

# Silaproline, a Silicon-Containing Proline Surrogate

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**Abstract** Silaproline (Sip) is a proline analogue that exhibits similar conformational properties as the natural amino acid in peptides. Moreover, the presence of a dimethylsilyl group confers to silaproline higher lipophilicity as well as improved resistance to biodegradation. The stereoselective synthesis of protected silaproline and two routes to obtain Fmoc-(*L*)Sip-OH on gram scale using chiral HPLC resolution are reported. Silaproline was introduced into the sequences of various natural peptides, and the influence of the silylated proline analogues on bioactivity was studied. In particular, considering the importance of polyproline II helices (PPII) in protein–protein molecular interactions and biology, a series of silaproline oligomers from dimer to pentamer were studied and shown to preferentially populate the polyproline type II secondary structure in both chloroform-*d* and methanol-*d*<sub>4</sub> as shown by circular dichroism (CD), NMR spectroscopy, and molecular modeling.

**Keywords** Modified bioactive peptides · Peptide structure · Polyproline II helix · Proline surrogate · Silaproline

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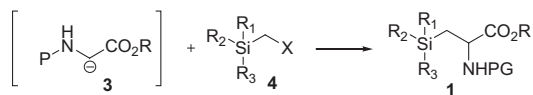
## 1 Introduction

The need to replace natural amino acids in peptides with non-proteinogenic counterparts, to obtain new pharmacological tools exhibiting better binding to specific receptors and more potent inhibitors of target enzymes, has stimulated a great deal of innovation on synthetic methods. The structural and functional diversity of such modified amino acids is unlimited and useful for the strategic development of new peptide analogues having potentially interesting biological activity.

Non-proteinogenic amino acids derived from natural amino acids and designed and synthesized by chemists are widely used for mimicking interactions of natural peptides [1]. They are important tools for studying the relationships between amino acid structure and peptide conformation [2–4]. Moreover, introduction of non-proteinogenic amino acids into peptides may provide resistance to enzymatic biodegradation [5]. In addition, they may be used either as chiral auxiliaries in asymmetric synthesis [6] or for the preparation of biopolymers [7]. Non-proteinogenic amino acid derivatives of poly- $\alpha$ -amino acids may be used either to prepare fibrous materials and films for studies in the solid state and in solution. For example, analogues of proline have been used in models of polyproline and collagen, which contain a high percentage of proline [8, 9].

The preparation of enantiopure unnatural amino acids remains of great importance in chemistry with wide applications in the life sciences [10–14]. Among the different families of unnatural amino acids ( $\beta$ -amino acids, homo-amino acids, D-amino acids, and *N*-methyl amino acids), we have targeted  $\alpha$ -amino acid analogues having a silicon atom serving as an isostere of carbon in order to study biopolymers. Silicon belongs to the crystallogen family and is the most abundant element in the earth's crust after oxygen. In medicinal chemistry, substitution of silicon by carbon within existing drugs is an approach for the synthesis of compounds with original biological properties [15]. In 1979, Tacke and Wannagat reviewed the concept of substituting a carbon atom by silicon [16].

Carbon and silicon share similarities; however, certain differences are notable. For example, although both are tetravalent, silicon is capable of forming penta- and hexa-coordinated complexes possessing stable charges. The size of the two atoms is different: 0.91 and 1.46 Å for the respective atomic radii of carbon and silicon. The lengths of the carbon–carbon and carbon–silicon bonds are, respectively, 1.54 and



**Scheme 1** Addition of a glycine anion equivalent to a halomethylsilane

1.87 Å. Silicon is more electropositive (1.8) than carbon (2.5), which induces a difference in bond polarization. Finally, lipophilicity is an important parameter, because silylated compounds are more lipophilic than their carbon analogues, which may facilitate ability to cross cell membranes. Consequently, these differences in size and shape between carbon and silicon influence the pharmacological and pharmacodynamic properties of compounds containing these elements.

Some limitations should be mentioned in incorporating silicon as a carbon isostere. The polarity of the silicon–hydrogen bond leads to an easily cleavable bond in water under non acidic conditions, forming the corresponding silanols. Increased lipophilicity limits water solubility, which is of major importance in medicinal chemistry. In this context, we extended our work to the synthesis and characterization of silaproline polypeptides.

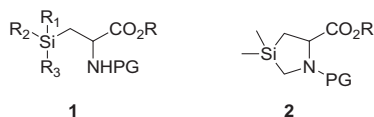
Several publications have described β-silyl α-amino acids with general structures **1** and **2**. The first reported silicon-containing amino acid belongs to this class of compounds, which are accessible using a wide variety of methods and reaction conditions [17, 18].

Moving the silicon atom one more atom further from the carbonyl increased the stability of the C–Si bond [19, 20]. The majority of methods used to synthesize β-silyl amino acids involve alkylation of a glycine anion equivalent **3** with a halomethylsilane **4** (Scheme 1). The resulting β-silyl α-amino acid **1** may undergo cyclization to provide **2** if another halogenated substituent is present on the electrophile.

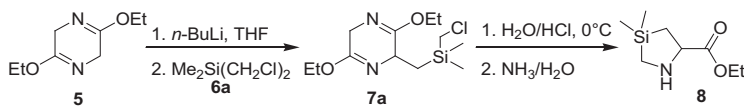
## 2 Synthesis of a Silicon-Containing Proline Surrogate

Silaproline (Sip, **2**, R=protecting group or H, Fig. 1) is a proline analogue in which the γ-carbon has been replaced by a dimethylsilyl group. As discussed below, X-ray analysis of model peptides has shown the carbon–silicon bonds in Sip are of about 0.35 Å longer than the carbon–carbon bonds of proline [21]. The C–Si–C angle is significantly small (~93°) relative to that of proline (105°). Furthermore, the presence of the dimethylsilyl group increased lipophilicity, as demonstrated by the octanol–water partition coefficient of Sip that was experimentally determined to be 14 times greater than that of Pro [21].

Increased lipophilicity may facilitate membrane permeability. Reduced sensitivity to enzymatic degradation may also arise from substitution of Sip for Pro in peptides. Moreover, similarities between the two ring systems may result in similar conformational properties for Sip- and Pro-containing peptide analogues.



**Fig. 1**  $\beta$ -Silyl amino acids



**Scheme 2** Synthesis of racemic silaproline starting from 2,5-dihydropyrazine

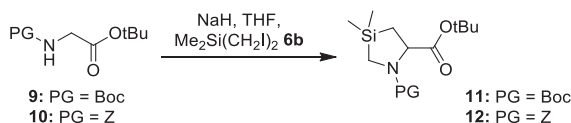
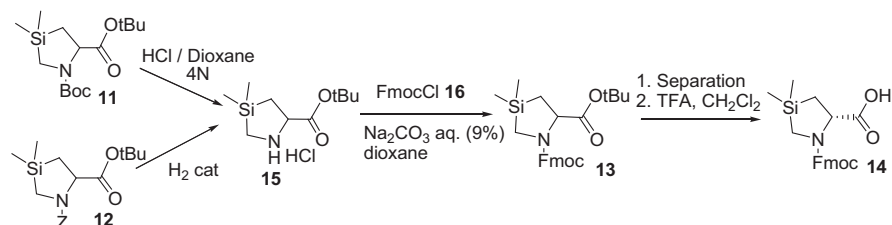
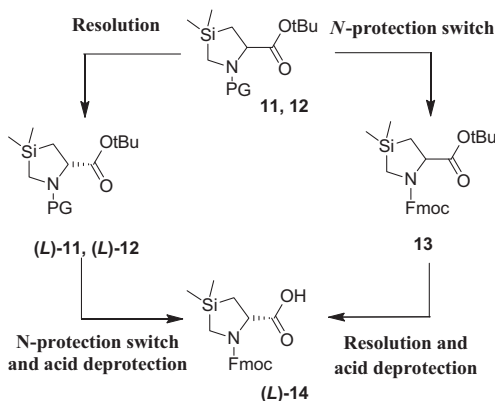
## 2.1 Racemic Silaproline Synthesis

Protected silaprolines were synthesized in racemic and enantiomerically pure forms by two different research teams in 2000 [22]. In both cases, a glycine equivalent was alkylated initially on carbon followed by nitrogen employing bis-(halomethyl) dimethylsilane. In the racemic case, Tacke and co-workers synthesized silaproline by a route featuring metallation of 2,5-dihydropyrazine **5** with *n*-butyllithium and alkylation with bis-(chloromethyl)dimethylsilane **6a** to afford (pyrazinyl)methylsilane **7a** (Scheme 2) [22]. Treatment with hydrochloric acid opened pyrazine **7a**, which upon washing with ammonia was converted to silaproline ethyl ester (H-(*D,L*)Sip-OEt) **8** in 20% overall yield for the two steps (Scheme 2).

Racemic *N*-(Boc)- and *N*-(Cbz)silaproline *tert*-butyl esters **11** and **12** have been synthesized recently by a strategy featuring alkylation of the corresponding protected glycines **9** and **10** using bis-(iodomethyl)dimethylsilane **6b** (Scheme 3) [23]. Treatment of glycines **9** and **10** in anhydrous THF with excess NaH, followed by the silane **6b**, gave, respectively, Boc-(*D,L*)Sip-OtBu (**11**) and Z-(*D,L*)Sip-OtBu (**12**) in 71% and 85% yields after chromatography.

Considering that *N*-(Fmoc)amino acids are commonly used in solid phase peptide synthesis, Fmoc-(*D,L*)Sip-OH (**13**) was prepared by protecting group shuffles from **11** and **12** (Scheme 4) [24], because loss of the base labile Fmoc group rendered alkylation of Fmoc-Gly-OtBu unrealistic. Selective removal of the Boc group from Boc-(*D,L*)Sip-OtBu (**11**) was achieved with anhydrous 4 N HCl in dioxane for 30 min. Alternatively, the Z protecting group was removed from Z-(*D,L*)Sip-OtBu (**12**) by hydrogenolysis. Fmoc-(*D,L*)Sip-OtBu (**13**) was then obtained from H-(*D,L*)Sip-OtBu (**15**) by protection using Fmoc-Cl **16**.

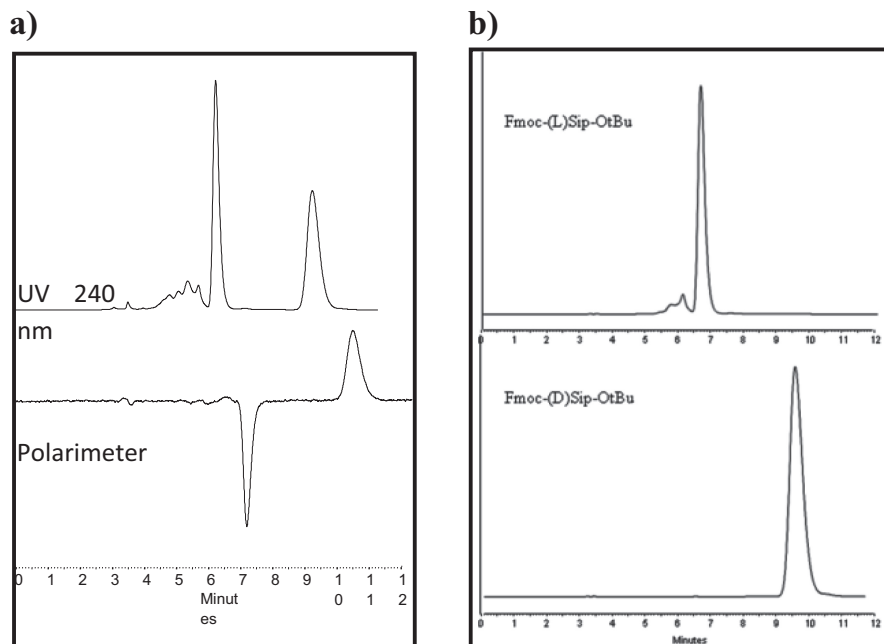
Different strategies were explored for the resolution of the protected racemic silaproline analogues (Fig. 2). For example, racemic Fmoc-(*D,L*)Sip-OtBu **13** was separated into its respective enantiomers by semi-preparative chromatography on a Chiralpak IC column using a mixture of hexane/isopropanol (70/30) as mobile phase. By this method, 200 mg of each enantiomer of **13** was recovered with an ee >99% (Fig. 3). After separation, the *tert*-butyl esters of Fmoc-(*L*)- and (*D*)Sip-

**Scheme 3** Synthesis of racemic silaproline starting from glycine**Scheme 4** Synthesis of Fmoc-(*L*)Sip-OH**Fig. 2** Strategies for the preparation of enantiomerically enriched Fmoc-(*L*)Sip-OH

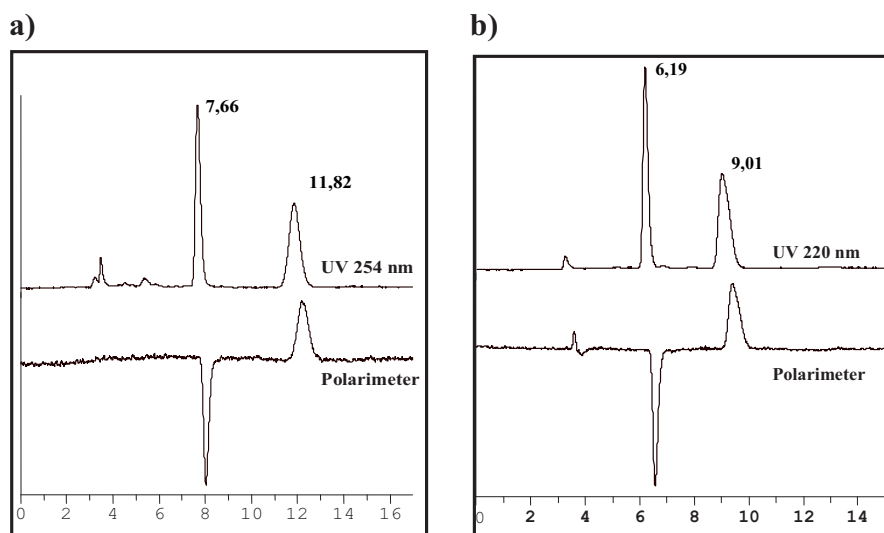
OtBu [(*L*)- and (*D*)-**13**] were removed quantitatively using TFA in dichloromethane for 30 min.

The second strategy for resolving racemic silaprolines consisted in separation of the enantiomers of Boc-(*D,L*)Sip-OtBu (**11**) and Z-(*D,L*)Sip-OtBu (**12**). Silaprolines **11** and **12** were respectively detected at 220 and 254 nm, as well as with a polarimeter. Employing a Chiralpak IC column and semi-preparative conditions, the enantiomers of **11** and **12** were respectively separated to afford 5 g quantities of each isomer (>99% ee, Fig. 4a,b). Subsequently, Boc-(*L*)Sip-OtBu was converted to Fmoc-(*L*)Sip-OH [(*L*)-**14**] by removal of the acid labile carbamate and ester using 6N HCl followed by introduction of the Fmoc group.

Notably, resolution by chiral HPLC circumvented the need for enantioselective synthesis providing multiple grams of each enantiomer of differently protected silaprolines. The syntheses of racemic Boc- and Z-protected silaprolines were



**Fig. 3** Separation of the two enantiomers of Fmoc-(*D,L*)Sip-OtBu (**a**) and chromatograms of pure Fmoc-(*L*)Sip-OtBu and Fmoc-(*D*)Sip-OtBu (**b**)



**Fig. 4** Separation of enantiomers of Boc-(*D,L*)Sip-OtBu (**a**) and *Z*-(*D,L*)Sip-OtBu (**b**)

straightforward, albeit exchange of protection was necessary for the Fmoc version. The best strategy for obtaining Fmoc-(*L*)Sip-OH [(*L*)-**14**] was to switch the *N*-protection from Boc-(*L*)Sip-OtBu [(*L*)-**11**] after resolution of the corresponding racemic mixture and then regenerate the carboxylic acid function on the enantiomerically pure compound, because separation on the preparative column was only possible with silaproline esters.

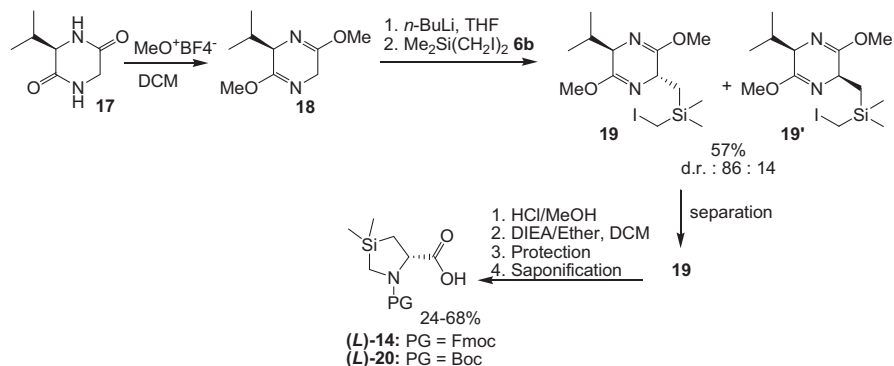
## 2.2 Diastereoselective Synthesis

Among various methods studied for the asymmetric synthesis of silaproline, the diastereoselective alkylation of the Schöllkopf bis-lactim was the most efficient [25]. The first step consisted in *O*-methylation of the (*D*)Val-Gly diketopiperazine **17** using trimethyloxonium tetrafluoroborate to afford bis-lactim ether **18**, which was deprotonated with *n*-BuLi and alkylated with bis-(iodomethyl)dimethylsilane **6b** to afford piperazine **19** as a 86:14 mixture of separable diastereomers (Scheme 5). After chromatography, each piperazine diastereomer **19** was hydrolyzed using hydrochloric acid, and cyclization with carbon–nitrogen bond formation was achieved by treating the ammonium hydrochloride intermediate with base. After *N*-protection and ester hydrolysis, *N*-(Fmoc)- and (Boc)silaprolines [(*L*)-**14** and (*L*)-**20**] were respectively obtained with 13 and 38% overall yields. Enantiopure silaproline has also been synthesized in 27% overall yield and >99% enantiomeric excess by a similar diastereoselective route from the 3,6-diethoxy-2,5-dihydropyrazine derived from (*D*)Val-Gly diketopiperazine using bis (chloromethyl)dimethylsilane **6a** in the alkylation step [22]. Optimized alkylation conditions of 3,6-dimethoxy-2,5-dihydropyrazine **18** in the presence of bis-(chloromethyl)dimethylsilane have been recently reported in a patent that claims to afford >100 g of enantiopure Boc-(*L*)Sip-OH [(*L*)-**20**] in 60% overall yield (Scheme 5) [26].

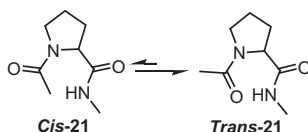
## 3 Conformational Studies

Proline is the only cyclic essential amino acid. Its pyrrolidine ring restricts local conformational freedom and lowers the molecular flexibility about its side chain and backbone dihedral angles to a fewer number of possible conformers. In addition, in contrast to the amide N-terminal to primary amino acids, which exist predominantly as the *trans*-isomer, the tertiary amide N-terminal to the pyrrolidine ring of proline, the so-called prolyl amide, may exist in *cis*- and *trans*-isomers separated by a relatively low isomerization barrier (Scheme 6) [27, 28].

Owing to these unique properties, proline plays an important role in biologically active peptides and biological processes, such as protein folding. Therefore, replacement of proline with substituted proline analogues may modify the flexibility of bioactive peptides, providing probes to study the influence of specific conformers on receptor recognition and affinity. Accordingly, numerous surrogates,



**Scheme 5** Diastereoselective synthesis of protected silaproline



**Scheme 6** *Cis*–*trans*-isomerization of the amide *N*-terminal to proline

mimetics, and analogues of proline have been developed. The first class contains constrained analogues, which are designed with the aim of governing the *cis*–*trans* ratio of peptide bond *N*-terminal to proline. The second class represents unconstrained analogues that conserve the native properties of proline. A typical example of a hindered substituted proline was offered by Lubell, who described  $\delta$ -*tert*-butylproline for constraining peptide conformation to favor proline-like turns [29, 30]. Pseudoproline residues have also been investigated to control prolyl amide geometry and have proven useful to circumvent solubility problems correlated with hydrophilic side chains during peptide synthesis [31].

The *cis*–*trans*-isomerization of the proline peptide bond has been extensively studied and depends on several factors such as solvent, aromaticity, and the configuration of the residue preceding proline. To modulate the proportion of *cis*- and *trans*-conformers, ring substituents have been used to orientate the amide bond geometry. For example,  $\delta$ -substituted prolines, such as  $\delta$ -*tert*-butylproline [29, 30] and  $\delta,\delta$ -dimethylproline [32], have been employed to augment the prolyl amide *cis*-isomer in peptides. Adding to this important field of modified prolines, several studies were reported to compare the structural influence of the replacement of proline by silaproline.

### 3.1 Model Peptides

To carry out conformational studies, we introduced silaproline in model peptides. We synthesized protected silaproline derivatives (Boc and Piv-Sip-NHPr),



dipeptides (Boc and Piv-Sip-Ala-NHiPr), and tripeptides containing silaproline, with two sets of epimers [Boc and Piv-(*L* or *D*)Ala-Sip-Ala-NHiPr]. Among the models, Piv-Sip-Ala-NHiPr crystallized and its X-ray structure showed a type II  $\beta$ -turn. This turn conformer is uncommon for homochiral dipeptides and may be stabilized in the crystal by intermolecular interactions between the middle amide groups of two neighboring molecules. Some ring properties due to the silicon atom included long carbon–silicon bonds and a small carbon–silicon–carbon intracyclic angle. The observed C $\beta$ -endo ring puckering consisted of an envelope conformation with four atoms in the same plane and the C $\beta$  atom pointing out of the plane toward the  $\alpha$ -carboxamide [33].

Conformational studies were performed on two sets of tripeptides using NMR spectroscopy in solution: Piv-(*L* and *D*)Ala-Pro-Ala-NHiPr and Piv-(*L* and *D*)Ala-Sip-Ala-NHiPr. The percentage of prolyl amide *cis*-isomer was unaffected by the change from proline to silaproline. Amide proton chemical shift differences obtained when switching from DMSO to chloroform and temperature coefficients  $\Delta\delta/\Delta T$  in DMSO- $d_6$  indicated solvent shielded and exposed hydrogens. Notably, both (*L*)Ala-peptides adopted extended conformations. Both (*D*)Ala-peptides adopted folded turn structures, albeit more predominantly for the proline than for the silaproline peptide analogue [21].

### 3.2 Diketopiperazines

The pyrrolidine ring shape of proline can modulate structural properties, such as the proportion of *cis*- and *trans*-amide isomers, that may play important roles in peptide biological activity. Diketopiperazine models were thus chosen to study the influence of the proline modification on ring shape. In this study, cyclo(Pro-Gly), cyclo(Sip-Gly), and cyclo(Thz-Gly) (Thz=thiazolidine-4-carboxylic acid) were studied by proton NMR spectroscopy (Fig. 5) [34]. We found that relative to proline, which typically exists in a dynamic equilibrium between C $\gamma$ -endo and C $\gamma$ -exo ring puckering, the analogues with  $\gamma$ -position heteroatoms (silicon and sulfur) displayed more rigid five-member rings that slowly interconverted between C $\beta$ -exo and C $\beta$ -endo conformations.

In both linear and cyclic Sip-peptide analogues, both C $\beta$ -exo and C $\beta$ -endo conformations were observed. The C $\delta$ -Si $\gamma$ -N-C $\alpha$  ring atoms were coplanar. The impact of the methyl groups on silicon does not seem to be important, because the sulfur in (Thz-Gly)DKP had practically the same conformational influence as the dimethylsilyl group in (Sip-Gly)DKP.

### 3.3 Homopolypeptides

In proline-rich regions, prolyl residues promote formation of extended helical secondary structures, such as type II polyproline (PPII) helices [35, 36]. Involved



**Fig. 5** Structure of diketopiperazines

in a wide range of molecular interactions important for biological function, PPII helices have been implicated in signaling, transcription, and immune response. They mediate protein–protein interactions [37] and facilitate cell penetration [38, 39]. These biological properties have stimulated chemists to synthesize PPII mimics for various applications, such as therapeutic agents.

Among proline containing oligomers that fold into conformationally ordered states, oligomers of Ser-Pro dipeptides [40], tricyclic Pro-Pro mimics [41], tri-proline mimics [42], and PTAAAs (proline-templated amino acids) [43–45] all have been used to design PPII helices with modified physical properties such as improved water solubility. A series of silaproline oligomers were synthesized to explore the influences of its alternative ring puckering and hydrophobic nature on PPII conformation.

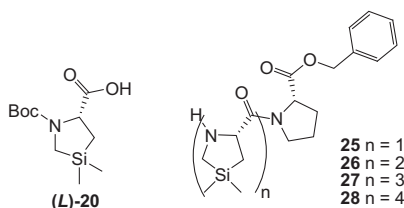
### 3.3.1 Polyproline Type II Helix (PPII Helix)

The typical PPII structure is a left-handed helix with all peptide bonds in *trans*-configuration ( $\omega = 180^\circ$ ) and  $\phi$ - and  $\psi$ -dihedral angle values of  $-75^\circ$  and  $145^\circ$ , respectively. Without intramolecular hydrogen bonds to stabilize the helical structure, backbone solvation has been suggested to be a major determinant of PPII formation [46]. Moreover, the PPII helix has been identified in peptides that do not contain proline residues but adopt similar dihedral angle values [47].

### 3.3.2 Monodisperse Homopolysilaprolines

Silaproline oligomers were synthesized in solution by a stepwise strategy using *N*-Boc-silaproline as monomer. The dimer was first made in solution by coupling equimolecular amounts of *N*-Boc-silaproline with proline benzyl ester hydrochloride in the presence of triethylamine. Chain extension was achieved by selective Boc-deprotection of the dimer and coupling with *N*-Boc-silaproline to afford the trimer. Longer chains were synthesized in a similar fashion (Fig. 6).

Polyproline has been observed to adopt PPII helices in methanol that exhibit far-UV (190–260 nm) CD spectra having two negative maxima at 200 and 232 nm and a positive maximum at 215 nm. Similarly, oligomers **25–28** in methanol exhibited CD curve shapes with negative and positive maxima in the 203–208 nm

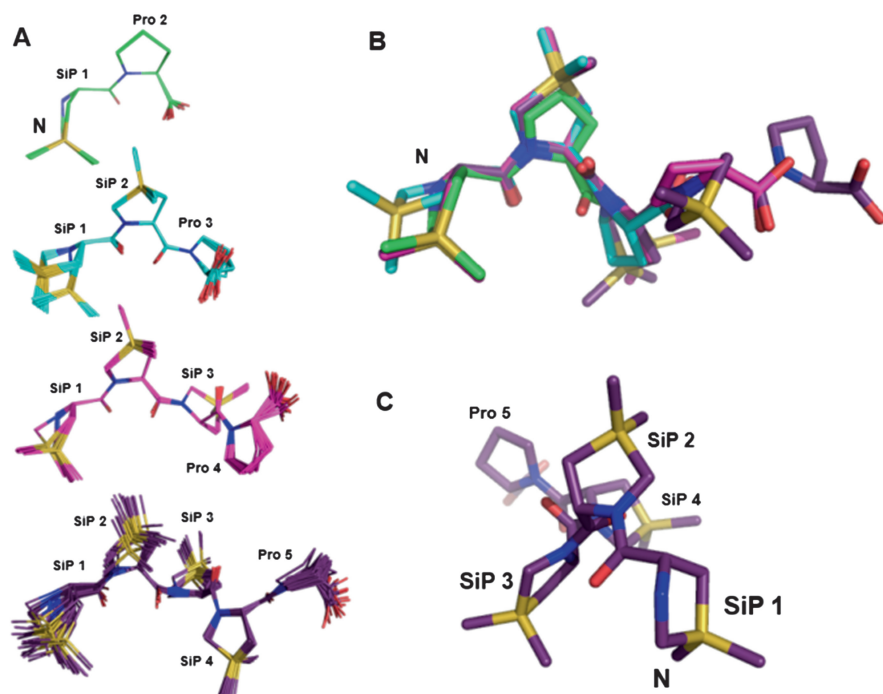


**Fig. 6** Silaproline building block and oligomers **25–28**

and 220–230 nm ranges, respectively (Fig. 3a). A subtle change in conformation with increasing chain length was detected by a redshift and increase in per residue molar ellipticity. Pentamer **28** exhibited a PPII-like CD signature with a negative maximum at 207 nm and a positive maximum at 229 nm, which were unaffected by increases in temperature from 20 to 55°C, suggesting a stable silaproline oligomer structure.

Studies of oligomers **25–28** by NMR spectroscopy were performed next in chloroform-*d* and in methanol-*d*<sub>4</sub>. A combination of COSY, ROESY, <sup>13</sup>C-HSQC, and <sup>13</sup>C-HMBC experiments was used to assign all of the <sup>1</sup>H and <sup>13</sup>C resonances. Better oligomer solubility and enhanced NMR spectral resolution were observed in methanol-*d*<sub>4</sub>, albeit strong NOEs between the <sup>α</sup>CH(*i*) and <sup>δ</sup>CH(*i* + 1) protons characteristic of the *trans*-conformation for all oligomers were observed in both solvents. Except in the case of the spectrum of dimer **25**, only the *trans*-conformer was detected in the <sup>1</sup>H NMR spectra of the longer oligomers both in chloroform and methanol. Dimer **25** exhibited *cis*- and *trans*-isomers about the amide bond between silaproline and proline, with ~7% *cis*-isomer detected by <sup>1</sup>H and <sup>29</sup>Si NMR in both solvents. Relative to the proline counterpart H-Pro-Pro-OBn, H-Sip-Pro-OBn exhibited more *trans*-isomer in methanol (85% and 93%, respectively). In the spectra of the high molecular weight proline oligomers (*n* = 3–5), the percentage of prolyl amide *trans*-isomer remained relatively constant (90%). As mentioned, no *cis*-isomer was detected in the longer silaproline oligomers starting from the trimer [48].

The NOE data from the NMR studies was used to add restraints in the calculations of the solution structures by a simulated annealing protocol with the AMBER 11 force field. The solution structures of **25**, **26**, **27**, and **28** in methanol were solved using 4, 12, 18, and 24 unambiguously restrained distances, respectively. The 20 lowest-energy NMR structures calculated for each compound demonstrated that the Sip oligomers converged toward PPII structures (Fig. 7). The root mean square deviations (RMSD) on all heavy atoms were 0.03, 0.31, 0.18, and 0.40 Å for **25**, **26**, **27**, and **28**, respectively, when the OBn capping group was omitted. Average values of the backbone dihedral angles for the Sip residue in the polysilaproline helix were  $\phi = -74.5 \pm 8.9^\circ$  and  $\psi = 143.6 \pm 13^\circ$  after optimization of the NMR structures using the B3LYP/6-31+G(d,p) method. The silaproline PPII helix was left-handed with an axial translation of 3.2 Å composed of three residues per turn, with all peptide bonds in *trans*-configuration ( $\omega = 170\text{--}175^\circ$ ).



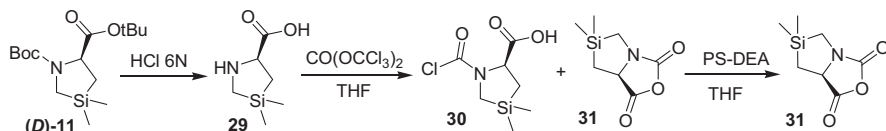
**Fig. 7** (a) NMR solution structures of dimer **25** (in *green*), trimer **26** (in *cyan*), tetramer **27** (in *pink*), and pentamer **28** (in *purple*). (b) Overlay of the structures of compounds **25–28** optimized by DFT. (c) Axial view of PPII helical structure of the tetramer **4**. Hydrogens and the disordered benzyl group of the C-terminal moiety were omitted for clarity

### 3.3.3 Polydispersed Polysilaproline Oligomers

To synthesize homopolysilaproline having longer chain lengths, the most extensively used method was based on polymerization of silaproline *N*-carboxyanhydride (Sip-NCA).

#### Synthesis of Sip-NCA

The synthesis of *N*-carboxyanhydrides (NCAs) of common amino acids has been well documented [49]. Reports of NCAs of amino acids with secondary amines are however more rare, in part due to the cyclic structure of such amino acids, which may impose conformational restrictions. For example, unsatisfactory results were obtained in attempt to prepare NCAs from proline by common methods [50], such as treatment with phosgene or a phosgene substitute such as triphosgene (Fuchs–Farthings method) [51]. In the latter case, the *N*-carbamoyl chloride intermediate was relatively stable and required addition of an HCl scavenger, such as silver oxide



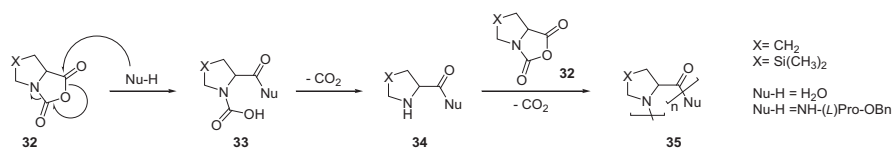
**Scheme 7** Synthesis of (D)Sip-NCA

or organic bases in order to cyclize. Recently, the synthesis of (*L*)Pro-NCA using triphosgene was achieved effectively with the assistance of *N,N*-diethanolaminomethyl polystyrene (DEAM-PS) to trigger the cyclization without diketopiperazine formation [52]. Removal of the polymer-supported ammonium hydrochloride by filtration facilitated purification of (*L*)Pro-NCA. Inspired by this method, NCAs of (*L*)- and (*D*)-Sip were made using triphosgene and diethylamine polystyrene, respectively. Greater spontaneous cyclization to form NCA was observed for the less constrained five-membered ring of Sip (50%) compared with proline (33%, Scheme 7). Among attempts to optimize cyclization, including variations of stoichiometry, reaction time, and temperature, only the addition of polystyrene-supported diethylaminomethylamine proved effective. With the optimized conditions, (*D*)Sip-NCA **30** was prepared in 72% yield and high purity, such that it could be crystallized and fully characterized [53].

## Homopolymerization

Homopolypeptides are commonly obtained by ring opening polymerization (ROP) of NCAs [54]. Several reagents can initiate the polymerization to afford polypeptides with a narrow molecular weight range that is essentially determined by the NCA to initiator ratio. Depending on the relative basicity and nucleophilicity of the initiator, two mechanisms have been described for this reaction: the normal amine mechanism [55] (NAM) and the activated monomer mechanism (AMM) [56]. In the AMM, a significantly basic initiator deprotonates an NCA bearing a proton on nitrogen and the resulting anion may serve as a nucleophile to initiate the ROP. More commonly, the initiator serves as a nucleophile and attacks the C-5 carbonyl of the NCA. Decomposition of the resulting carbamic acid with  $\text{CO}_2$  release results in a newly formed amine that propagates polymerization (Scheme 8).

In the particular cases of (*L*)Pro-NCA, and related imino acid NCAs, the AMM cannot occur, because no labile proton is available and the NAM is the only possible mechanism. Initially, precise amounts of water were employed as initiator in THF, at room temperature. Difficulty in verifying the degree of polymerization by NMR spectroscopy of the resulting carboxylic acid [57] and poor polymer purity using water as initiator prompted us to use H-(*L*)Pro-OBn to start the chain reaction, because integration of the benzyl group signal could be employed to check polymer purity and the degree of polymerization. Employing H-(*L*)Pro-OBn to initiate reactions of NCAs derived from (*L*)proline, (*L*)silaproline and



**Scheme 8** NAM mechanism for ring opening polymerization of *N*-carboxyanhydrides

(*D*)silaproline gave effectively homopolypeptides with a C-terminal proline benzyl ester.

### Conformational Studies

The degree of polymerization of the Pro and Sip homopolymers was measured by  $^1\text{H}$  NMR spectroscopy and integration of the aromatic protons of the C-terminal benzyl ester and the  $\text{C}_\alpha$  protons; the range of the latter, which included two additional protons for the benzyl methylene, was assigned accurately using heteronuclear single quantum coherence spectroscopy (HSQC) experiments. In all cases, this method evaluated consistently the degree of polymerization within the theoretical range determined by the reaction stoichiometry (Table 1).

Both MALDI (matrix assisted laser desorption ionization) and CD (circular dichroism) analyses have been widely used to characterize homopolypeptides. The molecular weight distribution of a homopolymer may be assigned based on the pattern of peaks for the mass of each component in the MALDI analysis. Sample preparation of the matrix influenced however the molecular weight determination of the MALDI-ToF MS experiment, resulting in lower molecular weights than those calculated by NMR spectroscopy. In addition, the abundances were suspected not to reflect the actual distribution, due to limitations of molecular discrimination by MALDI-ToF analysis during ionization.

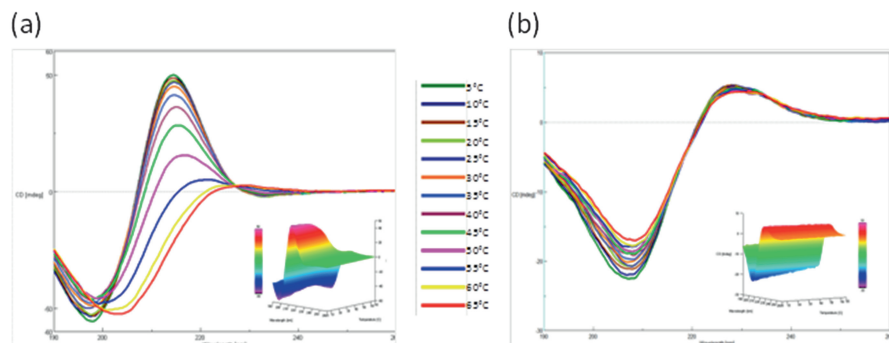
Polyproline oligomers are known to adopt a type II helical conformation (PPII) in polar solvents such as water, trifluoroethanol, and other fluorinated alcohols. The characteristic PPII circular dichroism signals include a strong negative maximum at 202–206 nm and a weak positive maximum at 225–229 nm [58]. As mentioned earlier, the lipophilic character of homopolysilaproline inhibited solubility in water. We investigated other polar solvents that induced PPII structure to solubilize the polysilaproline oligomers and found the best solvents to be HFIP and TFE; the latter was often used for thermal studies. We recorded circular dichroism spectra of all polymers in TFE at a concentration of 0.1 mg/mL.

The spectrum of polymer P7 showed the typical circular dichroism curve of PPII helix with a strong negative maximum at 207 nm and a weak positive maximum at 228 nm. These results indicated clearly that homopolysilaproline adopted a PPII conformation. Thermal denaturation of the PPII structure was studied next in TFE by heating over the range of 0–70°C in a sealed cell recording CD spectra at 5° intervals (Fig. 8). A slight diminution of the negative maximum at 207 nm was

**Table 1** Synthesis of homopolypeptides of different lengths

Polymer	Monomer	Initiator	Monomer/initiator	DPn <sup>a</sup>
P2	( <i>L</i> )Pro-NCA	( <i>L</i> )Pro-OBn	21	24
P4	( <i>D</i> )Sip-NCA	( <i>L</i> )Pro-OBn	5	5
P5	( <i>D</i> )Sip-NCA	( <i>L</i> )Pro-OBn	20	14
P6	( <i>D</i> )Sip-NCA	( <i>L</i> )Pro-OBn	50	44
P7	( <i>L</i> )Sip-NCA	( <i>L</i> )Pro-OBn	10	11

<sup>a</sup>Degree of polymerization was determined by NMR integration



**Fig. 8** CD spectra during thermal denaturation experiments of (a) oligoproline **P2** and (b) silaproline oligomers **P7** in TFE

observed, but the positive maximum was unaffected by increasing temperature. In comparison to the polyproline counterpart (Fig. 8a), the PPII helix of polysilaproline was clearly more thermally stable (Fig. 8b).

## 4 Silaproline Containing Biologically Active Peptides

Innovative building blocks have been used to replace natural amino acids in peptides with non-proteinogenic counterparts to obtain new pharmacological tools, exhibiting better binding to specific receptors and more potent enzyme inhibitors. Constrained amino acid analogues, particularly cyclic amino acid residues such as proline surrogates, have been used to study peptide geometry, because biological activity is often dependent on backbone conformation and side chain orientation [59, 60]. Moreover, unnatural residues may increase the stability and the bioavailability of modified peptides, presumably because they are not well recognized by proteolytic enzymes. In this light, the significance of Sip was studied after insertion into the sequence of several natural biologically active peptides.



**Fig. 9** Substance P (SP) sequence

### 4.1 Substance P

Substance P (SP) (Fig. 9) is a member of the tachykinin family of natural neuropeptides, which are characterized by a common C-terminal sequence that acts at three different receptor subtypes [61, 62]. Our study focused on SP using assays on the two binding sites associated with its NK-1 receptor, as well as secondary messenger assays on whole cells as previously described [63]. SP and [Pro<sup>9</sup>]SP were taken as reference peptides of the more abundant (NK-1M) binding site, and [pGlu<sup>6</sup>]SP(6–11) and [pGlu<sup>6</sup>, Pro<sup>9</sup>]SP(6–11) were selected as selective peptides of the less abundant (NK-1m) binding site.

Replacing glycine at position 9 by both proline and silaproline led respectively, to SP and C-terminal SP hexapeptide analogues retaining both the whole affinity and activity of the parent peptide. Moreover, in the C-terminal SP hexapeptide, replacement of Gly<sup>9</sup> with Sip gave a full agonist on the phospholipase C (PLC) pathway that exhibited partial agonist behavior on adenylate cyclase. This latter observation underlines that silaproline is a proline mimetic that may confer a different pharmacological pattern to a biologically active peptide [64].

The stability of the proline- and silaproline-containing SP analogues toward angiotensin-converting enzyme was also examined. After 1 h incubation at 37°C, 80% of SP was degraded; however, [Pro<sup>9</sup>]SP and [Sip<sup>9</sup>]SP remained uncleaved.

### 4.2 Octadecaneuropeptide (ODN)

The octadecaneuropeptide (ODN; QATVGDVNTDRPGLLDLK), which belongs to the endozepin family, has been previously shown to increase intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ) in cultured rat astrocytes through activation of a metabotropic receptor positively coupled to PLC [65]. The C-terminal octapeptide, called OP (Fig. 10), is the minimum active ODN sequence, and [(D)Leu<sup>5</sup>]OP possesses weak antagonistic activity [66].

Silaproline analogues of OP, [Sip<sup>2</sup>]OP and [Sip<sup>2</sup>, (D)Leu<sup>5</sup>]OP, were synthesized by conventional Fmoc solid phase methods and measured for ability to elevate intracellular calcium concentrations. Application of [Sip<sup>2</sup>]OP ( $10^{-8}$  M) in the vicinity of an astrocyte provoked a transient and robust increase in  $[\text{Ca}^{2+}]_i$  with an amplitude significantly higher than that induced by ODN at the same dose. A short pulse of [Sip<sup>2</sup>, (D)Leu<sup>5</sup>]OP ( $10^{-8}$  to  $10^{-6}$  M) close to an astrocyte did not affect basal calcium levels, but partially reduced ODN-evoked  $[\text{Ca}^{2+}]_i$  increases with the same efficacy as its non-silylated counterpart, [(D)Leu<sup>5</sup>]OP.



Fig. 10 OP sequence



### 4.3 Captopril

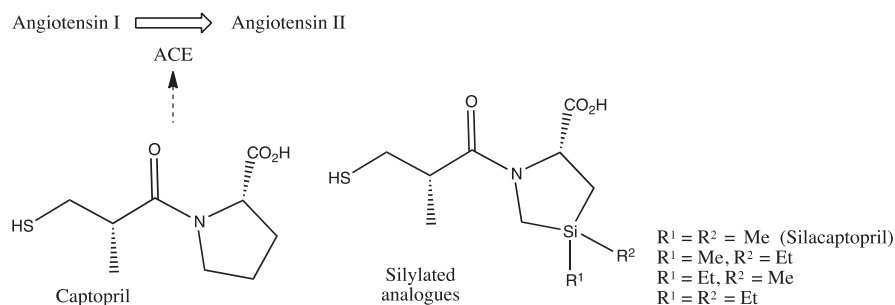
Angiotensin-I converting enzyme (ACE), a zinc metalloenzyme, plays a fundamental role in blood pressure regulation by converting the inactive decapeptide angiotensin I to the potent vasopressor octapeptide angiotensin II. Captopril [67] is currently used as an oral antihypertensive agent that competitively inhibits ACE [68]. Inhibitory potency is possibly mediated via a hydrophobic interaction with the enzyme. The ring conformation and orientation have been studied by annulation of cyclopropyl and cyclopentyl moieties onto the proline ring [69]. Studying the relevance of the hydrophobic character of the proline moiety, we replaced the proline residue in captopril by the more lipophilic silaproline. Employing the ACE crystal structure [70, 71], a set of silaproline Captopril analogues (Fig. 11) were docked in silico into the Zn catalytic site.

The less hindered analogue of the series (silacaptopril,  $R^1=R^2=\text{Me}$ ) was synthesized and tested in vitro for ability to inhibit ACE enzymatic activity. The observed similar inhibition compared to captopril indicated that hydrophobic interactions were either not of significance for interaction with ACE or tempered by steric hindrance, which hampered binding. Docking calculations supported the hypothesis, because silacaptopril exhibited the lowest docking energy in both ACE domains compared to all silylated analogues [72].

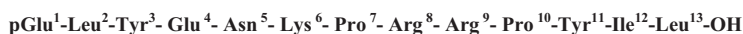
### 4.4 Neurotensin

Neurotensin (NT) (Fig. 12) is a tridecapeptide that is highly expressed in the central nervous system and was first isolated from bovine hypothalamus [73]. Following brain injection along with a cocktail of enzyme inhibitors, NT elicits hypothermic and analgesic responses [74]. The effects of NT have been shown to be differentially mediated by multiple receptors:  $\text{NTS}_{1-3}$ . For example,  $\text{NTS}_2$  has been reported to contribute to the protective effect of NT on pancreatic beta cells [75]. Structure–activity relationship studies have shown the minimal active sequence to be the C-terminal fragment NT(8–13) [76]. Replacement of the arginine residues of NT(8–13) by lysine gave H-Lys-Lys-Pro-Tyr-Ile-Leu-OH (JMV438), which had relatively better affinity and selectivity for  $\text{NTS}_2$  (Table 2).

Like many natural peptides, neurotensin has a short half-life in vivo, due to rapid enzymatic degradation. Stability studies in plasma using analysis by electrophoresis have highlighted specific enzymes that cleave specific peptide bonds. To overcome neurotensin instability, several NT analogues have been developed using different approaches including unnatural amino acid incorporations [77–82], peptide bond modifications [83–85], and cyclization [86, 87].



**Fig. 11** Role of ACE and structure of captopril



**Fig. 12** Neurotensin (NT) sequence

**Table 2** Binding of NT and analogues

Agonist	Sequence	NTS1 IC <sub>50</sub> (nM)	NTS2 IC <sub>50</sub> (nM)	Selectivity NTS2
NT	pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu-OH	0.4	4	0.1
NT (8–13)	H-Arg-Arg-Pro-Tyr-Ile-Leu-OH	0.3	2.7	0.11
JMV 438	H-Lys-Lys-Pro-Tyr-Ile-Leu-OH	6.3	2	3.15
JMV 2009	H-Lys-Lys-Sip-Tyr-Ile-Leu-OH	17.5	5	5.83

Few changes are tolerated for Pro<sup>10</sup>, which is important for folding into its active peptide conformation, yet is involved in amide bonds that are sensitive to cleavage by endoproteases. Replacement of Pro<sup>10</sup> by hydroxyproline, thioproline, and its 4 and 6 member ring counterparts, azetidine carboxylic acid and pipercolic acid, as well as cyclic aromatic derivatives such as Tic and Aic, all leads to conformational modifications and loss of affinity for the NT receptors. In general, substitutions of Pro<sup>10</sup> are more easily tolerated by amino acids that favor a reverse turn, rather than an extended conformation [88]. For example, information about the bioactive conformation of NT(8–13) has been gained using spirolactam conformational constraints [89]; however, substituted prolines have been tried without improvement in affinity [90].

The influence of silaproline in position 10 of NT(8–13) was evaluated by insertion into JMV438 to provide H-Lys-Lys-Sip-Tyr-Ile-Leu-OH (JMV2009), which exhibited improved NTS<sub>2</sub> selectivity, albeit with a 2.5-fold drop in affinity (Table 2). The conformations of JMV438 and JMV2009 appeared similar by NMR spectroscopic analysis. Moreover, Sip-containing NT analogue JMV2009

**Val-Arg-Lys-Pro-Pro-Sip(Val-Arg-Lys-Pro-Pro-Pro)<sub>2</sub>****Fig. 13** Silylated proline-rich peptide designed as CPP

maintained biological activity under conditions in which the natural peptide was rapidly degraded. Central administration of JMV2009 in rats induced dose-dependent antinociceptive responses in a variety of acute, tonic, visceral, and neuropathic pain models [91, 92].

### 4.5 Cell-Penetrating Peptides (CPPs)

The potential of cell-penetrating peptides (CPPs) to aid therapeutics to cross human membranes has attracted many scientists to the field of drug delivery [93]. Amphipathicity, charged residues, arginine moieties, and hydrophobicity are some of the claimed CPP properties suggested for a successful translocation through cell membranes. Among reported CPPs, proline-rich peptides that form amphipathic polyproline II helices have exhibited promising results as vectors [94, 95]. Replacement of a proline by silaproline on the hydrophobic face of a noncytotoxic CPP was examined to favor the interaction with the amphipathic environment of the cell membrane. Comparative studies using flow cytometry and confocal microscopy techniques showed a 20-fold enhancement in the internalization rate of the peptide Val-Arg-Lys-Pro-Pro-Sip(Val-Arg-Lys-Pro-Pro-Pro)<sub>2</sub> compared to its parent proline peptide [96]. Furthermore, like the parent peptide, the silaproline-containing CPP did not exhibit any cellular toxicity (Fig. 13).

The effect of replacement of Pro by Sip on the secondary structure and aggregation properties of the Pro-rich CPP was further studied by different biophysical techniques. Contact angle measurements, CD spectroscopy, and cellular uptake studies showed a good correlation between amphipathicity and cellular uptake. The CD spectra in water showed the same degree of PPII structure. The transmission electron microscopy (TEM) imaging results indicated the presence of fibrillar superstructures of ~20 nm width and variable length similar to those observed for (Val-Arg-Lys-Pro-Pro-Pro)<sub>3</sub> [97]. The higher hydrophobicity afforded by the Sip derivative resulted in a higher internalization inside HeLa cells due to a retained PPII conformation with increased amphipathic character.

## 5 Summary

We have described the preparation of enantiomerically pure silaproline (Sip), both by the first diastereoselective synthesis and by large-scale racemic synthesis, followed by resolution using preparative HPLC. Different *N*-protected versions of Sip, useful for the synthesis of oligomers and modified peptides, and the free amino

acid were made by these two methods. Silaproline oligomers were prepared in solution using a stepwise strategy and by polymerization of Sip-NCA. Compared to homopolypoline, homopolysilaproline was found to adopt a more stable PPII structure by CD spectroscopic analysis. Replacement of proline by silaproline in several biologically active peptides led generally to analogues retaining the activity of the parent peptide, as well as greater resistance toward enzymatic degradation. In the case of NT-containing Sip analogues, the *in vivo* biological response was improved, such that they could be used in the absence of a cocktail of enzyme inhibitors. Finally, the higher hydrophobicity afforded by Sip improved internalization of amphipathic proline-rich peptides inside HeLa cells. Considering the power of silaproline to enhance the conformational stability, biological activity, and cell permeability of proline-containing peptides, further use of this silicon-bearing heterocyclic amino acid is merited in applications to make peptide-based probes, receptor ligands, and enzyme inhibitors.

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