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NOTE

# Precursor-directed biosynthesis of new sansanmycin analogs bearing *para*-substituted-phenylalanines with high yields

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Sansanmycins are members of uridyl-peptide antibiotics (UPAs) isolated from *Streptomyces* sp. SS (CPCC 200442) in our previous researches.[1–4] More than 10 related compounds have been identified, which share a common structure with pacidamycin,[5] mureidomycin[6] and napsamycin.[7] They are composed of a 3′-deoxyuridyl attached via an unusual exocyclic enamide to a penta- or tetra-pseudopeptide backbone assembled by nonribosomal peptide synthetases.[8] The uridyl-peptide antibiotics represent a family of promising leading compounds that they target a clinic unexplored target, MraY translocase, to inhibit the bacterial cell-wall synthesis and show good activity against highly refractory pathogens *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*.[9]

In our previous research, we have noted that the substrate specificity of sansanmycin biosynthesis pathway is markedly different from that of other classes of UPAs in the case of the C-terminus.[1] Although all classes of UPAs can select a hydroxylphenylalanine as the C-terminus, sansanmycin chooses 4-hydroxylphenylalanine while the others choose 3-hydroxylphenylalanine (Figure 1). We were therefore curious about whether this difference in substrate specificity could lead to new sansanmycin analogs by precursor-directed biosynthesis, a simple but effective method for generating modified natural products in the producing organism itself. So five phenylalanine derivatives (4-F–, 4-Cl–, 4-Br–, 4-Me– and 4-MeO–phenylalanines) were picked out and tested for their incorporation, which led to four new sansanmycin analogs. Here we report on their precursor-directed biosynthesis, isolation, structure elucidation and antibacterial activity.

*Streptomyces* sp. SS. was cultivated on an ISPII slant containing yeast extract 0.4%, glucose 0.4%, malt extract 1%, agar 1.5% at 28 °C for 7 days, then inoculated into the sansanmycin production medium, consisting of glucose 3%, starch 0.5%, peptone 0.6%, $(NH_4)_2SO_4$ 0.7%, and $CaCO_3$ 0.2%, and incubated at 28 °C, 180 r.p.m. for 48 h. The obtained preculture was further inoculated into the sansanmycin production medium supplementary with the selected phenylalanine derivatives at a final concentration of 3 mM and incubated for 5 days. The cultures were enriched by Sep-Pak $C_{18}$ Cartridges (WATERS, Milford, MA, USA) and then analyzed by liquid chromatography-mass/mass spectra for the presence of the expected products. Liquid chormatography-mass spectra was conducted on an Agilent 1200 instrument (Agilent Technologies, Santa Clara, CA, USA) coupled to a Finnigan LTQ XT ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) by using negative electrospray ionization and an XBridge $C_{18}$ column (Waters; 4.6 × 150 mm, 3.5 μm, solvent A: 0.1% (w/v) $(NH_4)_2CO_3$), solvent B: MeOH; flow rate, 1 ml $min^{-1}$; 0 – 40 min, 20 – 45% B (linear gradient), 40 – 50 min, 45% B). The results indicated that four precursors (4-Cl–, 4-Br–, 4-Me– and 4-MeO–phenylalanines) were incorporated into the C-terminus of the peptide backbone of sansanmycin to generate corresponding analogs at higher levels than sansanmycin H (Figure 2). Especially, the yield of [4-Me–Phe]-sansanmycin H was ~19 times more than that of sansanmycin H. Then, to access enough amount of material for further structure elucidation and bioassay, fermentations with these four precursors were scaled up to volumes ranging from 2 –5 l according to the yields of the corresponding products. The expected sansanmycin analogs were isolated and purified according to the reported method with minor modifications.[1] Briefly, the sansanmycin analogs were enriched from the fermentation broth by macroporous absorbent resin 4006 column. The obtained crude was subsequently applied on Toyopearl DEAE 650 M column and eluted with Tris-HCl (20 mM, pH 8.5) plus NaCl and monitored by ultra-violet to yield expected products. Finally, the obtained crude products were further purified using high performance liquid chromatography (HPLC) (YMC-Pack ODS-A 5 μm, 250 × 20 mm column, 0.1% (w/v) $(NH_4)_2CO_3$ – MeOH; flow rate, 5 ml $min^{-1}$).

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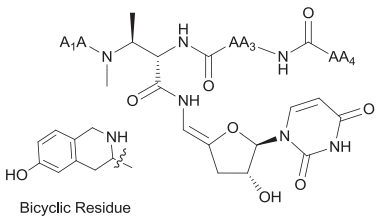
The yields of [4-R–Phe]-sansanmycin H (R = Cl, Br, Me, MeO) were 2.0, 1.9, 6.2 and 1.7 mg $l^{-1}$, respectively. Nuclear magnetic resonace (NMR) data of these purified compounds were obtained with Varian Mercury 600 spectrometers (Varian, Palo Alto, CA, USA) using $D_2O$ (pD = 8.0) or dimethylsulfoxide (DMSO) as solvent.

[4-Me–Phe]-sansanmycin H (**1**) is a white powder (melting point 204–206 °C) with a molecular weight of 838, two mass units smaller than that of sansanmycin H, which hinted at the replacement of the hydroxyl group in sansanmycin H with a methyl group in compound **1**. Furthermore, the daughter ion *m/z* 658, corresponding to the loss of C-terminal amino acid of sansanmycin H, also appeared as base peak in the electrospray ionization mass/mass spectrum of compound **1**, which indicated the 4-methylphenylalanine was incorporated into the C-terminus of **1**. Compared with the $^1$H NMR spectrum of sansanmycin H,[1] that of **1** showed an extra methyl proton signal at δ 2.29 (s, Ar − $CH_3$), which further confirmed the above hypothesis. Interpretation of the 2D NMR and ESIMS/MS data (Table 1 and Figure 2) also confirmed this expected structure.




| | AA1 | AA3 | AA4 |
|---|---|---|---|
| Sansanmycin A | *m*-Tyr | Met | Trp |
| Sansanmycin H | *m*-Tyr | Met | Tyr |
| Mureidomycins | *m*-Tyr | Met | *m*-Tyr |
| Pacidamycins | *m*-Tyr/Ala | Ala | Trp/Phe/*m*-Tyr |
| Napsamycins | Bicyclic Residue | Met | *m*-Tyr |
| **1** | *m*-Tyr | Met | 4-Me-Phe |
| **2** | *m*-Tyr | Met | 4-Cl-Phe |
| **3** | *m*-Tyr | Met | 4-Br-Phe |
| **4** | *m*-Tyr | Met | 4-MeO-Phe |

**Figure 1** Structures of uridyl-peptide antibiotics.

[4-Cl–Phe]- (**2**), [4-Br–Phe]- (**3**) and [4-MeO–Phe]-sansanmycin H (**4**) have expected molecular weights of 858, 902 and 854, respectively. Moreover, the ESIMS/MS of all three compounds showed a diagnostic daughter ion, *m/z* 658, for the loss of the C-terminal amino acid, and the ESIMS of **3** and **4** exhibited characteristic isotope patterns of chlorinated and brominated sansanmycin H, separately. All of these indicated that the precursors added were incorporated into the C-terminal part of sansanmycin. Interpretation of the NMR and ESIMS/MS data (Table 1 and Figure 2) further identified these compounds as expected structures.

The antibacterial activities of compounds (**1**–**4**) were assayed and listed in Table 2 in comparison to sansanmycin H, which served as a reference. While the majority of obtained compounds performed as well as or worse than their natural parent, sansanmycin H, one noteworthy improvement from the suite of sansanmycin H derivatives was identified; both [4-Br–Phe]- and [4-Cl–Phe]-sansanmycin H exhibited fourfold increase in potency over sansanmycin H against *Staphylococcus aureus* (the minimal inhibitory concentration (MIC) 32 vs 128 μg $ml^{-1}$). This improvement might be explained by the fact that both [4-Br–Phe]- (log $P$ = 1.08) and [4-Cl–Phe]- (log $P$ = 0.91) sansanmycin H had higher lipophilicity than their parent (log $P$ = −0.42), which could lead to an increase of passive diffusion through the cytoplasmic membrane of *S. aureus*. Another notable observation was that all tested compounds were potential against *E. coli* Δ*tolC* mutant (MIC = 1–4 μg $ml^{-1}$) while inactive against

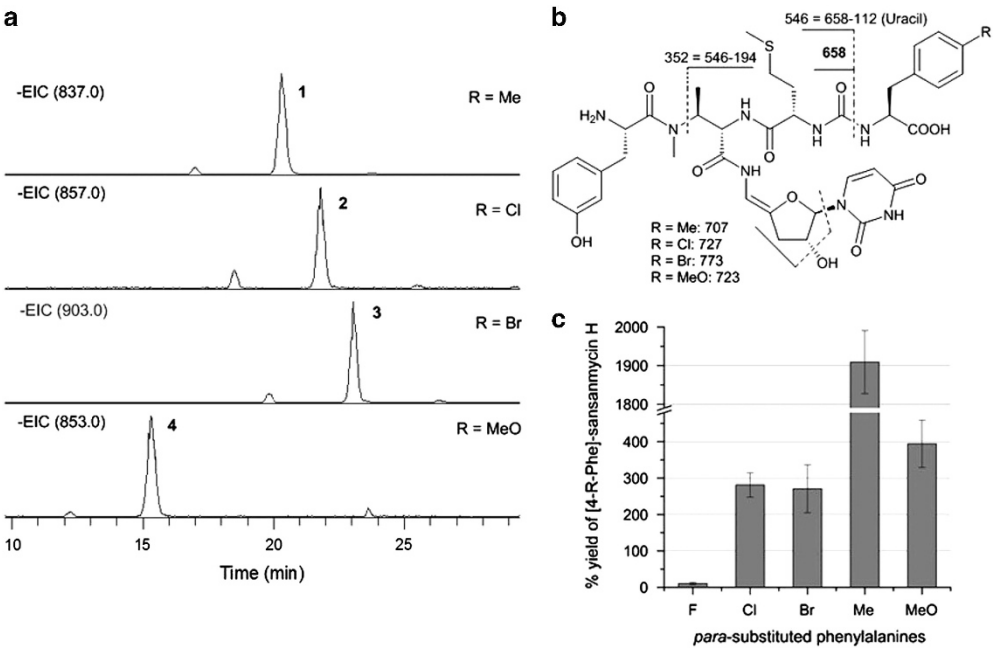


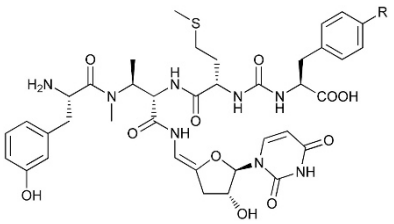

**Figure 2** Extracted-ion chromatograms (**a**) and MS/MS analyzes (**b**) of compounds (**1**–**4**). (**c**) The yields of [4-R–Phe]-sansanmycin H relative to sansanmycin H formed in the control. Experiments were carried out in triplicate with standard deviation indicated by error bars.

**Table 1** $^{1}H$ NMR (600 MHz) and $^{13}C$ NMR (125 MHz) data for [4-R–Phe]-sansanmycin H (1–4)

| | | *Comp. 1* | | *Comp. 2* | | *Comp. 3* | | *Comp. 4* | |
|---|---|---|---|---|---|---|---|---|---|
| *Position*[a] | *Multi* | δ*C*( )[b] | δ*H*( )[b] | δ*C*( )[b] | δ*H*( )[b] | δ*C*( )[b] | δ*H*( )[b] | δ*C*( )[b] | δ*H*( )[b] |
| Uracil-2 | N–CO–N | 147.8(147.0) | | 150.6 | | 150.5 | | 150.8 | |
| Uracil-4 | CO–N | 160.9 | | 163.4 | | 163.1 | | 163.8 | |
| Uracil-5 | CH | 105.7(105.5) | 5.83(5.55) | 102.1(102.2) | 5.63(5.45) | 102.1 | 5.64(5.46) | 102.0(102.2) | 5.61(5.45) |
| Uracil-6 | CH | 141.9(141.6) | 7.25(6.89) | 139.9 | 7.44(7.11) | 140.0(139) | 7.45(7.11) | 139.8(139.2) | 7.43(7.11) |
| Sugar-1 | O–CH–N | 96.0(96.6) | 6.09(6.11) | 92.3(91.8) | 5.96(6.00) | 92.3(91.7) | 5.96(5.99) | 92.4(91.9) | 5.96(5.99) |
| Sugar-2 | O–CH | 75.9(75.4) | 4.49(4.15) | 71.7(71.6) | 4.38(4.26) | 71.7(71.6) | 4.38(4.280 | 71.8(71.6) | 4.37(4.28) |
| Sugar-3 | $CH_2$ | 32.2 | 2.46 | 33.9 | 2.84 | 33.9 | 2.86 | 33.9 | 2.82 |
| | | | | | 2.51 | | 2.49 | | 2.49 |
| Sugar-4 | >C= | 141.2 | | 141.1 | | 141.1 | | 141.1(166.5) | |
| Sugar-5 | –CH= | 98.9(99.3) | 5.93(5.96) | 96.7 | | 96.7 | 5.84(5.82) | 96.8 | 5.83 |
| DABA-1 | CO–N | 170.3(170.0) | | 166.8(166.5) | 5.83(5.81) | 166.9(166.5) | | 166.9 | |
| DABA-2 | CH | 58.9 | 4.49 | 54.7 | 4.66 | 54.7 | 4.66 | 54.8 | 4.65 |
| DABA-3 | CH | 56.2 | 4.15 | 50.1 | 4.85 | 50.1 | 4.85 | 50.7(50.1) | 4.14 |
| DABA-4 | $CH_3$ | 16.1(16.6) | 1.19(0.59) | 13.8(14.8) | 1.07(0.64) | 13.8(14.8) | 1.07(0.65) | 13.8(14.9) | 1.07(0.61) |
| DABA–N–$CH_3$ | N–$CH_3$ | 33.1 | 2.93 | 30.1 | 2.91 | 27.0(29.7) | 2.55(2.91) | 30.0 | 2.91 |
| Ureido | N–CO–N | 160.9 | | 157.3 | | 157.3(157.1) | | 157.3 | |
| 4-R–Phe-1 | –COOH | 180.1 | | 173.6 | | 173.6 | | 175.6 | |
| 4-R–Phe-2 | CH | 54.7(54.4) | 4.26(4.22) | 55.6(53.2) | 3.90(4.00) | 55.8 | 3.91 | 54.7 | 3.90(4.14) |
| 4-R–Phe-3 | $CH_2$ | 40.8(44.0) | 2.85(2.67) 3.07 | 37.7 | 2.99 | 37.5 | 2.97 | 37.3 | 2.72 |
| | | | | | 2.89 | | 2.88 | | 2.94 |
| 4-R–Phe-1′ | ArC | 137.7(137.6) | | 139.3(138.9) | | 139.5 | | 139.8(140.4) | |
| 4-R–Phe-2′ | ArCH | 132.1 | 7.12 | 127.2(127.5) | 7.24(7.19) | 130.4(130.1) | 7.38(7.32) | 130.0 | 7.11 |
| 4-R–Phe-3′ | ArCH | 131.9 | 7.17 | 131.1(131.4) | 7.21(7.14) | 131.5(131.9) | 7.15(7.08) | 128.5 | 6.78 |
| 4-R–Phe-4′ | ArC | 139.3 | | 138.7 | | 118.6 | | 138.8 | |
| 4-R–Phe-5′ | ArCH | 131.9 | 7.17 | 131.1(131.4) | 7.21(7.14) | 131.5(130.9) | 7.15(7.08) | 128.5 | 6.78 |
| 4-R–Phe-6′ | ArCH | 132.1 | 7.12 | 127.2(127.5) | 7.24(7.19) | 130.4(130.1) | 7.38(7.32) | 130.0 | 7.11 |
| 4-Me–Phe–Me/4–MeO–Phe–OMe | –$CH_3$ | 23.0 | 2.29 | | | | | 54. 8 | 3.71 |
| *m*-Tyr-1 | CO–N | 179.4 | | 175.5 | | 175.5 | | 174.8 | |
| *m*-Tyr-2 | CH | 54.4(40.8) | 3.97(4.26) | 52.1 | 3.66(4.13) | 52.3 | 3.67 | 52.4(50.8) | 3.68 |
| *m*-Tyr-3 | $CH_2$ | 42.9(44.0) | 2.46(2.66) | 40.9 | 2.20(2.56) 2.71 | 40.9(42.0) | 2.71(2.55) | 40.9(42.1) | 2.20(2.57) |
| | | | | | | | | | 2.71(2.50) |
| | | | 2.85 | | | | 2.20 | | |
| *m*-Tyr-1′ | ArC | 141.4 | | 138.6 | | 139.0 | | 140.5 | |
| *m*-Tyr-2′ | CH | 118.1(117.9) | 6.67(6.71) | 116.1(116.0) | 6.40(6.68) | 119.6(116.1) | 6.40(6.68) | 116.2(116.1) | 6.69 |
| *m*-Tyr-3′ | Ar–C–O | 161.3(161.2) | | 158.3(157.3) | | 158.1 | | 157.3(157.2) | |
| *m*-Tyr-4′ | ArCH | 120.2 | 6.45 | 113.2(113.0) | 6.47(6.65) | 113.2(113.0) | 6.48(6.63) | 113.2(112.9) | 6.63(6.72) |
| *m*-Tyr-5′ | ArCH | 133.0 | 7.15 | 128.5 | 6.94(7.05) | 128.5(128.8) | 6.94(7.04) | 130.4 | 7.05 |
| *m*-Tyr-6′ | ArCH | 121.9(120.1) | 6.63(6.69) | 119.6(119.0) | 6.60(6.63) | 119.7 | 6.64(6.62) | 119.5 | 6.61 |
| Met-1 | CO–N | 177.4 | | 174.8 | | 172.3(174.8) | | 174.4 | |
| Met-2 | CH | 55.8(55.9) | 4.22 | 53.1 | 4.03 | 52.5 | 4.04(4.13) | 53.2 | 4.02 |
| Met-3 | $CH_2$ | 33.8(33.7) | 1.85 | 31.8(31.1) | 1.85 | 31.8(31.1) | 1.86(1.85) | 31.6 | 1.85 |
| | | | 1.94 | | 1.75 | | 1.75 | | 1.76 |
| Met-4 | $CH_2$ | 30.7 | 2.68 | 30.0 | 2.41 | 30.0 | 2.43 | 30.0(29.7) | 2.47(2.38) |
| Met–S–$CH_3$ | $CH_3$ | 17.0 | 2.05 | 14.4(14.5) | 1.99 | 14.5 | 1.99 | 14.5 | 1.99 |

The spectra of compound **1** were recorded in $D_2O$ (pD = 8) and the others in DMSO. The chemical shifts (δ) are given in p.p.m.
[a]Abbreviations for the structure units are: *m*-Tyr, *meta*-tyrosine; 4-R–Phe = 4-R-phenylalanine and R = Me for Comp. **1**, Cl for Comp. **2**, Br for Comp. **3** and MeO for Comp. **4**, DABA, 2-amino-3-methyl-aminobutyric acid, Met, methionine.
[b]The data in the parentheses are the extra signals due to rotational conformers around the amide bonds, which is often observed in peptides especially those containing *N*-methylated amino acids or proline.

**Table 2 Activity of precursor-directed biosynthetic sansanmycin analogs**



| | | | MIC ($\mu g\ ml^{-1}$) | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | *E. coli* | |
| *Compd* | *R* | *Log P*[a] | *PA*[b] | *SA*[b] | *MS*[b] | *Wild type* | *ΔtolC mutant* |
| **1** | Me | 0.77 | 16 | 64 | >128 | >128 | 2 |
| **2** | Cl | 0.91 | 16 | 32 | >128 | >128 | 1 |
| **3** | Br | 1.08 | 16 | 32 | >128 | >128 | 1 |
| **4** | MeO | 0.23 | 32 | 128 | >128 | >128 | 4 |
| SSH | OH | –0.42 | 4 | 128 | 64 | >128 | 1 |

[a]Log *P* values were calculated by ACD/ChemSketch software (12.0); Advanced Chemistry Development, Inc., Toronto, ON, Canada.
[b]PA, *P. aeruginosa* 11 (wild type); SA, *S. aureus* CPCC100051 (wild type); MS, *M. smegmatis* CPCC240556 (wild type).

wild-type *E. coli* (MIC $>64\ \mu g\ ml^{-1}$). This is the same as the situation of mureidomycin A and C, in which the expression of multidrug efflux system AcrAB-TolC was proved to mediate the intrinsic resistance of *E. coli* to mureidomycin.[10] All of these results hinted that the uptake and the efflux might be limiting factors in the antibacterial activities of sansanmycin.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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