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Microwave-Mediated Reduction of Disulfide Bridges with Supported (Tris(2-carboxyethyl)phosphine) as Resin-Bound Reducing Agent

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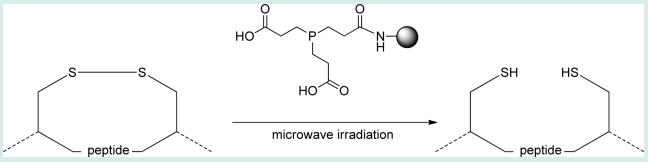
Supporting Information

ABSTRACT: We report on the synthesis and use of a new supported reagent consisting in tris(2-carboxyethyl)phosphine (TCEP) immobilized on hydrophilic PEG based resin beads. Used in conjunction with a 5 min microwave (MW) irradiation, "supported TCEP" reduced disulfide bridges in free thiols in peptides having two or more cysteine residues. Separation of reaction products from reducing agent was easily performed by simple filtration.



KEYWORDS: *disulfide bond, cysteine, supported reagent, reduction, thiol, TCEP*

Tris(2-carboxyethyl)phosphine (TCEP) is a very useful reducing agent used in biochemistry and chemistry laboratories. Odorless, water-soluble, and stable in a large range of pH,[1] it is the reactant of choice to denaturate proteins, breaking the tertiary structure established by disulfide bonds between cysteine prior to SDS-PAGE analysis. This reagent is particularly suitable when free thiol have to be obtained as reactive moieties for their immobilization onto maleimide or sulfhydryl functionalized supports[2] or for further chemical labeling.[3] Indeed, compared to sulfur-containing agents, such as ethanedithiol (EDT) or dithiothreitol (DTT), TCEP will not compete with thiols as a nucleophile in most chemical modifications of cysteines. Similarly, TCEP is also used to keep reductive conditions when free thiols are used reagents, for example, Michael addition on dehydroalanine residues[4] or on maleimide conjugates.[5,6]

Mild reductive agents such TCEP are also useful in peptide chemistry for bioconjugation purposes. Cysteines are often introduced at the C- or N-terminus of a bioactive sequence as a template for conjugation with a fluorescent probe, or another bioactive moiety (cell penetrating, homing peptide, DNA, ...). The cysteine side chain was also used to graft peptides and proteins on micro or nanoscale objects, such as polymers,[7] liposomes,[8] gold nanoparticles,[9] and glass microarrays.[10] Covalent linkage can be achieved via thiol nucleophilic substitution on a halogenated derivative,[11] 1,4-Michael addition[12] or hetero-disulfide bond formation by reacting with an activated disulfide (e.g., NPys).[13]

Thiol-free reductive conditions afforded by TCEP are of high interest for native chemical ligation (NCL). An unprotected peptide fragment bearing a N-terminus cysteine may react chemoselectively with a C-terminus thioester peptide yielding an amide bond after a S−N acyl transfer.[14] NCL is still extensively used and developed, and remains the method of choice for the chemical synthesis of proteins. In addition, an efficient and very elegant methodology has been recently developed for the Fmoc/tBu solid phase synthesis of C-terminus peptide thioester precursors.[15] C-terminal bis(2-sulfanylethyl)amido peptides are cleaved from the support and purified as stable cyclic disulfides. Reduction using TCEP opens the disulfide bridge, initiating the shift between amide and thioester suitable for disulfide exchange and NCL.[16]

In this case, TCEP removal is achieved by a preparative HPLC. To simplify the workup and speed of the treatments, the whole synthetic process using solid supported reagents constitutes an attractive alternative. Actually, only TCEP immobilized on cross-linked agarose gel has been described. However its use is limited to aqueous solutions. Moreover, the agarose gel loading, given by supplier datasheet, is quite low (8 μmol/mL) and is not convenient in the context of TCEP applications involving gram- or even milligram-scale samples. As an example, 125 μL of gel are required to reduce 1 mg of a peptide of MW 1000 g mol^{-1}. At least, the reduction speed decreases quickly with the sample concentration: 15 min are required to reduce protein samples at a concentration of 0.1 mg/mL but required 1 h at 1 mg/mL.[17]

In this context, we first designed a high loaded supported reducing agent consisting in TCEP immobilized on hydrophilic cross-linked PEG resin beads ChemMatrix (CM). Then, we used this new resin, both in aqueous or organic solvents, to quickly reduce disulfide bonds under short microwave irradiation.

Cross-linked polymer resin beads are useful auxiliaries both used for the synthesis of supported reagents or as solid supports for organic synthesis including peptide synthesis. The nature of polymer matrix is as important as the solvent used for a given

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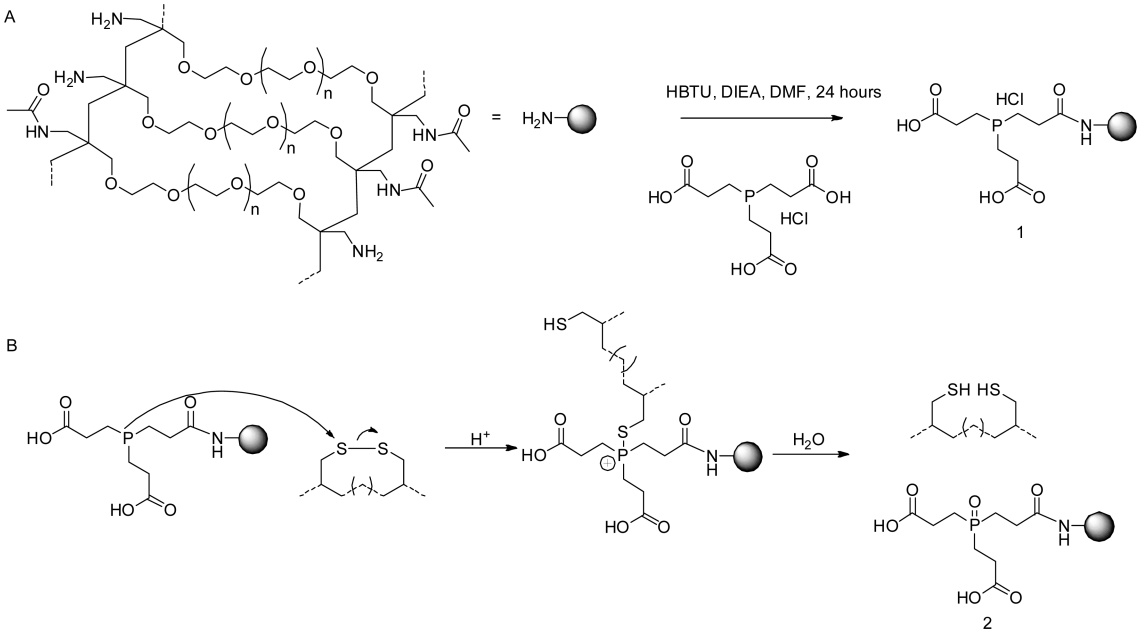
Scheme 1. (A) Synthesis of TCEP-ChemMatrix-Supported Reducing Agent and (B) TCEP-CM-Mediated Reduction Mechanism



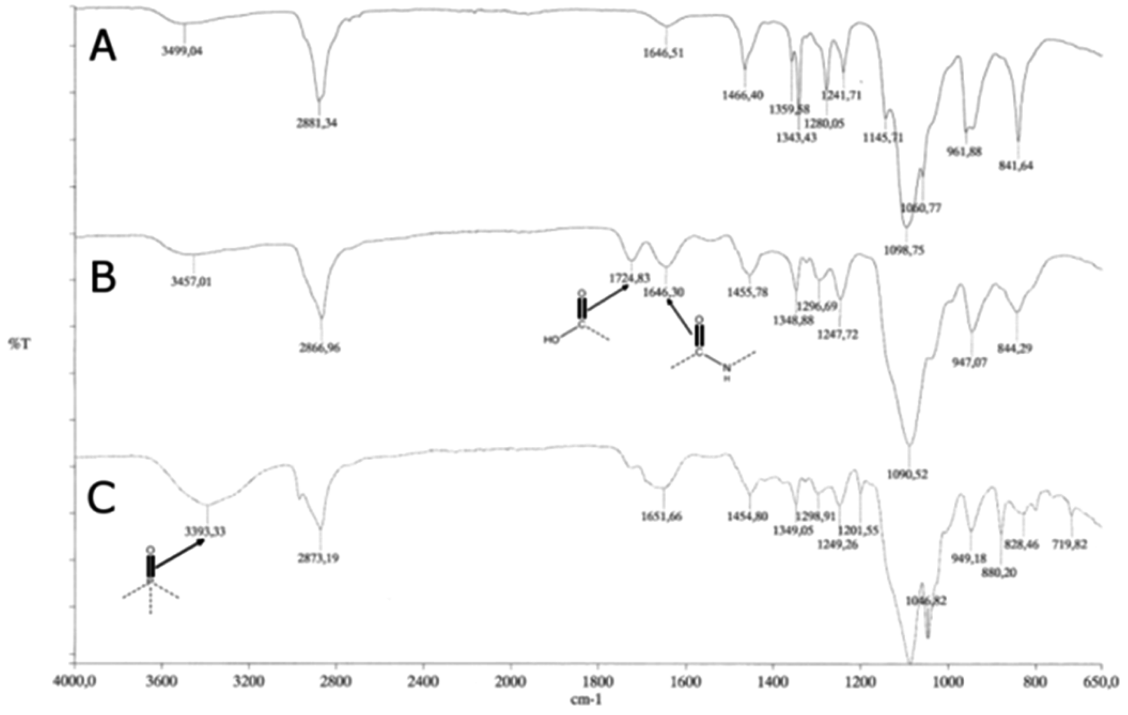


Figure 1. FT-IR spectra of (A) starting ChemMatrix resin, (B) supported-TCEP resin 1, and (C) TCEP bound ChemMatrix **2** resin after reduction of disulfide bridge.

reaction.[18] Because most of reactive sites are buried inside the resin bead, swelling of beads is essential for optimal penetration of reagents within the cross-linked matrix.[19] Aminomethyl ChemMatrix was chosen for supported TCEP preparation. In contrast to resins traditionally used for the synthesis of peptides and heterocycles, this hydrophilic cross-linked resin is exclusively composed of PEG. It has been used successfully for the synthesis of long and difficult peptides[20] yielding significant improvement of crudes. Its excellent swelling properties, both in water or in aprotic polar solvents, such as dimethyl formamide (DMF) and *N*-methyl pyrrolidone (NMP), makes ChemMatrix a matrix of choice for the applications foreseen for supported TCEP reducing agent.

TCEP-ChemMatrix Preparation and Characterization. TCEP-CM **1** was prepared straightforward by activating one carboxylic function of TCEP with one equivalent of HBTU in the presence of DIEA. HBTU was introduced dropwise on TCEP solution along with 1 equiv of DIEA to activate only one carboxylic function, thus minimizing the possibility of cross-linking TCEP on aminomethyl ChemMatrix (AM-CM).

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Table 1. Reduction of Disulfide Bridges with Supported TCEP-CM: (A) Starting Oxidized Peptides 3−9, (B) Reduced Peptides 3′−6′, and (C) Reduced Longer Peptides 7′−9′

A

	disulfide compound	RT[a] (min)	purity[a] (%)	MW calcd	$[M + H]^+$ m/z found
3	H-LCAGPCL-NH_2	0.93	76	672.3	673.3
4	H-MCAGMCL-NH_2	0.98	90	724.3	725.3
5	H-PFCNAFTGC-NH_2	1.07	75	955.4	956.5
6	(Boc-Tyr-NH-C_2H_4-S)$_2$	1.59	93	678.3	679.3
7	pGlu-CRRLCYKQRCVTYCRGR-NH_2 (gomesin)	2.63	83	2269.1	2270.2[d]
8	H-KWCFRVCYRGICYRRCR-OH (tachyplesin acid)	2.72	91	2263.1	2264.0[d]
9	H-KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW-OH (mesenthericin)	3.07	95	3865.8	3866.6[d]

B

reduced compound[c]	solvent	time required for complete conversion (μwave/RT)	RT[a] (min)	purity[a] (%)	MW calcd	ESI+ $[M + H]^+$ m/z found
3′	EtOH/H_2O/AcOH (6/3/1)	5 min/60 min	0.93	72	674.3	675.3
4′	EtOH/H_2O/AcOH (6/3/1)	5 min/*b*	0.97	85	726.3	727.3
5′	EtOH/H_2O/AcOH (6/3/1)	5 min/60 min	1.06	70	957.4	958.6
6′	DMF/H_2O/AcOH (6/3/1)	30 s/20 min	1.23	92	340.2	341.2
6′	THF/H_2O/AcOH (6/3/1)	30 s/20 min	1.23	90	340.2	341.2
6′	EtOH/H_2O/AcOH (6/3/1)	30 s/20 min	1.23	92	340.2	341.2
6′	H_2O	5 min/60 min	1.23	90	340.2	341.2

C

reduced compound[c]	solvent	time required for complete conversion (μwave/RT)	MW calcd	MALDI-MS $[M + H]^+$ m/z found
7′	EtOH/H_2O/AcOH (6/3/1)	5 min/*b*	2273.1	2274.0
8′	EtOH/H_2O/AcOH (6/3/1)	5 min/*b*	2267.1	2268.0
9′	EtOH/H_2O/AcOH (6/3/1)	5 min/*b*	3867.8	3868.9

[a]RT and purity % were calculated by peak integration at 214 nm during LC analyses. Compounds **3**−**6** and longer peptides **7**−**9** were analyzed in different conditions (see Supporting Information). [b]Complete conversion was not observed at RT after one hour. [c]**6′** corresponds to the monomer Boc-Tyr-NH-$(CH_2)_2$-SH. [d]m/z of the molecular ion was determined by MALDI-TOF mass spectrometry.

Activated TCEP was coupled to free amino groups of AM-CM (Scheme 1A).

The loading of **1** (0.42 mmol/g) was determined by phosphorus elemental analysis (see Supporting Information).

Resin **1** was also characterized by FT-IR and compared with starting CM (Figure 1). Characteristic vibration bands of carbonyl bonds appeared at 1646 and 1725 cm^{-1} corresponding to amide linkage and carboxylic acids. They confirmed the linkage of TCEP on the polymer matrix. Resin 1 was stored at room temperature with no particular precaution and was proved to be stable to oxidation. Indeed, FT-IR was recorded after 3 and 10 weeks and no difference was observed compared to the freshly prepared TCEP supported polymer. In particular, no difference in the baseline was observed at 3400 cm^{-1}, indicating no phosphine oxide (P═O) was present.

Cyclic peptides **3**, **4**, and **5** (Table 1) containing two cysteine residues linked via a disulfide bond were selected as model for TCEP-CM reduction. It is worth noting that the free thiol linear analogues of the model peptides (**3′**, **4′**, **5′**) could be separated using RP-HPLC (3 min, gradient water/acetonitrile containing 0.1% TFA on RP C18 column) allowing a straightforward monitoring of the reduction experiment courses. Three longer peptides of biological relevance were also prepared, including 18-mer gomesin[21] **7**, 17-mer tachyplesin acid[22] **8**, and 37-mer mesentericin[23] **9**. It is worth noting that peptides **7**−**8** display two disulfide bridges in their sequences. Peptides **3′**, **4′**, **5′**, and **7′**were synthesized on Rink amide PS resin and **8′** and **9′** were synthesized on 2-chlorochlorotrityl PS resin. All the syntheses were performed using microwave-assisted Fmoc SPPS[19] on various solid supports (see Supporting Information). They were cleaved from the support by TFA cocktail treatment, and precipitated in diethyl ether. Peptides were dissolved in water/acetonitrile (1/1 v/v) and freeze-dried. Compounds **3′**−**6′** and **9′** (with two cysteine residues in their sequences) were dissolved in H_2O/DMSO (9/1 v/v) at a 3 mM concentration and vigorously stirred for 12h to yield cyclic oxidized peptides. On the other hand, peptides **7′** and **8′** having 4 cysteine residues were dissolved at 30 μM concentration in ammonium acetate buffer (0.1 M, pH 8) and stirred vigorously for 48 h. pH was then lowered to 4 by addition of acetic acid and the media was finally desalted by RP chromatography. All oxidized peptides were purified straightforwardly by preparative RP-HPLC. They were analyzed by LC/MS (see Table 1A and Supporting Information for spectra and chromatograms).

(BocTyr-NH-C_2H_4-S-)$_2$ dimer **6** was also chosen as linear model. It was prepared by coupling Boc-Tyr-OH with cystamine using BOP/DIPEA activation. After extraction with ethyl acetate and solubilization with ethanol, **6** was precipitated by addition of water (yield 84%, purity 93%). It was analyzed by LC/MS (see Table 1 and Supporting Information for spectra and chromatograms).

Reduction of all peptides was carried out in EtOH/H_2O/AcOH mixture at 0.1 mg/mL which correspond to very common concentration of peptide samples for analytical or proteomic studies (i.e., trypsin digestion). Ethanol was used essentially to ensure a good solubility of deprotected peptides while acidic water solution was required to quantitatively

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generate free thiol groups from the thiolate intermediate and the supported P−S species. Other organic solvents and water were also used with compound **6** (Table 1). TCEP-CM resin **1** was added to peptide solutions (20 eq. excess). Reactions were performed at room temperature or under microwave irradiation. TCEP-CM **1** was removed by a simple filtration step. For compounds **3**−**6**, the completeness of the reaction was checked by LC/MS. Single ion recording chromatograms of oxidized compounds confirmed the complete disappearance of starting material within 5 min using microwave irradiation. Advantageously, longer peptide sequences **7**−**9** were analyzed by MALDI-TOF MS to obtain a sufficient resolution required to distinguish clearly the isotopic pattern of the oxidized and reduced species. Thus, as already observed for compounds **3**−**6**, a 5 min MW irradiation used along with TCEP-CM was required to observe the total disappearance of the molecular ion corresponding to oxidized species to the profit of reduced peptide (see Supporting Information for MALDI-TOF mass spectra).

Complete reduction of compound **6** was even quicker as it was obtained in 20 s under microwave irradiation whatever the solvent used. On the contrary, reductions at room temperature proceeded slower. For example, RT reduction of compounds **3**, **5**, and **6** were complete after 1h but reduction of compounds **4**, **7**, **8**, and **9** do not reach completion even after 1 h reaction.

After MW-mediated TCEP-CM reduction, reduced compounds can be isolated by freeze-drying, without any chromatography step. After reduction, the resin was recovered and analyzed by FT-IR (Figure 1 C). As expected, a characteristic phosphine oxide broad band at 3400 cm^{-1} appeared in the spectra.

Summing-up, we reported the straightforward and simple synthesis of a new supported reagent TCEP-CM for reduction of disulfide bridges. Immobilized on a hydrophilic polymer matrix, TCEP-CM could be used in aqueous media, compatible with the solubility of unprotected peptides. We showed that it was also possible to use mixtures of organic solvents as far as they are miscible with water, broadening the range of TCEP-CM potential applications. Combined with microwave irradiation, quantitative reduction occurred in 5 min, even on the 37-mer mesentericin **9** or on peptides **7** and **8** displaying four cysteines (gomesin and tachyplesin). Separation of free thiol-containing compounds from the reducing agent was easily done by simple filtration. The high loading (0.42 mmol/g) of this resin is particularly advantageous because TCEP-CM mediated reductions require a small quantity of solid support related to the sample volume. TCEP-CM could also be a particularly attractive additive to protect liquid biological samples from oxidation.

EXPERIMENTAL PROCEDURES

Analytical Methods. HPLC analyses were performed on a Beckman Gold apparatus for compounds **3**−**6** and **3′**−**6′** and on a Agilent 1100serie apparatus for peptides **7**−**9**. Beckman Gold apparatus is composed of the 126-solvent module, the 168 detector, and the 32 Karat software, using a 50 mm ×3.9 mm a C18 reversed-phase column VWR chromolith column. Standard conditions were eluent system A (water/0.1% TFA) and system B (acetonitrile/0.1% TFA). A flow rate of 5 mL/min and a gradient of (0−100)% B over 3 min were used. The Agilent 1100 series apparatus is composed of the G137 degasser, the G1376A capillary pump, the μ-WPS G1377A injection system, the MWD G1365B detector, and the ChemStation A.10.02 software, using a 50 mm ×3 mm C18 reversed-phase column PHENOMENEX Kinetex column. Standard conditions were eluent system A (water/acetonitrile/MSA 98/2/0.1) and system B (acetonitrile/water/MSA 98/2/0.1). A flow rate of 500 μL/min and a gradient of (0−100)% B over 5 min were used. Detection and purity determination by peak area integration was performed at 214 nm.

LC/MS Analyses. Samples were prepared in an acetonitrile/water mixture (50/50 v/v), containing 0.1% TFA. The LC/MS system consisted of a Waters Alliance 2695 HPLC, coupled to a Micromass (Manchester, U.K.) ZQ spectrometer (electrospray ionization mode, ESI+). All the analyses were carried out using a Merck Chromolith Speed rod C18, 25 × 4.6 mm reversed-phase column. A flow rate of 3 mL/min and a gradient of (0−100)% B over 3 min (or over 15 min) were used. Eluent A: water/0.1% HCO_2H; eluent B: acetonitrile/0.1% HCO_2H. Retention times (RT) are reported in minutes. Positive ion electrospray mass spectra were acquired at a solvent flow rate of 200 μL/min. Nitrogen was used for both the nebulizing and drying gas. The data were obtained in a scan mode ranging from 100 to 1000 m/z in 0.1 s intervals; 10 scans were summed up to get the final spectrum.

MALDI mass spectra were recorded on an Ultraflex III TOF/TOF instrument (Bruker Daltonics, Wissenbourg, France). α-Cyano-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid were used as matrix. A mixture of crude reduced peptide and matrix solution were deposited onto the MALDI target according to the dried droplet procedure.

RP-preparative HPLC purification was performed on a Waters HPLC 4000 instrument, equipped with a UV detector 486 and Waters Delta-Pack 40 × 100 mm, 15 Å, 100 μm, reversed-phase column. Standard conditions were eluent system A (water/0.1% TFA) and eluent system B (acetonitrile/0.1% TFA). A flow rate of 50 mL/min and a gradient of (10−70)% B over 30 min were used, detection 214 nm.

Solvents used for HPLC and LC/MS were of HPLC grade. IR spectra in solid state were recorded on a FTS 575C spectrometer Bio Rad.

ICP AES Mass Spectrometry was performed on ICAP instrument (ThermoFisherScientific). Twenty mg of TCEP-CM were mineralized in 6 mL of nitric acid/H_2O_2 (5/1 v/v) before ICP-AES introduction. Analyses were subcontracted to CNRS-service central d'analyses UMR 5280, Chemin du canal 69360 SOLAIZE, France.

Synthesis of Supported TCEP Resin 1. Three hundred milligrams of 1.14 mmol/g loaded ChemMatrix resin was swollen in DCM. TCEP·HCl (98 mg, 0.342 mmol) was dissolved in DMF containing DIPEA (117 μL, 0.684 mmol). HBTU in DMF was added dropwise (130 mg, 0.342 mmol), and the mixture was allowed to react for 5 min. Then the mixture was put onto the resin under mechanical stirring for 24 h. Resin was washed with 3 × 10 mL DMF (3 min), 1 × 10 mL HCl 1 M (3 min), 1 × 10 mL methanol (3 min), 1 × 10 mL Et_2O (3 min), 2 × 10 mL DCM (3 min) and finally dried under vacuum. About 320 mg of resin 1 was obtained. The loading was calculated from data obtained by phosphorus elemental analysis performed by inductively coupled plasma-atomic emission spectrometry (ICP-AES). 1.30% (weight) of phosphorus was detected. The loading of TCEP-CM 1 was determined as 0.42 mmol/g. (See calculations in Supporting Information.)

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General Protocol for Reduction of Disulfide Bonds in Peptides. Five milligrams of TCEP-supported reagent 1 (2.1 μmole, 20 equiv) was introduced into a syringe equipped with frit. Oxidized cyclic peptide (0.1 mg, ∼0.1 μmol, 1 equiv) was dissolved in 1 mL of ethanol/water/acetic acid (6/3/1, v/v/v) at a concentration of 0.1 mg/mL. The oxidized peptide solution was added onto resin **1**. The suspension was submitted to microwave irradiation (15 W, 70 °C, 5 min) or without microwave irradiation at room temperature for 1 h. Resin was removed by filtration and washed twice with the solution used for solubilization. Solution was then freeze-dried to yield the reduced peptide.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed SPPS protocols, loading calculation, LC chromatograms, and mass spectra. This information 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.

■ ABBREVIATIONS

ACN, acetonitrile; AcOH, acetic acid; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate; CM, ChemMatrix; DIPEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; DMF, dimethylformamide; EDT, ethanedithiol; EtOH, ethanol; Fmoc, fluorenylmethyloxycarbonyl; HBTU, *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate; MSA, methanesulfonic acid; MW, microwave; NCL, native chemical ligation; NMP, *N*-methyl-2-pyrolidone; PEG, polyethylene glycol; TCEP, tris(2-carboxyethyl)phosphine; RT, retention time; RP-HPLC, reverse-phase high-performance liquid chromatography; SPPS, solid-phase peptide synthesis

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