Pseudo-Prolines as a Solubilizing, Structure-Disrupting Protection Technique in Peptide Synthesis

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Abstract: Serine-, threonine-, and cysteine-derived cyclic building blocks (pseudo-prolines, ΨPro) serve as reversible protecting groups for Ser, Thr, and Cys and prove to be versatile tools for overcoming some intrinsic problems in the field of peptide chemistry. The presence of ΨPro within a peptide sequence results in the disruption of β-sheet structures considered as a source of intermolecular aggregation during chain elongation, thus increasing solvation and coupling kinetics in peptide assembly. Due to their easy synthetic access and variability in the chemical stability by modifications introduced in the C-2 position of the oxazolidine/thiazolidine ring system, this protection technique is adaptable to all common strategies in peptide synthesis. We describe new types of ΨPro building blocks suitable for standard Fmoc/*t*Bu-based solid phase peptide synthesis, convergent strategies, and chemoselective ligation techniques as well as their use as a structure-disrupting, solubilizing protection technique for the example of peptides generally considered as "difficult sequences".

Introduction

Since the advent of solid phase peptide synthesis (SPPS),[1] a multitude of synthetic peptides have been produced using this approach. Subsequently, many efforts have been devoted to improving specific protecting groups, support systems, activation methods, and the automation of protocols. However, successful peptide assembly is still hampered by inherent problems such as poor solvation of the growing peptide chain during solid phase synthesis as well as limited solubility of fully protected peptide fragments in the solution approach, often leading to incomplete coupling steps.[2] These undesirable physicochemical problems originate from intermolecular hydrophobic aggregation of the protected peptide chains and/or the formation of secondary structures, most notably of β-sheets.[3] Reported attempts to suppress the degenerative effect of such associations during aminoacylation reactions involve essentially "external factors" like solvent composition,[4] elevated temperature,[5] and use of chaotropic salts[6] or solubilizing protecting groups[7] which have been shown to have variable efficiencies. Hydrogen-bonded association has also been prevented by the introduction of an amide protecting group within the peptide chain.[8]

Recently, we reported that Ser/Thr-derived oxazolidine and Cys-derived thiazolidine derivatives (pseudo-prolines, ΨPro) exert a pronounced effect upon backbone conformation due to their structural similarity with proline itself (Scheme 1).[9] Due to the induction of a "kink" conformation in the peptide backbone, originating in the preference for *cis* amide bond formation, ΨPro prevent peptide aggregation, self-association, and β-structure formation, thus improving the solvation and coupling kinetics of the growing peptide chain considerably. These new building blocks are readily accessible by cyclization of Ser, Thr, or Cys with aldehydes or ketones (Scheme 1) and serve as a reversible protecting group in peptide synthesis. As a particular feature, variation of the C-2 substituents directly affects the ring stability, thus allowing for differential chemical stabilities in a variety of synthetic strategies.

Consequently, pseudo-prolines represent a valuable tool for combining the protection of Ser, Thr, and Cys residues with the simultaneous solubilization of the peptide chain. After having established some basic structural features of ΨPro and their potential in accessing difficult sequences, we elaborate here on the synthesis and chemical stability of ΨPro derivatives specifically designed for standard SPPS and for convergent strategies. In particular, their incorporation as dipeptide building

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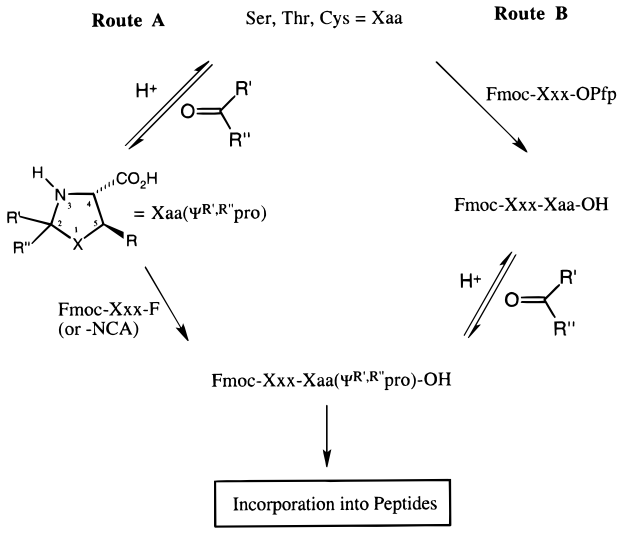
Scheme 1. Pseudo-Proline Unit and Overview of the ΨPro Building Block Synthesis According to Routes A and B[a]



[a] L-serine (X = O, R = H) and L-threonine (X = O, R = methyl) derived 4-oxazolidinecarboxylic acid (Ser($\Psi^{R',R''}$pro); Thr($\Psi^{R',R''}$pro)); L-cysteine (X = S, R = H) derived 4-thiazolidinecarboxylic acid (Cys($\Psi^{R',R''}$pro)); R′ and/or R″ = alkyl, aryl.

Table 1. Overall Yields of Synthesis Calculated from Ser, Thr, or Cys and Cleavage Conditions of Prototype ΨPro Building Blocks Compatible with Fmoc-Peptide Synthesis Strategies[a]

prototype ΨPro building blocks	overall yield (%)	cleavage conditions
1, Fmoc-Xxx-Cys($\Psi^{Me,Me}$pro)-OH[b]	60−80	TFA within hours
2, Fmoc-Xxx-Cys($\Psi^{H,R}$pro)-OH[b,c]	85−95	TFA within minutes
3, Fmoc-Xxx-Cys($\Psi^{H,H}$pro)-OH	85−95	stable to strong acids
4, Fmoc-Xxx-Ser($\Psi^{Me,Me}$pro)-OH	70−90	TFA within minutes
5, Fmoc-Xxx-Thr($\Psi^{Me,Me}$pro)-OH	70−90	TFA within minutes
6, Fmoc-Xxx-Ser($\Psi^{H,H}$pro)-OH	60−80	TFMSA within hours
7, Fmoc-Xxx-Thr($\Psi^{H,H}$pro)-OH	60−80	TFMSA within hours

[a] ΨPro building blocks **1**, **2**, **3**, **6**, and **7** synthesized according to route A, **4** and **5** according to route B (Scheme 1). [b] Structural formula; see Scheme 1. [c] R = 2,4-dimethoxyphenyl.

blocks into peptide backbones and their differential cleavage in connection with commonly applied protecting groups in peptide synthesis are exemplified for various structurally and functionally important peptides.

On the basis of the results obtained in these studies, ΨPro are established as a convenient side-chain protection technique for Ser, Thr, and Cys adaptable to most common strategies for peptide synthesis, opening new prospects for the chemical preparation of large peptides and proteins.

Results

Pseudo-prolines. The pseudo-proline (ΨPro)-containing dipeptides presented in Table 1 emerged as the most suitable building blocks compatible with the Fmoc stepwise synthetic approach as well as for the segment assembly approach. Their chemical stability toward acid, which predetermines the deprotection (ring-opening) conditions, largely depends on the nature of the C-2 substituents, due to the strong relationship between electronic effects of the substituents and ring stability: Whereas dilute TFA[10] was sufficient to open the ring in the 2,2-dimethyloxazolidine-dipeptide **4**, strong acids like TFMSA[10] were required to deprotect the exceptionally stable unsubstituted oxazolidine as in dipeptide **6**. Furthermore, it has been estimated that the relative stability of the thiazolidine ring is more than 10^4 times higher than that of the corresponding oxazolidine.[11] This was confirmed by attempts to cleave the thiazolidine ring system, as in **3**, with strong acids. Consequently, rather than using the C-2-unsubstituted ΨPro as a temporary protection technique, these building blocks may serve as proline mimics. In contrast, by introducing C-2 substituents as in compound **1** or **2**, ring opening can be achieved under moderately strong acidic conditions. The kinetics of the ring opening turns out to be strongly dependent on the acid/solvent system.

For example, the acidolysis of Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-NHBzl in methanol at room temperature was complete within minutes in a 70% TFA/methanol solution, whereas a 10% decrease in acid concentration resulted in an increase of the deprotection time by a factor >10 (Figure 1a). The same observations were made for the Ser and Cys analogues. The solvent dependency of the ring-opening reaction was investigated in methanol, THF, and DCM with the model dipeptide Fmoc-Ala-Ser($\Psi^{Me,Me}$pro)-NHBzl (Figure 1c). DCM was found to be the appropriate solvent for rapid deprotection, since a 5% TFA/DCM solution led to quantitative ring opening within minutes. In 50% TFA/THF and 60% TFA/methanol the cleavage time increased to 90 min and 24 h, respectively. Alternatively, TFA can be replaced by Lewis acids such as boron trifluoride which was shown to catalyze the deprotection reaction of Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-NHBzl even more efficiently (Figure 1b). Whereas the various ΨPro derivatives are effectively cleaved by acids, they proved to be stable toward the conditions normally used for *N*α-Fmoc deprotection (e.g., 20% piperidine in DMF).

The convenient preparation of oxazolidine- or thiazolidine-containing dipeptides allows for tailoring the chemical stability according to the strategy used for peptide synthesis. Hence, the ΨPro building blocks **2**, **4**, and **5** (Table 1) are compatible with standard Fmoc/*t*Bu protection (cleavable with 90% TFA within minutes) whereas the ΨPro ring systems **1**, **6**, and **7** are stable under *t*Bu/Boc cleavage conditions; here ring opening needs strong acidic conditions and proceeds within hours or days, depending on the sequence and chain length of the peptide (see below).

The incorporation of ΨPro residues into a growing peptide chain in SPPS proceeds preferentially *via* their preformed dipeptide derivatives of the type Fmoc-Xxx-Xaa($\Psi^{R',R''}$pro)-OH (see Table 1). These commercially available[12] ΨPro-containing building blocks can be coupled without racemization (in analogy to Pro) according to standard procedures (see below). The synthesis of C-2-unsubstituted Ser- and Thr-derived oxazolidine dipeptides is readily accomplished by *in situ N*-acylation with *N*α-Fmoc-protected amino acid fluorides or *N*-carboxyanhydrides (route B, Scheme 1), whereas the 2,2-dimethyloxazolidine building blocks **4** and **5** from Table 1 were obtained by direct insertion of oxazolidine in the suitably protected preformed dipeptides[9b] (route B, Scheme 1). In contrast, thiazolidine building blocks **1**, **2**, and **3** could readily be obtained by direct *N*-acylation of the stable 1,3-thiazolidine-4-carboxylic acids with *N*α-Fmoc-protected amino acid fluorides or *N*-carboxyanhydrides (route A, Scheme 1) with yields ranging from 60 to 95%. It is noteworthy that the condensation of L-cysteine with alde-

(10) The following abbreviations were used: ATR, attenuated total reflection; Bum, *tert*-butoxymethyl; CD, circular dichroism; DIC, *N*,*N*′-diisopropylcarbodiimide; DIEA, *N*,*N*-diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; MALDI, matrix-assisted laser desorption time of flight; NCA, *N*-carboxyanhydride; Pfp, pentafluorophenyl; ΨPro, pseudoproline; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; RP-HPLC, reversed phase high-performance liquid chromatography; R_t, retention time; TASP, template-assembled synthetic protein; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TFMSA, trifluoromethanesulfonic acid; Trt, trityl; Xaa, Xxx, amino acid.

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(12) Fmoc-ΨPro building blocks are now readily available from a commercial source (Calbiochem-Novabiochem) for routine use in peptide synthesis.

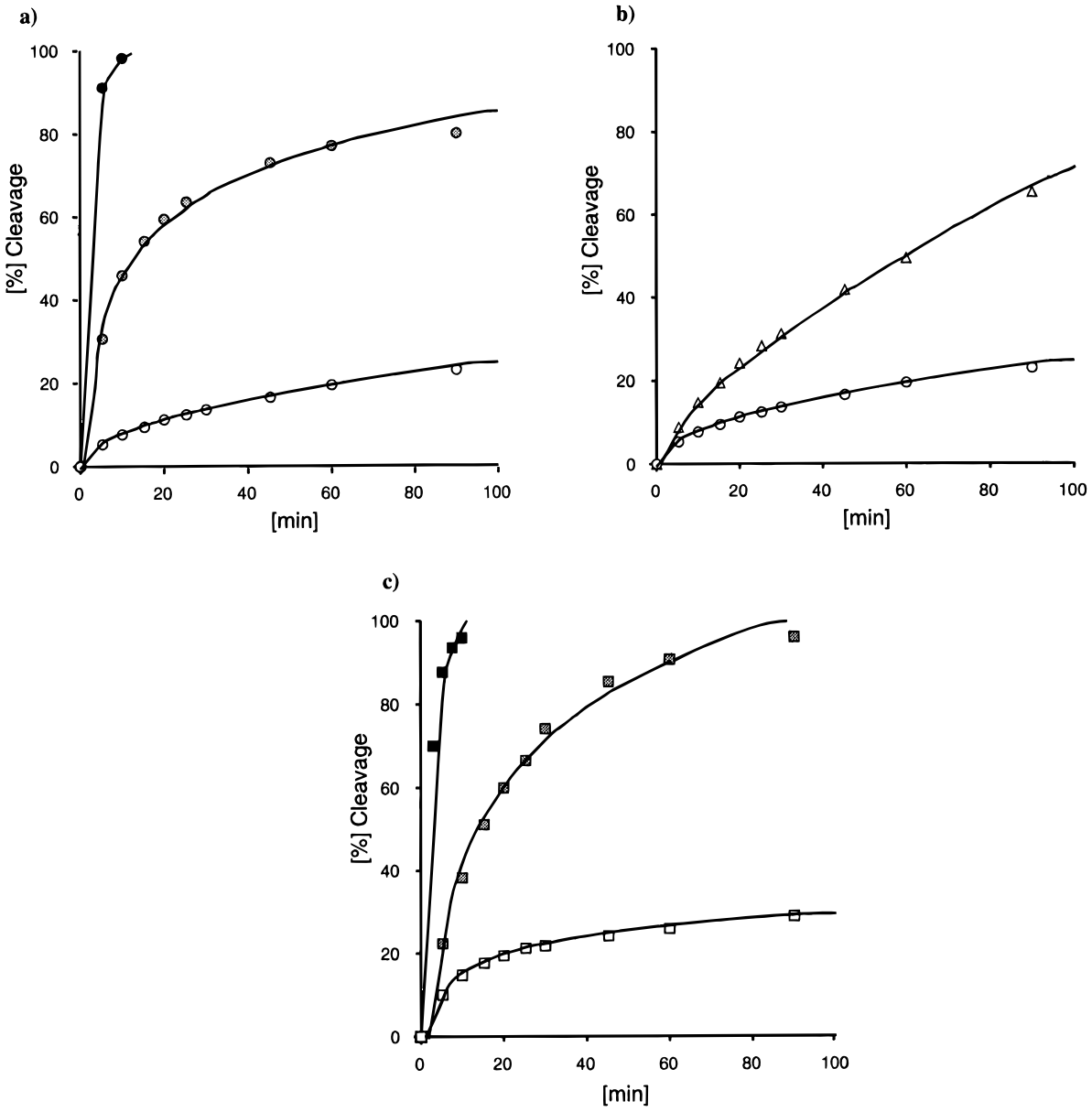

Figure 1. Kinetics of the deprotection of Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-NHBzl in solution (9.2 μmol/5 mL) at room temperature as monitored by RP-HPLC: (a) effect of increasing TFA concentration on ΨPro cleavage (deprotection); ○ in 50% TFA/MeOH, shaded circle, in 60% TFA/MeOH, ●, in 70% TFA/MeOH; (b) comparison of TFA- and BF_3-mediated deprotection; ○, Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-NHBzl in 5% $BF_3 \cdot C_4H_{10}O$/MeOH; △, Fmoc-Ala-Thr($\Psi^{Me,Me}$pro)-NHBzl in 50% TFA/MeOH; (c) influence of solvent choice on deprotection; □, in 5% TFA/DCM, shaded box, in 50% TFA/THF, ■, in 60% TFA/MeOH.

hydes or unsymmetrical ketones generates a mixture of C-2 epimers (2*S*,4*R*) and (2*R*,4*R*) as in ΨPro building blocks **2**. Although the diastereoisomeric mixture could be separated by RP-HPLC or crystallization, this was generally not undertaken since acidic deprotection results in the loss of the C-2 chiral center.

Oxazolidine- or thiazolidine-containing dipeptides present a much greater polar character than conventionally protected Ser, Thr, or Cys derivatives, eluting at retention times similar to those of the parent free Fmoc amino acids on RP-HPLC columns but earlier, for example, than *t*Bu- or trityl-protected Fmoc-amino acids. This finding can be rationalized by the formation of hydrogen bonds to the solvent-exposed ring heteroatom O or S similar to cyclic ethers. Consequently, ΨPro act as a polar protecting technique contributing to higher solvation of protected peptides.

All the ΨPro derivatives investigated showed remarkable thermodynamic stability. They were readily characterized using CI-MS and proved to be stable when stored over a long period of time at room temperature. In addition, ΨPro dipeptides are generally isolated as crystalline compounds and prove to be readily soluble in solvents used in peptide synthesis.

As well as the influence upon ring stability, the ΨPro C-2 substituents exert strong influence upon the conformation of the Xxx-Xaa(ΨPro) peptide bond. NMR studies followed by NOE experiments revealed that ΨPro dipeptides existed in two distinctive rotameric forms around the tertiary amide bond commonly assigned as *cis* and *trans* isomers.[13] However, differences were observed for C-2-disubstituted ΨPro as for example Ac-Ala-Ser($\Psi^{Me,Me}$pro)-NH-Me (data not shown). The absence of two sets of signals at room temperature indicated a stable conformer with a high activation barrier for the isomerization about the ΨPro peptide bond. By irradiating the $C\alpha_i$-proton and the NH_i proton specifically, a NOE upon the $C\alpha_{(i+1)}$-proton was observed, thus indicating a preference for the *cis*

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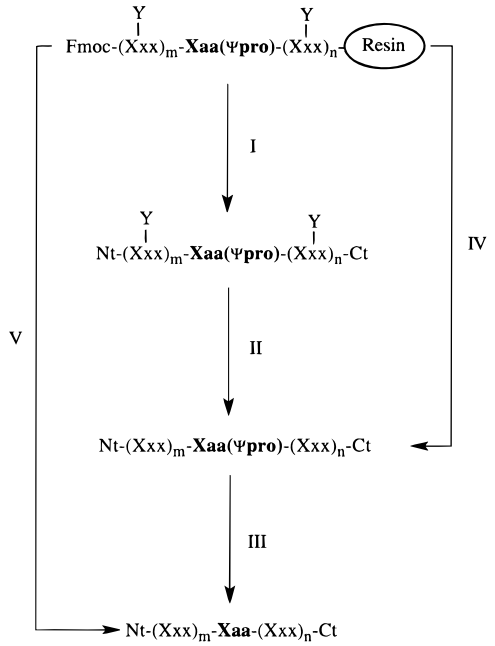

Figure 2. Strategic use of ΨPro building blocks in peptide chemistry according to standard Fmoc SPPS and convergent approaches using fully protected peptides or with ΨPro-containing deprotected peptides. Synthetic pathways I−V are described in the text. Abbreviations: Nt, Ct, N-terminal and C-terminal functional groups; Y, conventional protecting group; Xaa(Ψpro), Cys, Ser, or Thr protected as pseudoproline; Xxx, any amino acid.

conformation. Subsequent molecular dynamics calculations as well as X-ray diffraction analysis supported these findings.[14]

As a major goal of this study, general strategies for using ΨPro building blocks (Table 1) in peptide synthesis have been elaborated. When applied to segment condensation (synthetic route I, Figure 2), ΨPro have the potential to simplify the purification of peptide fragment intermediates and to enhance the segment coupling kinetics.[15] Here, the synthesis of the fully protected peptide is achieved by selective cleavage of the protected peptide fragment from super acid labile resins, for example, the 2-Cl-Trt[16] resin. Indeed, under these mild cleavage conditions (20% acetic acid), the C-2-substituted ΨPro blocks (e.g., 2,2-dimethyloxazolidine-4-carboxylic acid or 2-(2,4-dimethoxyphenyl)thiazolidine-4-carboxylic acid residues) remain stable. Similarly, ΨPro building blocks **2**, **4**, or **5** (Table 1) can be used with the Sasrin[17] resin if the cleavage conditions (1% TFA) are carefully controlled.

In some cases (e.g., in chemoselective ligation techniques)[18] it is desirable to preserve the ΨPro ring system intact while the other amino acid side chains are deprotected (strategy IV in Figure 2). In these circumstances, orthogonal side chain protecting groups Y to ΨPro, for instance, the benzylic,[19] allylic,[20] or Dde[21] protecting groups are applied. Alternatively, the use of the TFA stable proline isosteric oxazolidine or thiazolidine building blocks allows the preservation of intact

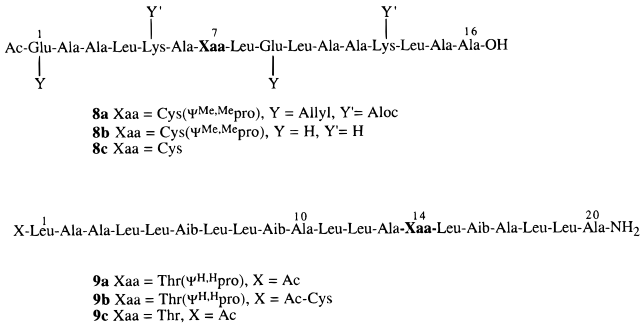

Figure 3. Peptides containing ΨPro building blocks for studying the solubilizing and conformational effects during SPPS and in subsequent segment assembly steps.

ΨPro residues in the fully deprotected peptide. Finally, by concomitant cleavage of the acid labile protecting groups Y (e.g., *t*Bu, Trt), the resin anchor (e.g., Wang, Sasrin, Rink), and the ΨPro ring system, a fully deprotected peptide can be obtained in one single step according to route V, Figure 2.

The strategies outlined in Figure 2 offer a large choice for accessing the beneficial properties of ΨPro building blocks in peptide synthesis. First, when used in conventional SPPS as a temporary protection technique for Ser, Thr, and Cys, the stepwise elongation of the peptide chain is strongly facilitated by the pronounced solubilizing effect of ΨPro building blocks. Cleavage of the ΨPro-containing, fully protected peptides (strategy I) allows the further condensation of hydrophobic peptides to larger polypeptides, thus increasing the potential of convergent strategies for the synthesis of proteins. Finally, differential cleavage of the side chain protecting groups (in preserving the ΨPro system) gives access to water soluble peptide fragments as required in chemoselective ligation techniques, preventing the problem of self-association and aggregation due to the secondary structure-disrupting effect of ΨPro. These various applications of ΨPro are exemplified by SPPS of some representative peptides, as described below.

ΨPro as a Solubilizing Protection Technique in Convergent Syntheses. (1) Bis-Amphiphilic Secondary Structure Forming Peptides (Switch Peptides). The potentially secondary structure forming peptide **8c** was designed according to the general principles of switch peptides[22] and included a Cys, protected as a ΨPro derivative at position 7 (Figure 3) for probing the effects of ΨPro.

The synthesis of peptide **8a** (Figure 3) was performed on a Fmoc-Ala-Sasrin resin using Fmoc chemistry and allyl protecting groups for Lys and Glu. A 4-fold orthogonality was used for preserving the potential of differential cleavage in **8a**. The incorporation of the ΨPro residue proceeded smoothly *via* the Fmoc-Ala-Cys(ΨMe,Mepro)-OH dipeptide and DIC/HOBt activation; the fully protected peptide **8a** was obtained in high yield after RP-HPLC purification. In contrast to the corresponding switch peptide devoid of a ΨPro residue (Cys(ΨMe,Mepro) replaced by Ala[22]), peptide **8a** proved to be soluble in polar organic solvents like methanol, DMF, or water/acetonitrile mixtures reflecting the ΨPro-induced conformational effects. Peptide **8b** was obtained after selective cleavage of the allyl protecting groups with a Pd^0/tributyltin hydride treatment[20] and subsequent RP-HPLC purification. Finally, the fully deprotected peptide **8c** was obtained by cleavage of the 2,2-dimethylthi-

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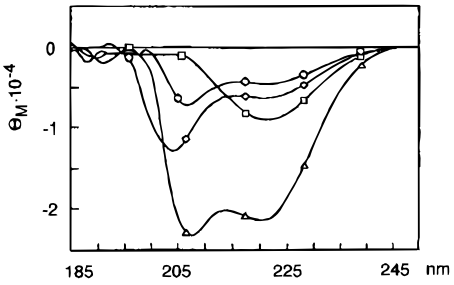

Figure 4. CD spectra of peptide **8b** (pH 4, 11) and peptide **8c** (pH 4, 11) (c = 0.25 mM).

azolidine ring in TFA for 4 h. The intrinsic capability of peptide **8c** to undergo medium dependent conformational transitions[22] was demonstrated by CD spectroscopy, as shown in Figure 4. Peptide **8c** adopts a β-structure conformation at pH 4, and shows a transition to a partially helical conformation at pH 11. It is significant to note that the replacement of Ala-7 with a Cys residue had no marked influence on the conformational properties of the peptide. However, the insertion of a single ΨPro residue within the peptide sequence as in **8b** results in a complete disruption of secondary structures (Figure 4) paralleled by increased solvation of the ΨPro-containing peptide.

The solubilizing power of ΨPro in peptide **8a** was further exemplified in the convergent synthesis of a TASP (template-assembled synthetic protein)[23] molecule. In this case, the fully protected peptide fragments **8a** were ligated to a topological template including the β-turn mimetics AMTA (8-(aminomethyl)-5,6,7,8-tetrahydro-2-naphthoic acid)[24] *via* amide bond formation to the lysine side chains. Due to the solubilizing effect of the ΨPro, peptide **8a** was readily soluble in a minimum volume of DMF, thus allowing the condensation reaction (PyBOP-mediated activation) to proceed to completion after 3 h as confirmed by analytical RP-HPLC and ES-MS.

(2) Transmembrane Peptides. The synthesis of a strongly hydrophobic sequence such as the transmembrane peptide **9c** (Figure 3) is generally classified as a "difficult sequence", essentially due to aggregation and β-sheet formation of the growing peptide chain resulting in low solvation and poor coupling efficiency. In order to circumvent this problem, we introduced a ΨPro moiety at position 14, as a structure-disrupting, solubilizing protection technique for Thr. The transmembrane peptide was synthesized on a Rink amide resin following standard Fmoc chemistry.[25] The incorporation of the pseudo-proline residue was effected *via* the dipeptide Fmoc-Ala-Thr($\Psi^{H,H}$pro)-OH (2.7 equiv) mediated by HOBt/DIC activation. Aib units were introduced as dipeptides Fmoc-Leu-Aib-OH in a similar fashion. Most significantly, the coupling of the ΨPro-containing dipeptide derivative as well as the following coupling steps proceeded smoothly to quantitative yields according to UV measurement of Fmoc release. After resin cleavage by TFA/H_2O (95/5) the ΨPro-containing peptide **9a** was obtained in high yield (86%) as shown by analytical HPLC of the crude product (Figure 5a). It is noteworthy that the HPLC conditions had to be adapted to the hydrophobic character of the peptide. The peptide products were best eluted with a linear gradient of 90% 2-propanol/10% water (0.09% TFA) (10−60%, 30 min) in 90% CH_3CN/10% water (0.09%

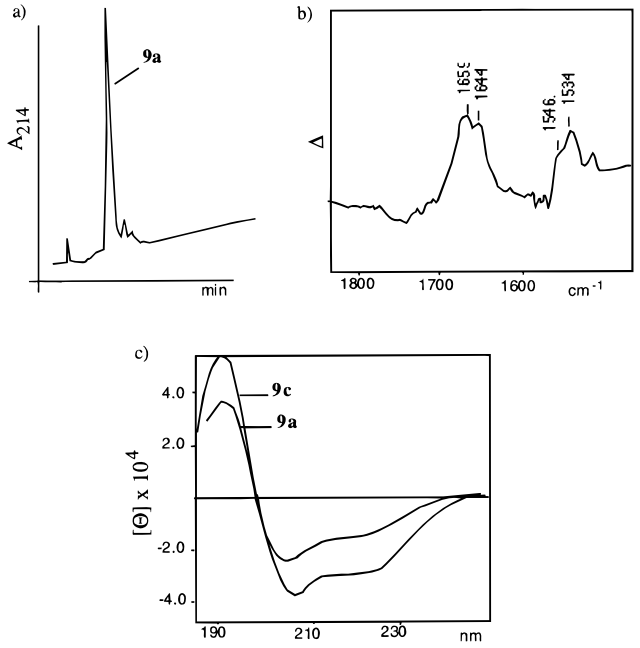

Figure 5. (a) RP-HPLC spectrum of the crude transmembrane peptide **9a** after resin cleavage. Column nucleosil 300-5C_4 (solvent A, 2-propanol, 90%/water, 10% (0.09% TFA); solvent B, acetonitrile, 90%/water, 10% (0.09% TFA)); 1 mL/min. (b) ATR-FT-IR of **9a** attached to the Rink amide resin. (c) CD spectra of peptides **9a** and **9b** in TFE (c = 1 mg/mL).

TFA) with a C_4 reversed phase column. The secondary structure-disrupting effect of the ΨPro residue was confirmed by the absence of β-sheet formation as seen from the ATR-IR spectrum of the resin bound sequence (Figure 5b). Furthermore, the ΨPro-containing peptide **9a** was readily soluble in a large number of organic solvents such as methanol, 2-propanol, DMF, DCM, and chloroform, thereby facilitating the purification protocol.

Selective cleavage of the oxazolidine ring system at position 14 was effected by treatment with strong acid (10% TFMSA in TFA) at room temperature for 48 h, producing unprotected peptide **9c**. Peptide **9c** showed a significantly lower solubility in organic solvents and in water/TFE mixtures compared to the precursor peptide **9a**. The CD spectra of transmembrane peptides **9c** and its ΨPro-containing precursor **9a** clearly reveal the helix-disrupting effect of pseudo-prolines: The observed helicity of the [ΨPro-14]-peptide **9a** increased significantly by transforming the Thr(ΨPro) building block to a regular Thr by ring opening (Figure 5c).

The solubilizing properties of ΨPro were further demonstrated for the convergent synthesis of an integral membrane TASP molecule[23,26] by chemoselective ligation. Peptide **9b** was found to be readily soluble in DMF, thus allowing the condensation reaction with template **10**[24] to proceed homogeneously. Thioether formation proceeded with 5 equiv of peptide **9b** at high dilution in DMF in the presence of DIEA (Figure 6). The condensation reaction was complete after 10 h as followed by analytical RP-HPLC. The transmembrane TASP protein **11** was finally obtained in 68% yield after RP-HPLC purification and proved to be soluble in most organic solvents.

ΨPro as Temporary Protection for Cys-Containing Peptides. The synthesis of [ΨPro-7]Sarafotoxin-S6b, a bicyclic 21 amino acid peptide isolated from the venom glands of the Israeli burrowing asp *Atractaspis engaddensis*[27] was used for

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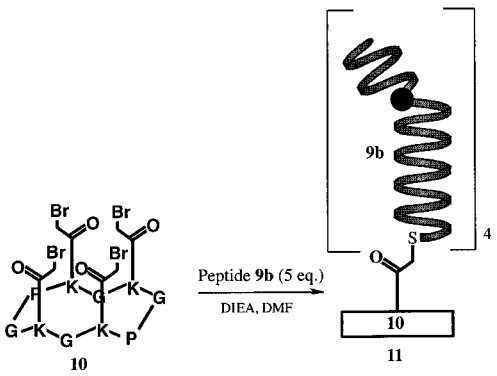

Figure 6. Synthesis of a four α-helix transmembrane TASP molecule (TASP T_4-($4\alpha_{20}$)) by chemoselective ligation via thioether formation. Template **10** was prepared as described previously.[24]

probing ΨPro as a temporary Cys protection technique in SPPS. In the strategy employed, Cys-3, -11, and -15 were protected as TFA labile 2,2-dimethylthiazolidines during the course of chain assembly, thereby preventing peptide aggregation on the solid support and allowing simultaneous deprotection of the thiol side chains during resin cleavage. Additionally, a TFA stable ΨPro residue (2,2-dihydrooxazolidine-4-carboxylic acid) was placed at the strategic position 7 for inducing backbone conformations (e.g., turns, kinks) favorable for the correct disulfide formation (isomer A, Figure 7).

The synthesis of Sarafotoxin-S6b (SRTX-b) was carried out according to Fmoc-PyBOP stepwise solid phase chemistry starting with a Fmoc-Trp(Boc)-functionalized Wang type resin. The amino acid side chain protections were chosen as follows: *t*Bu (Asp, Tyr, Ser, Glu), Boc (Lys), Bum (His), Trt (Gln, Cys-1). Thr-7, Cys-3, -11, and -15 were introduced as dipeptides in their pseudo-proline form. The progress of the assembly was monitored by UV detection of the dibenzofulvene adduct from the Fmoc deprotection step. The step-by-step synthesis proceeded smoothly since no coupling reaction needed to be repeated following the ninhydrin test. ATR-IR of resin samples revealed an absorption band at 1641 cm^{-1} (amide I band) throughout the build-up, consistent with the absence of β-sheet formation and pointing to the benefit of ΨPro in preventing peptide aggregation. Cleavage and deprotection of the peptide resin was effected by treatment with TFA/ethanedithiol/thioanisole/water/phenol (82.5/2.5/5/5/5) for 2 h under argon at room temperature. After filtration and washing with TFA and DCM, the crude product was precipitated with cold *tert*-butyl methyl ether. A further treatment for 32 h in TFA/water (95/5) was required to cleave the 2,2-dimethylthiazolidine ring completely. Surprisingly, the oxazolidine ring in position 7 was found to be stable when treated with the standard method for ring opening (10% TFMSA/90% TFA) compared to a corresponding dipeptide model. The preservation of a ΨPro system at position 7 turned out to be advantageous since the insolubility problems often encountered in SRTX or Endothelin[28] intermediates were eliminated, providing simple and efficient purification steps. The crude product was purified by preparative RP-HPLC to give the pure [ΨPro-7]-tetrathiol peptide in a very satisfactory overall yield of 18%, based on the resin loading (Figure 8b). The reduced peptide was subjected to air oxidation for 3 h under high dilution. As expected, the peptide with the correct disulfide bridge (isomer A) eluted earlier by RP-HPLC. Whereas an isomer ratio of 3/1 of the correct form A over B was obtained, a small peak characterized as Met(O)-peptide was also observed (Figure 8c). The folded peptides were isolated by RP-HPLC, lyophilized, and analyzed by analytical HPLC. ESI-MS analysis of the bicyclic peptides confirmed the integrity of the target molecule.

Discussion

In this paper we have presented ΨPro building blocks as a tool for modulation of the conformational and physicochemical properties of peptides. ΨPro primarily offer a convenient temporary protection technique for Ser, Thr, or Cys combined with several attractive inherent features useful for the design and synthesis of peptides. Most notably, ΨPro act as strongly solubilizing building blocks during peptide synthesis and in convergent strategies for the synthesis of large peptides.

ΨPro consist of oxazolidine- and thiazolidine-4-carboxylic acid ring systems which can readily be prepared from Thr, Ser, or Cys by cyclocondensation with aldehydes or ketones.[29] The cyclic structure is in equilibrium with the open chain imine form governed by the ring-chain tautomeric equilibrium constant of oxazolidines and thiazolidines. Furthermore, the presence of a heteroatom in position 1 of the ring system reduces the nucleophilic character of the nitrogen atom in position 3, resulting in poor acylation yields of a N-terminal ΨPro peptide. Consequently, rather than applying ΨPro in their monomeric form, they are introduced in a peptide preferably as preformed dipeptides of the type shown in Table 1. ΨPro building blocks have been developed for Fmoc synthetic strategies; however, they offer a degree of adaptability to other strategies as well, since varying the C-2 substituents directly affects the acid lability of the ring system. Besides their easy access, ΨPro building blocks are efficiently incorporated into solid phase peptide assembly according to conventional procedures such as HOBt activation, thus making their use as a routine technique (e.g., for automatic synthesis) very attractive. When applied in convergent strategies, ΨPro offer the additional advantage of increasing the choice of segmentation sites of the target sequence. Indeed, the coupling of C-terminal ΨPro-protected peptide fragments proceeds without racemization as for C-terminal proline or glycine fragments. Furthermore, it was demonstrated that ΨPro greatly expand our ability to synthesize so far inaccessible polypeptides in solubilizing hydrophobic protected segments and in preventing self-association of peptide fragments in convergent strategies or in chemoselective ligation approaches. The synthesis of a bis-amphiphilic peptide (switch peptide), designed for the investigation of the structural and dynamic factors involved in secondary and tertiary structure formation of peptides and proteins,[22] provided an ideal model for studying the influence of ΨPro upon secondary structure formation and self-association. Here, the solid phase assembly of the medium-sized fully protected peptide **8a** proceeded more efficiently and to higher purity compared to its analogue devoid of a ΨPro building block.[22] Owing to the solubilizing effect of the incorporated ΨPro residue, the fully protected fragment **8b** could be efficiently coupled in solution to a topological template, resulting in a four-bundle TASP molecule of $M >$ 8000.[30] This representative example shows that the general problem of low solvation in segment assembly strategies can be overcome with the use of the ΨPro protection technique.

Due to the structural and functional importance of membrane-spanning peptides, much attention has been devoted to the

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(30) The conformational properties of this switch TASP molecule will be the subject of another publication.

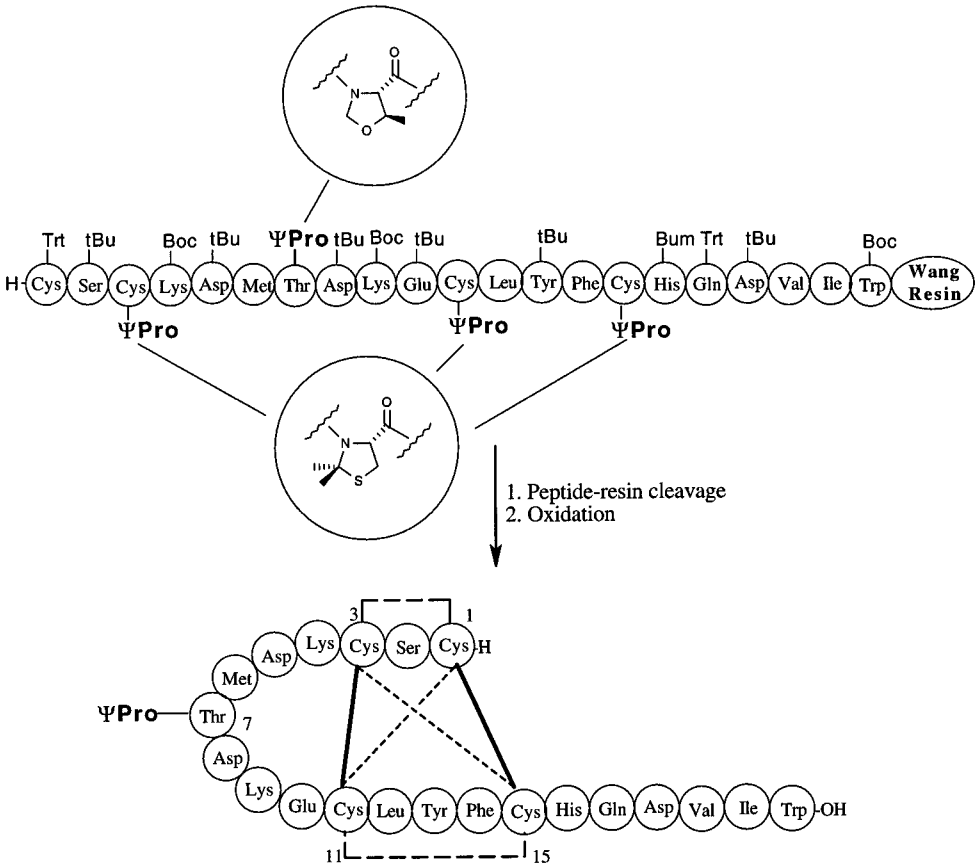

Figure 7. Solid phase synthesis of [Ψpro-7]Sarafotoxin-S6b with Cys-3, -11, and -15 protected as ΨPro followed by resin cleavage and random air oxidation of the Cys side chains, giving the three possible disulfide isomers: —, 1−15, 3−11, A, - - -, 1−11, 3−15, B; — — —, 1−3, 11−15, C.

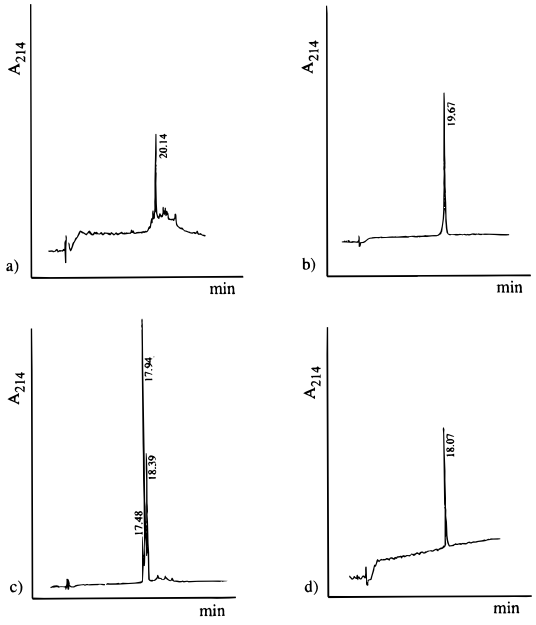

Figure 8. Analytical RP-HPLC of [ΨPro-7]SRTX-b: (a) crude, (b) purified tetrathiol, (c) after air oxidation, (d) after HPLC purification (conditions: Nucleosil C_{18} (250 × 4 mm) column; gradient of 10−60% B in 30 min (solven A, 0.09% TFA in water; solvent B, acetonitrile/water/TFA (90/10/0.09)); $\lambda = 214$ nm).

design, synthesis, and biostructural characterization of this particular class of peptides. Due to their hydrophobic nature, the chemical synthesis and purification protocols prove to be extremely difficult. For example, transmembrane peptides of the type $(Val)_n-(Ser)_m$ could not be prepared according to standard protocols of SPPS.[31] As part of our on-going structural studies directed toward the construction of supramolecular assembly systems using TASP molecules,[23c] the ΨPro concept appeared to be an ideal tool for overcoming these intrinsic problems in the synthesis of fragments derived from integral membrane proteins.[32] For this, we modified a hydrophobic transmembrane segment reported recently[32] by incorporating a ΨPro residue in a central position of the peptide **9a**. As well as the expected solubilizing effect, the incorporation of ΨPro as a proline mimetic provided an elegant tool for probing the structural and functional role of a Pro residue in a transmembrane helical peptide[33] (induction of a "kink", Figure 6). α-Aminoisobutyric acid (Aib) residues at positions 6, 9, and 16 were incorporated in order to enhance the helicity of this segment. As before, the solid phase assembly of this strongly hydrophobic sequence proceeded in single coupling steps to completion. In particular, the introduction of the ΨPro dipeptide was achieved according to standard protocols of automated SPPS; the target peptide **9c** containing the ΨPro building block and a N-terminal Cys was readily purified and coupled to a topological template *via* thioether formation. The presence of a single ΨPro residue prevented this hydrophobic peptide from intermolecular aggregation, thus providing excellent solubility in organic solvents for chemoselective thioether formation. The resulting TASP molecule will be the subject of detailed biofunctional investigations. The structure and biological function of Sarafotoxin-S6b (SRTX-b), Figure 7, are closely related to those of the mammalian family of vasoconstrictor

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peptides endothelins (ET-1, ET-2, ET-3),[28] and SRTX-b was chosen as a model for the use of Cys-derived ΨPro residues for improving the SPPS of bioactive peptides of larger size. So far, there has been no consensus as to the three-dimensional structure of SRTX-b, but it is suggested to be potentially identical to ET-1.[28] The secondary structure would therefore be characterized by an α-helix spanning from Lys-9 to Cys-15 and an extended strand of Cys-1 to Cys-3. In the natural form both structures are stabilized by disulfide bonds pairing Cys-1−15 and Cys-3−11, joining the two segments in antiparallel alignments. These two segments are linked by the β-turn-forming peptide Lys-4-Asp-Met-Thr-7 moiety. Although three disulfide isomers are possible (isomer C, 1−3, 11−15; isomer B, 1−11, 3−15; isomer A, 1−15, 3−11) as shown in Figure 7, only the A and B types are formed during random oxidation of the tetrathiol product. It has been shown for ET-1 that the exchange of amino acids may result in analogues, producing an altered isomer distribution during thiol oxidation. We therefore introduced a Thr-derived ΨPro of orthogonal chemical stability at position 7 for inducing backbone conformations promoting the correct disulfide formation. However, the observed isomer ratio (A/B = 3/1) was identical to the reported ratio for the oxidation of reduced sarafotoxin,[34] indicating that the conformational restriction induced by the ΨPro residue at position 7 did not significantly affect disulfide bridge formation. The stepwise synthesis of SRTX-b including ΨPro dipeptide derivatives as temporary protection for Cys-3, -11, and -15 proceeded smoothly and in high yields due to the solubilizing, β-sheet-disrupting effect of ΨPro. Most notably, the strategy applied here adapts perfectly to standard Fmoc/*t*Bu protocols and provides a general scheme for the SPPS of Cys-containing peptides. Obviously, by incorporating Cys-derived ΨPro building blocks of differential chemical stability, the ΨPro concept enlarges the potential for selective disulfide formation in bioactive peptides.

The above examples clearly demonstrate the crucial role that ΨPro may play in the stepwise synthesis of Thr-, Ser-, or Cys-containing peptides. Moreover, ΨPro have been essential in solubilizing protected segments in convergent strategies and in increasing the solvation of unprotected fragments for chemoselective ligation techniques. From the results obtained in this study, the following general features of the ΨPro concept in peptide synthesis can be derived: (1) ΨPro represent a convenient, cheap, and easily accessible temporary protection technique for Ser, Thr, and Cys of comparable chemical stability as *t*Bu or Trt side chain protecting groups.[12] (2) ΨPro building blocks are compatible with common strategies for peptide synthesis. (3) ΨPro are introduced in peptides as N-protected dipeptides following the same protocols as conventional Fmoc-amino acids (e.g., HOBt/DIC) without racemization, essentially because of their C-terminal proline-like structure. (4) The coupling of dipeptides or fragments containing C-terminal ΨPro residues proceeds without racemization, offering a new tool in the convergent synthesis of peptide fragments exhibiting C-terminal Ser, Thr, or Cys. (5) ΨPro disrupt secondary structures (most notably β-sheets) and increase the solvation of the growing peptide chain during solid phase assembly. This solubilizing effect may be preserved in fragment condensation or chemoselective ligation procedures by selective cleavage protocols.

Besides these practical applications, ΨPro as reversible mimetics of Pro provide an interesting source for structure−function studies in peptide and protein chemistry. Moreover, the introduction of reactive sites into the ring system of ΨPro (e.g., by using multifunctional aldehydes or ketones) opens new prospects in peptide modification and targeting and in the *de novo* design of proteins.

Experimental Section

Materials and Methods. All protected amino acids were purchased from Calbiochem-Novabiochem AG (Läufelfingen, CH) except for *N*α-Fmoc-Xaa-NCA (Propeptide, Vert-le-Petit, F) and *N*α-Fmoc-Xaa-Pfp (Bachem, Bübendorf, CH). Reagents and solvents were purchased from Fluka (Buchs, CH) and used without further purification. HPLC was performed on Waters equipment using columns packed with Vydac Nucleosil 300 Å 5 μm C_{18} particles unless otherwise stated. The analytical column (250 × 4.6 mm) was operated at 1 mL/min and the preparative column (250 × 21 mm) at 18 mL/min, monitoring at 214 nm. Solvent A consisted of 0.09% TFA and solvent B of 0.09% TFA in 90% acetonitrile unless otherwise stated. TLC was performed on silica gel plates, Merck 60 F254, detection by UV and 5% vanillin in concentrated sulfuric acid. Flash chromatography was performed with Merck silica gel 60 (40−63 mesh). Amino acid analyses were performed after 24 h of hydrolysis in 4 N methanesulfonic acid on a Perkin-Elmer HPLC. Mass spectra were obtained by laser desorption ionization (LDI-MS) with an LDI-1700 mass monitor, by electron spray ionization (ESI-MS) on a Finnigan MAT SSQ 710C, or by chemical ionization (CI-MS) with a Nermag R10-10C. Circular dichroism (CD) spectra were recorded at room temperature on a Jobin Yvon CD Mark V spectropolarimeter using quartz cells of 0.1 mm path length. The instrument was calibrated with D-10 camphorsulfonic acid. ^{1}H-NMR spectra were obtained on a Bruker-WH250 FT or Bruker DPX-400 with trimethylsilane as the internal standard. Infrared spectra were recorded on a Perkin-Elmer Paragon 1000 FT-IR spectrometer using KBr pills. Melting points are uncorrected values measured with a Büchi 510 melting point apparatus.

Peptide Synthesis. All the peptides described hereby were synthesized on a semiautomatic Advanced ChemTech ACT 200 peptide synthesizer using standard Fmoc solid-phase peptide synthetic protocols.[25] The resins were purchased from Calbiochem-Novabiochem AG (Läufelfingen, CH) with the C-terminal *N*α-Fmoc-amino acid already attached to the anchor group. Before starting a peptide synthesis, the resins were swollen in dichloromethane (DCM) for about 30 min. Commercial *N*,*N*-dimethylformamide (DMF) was degassed for several hours with nitrogen. The peptide chains were then assembled by sequential couplings of preactivated *N*α-Fmoc-amino acid (3 equiv) for 45−60 min at room temperature. The preactivation was carried out manually in DMF in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC) (3 equiv), *N*-hydroxybenzotriazole (HOBt) (3 equiv), and *N*,*N*-diisopropylethylamine (3 equiv) (DIEA) for 15 min at room temperature. The completeness of each coupling was verified by the Kaiser test. A positive Kaiser test was usually followed by an additional coupling cycle and if unsatisfactory the remaining free amino groups were blocked (capping step) by acylation with acetic anhydride (15 equiv) and pyridine (15 equiv) in DMF for 15 min. The *N*α-Fmoc deprotection was carried out by treatment with piperidine (20% v/v in DMF) (1 × 3 min and 1 × 15 min) and assessed by UV analysis of the effluents. All the wash steps were performed with the automated protocols of the peptide synthesizer. The protected peptides were cleaved from the resin with trifluoroacetic acid (TFA) and appropriate scavengers as indicated, concentrated, and precipitated with diethyl ether. The crude products were purified by preparative reversed phase HPLC to purities greater than 95% homogeneity.

General Procedure for the Preparation of ΨPro Building Blocks. Fmoc-Xxx-Cys($\Psi^{Me,Me}$pro)-OH (1), Fmoc-Xxx-Cys($\Psi^{H,R}$pro)-OH (2), and Fmoc-Xxx-Cys($\Psi^{H,H}$pro)-OH (3). 1,3-Thiazolidine-4-carboxylic acid, 2,2-Dimethyl-1,3-thiazolidine-4-carboxylic acid, and 2-(2,4-Dimethoxyphenyl)-1,3-thiazolidine-4-carboxylic acid were readily prepared according to published procedures.[35] The appropriate thiazolidine derivative (10 mmol) was dissolved in dry DMF (100 mL) in the presence of DIEA (19 mmol). A solution of Fmoc-Xxx-NCA or

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Fmoc-Xxx-F[36] (9 mmol) in dry DMF (100 mL) was added dropwise to the reaction mixture, and the mixture was stirred for 2 h at room temperature. The solution was then evaporated and the residue taken up in ethyl acetate, washed with aqueous 5% citric acid and brine, and dried over sodium sulfate. The solvents were evaporated to dryness, and the residue was purified by flash chromatography over silica gel, eluting with petroleum ether/ethyl acetate/acetic acid to give a white powder in 60−95% yield.

Fmoc-Xxx-Ser($\Psi^{Me,Me}$pro)-OH (4) and Fmoc-Xxx-Thr($\Psi^{Me,Me}$pro)-OH (5). L-Serine and L-threonine (30 mmol), respectively, were dissolved in a minimal volume of aqueous sodium carbonate (10% w/v) at pH 9, and the solution was added to a suspension of Fmoc-Xxx-OPfp (10 mmol) in acetone (80 mL). After completion of the reaction according to HPLC, the reaction mixture was acidified with aqueous HCl (5% w/v) to pH ∼1 in an ice bath. The solution was concentrated in vacuo to half the initial volume and the product extracted with ethyl acetate (2 × 150 mL). The organic solution was washed with water (100 mL) and brine (2 × 100 mL), dried over $MgSO_4$, and finally evaporated to dryness. The residue was recrystallized from ethyl acetate/hexane to give pure Fmoc-Xxx-Ser-OH or Fmoc-Xxx-Thr-OH as a white powder.

The dipeptide (5.0 mmol) was then suspended in dry THF (100 mL). Pyridyl toluene-4-sulfonate (250 mg, 1.0 mmol) and 2,2-dimethoxypropane (3.0 mL, 25.0 mmol) were added. The suspension was subsequently heated to reflux for several hours under an argon atmosphere, the condensate being bypassed over molecular sieves (4 Å). After cooling, the yellow solution was added with triethylamine (0.21 mL, 1.5 mmol) and evaporated to dryness. The residue was taken up in ethyl acetate (150 mL), washed with water (3 × 70 mL), dried over $MgSO_4$, and evaporated to dryness. The foamy residue was purified by flash chromatography over silica gel or crystallized from ethyl acetate/hexane to give **4** or **5** as a white powder in 70−90% yield.

Fmoc-Xxx-Ser($\Psi^{H,H}$pro)-OH (6) and Fmoc-Xxx-Thr($\Psi^{H,H}$pro)-OH (7). L-Serine and L-threonine (10.3 mmol), respectively, were dissolved in aqueous Na_2CO_3 (32 mL, 2.5 M). Then an aqueous solution of formaldehyde (19.3 mL, 260 mmol, 37%) was added dropwise under vigorous stirring and the resulting solution stored overnight at 4 °C, after which time the pH was 8. In order to perform the acylation, a solution of *N*α-Fmoc-Xxx-F[36] or *N*α-Fmoc-Xxx-NCA (12.8 mmol, 1 M) in acetone was added dropwise over a period of 70 min while the pH was adjusted between 8 and 9 with sodium carbonate (∼1 g). The reaction mixture was stirred for another 30 min at room temperature and then cooled to 0 °C. The solution was acidified with concentrated hydrochloric acid (16 mL). The white suspension at pH 3−4 was extracted with ethyl acetate (4 × 50 mL). The organic phases were combined and washed with brine (3 × 80 mL) and dried over magnesium sulfate. After evaporation the crude product was purified by flash chromatography and lyophilized from acetonitrile/water to give a white powder in 60−80% yield.

Synthesis of Switch Peptides 8a−c. Peptide **8a** was synthesized on *N*α-Fmoc-Ala-Sasrin resin (1.0 g, 0.68 mmol) according to the general procedure described above. The following protected side chain amino acids were used: *N*α-Fmoc-Glu(OAll)-OH, *N*α-Fmoc-Lys-(Aloc)-OH, and *N*α-Fmoc-Ala-Cys($\Psi^{Me,Me}$pro)-OH.

*N*α-Fmoc-Ala-Cys($\Psi^{Me,Me}$pro)-OH was prepared according to the general procedure described above, starting from Fmoc-Ala-F to give the target compound in 62% yield. Mp: 107−112 °C. R_f (petroleum ether/ethyl acetate/acetic acid, 8/8/1) = 0.53. HPLC (C_{18}, 214 nm, gradient of 40−100% solvent B over 30 min): R_t = 17.5 min (100%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.52 (d, ^3J = 6.5 Hz, 3H, Ala-CH_3), 1.99 (s, 3H, C2-CH_3), 2.10 (s, 3H, C2-CH_3), 3.36 (dd, ^2J = 11.5 Hz, ^3J = 5.6 Hz, 1H, C5-H), 3.54 (d, ^2J = 11.5 Hz, 1H, C5-H), 4.22−4.34 (m, 2H, Fmoc-CH_2), 4.43 (m, 1H, Fmoc-CH), 4.65 (t, ^3J = 7.0 Hz, 1H, Ala-Cα-H), 4.92 (d, ^3J = 5.0 Hz, 1H, C4-H), 6.14 (d br, ^3J = 6.8 Hz, 1H, NH), 7.27−7.72 (m, 8H, Fmoc-Ar-H). $C_{24}H_{26}N_2O_5S$ (454.55). ESI-MS: *m/z* = 455.5 [M + H$^+$].

The ΨPro dipeptide was introduced by single coupling according to the standard procedure described above. Double coupling was necessary for Ala in position 5 and Leu in position 6. The N-terminus position was acetylated according to the capping procedure described above. The protected peptide **8a** was finally cleaved from the resin by successive treatment (5 min) with a TFA solution (1% v/v in DCM), and the filtrates were neutralized with one equimolar amount of pyridine. The combined filtrates were precipitated with diethyl ether, centrifuged, and washed three times with diethyl ether. The crude product (990 mg, 0.51 mmol, 72%) was purified by preparative RP-HPLC with a gradient of 10−40% solvent B over 30 min. After lyophilization the purity of the peptide (95%) was confirmed by analytical HPLC with the same gradient at a retention time (R_t) of 23.2 min. Using a RP-C_4 column with an identical gradient, the R_t was 33.5 min. The desired pure peptide was obtained as a white powder (728 mg, 65%). IR (cm^{-1}, KBr): 3293, 1652 (amide I), 1547 (amide II). LDI-MS: *m/z* = 1916 (M$^+$), 1939 (M + Na), 1955 (M + K). ASA: Glu, 1.76 (2), Ala, 6.43 (7), Leu, 4.27 (4), Lys, 2.25 (2).

Peptide **8b** was obtained from **8a** after deprotection of the allyl side chain protecting groups according to the literature.[20] Protected peptide **8a** (20 mg, 0.01 mmol) was dissolved in dimethyl sulfoxide (DMSO) (20 mL) and the solution treated with bis(triphenylphosphine)palladium-(II) dichloride (0.58 mg, 0.08 equiv), tributyltin hydride (2.7 mL of a 1% v/v solution in DCM, 10 equiv), and acetic acid (0.68 mL of a 1% v/v solution, 12 equiv). Allyl deprotection was complete after 30 min of reaction time. Partially deprotected peptide **8b** was isolated by preparative HPLC to give a white powder (14 mg, 8.4 μmol, 81%). The purity was confirmed by analytical HPLC, with a gradient 20−80% solvent B over 40 min; the R_t was 21 min. LDI-MS: *m/z* = 1664.4 (M$^+$).

Peptide **8b** (5 mg, 3 μmol) was subsequently treated with TFA/water (98/2) under nitrogen for 10 h. Peptide **8c** was finally obtained by diethyl ether precipitation and subsequently used as such (4 mg, 2.24 μmol, 82%). ESI-MS: *m/z* = 813 ((M + 2H$^+$)/2).

Synthesis of Transmembrane Peptides 9a−c. Peptide **9a** was synthesized on Rink Amide MBHA resin (1.0 g, 0.30 mmol) according to the general procedure described above. Fmoc-Ala-Thr($\Psi^{H,H}$pro)-OH was prepared according to the general procedure described above, starting from Fmoc-Ala-NCA. The target compound was obtained in 60% yield. Mp: 103−108 °C (lyophilized). R_f (chloroform/methanol/acetic acid, 85/15/5) = 0.32. HPLC (C_{18}, 214 nm, gradient of 40−100% solvent B over 30 min): R_t = 13.4 min. ^{1}H-NMR (250 MHz, $CDCl_3$, two conformeres: (*major (80%)*, minor (20%)): δ 7.69−7.08 (m, 8 arom H), 6.06 (d, *J* = 9.0, HN), *5.85* (d, *J* = 7.8, HN), *5.31* (d, *J* = 3.9, H_{Re}-C2), 5.22 (d, *J* = 5.0, H_{Re}-C2), *4.85* (d, *J* = 4.0, H_{Si}-C2), 4.83 (H_{Si}-C2), 4.35−3.91 (m, 6 H, H-C_{Fmoc}, H_2-C_{Fmoc}, H-C^{α}_{Ala}, H-C4, H-C5), *1.39* (d, *J* = 5.9, H_3-C5), 1.38 (H_3C-C5), *1.29* (d, *J* = 7.0, H_3-C^{β}_{Ala}), 1.26 (H_3-C^{β}_{Ala}). $C_{23}H_{24}N_2O_6$ (424.45). CI-MS (NH_3): *m/z* = 425 (0.5, [M + 1]$^+$), 178 (100).

Double coupling was performed for every step except for the dipeptide Fmoc-Ala-Thr($\Psi^{H,H}$pro)-OH, where a single coupling was made with a prolonged reaction time (20 h). The N-terminal position was acetylated according to the capping procedure described above. The cleavage of the peptide from the resin (200 mg) was achieved by successive treatment (3 × 10 min) with a mixture of TFA/water (95/5), and the combined filtrates were concentrated *in vacuo*. The crude product was dissolved in a mixture of acetonitrile/methanol (2/1), precipitated with water, centrifuged, and washed two times with water. The crude peptide **9a** was dried *in vacuo* to give a white powder (58 mg, 86%) which was found to be 96% pure by analytical HPLC at R_t = 9.6 min using a C_4 column with a gradient 10−60% solvent B over 30 min (solvent A, 90% 2-propanol/10% water (0.09% TFA); solvent B, 90% CH_3CN/10% water (0.09% TFA). ESI-MS: *m/z* calculated for $C_{97}H_{173}N_{21}O_{22}$ 1983, found 993.9 ([M + 2H$^+$]/2). MALDI-MS: 2025.1 (corresponding to [M + 2Na − 4]). ASA: Thr, 0.73 (1); Ala, 5.88 (6); Leu, 10.71 (10); Aib, 3.57 (3).

Peptide **9c** was prepared from peptide **9a** (3.0 mg, 1.5 μmol) which was treated with 10% TFMSA in TFA (700 μl) at rt for 48 h. Completion of the reaction was monitored by HPLC. After evaporation of TFA, pyridine (56 μL) was added at 0 °C to neutralize TFMSA, and the whole was dissolved in water (1 mL). The precipitate thus formed was centrifuged, washed three times with water, and dried *in vacuo* overnight to give the deprotected peptide **9c** (1.0 mg, 33%) as a pale yellow solid. Analytical HPLC: R_t = 23.2 min using a C_4 column with a linear gradient of 10−60% solvent B over 30 min (solvent A, 90% 2-propanol/10% water (0.09% TFA); solvent B, 90% CH_3CN/10% water (0.09% TFA). ESI-MS: *m/z* calculated for $C_{96}H_{173}N_{21}O_{22}$ 1971, found 987.6 ([M + 2H$^+$]/2).

(36) Carpino, L. A.; Aalaee, D. S.; Chao, H. G.; De Selms, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 9751−9752.

Peptide **9b** was prepared from the nonacetylated precursor resin-bound peptide of **9a**. Prior to N-terminal acetylation of **9a** the peptidyl resin, an *S*-trityl-protected cysteine residue, was further coupled and acetylated according to standard protocols described above. The resin-bound peptide **9b** thus obtained (355 mg, substitution of 0.24 mmol/g after the last UV absorption of *N*-fluorenylmethylpiperidine) was subjected to successive treatment (3 × 10 min) with a mixture of TFA/triisopropylsilane/water (95/2.5/2.5) (3 × 3 mL) and washing with TFA (3 × 1 mL). The combined filtrates were concentrated *in vacuo*, and the resulting crude material was purified by semipreparative HPLC (C_4 column eluted with a linear gradient of 10−70% solvent B over 30 min (solvent A, 90% 2-propanol/10% water (0.09% TFA); solvent B, 90% CH_3CN/10% water (0.09% TFA) to give the peptide **9b** (76 mg, 71% based on the resin, substitution of 0.24 mmol/g). Analytical HPLC: R_t = 8.9 min using a C_4 column with a linear gradient of 10−60% solvent B over 30 min (solvent A, 90% 2-propanol/10% water (0.09% TFA); solvent B, 90% CH_3CN/10% water (0.09% TFA). ESI-MS: *m/z* calculated for $C_{100}H_{178}N_{22}O_{23}S$ 2086, found 1045.0 ([M + $2H^+$]/2).

Synthesis of Template 10. The bromoacetylated template **10** was prepared from the precursor Z-protected template previously described in the literature.[24] The Z protection was removed by treatment with hydrogen/10% Pd−carbon/concentrated HCl (6 equiv) in MeOH, affording the deprotected template (4 × HCl salt) quantitatively. The template thus obtained (30 mg, 27.7 μmol) was treated with bromoacetic anhydride (333 μmol, prepared from bromoacetic acid (92.4 mg, 665 μmol) and *N*,*N*′-dicyclohexylcarbodiimide (69.0 mg, 333 μmol) in dichloromethane (2 mL)) in the presence of DIEA (14.3 mg, 111 μmol) in DMF (5 mL) at rt for 20 h. The volatiles were evaporated, and the residue was again dissolved, filtered to remove a small amount of dicyclohexylurea, and concentrated. The crude product was purified by semipreparative HPLC using a C_4 column eluted with a linear gradient of 0−100% solvent B over 30 min to give the bromoacetyl template **10** (22 mg, 56%). Analytical HPLC: R_t = 19 min using a C_{18} column with a linear gradient 10−100% solvent B over 30 min. ESI-MS: *m/z* calculated for $C_{50}H_{78}B_{14}N_{14}O_{14}$ 1417, found 1419.1, 1420.4, 1421.4, 1422.6, 1423.4 in the ratio of 1/4/6/4/1 ([M + H]$^+$), 711.3 ([M + 2H]$^+$/2).

Synthesis of 4α-Helix Transmembrane TASP. To the bromoacetyl template **10** (2.0 mg, 1.4 μmol) were added sequentially a solution of cysteinyl peptide **9b** (14.6 mg, 7.0 μmol) in DMF (2 mL) and DIEA (905 μg, 7.0 μmol) at rt (the pH of the solution was 8.2−8.5). The reaction mixture was further stirred for 16 h. The solvent was evaporated, and the resulting crude mixture was purified by semi-preparative HPLC using a C_4 column eluting with a linear gradient of 10−80% solvent B over 30 min, to give the TASP molecule **11** (9.0 mg, 68%). Analytical HPLC: R_t = 30.6 min using a C_4 column with a linear gradient 10−70% solvent B over 30 min followed by 70−80% solvent B over 10 min. MALDI-MS: *m/z* calculated for $C_{450}H_{786}N_{102}O_{106}S_4$ 9438, found 9561.0.

Synthesis of [ΨPro-7]Sarafotoxin-S6b. Peptide assembly was carried out on a Fmoc-Trp(Boc)-functionalized Wang type resin (1.88 g, 0.31 mmol/g resin loading) according to Fmoc-PyBOP chemistry. The amino acid side chain protections were chosen as follows: *t*Bu (Asp, Tyr, Ser, Glu), Boc (Lys), Bum (His), Trt (Gln, Cys-1). Thr-7 and Cys-3, -11, and -15 were introduced as dipeptides Fmoc-Met-Thr-($\Psi^{H,H}$pro)-OH, Fmoc-Ser(*t*Bu)-Cys($\Psi^{Me,Me}$pro)-OH, Fmoc-Glu(OtBu)-Cys($\Psi^{Me,Me}$pro)-OH, and Fmoc-Phe-Cys($\Psi^{Me,Me}$pro)-OH, respectively. These ΨPro building blocks were readily prepared according to the procedures described above.

Fmoc-Met-Thr($\Psi^{H,H}$pro)-OH. Starting from Fmoc-Met-F, the target compound was obtained in 80% yield. Mp: 105−115 °C. R_f (ethyl acetate/hexane/acetic acid, 90/10/1) = 0.27. HPLC (C_{18}, 214 nm, gradient of 40−100% solvent B over 30 min): R_t = 17 min. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.77−7.30 (m, 8 arom H), 6.05 (d, *J* = 8.8, HN), 5.9 (d, *J* = 8.4, HN), 5.53 (d, *J* = 4.1, H_{Re}-C2), 5.3 (d, *J* = 5.4, H_{Re}-C2), 5.05 (d, *J* = 4.1, H_{Si}-C2), 4.95 (d, *J* = 5.4, H_{Si}-C2), 4.57−2.57 (m, 6 H, H-C_{Fmoc}, H_2-C_{Fmoc}, H-C^{α}_{Met}, H^{β}_{Thr}, H^{α}_{Thr}), 2.08 (s, CH_3-Met), 2.1−1.89 (m, H^{β}_{Met}) 1.49 (d, *J* = 6.1, CH3-Thr). $C_{23}H_{24}N_2O_6$ (424.45). EI-MS: 485 [M + H^+].

Fmoc-Ser(*t*Bu)-Cys($\Psi^{Me,Me}$pro)-OH. Starting from Fmoc-Ser(*t*Bu)-F, the target compound was obtained in 64% yield. Mp: 110−112 °C. R_f (petroleum ether/ethyl acetate/acetic acid, 14/5.5/0.5) = 0.32. HPCL (C_{18}, 214 nm, gradient of 40−100% solvent B over 30 min): R_t = 26.0 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.16 (s, 9H, *t*Bu-CH_3), 1.84 (s, 3H, $-CH_3$), 1.91 (s, 3H, $-CH_3$), 3.18−3.61 (m, 4H, Ser-CH_2, C5-H), 4.16 (m, 1H, Fmoc-CH), 4.30 (m, 2H, Fmoc-CH_2), 4.65 (m, 1H, Ser-Cα-H), 5.52 (m, 1H, C4-H), 7.27−7.75 (m, 8H, Fmoc-Ar-H). $C_{28}H_{34}N_2O_6S$ (526.66). ESI-MS: *m/z* = 527.5 [M + H^+].

Fmoc-Glu(OtBu)-Cys($\Psi^{Me,Me}$pro)-OH. Starting from Fmoc-Glu-(OtBu)-F, the target compound was obtained in 64% yield. Mp: 94−95 °C. R_f (petroleum ether/ethyl acetate/acetic acid, 14/5.5/0.5) = 0.31. HPCL (C_{18}, 214 nm, gradient of 40−100% solvent B over 30 min): R_t = 24.8 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.44 (s, 9H, O*t*Bu-CH_3), 1.81 (s, 3H, $-CH_3$), 1.91 (s, 3H, $-CH_3$), 2.23−2.44 (m, 4H, Glu-CH_2), 3.28 (m, 1H, C5-H), 3.42 (m, 1H, C5-H), 4.11 (m, 1H, Fmoc-CH), 4.18−4.32 (m, 2H, Fmoc-CH_2), 4.58 (m, 1H, Glu-Cα-H), 5.93 (d, ^3J = 8.9 Hz, 1H, C4-H), 7.24−7.77 (*m*, 8H, Fmoc-Ar-H). $C_{30}H_{36}N_2O_7S$ (568.69). ESI-MS: *m/z* = 569.6 [M + H^+].

Fmoc-Phe-Cys($\Psi^{Me,Me}$pro)-OH. Starting from Fmoc-Phe-F, the target compound was obtained in 79% yield. Mp: 106−110 °C. R_f (petroleum ether/ethyl acetate/acetic acid, 14/5.5/0.5) = 0.30. HPCL (C_{18}, 214 nm, gradient of 40−100% solvent B over 30 min): R_t = 24.7 min. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.74 (s, 3H, $-CH_3$), 1.86 (s, 3H, $-CH_3$), 2.35 (m, 1H, Phe-CH_2), 2.90 (m, 1H, C5-H), 3.06 (m, 2H, Phe-CH_2, C5-H), 4.06−4.30 (m, 4H, Fmoc-CH, Fmoc-CH_2, Phe-Cα-H), 4.79 (m, 1H, C4-H), 7.23−7.73 (m, 13H, Phe-Ar-H, Fmoc-Ar-H). $C_{30}H_{30}N_2O_5S$ (530.65). ESI-MS: *m/z* = 531.1 [M + H^+].

The step by step assembly proceeded smoothly with single couplings. Cleavage and deprotection of the peptide resin (1.0 g) was carried out by treatment with a freshly prepared solution of 82.5% TFA/2.5% ethanedithiol/5% thioanisole/5% water/5% phenol (7 mL) for 2 h under argon at room temperature. After filtration and washing with TFA (2 × 3 mL) and DCM (3 mL), the volatiles were evaporated and the crude peptide material was precipitated with chilled *tert*-butyl methyl ether and isolated by centrifugation. After HPLC analysis, a further treatment of 32 h in 95% TFA/5% water (5 mL) was carried out followed by precipitation in cold *tert*-butyl methyl ether and isolated by centrifugation as a white solid. The crude product was subsequently purified by preparative RP-HPLC using a C18 column with a gradient of 10−60% solvent B over 30 min to give the pure $C^{1,3,11,15}$-thiol, T^7-ΨPro peptide in 18% yield (80 mg) after lyophilization. Analytical HPLC: R_t = 19.7 min using a C_{18} column with a linear gradient of 10−60% solvent B over 30 min. ESI-MS: found ((M + 3)/3) 860.8, calculated 861.0. ASA: Asp + Asn, 3.06 (3); Glu + Gln, 1.98 (2); Ser, 0.64 (1); Thr, 1.00 (1); Val, 0.71 (1); Met, 1.07 (1); Ile, 0.68 (1); Leu, 0.98 (1); Phe, 1.09 (1); Lys, 2.19 (2); His, 0.95 (1); Tyr, 0.60 (1).

The reduced peptide (50 mg) was dissolved at a concentration of 10^{-4} M in Tris·HCl (10 mM) and EDTA (1 mM) buffer at pH 8.0 for 3 h. After lyophilization the two oxidized forms of [ΨPro-7]-Sarafotoxin-S6b (isomers A and B) were isolated by semipreparative RP-HPLC using a C_{18} column with a linear gradient of 10−60% solvent B over 30 min, and lyophilized. Analytical HPLC of isomers A and B: R_t (A) = 18.1 min, R_t (B) = 18.5 min, using a C_{18} column with a linear gradient of 10−60% solvent B over 30 min. ESI-MS: found ((M + 3)/3) 859.1, calculated 859.6.

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