ChemPubSoc Europe

DOI: 10.1002/chem.201600445

CHEMISTRY A European Journal Full Paper

Sila-Substitution

Incorporation of β-Silicon-β3-Amino Acids in the Antimicrobial Peptide Alamethicin Provides a 20-Fold Increase in Membrane Permeabilization

Julie L. H. Madsen,[a] Claudia U. Hjørringgaard,[a] Brian S. Vad,[b] Daniel Otzen,*[b] and Troels Skrydstrup*[a]

Abstract: Incorporation of silicon-containing amino acids in peptides is known to endow the peptide with desirable properties such as improved proteolytic stability and increased lipophilicity. In the presented study, we demonstrate that incorporation of β-silicon-β3-amino acids into the antimicrobial peptide alamethicin provides the peptide with improved membrane permeabilizing properties. A robust synthetic procedure for the construction of β-silicon-β3-amino acids was developed and the amino acid analogues were incorporated into alamethicin at different positions of the hydrophobic face of the amphipathic helix by using SPPS. The incorporation was shown to provide up to 20-fold increase in calcein release as compared with wild-type alamethicin.

Introduction

The introduction of silicon in place of carbon in biologically active molecules, known as sila-substitution, has, during the preceding decades, become a rapidly growing field of research.[1] The slightly altered physiochemical properties of silicon compared with carbon facilitates desirable properties with no or only little interruption of the biological activity. Incorporation of silicon into amino acids is one example of sila-substitution.[2] The unique properties of silicon-containing amino acids, such as increased lipophilicity,[3] make them desirable synthetic targets. Considerable efforts in this field of research has focused on the synthesis of silicon-containing α-amino acids with silicon in either the α- or β-position (Figure 1). The first example of β-trimethylsilyl alanine **1** synthesis was published in 1956.[4] Later, different asymmetric syntheses of β-trimethylsilyl alanine **1** have been reported,[5] as well as different types of silicon-containing amino acids, such as α-silyl-α-amino acids **2**,[6] and silaproline **3**.[7]

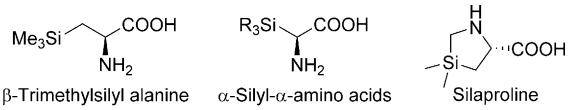


Figure 1. Examples of silicon-containing α-amino acids.

Incorporation of silicon-containing amino acids into peptides has been shown to alter the properties of these biomolecules. Due to an unstable carbon–silicon bond, no α-silicon-α-amino acids have been incorporated into functionalized peptide structures; however, TMS-alanine **1** and silaproline **3** are stable and have been used in studies of sila-substitution of several biologically important peptides. Examples include[8] incorporation of TMS-alanine in place of a phenylalanine in three renin inhibitors, which was shown to reduce the enzyme affinity only slightly and increase the α-chymotrypsin resistance of the peptides significantly (Figure 2, peptides **4a** and **4b**).[5a] In a second example, incorporation of TMS-alanine into the gonadotropin-releasing hormone (GnRH) antagonist Cetrorelix, led to an increased duration of effect compared with the carbon analogue (Figure 2, peptides **5a** and **5b**).[5c] Furthermore, incorporation of silaproline into a proline-rich cell-penetrating peptide was found to improve the peptide uptake into human cancer cells by 20-fold due to an increased overall hydrophobicity of the sila analogue.[9]

In a recent study, the α,α-disubstituted disilylated amino acid TESDpg was synthesized and incorporated into different positions in the antimicrobial peptide alamethicin.[10] The study showed that the α-helical structure of alamethicin was retained; however, the antimicrobial activity against *B. subtilis* was lost for all the peptide analogues, possibly due to steric

[a] *Dr. J. L. H. Madsen, Dr. C. U. Hjørringgaard, Prof. Dr. T. Skrydstrup*
Department of Chemistry and
Interdisciplinary Nanoscience Center
Center for Insoluble Protein Structures
Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C (Denmark)
E-mail: ts@chem.au.dk

[b] *Dr. B. S. Vad, Prof. Dr. D. Otzen*
Department of Molecular Biology and Genetics and
Interdisciplinary Nanoscience Center
Center for Insoluble Protein Structures
Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C (Denmark)
E-mail: dao@inano.au.dk

Supporting information and ORCID(s) from the author(s) for this article are available on the WWW under http://dx.doi.org/10.1002/chem.201600445.

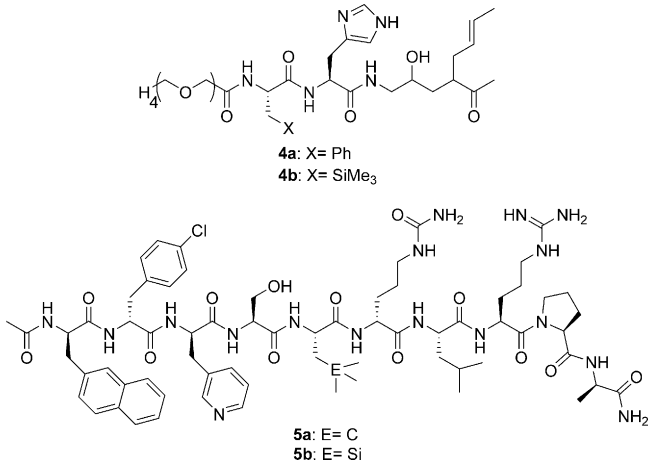


Figure 2. Examples of the incorporation of silicon-containing amino acids in peptides.

hindrance from the bulky amino acid preventing self-association of the peptide in the membrane.

In the present study, we wished to investigate the effects of incorporation of β-silicon-β3-amino acids on the properties of peptides. The antimicrobial peptide alamethicin was used as a model peptide for these studies. This peptide belongs to the family of peptaibols, asserting its antimicrobial effect through pore formation in the cell membrane of bacteria.[11] Upon interaction with the cell membrane, alamethicin folds up into an amphipathic α-helical structure, which inserts into the membrane and self-assembles into pore structures.[12] A helical wheel projection of alamethicin illustrates the amphipathic nature of the peptide (Figure 3 A).[13] We report the synthesis of three β-silicon-β3-amino acids and their incorporation into different positions at the hydrophobic face of alamethicin by using solid-phase peptide synthesis (SPPS). The effect of incorporation was studied by using RP-HPLC, far-UV circular dichroism, and calcein release assays. These studies revealed an increased lipophilicity, a secondary fold resembling the membrane-induced fold of alamethicin, as well as an up to 20-fold increase in calcein release as compared with wild-type alamethicin (WT Alm).

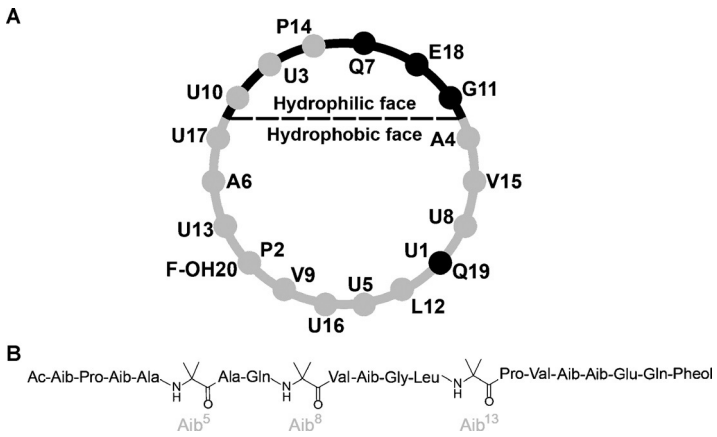


Figure 3. A) Amphipathic nature of alamethicin presented by an α-helical wheel; B) Alamthicin sequence with the three sites of substitution, Aib^5, Aib^8, and Aib^{13}, highlighted (U, Aib: α-Aminoisobutyric Acid; F-OH, Pheol: Phenylalaninol).

Results and Discussion

Design and synthesis

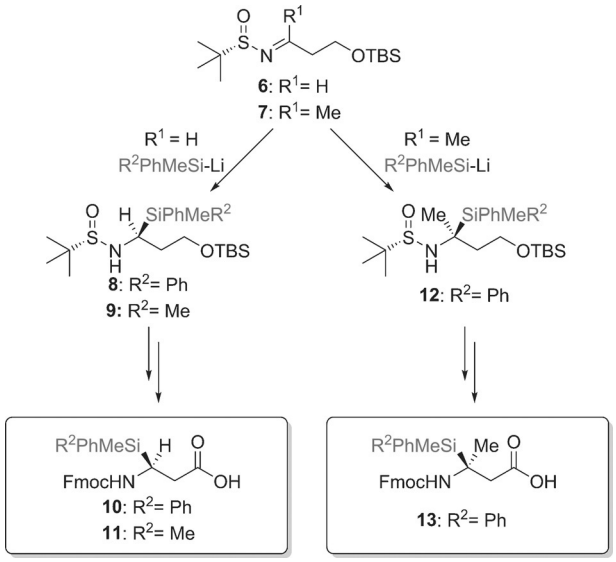
One characteristic feature of alamethicin is its high content of the non-ribosomally synthesized amino acid, α-aminoisobutyric acid (Aib, U). This amino acid is known to have a helix-inducing effect,[14] and its high content in alamethicin is suggested to assists in the formation of the amphipathic helix upon interaction with the cell membrane. In the presented study, three different positions for incorporation of β-silicon-β3-amino acids in alamethicin were investigated, namely Aib^5, Aib^8, and Aib^{13}, which are all positioned at the hydrophobic face of the helix (Figure 3 A and B). Incorporation of the lipophilic silicon-containing amino acids on the hydrophobic face of the helix could lead to an improvement of both the overall hydrophobicity and amphipathicity of the peptide. An increased hydrophobicity would facilitate membrane permeabilization, whereas, an increased amphipathicity would stabilize the pore structures in the membrane. Overall, this could lead to improvement of the antimicrobial action. The displacement of the three Aib residues with the synthesized β-silicon-β3-amino acids will result in two changes to the specific position of the peptide: alteration of the backbone, from α-amino acid to β-amino acid, and incorporation of silicon in the sidechain. The change in backbone may lead to alterations in the helicoidal conformation,[15] whereas, positioning silicon in the sidechain will provide the desired changes to the hydrophobicity and amphipathicity of the peptide. The overall effect of the two changes was investigated by using a range of biophysical techniques, as discussed below.

Synthetic strategy

The chemical structure and synthetic strategy for the three β-silicon-β3-amino acid analogues, **10**, **11**, and **13**, investigated in this study, are depicted in Scheme 1. The amino acid analogues were all designed to contain silicon in the β3-position to avoid the presence of an unstable α-carbonyl Si–C bond.[6e,16] The β,β-disubstituted amino acid **13** was designed to mimic the disubstituted nature of the native amino acid Aib.

The key step of the synthetic route to the silicon-containing amino acids **10** and **11** was the addition of a silyl lithium reagent to an Ellman sulfinimine,[17] which is a method previously reported by Ballweg et al.[18] and later by our group[19] for the preparation of silanediol isosters.[20] This addition reaction generally proceeds with a high diastereoselectivity in the order of $\geq$ 95:5.[21]

Synthesis of the β,β-disubstituted amino acid **13**, proceeds via a sulfinyl ketimine intermediate **7**. One reported example of silyl lithium addition to sulfinyl ketimines exists, in which the addition of diphenyl-

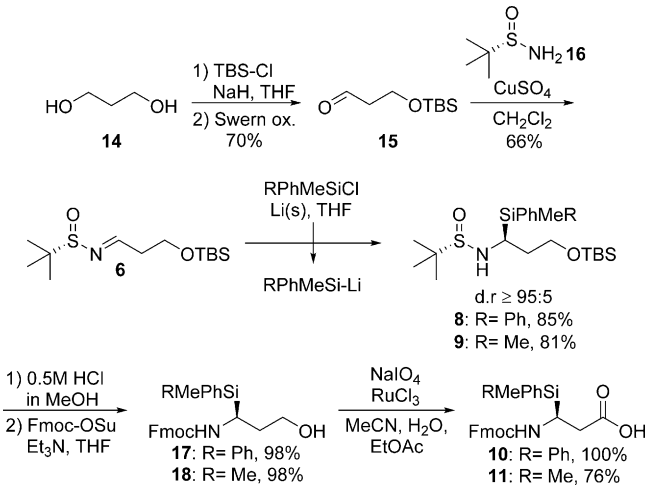


Scheme 1. General synthetic strategy for the synthesis of the three β-silicon-β3-amino acids.

methylsilyl lithium to a symmetrical sulfinyl ketimine provided the sulfinamide in 30% yield.[19a] The main challenges for this synthetic route are the *E/Z* selectivity of the sulfinyl ketimine formation,[22] as well as the resulting stereoselectivity of the silyl anion addition to the sulfinyl ketimine.[23]

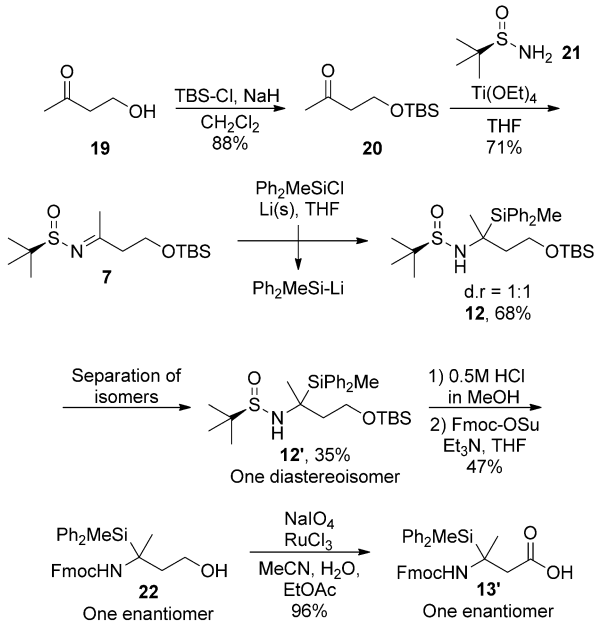
Synthesis of β-silicon-β3-amino acids

The synthesis of amino acids **10** and **11** is presented in Scheme 2. An initial monoprotection and Swern oxidation of 1,3-propanediol **14** afforded aldehyde **15**, which, upon copper sulfate mediated condensation with sulfinamide **16**, led to sulfinimine **6** in a 66% yield.[22] Lithiation of the chlorosilanes[24] followed by addition to the sulfinimine furnished sulfinamide **8** and **9** in yields of 85 and 81%, respectively, with excellent diastereoselectivity in both cases. Cleavage of the sulfur–nitrogen bond and liberation of the primary alcohol was accomplished by treatment with methanolic hydrochloric acid. The resulting free amine was Fmoc protected using Fmoc-succinimide leading to compounds **17** and **18**. A final oxidation step of the primary alcohol to the corresponding carboxylic acid provided amino acids **10** and **11** in good to excellent yields.

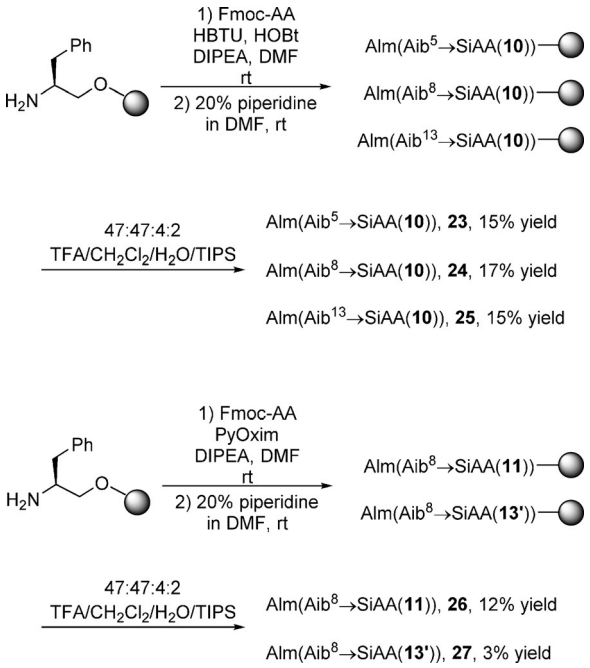


Scheme 2. Synthesis of β-silicon-β3-amino acids **10** and **11** via a sulfinyl-aldimine.

For the synthesis of the β,β-disubstituted amino acid **13**, addition of the silyl lithium reagent to a sulfinyl ketimine was performed (Scheme 3). Starting from 4-hydroxybutan-2-one (**19**), protection of the alcohol as its TBS ether was initially performed. Condensation with sulfinamide **21** was mediated by titanium(IV) ethoxide,[22] furnishing the sulfinyl ketimine **7** as only one isomer as observed in its ^{1}H NMR spectrum. Although we were not able to determine the stereochemistry of the imine double bond, the *E*-isomer is most likely to be the product obtained from this condensation. Unfortunately, addition of the silyl lithium reagent to the sulfinyl ketimine displayed no diastereoselective control, and the reaction resulted in a 1:1 mixture of diastereoisomers in a total yield of 68%. The two diastereoisomers could nevertheless be separated by flash chromatography, and a pure isomer **12′** was brought through the final protecting group manipulations and oxidation, affording an enantiopure analogue of the β,β-disubstituted amino acid **13′** of which the absolute stereochemistry was, however, not determined. In contrast to the observed unselective addition of silyllitium to the ketimine, the more recently developed copper-catalyzed reactions have proven to provide good selectivity in the addition to the challenging ketimines substrates.[21a,d]



Scheme 3. Synthesis of β-silicon-β3-amino acid **13′** via a sulfinyl-ketimine.



Scheme 4. Solid-phase peptide synthesis of sila-substituted alamethicins **23**–**27**.

Synthesis of alamethicin analogues

The three amino acid analogues were then incorporated into alamethicin by using solid-phase peptide synthesis (SPPS) (Scheme 4). Amino acid **10** was first incorporated at the three different positions corresponding to Aib^5, Aib^8, and Aib^{13}, affording peptides **23**, **24**, and **25**, respectively. This was performed to establish which position would provide the best effect of the sila-substitution. Initial calcein-release experiments revealed that the most prominent effect was observed by substitution at the Aib^8 position (see below). Amino acid analogues **11** and **13′** were subsequently incorporated into alamethicin at that position only, affording peptides **26** and **27**.

Peptides **23**–**25** were synthesized according to a modification of a previously reported method.[25] HBTU was applied as the coupling reagent for the ribosomal amino acids, whereas the sterically hindered Aib and Val residues were coupled as the pre-synthesized amino acid fluorides.[26] Peptides **23**–**25** were isolated by semipreparative RP-HPLC in a yield of 15–17%. Peptides **26** and **27** were synthesized by using PyOxim as the coupling reagent for the incorporation of all amino acid residues. The higher reactivity of the PyOxim coupling reagent meant that application of preformed Val and Aib amino acid fluoride was not necessary. This synthetic protocol afforded peptides **26** and **27** in a purified yield of 12 and 3%, respectively.

Structural and functional properties

The five alamethicin analogues were studied by using RP-HPLC, far-UV circular dichroism,[27] and calcein release assays[28] to investigate changes in lipophilicity, secondary structure, and membrane permeabilizing ability, respectively.

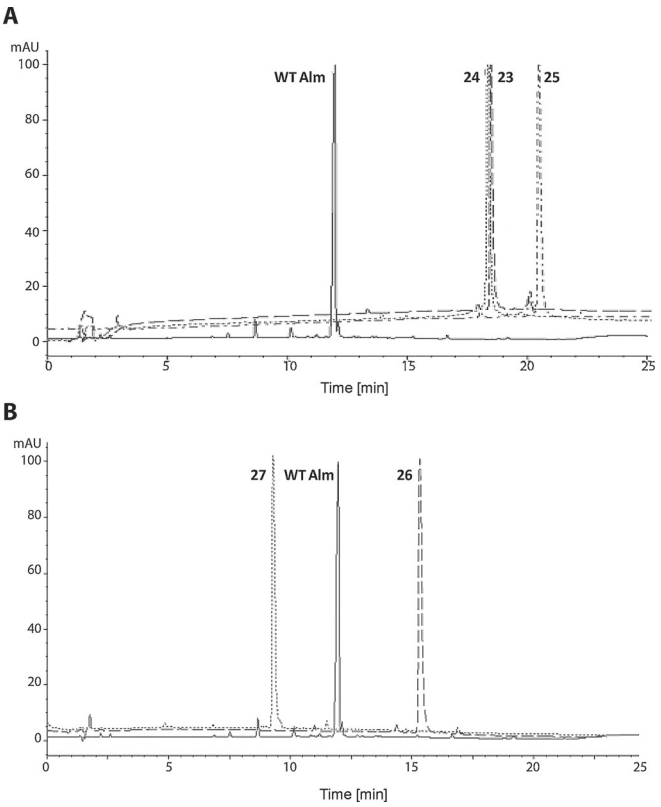


Figure 4. Analytical RP-HPLC analysis (solvent gradient: 5–95% MeCN in H_2O (0.1% TFA) over 20 min) showing change in overall peptide lipophilicity as compared with wild-type alamethicin (WT Alm). A) Comparing analogues **23**, **24**, and **25** to WT Alm. B) Comparing analogues **26** and **27** to WT Alm.

Changes in lipophilicity

The change in lipophiliciy of the alamethicin analogues was studied by analytical RP-HPLC and the results were compared with WT Alm (Figure 4). Incorporation of silicon-containing amino acid **10** into positions Aib^5, Aib^8, and Aib^{13} provided an increase in overall lipophilicity of the peptides. The retention times were increased by 6–9 min (Figure 4A). Incorporation of amino acid **11** likewise increased the lipophilicity with an observed increase in retention time of 4 min (Figure 4B). In contrast, incorporation of amino acid **13′** afforded a more hydrophilic peptide with a 3 min reduction in retention time. This reduction could be an effect of a difference in secondary structure (see below), potentially exposing different residues of the peptide.

Secondary structure of the peptides in the absence and presence of lipid vesicles

The mean residual ellipticity was measured at three different conditions; in a TRIS buffered solution, in the presence of zwitterionic dioleylphosphatidylcholine (DOPC) lipids, and in the presence of an anionic 2:8 mixture of dioleylphosphatidylgly-

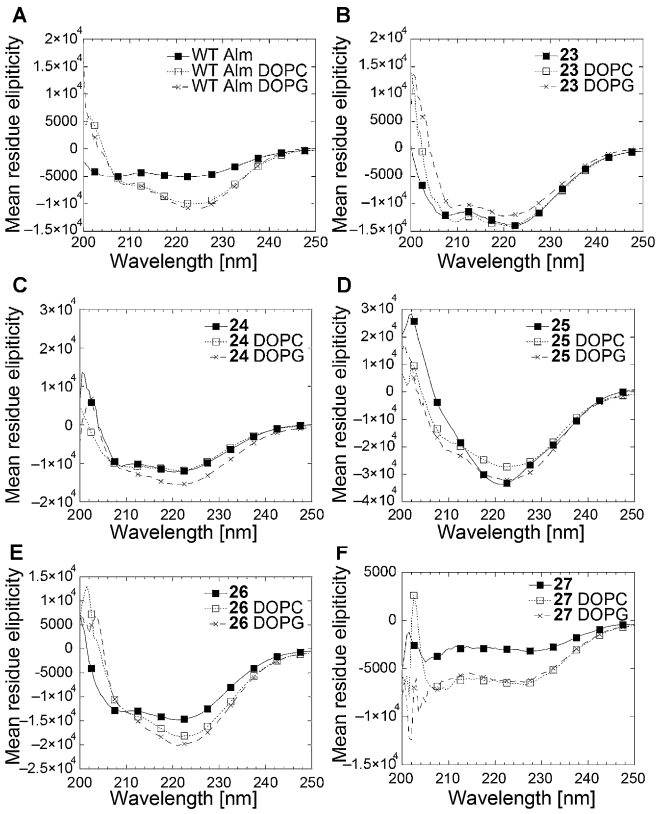


Figure 5. Circular dichroism of wild-type alamethicin, and peptide analogues **23**–**27**.

cerol (DOPG)/DOPC lipids (Figure 5). The two lipid-containing conditions should mimic the presence of either a mammalian cell membrane (mainly zwitterionic) or a bacterial cell membrane (which contains a significant amount of anionic lipids). The circular dichroism spectrum of WT Alm in a TRIS-buffered solution revealed two minima of equal magnitude at 205 and 222 nm, corresponding to the spectrum of an α-helix (Figure 5 A). Upon interaction with either pure DOPC or DOPG/DOPC mixed lipids, a large spectroscopic change was observed, resulting in a pronounced negative Cotton effect at 225 nm with a shoulder at 208 nm. This spectroscopic change is in agreement with previous reports.[29] Several explanations for this spectroscopic change have been proposed, including self-association of the peptides with or without changes in secondary structure or other changes in the chromophore environment.[30]

The spectra obtained for the alamethicin analogues **23**, **24**, and **25**, containing sila-amino acid **10** at positions Aib^5, Aib^8, or Aib^{13}, respectively, are shown in Figure 5 B, C, and D. They reveal a less pronounced spectroscopic change upon lipid interaction than was observed for WT Alm. The spectra resemble the lipid-induced spectra of WT Alm with the deepest minimum at 225 nm. This indicates that the incorporation of sila-amino acid **10** into the structure has induced a conformation, which, even in the absence of the membrane lipids, resembles the conformation of the membrane-interacting alamethicin.

In contrast, alamethicin analogues **26** and **27**, containing sila-amino acids **11** or **13**′, respectively, at position Aib^8, do not follow the same trend (Figure 5 E and F). The spectrum obtained for peptide **26** displays a similar, though smaller, conformational change as was observed for WT Alm. Upon lipid interaction, the spectra changes from a typical α-helix to the conformation showing a deep minimum at 225 nm and a shoulder at 208 nm. Incorporation of sila-amino acid **11** therefore seems to induce no or only little change in secondary structure of the peptide. Finally, analogue **27** was shown to undergo no structural change upon lipid interaction, in contrast to the other peptides. In a TRIS-buffered solution, the spectrum shows an α-helical conformation with weak minima at 205 and 222 nm. The minima are enhanced upon lipid interaction; however, the membrane-induced conformation of alamethicin is not obtained.[31]

Calcein-release assay

The membrane permeabilizing properties of the analogues were studied by using a calcein-release assay (Figure 6 and Figure 7). Calcein is trapped inside the aqueous interior of the vesicles at self-quenching concentrations, and peptide permeabilization of the membrane results in release of calcein to the exterior and a consequential increase in fluorescence.[32] The calcein release was measured as a function of peptide concentration from two different vesicles, zwitterionic DOPC and anionic DOPG/DOPC vesicles. The concentration of WT Alm producing a 50% calcein release (EC_{50}) was found to be 1 μg mL^{-1} in both zwitterionic DOPC and anionic DOPG/DOPC vesicles (Figure 6 and 7).

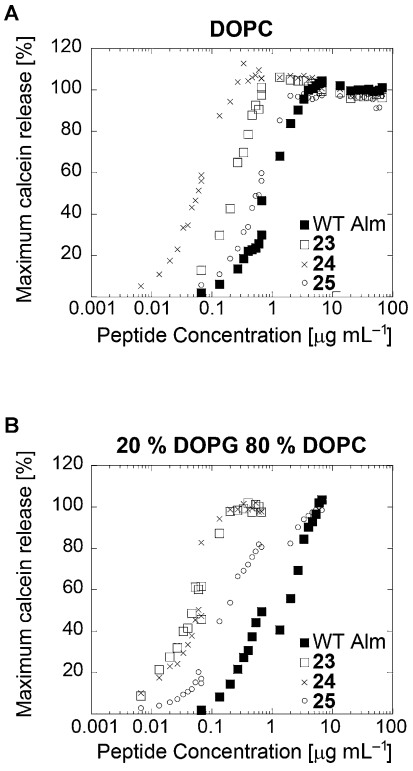


Figure 6. Calcein release assay of wild-type alamethicin, and peptide analogues **23**–**25**.

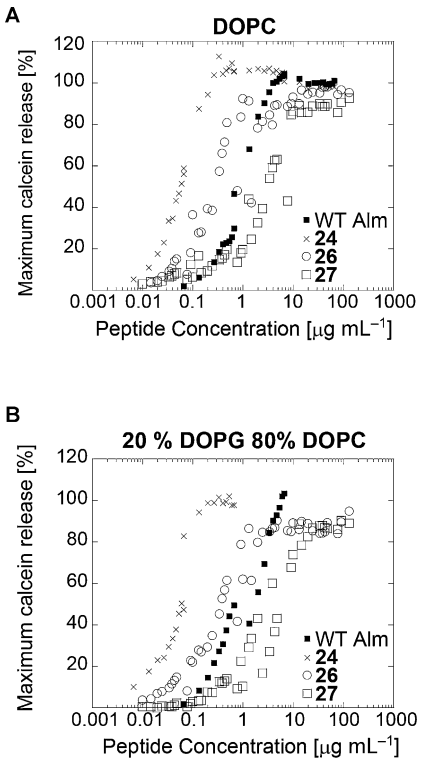


Figure 7. Calcein release assay of wild-type alamethicin, and peptide analogues **24**, **26** and **27**.

In the zwitterionic vesicles, analogue **24**, containing sila-amino acid **10** at the Aib[8] position showed the best pore-forming ability, providing a 50% calcein release at a concentration of 0.05 μg mL^{-1} (Figure 6A). This corresponds to a 20-fold increase in the pore-forming ability compared with WT Alm. Analogue **23** showed a fourfold increase and analogue **25** a twofold increase. In the anionic DOPG/DOPC vesicles (Figure 6B), both analogue **23** and **24** exhibited a 15-fold increase, whereas, analogue **25** showed a sevenfold increase. These results demonstrate that an incorporation of sila-amino acid **10** at position Aib[8] has the greatest effect on the membrane permeabilizing abilities of alamethicin. The effect is similar in both zwitterionic and anionic vesicles. The results furthermore indicate that incorporation at position Aib[5] could potentially result in a higher selectivity towards bacterial membranes, because analogue **23** showed a higher pore-forming ability in the anionic DOPG/DOPC vesicles compared with the zwitterionic DOPC vesicles.

When comparing the membrane permeabilizing ability of analogues **24**, **26**, and **27**, containing sila-amino acid analogues **10**, **11**, and **13′**, respectively, at position Aib[8], peptide **24** showed the highest potency (Figure 7). Exchanging one phenyl substituent on silicon with a methyl, peptide **26**, resulted in an EC_{50}-value between that of WT Alm and peptide **24**. Incorporation of the β-disubstituted sila-amino acid **13′**, peptide **27**, resulted in a lowered membrane permeabilization as compared with WT Alm. This low activity of peptide **27** is consistent with the results obtained from circular dichroism, indicating the inability of peptide **27** to adopt the membrane-induced conformation of alamethicin.

The overall picture of the biophysical studies shows that incorporation of the β-silicon-β3-amino acids into alamethicin is beneficial for the membrane permeabilizing properties of the peptide. An exception was found for the more bulky β,β-disubstituted amino acid **13′**, which was found by circular dichroism to prevent formation of the active membrane-induced conformation of the peptide. This could potentially be due to the amino acid being too sterically hindered or possessing a stereochemistry that does not facilitate the correct fold. The observed higher EC_{50} value of peptide **27** is in agreement with this inability of the peptide to adopt the membrane-induced fold.

The positive effect observed for the monosubstituted amino acid analogues was found to be dependent on both the position of the silicon-containing amino acid in the peptide sequence and the substituents on silicon. Incorporation at position Aib[8] and the presence of two phenyl substituents on silicon provided the most active peptide analogue. A positive correlation between peptide lipophilicity (represented by their retention times on the RP-HPLC column) and their ability to permeabilize DOPC vesicles was found (Figure 8). Consistent

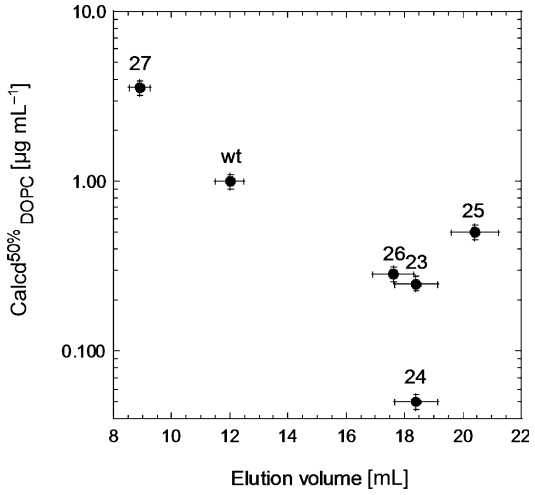


Figure 8. The peptide concentration resulting in 50% calcein release from DOPC vesicles as a function of the elution volume for the peptides; wild-type alamethicin, **23**–**27**.

with this, previous studies have shown a correlation between an increased hydrophobicity and hemolytic activity of antimicrobial peptides.[33]

Conclusion

In the presented study, three analogues of β-silicon-β3-amino acids were prepared. Addition of a silyl lithium reagent to a sulfinyl aldimine afforded amino acids **10** and **11**, whereas amino acid **13′** was synthesized via a more challenging sulfinyl ketimine intermediate. The three β-silicon-β3-amino acid ana-

logues were successfully incorporated into different positions of the antimicrobial peptide alamethicin by using SPPS.

Incorporation of amino acid **10** at position Aib[8] provided peptide **24**, which exhibited the best membrane-permeabilizing properties among the analogues, namely an impressive 20-fold increase in calcein release as compared with WT Alm. The CD spectrum of peptide **24** indicated a secondary structure resembling the membrane-induced conformation of WT Alm, even in the absence of vesicles, indicating an already structurally prepared peptide for membrane insertion prior to contact with the membrane. The results suggest that incorporation of β-silicon-β3-amino acids at the hydrophobic face of alamethicin provides a peptide with improved antimicrobial activity. This may be partially attributed to the increase in lipophilicity, although other factors may also be at play, given the imperfect correlation at high lipophilicity presented in Figure 8. Future microbiological studies will reveal whether the observed increase in membrane permeabilization provides a similarly increased antimicrobial activity. Furthermore, studies are ongoing to elucidate whether both alterations to the peptide, backbone and sidechain, are necessary to obtain the observed improvement in permeability.

Experimental Section

Organic synthesis

General procedures: Solvents were dried according to standard procedures. All commercially available reagents were used as received without further purification unless otherwise noted. All resins, coupling reagents and amino acids (except acid fluorides) were purchased from Iris Biotech, Germany. All reactions were performed under inert atmosphere unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC) analysis. Flash chromatography was performed using silica gel 60 (230–400 mesh). NMR spectra were recorded with a Varian Gemini 400 spectrometer. ^{1}H NMR was recorded at 400 MHz, ^{13}C NMR at 100 MHz. The chemical shifts are reported in ppm relative to solvent residual peak.[34] Multiplicities are reported with the following abbreviations: s = singlet, br s = broad singlet, d = doublet, dd = double of doublets, t = triplet, dt = double of triplets, q = quartet, quin = quintet, hex = hextet, m = multiplet.

General procedure A—addition of silyllithium to *N*-sulfinyl aldimine: Lithium bands (17.45 mmol, 12 equiv.) were suspended in anhydrous, degassed THF (9 mL). Chlorosilane (4.35 mmol, 3 equiv.) was added and the mixture was stirred for 4 h. In a separate flask, sulfinimine (1.45 mmol, 1 equiv.) was dissolved in anhydrous, degassed THF (6 mL) and cooled to −78 °C. To this cooled solution, the silyllithium solution was added dropwise over 5 min and the reaction was stirred at −78 °C for 18 h. H_2O (5 mL) was added and the mixture was allowed to warm to RT, poured into H_2O, extracted with EtOAc, dried over $MgSO_4$ and evaporated in vacuo. The crude mixture was purified by FC affording the product.

General procedure B—sulfinamide and TBS-deprotection, followed by Fmoc-protection: A solution of sulfinamide (0.28 mmol, 1 equiv.) in 0.5 M HCl in MeOH (5 mL) was stirred for 5 h. The solvent was removed in vacuo and the mixture was redissolved in anhydrous THF (3 mL). The solution was cooled to 0 °C, triethylamine (0.69 mmol, 2.5 equiv.) and 9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide (0.41 mmol, 1.5 equiv) was added and the reaction was stirred for 1 h at 0 °C, allowed to warm to RT and stirred overnight. EtOAc (4 mL) and sat. NH_4Cl (aq. 4 mL) were added and the layers were separated. The organic phase was washed with $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated in vacuo. The crude mixture was purified by FC.

General procedure C—oxidation of primary alcohol: To a solution of 3-aminoalcohol (0.41 mmol, 1 equiv.) in 2:2:7 acetonitrile/EtOAc/H_2O (7 mL) was added sodium periodate (1.66 mmol, 4 equiv.) and a catalytic amount of ruthenium(III) chloride (10 mol%) and the reaction was stirred for 2.5 h. H_2O was then added and the mixture was extracted with EtOAc. The combined organic phases were washed thoroughly with H_2O until no brown color persisted, dried over $MgSO_4$ and evaporated in vacuo affording the product.

(*S*)-*N*-{(*S*)-3-[(*tert*-Butyldimethylsilyl)oxy]-1-(methyldiphenylsilyl)-propyl}-2-methylpropane-2-sulfinamide (8): The compound was synthesized according to general procedure A from diphenylmethylchlorosilane (0.92 mL, 4.35 mmol) and sulfinimine **6** (424 mg, 1.45 mmol). The product was obtained by FC (EtOAc/pentane, 15%) as a clear oil (653 mg, 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.59–7.54 (m, 4H), 7.42–7.32 (m, 6H), 3.91–3.84 (m, 1H), 3.80–3.73 (m, 1H), 3.61 (dt, J = 3.2, 9.2 Hz, 1H), 3.01 (d, J = 9.2 Hz, 1H), 2.06–2.00 (m, 1H), 1.73–1.64 (m, 1H), 1.01 (s, 9H), 0.90 (s, 9H), 0.63 (s, 3H), 0.06 (s, 3H), 0.03 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.2 (2C), 135.1 (2C), 134.9, 134.6, 129.82, 129.79, 128.136 (2C), 128.125 (2C), 61.4, 56.3, 43.6, 36.4, 26.2 (3C), 22.8 (3C), 18.5, −5.0, −5.1, −5.2 ppm; HRMS: *m/z* calcd for $C_{26}H_{43}NO_2SSi_2$: 512.2451 $[M+Na]^+$; found: 512.2443.

(*S*)-*N*-{(*S*)-3-[(*tert*-Butyldimethylsilyl)oxy]-1-[dimethyl(phenyl)silyl]propyl}-2-methylpropane-2-sulfinamide (9): The compound was synthesized according to general procedure A from dimethylphenylchlorosilane (0.26 mL, 1.70 mmol) and sulfinimine **6** (165 mg, 0.57 mmol). The product was obtained by FC (10%, EtOAc/pentane) as a clear oil (196 mg, 81%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.53–7.50 (m, 2H), 7.38–7.34 (m, 3H), 3.81–3.76 (m, 2H), 3.13 (dt, J = 3.5, 8.5 Hz, 1H), 3.08 (d, J = 9.0 Hz, 1H), 1.93 (dddd, J = 3.3, 6.6, 7.8, 14.4 Hz, 1H), 1.67 (ddt, J = 5.1, 8.2, 14.2 Hz, 1H), 1.11 (s, 9H), 0.88 (s, 9H), 0.370 (s, 3H), 0.368 (s, 3H), 0.05 (s, 3H), 0.03 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 136.6, 134.2 (2C), 129.6, 128.1 (2C), 61.8, 56.2, 45.4, 36.0, 26.2 (3C), 22.9 (3C), 18.5, −3.9, −4.4, −5.10, −5.14 ppm; HRMS: *m/z* calcd for $C_{21}H_{41}NO_2SSi_2$: 450.2294 $[M+Na]^+$; found: 450.2280.

(*S*)-3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-3-(methyldiphenylsilyl)propanoic acid (10): The compound was synthesized according to general procedure C from carbamate **17** (200 mg, 0.41 mmol). The product was obtained as a pale-yellow foamy solid (202 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.74 (d, J = 7.6 Hz, 2H), 7.62–7.20 (m, 16H), 4.97 (d, J = 9.6 Hz, 1H), 4.40–4.10 (m, 5H), 2.68 (dd, J = 3.2, 15.8 Hz, 1H), 2.49 (dd, J = 8.0, 15.8 Hz, 1H), 0.67 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.3, 156.6, 144.0, 143.9, 141.4 (2C), 135.0 (4C), 133.71, 133.67, 130.2, 130.1, 128.4 (2C), 128.3 (2C), 127.7 (2C), 127.1 (2C), 125.2 (2C), 120.0 (2C), 67.1, 47.3, 36.8, 36.4, −5.2 ppm; HRMS: *m/z* calcd for $C_{31}H_{29}NO_4Si$: 530.1764 $[M+Na]^+$; found: 530.1764.

(*S*)-3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-3-[dimethyl(phenyl)silyl]propanoic acid (11): The compound was synthesized according to general procedure C from carbamate **18** (196 mg, 0.45 mmol). The product was obtained as a pale-yellow foamy solid (154 mg, 76%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (d, J = 7.6 Hz, 2H), 7.56–7.48 (m, 4H), 7.41–7.35 (m, 5H), 7.28 (t, J = 7.4 Hz, 2H), 4.95 (d, J = 9.5 Hz, 1H), 4.37 (quin, J = 7.7 Hz, 2H), 4.17 (t, J = 6.8 Hz, 1H), 3.74–3.65 (m, 1H), 2.58 (dd, J = 4.3, 16.2 Hz, 1H), 2.43 (dd, J = 8.3, 16.1 Hz, 1H), 0.39 ppm (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 177.6, 156.7, 144.1, 143.9, 141.4 (2C), 135.5, 134.1 (2C),

129.9 (2C), 128.2 (2C), 127.8 (2C), 127.2, 125.20, 125.16, 120.0 (2C), 66.9, 47.4, 38.1, 35.9, −4.4, −4.6 ppm; HRMS: *m/z* calcd for $C_{26}H_{27}NO_4Si$: 468.1607 $[M+Na]^+$; found: 468.1588.

(*R*)-*N*-{(*R*/*S*)-4-[(*tert*-Butyldimethylsilyl)oxy]-2-(methyldiphenylsilyl)butan-2-yl}-2-methylpropane-2-sulfinamide (12′): Lithium bands (78 mg, 11.3 mmol) were suspended in anhydrous, degassed THF (4 mL), diphenylmethylchlorosilane (659 mg, 2.83 mmol) was added and the mixture was stirred for 4 h. In a separate flask, sulfinyl ketimine **7** (172 mg, 0.57 mmol) was dissolved in anhydrous, degassed THF (4 mL) and cooled to −78 °C. To this cooled solution, the silyllithium solution was added dropwise over 5 min and the reaction was stirred at −78 °C for 18 h. H_2O (5 mL) was added and the mixture was allowed to warm to RT, poured into H_2O, extracted with EtOAc, dried over $MgSO_4$, and evaporated in vacuo. The crude mixture contained a 1:1 mixture of the two diastereoisomers. The two diastereoisomers were separated by FC (EtOAc/pentane, 10%) to afford the product diastereoisomer **12′** (99 mg, 35%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.73 (dd, *J* = 1.6, 7.6 Hz, 2H), 7.63 (dd, *J* = 1.7, 7.4 Hz, 2H), 7.43–7.33 (m, 6H), 3.63 (ddd, *J* = 1.7, 6.6, 8.2 Hz, 2H), 3.36 (s, 1H), 2.16 (ddd, *J* = 6.3, 8.3, 13.9 Hz, 1H), 2.05 (ddd, *J* = 6.6, 8.4, 13.8 Hz, 1H), 1.59 (s, 3H), 1.10 (s, 9H), 0.81 (s, 9H), 0.74 (s, 3H), −0.08 (s, 3H), −0.09 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 135.7 (2C), 135.5 (2C), 134.4, 134.0, 129.9 (2C), 128.2 (2C), 128.1 (2C), 60.4, 56.1, 48.3, 41.9, 26.1 (3C), 25.8, 22.9 (3C), 18.4, −5.2, −5.3, −5.4 ppm; HRMS (-TBS-group): *m/z* calcd. for $C_{21}H_{31}NO_2SSi$: 428.1482 $[M+K]^+$; found: 428.1839.

3-({[(9*H*-Fluoren-9-yl)methoxy]carbonyl}amino)-3-(methyldiphenylsilyl)butanoic acid (13′): The compound was synthesized according to general procedure C from carbamate **22** (98 mg, 0.19 mmol). The product was obtained as a pale-yellow foamy solid (94 mg, 94%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.75 (d, *J* = 7.6 Hz, 2H), 7.61 (dd, *J* = 1.6, 4.7 Hz, 2H), 7.60 (dd, *J* = 2.0, 4.5 Hz, 2H), 7.49 (t, *J* = 7.4 Hz, 2H), 7.41–7.32 (m, 8H), 7.29 (tt, *J* = 1.2, 7.4 Hz, 2H), 5.21 (br s, 1H), 4.27 (dd, *J* = 7.2, 10.6 Hz, 1H), 4.21 (dd, *J* = 6.8, 10.6 Hz, 1H), 4.04 (t, *J* = 6.8 Hz, 1H), 3.16 (d, *J* = 10.0 Hz, 1H), 2.55 (d, *J* = 14.9 Hz, 1H), 1.45 (s, 3H), 0.79 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 176.6, 155.9, 144.05, 143.99, 141.35, 141.33, 135.3 (4C), 129.71 (2C), 129.68 (2C), 128.05 (2C), 128.03 (2C), 127.7 (2C), 127.1 (2C), 125.28, 125.25, 120.0 (2C), 66.7, 47.2, 43.6, 41.1, 23.6, −4.0 ppm; HRMS: *m/z* calcd for $C_{32}H_{31}NO_4Si$: 544.1920 $[M+Na]^+$; found: 544.1633.

(*S*)-(9*H*-Fluoren-9-yl)methyl [3-hydroxy-1-(methyldiphenylsilyl)-propyl]carbamate (17): The compound was synthesized according to general procedure B from sulfinamide **8** (135 mg, 0.28 mmol). The product was obtained by FC (EtOAc/pentane, 5–33%) as a colorless foamy solid (146 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.6 Hz, 2H), 7.58–7.49 (m, 5H), 7.46–7.36 (m, 10H), 7.32–7.23 (m, 1H), 4.49–4.44 (m, 2H), 4.39–4.34 (m, 1H), 4.18 (t, *J* = 6.8 Hz, 1H), 4.02 (ddd, *J* = 2.8, 10.4, 12.8 Hz, 1H), 3.57 (ddd, *J* = 2.4, 5.2, 12.0 Hz, 1H), 3.48 (dt, *J* = 2.8, 11.2 Hz, 1H), 1.93–1.84 (m, 1H), 1.43 (tt, *J* = 2.4, 13.6 Hz, 1H), 0.63 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.6, 143.9, 143.7, 141.4 (2C), 134.99 (2C), 134.97 (2C), 133.7, 133.5, 130.1 (2C), 128.41 (2C), 128.38 (2C), 127.81 (2C), 127.78 (2C), 127.2 (2C), 127.1 (2C), 67.1, 58.3, 47.4, 34.9, 34.3, −5.4 ppm; HRMS: *m/z* calcd for $C_{31}H_{31}NO_3Si$: 516.1971 $[M+Na]^+$; found: 516.1974.

(*S*)-(9*H*-Fluoren-9-yl)methyl {1-[dimethyl(phenyl)silyl]-3-hydroxypropyl}carbamate (18): The compound was synthesized according to general procedure B from sulfinamide **9** (179 mg, 0.42 mmol). The product was obtained by FC (MeOH/CH_2Cl_2, 1%) as a colorless foamy solid (146 mg, 98%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.77 (d, *J* = 7.6 Hz, 2H), 7.55–7.49 (m, 4H), 7.43–7.38 (m, 5H), 7.31 (ddd, *J* = 1.1, 3.0, 7.4 Hz, 2H), 4.44 (ddt, *J* = 6.8, 6.8, 10.8 Hz, 2H), 4.38 (d, *J* = 10.2 Hz, 1H), 4.19 (t, *J* = 6.7 Hz, 1H), 3.55 (ddt, *J* = 2.9, 10.0, 13.0 Hz, 2H), 3.43 (dt, *J* = 3.0, 11.2 Hz, 1H), 1.84–1.75 (m, 1H), 1.34 (tt, *J* = 2.7, 13.4 Hz, 1H), 0.38 (s, 3H), 0.30 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 158.6, 143.9, 143.8, 141.5, 153.2, 134.1 (2C), 130.0 (2C), 128.4 (2C), 127.8 (2C), 127.20, 127.17, 125.05, 125.02, 120.13, 120.11, 67.0, 58.3, 47.5, 36.3, 34.0, −4.8, −4.9 ppm; HRMS: *m/z* calcd for $C_{26}H_{29}NO_3Si$: 454.1814 $[M+Na]^+$; found: 454.1800.

(*S*/*R*)-(9*H*-Fluoren-9-yl)methyl [4-hydroxy-2-(methyldiphenylsilyl)-butan-2-yl]carbamate (22): The compound was synthesized according to general procedure B from sulfinamide **12′** (208 mg, 0.41 mmol). The product was obtained by FC (EtOAc/pentane, 10–20%) as a colorless foamy solid (98 mg, 47%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.76 (d, *J* = 7.6 Hz, 2H), 7.64 (d, *J* = 7.6 Hz, 2H), 7.59 (d, *J* = 6.9 Hz, 2H), 7.50 (d, *J* = 7.4 Hz, 2H), 7.42–7.26 (m, 10H), 5.32 (br s, 1H), 4.28–4.17 (m, 2H), 4.03 (t, *J* = 6.8 Hz, 1H), 3.83–3.69 (m, 2H), 2.33–2.21 (m, 1H), 1.81–1.70 (m, 1H), 1.46 (s, 3H), 0.77 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 155.9, 144.16, 144.14, 141.39, 141.38, 135.3 (4C), 129.5 (2C), 128.0 (4C), 127.7 (2C), 127.10 (2C), 127.08 (2C), 125.24, 124.21, 120.0 (2C), 66.3, 59.1, 47.4, 45.1, 39.5, 22.7, −4.1 ppm; HRMS: *m/z* calcd. for $C_{32}H_{33}NO_3Si$: 530.2127 $[M+Na]^+$; found: 530.2124.

Solid-phase peptide synthesis

General procedure for synthesis of peptides 23, 24, and 25: Compounds were synthesized according to a modification of the reported procedure.[25] The peptide was synthesized in a fritted syringe on a 2-chlorotrityl chloride resin preloaded with phenylalaninol (0.15 mmol) by following Method B with a few modifications;[25] α-aminoisobutyric acid (**5**, **8** or **13**) was substituted for **10**, which was single coupled by using a modified single coupling cycle (shaking at RT for 60 min instead of MW heating). The two valines at positions 15 and 9 were coupled as preformed acid fluorides by using a modified acid fluoride coupling cycle (shaking at RT for 60 min instead of MW heating). Alanine (**4**) was coupled by using a modified double coupling cycle (shaking at RT for 60 min instead of MW heating). The peptide was purified by semipreparative RP-HPLC (Zorbax column, using a linear gradient from 50–90% B in A over 25 min with a flow rate of 5 mL min^{-1}).

Peptide 23: The product was obtained as a lyophilized powder (50.4 mg, 15% yield, 93% purity). MALDI-TOF MS: *m/z* calcd. for $C_{104}H_{160}N_{22}O_{25}Si$: 2168.1592 $[M+Na]^+$; found: 2168.531.

Peptide 24: The product was obtained as a lyophilized powder (54.2 mg, 17% yield, 91% purity). MALDI-TOF MS: *m/z* calcd. for $C_{104}H_{160}N_{22}O_{25}Si$: 2168.1592 $[M+Na]^+$; found: 2168.196.

Peptide 25: The product was obtained as a lyophilized powder (48.0 mg, 15% yield, 99% purity). MALDI-TOF MS: *m/z* calcd. for $C_{104}H_{160}N_{22}O_{25}Si$: 2168.1592 $[M+Na]^+$; found: 2168.391.

General procedure for synthesis of peptides 26 and 27: The peptides were synthesized in a fritted syringe on a 2-chlorotrityl chloride resin preloaded with phenylalaninol (0.2 mmol). The resin was swelled in 1:1 DMF/CH_2Cl_2 for 30 min and washed three times with DMF. Acylations were performed by treating the resin with a solution of Fmoc-amino acid (3 equiv.), PyOxim (3 equiv.), and DIPEA (6 equiv.) in DMF (1.2 mL) for 30 min [the following amino acids were double coupled: Aib(16), Val(15), Aib(13), Leu(12), Val(9), Gln(7), Ala(4), Pro(2) and Aib(1); **11**(8) was coupled for 60 min]. A wash cycle of 3×DMF was applied after each operation. Fmoc-deprotection was performed by treating the resin with a solution of 20% piperine in DMF for 2×10 min. Acetylation of the N-terminus was performed by treating the resin with a solution of acetic anhydride (7 equiv.) and DIPEA (14 equiv.) in DMF (1.2 mL) for 2× 10 min. Cleavage and deprotection of the peptide was performed

by treating the resin with 5 mL of a 47:47:4:2 (v/v) mixture of trifluoroacetic acid/CH_2Cl_2/H_2O/triisopropylsilane for 60 min. The cleavage mixture was concentrated in vacuo to 1 mL and precipitated by using cold *tert*-butyl methyl ether. Three times of centrifugation, decantation, and trituration followed by lyophilization afforded the crude peptide, which was purified by semipreparative RP-HPLC, Jupiter column, using a linear gradient from 50–90% B in A over 25 min with a flow rate of 5 mL min^{-1}.

Peptide 26: The product was obtained as a lyophilized powder (50 mg, 12% yield, 95% purity). MALDI-TOF MS: *m/z* calcd. for $C_{99}H_{158}N_{22}O_{25}Si$: 2106.1435 $[M+Na]^+$; found: 2106.317.

Peptide 27: The product was obtained as a lyophilized powder (12 mg, 3% yield, 96% purity). MALDI-TOF MS: *m/z* calcd. for $C_{105}H_{162}N_{22}O_{25}Si$: 2182.1748 $[M+Na]^+$; found: 2182.148.

Biophysical studies

Circular dichroism: The experiments were carried out in 20 mM Tris-HCl pH 8.0, at 25 °C with a 1 mm quartz cuvette. Alamethicin or alamethicin analogues were mixed with lipid and incubated for 1 min before spectra were measured with a Jasco J-810 (Tokyo, Japan). At least three accumulations were acquired, using a scanning speed of 100 nm min^{-1} and 0.2 nm data pitch. The data are reported as mean residue ellipticity: $MRE = 100\theta/(cdN)$, where θ is the measured ellipticity in degrees, c is the peptide concentration in mol/L, d is the light path in cm, and N is the number of residues.

Calcein release: LUVs (large unilamellar vesicles) containing 70 mM calcein were prepared as described previously,[35] using desiccated lipids, which were resuspended by vortexing. The samples were exposed to at least seven cycles of freezing and thawing before extrusion through a 200 nm pore filter. Free calcein was removed by gel filtration, and the vesicles were diluted to a final concentration of 45 μM in 50 mM tris pH 7.5. The vesicle solution was transferred by using a pipette into a 96-well plate and measured with a GENios Pro Plate Reader (TECAN, Männedorf, Germany). To monitor the release of free calcein from the vesicles and the following rise in fluorescence, the solution was excited at 490 nm, measuring emission at 515 nm. Spectra were normalized with regards to maximum fluorescence by the subsequent addition of Triton X-100 and values used in Equation (1), in which F is the fluorescence intensity achieved by peptide addition, and F_0 and F_t are fluorescence intensities for calcein solution and calcein solution with the addition of Triton X-100, respectively.

$$\text{Dye leakage } (\%) = 100 \times (F-F_0)/(F_t-F_0) \quad (1)$$

Acknowledgements

We thank the Danish National Research Foundation (Grant number DNRF59), the Lundbeck Foundation, the Carlsberg Foundation, and Aarhus University for generous financial support of this work.

Keywords: amino acids · antibiotics · membrane proteins · peptidomimetics · silicon

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Received: January 30, 2016
Published online on May 4, 2016