Structural Characterization of Daptomycin Analogues A21978C$_{1-3}$(D-Asn$_{11}$) Produced by a Recombinant *Streptomyces roseosporus* Strain

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Received October 16, 2006

Three daptomycin-related lipopeptides, A21978C$_{1-3}$(D-Asn$_{11}$) (**2**−**4**), were purified from the fermentation broth of a recombinant *Streptomyces roseosporus* strain. Their chemical structures were determined by analyses of the biosynthetic pathway, chemical transformations, D,L-amino acid quantitation by enantiomer labeling, tandem LC-MS/MS, and 2D-NMR techniques. Compounds **2**−**4** exhibited potent antibacterial activity against *Staphylococcus aureus* with MIC values of 0.6, 0.3, and 0.15 μM, respectively, well correlated to the acyl tail chain length.

Cubicin (daptomycin-for-injection) is a first-in-class cyclic 13-membered lipopeptide antibiotic approved in the United States for the treatment of complicated skin and skin structure infections caused by Gram-positive pathogens including methicillin-resistant *Staphylococcus aureus* (MRSA).[1] Recently, daptomycin has been further approved for treatment of bloodstream infections (bacteremia/endocarditis).[2] However, daptomycin is not indicated for community-acquired pneumonia (CAP).[3] To design second-generation daptomycin-related antibiotics with an improved efficacy in CAP and other indications, it is necessary to have a better understanding of the structure−activity relationships (SARs) of the daptomycin scaffold. Daptomycin (**1**) is a member of the A21978C family of antibiotics produced by a nonribosomal peptide synthetase (NRPS) in *Streptomyces roseosporus* NRRL 11379.[4] The A21978C lipopeptides contain a 13 amino acid chain linked by an ester bond between the carboxyl of kynurenine (Kyn) and the hydroxyl of Thr$_4$ to form a 10 amino acid ring with a three amino acid exocyclic tail.[5] The three prominent factors, A21978C$_{1-3}$, have *anteiso*-undecanoic (*a*C$_{11}$), *iso*-dodecanoic (*i*C$_{12}$), and *anteiso*-tridecanoic (*a*C$_{13}$) acids, respectively, attached to the terminal amino group of Trp$_1$. Previous semisynthetic modifications of daptomycin were focused on either the lipid tail or the NH$_2$ group of ornithine (Orn).[6,7] Nevertheless, none of these derivatives have advanced to clinical studies. Several daptomycin analogues were prepared by chemoenzymatic synthesis with glutamic acid, which was used to substitute nonproteinogenic *threo*-3-methylglutamic acid (3mGlu) of **1**, even though antibacterial activity decreased significantly.[8]

Genetic manipulation of the related lipopeptide biosynthetic gene clusters of daptomycin (*dpt*),[9] A54145 (*lpt*),[10] and calcium-dependent antibiotic (*cda*)[11] has generated novel daptomycin analogues.[12−14] Nguyen et al.[14] described a method to generate a series of hybrid daptomycin analogues by combinatorial biosynthesis using NRPS module exchanges combined with subunit exchanges and deletion of a tailoring enzyme. Several hybrid NRPS pathways constructed by replacing the module D-Ser$_{11}$ of daptomycin NRPS by the module D-Asn$_{11}$ from the A54145 NRPS using several fusion sites were able to confer production of novel compounds **2**−**4** (Figure 1) as verified by ESIMS. More importantly, compound **2** exhibited significant antibacterial activities against a panel of pathogens including two MRSA strains, *S. aureus* 399 (ATCC 43300) and *S. aureus* 1118 (MW2), with MIC values of 1.0 μg/mL.[14] In the present study, we present the purification and structural characterization of **2**−**4** after exploration of another set of fusion sites for the D-Ser$_{11}$ to D-Asn$_{11}$ module exchange to produce **2**−**4**.

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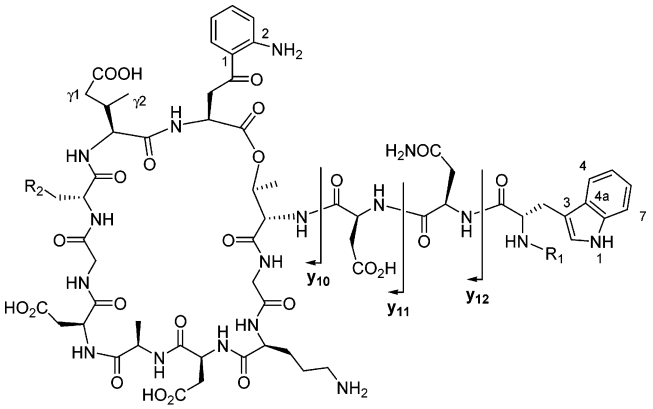

1 R_1 = $CO(CH_2)_8CH_3$, R_2 = OH
2 R_1 = $CO(CH_2)_8CH(CH_3)CH_2CH_3$, R_2 = $CONH_2$
3 R_1 = $CO(CH_2)_8CH(CH_3)_2$, R_2 = $CONH_2$
4 R_1 = $CO(CH_2)_8CH(CH_3)CH_2CH_3$, R_2 = $CONH_2$

Figure 1. Chemical structures of **1**−**4** and three product ions y$_{10}$−y$_{12}$ for their LC-MS-MS spectra.

Results and Discussion

The daptomycin NRPS was modified by module exchanges essentially as previously described to generate daptomycin analogues **2**−**4** in which D-Ser$_{11}$ was replaced by D-Asn$_{11}$.[14] As briefly described in the Experimental Section, the module D-Ser$_{11}$ of DptBC was deleted and replaced by the module D-Asn$_{11}$ from the A54145 pathway using fusion sites TC$_1$ (located in the T−C linker)[14] and SuE (*su*bunit *e*nd, located after the end of the DptBC subunit). A recombinant *S. roseosporus* strain (KN362) that carries the modified *dptBC* gene was fermented in 125 mL shake flasks, and fermentation broth was harvested after 6 days. A disc diffusion test[15] showed that the crude broth inhibited growth of *S. aureus* (data not shown). HPLC analysis identified three compounds eluting as a cluster of peaks with retention times of 10.75, 11.22, and 11.61 min. All three exhibited daptomycin-like UV spectra: λ_{max} 221, 262, and 365 nm[12] and ESIMS data at *m*/*z* 1661.6, 1675.7, and 1689.8 for the $[M + H]^+$ ion.

To generate sufficient quantities of lipopeptides for characterization, structure elucidation, and assessment of biological activities, 7 L of a fermentation broth of KN362 was sequentially fractionated by Diaion HP-20 resin column chromatography, Sephadex LH-20 gel filtration size exclusion chromatography (SEC), and reversed-phase C$_8$ HPLC to yield three compounds, **2** (20 mg), **3** (12 mg), and **4** (9 mg).

Compound **2**, $[\alpha]^{25}_D$ +26 (*c* 0.1, H$_2$O), was obtained as a yellowish, amorphous powder. The molecular formula was deduced

10.1021/np0605135 CCC: $37.00
Published on Web 02/07/2007

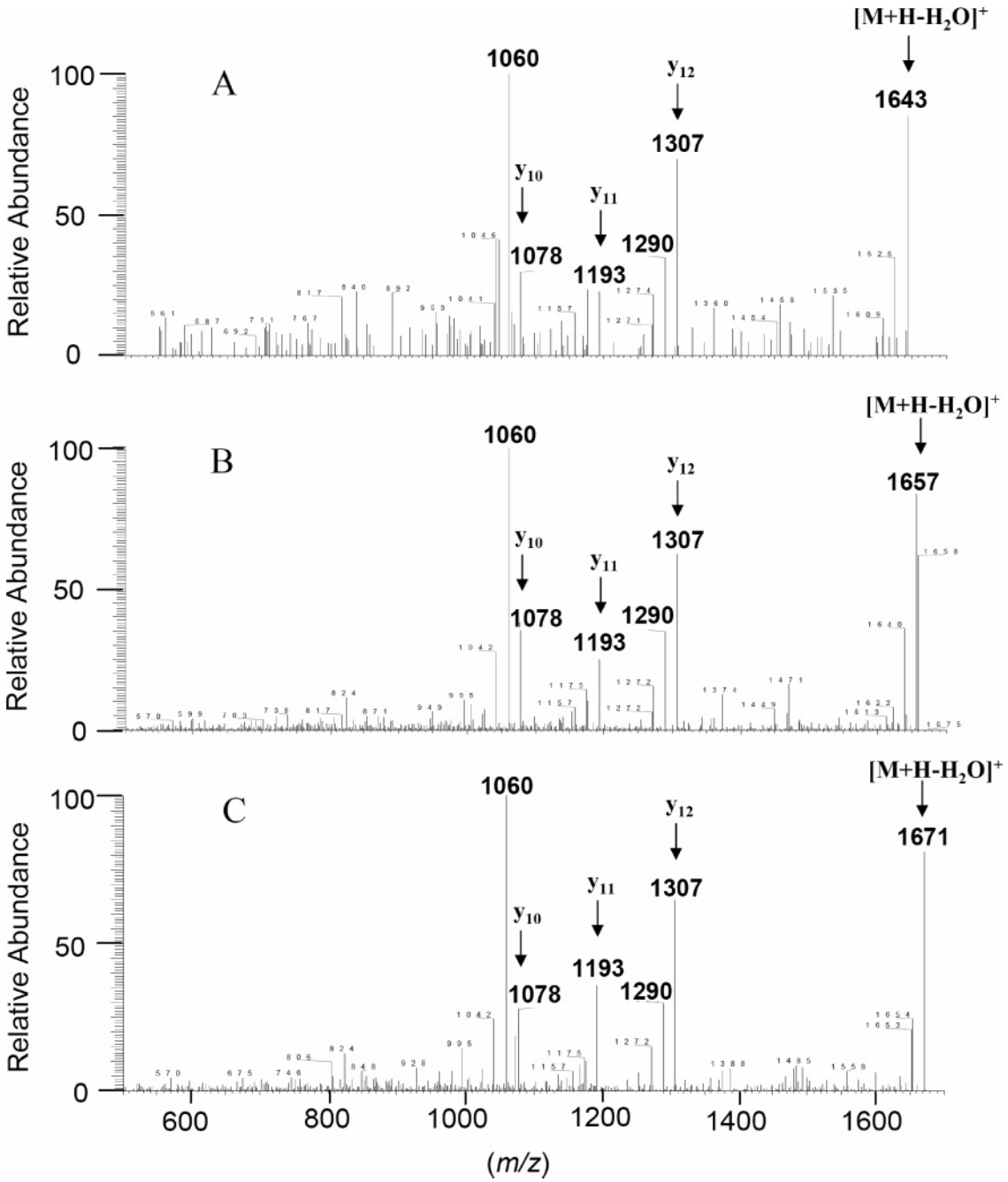

Figure 2. MS-MS spectra of **2** (A), **3** (B), and **4** (C) and assignment of key product ions.

as $C_{74}H_{104}O_{26}N_{18}$ from the HRESIMS data at *m*/*z* 1661.7413 [M + H]$^+$ ($C_{74}H_{105}O_{26}N_{18}$, calcd for 1661.7442) in conjunction with the predicted biosynthetic pathway product. Analysis of the IR spectrum suggested that it contained one or more amine groups (3289 cm^{-1}) and amide functionalities (1651 cm^{-1}). The UV absorption at λ_{max} 221, 262, and 365 nm was consistent with the presence of conjugation systems contributed by the sum of Trp and Kyn moieties.[12] Standard amino acid analysis of **2** indicated the presence of five Asx (aspartic acid and/or asparagine), two Gly, one Ala, one Thr, and four unidentified amino acid residues due to the lack of amino acid standards. The results confirmed that compound **2** contains one additional Asx and no Ser residue in comparison with **1**. The amino acid sequence of **2** was determined by the MS-MS data of the parent molecule (Figure 2A) and its linear hydrolysate **2a** (Table 1, Figures 3 and S1). The nomenclature for the amino acid sequence-determining fragmental ions was described elsewhere.[16,17] As shown in Figure 2A, the MS-MS spectrum of **2** provided distinct fragment peaks at *m*/*z* 1643 ([M + H − H_2O]$^+$), 1307 (y_{12}), 1193 (y_{11}), and 1078 (y_{10}) along with their corresponding [M − 18] fragmental ions. The three fragments y_{12}, y_{11}, and y_{10} confirmed the side chain amino acid sequence of **2** as *N*-undecanoyl-Trp-Asn-Asp. Furthermore, the amino acid sequence of **2** was determined by the analysis of the MS-MS data of the linear lipopeptide **2a** (*m*/*z* 1679.8 [M + H]$^+$), which was produced by hydrolysis of **2** with lithium hydroxide. As demonstrated in many studies,[18−21] sequential cleavage of amide bonds of a linear peptide allowed observations of two sets of fragmental ions y_n and b_n resulting from both C- and N-termini, respectively, to identify the amino acid sequence.[16] As shown in Figures 2 and S1 and summarized in Table 1, experimental values of y_n and b_n (n = 1 to 12) agreed with their respective theoretical fragment ions. Therefore, the amino acid sequence of **2** was strongly supported by its linear hydrolysate **2a** as *N*-undecanoyl-Trp-Asn-Asp-Thr-Gly-Orn-Asp-Ala-Asp-Gly-Asn-3mGlu-Kyn.

Daptomycin (**1**) possesses three D-configured amino acids including D-Asn_2, D-Ala_8, and D-Ser_{11}. In 1987, Debono et al.

Table 1. Ions Observed in MS-MS Sequence Analysis of **2a**−**4a**

ion	**2a** (*m*/*z*)	**3a** (*m*/*z*)	**4a** (*m*/*z*)
b_1	355.1	369.1	383.1
b_2	469.1	483.1	497.1
b_3	584.0	598.0	612.1
b_4	685.1	699.2	713.1
b_5	742.1	756.1	770.4
b_6	856.2	870.1	884.1
b_7	971.2	985.3	999.2
b_8	1042.2	1056.2	1070.2
b_9	1157.2	1171.2	1185.2
b_{10}	1214.3	1228.1	1242.2
b_{11}	1328.3	1342.1	1356.2
b_{12}	1471.2	1485.3	1499.2
y_1	209.1	209.1	209.1
y_2	352.1	352.1	355.2
y_3	466.1	466.1	466.1
y_4	523.1	523.1	523.1
y_5	623.2	623.2	623.1
y_6	709.1	709.1	709.1
y_7	824.0	824.1	824.2
y_8	938.1	938.2	938.2
y_9	995.2	995.1	995.2
y_{10}	1096.2	1096.1	1096.2
y_{11}	1211.2	1211.2	1211.2
y_{12}	1325.1	1325.2	1325.2

determined the D-Ala and D-Ser residues by using enzymatic digestion of daptomycin hydrolysates with L- and D-amino acid oxidase.[4] Recently, Miao et al. found epimerase domains in modules 2, 8, and 11 of the daptomycin NRPS, suggesting the presence of D-Asn_2, D-Ala_8, and D-Ser_{11}.[9] The presence of D-Asn_2 in **1** was further confirmed by comparing stereospecific synthetic peptides and the naturally occurring daptomycin chemically and microbiologically.[9] In this study, to confirm the presence of D-Asn_2, D-Ala_8, and D-Asn_{11} in **2** as predicted by genetic information of the modified *dptBC*, Asx and Ala were specifically selected for determination of their optical purity using an enantiomer-labeling method.[22] The GC-MS results showed that compound **2** contained D-Asx (33% optical purity) and D-Ala (99% optical purity). Two D-Asx could be reasonably interpreted for 33% optical purity from the contribution of Asn_2 and Asn_{11}, while three L-Asp (67% optical purity) from the Asp residue at positions 3, 7, and 9. As a result, compound **2** contains three D-amino acid residues (two D-Asn and one D-Ala).

Naturally occurring A21978C_{0-3} have acyl groups that consist of *n*-decanoyl (*n*C_{10}), *anteiso*-undecanoyl (*a*C_{11}), *iso*-dodecanoyl (*i*C_{12}), and *anteiso*-tridecanoyl (*a*C_{13}), respectively.[4] The acyl substituent of **2** was determined by acidic hydrolysis of the lipopeptide followed by coupling the resultant fatty acid (FA) with Trp to produce a corresponding FA-Trp amide. The HPLC retention time (t_R) and UV spectrum of the FA-Trp amide from **2** (Figure 4B) were compared with those of amides from a library of authentic fatty acids (Figure 4A). In addition, the identification of the acyl group was further confirmed by observing a homogeneous peak at t_R 7.01 min after spiking the reaction resultant of **2** with the library mixture (data not shown). As a result, the *anteiso*-undecanoyl (*a*C_{11}) group was identified in **2**.

To identify and sequence-specifically assign the amino acid residues of **2**, NMR spectra including ^1H, ^{13}C, gHSQC-DEPT, gHMBC, gDQF-COSY, TOCSY, and ROESY were recorded in DMSO-d_6 at room temperature (Figures S2 to S8, respectively). By comparison of the NMR data of **2** (Table 2) with those of **1**,[23] the characteristic Ser_{11} resonances of **1** at δ_H 4.58 (H-α), 3.88 (H_2-*β*) and δ_C 64.19 (C-*β*) were missing as predicted for **2**. To identify amino acid residues of **2**, analysis of the gDQF-COSY spectrum resulted in the assignment of aromatic moieties of Trp and Kyn by analysis of the scalar correlated coupled proton connectivity, $^3J_{H-H}$. For example, the indole N-proton at δ_H 10.75 was correlated with δ_H 7.14 (H-2) in the Trp_1 residue. In turn, four amino acid residues

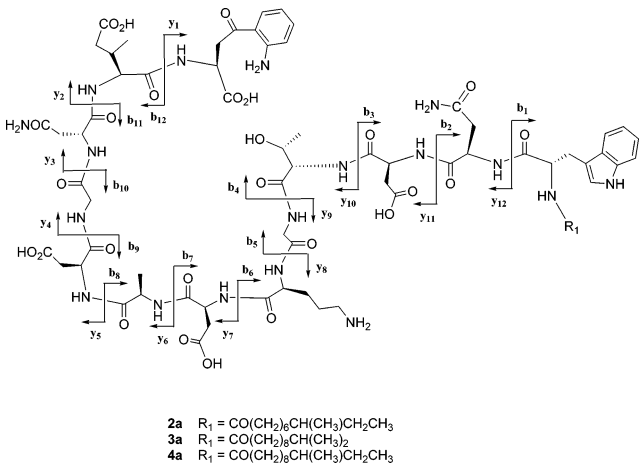

Figure 3. Chemical structures of **2a**−**4a** and their MS-MS fragmentation patterns.

of **2** including Thr, Orn, Ala, and 3mGlu were unambiguously identified after analysis of its TOCSY and gHSQC-DEPT data. In these cases, the TOCSY spectrum shows their intraresidue scalar correlation of exchangeable backbone amide protons with the respective nonexchangeable side chain protons of each residue. gHSQC-DEPT is a 2D spectroscopy similar to HSQC with additional DEPT-like information (CH and CH_3 in one phase and CH_2 in another phase). For example, the amino acid residue Thr of **2** was identified on the basis of its TOCSY data, which revealed a spin system inclusive of an exchangeable NH at δ 7.89 d (J = 8.0 Hz), H-α at δ 4.48, H-*β* at δ 5.14 m, and H-*γ* at δ 1.07 d (J = 6.1 Hz). Assignment of the Thr residue was further confirmed by gHSQC-DEPT data at C-α at δ 57.2 (CH), oxygenated C-*β* at δ 73.1 (CH), and C-*γ* at δ 18.2 (CH_3). In addition, two Gly residues were also identified from the gHSQC-DEPT spectrum by α-position resonances at δ_H 3.72 and 3.80, both coupled with δ_C 45.3 (t), which were characteristic in upfield, relative to α-position, chemical shifts of the other amino acid residues. Nevertheless, assignments of the remaining Asp and Asn residues were challenging due to heavily overlapping H-α and H_2-*β* signals contributed by several repeating units and the same spin system pattern. To resolve this issue, the ROESY data were collected to trace the sequence-specific resonance assignment of **2**, especially useful for two Gly, three Asp, and two Asn residues. As summarized in Figure 5, most backbone connectivities were confirmed by the inter-residue dipolar correlations (NOE) of amide protons with the side chain protons of the neighboring residues in the sequence. More importantly, spatial correlations between H-α at δ 4.34 of Trp_1 and NH at δ 8.35 (D-Asn_2) led to the assignment of NMR resonances of the D-Asn_2 residue after carefully deducing the spin system containing the NH at δ 8.35 from the TOCSY spectrum. Using a similar approach, NMR resonances of Asp_3, Gly_5, Asp_7, and Asp_9 residues were individually assigned on the basis of their backbone NOE correlations between H-α and neighboring amide protons. Finally, NMR data of the remaining Gly_{10} and D-Asn_{11} of **2** were individually deduced after subtracting other assigned residues from NMR spectra. Furthermore, a series of NOEs (Figure 5) observed for **2** should facilitate the calculation of the 3D-structure of **2** in the future. Thus, the structure of **2** was determined as *anteiso*-undecanoyl-L-tryptophanyl-D-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-L-aspartylglycyl-D-asparaginyl-*threo*-3-methyl-L-glutamyl-3-anthraniloyl-L-alanine-ϵ1-lactone, to which the trivial name A21978C_1(D-Asn_{11}) has been assigned.

Compound **3** was obtained as a yellowish, amorphous powder. Its molecular formula was deduced as $C_{75}H_{106}O_{26}N_{18}$ from the HRESIMS data at *m*/*z* 1675.7572 $[M + H]^+$ ($C_{75}H_{107}O_{26}N_{18}$, calcd for 1675.7598). The MS-MS spectrum of **3** (Figure 2B) showed dominant fragments at *m*/*z* 1657 ($[M + H - H_2O]^+$), 1307 (y_{12}),

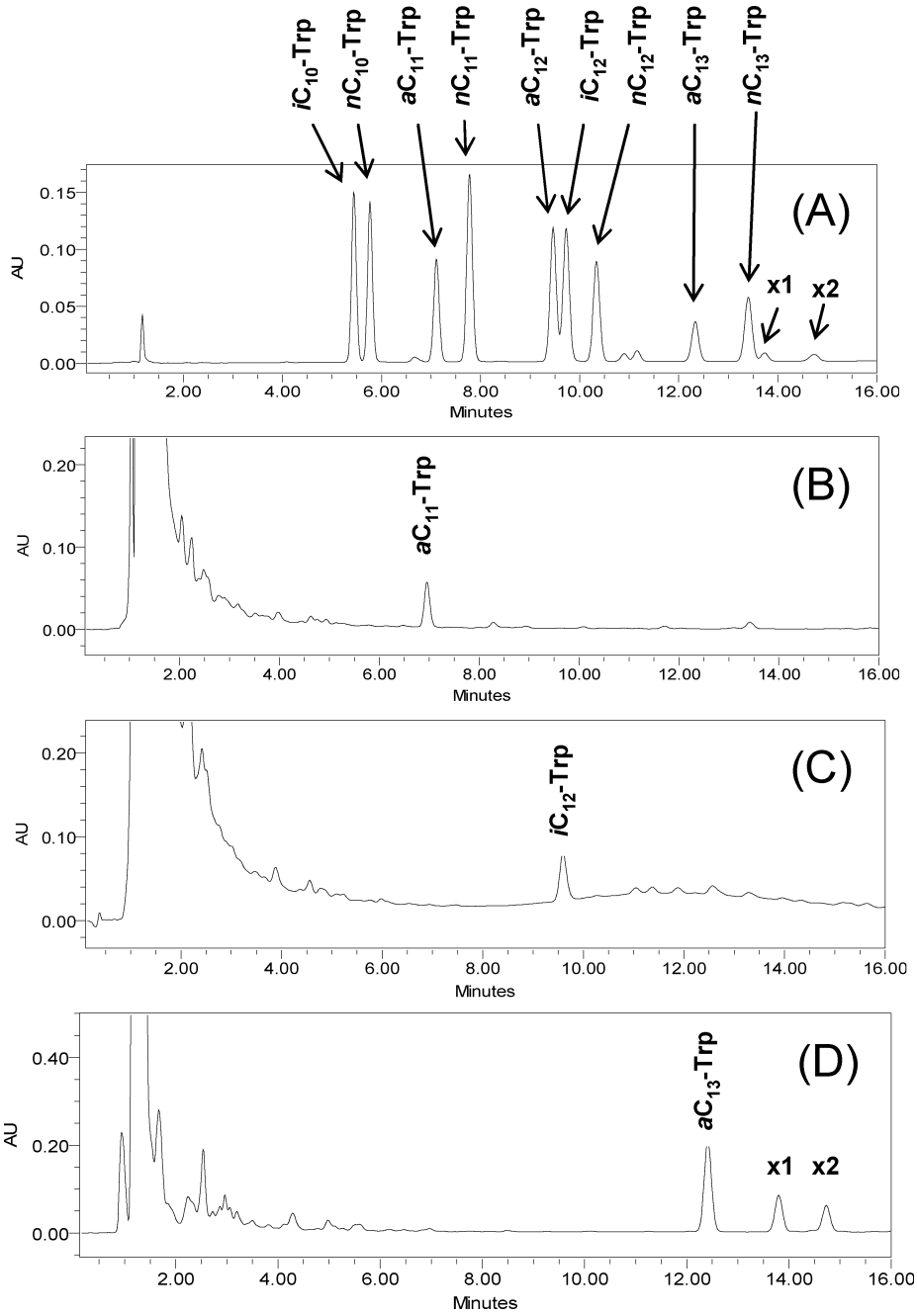

Figure 4. Determination of *N*-acyl substituents of **2**−**4** by HPLC analyses of fatty acid (FA)-Trp amides. (A) HPLC profile of a panel of authentic FA-Trp amides. (B−D) HPLC profiles of Trp coupling resultants for compounds **2**−**4**, respectively. Assignment of *a*C_{11}-Trp in (B), *i*C_{12}-Trp in (C), and *a*C_{13}-Trp in (D) was confirmed by observation of the corresponding homogeneous peak after spiking the individual tested lipopeptide reaction mixture into a panel of authentic FA-Trp amides (data not shown). x1 and x2 stand for two unidentified side products in both A and B.

1193 (y_{11}), and 1078 (y_{10}) along with their corresponding [M − 18] ions. Comparison of the MS-MS data of **3** with those of **2** indicated that compound **3** has a 14-mass-unit heavier fatty acid moiety than **2**. This was further confirmed by comparison of the MS-MS data of linear hydrolysates **3a** (Figure S9) and **2a** (Figure S1). As summarized in Table 1, highly comparable N-terminal fragments of **3a** and **2a** (y_1 to y_{12} ions) suggested that both possess the identical peptide core. Each of the observed C-terminal fragments of **3a** (b_1 to b_{12} ions) were observed to be 14 mass units heavier than the respective ions of **2a**. These data strongly suggested that compound **3** consists of a dodecanoyl fatty acid residue. By utilizing a similar approach for **2**, the acyl group of **3** was determined as *iso*-dodecanoyl (*i*C_{12}) at t_R 9.59 min (Figure 4C). Moreover, analysis of TOCSY and ROESY data of **3** led to the assignment of the ^{1}H NMR data (Table S1 and Figure S10). Thus, the structure of **3** was determined as *iso*-dodecanoyl-L-tryptophanyl-D-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-L-aspartylglycyl-D-asparaginyl-*threo*-3-methyl-L-glutamyl-3-anthraniloyl-L-alanine-ϵ1-lactone, to which the trivial name A21978C_2(D-Asn_{11}) has been assigned.

Compound **4**, a yellowish, amorphous powder, showed similar UV and IR spectra to **3**. The molecular formula of **4** was established as $C_{76}H_{108}O_{26}N_{18}$ by positive mode HRESIMS at *m*/*z* 1689.7736 $[M + H]^+$ ($C_{76}H_{109}O_{26}N_{18}$, calcd for 1689.7755). In a similar approach to the identification of **3**, analysis of the MS-MS data of **4** (Figure 2C) and its linear hydrolysate **4a** (Table 1 and Figure S11) strongly suggested that **4** contains a tridecanoyl group with the same tridecadepsipeptide core as in **3**. The acyl substituent of

Table 2. ^{1}H and ^{13}C NMR Data for **2** in DMSO-d_6 (δ in ppm, J in Hz)[a]

residue	position	δ_C	δ_H	residue	position	δ_C	δ_H
Trp_1	NH		7.99 d (6.3)	Asp_9	NH		8.47[c]
	α	57.3 d	4.34 ddd (6.3, 7.0)		α	52.9 d	4.53[c]
	β	29.9 t	2.90 dd (7.0, 15), 3.01 dd (4.8, 15)		*β*	38.9 t	2.53,[c] 2.68 m
	1		10.75 br s		C═O	174.3 s	
	2	126.6 d	7.14 d (1.9)		COOH	174.9 s	12.27 br s
	3	112.7 s		Gly_{10}	NH		8.05[c]
	4	121.2 d	7.55 d (7.8)		α	45.3 t	3.72[c]
	4a	130.1 s			C═O	173.4 s	
	5	121.1 d	6.94 dd (7.8)	Asn_{11}	NH		8.17[c]
	6	123.7 d	7.03 dd (7.8)		α	53.0 d	4.61[c]
	7	114.2 d	7.30 d (7.8)		*β*	41.6 t	2.51,[c] 2.74[c]
	7a	139.0 s			C═O	173.5 s	
	C═O	174.9 s			$CONH_2$	174.2 s	6.98 br s, 7.39 br s
Asn_2	NH		8.35[c]	$3mGlu_{12}$	NH		7.90[c]
	α	52.9 d	4.55[c]		α	58.1 d	4.45[c]
	β	40.0 t	2.39 m, 2.47[c]		*β*	36.4 d	2.33[c]
	C═O	174.9 s			*γ*1	40.9 t	1.90 m, 2.32[c]
	$CONH_2$	174.3 s	6.87 br s, 7.29 br s		*γ*2	17.3 q	0.79 d (6.3)
Asp_3	NH		8.05[c]		C═O	173.1[b]	
	α	52.5 d	4.61[c]		COOH	176.5 s	
	β	40.4 t	2.48,[c] 2.84[c]	Kyn_{13}	NH		8.37[c]
	C═O	173.5 s			α	51.0 d	4.79 m
	COOH	174.9 s	12.27 br s		*β*	42.9 t	3.50 m, 3.58 m
Thr_4	NH		7.89 d (8.0)		*γ*	174.6[b]	
	α	57.2 d	4.48[c]		1	119.0 s	
	β	73.1 d	5.14 m		2	154.0 s	
	γ	18.2 q	1.07 d (6.1)		3	119.8 d	6.74 d (8.0)
	C═O	174.6[b]			4	137.3 d	7.22 dd (8.0)
Gly_5	NH		8.04[c]		5	117.5 d	6.53 dd (7.7, 8.0)
	α	45.3 t	3.80[c]		6	134.3 d	7.75 d (7.7)
	C═O	173.9[b]			C═O	174.6[b]	
Orn_6	NH		8.16 d (7.5)		NH_2		7.09 br s
	α	55.4 d	4.19 m	*a*C11	C═O	175.9 s	
	β	31.2 t	1.71 m, 1.76 m		2	37.9 t	2.02 t (7.0)
	γ	26.4 t	1.61 m		3	27.9 t	1.35 m
	δ	41.9 t	2.81[c]		4	31.6 t	1.08[c]
	NH_2		7.55 br s		5	29.3 t	1.14[c]
	C═O	174.5 s			6	31.6 t	1.11[c]
Asp_7	NH		8.43[c]		7	38.9 t	1.19[c]
	α	52.7 d	4.59[c]		8	36.6 d	1.23[c]
	β	38.7 t	2.33,[c] 2.62 m		9	31.8 t	1.26,[c] 1.06[c]
	C═O	172.9 s			10	14.1 q	0.79 t (6.8)
	COOH	174.9 s	12.27 br s		CH_3-8	14.1 q	0.78 d (6.8)
Ala_8	NH		7.95 d (7.3)				
	α	51.5 d	4.21 m				
	β	20.8 q	1.19 d (7.0)				
	C═O	175.9 s					

[a] Assignments were made on the basis of gDQF-COSY, TOCSY, gHSQC-DEPT, gHMBC, and ROESY data. [b]Interchangeable assignment due to lack of observed HMBC correlations. [c]Overlapped signals.

4 was identified as *anteiso*-tridecanoyl (*a*C_{13}) at t_R 12.18 min (Figure 4D). The ^{1}H NMR data (Figure S12) of **4** were assigned in Table S2 by analysis of the TOCSY and ROESY spectra and comparison with those of **3**. Thus, the structure of **4** was determined as *anteiso*-tridecanoyl-L-tryptophanyl-D-asparaginyl-L-aspartyl-L-threonylglycyl-L-ornithyl-L-aspartyl-D-alanyl-L-aspartylglycyl-D-asparaginyl-*threo*-3-methyl-L-glutamyl-3-anthraniloyl-L-alanine-ϵ1-lactone, to which the trivial name A21978C_3(D-Asn_{11}) has been assigned.

The three metabolites **2**−**4** obtained from KN362 were tested for *in vitro* antibacterial activity against *S. aureus* using a microdilution technique following CLSI standards as described.[24] Compounds **2**−**4** exhibited potent inhibitory activity with MIC values of 0.6, 0.3, and 0.15 μM, respectively. The results showed that essentially equivalent or superior antibacterial activities of **2**−**4** were evident in comparison with the MIC value of 0.6 μM for daptomycin, which was used as a positive control. Sequential reduction of MICs of **2**−**4** was consistent with previous SARs of varying acyl chain length, i.e., antibacterial potency increases up to chain lengths of 12−13 carbons for A21978C.[5] Compounds **2**−**4** may warrant further investigation to evaluate their *in vivo* efficacy in animal models.

This investigation established a template for characterizing a library of novel daptomycin analogues by combined chromatographic, spectroscopic, and chemical transformation methods. In summary, key structure-determination techniques included amino acid composition analysis, D,L-amino acid enantiomeric purity evaluation, acyl group identification by comparative HPLC, amino acid sequence determination based on analysis of the MS-MS data of the parent compound and its linear hydrolysate, and 2D-NMR spectroscopic data including TOCSY and ROESY. The current study supports the predictions made on the basis of genetic information of the hybrid NRPS, enabling rapid determination of complex structures. Of particular note, the present work confirms the accuracy of kinetic proofreading of the C domain of $3mGlu_{12}$ module in this hybrid pathway, in that only the D-isomer of Asn_{11} was condensed into products. This bodes well for applying molecular genetic approaches to produce novel lipopeptides and other peptides with specific chirality in the peptide core.

Experimental Section

General Experimental Procedures. Melting points were determined using a Thomas-Hoover capillary melting point apparatus and are

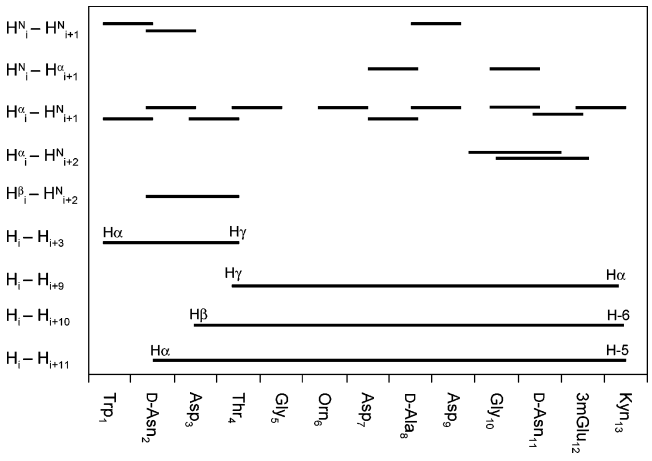

Figure 5. Inter-residue NOEs observed in the ROESY data set of **2** at a mixing time of 220 ms. H^N_i stands for the amide bond proton of a certain amino acid residue within the molecule. H^α_i and H^β_i stand for protons at positions α and β in an amino acid residue.

uncorrected. Optical rotations were recorded by an Autopol III automatic polarimeter (Rudolph Research Analytical, Flanders, NJ). UV spectra were measured on an Evolution 300 UV spectrophotometer (Thermo Corporation, Waltham, MA) and IR spectra on a Thermo Nicolet 4700 FT-IR spectrometer. NMR spectra were recorded on a Varian 600 MHz NMR spectrometer (Varian, Lake Forest, CA) with standard pulse programs, and 1D- and 2D-NMR data were processed by MestRec software (Mestrelab Research S.L., A Coruña, Spain). HRESIMS were obtained from a Finnigan LTQ Orbitrap mass spectrometer (Thermo Corporation). Column chromatography was conducted on Diaion HP20 resins (Mitsubishi Chemical Corporation, Tokyo, Japan) and Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden). Analytical HPLC was performed at ambient temperature using a Waters Alliance 2690 HPLC system and a 996 photodiode array detector (Waters, Milford, MA). Semipreparative HPLC was performed on a Varian system equipped with two Model SD-1 PrepStar solvent delivery modules, a PDA ProStar detector, a ProStar injector, and a Model 701 fraction collector.

Media, Strains, Construction of Mutants, and Expression Plasmids. Experimental conditions were as described.[12] The module D-Ser_{11} of the daptomycin NRPS was deleted and replaced with module D-Asn_{11} from the A54145 NRPS pathway using fusion sites TC_1 (located in the T−C linker)[14] and SuE (*su*bunit *e*nd, located after the end of the DptBC subunit). Genetic manipulations including deletion of D-Ser_{11} module by an antibiotic-resistant gene flanked by universal linkers and cloning of the replacing D-Asn_{11} module by a gap repair method were mediated by the λ−Red recombination as previously described.[14] Two primers (sequences of universal linkers underlined) used for the deletion of module D-Ser_{11} were 8_B-AvrII (5′-TTG TTC GAG GCG CCG ACG GTG AGC CGT TTG GAG CGG TTG CTG CGG GAG CGC CTA GGA CGT TGA CAC CAT CGA ATG G-3′) and 11_SUE-PmeI (5′-CAG CTC GCT GAT GAT ATG CTG ACG CTC AAT GCC GTT TGG CCT CCG ACT AAG TTT AAA CCC TCA TTC ATC GGG CGA AAG-3′). Two primers (sequences annealed to pBR322 sequences underlined) used for gap repair cloning of module D-Asn_{11} were Lpt-N11-B-P13 (5′-TCG GGG CGC GGG TCG GCG GGG CGC AGC CGG GGT CCG GCC TCG CCC GCT AGC TTC TTA GAC GTC AGG TGG CAC-3′) and Lpt-N11-SUE-P14 (5′-CAC CGA ACT CGA CCA GCT CGA AGC AGA GTG GAA GGC CGG CTG ATG GTT AAC CGA TAC GCG AGC GAA CGT GA-3′). The recombinant *S. roseosporus* KN362 strain was predicted to produce the A21978C(D-Asn_{11}) hybrid depsipeptide core along with natural variations in lipid side chain.

Fermentation. Small-scale fermentations (20 mL broth) were conducted in 125 mL baffled shaken flasks at 200 rpm, 30 °C. Starter cultures were grown in trypticase soy broth (TSB) for 48 h, and 1 mL of culture was transferred to A355 medium[9] and incubated for 24 h to obtain seed culture. The seed was transferred into F10A medium[9] for production. Cultures were sampled 4−6 days later for quantitative HPLC analysis and LC-ESIMS identification. Large-scale fermentation was conducted in a 7 L fermenter stirred at 500 rpm, aerated at 1.0 vvm, and incubated at 30 °C. Seed was grown by inoculating mycelium from a well-grown plate culture into 250 mL of A355 medium in a 2 L baffled shaken flask at 240 rpm, 30 °C for 24 h. The whole seed culture was transferred to the fermenter containing 5 L of A354 medium,[9] and the broth was harvested after 7 days.

HPLC Analyses. Target compounds **2**−**4** were analyzed by a Waters HPLC system with a Waters Symmetry C_8 column (4.6 × 250 mm, 5 μm) and a Waters Guard Symmetry C_8 cartridge (4.6 × 20 mm, 5 μm). Mobile solvent systems included acetonitrile buffered with 0.01% TFA (solvent A) and H_2O buffered with 0.01% TFA (solvent B). The mobile phase, flowing at 1.5 mL/min, was initially held at 10% A (2 min), linearly changed to 90% A over 18 min, held at 90% A (3 min), and equilibrated with 10% A (3 min). In HPLC chromatograms, a cluster of peaks with retention times (t_R) at 10.75, 11.22, and 11.61 min were identified as the predicted lipopeptides **2**−**4**, respectively, by their characteristic UV spectra (all exhibited a daptomycin-like spectrum: λ_{max} 221, 262, and 365 nm) and ESIMS data at *m*/*z* 1661.6, 1675.7, and 1689.8 for each $[M + H]^+$.

Extraction and Isolation. Production culture broth of KN362 (7 L) was centrifuged to remove biomass, and the supernatant was loaded onto an open glass column packed with preconditioned 500 mL Diaion HP20 resin (60 × 500 mm) in water. The column was sequentially eluted with 1.5 L each of H_2O, 10% MeOH, 30% MeOH, and MeOH. The MeOH eluate was concentrated by rotary evaporation and lyophilized to give 2 g of crude material, which was further subjected to a 500 mL Sephadex LH-20 column chromatography eluting with a mixture of MeOH−H_2O (1:1). Fractions containing target components were collected to yield 300 mg of lyophilized powder. Further purification was achieved by semipreparative HPLC using a Waters SymmetryPrep C_8 column (19 × 300 mm, 7 μm) at a flow rate of 20 mL/min with a linear gradient of 36% A to 40% A in 20 min, washing the column with 90% A for 4 min, and equilibrating with 36% A for 6 min. HPLC traces were recorded at UV 220 nm. Compounds **2** (20 mg), **3** (12 mg), and **4** (9 mg) were obtained at t_R 10.9, 15.5, and 21.1 min, respectively.

A21978C_1(D-Asn_{11}) (2): yellowish, amorphous powder; mp 186 °C (dec); $[\alpha]^{25}_D$ +26 (*c* 0.1, H_2O); UV (H_2O) λ_{max} (log ε) 221 (4.62), 262 (3.93), 289 (3.63), 365 (3.56) nm; IR (dried film) ν_{max} 3289, 1651, 1526, 1407, 1272, 1200, 1135 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 1661.7413 $[M + H]^+$ (calcd for $C_{74}H_{105}O_{26}N_{18}$, 1661.7442).

A21978C_2(D-Asn_{11}) (3): yellowish, amorphous powder; mp 200 °C (dec); $[\alpha]^{25}_D$ +28 (*c* 0.078, H_2O); UV (H_2O) λ_{max} (log ε) 220 (4.65), 261 (3.98), 281 (3.77), 364 (3.58) nm; IR (dried film) ν_{max} 3288, 1651, 1526, 1407, 1272, 1201, 1136 cm^{-1}; ^1H NMR data, see Table S1; HRESIMS *m*/*z* 1675.7572 $[M + H]^+$ (calcd for $C_{75}H_{107}O_{26}N_{18}$, 1675.7598).

A21978C_3(D-Asn_{11}) (4): yellowish, amorphous powder; mp 200 °C (dec); $[\alpha]^{25}_D$ +25 (*c* 0.075, H_2O); UV (H_2O) λ_{max} (log ε) 219 (4.57), 261 (3.92), 281 (3.72), 365 (3.52) nm; IR (dried film) ν_{max} 3290, 1651, 1527, 1407, 1272, 1201, 1135 cm^{-1}; ^1H NMR data, see Table S2; HRESIMS *m*/*z* 1689.7736 $[M + H]^+$ (calcd for $C_{76}H_{109}O_{26}N_{18}$, 1689.7755).

Alkali Hydrolysis of 2−4. Each aliquot of **2**−**4** (10 μg/mL) was prepared in 1.5 mL vials, to which 1 mg of $LiOH·H_2O$ was individually added. After 20 min at room temperature, the hydrolysis reaction was quenched by adding 10 μL of formic acid, and hydrolysates **2a**−**4a** were subjected to LC-MS-MS analysis.

LC-MS-MS Analyses of 2a−4a. LC-MS-MS analysis was carried out using a Finnigan Surveyor HPLC system interfaced to a Finnigan LTQ Orbitrap mass spectrometer. A Waters Sunfire C_8 column (2.1 × 100 mm, 3.5 μm) was used for HPLC separations. The sample injection volume was 10 μL. The solvent systems were water containing 0.1% formic acid (solvent C) and acetonitrile containing 0.1% formic acid (solvent D). The linear gradient for separation was set up as 10% D to 90% D in 10 min, and then the column was cleaned with 90% D for 2 min and equilibrated with 10% D for 3 min. The flow rate was 350 μL/min. Positive ESI source conditions were sheath gas flow rate at 30, auxiliary gas flow rate at 2, ion spray voltage at 2 kV, capillary temperature at 350 °C, capillary voltage at 40 V, and tube lens voltage at 250 V. Normalized collision energy was 35%.

MS-MS data used for amino acid sequence analysis of **2**−**4** were acquired from their individual hydrolysates **2a**−**4a**, which were

confirmed by their respective $[M + H]^+$ *m/z* at 1679.8, 1693.8, and 1707.8 along with their characteristic UV spectra at λ_{max} 222, 261, 281, and 366 nm.

Analyses of Amino Acid Optical Purity. A modified enantiomer-labeling method was employed to quantitatively determine the enantiomer purity of amino acids according to previously established protocols.[22] In brief, compound **2** (100 nmol) was hydrolyzed in 0.5 mL of 6 N DCl in D_2O at 110 °C for 24 h and dried under a stream of nitrogen. The residue was esterified with 0.5 mL of 4 N DCl in MeOH at 110 °C for 15 min. After drying by a gentle nitrogen steam, the ester was acylated with 0.25 mL of trifluoroacetic anhydride in ethyl trifluoroacetate (1:1, v/v) at 130 °C for 10 min. After the excess of reagents was again removed by a stream of nitrogen, the residue was dissolved in 0.15 mL of toluene and injected into the gas chromatograph. All of the GC-MS analyses were performed using a Varian Saturn 2000/HP5973 GC-MS system (Varian, Lake Forest, CA) including a Saturn 2000 software/HP Chem integrator. The gas chromatograph was fitted with a deactivated glass capillary column (20 m × 0.31 mm) coated with Chirasil-Val (film thickness 0.2 μm) (Applied Sciences Laborites Inc., State College, PA). Hydrogen was used as the carrier gas at a flow rate of 1.5 mL/min. Sample introduction (0.5 μL) was made in split mode with a 25:1 split ratio and an injector temperature of 190 °C. The temperature of the column oven was programmed at 65 °C for 3 min, followed by a linear gradient of 65 °C to 190 °C over 31 min. To confirm the presence of D-configuration amino acids of **2**, two residues (aspartic acid and alanine) were specifically chosen for evaluation of their enantiomer purity based on their biosynthetically predicted structure. In this method, optical purity of Asp was contributed by both Asp and Asn since Asn was converted to Asp during the DCl/D_2O hydrolysis of the lipopeptide.

Determination of *N*-Acyl Substituents. All chemicals used for coupling reactions were obtained in analytical-grade. Decanoic (*n*C_{10}), lauric (*n*C_{12}), and tridecanoic (*n*C_{13}) acids, L-tryptophan (Trp), 1-hydroxybenzotriazole hydrate (HOBt), and *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium PF6 (HATU) were purchased from Sigma-Aldrich Corporation (Saint Louis, MO). 8-Methylnonanoic acid (*i*C_{10}) and undecanoic acid (*n*C_{11}) were bought from Fisher Scientific (Pittsburgh, PA). 8-Methyldecanoic (*a*C_{11}), 10-methyldodecanoic (*a*C_{13}), and 10-methylundecanoic (*i*C_{12}) acids were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). 9-Methylundecanoic acid (*a*C_{12}) was provided by Dr. Wai-Kit Pang (Medicinal Chemistry Department, Cubist Pharmaceuticals, Inc.). Authentic fatty acid-Trp (FA-Trp) amides were synthesized in 4 mL vials after shaking at 200 rpm for 1 h at ambient temperature using a mixture of 22 mM fatty acid, 27 mM Trp, 27 mM HOBt, 27 mM HATU, 1.5 mL of DMF, and 14 μL of DIPEA. Each reaction mixture was subjected to semipreparative HPLC for purification of a target FA-Trp amide. Semipreparative HPLC conditions including column, flow rate, and monitoring UV wavelength were the same as in the Extraction and Isolation section. Mobile phase solvent systems were acetonitrile containing 0.01% TFA (solvent A) and H_2O containing 0.01% TFA (solvent B). Nine target FA-Trp amides were purified by three linear gradient programs over 20 min including 50% A to 80% A for *i*C_{10}-, *n*C_{10}-, *a*C_{11}-, and *n*C_{11}-Trp amides, 60% A to 90% A for *a*C_{12}-, *i*C_{12}-, and *n*C_{12}-Trp amides, and 70% A to 90% A for *a*C_{13}- and *n*C_{13}-Trp amides. All of the purified FA-Trp amides were identified by tryptophan-like UV spectra at λ_{max} 221, 281, and 290 nm as well as their respective ESIMS data $[M + H]^+$ at *m/z* 359.2 for *i*C_{10}-Trp and *n*C_{10}-Trp, 373.2 for *a*C_{11}-Trp and *n*C_{11}-Trp, 387.3 for *a*C_{12}-, *i*C_{12}-, and *n*C_{12}-Trp amides, and 401.3 for *a*C_{13}-Trp and *n*C_{13}-Trp. Analytical HPLC was performed on a YMC-Pack ODS-A column (4.6 × 150 mm, 5 μm) and monitored at 280 nm. The mobile phase was linearly delivered from 55% A to 70% A over 15 min. At a flow rate of 1.5 mL/min, typical retention times were observed for nine authentic FA-Trp amides including *i*C_{10}-Trp at 5.37 min, *n*C_{10}-Trp at 5.69 min, *a*C_{11}-Trp at 7.01 min, *n*C_{11}-Trp at 7.67 min, *a*C_{12}-Trp at 9.33 min, *i*C_{12}-Trp at 9.59 min, *n*C_{12}-Trp at 10.20 min, *a*C_{13}-Trp at 12.18 min, and *n*C_{13}-Trp at 13.25 min.

N-Acyl substituents of **2**−**4** were determined by acidic hydrolysis of these lipopeptides to produce the corresponding fatty acid residue followed by coupling with Trp to further produce the resultant tryptophanyl amides. The HPLC retention times (t_R) and UV spectra were compared with those of authentic FA-Trp amides. In a typical example, compound **2** (3 mg) was dissolved with 10 N HCl (1 mL) in a 1 mL vacuum hydrolysis tube. The tube was evacuated and then placed in a 110 °C heating block for 16 h. After cooling, CH_2Cl_2 (1 mL × 3 times) was added to extract the resultant fatty acid. After the CH_2Cl_2 extract was dried under a stream of N_2, 10 mM Trp, 10 mM HOBt, 10 mM HATU, 0.75 mL of DMF, and 10 μL of DIPEA were added sequentially. After 2 h incubation at room temperature with shaking at 200 rpm, a 10 μL aliquot of the reaction mixture was analyzed by analytical HPLC as described above to acquire the t_R and UV spectrum. To confirm the presence of the *N*-acyl group, a panel of authentic FA-Trp amides was further spiked into the lipopeptide reaction mixture for HPLC analysis.

Bioassays. Minimum inhibitory concentrations (MICs) of daptomycin and its analogues against *S. aureus* 42 were determined by the broth microdilution method according to NCCLS guidelines,[15] except that Mueller-Hinton broth was supplemented with 50 mg/L Ca^{2+} and the assays were performed at 37 °C.[24] Daptomycin (Manufacturing Department, Cubist Pharmaceuticals, Inc.) was used as a positive control.

Acknowledgment. We are grateful to N. Cotroneo and J. Silverman for MIC testing, X. He for small-scale fermentation, H. Cheng for initial LC-MS analyses, and M. Varoglu, W.-K. Pang, P. Herradura, and C. Li for technical assistance. We acknowledge G. J. Heffron of Harvard Medical School NMR facility for recording NMR spectra, W. S. Lane of Harvard Microchemistry and Proteomics Analysis Facility for standard amino acid analysis, and J. Gerhardt of C.A.T. GmbH & Co, Tübingen, Germany, for determination of optical purity of selected amino acids. We also thank D. Baker for comments on the manuscript.

Supporting Information Available: Copies of various NMR spectra of **2**−**4**, LC-MS-MS spectra of **2a**−**4a**, and ^1H NMR data of **3** and **4** summarized in Tables S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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NP0605135