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Chemical synthesis of the S-linked glycopeptide, sublancin

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Sublancin is an S-linked glycopeptide produced by *Bacillus subtilis* 168 and consists of 37 amino acid residues with two disulfide bonds. In this study, we synthesized sublancin by Fmoc-based solid-phase peptide synthesis and chemoselective disulfide formation reactions. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: S-linked glycopeptide; sublancin; solid-phase peptide synthesis; disulfide bond

Sublancin is originally characterized as a lantibiotic produced by *Bacillus subtilis* 168 in 1998 [1]. The proposed structure consisted of 37 amino acid residues with five Cys; four of them formed two disulfide bonds between Cys^{7}-Cys^{36} and Cys^{14}-Cys^{29} and the other, Cys^{22}, linked with Abu^{19} by thioether bond forming methyllanthionine residue. However, the calculated mass number disagreed with the result of mass analysis, and sublancin was presumed to have an additional modification. Recently, sublancin (**1**) was shown not to be a lantibiotic but to be an S-linked glycopeptide containing a glucose attached to Cys^{22} residue as shown in Figure 1 [2]. *S*-glycoside moiety of sublancin was shown to be essential for conferring antimicrobial activity [2]. This S-glycosylation is a quite unique posttranslational modification, and except for sublancin, only one S-linked glycopeptide, GccS, has been reported [3]. This S-linked glycopeptide is an attractive target for chemical synthesis. Until now, several *S*-glycopeptides have been synthesized as analogues of *O*-linked glycopeptides [4–7]. However, all of these analogues were small and linear, and no S-linked glycopeptide with disulfide bonds has not yet been synthesized. In this study, we synthesized sublancin by SPPS and chemoselective disulfide bond formation reactions.

We synthesized the glycoamino acid unit, Fmoc-Cys(Glc·Ac$_4$)-OH (**2**), for Fmoc-based SPPS strategy at first by the procedure shown in Scheme 1. The carboxyl group of Fmoc-Cys(Trt)-OH (**3**) was protected by allyl group using allyl bromide in DMF„ and trityl group on sulfur atom was then removed by TFA/triisopropylsilane (TIS) treatment, giving Fmoc-Cys-OAll (**4**) in 73% yield. Penta-*O*-acetyl-D-glucopyranoside was then treated in 1,2-dichloroethane under acidic conditions, giving tetra-*O*-acetyl-β-D-glucopyranosyl cysteine derivative **5a** in 32% yield. At the same time, α-isomer **5b** was also obtained in 20% yield. Allyl group of **5a** was cleaved by 5,5-dimethyl-1,3-cyclohexanedione and tetrakis(triphenylphosphine) Pd(0) in DME, to give a desired product **2** in 81% yield.

Using this protected glycoamino acid **2**, we attempted to synthesize sublancin **1** by the procedure as shown in Scheme 2. To form the disulfide bonds chemoselectively, Fmoc-Cys(Trt)-OH was used at Cys^{7} and Cys^{36}, and Fmoc-Cys(Acm)-OH was used at positions 14 and 29 in Fmoc-SPPS. After the Fmoc group of Fmoc-Arg(Pbf)-Wang resin was removed by piperidine treatment, Fmoc-Cys(Trt)-OH was introduced by DCC/HOBt method. At this point, to reduce the loading amount of peptide chain, Fmoc-Cys(Trt)-OH was restricted to a half amount of Arg redisue on the resin, and the nonreacted amino group was capped by acetic anhydride treatment. Then, Fmoc-Phe-OH was introduced to the resin by the DCC/HOBt method, and Fmoc-Phe-Cys(Trt)-Arg(Pbf)-OCH$_2$-resin was obtained. The Phe/Arg ratio on the resin measured by amino acid analysis demonstrated that Phe/Arg was 0.33/1, and the loading amount of peptide on the resin reduced to one-third compared with the initial amount. The peptide chain was then elongated manually by the DCC/HOBt method, and the protected peptide resin corresponding to the sublancin sequence was obtained. The peptide was cleaved from the resin using Reagent K [8], and the crude peptide was then treated with 5% hydrazine/10% DMSO aqueous solution containing 6 M urea. This step cleaved acetyl groups on the carbohydrate moiety, and the first disulfide bond between Cys^{7}-Cys^{36} was simultaneously formed by DMSO-assisted oxidation. Cys^{14,29}(Acm)-Sublancin **6** with one disulfide bond was purified by RP-HPLC in 5.0% yield on the basis of the amount of Phe residue of Fmoc-Phe-Cys(Trt)-Arg(Pbf)-OCH$_2$-resin (Figure 2). During these deprotection and oxidation steps, no significant side reaction was observed, and the desired product was obtained in an acceptable yield.

The second disulfide bond between Cys^{14}-Cys^{29} was formed by iodine oxidation. Peptide **6** was dissolved in distilled water, and the solution was added dropwise to an iodine/CH_3OH solution containing HCl. Unexpectedly, the reaction proceeded slower than the usual cases [9,10] and did not finish within 1 h at room temperature. So, we carried out this reaction at 40 °C. The reaction was

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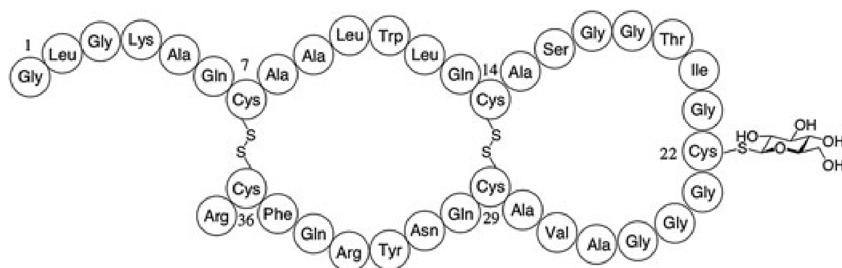


Figure 1. Chemical structure of sublancin **1**.

almost complete within 1 h without significant side reaction, and the desired product, sublancin **1**, was obtained by RP-HPLC purification in 20% yield.

In conclusion, we successfully synthesized S-glycosylated peptide, sublancin, in an acceptable yield. This is the first report of total chemical synthesis of native glycopeptide carrying *S*-glycoside. This synthetic strategy described here might be useful not only for the chemical synthesis of sublancin and its analogues but also for that of the other S-glycosylated peptides such as GccF [3]. Along this line, further synthetic studies of *S*-glycopeptides are now in progress.

Experimental

General

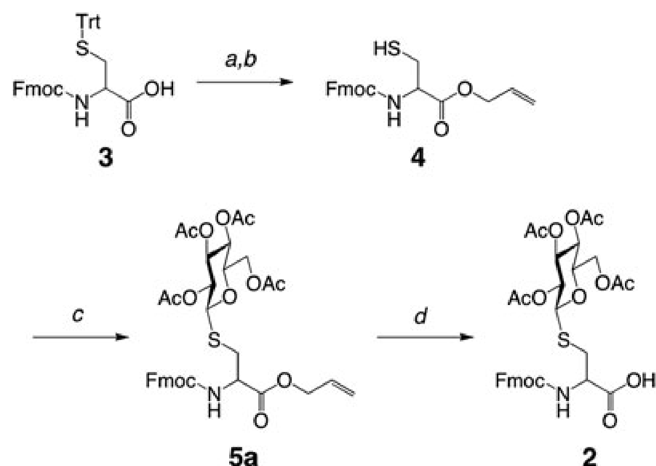
MALDI-TOF mass spectra were recorded using a Voyager DE PRO spectrometer (Applied Biosystems, Carlsbad, CA, USA). Amino acid composition was determined using a LaChrom amino acid analyzer (Hitachi, Tokyo, Japan) after hydrolysis with a 6 M HCl solution at 150 °C for 2 h in a vacuum-sealed tube.

N-(9-fluorenylmethoxycarbonyl)-L-cysteine allyl Ester 4

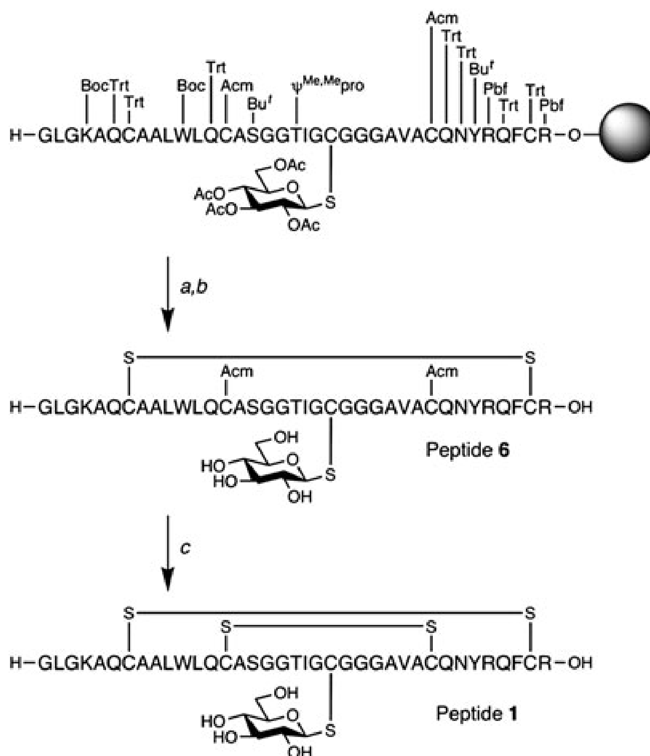
Fmoc-Cys(Trt)-OH **3** (1.2 g, 2.0 mmol) and allyl bromide (0.24 ml, 2.8 mmol) was dissolved in DMF (5 ml) containing DIEA (0.70 ml, 4.0 mmol) and stirred at room temperature overnight. The solution was diluted with ethyl acetate (EtOAc), washed with 1 M HCl, H_2O, and brine and dried over Na_2SO_4. After filtration and concentration in vacuo, the residue was dissolved in 20% TFA/DCM (10 ml) containing TIS (0.82 ml, 4.0 mmol). After stirring at room temperature for 30 min, the solvent was removed under reduced pressure. The residue was chromatographed on silica gel with hexane/EtOAc (75/25) to give Fmoc-Cys-OAll **4** (560 mg, 73%). R_f 0.27 (hexane/EtOAc, 75/25). ^{1}H-NMR ($CDCl_3$): δ 7.79–7.31 (m, 8H, Ar), 5.93 (m, 1H, CH_2 C*H*–), 5.69 (d, 1H, *J* = 7.2 Hz, N*H*), 5.37 (d, 1H, *J* = 17.2 Hz, C*H*$_2$ CH–), 5.30 (d, 1H, *J* = 10.4 Hz, C*H*$_2$ CH–), 4.69 (m, 3H, Cα*H*, –C*H*$_2$–O–), 4.44 (m, 2H, Ar_2CH–C*H*$_2$–), 4.24 (t, 1H, *J* = 6.8 Hz, Ar_2C*H*–CH_2–), 3.02 (m, 2H, CβH). MALDI-TOF mass, found: *m/z* 384.0, calcd: 384.1 for $(M + H)^+$.

N-(9-fluorenylmethoxycarbonyl)-*S*-(tetra-O-acetyl-*β*-D-glucopyranosyl)-L-cysteine Allyl Ester 5a

Compound **4** (190 mg, 0.50 mmol) and penta-*O*-acetyl-D-glucopyranoside (230 mg, 0.60 mmol) were dissolved in 1,2-dichloroethane (2 ml) under Ar atmosphere, and trimethylsilyl triflate (0.11 ml, 0.60 mmol) was then added. The reaction mixture was stirred at 50 °C for 48 h. After the solvent was removed under reduced pressure, the residue was dissolved



Scheme 1. Synthetic route of Fmoc-Cys(Glc·Ac$_4$)-OH **2**. Reaction conditions: (a) All-Br, (1.4 eq.) DIEA (2.0 eq.) in DMF, RT, o/n; (b) TIS (2.0 eq.) in 20% TFA/CH_2Cl_2, RT, 30 min (73% in 2 steps); (c) penta-*O*-acetyl-D-glucopyranoside (1.2 eq.), TMSOTf (1.2 eq.) in 1,2-dichloroethane, 50 °C, 48 h (32%); (d) 5,5-dimethyl-1,3-cyclohexanedione (5.0 eq.), tetrakis(triphenylphosphine)Pd(0) in DME, RT, 1 h (81%).



Scheme 2. Synthetic route of sublancin **1**. Reaction conditions: (a) Reagent K, RT, 2 h; (b) 5% hydrazine/10% DMSO/6 M urea, RT, o/n (5.0% based on Phe[35]); (c) I_2/HCl/CH_3OH/H_2O, 40 °C, 60 min (20%).

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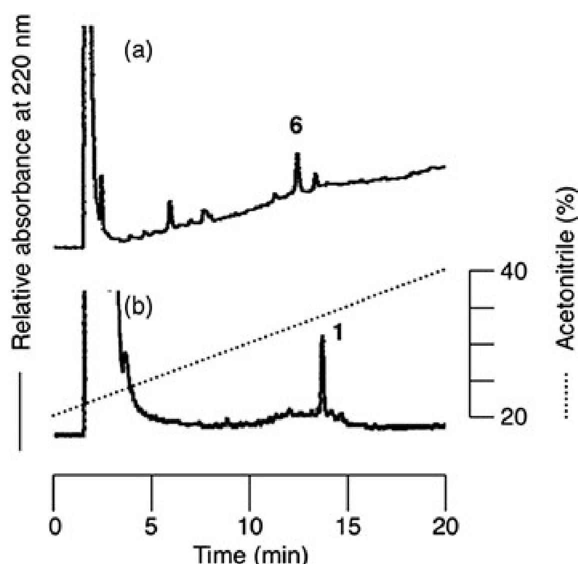

Figure 2. RP-HPLC elution profiles of sublancin. (a) Crude peptide after deprotection and oxidation reactions. (b) After iodine oxidation.

in EtOAc, washed with saturated $NaHCO_3$ aqueous solution and brine, and dried over Na_2SO_4. After filtration, the solution was concentrated in vacuo, and the residue was chromatographed on silica gel with hexane/EtOAc (50/50) to give the desired β-glucosylated compound, Fmoc-Cys(Glc·Ac_4)-OAll **5a** (115 mg, 32%). At the same time, α-glucosylated by-product **5b** was also obtained (71 mg, 20%). R_f 0.30 (hexane/EtOAc, 50/50). ^{1}H-NMR ($CDCl_3$): δ 7.78–7.30 (m, 8H, Ar), 5.92–5.87 (m, 2H, N*H*, CH_2 C*H*–), 5.36–5.21 (m, 3H, *H*-3, C*H*$_2$ CH–), 5.09–4.99 (m, 2H, *H*-2, *H*-4), 4.66 (d, 2H, *J* = 6.0 Hz, –C*H*$_2$–O–), 4.60 (m, 1H, Cα*H*), 4.52 (d, 1H, *J* = 9.6 Hz, *H*-1), 4.49–4.24 (m, 3H, Ar_2C*H*–C*H*$_2$–), 4.12 (m, 2H, *H*$_2$-6), 3.68 (m, 1H, *H*-5), 3.27 (dd, 1H, *J* = 4.4, 14.0 Hz, CβH), 3.05 (dd, 1H, *J* = 6.8, 14.0 Hz, CβH). MALDI-TOF mass, found: *m/z* 736.1, calcd: 736.2 for $(M + Na)^+$.

N-(9-fluorenylmethoxycarbonyl)-*S*-(tetra-O-acetyl-β-D-glucopyranosyl)-L-cysteine 2

Compound **5a** (30 mg, 42 μmol) and 5,5-dimethyl-1,3-cyclohexanedione (30 mg, 210 μmol) were dissolved in DME under Ar atmosphere, and a small amount of tetrakis(triphenylphosphine)Pd(0) was then added to the solution. After stirring at room temperature for 1 h, the solution was diluted with EtOAc, washed with 1 M HCl and brine, and dried over Na_2SO_4. After filtration, the solution was concentrated in vacuo, and the residue was chromatographed on silica gel with toluene/EtOAc/AcOH (50/50/1) to give Fmoc-Cys (Glc·Ac_4)-OH **2** (23 mg, 81%). R_f 0.12 (toluene/EtOAc/AcOH, 50/50/1). ^{1}H-NMR ($CDCl_3$): δ 7.78–7.30 (m, 8H, Ar), 5.96 (d, 1H, *J* = 7.2 Hz, N*H*), 5.22 (t, 1H, *J* = 9.4 Hz, *H*-3), 5.05 (t, 1H, *J* = 9.8 Hz, *H*-4), 4.98 (t, 1H, *J* = 9.8 Hz, *H*-2), 4.65 (m, 1H, Cα*H*), 4.54 (d, 1H, *J* = 9.6 Hz, *H*-1), 4.44 (m, 2H, Ar_2CH–C*H*$_2$–), 4.26–4.15 (m, 3H, *H*$_2$-6, Ar_2C*H*–CH_2–), 3.64 (m, 1H, *H*-5), 3.30 (dd, 1H, *J* = 4.0, 14.0 Hz, CβH), 3.13 (dd, 1H, *J* = 4.0, 14.4 Hz, CβH). MALDI-TOF mass, found: *m/z* 674.1, calcd: 674.2 for $(M + H)^+$.

$Cys^{14,29}$(Acm)-Sublancin 6

Fmoc-Arg(Pbf)-Wang resin (0.56 mmol/g, 360 mg, 0.20 mmol) was swelled in 1-methyl-2-pyrrolidinone (NMP) for 30 min and treated with 20% piperidine/NMP for 5 and 15 min. After washing with NMP, Fmoc-Cys(Trt)-OBt, which was prepared by mixing Fmoc-Cys (Trt)-OH (0.1 mmol), 1 M DCC/NMP (150 μl), and 1 M HOBt/NMP (150 μl) at room temperature for 30 min, was added, and the reaction mixture was vortexed at 50 °C for 1 h. The resin was washed with NMP and 50% DCM/CH_3OH, treated with 10% Ac_2O/5% DIEA/NMP for 5 min, and washed with NMP. Fmoc-Phe-OH was then introduced to the resin by the DCC/HOBt method using Fmoc-Phe-OH (0.4 mmol), and Fmoc-Phe-Cys(Trt)-Arg(Pbf)-OCH_2-resin was obtained. At this stage, Phe/Arg ratio on the resin was measured by amino acid analysis. The result showed that Phe/Arg was 0.33/1, and the total loading amount of Phe residue on the resin was estimated at 66 μmol. After washing the resin with NMP, the peptide chain was elongated manually by the Fmoc-SPPS. The amino acids (0.30 mmol each) were activated by mixing with 1 M DCC/NMP (0.4 ml) and 1 M HOBt/NMP (0.4 ml) at room temperature for 30 min, and the coupling reaction was carried out at 50 °C for 1 h, giving the protected peptide resin corresponding to the sublancin(23–37) sequence. After a half amount of the resin was removed, the peptide chain elongation was continued by the same manner as described previously, except that Fmoc-Gly-Thr($\psi^{Me,Me}$pro)-OH dipeptide unit (0.07 mmol) was used as a building block at Gly^{18}-Thr^{19} site. After elongation, H-Gly-Leu-Gly-Lys(Boc)-Ala-Gln(Trt)-Cys(Trt)-Ala-Ala-Leu-Trp (Boc)-Leu-Gln(Trt)-Cys(Acm)-Ala-Ser (Bu^t)-Gly-Gly-Thr($\psi^{Me,Me}$pro)-Ile-Gly-Cys(Glc·Ac_4)-Gly-Gly-Gly-Ala-Val-Ala-Cys(Acm)-Gln(Trt)-Asn(Trt)-Tyr(Bu^t)-Arg(Pbf)-Gln(Trt)-Phe-Cys (Trt)-Arg(Pbf)-OCH_2-resin (388 mg) was obtained. A part of the resin (10 mg) was treated with Reagent K (200 μl) at room temperature for 2 h. TFA was removed under nitrogen stream, and the peptide was precipitated with diethyl ether. After washing twice with ether, the precipitant was dried under vacuum. The crude peptide was dissolved in 50% CH_3CN/0.1% TFA aqueous solution, and the insoluble material was removed by filtration. After lyophilization, the crude peptide was dissolved in 6 M urea/5% hydrazine/10% DMSO aqueous solution (2 ml), and the solution was stirred at room temperature for overnight. The reaction mixture was separated by RP-HPLC on a Mightysil RP-18 GP column (Kanto Kagaku, Tokyo, Japan) with a linear gradient of acetonitrile containing 0.1% TFA, to give peptide **6** (43 nmol, 5.0% yield). MALDI-TOF mass, found: *m/z* 4020.9, calcd: 4020.9 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{1.04}Thr_{0.70}Ser_{0.77}Glu_{4.86}Gly_{7.71}Ala_6Cys_{0.39}Val_{1.10}Ile_{0.86}Leu_{2.47}Tyr_{1.03}Phe_{1.08}Lys_{0.79}Arg_{2.05}$.

Sublancin 1

Peptide **6** (19 nmol) was dissolved in distilled water (200 μl), and the solution was added dropwise to CH_3OH containing 20 mM I_2/CH_3OH (80 μl) and 6 M HCl (20 μl) within 5 min with mixing. After the resultant solution was stirred for another 55 min at 40 °C, the reaction was quenched by adding ascorbic acid aqueous solution. The reaction mixture was separated by RP-HPLC on a Mightysil RP-18 GP column with a linear gradient of acetonitrile containing 0.1% TFA, to give the desired product **1** (3.8 nmol, 20% yield). MALDI-TOF mass, found: *m/z* 3876.4, calcd: 3876.8 for $(M + H)^+$ (average). Amino acid analysis: $Asp_{1.14}Thr_{0.91}Ser_{1.28}Glu_{4.52}Gly_{8.55}Ala_6Cys_{0.89}Val_{0.97}Ile_{0.94}Leu_{2.88}Tyr_{0.99}Phe_{1.08}Lys_{0.98}Arg_{2.02}$.

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