, dq, J = 6.7, 3.6 Hz), 3.48 (3H, s), 3.42 (1H, dq, J = 6.6, 2.4 Hz), 3.27 (1H, d, J = 10.0 Hz), 2.28 (1H, dddd, J = 10.8, 10.7, 10.5, 4.3 Hz), 2.32−2.23 (1H, m), 2.16−2.07 (1H, m), 2.07−1.97 (1H, overlapped), 1.96−1.82 (1H, overlapped), 1.86 (1H, ddd, J = 10.7, 10.6, 3.7 Hz), 1.82−1.74 (1H, m), 1.64−1.50 (5H, overlapped), 1.49−1.29 (4H, overlapped), 1.29−1.01 (4H, overlapped), 1.19 (3H, d, J = 6.5 Hz), 1.17 (9H, s), 0.98 (3H, d, J = 6.7 Hz), 0.92 (3H, d, J = 6.6 Hz), 0.67 (3H, d, J = 6.5 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz): δ 201.14, 180.20, 179.05, 173.61, 154.23, 145.14, 134.51, 133.15, 127.34, 96.80, 89.93, 88.43, 79.27, 79.17, 77.85, 59.62, 57.48, 47.16, 44.00, 39.28, 38.86, 37.46, 35.55, 35.39, 34.20, 32.94, 32.12, 31.64, 31.22, 29.84, 27.25, 27.19, 25.82, 19.78, 18.15, 10.13, 8.85; ESI-MS m/z 688.4422 $[M + NH_4]^+$ (calcd. for $C_{39}H_{62}NO_9$, m/z 688.4425 $[M + NH_4]^+$).

Antimicrobial Activity. The antimicrobial activity against *S. aureus* ATCC43300 was determined using the paper-disc method (6 mm discs from Advantec Co., Ltd., Tokyo, Japan). Sterile filter discs impregnated with compound solutions were placed on agar plates, and the plates were incubated. After incubation, inhibitory zones were determined. Culture conditions were as follows: 3.8% Mueller Hinton II agar (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 1.0% inoculation, 37 °C, 16 h.

The MICs of **1**, its derivatives, vancomycin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan; broad-spectrum activity against drug-resistant Gram-positive bacteria) and linezolid (Pfizer, Inc., NY, USA; used as a treatment for VRE and MRSA) were determined against 27 bacterial strains employing the agar dilution method using Mueller Hinton agar (Nippon Becton Dickinson Company, Ltd., Tokyo, Japan) according to the Clinical and Laboratory Standards Institute manuals.[19]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00651.

^{1}H and ^{13}C NMR spectra of compounds **2**−**7** and HSQC, COSY, and HMBC spectra of compound; comparison of biosynthetic gene clusters of tetronomycin between *Streptomyces* sp. K20-0247 and *Streptomyces* sp. NRRL 11266; annotation of biosynthetic gene clusters of tetronomycin in *Streptomyces* sp. K20-0247; results of determination of Ca^{2+} ionophobic activities of

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1, 4, and EDTA and information on strains used in this study (PDF)

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Notes

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ABBREVIATIONS

AMR	antimicrobial resistance
COSY	correlated spectroscopy
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence spectroscopy
MIC	minimal inhibitory concentration
MRSA	methicillin-resistant *Staphylococcus aureus*
SAR	structure−activity relationship
TFA	trifluoroacetic acid
TLC	thin-layer chromatography
VISA	vancomycin-intermediate *Staphylococcus aureus*
VRE	vancomycin-resistant *Enterococci*

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