Organic & Biomolecular Chemistry

PAPER

View Article Online
View Journal | View Issue

Cite this: *Org. Biomol. Chem.*, 2019, **17**, 10228

A trimethyllysine-containing trityl tag for solubilizing hydrophobic peptides†

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Received 17th October 2019,
Accepted 20th November 2019

DOI: 10.1039/c9ob02253h

rsc.li/obc

Hydrophobic membrane peptides/proteins having low water solubility are often difficult to prepare. To overcome this issue, temporal introduction of solubilizing tags has been demonstrated to be beneficial. Following our recent work on the solubilization of a difficult target by using a hydrophilic oligo-Lys tag bearing a trityl linker (Trt-K method), this paper describes a comparative study of the solubilizing abilities of several peptidic trityl tags containing Lys, Arg, Glu, Asn, N^{ε}-tri-Me-Lys or Cys-sulfonate using two hydrophobic model peptides. Among the tags evaluated, that containing N^{ε}-tri-Me-Lys exhibits superior solubilizing ability.

Introduction

Chemical peptide/protein synthesis mainly consists of two steps: peptide chain construction and purification using high performance liquid chromatography (HPLC). The first step is well established and based on methods such as solid-phase peptide synthesis (SPPS),[1] native chemical ligation (NCL),[2] and related techniques.[3] In contrast, there is still room for improvement in the second step, since hydrophobic membrane peptides/proteins having low water solubility are difficult to purify by HPLC.[4] Additionally, the low solubility of these compounds also hampers further development of NCL processes in which unprotected peptide segments are ligated in an aqueous solvent.[5] To solve this problem, many researchers developed solubilizing tags such as a phenylacetamidomethyl linker method,[6g] a removable backbone modification method,[6h] and a canaline tag.[6m] In this context, we recently developed a simple solubilizing trityl-type tag system (Trt-K, where Trt and K denote trityl and Lys, respectively) (Fig. 1),[7] which is characterized by the following features: (1) an introducing reagent for the trityl alcohol-type tag, "Trt(OH)-K_n", can be readily prepared using commercially available 4-(diphenylhydroxymethyl) benzoic acid; (2) the tag can be attached to the thiol group in the unprotected peptide by simply adding the introducing reagent under acidic conditions such as neat 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), which can dissolve hydrophobic and practically insoluble peptides,[8] or an acidified thiol-additive-free NCL reaction mixture in a one-pot manner; (3) the tag can be quickly and cleanly detached by a standard deprotection approach using trifluoroacetic acid (TFA) with a cation scavenger such as triisopropylsilane (TIS). Unlike the other known solubilizing tag systems,[6] the solubilizing tag in our method can be attached "on demand", without the need for starting a new preparation when the target peptide segment is found insoluble. This is a great advantage because it is generally difficult to estimate the solubility of each protein intermediate prior to the synthesis. To broaden the scope of our method and facilitate its further application to challenging targets, we herein report a comparative study of the solubilizing abilities of ionic/nonionic peptide tags having different structures. We compare several peptide tags composed of basic residues (Lys/Arg), an acidic residue (Glu), or a nonionic residue (Asn). Furthermore, peptide tags composed of N^{ε}-tri-Me-Lys or Cys-sulfonate as pH-independent analogs are evaluated. As a result, the N^{ε}-tri-Me-Lys-containing tag is demonstrated to exhibit the best solubilizing ability in solution.

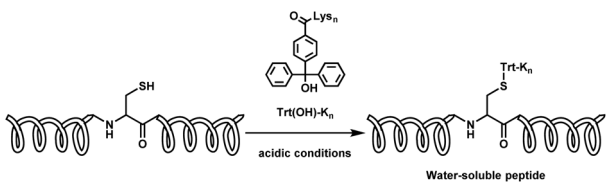

Fig. 1 Solubilizing trityl-type tag system.

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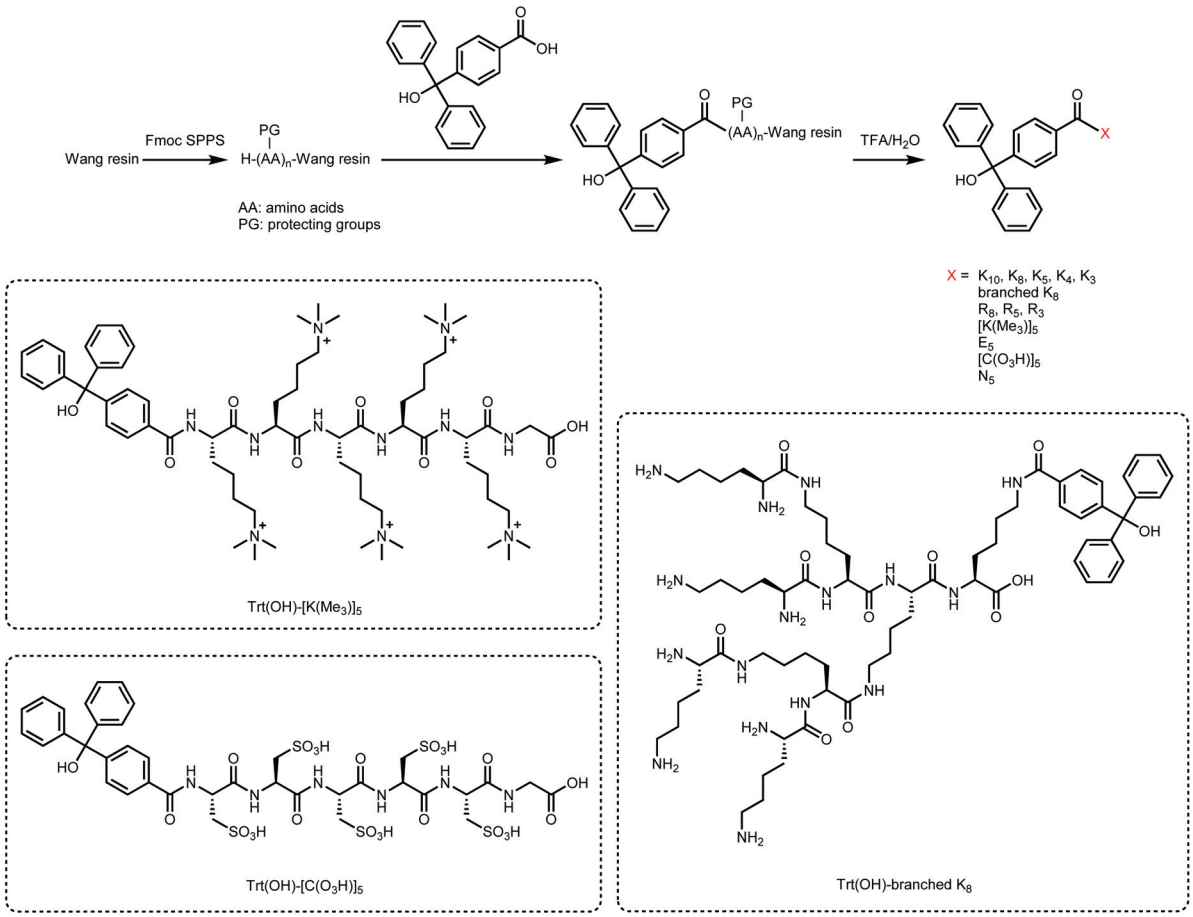
† Electronic supplementary information (ESI) available: Solubility of tagged SPP4 in different solvents for various ligation techniques, and characterization data such as NMR spectra, HPLC traces, and MS spectra. See DOI: 10.1039/c9ob02253h

Results

Preparation of tag-introducing reagents

To compare the solubilizing abilities of several Trt-X tags, we designed several examples: Trt-K_n, where K denotes Lys and n = 10, 8, 5, 4, 3, branched 8;[9] Trt-R_n, where R denotes Arg and



Scheme 1 Synthesis of Trt-X tags and structures of selected solubilizing tags.

n = 8, 5, 3; Trt-E_5, where E denotes Glu; Trt-N_5, where N denotes Asn;[10] Trt-[K(Me_3)]$_5$, where K(Me_3) denotes N^{ε}-tri-Me-Lys; and Trt-[C(O_3H)]$_5$, where C(O_3H) denotes Cys-sulfonate. Each tag-introducing reagent was prepared straightforwardly by a standard Fmoc SPPS using Wang resin (Scheme 1). After final acylation using commercially available 4-(diphenylhydroxymethyl)benzoic acid, TFA treatment without a cation scavenger such as TIS followed by RP-HPLC purification afforded each desired trityl alcohol-type tag-introducing reagent successfully.

Solubilization of Ac-Val-Val-Cys-Val-Val-NH_2

Having several tag-introducing reagents in hand, we proceeded with their attachment under standard HFIP conditions to the first solubilizing target Ac-Val-Val-Cys-Val-Val-NH_2 (**1**), which is a highly hydrophobic model peptide.[11] The different tags were directly attached to crude **1** after Fmoc SPPS using Rink amide resin, and the respective tagged peptides were purified by RP-HPLC. The resulting HPLC profiles are summarized in Fig. 2. As we expected, the longer Lys/Arg-tagged peptide was eluted faster than its shorter counterpart. Moreover, the branched tagged peptide (branched K_8) was eluted later than the linear derivative. The sulfonate-containing tagged peptide[12] was eluted very fast because it remains

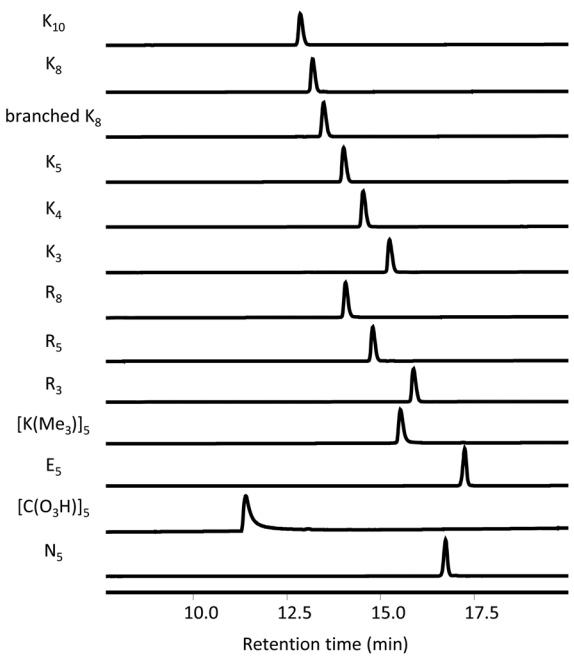
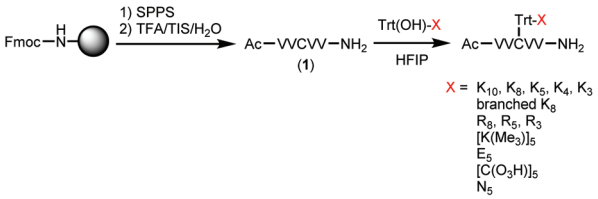


Fig. 2 HPLC profiles of tagged Ac-VVCVV-NH_2. HPLC conditions: column, YMC-Pack ODS-A (4.6 × 150 mm); elution, 10%–60% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL min^{-1}; detection, 220 nm.



X	Retention time / min	Solubility / mM: 0.2 M phosphate pH 7	Solubility / mM: 0.1% TFA aq.
K_{10}	12.9	> 13.00	> 13.00
K_8	13.2	> 13.00	> 13.00
branched K_8	13.5	4.09	> 13.00
K_5	14.0	9.29	> 13.00
K_4	14.5	-[a]	> 13.00
K_3	15.2	0.03	> 13.00
R_8	14.1	0.01	> 13.00
R_5	14.8	0.02	> 13.00
R_3	15.9	0.01	> 13.00
$[K(Me_3)]_5$	15.5	> 13.00	> 13.00
E_5	17.2	> 13.00	0.09
$[C(O_3H)]_5$	11.4	> 13.00	> 13.00
N_5	16.7	0.00	0.00

Fig. 3 Solubilization ability of tagged Ac-VVCVV-NH_2. Ac-VVCVV-NH_2 (**1**) was not soluble (0.00 mM) without a tag. [a] Gel was formed to prevent solubility evaluation.

in anionic form under 0.1% TFA aq conditions and it does not form TFA salt during HPLC. In contrast, the Glu-containing peptide was eluted very late, since it is not anionic under the HPLC conditions. The Asn-containing peptide was also eluted late. With respect to the solubilization ability under acidic conditions (Fig. 3), all basic tags [K, R, K(Me_3)] and the strongly acidic tag C(O_3H) solubilized **1** well. In contrast, the weakly acidic tag E and the nonionic tag N did not solubilize **1** efficiently under these acidic conditions. Under neutral conditions, we observed a dependence of the solubilizing ability on the peptide length of linear K_n and R_n, and linear K_8 solubilized **1** better than branched K_8. The K_n tags exhibited higher solubilizing ability than the corresponding R_n tags.[13] Furthermore, the $[K(Me_3)]_5$ tag solubilized **1** more than the corresponding K_5/R_5 tags. Additionally, we observed that both strongly and weakly acidic tags [C(O_3H) and E] solubilized **1** well, whereas the nonionic tag N did not work under neutral conditions.

Solubilization of SPP4

Next, we evaluated the solubilizing ability using as a second target peptide SPP4 (**2**), which is a known hydrophobic peptide found in membrane proteins.[6d,14] In a similar manner to that described for model peptide **1**, several basic tags were attached to crude **2**, and the tagged series **2** was purified by RP-HPLC. In this case, we could not prepare a sulfonate-containing

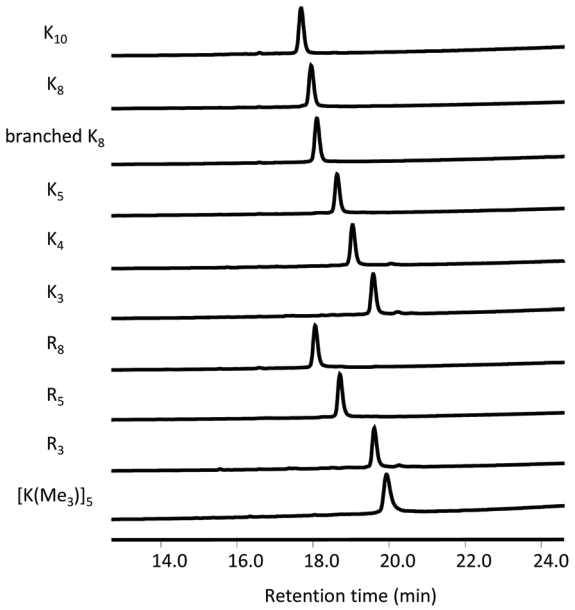
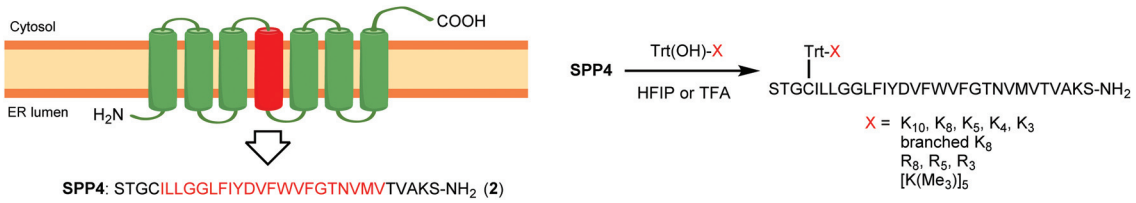


Fig. 4 HPLC profiles of tagged SPP4. HPLC conditions: column, YMC-Pack ODS-A (4.6 × 150 mm); elution, 10%–98% CH_3CN in 0.1% TFA (25 min) at 40 °C; flow rate, 1.0 mL min^{-1}; detection, 220 nm.

tagged **2** because its purification by RP-HPLC was difficult.[12] As shown in Fig. 4, the elution profile obtained by HPLC was similar to that of **1**. Thus, the longer Trt-K/R-tagged peptides eluted faster than the shorter Trt-K/R-tagged peptides, the branched tagged peptide eluted later than the linear one, and the Trt-K(Me_3)-tagged peptide eluted later than the corresponding nonmethylated derivative. With respect to the solubilization ability (Fig. 5), a similar tendency to that of peptide **1** was observed. The Trt-K/R-tagged peptides were found to dissolve well in solutions of low pH but not under neutral conditions, and the longer Trt-K/R tags solubilized **2** more than their shorter counterparts. Furthermore, the Trt-K tags showed better solubilizing ability than the Trt-R tags. Additionally, the Trt-$[K(Me_3)]_5$-tagged peptide was more soluble than the Trt-K_5/R_5-tagged peptides.

Discussion

In this paper, we compare the solubilizing abilities of a series of Trt-based tags, *i.e.*, Trt-K/R/K(Me_3)/C(O_3H)/E/N, which is expected to depend on the electrical properties derived from their different pK_a values.[15] Accordingly, basic tags [Trt-K/R/K(Me_3)] are cationic under both neutral and acidic conditions; the strongly acidic tag Trt-C(O_3H) is anionic under both neutral and cationic conditions; the weakly acidic tag Trt-E is anionic under neutral conditions but not under acidic conditions; and the neutral tag Trt-N is nonionic regardless of the solution pH. The results obtained for the retention times and solubilization abilities of the two hydrophobic peptides evaluated in this study are basically in agreement with that theory; longer/ionized tags showed good solubilization ability com-



		Solubility / mM					
		0.2 M phosphate					
X	Retention time / min	pH 3	pH 4	pH 5	pH 6	pH 7	0.1% TFA aq.
K_{10}	17.7	> 5.00	4.28	3.43	0.35	0.25	> 5.00
K_8	18.0	4.76	3.31	1.00	0.39	0.22	> 5.00
branched K_8	18.1	2.69	1.50	0.79	0.01	0.00	> 5.00
K_5	18.6	1.34	0.57	0.31	0.11	0.07	> 5.00
K_4	19.0	1.31	0.46	0.21	0.01	0.00	> 5.00
K_3	19.6	0.25	0.16	0.07	0.01	0.00	0.83
R_8	18.1	0.56	0.45	0.01	0.00	0.00	4.21
R_5	18.7	0.29	0.14	0.00	0.00	0.00	1.29
R_3	19.6	0.10	0.03	0.00	0.00	0.00	0.42
$[K(Me_3)]_5$	19.9	3.38	3.61	-[a]	-[a]	3.42	> 5.00

Fig. 5 Solubilization ability of tagged SPP4. SPP4 (**2**) was not soluble (0.00 mM) without a tag. [a] Not tested.

pared with shorter/nonionized tags. However, to our surprise, the Trt-K/R tag did not work well in solution under neutral conditions. In fact, the Trt-K(Me_3) tag showed higher solubilization ability than the Trt-K/R tags having the same length under neutral conditions. These results cannot be explained simply by the pK_a theory. On the basis of the pK_a of Lys and Arg, the Trt-K/R tags would be protonated consistently under neutral and acidic conditions, and should have similar solubilization abilities regardless of the solution pH. This contradiction can be rationalized in terms of the crowd effect (Fig. 6); under crowded conditions such as those found in the Trt-X tags, the interaction between functionalities would affect the protonation state. Similar protonation inhibitions are well known in peptide chemistry as a neighboring effect; for example, N-terminal Asn(Trt) deprotection is slow during final TFA treatment in Fmoc SPPS,[16] and N^{α}-Boc deprotection from N-terminal Boc-His(Bom) on the resin is slow during Boc SPPS.[17] In these cases, a protonated base prevents the protonation of a neighboring base, which is essential for the reaction progress. In our case, especially under neutral conditions, the crowd effect would decrease the solubilizing ability of Trt-K/R. In addition, the side-chain guanidine groups of Arg would tend to aggregate *via* π–π stacking under neutral conditions,[18] thereby mitigating the solubilizing ability. Such stacked structures have been crystallographically observed.[19] This phenomenon can also be invoked to explain the lower solubilizing ability of the branched K_8 tag compared to that of linear K_8. In the case of the branched tag, α-amino groups possessing relatively weaker basicity than ε-amino groups are present on the surface, and their protonation would be inhibited by the crowd effect. Although the anionic Trt-C(O_3H) tag has good solubilizing ability, its purification is difficult. The Trt-E tag does not solubilize the peptide under acidic conditions, and the Trt-N tag has no obvious solubilizing ability. From these results, it can be concluded that Trt-K(Me_3) is the best tag among those evaluated in this study.

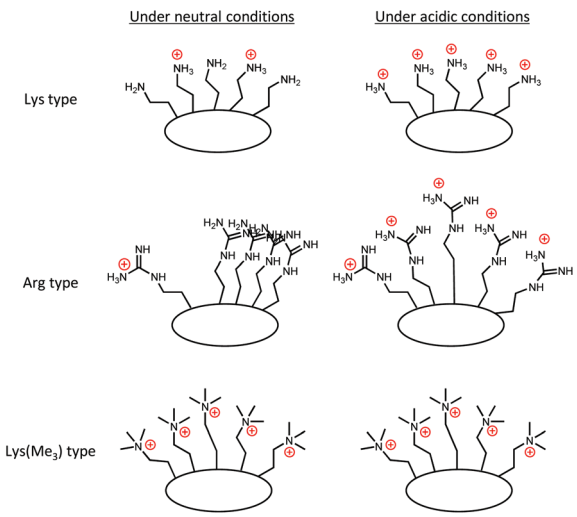


Fig. 6 Proposed structures of tags in the protonation state.

Conclusions

In this study, we compared the solubilizing abilities of several Trt-X tags using two hydrophobic peptides. The results obtained indicate that the Trt-K(Me_3) tag is superior with respect to solubilizing ability among the tags examined. Additionally, the sulfonate-containing tag Trt-C(O_3H) solubilizes the hydrophobic peptide well; however, its HPLC purification is difficult. These results contribute to further development of chemical hydrophobic peptide/protein preparation.

Experimental

General information

Materials. All reagents and solvents were obtained from Peptide Institute, Inc. (Osaka, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Tokyo Chemical Industry Co., Ltd (Tokyo, Japan), Nacalai Tesque, Inc. (Kyoto, Japan), Watanabe Chemical Industries, Ltd (Hiroshima, Japan), Merck KGaA (Darmstadt, Germany) and Sigma-Aldrich Co. LLC (St Louis, MO).

HPLC, MS and NMR. Preparative HPLC was carried out on a Shimadzu liquid chromatograph Model LC-8A (Kyoto, Japan) with a YMC-Pack ODS-A (30 × 250 mm) or a YMC-Actus Triart C18 (30 × 250 mm) and the following solvent systems: 0.1% TFA in H_2O and 0.1% TFA in CH_3CN or 0.1 M NH_4OAc buffer (pH 7.0) and 60% CH_3CN/0.1 M NH_4OAc buffer (pH 7.0) at a flow rate of 20 mL min^{-1} with detection at 220 nm. Analytical HPLC was performed on a Shimadzu liquid chromatograph Model LC-10A (Kyoto, Japan) with a YMC-Pack ODS-A (4.6 × 150 mm) or a YMC-Triart C18 (4.6 × 150 mm) and the following solvent systems: 0.1% TFA in H_2O and 0.1% TFA in CH_3CN or 0.1 M NH_4OAc buffer (pH 7.0) and 60% CH_3CN/0.1 M NH_4OAc buffer (pH 7.0) at a flow rate of 1 mL min^{-1} (40 °C) with detection at 220 nm. Low resolution mass spectra (LRMS) were observed with an Agilent G1956B LC/MSD detector using an Agilent 1100 series HPLC system; for deconvolution, the observed masses (most abundant masses) were derived from the experimental *m*/*z* values for each protonation state of a target peptide. ^{1}H-/^{13}C-NMR spectra were recorded on a JEOL-ECX400 spectrometer (Tokyo, Japan), as solutions in deuterated solvents as specified. Chemical shift values (δ) are given in parts per million (ppm) using the residual solvent as the internal standard.

SPPS. Automated Fmoc SPPS was performed on an ABI 433A peptide synthesizer (Applied Biosystems, USA). The peptide chains except Trt(OH)-[K(Me_3)]$_5$ and Trt(OH)-[C(O_3H)]$_5$ were elongated using the coupling protocol of Fmoc-amino acid/DIC/OxymaPure.[20] Trt(OH)-[K(Me_3)]$_5$ and Trt(OH)-[C(O_3H)]$_5$ were manually elongated using the coupling protocol of HCTU/DIEA.[21] The following side-chain-protected amino acids and pseudoproline unit were employed: Arg(Pbf), Asn(Trt), Asp(O*t*Bu), Glu(O*t*Bu), Ser(*t*Bu), Cys(Trt), Lys(Boc), Lys(Fmoc), Ser(*t*Bu), Thr(*t*Bu), Trp(Boc), Tyr(*t*Bu), and Val-Thr($\Psi^{Me,Me}$pro).

Experimental section

Synthesis of Fmoc-amino acid and peptides

Fmoc-Cys(O_3H)-OH. To a solution of cysteic acid (1.0 g, 5.91 mmol) and Na_2CO_3 (0.626 g, 5.91 mmol) in H_2O (25 mL) was added Fmoc-OSu solution (2.39 g, 7.09 mmol in 25 mL acetone). The reaction mixture was stirred at room temperature for 2 h, and then acetone was removed under reduced pressure. The aqueous residue was washed with AcOEt/hexane (v/v, 1/1). The aqueous layer was acidified with 1 M HCl to pH 2 and then subjected to preparative HPLC to yield the title compound (1.57 g, 80%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 2.86 (dd, J = 13.7 and 5.0 Hz, 1H), 2.90 (dd, J = 13.7 and 6.4 Hz, 1H), 4.10–4.33 (m, 4H), 7.28–7.37 (m, 2H), 7.42 (t, J = 7.3 Hz, 2H), 7.47 (d, J = 6.9 Hz, 1H) 7.70 (dd, J = 7.3 and 2.7 Hz, 2H), 7.89 (d, J = 7.3 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 46.7, 51.0, 51.4, 65.9, 120.2, 125.3, 125.4, 127.2, 127.7, 140.7, 140.8, 143.8, 143.9, 155.7, 172.3; LRMS (M − H) calcd for $C_{18}H_{16}NO_7S$ 390.1, found 390.1.

*Ac-VVCVV-NH_2 (**1**).* The peptide was assembled on Rink amide resin (0.40 mmol) using an automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/TIS/H_2O (v/v, 95/2.5/2.5) for 1.5 h to give a crude product. The peptide was used to the next reaction without further purification. Analytical HPLC: t_R = 14.5 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{25}H_{47}N_6O_6S$ 559.3, found 559.3.

*STGCILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4 (**2**).* The peptide was assembled on Rink amide resin (0.25 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa and Fmoc-Val-Thr($\Psi^{Me,Me}$pro): 4 equiv.). The subsequent deprotection of the resin was carried out with TFA/TIS/H_2O/DMB (v/v, 92.5/2.5/2.5/2.5) for 1.5 h to give a crude product. The peptide was used for the next reaction without further purification. Analytical HPLC: t_R = 22.3 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{153}H_{234}N_{34}O_{39}S_2$ 3236.7, found 3237.6.

Trt(OH)-K_{10}. The peptide was assembled on Fmoc-Lys(Boc)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Lys(Boc): 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 30 min to give a crude product, which was purified by preparative HPLC to yield the title compound (641 mg, 59%). Analytical HPLC: t_R = 14.4 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{80}H_{137}N_{20}O_{13}$ 1586.1, found 1586.0.

Trt(OH)-K_8. The peptide was assembled on Fmoc-Lys(Boc)-Wang resin (0.25 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 4 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 40 min to give a crude product, which was purified by preparative HPLC to yield the title compound (361 mg, 64%). Analytical HPLC: t_R = 14.8 min (1–40% CH_3CN/

0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{68}H_{113}N_{16}O_{11}$ 1329.9, found 1329.8.

Trt(OH)-K$_5$. The peptide was assembled on Fmoc-Lys(Boc)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Lys(Boc): 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 40 min to give a crude product, which was purified by preparative HPLC to yield the title compound (395 mg, 65%). Analytical HPLC: t_R = 15.7 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{50}H_{77}N_{10}O_8$ 945.6, found 945.5.

Trt(OH)-K$_4$. The peptide was assembled on Fmoc-Lys(Boc)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound (328 mg, 64%). Analytical HPLC: t_R = 16.3 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{44}H_{65}N_8O_7$ 817.5, found 817.4.

Trt(OH)-K$_3$. The peptide was assembled on Fmoc-Lys(Boc)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 40 min to give a crude product, which was purified by preparative HPLC to yield the title compound (202 mg, 49%). Analytical HPLC: t_R = 17.4 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{38}H_{53}N_6O_6$ 689.4, found 689.4.

Trt(OH)-R$_8$. The peptide was assembled on the Fmoc-Arg(Pbf)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O/thioanisole (v/v/v, 92.5/2.5/5) for 25 min to give a crude product, which was purified by preparative HPLC to yield the title compound (202 mg, 20%). Analytical HPLC: t_R = 16.8 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{68}H_{113}N_{32}O_{11}$ 1553.9, found 1553.9.

Trt(OH)-R$_5$. The peptide was assembled on the Fmoc-Arg(Pbf)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O/thioanisole (v/v/v, 95/2.5/2.5) for 25 min to give a crude product, which was purified by preparative HPLC to yield the title compound (248 mg, 37%). Analytical HPLC: t_R = 17.4 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{50}H_{77}N_{20}O_8$ 1085.6, found 1085.5.

Trt(OH)-R$_3$. The peptide was assembled on the Fmoc-Arg(Pbf)-Wang resin (0.40 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O/thioanisole (v/v/v, 95/2.5/2.5) for 25 min to give a crude product, which was purified by preparative HPLC to yield the title compound (265 mg, 59%). Analytical HPLC: t_R = 18.8 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{38}H_{53}N_{12}O_6$ 773.4, found 773.4.

Trt(OH)-E$_5$. The peptide was assembled on the Fmoc-Glu(O*t*Bu)-Wang resin (0.25 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 4 equiv., 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound (144 mg, 61%). Analytical HPLC: t_R = 14.3 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M − H) calcd for $C_{45}H_{50}N_5O_{18}$ 948.3, found 948.2.

Trt(OH)-N$_5$. The peptide was assembled on Rink amide resin (0.4 mmol) using the automated Fmoc SPPS procedure as described in General information (Fmoc-Xaa: 2.5 equiv., Fmoc-Asp-O*t*Bu is used as C-terminal Xaa, 4-(diphenylhydroxymethyl)benzoic acid: 2.5 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound (234 mg, 67%). Analytical HPLC: t_R = 12.9 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M − H) calcd for $C_{40}H_{45}N_{10}O_{13}$ 873.3, found 873.3.

Trt(OH)-branched K$_8$. The peptide was assembled on the Fmoc-Lys(Aloc)-Wang resin (0.25 mmol) using a manual Fmoc SPPS procedure (Fmoc-Lys(Fmoc): 4 equiv. to amino group) without final Fmoc deprotection. The resin was treated with $Pd(PPh_3)_4$ (72.2 mg, 0.0625 mmol) and $PhSiH_3$ (1.53 mL, 12.5 mmol) in CH_2Cl_2 for 1 h. Then, 4-(diphenylhydroxymethyl)benzoic acid was coupled using the DIC/Oxyma method, followed by deprotection of the Fmoc group with 20% piperidine/DMF. The obtained resin was treated with TFA/H_2O (v/v, 95/5) for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound (77.8 mg, 14%). Analytical HPLC: t_R = 15.9 min (1–40% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{68}H_{113}N_{16}O_{11}$ 1329.9, found 1329.8.

Trt(OH)-[K(Me$_3$)]$_5$. The peptide was assembled on the Fmoc-Gly-Wang resin (0.1 mmol) using the manual Fmoc SPPS procedure (Fmoc-Lys(Me_3)·Cl: 2 equiv., 4-(diphenylhydroxymethyl) benzoic acid: 2 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound (72.6 mg, 41%). Analytical HPLC: t_R = 12.1 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M) calcd for $C_{67}H_{114}N_{11}O_9^{5+}$ 243.4, found 243.5.

Trt(OH)-[C(O$_3$H)]$_5$. The peptide was assembled on the Fmoc-Gly-Wang resin (0.25 mmol) using the manual Fmoc SPPS procedure (Fmoc-Cys(O_3H): 2 equiv., 4-(diphenylhydroxymethyl) benzoic acid: 2 equiv.). The subsequent deprotection of the resin was carried out with TFA/H_2O (v/v, 95/5) for 1 h to give a crude product, which was purified by preparative HPLC to yield the title compound (96.9 mg, 35%). Analytical HPLC: t_R =

9.2 min (1–60% CH_3CN/0.1% TFA for 25 min); LRMS (M − H) calcd for $C_{37}H_{43}N_6O_{24}S_5$ 1115.1, found 1115.0.

Attachment of Trt(OH)-X to Ac-VVCVV-NH_2

General procedure. Ac-VVCVV-NH_2 (1 equiv., 10 mM) and Trt(OH)-X (1.1 equiv.) were dissolved in HFIP.[6] After stirring for 1 h at room temperature, the reaction mixture was concentrated and subjected to preparative HPLC.

Ac-VVC(Trt-K_{10})VV-NH_2. 27.8 mg, 85% isolated yield. Analytical HPLC: t_R = 12.9 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{105}H_{181}N_{26}O_{18}S$ 2126.4, found 2126.3.

Ac-VVC(Trt-K_8)VV-NH_2. 25.6 mg, 92% isolated yield. Analytical HPLC: t_R = 13.2 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{93}H_{157}N_{22}O_{16}S$ 1870.2, found 1870.1.

Ac-VVC(Trt-branched K_8)VV-NH_2. 25.1 mg, 90% isolated yield. Analytical HPLC: t_R = 13.5 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{93}H_{157}N_{22}O_{16}S$ 1870.2, found 1870.1.

Ac-VVC(Trt-K_5)VV-NH_2. 26.1 mg, 85% isolated yield. Analytical HPLC: t_R = 14.0 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{75}H_{121}N_{16}O_{13}S$ 1485.9, found 1485.8.

Ac-VVC(Trt-K_4)VV-NH_2. 24.0 mg, 88% isolated yield. Analytical HPLC: t_R = 14.5 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{69}H_{109}N_{14}O_{12}S$ 1357.8, found 1357.8.

Ac-VVC(Trt-K_3)VV-NH_2. 26.3 mg, 84% isolated yield. Analytical HPLC: t_R = 15.2 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{63}H_{97}N_{12}O_{11}S$ 1229.7, found 1229.6.

Ac-VVC(Trt-R_8)VV-NH_2. 22.1 mg, 73% isolated yield. Analytical HPLC: t_R = 14.1 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{93}H_{157}N_{38}O_{16}S$ 2094.2, found 2094.2.

Ac-VVC(Trt-R_5)VV-NH_2. 29.1 mg, 88% isolated yield. Analytical HPLC: t_R = 14.8 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{75}H_{121}N_{26}O_{13}S$ 1625.9, found 1625.9.

Ac-VVC(Trt-R_3)VV-NH_2. 27.6 mg, 83% isolated yield. Analytical HPLC: t_R = 15.9 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{63}H_{97}N_{18}O_{11}S$ 1313.7, found 1313.7.

Ac-VVC(Trt-E_5)VV-NH_2. 16.5 mg, 74% isolated yield. Analytical HPLC: t_R = 17.2 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M − H) calcd for $C_{70}H_{94}N_{11}O_{23}S$ 1488.6, found 1488.5.

Ac-VVC(Trt-N_5)VV-NH_2. 7.1 mg, 70% isolated yield. Analytical HPLC: t_R = 16.7 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M + H) calcd for $C_{65}H_{91}N_{16}O_{18}S$ 1415.6, found 1415.6.

Ac-VVC{Trt-[K(Me_3)]$_5$}VV-NH_2. 21.3 mg, 81% isolated yield. Analytical HPLC: t_R = 15.5 min (10–60% CH_3CN/0.1% TFA for 25 min); LRMS (M) calcd for $C_{92}H_{158}N_{17}O_{14}S^{5+}$ 351.4, found 351.6.

Ac-VVC{Trt-[C(O_3H)]$_5$}VV-NH_2. The title peptide was isolated using a 0.1 M NH_4OAc buffer (pH 7) solvent system as a single peak (8.8 mg, 35% isolated yield). Analytical HPLC: t_R = 14.2 min (10–60% CH_3CN/0.1 M NH_4OAc buffer (pH 7) for 25 min); LRMS (M − H) calcd for $C_{62}H_{87}N_{12}O_{29}S_6$ 1655.4, found 1655.3.

Attachment of Trt(OH)-X to SPP4

General procedure. SPP4 (1 equiv., 10 mM) and Trt(OH)-X (1.1 equiv.) were dissolved in HFIP or TFA. After stirring for 1 h at room temperature, the reaction mixture was concentrated and subjected to preparative HPLC.

STGC(Trt-K_{10})ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-K_{10}. 46.1 mg, 35% isolated yield. Analytical HPLC: t_R = 17.7 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{233}H_{368}N_{54}O_{51}S_2$ 4804.7, found 4805.4.

STGC(Trt-K_8)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-K_8. 37.3 mg, 33% isolated yield. Analytical HPLC: t_R = 18.0 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{221}H_{344}N_{50}O_{49}S_2$ 4548.5, found 4549.0.

STGC(Trt-branched K_8)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-branched K_8. 31.6 mg, 34% isolated yield. Analytical HPLC: t_R = 18.0 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{221}H_{344}N_{50}O_{49}S_2$ 4548.5, found 4549.1.

STGC(Trt-K_5)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-K_5. 27.9 mg, 28% isolated yield. Analytical HPLC: t_R = 18.6 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{203}H_{308}N_{44}O_{46}S_2$ 4164.3, found 4164.5.

STGC(Trt-K_4)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-K_4. 12.8 mg, 14% isolated yield. Analytical HPLC: t_R = 19.0 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{197}H_{296}N_{42}O_{45}S_2$ 4036.2, found 4036.6.

STGC(Trt-K_3)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-K_3. 15.8 mg, 18% isolated yield. Analytical HPLC: t_R = 19.6 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{191}H_{284}N_{40}O_{44}S_2$ 3908.1, found 3908.1.

STGC(Trt-R_8)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-R_8. 32.8 mg, 28% isolated yield. Analytical HPLC: t_R = 18.1 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{221}H_{344}N_{66}O_{49}S_2$ 4772.6, found 4773.2.

STGC(Trt-R_5)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-R_5. 27.5 mg, 27% isolated yield. Analytical HPLC: t_R = 18.7 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{203}H_{308}N_{54}O_{46}S_2$ 4304.3, found 4304.6.

STGC(Trt-R_3)ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-R_3. 15.9 mg, 17% isolated yield. Analytical HPLC: t_R = 19.6 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (ESI) calcd for $C_{191}H_{284}N_{46}O_{44}S_2$ 3992.1, found 3992.2.

STGC{Trt-[K(Me_3)]$_5$}ILLGGLFIYDVFWVFGTNVMVTVAKS-NH_2 = SPP4-Trt-K[(Me_3)]$_5$. 11.5 mg, 13% isolated yield. Analytical HPLC: t_R = 19.9 min (10–98% CH_3CN/0.1% TFA for 25 min); LRMS (M) calcd for $C_{220}H_{346}N_{45}O_{47}S_2^{5+}$ 887.3, found 887.3.

Evaluation of the solubility of Trt-X tagged peptides. The solubility of Trt-X tagged peptides was determined by calculation of peak areas of standard solutions and the supernatant of saturated solutions using analytical HPLC. The standard solutions were prepared by dissolving the peptides at 1 mg mL^{-1} in DMSO. The saturated solutions were prepared by adding small aliquots of buffers into the peptides. The satu-

rated solutions were centrifuged and the supernatants were diluted with 50% AcOH/H_2O or 3 M Gn·HCl in 50% AcOH/H_2O, respectively. The standard solutions and the diluted supernatant solutions were compared by peak areas using analytical HPLC.

Conflicts of interest

There are no conflicts to declare.

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