JOC The Journal of Organic Chemistry

Article

pubs.acs.org/joc

Synthesis of Cyclic Peptides Containing a Thioester Handle for Native Chemical Ligation

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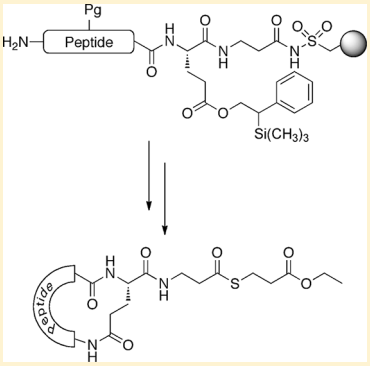
Supporting Information

ABSTRACT: The synthesis of cyclic peptides containing a thioester handle using a sulfo-click linker is reported. These cyclic peptides can be coupled to N-terminal cysteine-containing constructs via native chemical ligation. A successful application of a cyclic peptide bearing a thioester handle in native chemical ligation is shown by a high yielding ligation.

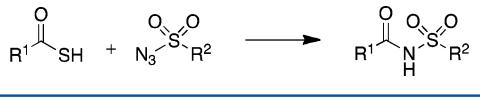


INTRODUCTION

C-Terminal peptide thioesters are crucial intermediates in the synthesis of peptides and proteins by native chemical ligation (NCL), which is a chemoselective reaction between two unprotected peptides, one containing an N-terminal cysteine residue and the other containing a C-terminal peptide thioester. Native chemical ligation starts with a reversible transthioesterification reaction between a C-terminal thioester and the sulfhydryl group of the N-terminal cysteine residue, followed by a rapid and irreversible reaction comprising a *S*- to *N*-acyl shift to yield a native peptide bond.[1] While C-terminal peptide thioesters traditionally have been accessed by Boc-based solid-phase peptide synthesis (SPPS),[2] their synthesis with Fmoc-based methods is difficult due to reaction of the thioesters with piperidine during the Fmoc deprotection step. To circumvent this problem, safety-catch linkers were developed for the Fmoc-based SPPS of peptide thioesters.[3] An excellent example of such a linker is the *N*-acyl sulfonamide linker, developed by Kenner et al.[4] This linker is completely stable toward basic or strongly nucleophilic conditions and thus compatible with Fmoc-based SPPS. Only after N-alkylation of the *N*-acyl sulfonamide does the linker become sensitive to mild nucleophilic cleavage, for example by a thiol to yield a C-terminal peptide thioester.

The original synthesis of the Kenner linker resulted in poor loading efficiencies and racemization in the loading step. Backes and Ellman addressed these issues by using a more nucleophilic aliphatic *N*-acyl sulfonamide and by lowering the temperature during the loading step to minimize the amount of racemization.[5] Although these modifications meant a large improvement compared to the original protocol, the loading yield and epimerization of the first amino acid was still a major concern. To address these issues we have previously developed a method in which the "sulfo-click" reaction was used to obtain an *N*-acyl sulfonamide linker. The sulfo-click reaction is a fast, high yielding, and chemoselective reaction between a sulfonyl azide and a thioacid, which yields an *N*-acyl sulfonamide (Scheme 1).[6−8] For preparation of the resin-bound linker, a resin-bound sulfonyl azide was allowed to react with an N-terminal-protected amino thioacid to form an *N*-acyl sulfonamide linker with high loading efficiencies and without epimerization of the first amino acid.[9]

Scheme 1. Reaction between a Sulfonyl Azide and a Thioacid[6] Denoted as the "Sulfo-Click" Reaction[7,8]



To our knowledge, synthesis of C-terminal peptide thioesters has not been applied to more complex systems such as cyclic peptides, containing a thioester handle. Cyclization of peptides is believed to lead to increased potency, metabolic stability, bioavailability, and receptor selectivity.[10] Thus, cyclic peptides are an important class in peptide drug design. The inclusion of a thioester functionality allows, for example, the facile introduction of an additional peptide sequence (vide infra), a label, or chelating moiety.

Received: July 23, 2012
Published: October 18, 2012

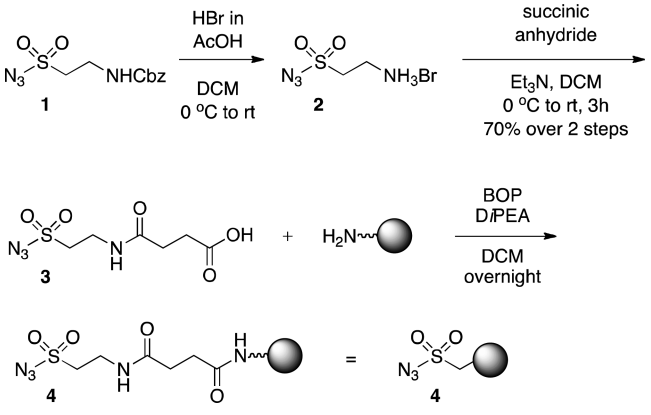
RESULTS AND DISCUSSION

In this paper we describe a general method for the SPPS of cyclic peptides containing a thioester handle. This approach provides access to cyclic peptides, which can be coupled to N-terminal cysteine-containing molecular constructs via native chemical ligation. To illustrate the general applicability of this method, three different cyclic peptide sequences were synthesized corresponding to the loops present in the HIV-gp120 interacting with CD4 as was found in the X-ray structure of the gp120-CD4 complex. On the basis of this structure, the ^{365}SGGDPEIVT^{373}, ^{424}INMWQEVGKA^{433}, and ^{454}LTRDGGN^{460} peptide sequences were selected for preparation of cyclic peptide thioesters.[11,12] By attachment to the CD4 receptor, HIV-gp120 plays a crucial role in the first steps of HIV-infection. Preventing attachment of gp120 to cells and/or using gp120 as a starting point to develop a vaccine may represent alternative approaches to avoid further spread of HIV.

Crucial for a successful solid-phase approach for obtaining cyclic peptide thioesters is a linker, which is high yielding, stable during SPPS conditions, gives no racemization, and liberates C-terminal thioester after a mild cleavage. Although the Backes and Ellman protocol largely meets these requirements, we found that the sulfo-click approach, was especially useful for obtaining good loading yields.[9]

The Cbz-protected sulfonyl azide **1** required for the linker was convienently synthesized on a large scale starting from taurine.[13] Next, the Cbz group was cleaved by using hydrogen bromide in acetic acid, followed by a reaction with succinic anhydride to yield sulfonyl azide **3**. Attachment of **3** to an amino methyl resin, using a BOP coupling, gave sulfo-click linker-containing resin **4** (Scheme 2).

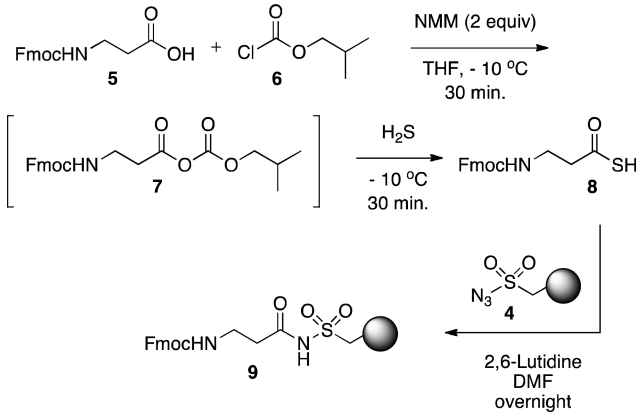
Scheme 2. Synthesis of the Sulfo-Click Linker



Next, a sulfo-click reaction was performed between the resin-bound sulfonyl azide **4** and a spacer amino thioacid, Fmoc-β-Ala-SH (**8**), to obtain *N*-acyl sulfonamide resin **9**. Fmoc-β-Ala-SH (**8**) was obtained in a one-pot synthesis, starting from the corresponding amino acid **5** via a mixed anhydride **7**, followed by a treatment with hydrogen sulfide (Scheme 3).[14] In general, thioacids are instable due to dimerization and hydrolysis. Thus, it was decided to use the relatively pure crude Fmoc-β-Ala-SH directly in the chemoselective sulfo-click reaction. The loading of this resin was obtained by an Fmoc determination and was 0.19 mmol·g^{-1}, indicative of a yield of 94% per reaction step in this three-step sequence.

For the preparation of the cyclic peptides, we decided to use the previously applied on-resin tail-to-side chain cyclization

Scheme 3. Preperation of the Amino Thioacid and the Resin-Bound *N*-Acyl Sulfonamide



method.[11] In this approach a glutamic acid residue containing a (2-phenyl-2-trimethylsilyl)ethyl (PTMSE) protecting group was used as a cyclization hinge. The PTMSE group is orthogonal to the acid-labile amino acid side-chain protecting groups.[11,15]

Thus, after introduction of the Glu(OPTMSE) residue on *N*-acyl sulfonamide resin **9**, the peptide sequence corresponding to each of the loops was assembled by SPPS. Selective cleavage of the PTMSE group by TBAF in DCM was followed by N-terminus-to-side chain cyclization of the resin-bound peptide (**11a**–**c**) using BOP and HOBt. The *N*-acyl sulfonamide linker (**12a**–**c**) was then alkylated by treatment with trimethylsilyldiazomethane, followed by cleavage of the protected cyclic peptide thioester (**13a**–**c**) from the resin using a mixture of ethyl 3-mercaptopropionate, D*i*PEA, and sodium thiophenolate in DMF. Finally, the acid-labile side-chain protecting groups of peptide (**13a**–**c**) were removed without affecting the thioester and the resulting free cyclic peptide thioesters **14a**–**c** were purified by preparative HPLC (Scheme 4).

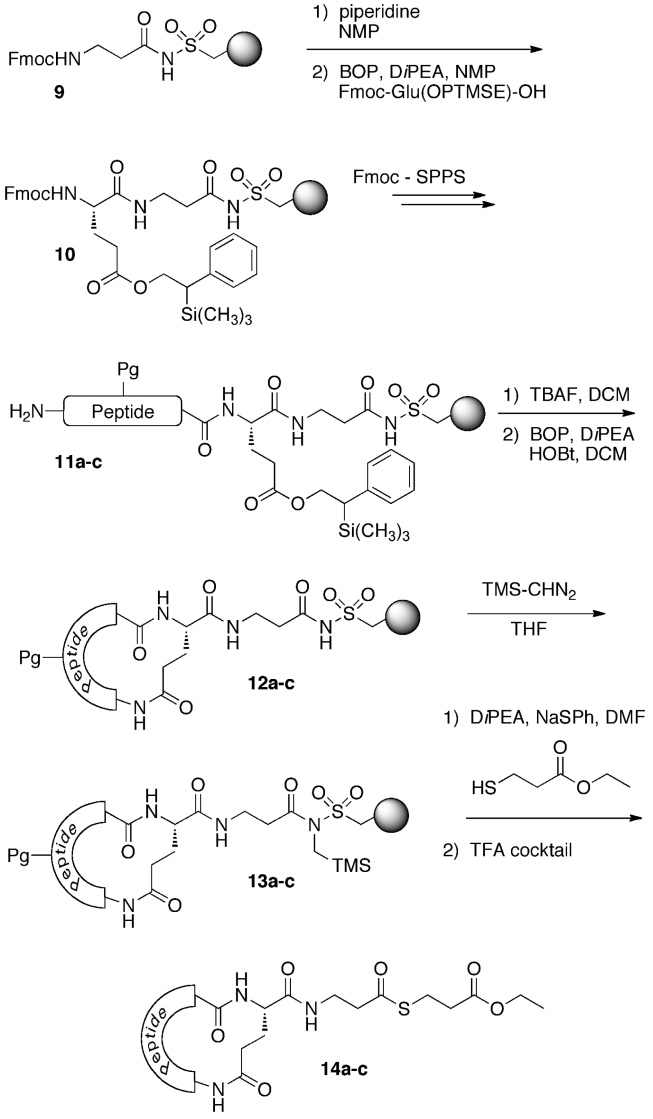
It was found that it was necessary to repeat the thiolysis step at least twice to obtain an optimal yield of the cyclic peptide thioester. This rather difficult thiolysis might be attributed to the large sterical hindrance imposed by the tertiary amide to be cleaved by the thiolate nucleophile. Nevertheless, in this way cyclic peptide thioesters having amino acid sequences SGGDPEIVT (**14a**), INMWQEVGKA (**14b**), and LTRDGGN (**14c**) were obtained in overall yields of 6.7%, 5.6%, and 7.0%, respectively. This corresponded to a good average yield per step of 90%, 89%, and 87%, respectively (Figure 1).

To illustrate the ability of these cyclic peptide thioesters to act as a substrate, NCL was carried out with cyclic peptide thioester **14c** and N-terminal cysteine-containing antimicrobial HHC-10 peptide (H-CKRWWKWIRW-NH$_2$, **15**).[16] Ligation, performed under conditions comprising 1 mM peptide concentration in ligation buffer (200 mM sodium phosphate, 200 mM 4-mercaptophenylacetic acid (MPAA), 40 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and pH 7.4) did not furnish the expected product. It was found that peptide **15** did not dissolve sufficiently in the ligation buffer and that addition of ca. 20% DMF to the ligation buffer resulted in a clear reaction mixture and a complete conversion to ligation product **16** within 3 h. After preparative HPLC, **16** was obtained in an excellent yield of 92% (Scheme 5).

Prior to purification of the ligated peptide **16**, the reaction mixture was first treated with an aqueous TFA solution,

dx.doi.org/10.1021/jo3015566 | J. Org. Chem. 2012, 77, 10058–10064

Scheme 4. General SPPS Procedure for the Preparation of Cyclic Peptides Containing a Thioester Handle



followed by an extraction with ether in order to completely remove MPAA, leading to a relatively clean crude product (Figure 2).[17]

In conclusion, we have described a general and efficient method for the SPPS of cyclic peptides containing a thioester handle featuring the use of a sulfo-click linker.[9] By this method, three different HIV-gp120 peptide loops present in the HIV-gp120−CD4 complex were successfully synthesized. The successful application in native chemical ligation was illustrated by a high yielding ligation of cyclic peptide thioester **14c** and N-terminal cysteine-containing antimicrobial HHC-10 peptide **15**.

EXPERIMENTAL SECTION

General Experimental Methods. All solvents and reagents were used without further purification. Side-chain-protected amino acids used were as follows: Fmoc-Ser(tBu)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH. To prevent aspartimide formation in the sequence of loop **14c** (^{457}Asp-Gly^{458}), Fmoc-(Dmb)Gly-OH was used instead of Fmoc-Gly-OH. Solid-phase peptide synthesis was

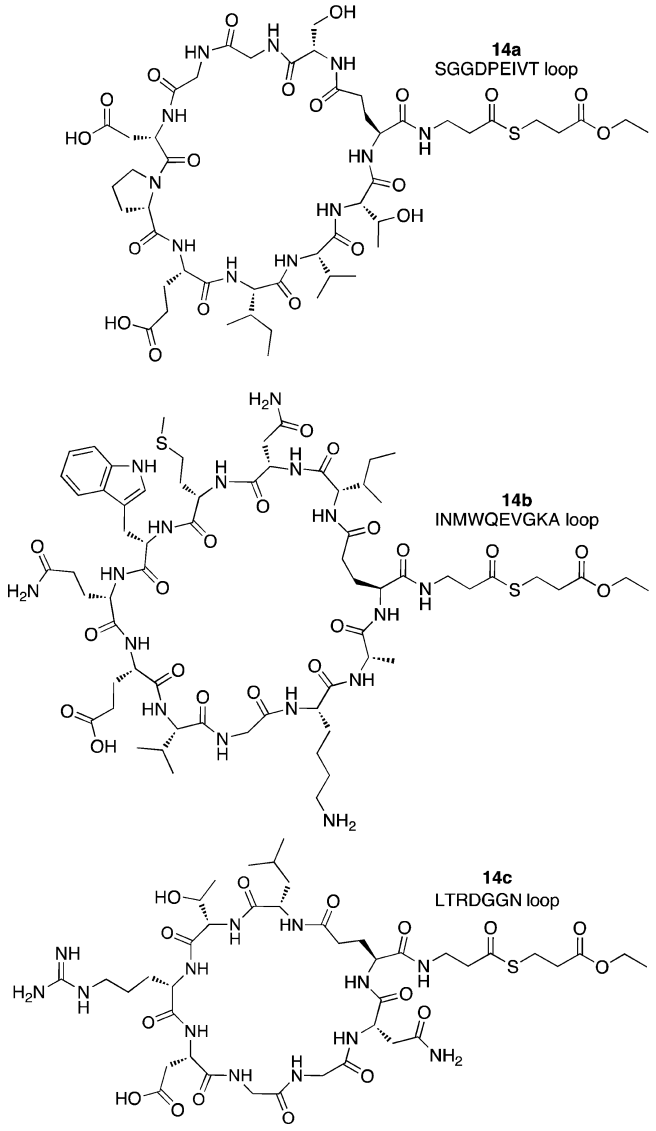
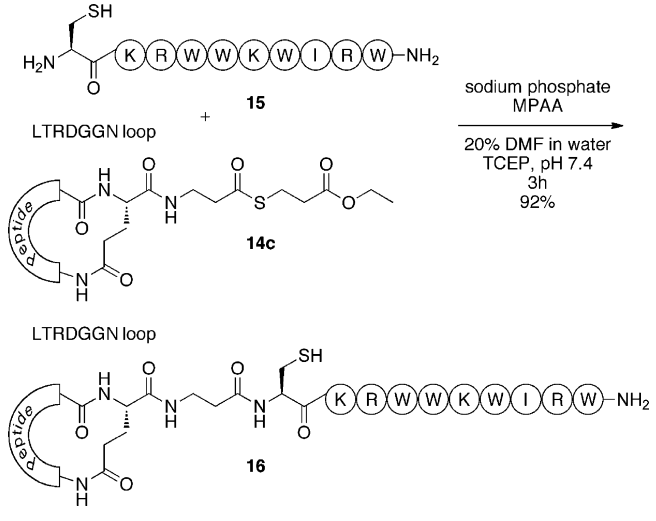


Figure 1. Structures of the SGGDPEIVT (**14a**), INMWQEVGKA (**14b**), and LTRDGGN (**14c**) peptide thioester loops.

Scheme 5. NCL between LTRDGGN Loop 14c and H-CKRWWKWIRW-NH$_2$ Peptide 15



dx.doi.org/10.1021/jo3015566 | J. Org. Chem. 2012, 77, 10058−10064

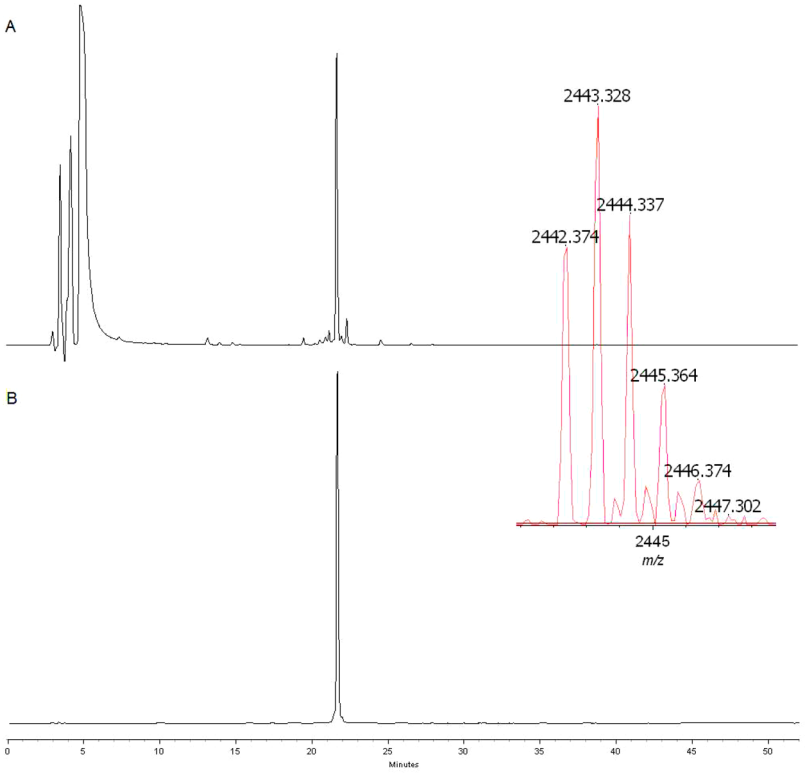


Figure 2. NCL between LTRDGGN loop **14c** and N-terminal cysteine-containing antimicrobial HHC-10 peptide **15**. Analytical HPLC profiles (λ = 214 nm) together with MALDI-TOF data. (A) Crude reaction mixture after 3 h. (B) Ligation product **16** after purification with preperative HPLC.

carried out on an amino methyl resin in a plastic syringe with a polyethylene frit. Loading of a resin sample was assessed by Fmoc-spectrophotometric quantification[18] of the absorbance of the dibenzofulvene−piperidine adduct at 300 nm in a UV/Vis spectrometer. Kaiser[19] and bromophenol blue tests[20] were used for detection on the solid phase of primary and secondary amines, respectively. The capping solution used was a mixture of 0.5 mol/L acetic anhydride, 0.125 mol/L D*i*PEA, and 0.015 mol/L HOBt in NMP. MALDI-TOF-MS spectra were obtained by using sinapic acid or α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix and synthetic peptide $P_{14}R$ (monoisotopic $[M + H]^+$ 1533.8582) as a reference. The high-resolution mass spectrum was obtained by electrospray ionization (ESI) in negative mode and calibrated with an ESI tuning mix. Reactions in solution were monitored by TLC analysis. Spots were visualized by heating plates after dipping in a ninhydrin solution or Cl_2/TDM[21] treatment. ^{1}H NMR data was acquired on a 300 MHz spectrometer in DMSO-d_6 as solvent. Chemical shifts (δ) are reported in ppm relative to TMS (0.00 ppm). ^{13}C NMR data was acquired on a 75 MHz spectrometer in DMSO-d_6 as solvent. Chemical shifts (δ) are reported in ppm relative to the solvent residual signal, DMSO-d_6 (39.52 ppm). 2-D NMR data (HSQC, HMBC, and TOCSY) were acquired on a 500 MHz spectrometer. ^{1}H NMR and ^{13}C NMR spectra of cyclic peptide thioesters **14a−c** were recorded on a 500 MHz spectrometer in H_2O/D_2O (9:1, v/v) using ^{1}H−^{13}C HSQC, NOESY, and TOCSY sequences at 283.15 K. Chemical shifts (δ) are reported in ppm relative to 1,4-dioxane (^{1}H NMR: 3.75 ppm, ^{13}C NMR: 67.19). Analytical HPLC was accomplished by using a C18 column (110 Å, 5 μm, 250 × 4.60 mm) at a flow rate of 1 mL min^{-1} using a standard protocol: 100% buffer A for 1 min, followed by a linear gradient of buffer B (0−100% in 30 min, method A) or 100% buffer A for 2 min, and next a linear gradient of buffer B (0−100% in 48 min, method B). The mobile phase was H_2O/CH_3CN/TFA (95:5:0.01, v/v/v, buffer A) and H_2O/CH_3CN/TFA (5:95:0.01, v/v/v, buffer B). Purification of the peptides was performed on a Prep LCMS system using a C18 column (10 μm, C18, 110 Å, 250 × 20 mm) at a flow rate of 12.5 mL min^{-1} using a standard protocol: 100% buffer A for 5 min followed by a linear gradient of buffer B (0−100% in 100 min) using the same buffers as described for analytical HPLC.

General Procedure for Fmoc Removal. The resin (1 equiv) was swollen in NMP (20 mL mmol^{-1} for 2 min). After the solvent was drained, the resin was shaken with piperidine/NMP (1:4, v/v, 3 × 40 mL mmol^{-1}, each 10 min) and washed with NMP (3 × 20 mL mmol^{-1}, each 2 min) and DCM (3 × 20 mL mmol^{-1}, each 2 min). A positive Kaiser test and/or bromophenol blue test indicated Fmoc removal.

General Procedure for Amino Acid Coupling. The Fmoc-deprotected resin (1 equiv) was swollen in NMP (20 mL mmol^{-1} for 2 min). After the solvent was drained, the resin was shaken with an Fmoc-amino acid (4 equiv), BOP (4 equiv), and D*i*PEA (8 equiv) in NMP (40 mL mmol^{-1}) for 90 min and washed with NMP (3 × 20 mL mmol^{-1}, each 2 min) and DCM (3 × 20 mL mmol^{-1}, each 2 min). Fmoc-Glu(OPTMSE)-OH was coupled overnight using 2 equiv of amino acid, 2 equiv of BOP, and 4 equiv of D*i*PEA with DCM as the solvent. Fmoc-Asp(OtBu)-OH was "double coupled" on DMB-Gly (loop **14c**), following the normal coupling procedure. A negative Kaiser test and/or bromophenol blue test indicated a complete coupling of the Fmoc-amino acid.

General Procedure for Capping of the Resin. The resin (1 equiv) was swollen in NMP (20 mL mmol^{-1} for 2 min), and the solvent was drained. The capping solution (20 mL mmol^{-1}) was added, and the resin was shaken for 30 min. The resin was washed with NMP (3 × 20 mL mmol^{-1}, each 2 min) and DCM (3 × 20 mL mmol^{-1}, each 2 min).

General Procedure for PTMSE Removal. The resin (1 equiv) was swollen in DCM (20 mL mmol^{-1} for 2 min). After the solvent was drained, the resin was shaken twice with TBAF·3H_2O (4 equiv) in DCM (40 mL mmol^{-1}) for 15 min. The resin was washed with DCM (4 × 20 mL mmol^{-1}, each 2 min).[11]

General Procedure for Solid-Phase Cyclization. The resin (1 equiv) was swollen in DMF (20 mL mmol^{-1} for 2 min), and the solvent was drained. BOP (4 equiv), HOBt (4 equiv), and DMF (40 mL mmol^{-1}) were added, the mixture was shaken until complete dissolution, D*i*PEA (8 equiv) was added, and the reaction was shaken

dx.doi.org/10.1021/jo3015566 | J. Org. Chem. 2012, 77, 10058−10064

overnight. The resin was washed with DMF (3 × 20 mL mmol^{-1}, each 2 min) and DCM (3 × 20 mL mmol^{-1}, each 2 min). A negative Kaiser test and bromophenol blue test indicated successful cyclization.

General Procedure for the Alkylation of the Resin with TMS-CHN$_2$. The resin (1 equiv) was swollen in THF (20 mL mmol^{-1} for 2 min). After the solvent was drained, the resin was shaken overnight with TMS-CHN$_2$ (50 equiv, 2 M solution in diethyl ether) in THF (20 mL mmol^{-1}) and then washed with THF (3 × 20 mL mmol^{-1}, each 2 min) and DCM (3 × 20 mL mmol^{-1}, each 2 min).

General Procedure for the Cleavage from the Resin. The resin (1 equiv) was swollen in DMF (20 mL mmol^{-1} for 2 min). After the solvent was drained, the resin was shaken overnight with ethyl 3-mercaptopropionate (50 equiv), D*i*PEA (10 equiv), and sodium thiophenolate (0.5 equiv) in DMF (20 mL mmol^{-1}). Finally, the resin was washed with DMF (4 × 20 mL mmol^{-1}, each 2 min), and the filtrates were combined and evaporated in vacuo.

General Procedure for the Side-Chain Deprotection of a Peptide Thioester. The crude protected peptide thioester (1 equiv) was treated with TFA/H$_2$O/TIPS (95:2.5:2.5, v/v/v) (20 mL mmol^{-1}) for 2 h. Next, the mixture was reduced to 2 mL by evaporation and was added dropwise to a cold (4 °C) solution of MTBE/hexanes (1:1, v/v). After centrifugation (3500 rpm, 5 min), the supernatant was decanted and the pellet was resuspended in MTBE/hexanes (1:1, v/v) and centrifuged again. Finally, the pellet was washed twice with MTBE/hexanes (1:1, v/v), each time collected by centrifugation, and dissolved in *t*BuOH/H$_2$O (1:1, v/v) followed by lyophilization.

Synthesis of Sulfonyl Azide 3. HBr (33 wt % in AcOH, 105 mL, 600 mmol, 8.6 equiv) was added dropwise to a solution of Cbz-protected taurylsulfonyl azide **1**[13] (19.90 g, 70 mmol, 1 equiv) in DCM (350 mL) at 0 °C. After being stirred for 3 h at room temperature, the reaction mixture was evaporated in vacuo to dryness and coevaporated with toluene. The residue was dissolved in H$_2$O and washed three times with DCM. The aqueous layer was evaporated in vacuo and coevaporated three times with toluene, yielding crude hydrobromide salt **2**, which was dried over P_2O_5 overnight. Next, succinic anhydride (7.00 g, 70 mmol, 1 equiv) and Et$_3$N (11.1 mL, 154 mmol, 2.2 equiv) were added to a solution of the crude hydrobromide **2** in DCM (350 mL) at room temperature, and the mixture was stirred overnight. The reaction mixture was acidified using HCl/Et$_2$O (100 mL), followed by the evaporation of the solvents in vacuo. The crude product was dissolved by heating in EtOAc and washed with 1 N HCl. The water layer was washed twice with EtOAc, after which the organic layers were combined, dried over Na_2SO_4, and evaporated in vacuo. The crude sulfonyl azide **3** was recrystallized from EtOAc–hexanes, giving sulfonyl azide **3** as a colorless crystalline solid (12.30 g, 70% over two steps). R_f = 0.41 (DCM/MeOH/AcOH, 89.9:10:0.1, v/v/v); mp = 127–129 °C; HPLC (see below): t_R = 11.6 min (method B); HRMS *m*/*z* calcd for $C_6H_9N_4O_5S$ $[M - H]^-$ 249.0299, found 249.0307; ^{1}H NMR (300 MHz, DMSO-d_6): δ 2.33 (t, J = 6.6 Hz, 2H, CH_2CH_2COOH), 2.42 (t, J = 6.7 Hz, 2H, CH_2CH_2COOH), 3.50 (q, J = 6.0 Hz, 2H, $N_3SO_2CH_2CH_2$), 3.83 (t, J = 6.3 Hz, 2H, $N_3SO_2CH_2CH_2$), 8.22 (bs, 1H, NHCO), 12.08 (bs, 1H, COOH); ^{13}C NMR (75 MHz, DMSO-d_6): δ 28.9 (CH_2CH_2COOH), 29.9 (CH_2CH_2COOH), 33.5 ($N_3SO_2CH_2CH_2$), 54.1 ($N_3SO_2CH_2CH_2$), 171.6 (NHCO), 173.8 (COOH). Moreover, HSQC, HMBC and TOCSY were in accord to the proposed structure (see the Supporting Information).

General Procedure for the Synthesis of Resin-Bound Sulfonyl Azide 4. Amino methyl resin (0.25 mmol·g^{-1}, 1 equiv) was swollen in NMP (20 mL mmol^{-1} for 10 min), and the solvent was drained. Subsequently, the resin was washed with HOAc/NMP (1:1, v/v, 2 × 40 mL mmol^{-1}, each 10 min), NMP (3 × 40 mL mmol^{-1}, each 2 min), D*i*PEA/NMP (1:4, v/v, 2 × 40 mL mmol^{-1}, each 10 min), NMP (3 × 40 mL mmol^{-1}, each 2 min), and DCM (3 × 40 mL mmol^{-1}, each 2 min).[9] A solution of compound **3** (2 equiv), BOP (2 equiv), and D*i*PEA (4 equiv) in DCM (40 mL mmol^{-1}) was stirred for 5 min. Next, the solution was added to the resin and the resin was shaken overnight. The resin was washed with DCM (4 × 20 mL mmol^{-1}, each 2 min), dried in vacuo, and stored for further use. Negative Kaiser and bromophenol blue tests indicated a successful coupling.

General Procedure for the Synthesis of Thioacid 8. Fmoc-β-Ala-OH (**5**, 1 equiv) was dissolved in THF (2.5 mL mmol^{-1}), and the mixture was cooled to −10 °C. Next, *N*-methylmorpholine (2 equiv) and isobutyl chloroformate (1 equiv) were added at −10 °C under N$_2$; immediately a white precipitate was formed. The mixture was stirred for 30 min under N$_2$ at −10 °C, and a slow stream of in situ-generated H$_2$S (prepared by dropwise addition of a solution of H$_2$SO$_4$/H$_2$O (1:1, v/v) to NaSH·*x*H$_2$O (10 equiv)) was bubbled through the solution.[14] After the reaction mixture was stirred for 30 min under N$_2$ at −10 °C, it was concentrated in vacuo. Next, the crude was dissolved in EtOAc, washed with 5% aqueous KHSO$_4$, dried over Na_2SO_4, and concentrated in vacuo to yield relatively pure crude thioacid **4**, which was used immediately in the sulfo-click reaction. R_f = 0.52 (EtOAc/AcOH, 99.9:0.1, v/v).

General Procedure for the Synthesis of *N*-Acylsulfonamide Resin 9. Resin-bound sulfonyl azide **4** (1 equiv) was swollen in DMF (20 mL mmol^{-1} for 10 min) after which it was drained. The resin was shaken with 2,6-lutidine (80 equiv), thioacid **8** (4 equiv), and DMF (20 mL mmol^{-1}) overnight. Next, the resin was washed with DMF (3 × 20 mL mmol^{-1}, each 2 min), DCM (3 × 20 mL mmol^{-1}, each 2 min), MeOH (3 × 20 mL mmol^{-1}, each 2 min), and diethyl ether (3 × 20 mL mmol^{-1}, each 2 min).[9] After the resin was dried in vacuo for 3 h, the loading was determined by an Fmoc-spectrophotometric quantification.[18] Starting from 0.5 mmol of amino methyl resin (0.25 mmol·g^{-1}), the found loading was 0.19 mmol·g^{-1} and corrected for the added mass 0.21 mmol·g^{-1}, indicative for a yield of 94% per reaction step in this three-step sequence.

Synthesis of Cyclic Peptide Thioester 14a. Following the above-described general procedures, crude lyophilized cyclic peptide thioester SGGDPEIVT **14a** was obtained. Crude peptide **14a** was dissolved in H$_2$O/CH$_3$CN/TFA (50:50:0.01, v/v/v) and purified by preparative HPLC. Fractions corresponding to cyclic peptide **14a** were pooled and lyophilized to yield SGGDPEIVT loop **14a** as a white fluffy solid (29.2 mg, 6.7% overall yield starting from 0.37 mmol of *N*-acyl sulfonamide resin **9**). t_R = 16.5 min (method A); MALDI-TOF MS *m*/*z* calcd for $C_{49}H_{78}N_{11}O_{20}S$ $[M + H]^+$ 1172.51, found 1172.59; calcd for $C_{49}H_{77}N_{11}NaO_{20}S$ $[M + Na]^+$ 1194.50, found 1194.61; calcd for $C_{49}H_{77}N_{11}KO_{20}S$ $[M + K]^+$ 1210.47, found 1210.56. ^{1}H NMR (500 MHz, H$_2$O/D$_2$O (9:1, v/v)): δ Ser-1: 8.37 (NH), 4.50 (αCH), 3.82/3.91 (βCH_2); Gly-2: 8.72 (NH), 3.93 (αCH_2); Gly-3: 8.44 (NH), 3.93 (αCH_2); Asp-4: 8.13 (NH), 5.05 (αCH), 2.81/3.03 (βCH_2); Pro-5: 4.45 (αCH), 1.99/2.30 (βCH_2), 2.03 (γCH_2), 3.84 (δCH_2); Glu-6: 8.01 (NH), 4.30 (αCH), 2.03/2.19 (βCH_2), 2.50 (γCH_2); Ile-7: 7.77 (NH), 4.20 (αCH), 1.92 (βCH), 0.91 (γCH_3), 1.16/1.44 (γCH_2), 0.86 (δCH_3); Val-8: 8.23 (NH), 4.17 (αCH), 2.15 (βCH), 0.97 (γCH_3), 0.97 (γCH_3); Thr-9: 7.90 (NH), 4.33 (αCH), 4.19 (βCH), 1.21 (γCH_3); Glu-10: 8.42 (NH), 4.23 (αCH), 1.96/2.13 (βCH_2), 2.46 (γCH_2); β-Ala-11: 8.17 (NH), 3.50 (αCH_2), 2.84 (βCH_2); thioester tail: 2.68 (CH_2), 3.11 (CH_2), 4.15 (CH_2), 1.24 (CH_3). ^{13}C NMR (500 MHz, H$_2$O/D$_2$O (9:1, v/v)): δ Ser-1: 56.2 (αCH), 62.3 (βCH_2); Gly-2: 43.1 (αCH_2); Gly-3: 43.1 (αCH_2); Asp-4: overlap with H$_2$O (αCH), 36.3 (βCH_2); Pro-5: 62.1 (αCH), 29.9 (βCH_2), 25.1 (γCH_2), 48.8 (δCH_2); Glu-6: 54.3 (αCH), 26.3 (βCH_2), 31.3 (γCH_2); Ile-7: 59.2 (αCH), 36.7 (βCH), 15.5 (γCH_3), 25.2 (γCH_2), 10.6 (δCH_3); Val-8: 60.9 (αCH), 30.5 (βCH), 18.9 (γCH_3), 18.9 (γCH_3); Thr-9: 59.9 (αCH), 67.4 (βCH), 19.7 (γCH_3); Glu-10: 53.8 (αCH), 27.5 (βCH_2), 32.3 (γCH_2); β-Ala-11: 36.3 (αCH_2), 43.1 (βCH_2); thioester tail: 34.5 (CH_2), 24.4 (CH_2), 62.6 (CH_2), 13.9 (CH_3).

Synthesis of Cyclic Peptide Thioester 14b. Following the above-described general procedures, crude lyophilized cyclic peptide thioester INMWQEVGKA **14b** was obtained. Crude peptide **14b** was dissolved in H$_2$O/CH$_3$CN/TFA (50:50:0.01, v/v/v) and purified by preparative HPLC. Fractions corresponding to cyclic peptide **14b** were pooled and lyophilized to yield INMWQEVGKA loop **14b** as a white fluffy solid (36.2 mg, 5.6% overall yield starting from 0.43 mmol of *N*-acyl sulfonamide resin **9**). t_R = 16.8 min (method A); MALDI-TOF MS *m*/*z* calcd for $C_{65}H_{101}N_{16}O_{19}S_2$ $[M + H]^+$ 1473.69, found 1473.61.

dx.doi.org/10.1021/jo3015566 | J. Org. Chem. 2012, 77, 10058–10064

^{1}H NMR (500 MHz, H_2O/D_2O (9:1, v/v)): δ Ile-1: 8.25 (NH), 4.17 (αCH), 1.82 (βCH), 0.90 (γCH_3), 1.18/1.44 (γCH_2), 0.85 (δCH_3); Asn-2: 8.55 (NH), 4.65 (αCH), 2.79 (βCH_2), 7.04/7.68 (δNH_2); Met-3: 8.26 (NH), 4.32 (αCH), 1.83/1.89 (βCH_2), 2.24 (γCH_2), 1.97 (εCH_3); Trp-4: 8.03 (NH), 4.63 (αCH), 3.30/3.35 (βCH_2), 7.27 (H2), 7.58 (H4), 7.15 (H5), 7.23 (H6), 7.48 (H7), 10.24 (NH); Gln-5: 8.15 (NH), 4.02 (αCH), 1.87/2.01 (βCH_2), 1.91/2.03 (γCH_2), 6.85/7.40 (εNH_2); Glu-6: 8.02 (NH), 4.37 (αCH), 1.96/2.07 (βCH_2), 2.42 (γCH_2); Val-7: 8.26 (NH), 4.07 (αCH), 2.07 (βCH), 0.96 (γCH_3), 0.92 (γCH_3); Gly-8: 8.62 (NH), 3.87/4.00 (αCH_2); Lys-9: 8.07 (NH), 4.36 (αCH), 1.73/1.84 (βCH_2), 1.39 (γCH_2), 1.64 (δCH_2), 2.95 (εCH_2), 7.58 (ζNH_2); Ala-10: 8.39 (NH), 4.25 (αCH), 1.36 (βCH_3); Glu-11: 8.36 (NH), 4.08 (αCH), 1.98 (βCH_2), 2.37/2.40 (γCH_2); β-Ala-12: 8.20 (NH), 3.46/3.54 (αCH_2), 2.85 (βCH_2); thioester tail: 2.66 (CH_2), 3.09 (CH_2), 4.14 (CH_2), 1.23 (CH_3). ^{13}C NMR (500 MHz, H_2O/D_2O (9:1, v/v)): δ Ile-1: 59.7 (αCH), 36.5 (βCH), 15.4 (γCH_3), 25.2 (γCH_2), 11.1 (δCH_3); Asn-2: 51.1 (αCH), 36.5 (βCH_2); Met-3: 54.2 (αCH), 30.2 (βCH_2), 29.3 (γCH_2), 14.6 (εCH_3); Trp-4: 55.6 (αCH), 26.6 (βCH_2), 125.0 (C2), 118.6 (C4), 119.9 (C5), 122.6 (C6), 112.6 (C7); Gln-5: 54.4 (αCH), 26.2 (βCH_2), 31.5 (γCH_2); Glu-6: 53.6 (αCH), 27.0 (βCH_2), 30.9 (γCH_2); Val-7: 60.6 (αCH), 30.2 (βCH), 18.7 (γCH_3), 18.8 (γCH_3); Gly-8: 43.1 (αCH_2); Lys-9: 53.6 (αCH), 31.0 (βCH_2), 22.6 (γCH_2), 26.8 (δCH_2), 40.0 (εCH_2); Ala-10: 50.4 (αCH), 17.0 (βCH_3); Glu-11: 54.2 (αCH), 27.5 (βCH_2), 31.8 (γCH_2); β-Ala-12: 36.2 (αCH_2), 43.0 (βCH_2); thioester tail: 34.5 (CH_2), 24.4 (CH_2), 62.5 (CH_2), 13.8 (CH_3).

Synthesis of Cyclic Peptide Thioester 14c. Following the above-described general procedures, crude lyophilized cyclic peptide thioester LTRDGGN **14c** was obtained. Crude peptide **14c** was dissolved in H_2O/CH_3CN/TFA (50:50:0.01, v/v/v) and purified by preparative HPLC. Fractions corresponding to cyclic peptide **14c** were pooled and lyophilized to yield LTRDGGN loop **14c** as a white fluffy solid (31.0 mg, 7.0% overall yield starting from 0.43 mmol of *N*-acyl sulfonamide resin **9**). t_R = 14.6 min (method A); MALDI-TOF MS *m*/*z* calcd for $C_{41}H_{68}N_{13}O_{16}S$ $[M + H]^+$ 1030.46, found 1030.40; calcd for $C_{41}H_{67}N_{13}NaO_{16}S$ $[M + Na]^+$ 1052.44, found 1052.39; calcd for $C_{41}H_{67}N_{13}KO_{16}S$ $[M + K]^+$ 1068.42, found 1068.35. ^{1}H NMR (500 MHz, H_2O/D_2O (9:1, v/v)): δ Leu-1: 8.39 (NH), 4.31 (αCH), 1.67/2.61 (βCH_2), 1.60 (γCH), 0.94 (δCH_3), 0.90 (δCH_3); Thr-2: 8.05 (NH), 4.43 (αCH), 4.32 (βCH), 1.17 (γCH_3); Arg-3: 8.44 (NH), 4.29 (αCH), 1.83 (βCH_2), 1.61 (γCH_3), 3.20 (δCH_2), 7.24 (εNH), 6.93 (ηNH), 6.51 (ηNH); Asp-4: 8.58 (NH), 4.71 (αCH), 2.79/2.95 (βCH_2); Gly-5: 8.31 (NH), 3.88/4.01 (αCH_2); Gly-6: 8.27 (NH), 4.00 (αCH_2); Asn-7: 8.40 (NH), 4.70 (αCH), 2.79/2.85 (βCH_2), 6.99/7.69 (δNH_2); Glu-8: 8.66 (NH), 4.20 (αCH), 1.99/2.09 (βCH_2), 2.40 (γCH_2); β-Ala-9: 8.25 (NH), 3.45/3.53 (αCH_2), 2.83 (βCH_2); thioester tail: 2.68 (CH_2), 3.12 (CH_2), 4.14 (CH_2), 1.23 (CH_3). ^{13}C NMR (500 MHz, H_2O/D_2O (9:1, v/v)): δ Leu-1: 54.1 (αCH), 40.3 (βCH_2), 24.8 (γCH), 22.8 (δCH_3), 21.2 (δCH_3); Thr-2: 59.2 (αCH), 68.1 (βCH), 19.4 (γCH_3); Arg-3: 54.8 (αCH), 28.0 (βCH_2), 25.1 (γCH_3), 41.2 (δCH_2); Asp-4: 51.3 (αCH), 36.4 (βCH_2); Gly-5: 43.6 (αCH_2); Gly-6: 43.1 (αCH_2); Asn-7: 51.3 (αCH), 36.6 (βCH_2); Glu-8: 54.3 (αCH), 26.9 (βCH_2), 32.0 (γCH_2); β-Ala-9: 36.4 (αCH_2), 43.1 (βCH_2); thioester tail: 34.5 (CH_2), 24.6 (CH_2), 62.6 (CH_2), 14.0 (CH_3).

Synthesis of N-Terminal Cysteine-Containing HHC-10 Peptide 15. Immobilized HHC-10 peptide **15** was available in our group. The peptide was assembled on an automatic ABI 433A peptide synthesizer using the ABI FastMoc 0.25 mmol protocol,[22] and synthesis was carried out on a RAM resin. The obtained anchored peptide was deprotected and cleaved from the solid support by treatment with TFA/H_2O/TIPS/EDT (90:5:2.5:2.5, v/v/v/v) (20 mL $mmol^{-1}$) for 2 h. The reaction mixture was filtered and washed with TFA (20 mL $mmol^{-1}$). The filtrates were combined, and the volume was reduced to 2 mL by evaporation. Next, the mixture was added dropwise to a cold (4 °C) solution of MTBE/hexanes (1:1, v/v). After centrifugation (3500 rpm, 5 min), the supernatant was decanted and the pellet was resuspended in MTBE/hexanes (1:1, v/v) and centrifuged again. Finally, the pellet was washed twice with MTBE/hexanes (1:1, v/v), each time collected by centrifugation, and dissolved in *t*BuOH/H_2O (1:1, v/v) followed by lyophilization to afford crude HHC-10 peptide **15**. Crude peptide **15** was dissolved in H_2O/CH_3CN/TFA (50:50:0.01, v/v/v) and purified by preparative HPLC. Fractions corresponding to peptide **15** were pooled and lyophilized to yield N-terminal cysteine-containing antimicrobial HHC-10 peptide **15** as a white fluffy solid. t_R = 21.6 min (method B); MALDI-TOF MS *m*/*z* calcd for $C_{77}H_{108}N_{23}O_{10}S$ $[M + H]^+$ 1546.84, found 1546.84.

Synthesis of Ligated Cyclic-Linear Peptide 16. N_2 was bubbled through a freshly prepared solution of $NaH_2PO_4 \cdot 2H_2O$ (200 mM, 62.4 mg), 4-mercaptophenylacetic acid (MPAA, 200 mM, 67.3 mg), and tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 40 mM, 22.9 mg), pH 7.4, in 2 mL of H_2O/DMF (4:1, v/v) for 5 min. Next, this solution (2 mL) was added to the TFA salt of cyclic peptide thioester LTRDGGN **14c** (1 mM, 2.52 mg, two TFA molecules were added to the molecular weight of the cyclic peptide) and HHC-10 peptide **15** (1 mM, 4.69 mg, seven TFA molecules were added to the molecular weight of the peptide), and the mixture was stirred. After 3 h, the reaction mixture was diluted with H_2O/TFA (9:1, v/v, 3 mL) to precipitate MPAA. After two extractions with Et_2O, TCEP was added and the solution was stirred for 5 min. After two additional extractions with Et_2O, purification by using preparative HPLC was performed. Fractions corresponding to ligated cyclic-linear peptide **16** were pooled and lyophilized to yield the TFA salt of ligation product **16** as a white fluffy solid (6.2 mg, 92%, eight TFA molecules were added to the molecular weight). t_R = 21.7 min (method B); MALDI-TOF MS *m*/*z* calcd for $C_{113}H_{165}N_{36}O_{24}S$ $[M + H]^+$ 2442.25, found 2442.37.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR, HSQC, HMBC, and TOCSY spectra of compound **3**. HSQC, NOESY, and TOCSY spectra of cyclic peptide thioesters **14a**–**c**. HPLC and MALDI-TOF analysis for compounds **3**, **14a**–**c**, **15**, and **16**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ing. Javier Sastre Toraño for the HRMS measurement and Rik T. C. Cleophas for providing the N-terminal cysteine-containing antimicrobial HHC-10 peptide **15**. This research was (partly) financed by Chemical Sciences of The Netherlands Organisation for Scientific Research (NWO).

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