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SPECIAL FEATURE: ARTICLE

Synthesis and biological activity of analogs of CPZEN-45, a novel antituberculosis drug

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Abstract

Analogs of CPZEN-45, which is expected to be a promising new antituberculosis drug that overcomes the shortcomings of caprazamycins, were synthesized and their biological activities were evaluated. The biological activity of analogs **1**–**3**, which converted the anilide portion, and analogs **4** and **5**, focusing on the seven-membered ring, were lower than that of CPZEN-45. These results suggest that the inhibitory activity of CPZEN-45 against TagO, an ortholog of WecA, has a strict structural limitation, and it was hoped for elucidation of the mode of action of CPZEN-45 using structural biology in the future.

Introduction

In reports from the World Health Organization, tuberculosis (TB) is listed as one of the ten leading causes of death worldwide in 2016 [1]. There are an estimated 10.4 million new incidents of TB annually, and the number of deaths is around 1.8 million. TB is one of the deadliest diseases caused by a single infectious agent, and is the single largest cause of death for individuals infected with human immunodeficiency virus. The number of new multidrug-resistant (MDR)-TB patients in 2016 was estimated to be 490,000, of whom about 6.2% are infected with extensively drug-resistant (XDR)-TB, against which existing anti-TB drugs are ineffective [1]. There is a clear and urgent need to develop new drugs for the treatment of this disease, in order to reduce the worldwide spread of TB infections.

To address this problem, we screened for novel antimycobacterial substances having an effective spectrum of activity with a new mode of action, from microbial products. As a part of this program, we discovered a structurally analogous mixture of caprazamycins (CPZs), a group of novel lipo-nucleoside antibiotics, in a culture broth of Actinomycete strain *Streptomyces* sp. MK 730-62F2 [2, 3]. Its major component, caprazamycin B (CPZ-B), showed excellent antimycobacterial activity in vitro against drug-susceptible and multidrug-resistant *Mycobacterium tuberculosis* strains, although the compounds do have some shortcomings, including its onerous separation from a complex mixture by using HPLC and its extremely poor water-solubility profile [4].

In the process of structural analysis of CPZs, two compounds with the core structure, caprazene and caprazol, were obtained very efficiently from the mixture, as shown in Scheme 1 [3]. By using advanced medicinal chemistry with core compounds, we aimed to create new derivatives that overcome the disadvantages of CPZs. As a result, CPZEN-45, which is expected to be a promising new anti-TB drug, was discovered [4].

CPZEN-45, a derivative of caprazene, showed excellent antibacterial activity against *M. tuberculosis* and MDR-TB strains, and showed excellent therapeutic efficacy in a murine TB model infected with an XDR-TB strain resistant to ten drugs [5]. Currently, CPZEN-45 is under development as an inhaled MDR/XDR-TB drug [6, 7].

In previous research, we showed that CPZEN-45 exhibits selective anti-TB activity by specifically inhibiting the

This article is dedicated to Dr. Kiyoshi Isono's 88 years anniversary, and his long-standing contribution to the study of antibiotics.

Supplementary information The online version of this article (https://doi.org/10.1038/s41429-019-0225-5) contains supplementary material, which is available to authorized users.

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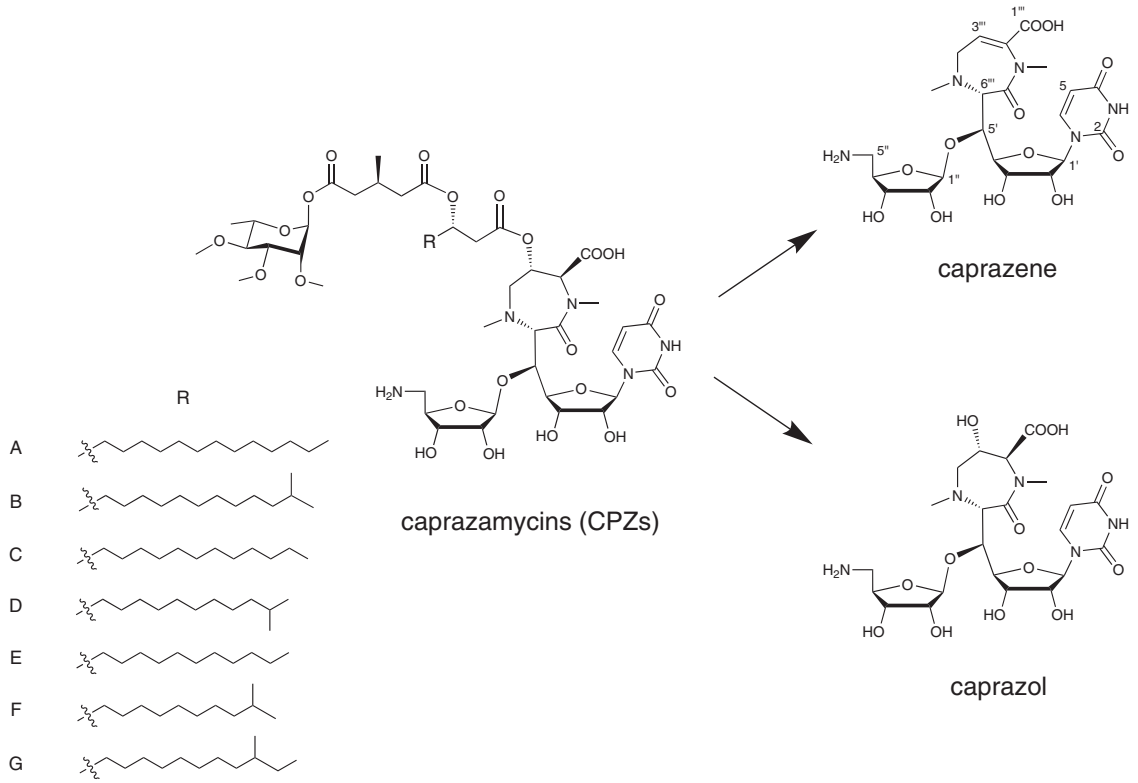


Scheme 1 Preparation of caprazene and caprazol from a mixture of caprazamycins A−G

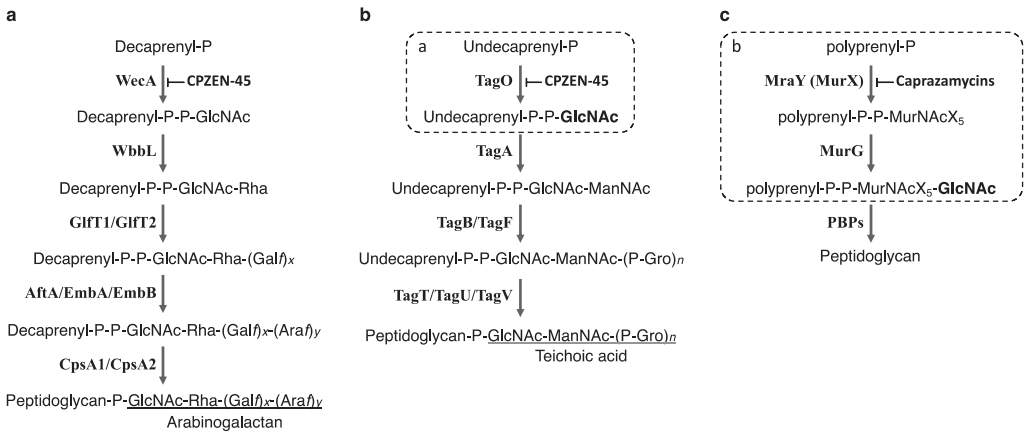


Fig. 1 Biosynthetic pathway of arabinogalactan in *M. tuberculosis* (**a**), teichoic acid in *B. subtilis* 168 (**b**), and bacterial peptidoglycan (**c**). Reactions 'a' and 'b' surrounded by dashed lines indicate the reactions observed in this report. P phosphate, GlcNAc *N*-acetyl-D-glucosamine, Rha rhamnose, Gal*f* galactofuranoside, Ara*f* arabinofuranoside, ManNAc *N*-acetyl-D-mannosamine, Gro glycerol, $MurNAcX_5$ *N*-acetyl-D-muramate pentapeptide, PBPs penicillin binding proteins. Since radiolabeled UDP-GlcNAc was used as the substrate for the enzyme assays in this report, MarY activity was evaluated with the subsequent reaction by MurG

phospho-*N*-acetyl glucosaminyl transferase, WecA [8] involved in arabinogalactan biosynthesis (Fig. 1) [9]. This mechanism is different from that of the parent compounds, CPZs, which acts on bacterial translocase I (also known as MraY in various bacteria, or MurX in *M. tuberculosis*), a paralog of WecA, which is involved in peptidoglycan biosynthesis (Fig. 1) [10]. WecA is a novel target as a drug for tuberculosis. It is very interesting that CPZEN-45, the 1‴-anilide derivative of caprazene, acquired WecA inhibitory activity, although the starting material, caprazene, has no enzyme inhibitory activity.

Because of its attractive biological activity and interest in the synthetic chemistry, total synthesis of CPZEN-45 using pure chemical methods has been reported [11, 12], but there

has not yet been any reports on the structure−activity relationships (SAR) of CPZEN-45. Herein, we describe the SAR of five analogs, **1**–**5**, of CPZEN-45 (Fig. 2), which were prepared by focusing on the seven-membered ring and an anilide moiety of CPZEN-45.

Results

Synthesis

As outlined in Scheme 2, compounds **2** and **3** were prepared according to the synthetic method [4] of CPZEN-45. The condensation reaction of *N*-Boc caprazene with 4-propylaniline or 2-butylaniline was effectively attained using 4-(4, 6-dimethoxy-1, 3, 5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) to produce amides **6** and **8**. Subsequent removal of the protecting group (Boc) from the primary amino group by trifluoroacetic acid efficiently produced analogs **2** and **3** of CPZEN-45.

On the other hand, treatment of 5″-*N*-Boc CPZEN-45 (**7**) [4] with sodium borohydride induced the reduction of the double bond on the seven-membered diazepinone ring, resulting in saturated compound **9** stereoselectively, with high yield. The *S* configuration at C-2‴ position of **9** was determined using X-ray crystallography (see Fig. S1 in Supplemental). The expression of this stereoselectivity is presumed to be due to the steric influence of uracil and *N*-protected 5-amino-5-deoxy-D-ribose moiety located in the beta face. Similar deprotection of the amino group as described above proceeded smoothly to produce **4**.

Compound **5** was prepared using caprazol, which is one of the core components obtained from a mixture of caprazamycins. Compound **5** corresponds to the 3‴-hydroxyl derivative of **4**. With respect to the seven-membered ring moiety, **5** has a structure closer to the parent natural product caprazamycins than CPZEN-45. As shown in Scheme 3, **5** was readily synthesized via 5″-*N*-*t*-butoxycarbonylation of caprazol, introduction of 4-butylaniline through an amide-linkage, and deprotection of the amino group.

Effects of the analogs of CPZEN-45 on enzyme activity

To investigate the SAR of CPZEN-45 analogs, we used an enzyme assay system we previously established, which uses

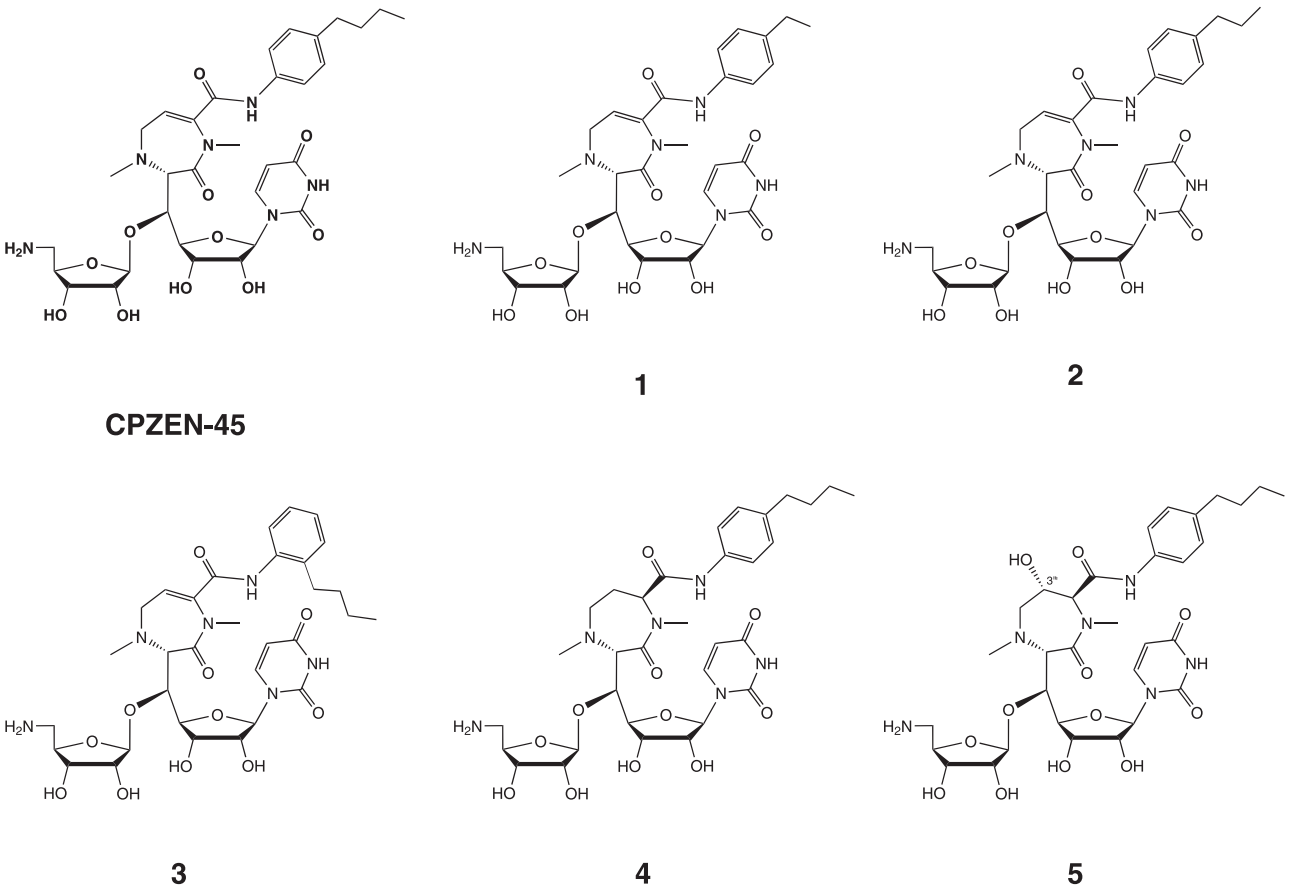
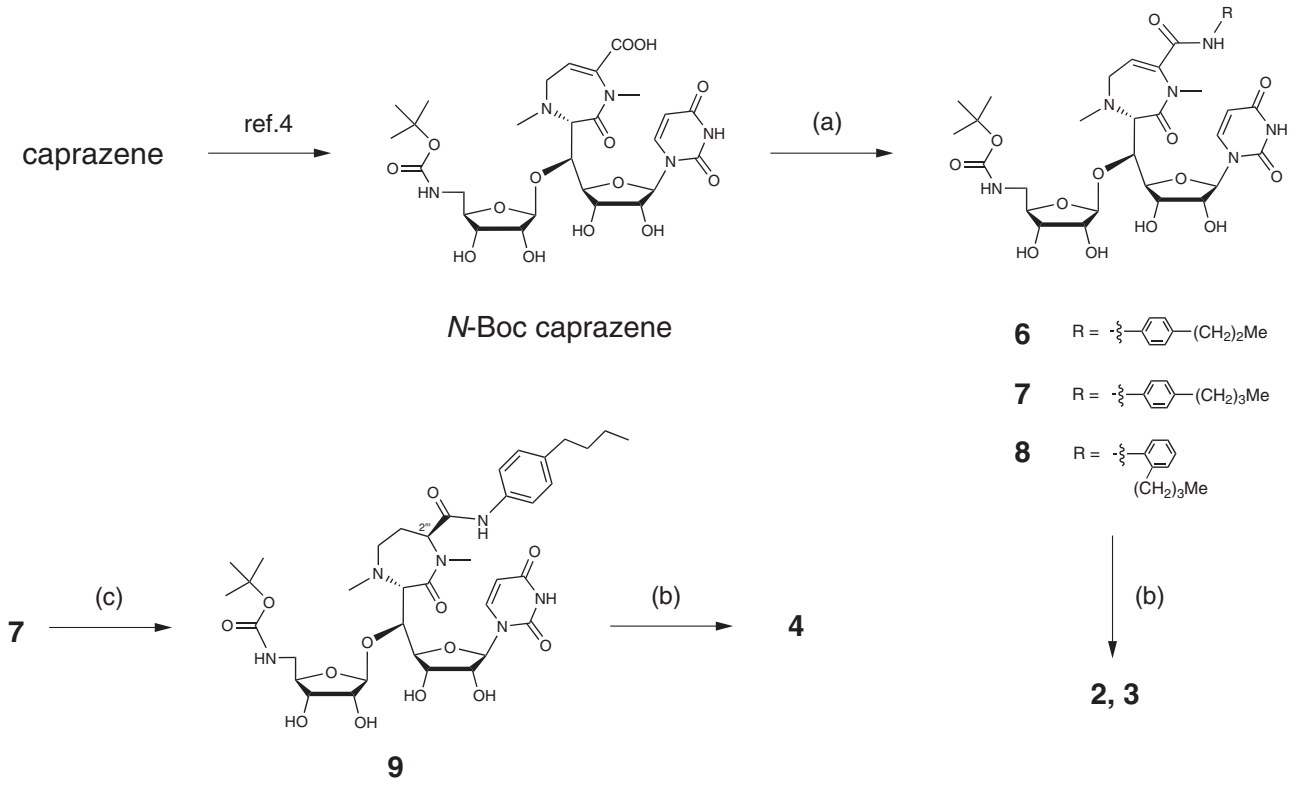
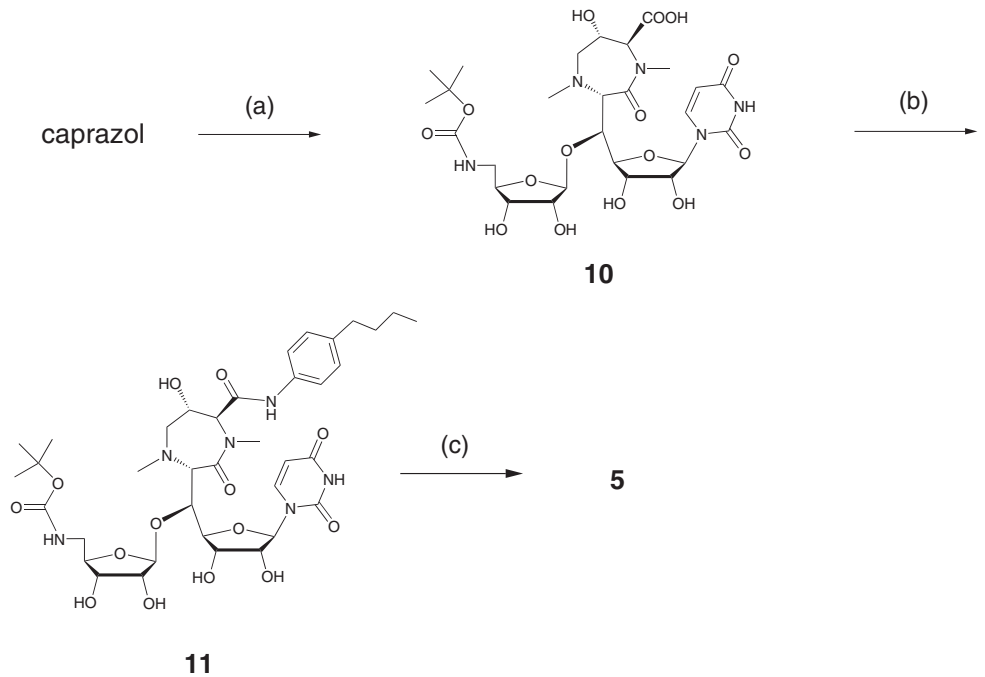


Fig. 2 Structures of CPZEN-45 and its analogs



Scheme 2 Synthesis of **2**–**4** from caprazene. Reagents and conditions: **a** RNH_2, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), 95% aqueous 2-propanol, room temperature; **b** CF_3COOH, MeOH, room temperature; **c** $NaBH_4$, MeOH, room temperature



Scheme 3 Synthesis of **5** from caprazol. Reagents and conditions: **a** Boc_2O, Et_3N, aqueous 1,4-dioxane, room temperature; **b** 4-butylaniline, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), 95% aqueous 2-propanol, room temperature; **c** CF_3COOH, MeOH, room temperature

cell lysates of *Bacillus subtilis* [9]. In addition to MraY, which is widely distributed among bacteria, *B. subtilis* also has a WecA ortholog, TagO, as the enzyme that catalyzes the first step of the biosynthesis of teichoic acid (Fig. 1) [13].

The effects of the analogs of CPZEN-45 on the activity of TagO were evaluated using sonicated cell lysate of BKKZ2202 as the enzyme. The IC_{50} value of CPZEN-45 against TagO was 55 nM, the strongest activity among the compounds tested. Compounds **1** and **2** were also effective, but their activity was inferior to that of CPZEN-45. Compound **3** showed moderate inhibition, but **4** and **5** were not effective (Fig. 3 and Table 1). No compounds tested inhibited MraY or MurG effectively, whereas caprazamycin B inhibits MraY and/or MurG at an IC_{50} of less than 500 nM. One thing to be noted is that MraY activity was evaluated by the subsequent reactions with MurG [14, 15] (see Materials and Methods and Fig. 1c), because radiolabeled UDP-GlcNAc was used as the substrate of this enzyme assay.

Effects of the analogs of CPZEN-45 on the viability of *B. subtilis* 168-derived strains

To evaluate the contribution of *tagO* and *mraY* to the resistance of the analogs of CPZEN-45, the MIC of these compounds against *B. subtilis* strains were determined (Table 1). CPZEN-45 showed the highest antibacterial activity among all of the compounds tested against the control strain BKZ2201, with an MIC of 8 μg/mL. The activities of the analogs of CPZEN-45 were 2−32 times lower than CPZEN-45. The *tagO*-overexpressing strain BKKZ2202 was at least four times as resistant, compared with BKKZ2201, as any of the analogs tested, except compound **5**. All of the analogs showed similar antibacterial activity against the *mraY*-overexpressing strain BKKZ2203 and BKKZ2392 strain which overexpresses *mraY* and *murG*, as BKKZ2201. The MICs of caprazamycin B against BKKZ2203 and BKKZ2392 were quite different from those against BKKZ2201.

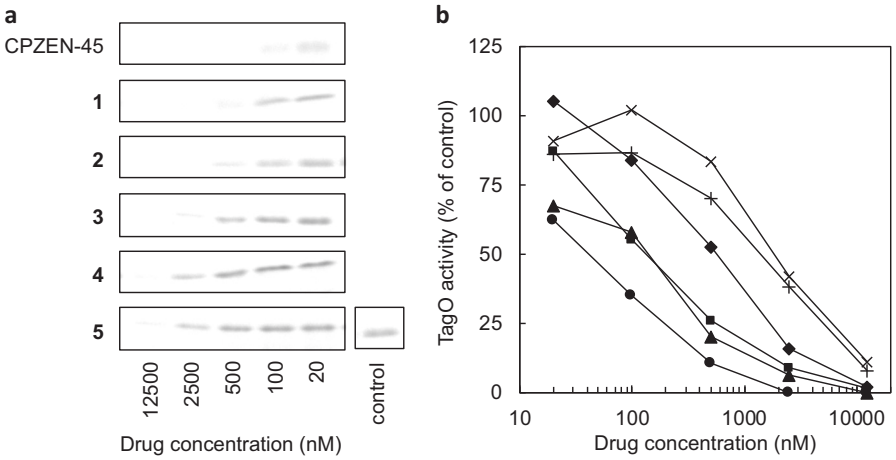


Fig. 3 Effect of the analogs of CPZEN-45 on the TagO activity of cell lysates of *B. subtilis* BKKZ2202 strain. **a** Autoradiographic image of TLC, developed and subjected to radioautography as described in Materials and methods, showing the effects of the analogs of CPZEN-45 on the synthesis of the product of TagO. **b** TagO activities calculated from the autoradiographic intensity of the TagO products shown in (**a**). Circle, CPZEN-45; triangle, **1**; square, **2**; diamond, **3**; cross, **4**; plus, **5**

Table 1 Antibacterial activities against *B. subtilis* strains and enzyme inhibition activities of CPZEN-45 and its analogs

	CPZEN-45	1	2	3	4	5	CPZ-B
MIC (μg/mL)							
BKKZ2201 (control)	8	32	16	32	256	128	4
BKKZ2202 (*tagO*)	64	256	128	256	>512	256	4
BKKZ2203 (*mraY*)	8	32	16	32	128	128	>128
BKKZ2392 (*mraY*, *murG*)	8	64	32	64	128	128	>128
IC_{50} (nM)							
TagO	55	190	180	630	2,100	1,700	not tested
MraY+MurG	7900	>12,500	>12,500	>12,500	>12,500	>12,500	<500

CPZ-B represents caprazamycin B

Table 2 Antibacterial activities of CPZEN-45 and its analogs against *Mycobacterium* strains

Test organisms	MIC (μg/mL)					
	CPZEN-45	**1**	**2**	**3**	**4**	**5**
Slowly growing mycobacteria						
M. avium subsp. *avium* ATCC 25291^T	0.5	16	8	64	>128	>128
M. avium subsp. *paratuberculosis* ATCC 43015	0.25	8	0.5	1	8	16
M. intracellulare JCM 6384^T	0.125	1	0.5	1	4	16
M. bovis BCG Pasteur	2	4	8	8	32	128
M. bovis BCG Tokyo 172–2	1	4	4	8	128	>128
Rapidly growing mycobacteria						
M. smegmatis ATCC 607^T	32	128	64	64	>128	64
M. fortuitum B-0701	>128	>128	>128	>128	>128	>128
M. vaccae ATCC 15483^T	32	128	64	64	128	64

Effects of the analogs of CPZEN-45 on the viability of mycobacterial strains

The antimycobacterial activity of CPZEN-45 and its analogs is shown is Table 2. In slowly growing mycobacteria, **1**, **2** and **3** showed similar or weaker activity at MIC values of 1−16 times higher, compared with CPZEN-45. **4** and **5** were not effective at an MIC of ≥4 μg/mL. In rapidly growing bacteria, all of the compounds tested were hardly effective, although CPZEN-45 had better activity than the other analogs.

Discussion

Although caprazene was inactive, antibacterial activity was restored for its 1‴-anilide derivatives, as exemplified by CPZEN-45, which contains a 4-butylaniline. It has previously been shown that, in the process of discovery of CPZEN-45, increasing the alkyl chain at the para-position of aniline slightly improves the antimicrobial activity, but at the same time enhances the hemolytic side effects [4]. Therefore, analogs **1** and **2** of CPZEN-45 with a shortened alkyl chain were synthesized, and the effect of the small structural change to CPZEN-45 on the antimycobacterial activity and inhibition of TagO activity was evaluated in sonicated cell lysates of *B. subtilis*. As the result, it is revealed that as the carbon chain become shorter, from butyl to propyl to ethyl, inhibitory activity against TagO and antimycobacterial activity decreased.

Because 4-butylanilide was shown to have more effective inhibitory activity, we next tested the effect of the position of the butyl group of the anilide moiety using data from CPZEN-45 and **3**. The inhibitory activity of **3** against TagO and its antibacterial activity against *Mycobacterium* strains were significantly reduced compared to that of CPZEN-45, indicating that the para substitution of the butyl group is superior to the ortho substitution.

We also checked the importance of the double bond on the seven-membered ring. Compounds **4** and **5**, with saturated ring systems whose stereochemistry is the same as that of caprazamycins, were prepared for the investigation of MraY and TagO inhibitory activity. The inhibitory activities of these compounds against TagO were notably lower than that of CPZEN-45, suggesting that the double bond on the seven-membered ring system is structurally important for binding to TagO. This observation also suggests that the synthesis of inhibitors of TagO or WecA utilizing caprazol, one of the core compounds of CPZs, is less promising than anticipated.

With respect to MraY/MurG subsequent reactions, none of the analogs tested inhibited these reactions effectively, whereas caprazamycin B, the parent compound of CPZEN-45, showed inhibitory effects against the subsequent reactions. Data from the enzyme assay described above correspond reasonably well with antibacterial activity against *B. subtilis* strains.

These results indicate that the presence of double bond at C-2‴ and C-3‴ on the seven-membered ring is crucial for inhibitory activity against TagO, and the position and chain length of the alkyl group attached to the anilide moiety is also important. This observation suggests that there is a strict structural limitation on the degree of inhibitory activity of CPZEN-45 for TagO.

This finding appeared to be mostly applicable to WecA, the ortholog of TagO, of slowly growing mycobacteria, according to data on the antibacterial activity of the analogs of CPZEN-45. In rapidly growing mycobacteria the analogs of CPZEN-45 were hardly effective. This lack may be because these compounds had lower affinity to WecA, or to lower permeability of the cell membrane, or because they were well recognized by an efflux pump in rapidly growing mycobacteria. Even though CPZEN-45 was not so effective, it showed better inhibitory activity against *M. smegmatis* and *M. vaccae* than the other analogs, supporting the superiority of this compound.

Although these observations should also be discussed from the viewpoint of structural biology, the crystal structure of TagO/WecA orthologs has not been reported in any species to date. Even for protein expression and purification, there is only one report using the thermophilic bacterium *Thermotoga maritima* [16]. We are now trying to overexpress WecA of *M. tuberculosis* using *E. coli* and *M. smegmatis*, in order to perform subsequent structural analysis. If this trial successful, we will be able to confirm the findings in this report, and elucidate the mode of mechanism of CPZEN-45 against bacterial WecA, using the structural information.

Materials and methods

General information

Melting point was determined on a Kofler block and is uncorrected. NMR spectra were recorded on an AVANCE III 600 spectrometer (^{1}H at 600 and ^{13}C at 150.9 MHz) (Bruker, Billerica, MA, USA) at 300 K, unless stated otherwise. The chemical shifts (δ) of ^{1}H and ^{13}C spectra were measured downfield from internal Me_4Si, and were confirmed, when necessary, by shift-correlated 2D spectra. The mass spectra were recorded using a Thermo Scientific LTQ Orbitrap XL mass spectrometer (HRESI-MS) or Thermo Scientific LTQ XL (ESI) spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Thin layer chromatography (TLC) was performed on a Kieselgel 60 F_{254} (Merck, Darmstadt, Germany), and column chromatography was carried out on a Wakogel C-200 (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Elemental analysis was performed using a Perkin Elmer PE 2400 II analyzer (PerkinElmer Inc., Waltham, MA, USA). Optical rotations were measured on a P-1030 polarimeter (JASCO Corporation, Tokyo, Japan).

Preparation of caprazene-1‴-anilides

5″-*N*-*t*-Butoxycarbonylcaprazene 4-propylanilide (6)

To a solution of *N*-Boc caprazene [4] (an addition salt of triethylamine, 602 mg, 0.793 mmol) in 2-propanol−water (19:1, 12 mL) were added 4-propylaniline (323 mg, 2.39 mmol) and 4-(4, 6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (329 mg, 1.19 mmol) and the mixture was stirred for 3 h at room temperature. Concentration gave a residue, which was dissolved in 5% aq potassium hydrogen sulfate (35 mL). The aqueous solution was washed with EtOAc and neutralized with sodium hydrogen carbonate (1.3 g). The precipitate obtained was extracted with EtOAc. The organic solution was washed with saturated aq sodium chloride, dried (Na_2SO_4), and concentrated. The residue was purified by silica gel column chromatography (7:1 $CHCl_3$−MeOH) to give **6** (516 mg, 84%) as a colorless solid. ESI-MS: *m/z* calcd for $C_{36}H_{50}N_6NaO_{13}$ $(M+Na)^+$ 797.3, found 797.3; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.87 [3H, t, $J = 7$ Hz, *CH*$_3$ $(CH_2)_2C_6H_4NH$], 1.31 [9 H, s, $(CH_3)_3C-O$], 1.56 (2H, m, $CH_3CH_2CH_2-$), 2.35 (3H, s, CH_3N-5‴), 2.94 (3H, s, CH_3N-8‴), 5.02 (1H, s, H-1″), 5.59 (1H, d, $J = 8$ Hz, H-5), 5.63 (1H, slightly br d, J = ~2 Hz, H-1′), 6.39 (1H, t, J = 6 Hz, H-3‴), 7.13 and 7.52 [each 2H, d, $J = 8.5$ Hz, CH_3 $(CH_2)_2C_6H_4NH$], 7.30 (1H, br s, HN-5″), 7.79 (1H, d, J = 8 Hz, H-6), 10.14 (1H, s, HN-1‴), 11.31 (1H, s, HN-3).

5″-*N*-*t*-Butoxycarbonylcaprazene 2-butylanilide (8)

To a solution of *N*-Boc caprazene (an addition salt of triethylamine, 600 mg, 0.791 mmol) in 2-propanol−water (19:1, 12 mL) were added 2-butylaniline (594 mg, 3.98 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (330 mg, 1.19 mmol) and the mixture was stirred for 2 h at room temperature. Concentration gave a residue, which was dissolved in 5% aq potassium hydrogen sulfate (35 mL). The aqueous solution was washed with EtOAc and neutralized with sodium hydrogen carbonate (1.3 g). The precipitate obtained was extracted with EtOAc. The organic solution was washed with saturated aq sodium chloride, dried (Na_2SO_4), and concentrated. The residue was purified by silica gel column chromatography (7:1 $CHCl_3$−MeOH) to give **8** (359 mg, 58%) as a colorless solid. ESI-MS: *m/z* calcd for $C_{37}H_{52}N_6NaO_{13}$ $(M+Na)^+$ 811.3, found 811.3; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.80 [3H, t, $J = 7$ Hz, *CH*$_3$ $(CH_2)_3C_6H_4NH$], 1.19 (2H, m, $CH_3CH_2CH_2CH_2-$), 1.36 [9H, s, $(CH_3)_3C$–O], 1.41 (2H, m, $CH_3CH_2CH_2CH_2-$), 2.36 (3H, s, CH_3N-5‴), 3.00 (3H, s, CH_3N-8‴), 5.03 (1H, s, H-1″), 5.60 (1H, d, $J = 8$ Hz, H-5), 5.61 (1H, slightly br d, J = ~2 Hz, H-1′), 6.47 (1H, t, $J = 6$ Hz, H-3‴), 7.37 (1H, br s, HN-5″), 7.82 (1H, d, $J = 8$ Hz, H-6), 9.72 (1H, s, HN-1‴), 11.33 (1H, s, HN-3).

Caprazene 4-propylanilide (2)

A solution of **6** (196 mg, 0.253 mmol) in trifluoroacetic acid −methanol (4:1, 6 mL) was kept for 2 h at room temperature. The reaction solution was concentrated, and to the resulting syrup was added diethyl ether. The precipitate obtained was thoroughly washed with diethyl ether to give **2** bis-trifluoroacetate (218 mg, 94%) as a colorless solid, $[\alpha]_D^{20} + 83°$ (*c* 1, MeOH); HRESI-MS: *m/z* calcd for $C_{31}H_{43}N_6O_{11}$ $(M + H)^+$ 675.2984, found 675.2987; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.87 (3H, t, $J = 7$ Hz, *CH*$_3$$CH_2CH_2-$), 1.56 (2H, m, $CH_3CH_2CH_2-$), 2.39 (3H, br

s, CH_3N-5‴), 2.96 (3H, s, CH_3N-8‴), 5.12 (1H, slightly br s, H-1″), 5.59 (1H, d, $J = 2$ Hz, H-1′), 5.61 (1H, slightly br d, $J = 8$ Hz, H-5), 6.40 (1H, br s, H-3‴), 7.14 and 7.52 [each 2H, d, $J = 8.5$ Hz, $CH_3(CH_2)_2C_6H_4$NH], 7.67 (1H, slightly br d, $J = 8$ Hz, H-6), 8.11 (3H, br s, NH_3^+), 10.17 (1H, s, HN-1‴), 11.33 (1H, s, HN-3). Anal Calc for $C_{31}H_{42}N_6O_{11}$ $2CF_3COOH$ H_2O: C 45.66, H 5.04, N 9.13. Found: C 45.39, H 5.23, N 9.02.

Caprazene 2-butylanilide (3)

A solution of **8** (226 mg, 0.286 mmol) in trifluoroacetic acid −methanol (4:1, 6.8 mL) was kept for 2 h at room temperature. The reaction solution was concentrated, and to the resulting syrup was added diethyl ether. The precipitate obtained was thoroughly washed with diethyl ether to give **3** bis-trifluoroacetate (253 mg, 94%) as a colorless solid, $[\alpha]_D^{20} + 76°$ (*c* 1, MeOH); HRESI-MS: *m/z* calcd for $C_{32}H_{45}N_6O_{11}$ $(M + H)^+$ 689.3141, found 689.3146; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.81 (3H, t, $J = 7$ Hz, $CH_3CH_2CH_2CH_2-$), 1.20 (2H, m, $CH_3CH_2CH_2CH_2-$), 1.42 (2H, m, $CH_3CH_2CH_2CH_2-$), 2.39 (3H, br s, CH_3N-5‴), 3.03 (3H, s, CH_3N-8‴), 5.13 (1H, slightly br s, H-1″), 5.59 (1H, d, $J = 2$ Hz, H-1′), 5.66 (1H, slightly br d, $J = 7$ Hz, H-5), 6.47 (1H, br s, H-3‴), 7.70 (1H, slightly br d, $J = 7$ Hz, H-6), 8.14 (3H, br s, NH_3^+), 9.72 (1H, slightly br s, HN-1‴), 11.35 (1H, s, HN-3). Anal Calc for $C_{32}H_{44}N_6O_{11}$ $2CF_3COOH$ H_2O: C 46.26, H 5.18, N 8.99. Found: C 45.95, H 5.56, N 8.99.

5″-*N*-*t*-Butoxycarbonyl-3‴-deoxycaprazol 4-butylanilide (9)

To an ice-cooled solution of **7** (986 mg, 1.25 mmol) in methanol (20 mL) was added sodium borohydride (711 mg, 18.7 mmol). After the mixture was stirred for 3 h at room temperature, the reaction was quenched by the addition of dry-ice. Concentration gave a residue, which was extracted with 1-butanol. The organic solution was washed with water and concentrated. The resulting solid was purified by column chromatography (upper layer of 5:1:1 EtOAc−1-BuOH −20% aq AcOH) to give **9** (831 mg, 81%) as a colorless solid. An analytical sample was prepared by crystallization from methanol−water, m.p. 163~164 °C; $[\alpha]_D^{20} - 9°$ (*c* 1, MeOH); ESI-MS: *m/z* calcd for $C_{37}H_{54}N_6NaO_{13}$ $(M+Na)^+$ 813.4, found 813.4; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.88 (3H, t, $J = 7$ Hz, $CH_3CH_2CH_2CH_2-$), 1.27 (2H, m, $CH_3CH_2CH_2CH_2-$), 1.34 [9H, s, $(CH_3)_3$C-O], 1.47 (2H, m, $CH_3CH_2CH_2CH_2-$), 1.95−2.07 (2H, m, H-3‴), 2.12 (3H, s, CH_3N-5‴), 2.44 (2H, m, $CH_3CH_2CH_2CH_2-$), 2.88 (1H, slightly br d, $J = 14.5$ Hz, H-4‴a), 3.00 (1H, slightly br d, $J = 14$ Hz, H-5″a), 3.06 (3H, s, CH_3N-8‴), ~3.35 (1H, H-5″b), 3.40 (1H, slightly br t, $J = 12$ Hz, H-4‴b), 3.71 (1H, d, $J = 10$ Hz, H-6‴), 3.73−3.77 (2H, H-2′ and H-2″), 3.78 −3.84 (2H, H-3′ and H-3″), 3.88 (1H, br, H-4″), 4.07 (1H, d, $J = 10$ Hz, H-5′), 4.16 (1H, d, $J = 8$ Hz, H-4′), 4.33 (1H, t, $J = 4$ Hz, H-2‴), 4.91 (1H, d, $J = 5.5$ Hz, HO-2″), 4.92 (1H, d, $J = 6$ Hz, HO-3″), 4.96 (1H, s, H-1″), 5.03 (1H, s, H-1′), 5.07 (1H, d, $J = 6$ Hz, HO-3′), 5.38 (1H, d, $J = 8$ Hz, H-5), 5.43 (1H, d, $J = 4$ Hz, HO-2′), 6.87 and 7.36 [each 2H, d, $J = 8.5$ Hz, $CH_3(CH_2)_3C_6H_4$NH], 7.02 (1H, slightly br d, $J = 8.5$ Hz, HN-5″), 7.61 (1H, d, $J = 8$ Hz, H-6), 9.39 (1H, s, HN-1‴), 11.16 (1H, s, HN-3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 13.7 ($CH_3CH_2CH_2CH_2-$), 21.3 (C-3‴), 21.6 ($CH_3CH_2CH_2CH_2-$), 28.1 [$(CH_3)_3C-O$], 32.8 ($CH_3CH_2CH_2CH_2-$), 33.6 (CH_3N-5‴), 34.1 ($CH_3CH_2CH_2CH_2-$), 36.6 (CH_3N-8‴), 41.8 (C-5″), 53.5 (C-4‴), 61.6 (C-2‴), 63.7 (C-6‴), 68.7 (C-3′), 71.1 (C-3″), 74.2 (C-5′), 74.6 (C-2′), 75.4 (C-2″), 77.5 [$(CH_3)_3C$-O], 82.1 (C-4′), 83.1 (C-4″), 89.0 (C-1′), 100.1 (C-5), 109.9 (C-1″), 120.5, 127.3, 135.8, and 137.4 (aromatic), 139.1 (C-6), 149.6 (C-2), 155.8 (COHN-5″), 163.1 (C-4), 167.4 (C-1‴), 171.0 (C-7‴). Anal Calc for $C_{37}H_{54}N_6O_{13}$ $1.5H_2O$: C 54.34, H 7.02, N 10.28. Found: C 54.22, H 7.23, N 10.19.

3‴-Deoxycaprazol 4-butylanilide (4)

A solution of **9** (223 mg, 0.273 mmol) in trifluoroacetic acid −methanol (4:1, 6.7 mL) was kept for 2 h at room temperature. The reaction solution was concentrated, and to the resulting syrup was added diethyl ether. The precipitate obtained was thoroughly washed with diethyl ether to give **4** bis-trifluoroacetate (250 mg, 98%) as a colorless solid, $[\alpha]_D^{20} + 22°$ (*c* 1, MeOH); HRESI-MS: *m/z* calcd for $C_{32}H_{47}N_6O_{11}$ $(M + H)^+$ 691.3297, found 691.3313; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.88 (3H, t, $J = 7$ Hz, $CH_3CH_2CH_2CH_2-$), 1.27 (2H, m, $CH_3CH_2CH_2CH_2-$), 1.47 (2H, m, $CH_3CH_2CH_2CH_2-$), 2.14 (3H, slightly br s, CH_3N-5‴), 2.44 (2H, m, $CH_3CH_2CH_2CH_2-$), 3.08 (3H, s, CH_3N-8‴), 4.40 (1H, s, H-2‴), 4.93 (1H, s, H-1′), 4.99 (1H, s, H-1″), 5.46 (1H, slightly br d, $J = 7.5$ Hz, H-5), 7.45 (1H, slightly br d, $J = 7.5$ Hz, H-6), 8.03 (3H, br s, NH_3^+), 9.47 (1H, s, HN-1‴), 11.18 (1H, s, HN-3). Anal Calc for $C_{32}H_{46}N_6O_{11}$ $2CF_3COOH$ H_2O: C 46.16, H 5.38, N 8.97. Found: C 46.29, H 5.49, N 8.90.

Preparation of caprazol-1‴-anilide

5″-*N*-*t*-Butoxycarbonylcaprazol (10)

To a suspension of caprazol [4] (tetrahydrate, 346 mg, 0.534 mmol) in water−1, 4-dioxane (2:1, 10 mL) were added triethylamine (145 mg, 1.43 mmol) and di-*t*-butyl dicarbonate (167 mg, 0.765 mmol) and the mixture was stirred for 1 h at room temperature. To the resulting solution was added 28% aq ammonia (0.1 mL) and the solution was concentrated to give a colorless solid of **10** (421 mg, 97%)

as an addition salt of triethylamine. This solid was used in the next reaction without further purification.

Compound **10** as an addition salt of triethylamine; ESI-MS m/z calcd for $C_{27}H_{41}N_5NaO_{15}$ $(M+Na)^+$ 698.3, found 698.3; ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.01 [9H, t, J = 7 Hz, $(CH_3CH_2)_3N$], 1.34 [9H, s, $(CH_3)_3C$–O], 2.31 (3H, s, CH_3N-5‴), 2.89 (1H, d, J = 15 Hz, H-4‴a), 2.98 (3H, s, CH_3N-8‴), 3.25 (1H, d, J = 15 Hz, H-4‴b), 3.66 (1H, d, J = 10 Hz, H-6‴), 4.94 (1H, s, H-1″), 5.55 (1H, d, J = 8 Hz, H-5), 5.59 (1H, d, J = 2.5 Hz, H-1′), 7.02 (1H, d, J = 8.5 Hz, HN-5″), 7.92 (1H, d, J = 8 Hz, H-6), 11.32 (1H, br s, HN-3). Anal Calc for $C_{27}H_{41}N_5O_{15}$ Et_3N $2H_2O$: C 48.76, H 7.44, N 10.34. Found: C 48.55, H 7.58, N 10.05.

5″-*N*-*t*-Butoxycarbonylcaprazol 4-butylanilide (11)

To a solution of **10** (an addition salt of triethylamine, 2.28 g, 2.80 mmol) in 2-propanol−water (10:1, 44 mL) were added 4-butylaniline (480 mg, 3.22 mmol) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (1.06 g, 3.83 mmol) and the mixture was stirred at room temperature. The same amounts of 4-butylaniline and DMT-MM were added after 5 and 15 h, and the mixture was further stirred for 2 days. Concentration gave a syrup, which was extracted with 5% aq potassium hydrogen sulfate (150 mL) and EtOAc (50 mL). The aqueous solution was washed with EtOAc and neutralized with sodium hydrogen carbonate (8.18 g). The precipitate obtained was extracted with EtOAc and the organic solution was washed with saturated aq sodium chloride, dried (Na_2SO_4), and concentrated. The residue was purified by silica gel column chromatography (6:1 $CHCl_3$−MeOH) to give **11** (1.03 g, 44%) as a colorless solid; $[\alpha]_D^{20}$ − 11° (*c* 0.5, MeOH); ESI-MS: *m/z* calcd for $C_{37}H_{54}N_6NaO_{14}$ $(M+Na)^+$ 829.4, found 829.3; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.88 [3H, t, J = 7 Hz, $CH_3(CH_2)_3C_6H_4NH$], 1.27 (2H, m, $CH_3CH_2CH_2CH_2$−), 1.37 [9H, s, $(CH_3)_3C$–O], 1.46 (2H, m, $CH_3CH_2CH_2CH_2$−), 2.37 (3H, s, CH_3N-5‴), 2.97 (1H, d, J = 15 Hz, H-4‴a), 3.13 (3H, s, CH_3N-8‴), 3.43 (1H, d, J = 15 Hz, H-4‴b), 3.65 (1H, d, J = 10 Hz, H-6‴), 4.08 (1H, d, J = 10 Hz, H-5′), 4.18 (1H, d, J = 8 Hz, H-4′), 4.37 (2H, s, H-2‴ and H-3‴), 4.87 (1H, s, H-1′), 4.94 (1H, s, H-1″), 5.35 (1H, d, J = 8 Hz, H-5), 6.85 and 7.35 [each 2H, d, J = 8.5 Hz, $CH_3(CH_2)_3C_6H_4NH$], 6.92 (1H, slightly br d, J = 8.5 Hz, HN-5″), 7.58 (1H, d, J = 8 Hz, H-6), 9.38 (1H, s, HN-1‴), 11.14 (1H, s, HN-3). Anal Calc for $C_{37}H_{54}N_6O_{14}$ $2H_2O$: C 52.72, H 6.94, N 9.97. Found: C 52.95, H 7.24, N 9.99.

Caprazol 4-butylanilide (5)

A solution of **11** (187 mg, 0.222 mmol) in trifluoroacetic acid−methanol (4:1, 5 mL) was kept for 2 h at room temperature. The reaction solution was concentrated, and to the resulting syrup was added diethyl ether. The precipitate obtained was thoroughly washed with diethyl ether to give **5** bis-trifluoroacetate (209 mg) as a colorless solid. The solid was dissolved in 1-BuOH and the solution was washed successively with 5% aq sodium hydrogen carbonate and water. Concentration gave a solid, which was chromatographed (upper layer of 2:1:1 EtOAc−1-PrOH−H_2O) to give **5** trifluoroacetate (114 mg, 59%) as a colorless solid; $[\alpha]_D^{20}$ + 17° (*c* 0.5, MeOH); HRESI-MS: *m/z* calcd for $C_{32}H_{47}N_6O_{12}$ $(M+H)^+$ 707.3246, found 707.3249; ESI-MS: m/z 819.3 $(M+CF_3COOH-H)^-$; ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.88 (3H, t, J = 7 Hz, $CH_3CH_2CH_2CH_2$−), 1.26 (2H, m, $CH_3CH_2CH_2CH_2$−), 1.46 (2H, m, $CH_3CH_2CH_2CH_2$−), 2.38 (3H, s, CH_3N-5‴), 2.43 (2H, m, $CH_3CH_2CH_2CH_2$−), 3.00 (1H, slightly br dd, J = ~2 and 14 Hz, H-4‴a), 3.05 (1H, slightly br dd, J = ~2 and ~13 Hz, H-5″a), 3.14 (1H, slightly br d, J = ~13 Hz, H-5″b), 3.15 (3H, s, CH_3N-8‴), 3.22 (1H, slightly br d, J = 14 Hz, H-4‴b), 3.58 (1H, d, J = 10 Hz, H-6‴), 3.66 (1H, ddd, J = 4.5, 6.5 and 9 Hz, H-3′), 3.73 (1H, t, J = 4.5 Hz, H-2′), 3.76 (1H, t, J = 4 Hz, H-2″), 3.98−4.04 (2H, m, H-3″ and H-4″), 4.09 (1H, d, J = 10 Hz, H-5′), 4.19 (1H, d, J = 9 Hz, H-4′), 4.37 (1H, m, H-3‴), 4.42 (1H, d, J = 5 Hz, H-2‴), 4.83 (1H, s, H-1′), 4.96 (1H, s, H-1″), 5.15 (1H, d, J = 6.5 Hz, HO-3′), 5.22 (1H, d, J = 5.5 Hz, HO-3″), 5.24 (2H, d, J = 4 Hz, HO-2″ and HO-3‴), 5.44 (1H, d, J = 8 Hz, H-5), 5.52 (1H, d, J = 4 Hz, HO-2′), 6.85 and 7.38 [each 2H, d, J = 8.5 Hz, $CH_3(CH_2)_3C_6H_4NH$], 7.42 (1H, d, J = 8 Hz, H-6), 8.02 (3H, br s, NH_3^+), 9.48 (1H, s, HN-1‴), 11.16 (1H, s, HN-3); ^{13}C NMR (150.9 MHz, DMSO-d_6) δ 13.7 ($CH_3CH_2CH_2CH_2$−), 21.7 ($CH_3CH_2CH_2CH_2$−), 32.7 ($CH_3CH_2CH_2CH_2$−), 34.0 ($CH_3CH_2CH_2CH_2$−), 36.3 (CH_3N-5‴), 38.8 (CH_3N-8‴), 39.6 (C-5″), 58.2 (C-4‴), 63.0 (C-6‴), 66.5 (C-2‴), 67.3 (C-3‴), 68.4 (C-3′), 69.6 (C-3″), 74.2 (C-2″), 74.4 (C-2′), 74.8 (C-5′), 78.0 (C-4″), 81.4 (C-4′), 89.6 (C-1′), 99.9 (C-5), 109.6 (C-1″), 117.3 (CF_3, q, $J_{C,F}$ = 302 Hz), 120.4, 127.2, 135.6, and 137.6 (aromatic), 138.9 (C-6), 149.5 (C-2), 157.6 (CF_3COOH, q, $J_{C,F}$ = 30 Hz), 163.2 (C-4), 167.2 (C-1‴), 169.8 (C-7‴). Anal Calc for $C_{32}H_{46}N_6O_{12}$ CF_3COOH $3H_2O$: C 46.68, H 6.11, N 9.61. Found: C 46.56, H 6.07, N 9.39.

Construction of *B. subtilis* 168 derivatives overexpressing *tagO*, *mraY* and *murG*

The *Bacillus subtilis-Escherichia coli* shuttle vector pHYermA was constructed using pHY300PLK (Takara bio Inc. Shiga, Japan) [17], as a source of replication origins, and erythromycin-resistant *ermA* gene from a *Staphylococcus aureus* strain. Fragments of *tagO* and *mraY* genes of *B. subtilis* were cloned into pHYermA, and the resulting plasmids were designated as pHYermA-tagO and

Table 3 *Bacillus subtilis* strains and plasmids used in this study

Strains/plasmids	Description	
B. subtilis		
168	*trpC2*	ATCC
BKKZ2201	168/pHYermA	This work
BKKZ2202	168/pHYermA-tagO	This work
BKKZ2203	168/pHYermA-mraY	This work
BKKZ2392	168/pHYermA-mraYmurG	This work
Plasmids		
pHY300PLK	*B. subtilis-E. coli* shuttle vector, p15A and pAMα1 replicons, Amp^R, Tet^R	Takara Bio Inc.
pHYermA	p15A and pAMα1 replicons, EM^R	This work
pHYermA-tagO	pHYermA carrying a *tagO* fragment	This work
pHYermA-mraY	pHYermA carrying an *mraY* fragment	This work
pHYermA-mraYmurG	pHYermA carrying an *mraY-murG* fragment	This work

Amp^R ampicillin resistant, *Tet^R* tetracycline resistant, *EM^R* erythromycin resistant

pHYermA-mraY, respectively. For the construction of plasmid pHYermA-mraYmurG, a DNA fragment containing *mraY-murD-spoVE-murG* genes (part of an operon consisting of ten genes) was amplified by PCR, inserted into pHYermA, and the *murD-spoVE* region was cut out using the restriction enzyme *Hin*dIII. *E. coli* HST08 strain (Takara Bio Inc., Shiga, Japan) was used for plasmid construction. Transformation of *B. subtilis* 168 strains using the constructed plasmids was performed as previously described [9]. Erythromycin at concentrations of 500 and 5 μg/mL was used for the selection of transformants of *E. coli* and *B. subtilis*, respectively. The resulting strains (Table 3) were used for MIC evaluation of CPZEN-45 analogs by microbroth dilution in LB medium, Miller (Thermo Fisher Scientific Kabushiki Kaisha, Kanagawa, Japan). The strains were incubated for 16 h at 37 °C, and the MIC values were evaluated.

Evaluation of enzyme activity of MraY and TagO of *B. subtilis*

The enzyme activity of TagO was measured as previously described, with some modifications [9]. Log phase cell culture ($OD_{600} \approx 0.4$) of *B. subtilis* BKKZ2202 was collected, washed twice with ice-cold buffer A (50 mM MOPS-KOH (pH 7.9), 10% Glycerol, 5 mM $MgCl_2$, 5 mM 3-mercapto-1,2-propanediol) and resuspended in 1/40 volume of buffer A. The cell suspensions were probe-sonicated five times for 60 s each with 60 s breaks on ice, and centrifuged at 3000 rpm to remove unbroken cells. The supernatant, which was used as the enzyme source, was stored at −80 °C until use. For the evaluation of enzyme activity, the reaction mixture, contained 250 ng of total protein/μL, 125 μM undecaprenyl phosphate (Larodan AB, Solna, Sweden), 4 μCi/mL uridine diphosphate *N*-Acetyl-[1-^{14}C] D-Glucosamine (American Radiolabeled Chemicals Inc., St. Louis, MO), and 0.1% CHAPS in a total volume of 0.05 mL of buffer A, followed by incubation for 2 h at 30 °C. The reaction was stopped by adding 0.25 mL of chloroform/methanol (2:1), mixed vigorously, centrifuged (3000 rpm, 15 min), and the resulting lower phase containing the radiolabeled product was washed with 50 μL of water and subsequent vigorous mixing. The resulting mixture was centrifuged, and the lower phase was chromatographed on a silica gel Kieselgel 60 F_{254} (Merck) by developing with $CHCl_3$ /MeOH/H_2O, ≈14 M NH_4OH (56:42:6.8:2.7) and subsequent autoradiography (Typhoon 9400, GE Healthcare). The intensity of the autoradiographic image was quantified using ImageJ software (National Institutes of Health).

The enzyme activity of MraY was evaluated by the subsequential reactions with another transglycosylase MurG, which catalyzes the addition of *N*-acetyl-D-glucosamine to the MraY product (Fig. 1). In this reaction, the same procedure as used in the TagO reaction was applied, but cell lysates of the BKKZ2392 strain, instead of the BKKZ2203 strain, were used, and 100 μM UDP-MurNAc-pentapeptide (UK Bacterial Cell Wall Biosynthesis Network, University of Warwick, Coventry, UK) was added. In this experiment, caprazamycin B was used as a control.

Antimycobacterial activity

The MICs against bacteria of CPZEN-45 and its analogs (Table 2) were measured using a serial agar dilution method using Middlebrook 7H10 agar (Thermo Fisher Scientific Kabushiki Kaisha) with glycerol and OADC enrichment (Thermo Fisher Scientific Kabushiki Kaisha). MIC values were measured after 2−14 days at 37 °C.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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