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Solution phase synthesis and purification of the minigramicidin ion channels and a succinyl-linked gramicidin

Hans-Dieter Arndt,[a] Andrea Vescovi,[a] Anna Schrey,[a] Jochen R. Pfeifer[a,b] and Ulrich Koert[a,b,*]

[a]*Institut für Chemie der Humboldt-Universität zu Berlin, Brook-Taylor-Str. 2, D-12489 Berlin, Germany*
[b]*Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Str., D-35032 Marburg, Germany*

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Abstract—Peptides with alternating D- and L-configured residues as found in the natural ion channel active peptide gramicidin A (gA) are important building blocks for artificially engineered ion channels. We detail an optimised solution phase synthesis employing a convergent assembly of peptide building blocks, giving access to the minigramicidines and a succinyl linked gA derivative on preparative scale. Moreover, the minigramicidines were investigated for secondary structure formation by CD-spectroscopy, which was discovered to be medium and capping-group dependent. A parallel, left-handed double-β-helix seems to be generally favoured in organic solvents for the minigramicidines.

1. Introduction

Ion channels allow the passive transport of ions through a phospholipid bilayer.[1] The driving force for the channel transport can be a concentration gradient or an electric potential. In line with progress in the structural understanding of biological ion channels[2] stands the active area of synthetic ion channels.[3] Two different approaches to synthesise ion channels were investigated in the past: a non-peptidic one,[4] using ether motifs, and a peptidic approach.[5] Recently hybrid channels have been reported, which combine peptides with synthetic subunits.[6]

Our interest in ion channels results from the possibility to understand a functional molecule on the basis of its molecular structure. The functional analysis of ion channels by conductivity measurements is well established.[7] In contrast, many questions remain unanswered concerning the nature of the distinct ion channel properties observed for the synthetic channels described so far. In our view, this is most likely due to the delicate self-assembly nature of many of the artificial systems reported. Many of them do need a membrane environment to assemble properly—but the fraction of properly assembled molecules may be minute. These facts severely complicate the characterisation of the channel active conformation by direct methods such as NMR or X-ray crystallography.

A possible solution of this general problem is to focus on *unimolecular channels*, i.e. to avoid intermolecular association equilibria, and to use a lead conformation with a *defined folding motif*, which will allow to study the action of deliberately shaped molecular interactions.

With these requirements in mind, we decided to explore gramicidin A (gA) as a structural lead (Fig. 1). This lipophilic peptide with alternating D- and L-configured residues is synthesised by the bacterium *bacillus brevis* in its sporulation phase.[8] The biological function of this antibacterial peptide remains unclear, but its potency for ion channel formation in cell membranes together with its commercial availability has made this compound the most prominent model substance for ion channel studies to date.[9] In a membrane-like environment gA folds into a head-to-head associated dimer of two right handed, single stranded β-helices with a pitch of 6.3 residues per turn.[10a] This ion-channel active conformation is populated in the membrane,[10b–d] while in organic solvents gA forms a multitude of generally dimeric β-helical species.[10e]

A covalent linker joining two N-termini of the D-L-peptide strands should severely confine the conformational space of the molecule. This will avoid the entropic cost for the head-to-head dimerisation of gA and decrease the portion of solvent-exposed backbone amide bonds. Considering the spatial structure of the gA dimer,[10a] a C_4-linker should be the optimal substitute for two formamides. The NMR-structure of succinyl-linked gA has been determined in SDS-micelles and its conformation was found to be similar to the gA dimer in this environment.[11a] Schreiber et al. have employed more elaborate C_4-linkers to connect two gA moieties and studied their influence on the channel

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* Corresponding author. Address: Fachbereich Chemie, Philipps-Universität Marburg, Hans-Meerwein-Str., D-35032 Marburg, Germany. Tel.: +49-6421-282-6970; fax: +49-6421-282-5677; e-mail: koert@chemie.uni-marburg.de

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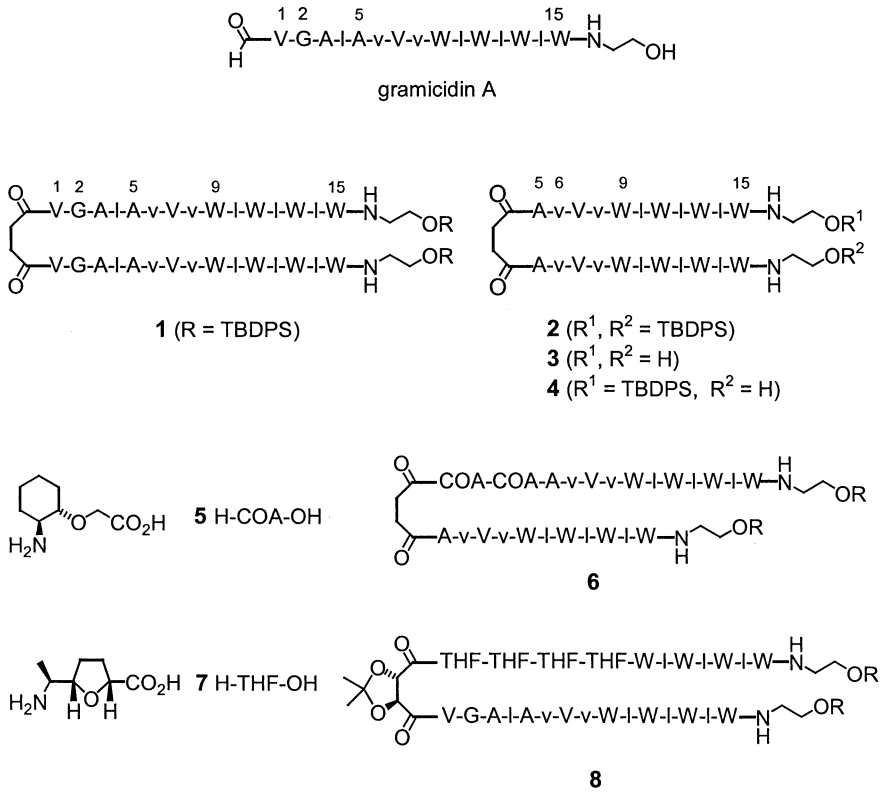


Figure 1. Molecular structure of gA and the N-terminally linked target structures **1**–**4**. The α-/δ-heteropeptides **6** and **8** employ ether-δ-amino acids and deliberately chosen fragments from the gA sequence.[6a,b]

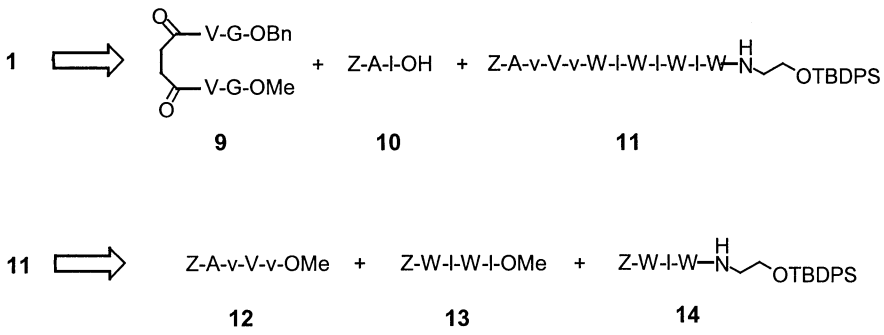
behaviour in some detail,[11b] but further information on covalently linked D-L-peptide ion channels is scarce.

In this report we give a detailed account on the high-yielding synthesis and purification of N-terminally succinyl-linked gramicidin **1** and the membrane-selective mini-gramicidines **2**–**4**,[12] which also provides important building blocks for the incorporation of the ether-δ-amino acids **5** and **7** into the backbone-modified α-/δ-heteropeptides **6** and **8**.[6a,b]

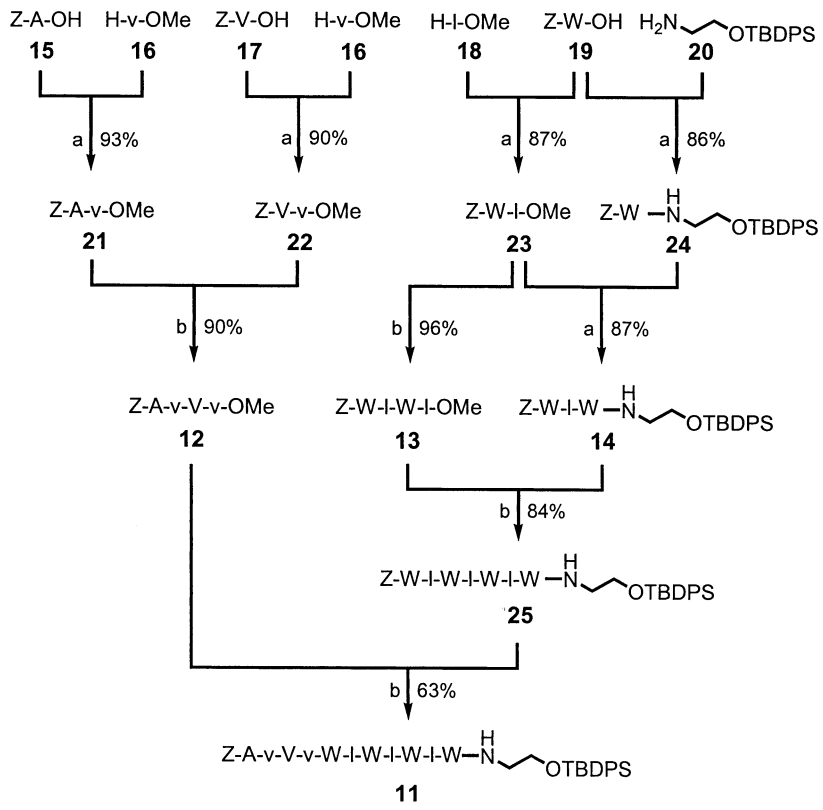
2. Synthetic considerations

In a seminal contribution, Sarges and Wittkop confirmed the molecular structure of gA by total synthesis using fragment couplings in solution.[13a] This strategy has later been explored to provide gA-analogues,[13b] but was abandoned with the advent of solid-phase peptide synthesis. Optimised protocols for the solid-phase assembly of gA are available.[14] But after some experimentation, we decided to refocus on a fragment coupling strategy for our D-L-peptide targets due to three main reasons:

1. It is by no means trivial to ensure that an observed specific ion channel property is not caused by an impurity. Due to the single-molecule resolution of the electrophysiological measurement techniques, highly active minor components can be seriously misleading. A coupling process employing pure fragments can be scrutinised by all analytical techniques available and separation problems, e.g. with co-eluting miscoupling products are minimised. This will become especially important for larger D-L-peptide fragments (>10), as these generally do not behave benign on both normal and reversed phase columns.



Scheme 1. Retrosynthetic disconnection of target **1** leading to the strategic peptide fragments.



Scheme 2. Synthesis of the undecamer **11**. (a) HOBt (1.5 equiv.), NEt_3 (1.5 equiv.), EDC (1.25 equiv.), CH_2Cl_2; (b) methylester deprotection: 2.5 equiv. LiOH, THF/H_2O 3:1, 0°C; *Z*-group cleavage: 0.02 M, 15 wt% Pd/C (5%), H_2 (1 bar); coupling: HOBt (2.5 equiv.), DIEA (2.5 equiv.), HBTU (1.5 equiv.), CH_2Cl_2/DMF 10:1, 0–20°C.

2. Larger amounts of materials have to be provided for structural investigations or studies with living cells. Solution phase chemistry is competitive on scale, especially if the amounts of resin, coupling reagents (>4 equiv.), D-amino acids (4 equiv., double couplings), and solvents are taken into account, which are routinely employed in solid phase synthesis.[14]
3. A convergent retrosynthetic strategy will provide a library of fragments, which can be used in multiple, interchanged or substituted by new entities when they are assembled in a block-wise fashion. This opens the door for hybrid channels like **6** or **8**.[6a,b]

Our retrosynthetic strategy is shown in Scheme 1. The end-capped succinyl-linked peptide dimer **1** is disconnected into a desymmetrised 'clamp' **9**, the interconnecting dipeptide **10**, and the undecamer **11**. This key compound will be assembled from three fragments of equal size **12**, **13** and **14**. All peptide fragments employ terminal Z/-OMe protection due to their availability and mild cleavage conditions, and they allow leaving the Trp side chains unprotected. The ethanolamine will carry a bulky, lipophilic TBDPS-protecting group to aid solubility and ease chromatographic purification of the intermediates.

3. Results and discussion

3.1. Synthesis of the minigramicidines

The synthesis of the key undecapeptide **11** is comprised in Scheme 2. The dipeptides **21**–**23** and ethanolamine-tagged **24** were synthesised from the respective monomers[15] **15**–**20** in very good yield and purity using HOBt/EDC-mediated couplings in CH_2Cl_2. Best results were obtained when the initial reaction temperature was kept at 0°C. The Z-deprotection of all fragments was accomplished by hydrogenolysis, but the polyaromatic compounds **14**, **25** and **11** behaved somewhat capricious in line with early findings.[13a] Generally the proper choice of catalyst (Degussa E101 NO/W), dilution (0.02 M in MeOH) and purity of starting material proved to be essential for a clean conversion, in some cases slightly elevated temperatures (30–40°C) and cosolvents (THF, DMF or H_2O) were beneficial.

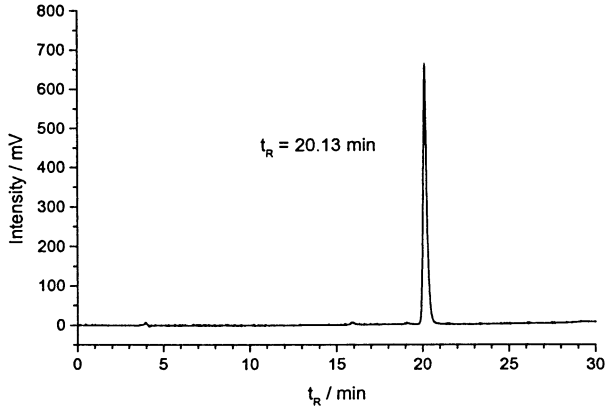


Figure 2. HPLC-trace of heptamer **25**: λ=280 nm, method M5, but 50→100% B in 25 min.

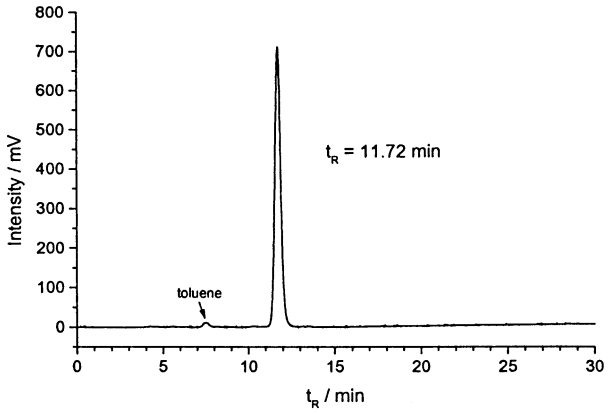


Figure 3. HPLC-trace of undecamer **11**: λ=280 nm, method M5, but 70→100% B in 25 min.

Neutral reaction conditions were preferred, as acidic hydrogenation- or transfer-hydrogenation protocols delivered less pure, coloured products. The Z-deprotection of heptamer **25** was best not driven to completion in order to minimise side product formation. In contrast, the LiOH-mediated[16] saponification of the methylesters performed smoothly with no detectable epimerisation even for the hindered tetramer **12**.

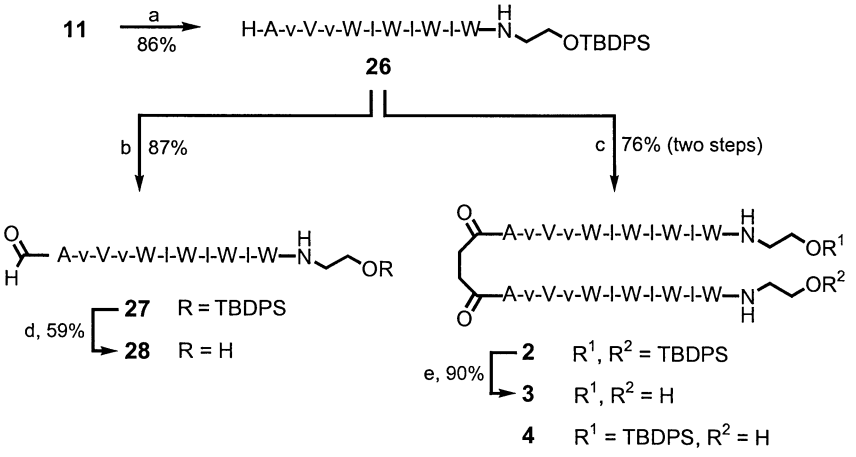
After some experimentation, a fragment coupling protocol was established employing HBTU/HOBt/DIEA in CH_2Cl_2/DMF-mixtures, which provided superior reactivity for these peptides even at 0°C. Epimerisation of residues was generally weak (<3%), only the difficult coupling leading to **11** was hampered by a decreased reaction rate and concomitant side product formation (majorly the ^{4}Val-epimer). All oligomers **12**–**14**, **25** and **11** were obtained diasteromerically pure after careful FCC as evidenced by ^{1}H NMR, MS and HPLC (Figs. 2 and 3). Following the procedure outlined in Scheme 2, multigram quantities of the stable key intermediate **11** could be prepared.

To proceed towards the minigramicidines,[12] undecamer **11** was deprotected and the resulting amine **26** formylated (HCOOH, DIEA, EDC) to yield TBDPS-protected minigramicidin **27** (Scheme 3). This was desilylated with HF in $CHCl_3$/CH_3CN to give minigramicidin **28** in 59% yield after silica gel chromatography. For the linked minigramicidin, amine **26** was derivatised with succinic anhydride, the resulting acid purified and homocoupled with a second equivalent of **26** using HATU/HOAt[17] in CH_2Cl_2/DMF to provide dimer **2** in excellent yield. MeOH as solvent was found to give the cleanest deprotection (HPLC-monitoring), and deprotected minigramicidin **3** could be obtained in a gratifying 90% yield. Interestingly, the linked minigramicidines **2**–**4** can be separated by FCC under optimised conditions, so the asymmetrically protected ion channel **4** could be isolated if the reaction was quenched at 50–70% conversion.

In the first instance, reaction monitoring and preparative purification of **11**, **27**, **28**, **2**–**4** were not straightforward. Schreiber et al. have given a procedure to purify gA by FCC on silica gel using $CHCl_3$/MeOH doped with HOAc and H_2O.[18] After a careful examination of solvent systems, mixtures of $CHCl_3$, MeOH (up to 10 vol%) and anhydrous HCOOH (up to 10 vol%) proved to have superior resolving power. They are easy to adjust and ensured good recovery on preparative scale for all the compounds described here, and gA itself. The HCOOH was removed by extraction with aqueous buffer before evaporation. With this optimised purification protocol, linked minigramicidin **2** can be provided on gram scale in >95% purity (600 MHz ^{1}H NMR) without the need for large scale HPLC purification. Preparative RP-HPLC purification of the ion channels **2**–**4** was frequently accompanied by significant losses of material and thus was only used to deliver high-purity material for ion-channel studies on the 1–5 mg scale.

3.2. CD-spectroscopy

To gain insight into the propensity of the minigramicidines for secondary structure formation, CD-spectra of ion channels **2**–**4** were recorded in several organic solvents (10^{-5} M). Each different conformer of gA including the ion-channel active conformation gives rise to a distinct CD-spectrum,[10e] so conformational preferences may be concluded by comparison. The bis-TBDPS protected



Scheme 3. Preparation of the minigramicidines **2**–**4**. (a) 25 wt% Pd/C (5%), H_2 (1 bar), MeOH/DMF 10:1, 40°C; (b) HCOOH (10 equiv.), DIEA (10 equiv.), cat. HOBt, EDC (6 equiv.), $CHCl_3$, 0→20°C; (c) (i) succinic anhydride (10 equiv.), pyridine, CH_2Cl_2/DMF 3:1, (ii) **26** (1 equiv.), HOAt (4 equiv.), DIEA (3 equiv.), HATU (2.5 equiv.), CH_2Cl_2/DMF 3:1, 0→20°C; (d) 2.5 vol% HF in CH_3CN/$CHCl_3$ 1:1; (e) 2.5 vol% HF in CH_3CN/MeOH 1:1.

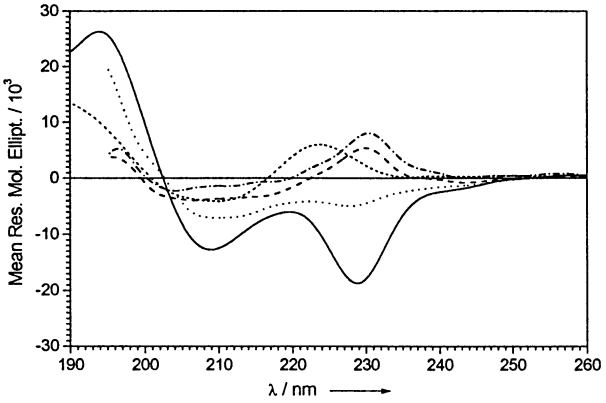


Figure 4. CD-spectra of diprotected minigramicidin **2** (10^{-5} M) in several organic solvents; — CH_3CN, ⋯ MeOH, - - - TFE, – – – *n*-hexane/*i*-PrOH 10:1, –·–·– *i*-PrOH.

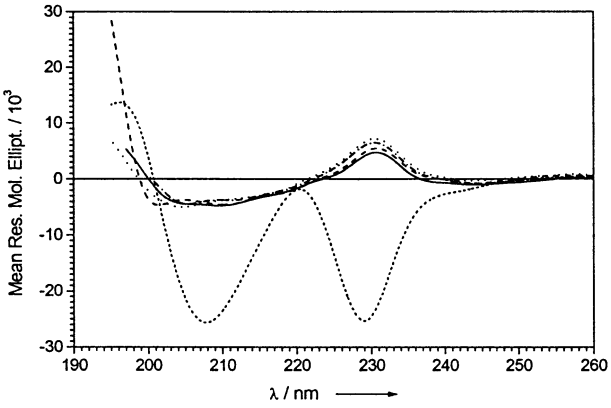


Figure 6. CD-spectra of minigramicidin **2** (10^{-5} M) in *i*-PrOH/H_2O 99:1 in the presence of different metal salts (1 mM); — *i*-PrOH/H_2O 99:1, – – – KPF_6, ⋯ CsCl, –·–·– $ZnBr_2$, - - - $Ca(ClO_4)_2$.

compound **2** shows different types of secondary structure, and the conformations may be addressed by the choice of solvent (Fig. 4). In MeOH, a mixture of conformations is present, which can also be concluded from the extremely complex ^{1}H NMR spectra in this solvent. A presently unknown right-handed species is favoured in *i*-PrOH, which can be concluded from the positive ellipticity at 230 nm. This conformation is also significantly populated in *n*-hexane (doped with *i*-PrOH for solubilisation). TFE is known to deaggregate D-L-peptides, so the spectrum obtained for **2** is somewhat similar to gA and is thought to represent a mixture of random-coil and right-handed $\beta^{4.4}$-helical species. But in acetonitrile and in aqueous *i*-PrOH (not shown), a left-handed parallel double helix is formed exclusively,[19] which indicates a symmetric pairing of two subunits.

The mono-deprotected compound **4** has rather weak ellipticities in organic solvents and probably exists mainly as a mixture of conformers (not shown). But the complete desilylation in the ion-channel **3** reduces the conformational freedom (Fig. 5): in all the solvents examined for **3**, a left-handed parallel double helical species predominates (*n*-hexane) or is the only one present (CH_3CN, *i*-PrOH and MeOH). Only TFE leads as expected to a merely random-coil conformation. The propensity to form aggregates may be the reason for the losses associated with the preparative RP-HPLC-purification of this compound, where strong denaturing conditions (low pH, TFE or DMSO as solvents) cannot be applied.

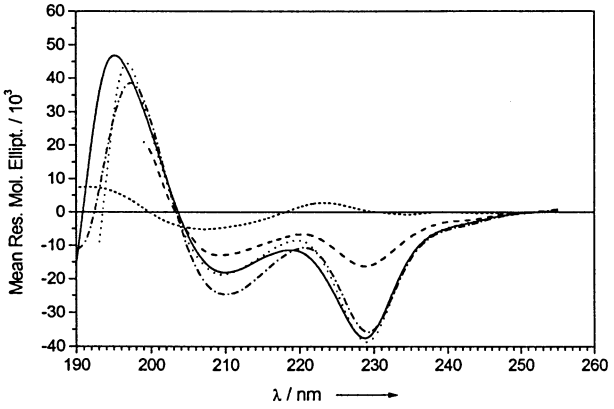
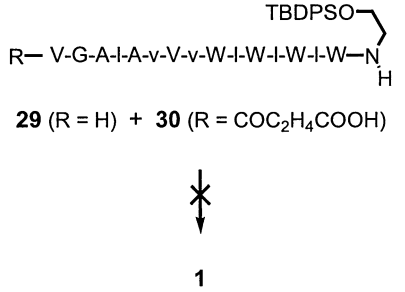


Figure 5. CD-spectra of desilylated minigramicidin **3** (10^{-5} M) in several organic solvents; — CH_3CN, ⋯ MeOH, - - - TFE, – – – *n*-hexane/*i*-PrOH 10:1, –·–·– *i*-PrOH.

The influence of metal ions on the conformation of **2** was briefly examined (Fig. 6). In *i*-PrOH (containing 1% H_2O to solubilise the salts), the presence of monovalent cations (1 mM) did not significantly change the CD-spectrum, and the LEWIS-acidic Zn^{2+} also did not have any influence. In contrast to the former, the presence of Ca^{2+} led to a characteristic change in the spectrum. Its appearance resembles the left-handed parallel double helix found for **2** in CH_3CN, but the doubled negative ellipticity at 208 nm and decreased positive ellipticity at 195 nm indicate moderate structural deviations from this type of secondary structure.[19] A lowered symmetry or differences in helical pitch may account for the difference.

3.3. Synthesis of the succinyl-linked gA (1)

In our initial attempts to prepare the succinyl-linked gramicidin **1**, we tried to assemble the two pentadecapeptide fragments **29** and **30** as shown in Scheme 4. To our surprise, we were hardly able to isolate any coupling product under the various conditions we tried. The HATU/HOAt conditions proven for the minigramicidines also did not lead to appreciable product formation, as evidenced by a HPLC-trace of the crude product mixture (Fig. 7, dotted trace). For whatever reason, the coupling of fragments to ^{1}Val seemed to be strongly retarded and we decided to shift the



Scheme 4. Attempted synthesis of succinyl-linked **1** by the union of two pentadecapeptide fragments **29** and **30**.

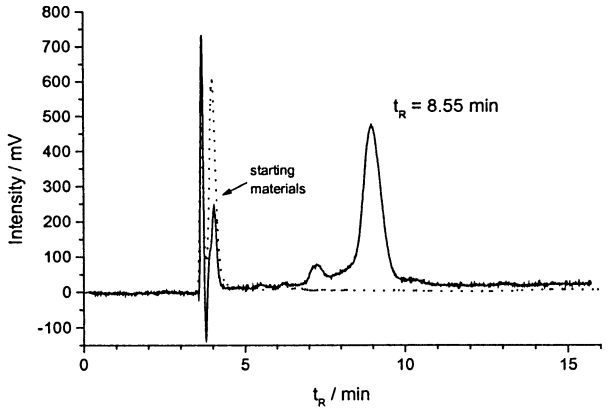


Figure 7. Overlay of the HPLC-traces of the attempted (dotted line) and productive (solid line) coupling experiments leading to **1** after gross removal of the coupling reagents by aqueous extraction (remainings at 3.6 min). λ=280 nm, method M3, column temperature 50°C.

final coupling position between ^{2}Gly and ^{3}Ala as shown in Scheme 1.

To this end, the undecamer amine **26** was elongated with the dimer **10**[20] to give the tridecamer **32** (Scheme 5). The clamp **9** was assembled from **31**[21] by deprotection, succination and coupling with H-Ala-Gly-OMe[22] under standard conditions. Even this coupling was extremely slow, supporting our notion of ^{1}Val being especially unreactive. Compound **9** is highly insoluble and had to be isolated by recrystallisation from boiling *i*-PrOH.

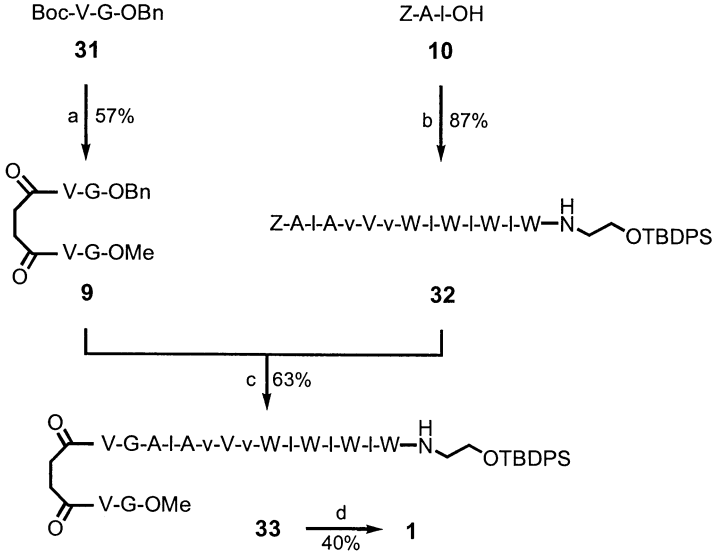
The union of **32** with the clamp **9** proceeded uneventfully to deliver the octadecamer **33** in 63% purified yield, which was coupled to the tridecamer **32** using the conditions previously established for the minigramicidines. In the event, the starting materials were consumed, furnishing the linked gramicidin **1** as the main product (Fig. 7, solid trace). The purification of compound **1** by preparative HPLC was difficult, because for an acceptable peak shape in the HPLC high column temperatures had to be applied (>50°C), which were not applicable for preparative purposes. After a combination of preparative TLC and size-exclusion chromatography, **1** was obtained in 40% yield with >95% purity (^{1}H NMR). 5 mg were purified further by repetetive preparative HPLC for ion channel analysis, until the ion channel activity did not increase further.

Compound **1** forms ion channels, which do hardly close again once they are open (Fig. 8). This observation and the minute amounts needed ($<10^{-14}$ M added to the bath solution) to induce ion channel formation indicate, that a chemically stable, simple succinate linker is fully sufficient for joining two gA units to form a stable, long-lived ion channel in the membrane.

4. Conclusions

For the synthesis of D-L-peptide ion channels a fragment coupling strategy was optimised. Solution-based chemistry ensured maximum purity of the products and gives access to the minigramicidines on a gram scale. The flexibility of this building block approach has already been demonstrated by the combination of ether-δ-amino acids with D-L-peptide fragments.[6a,b] In the synthesis of the linked gramicidin **1**, the disconnection into two equally sized gA-subunits was identified as a dead end and successfully overcome by a productive 13+5+13 clamping strategy. Using a simple, chemically robust succinate linker, D-L-peptide ion channels of membrane spanning dimension are obtained in excellent yield and purity.

CD-spectroscopy in organic solvents revealed, that the conformational space for the linked minigramicidines **2**–**4** appears to be much more confined than for natural gA. If the



Scheme 5. Productive synthesis of succinyl-linked gramicidin **1**. (a) (i) CH_2Cl_2/TFA 4:1, (ii) succinic anhydride (1.1 equiv.), CH_2Cl_2/DMF 5:1, 81%, (iii) H-Val-Gly-OMe (1.25 equiv.), HOBt (1.5 equiv.), NEt_3 (1.5 equiv.), EDC (1.25 equiv.), 20°C, 18 h, 70%; (b) **26**, HOBt (1.5 equiv.), NEt_3 (1.5 equiv.), EDC (1.25 equiv.), 0→20°C; (c) cleavage of *Z*- and benzyl-ester groups: 0.02 M in MeOH, 15 wt% Pd/C (5%), H_2 (1 bar), 40°C; coupling: HOBt (2.5 equiv.), DIEA (2.5 equiv.), HBTU (1.5 equiv.), CH_2Cl_2/DMF 10:1, 20°C; (d) methylester saponification: LiOH (2.5 equiv.), THF/H_2O 3:1; *Z*-group cleavage according to (c); coupling: HOAt (4 equiv.), DIEA (3 equiv.), HATU (2.5 equiv.), CH_2Cl_2/DMF 3:1, 20°C.

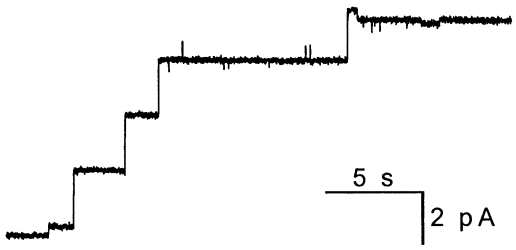


Fig. 8. Trace of a single channel conductance experiment in a black lipid bilayer (soy bean lecithin (symmetrical 1 M CsCl, 100 mV)). Each step in the diagram corresponds to a channel opening event. Open channels did not close any more during the period of observation (30 min).

termini are capped, a medium dependency of the secondary structure was evident. Generally, left-handed double-stranded β-helical species are favoured in organic solvents, especially for the uncapped compound **3**.

In our hands the minigramicidines can be provided in a pure state in preparative amounts and are potent, unimolecular ion channel formers in lipid bilayers.[12] If the constructs get larger, reliable purification becomes tedious and low yielding. Thus the minigramicidines are deemed to be most valuable subunits for the construction of more elaborate, unimolecular ion channel constructs.

5. Abbreviations

Amino acid residues, for the schemes the common one letter code was used, where capital letters denote L- and small letters D-configuration; V, L-Val; G, Gly; A, L-Ala; l, D-Leu; v, D-Val; W, L-Trp; SDS, sodium-dodecyl sulfate; Z, carboxy-benzyl; TBDPS, *t*-butyl-diphenyl-silyl; HOBt, 1-hydroxy-benzotriazole monohydrate; EDC, *N*-ethyl-*N*′-(3-dimethylamino-propyl)-carbodiimide hydrochloride; HBTU, *O*-(benzotriazol-1′-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate; DIEA, di-*iso*-propyl-ethylamin; HATU, *O*-(7′-aza-benzotriazol-1′-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate; HOAt, 1-hydroxy-7-aza-benzotriazole; FCC, flash column chromatography; TFE, trifluorethanol; DMSO, dimethylsulfoxide; DMF, dimethyl-formamide; sat., saturated solution.

6. Experimental

6.1. Analytical techniques

NMR-spectroscopy. Bruker AMX300 and DPX600. All resonances are given in ppm and referenced to residual solvent signals ($CDCl_3$: 7.25 ppm, DMSO-d_6: 2.49 ppm).

HPLC. Rainin Dynamax HPLC-System, DA-Detector. Method 1 (M1): Column Rainin Dynamax C8, 250×4 mm; solvent A: H_2O, solvent B: CH_3CN/*i*-PrOH 2:1; linear gradient 70⟶100% B in 20 min, flow 0.7 mL/min, column thermostat at 30°C. Method 2 (M2): Column Jupiter C5, 250×4 mm; A: H_2O, B: CH_3CN/*i*-PrOH 2:1, 75⟶85% B in 20 min, 0.7 mL/min, 30°C. Method 3 (M3): Column Jupiter C5, 250×4 mm; A: H_2O, B: CH_3CN/*i*-PrOH 2:1, 90⟶100% B in 15 min, 1 mL/min, 50°C. Method 4 (M4): Column Macherey and Nagel C8, 250×4 mm, A: H_2O, B: CH_3CN, 30°C. Method 5 (M5): Column Macherey and Nagel C8, 250×4 mm, A: H_2O, solvent B: CH_3CN/*i*-PrOH 2:1, 80% B⟶100% B in 25 min, 0.7 mL/min, 30°C. Retention times are uncorrected.

CD-spectroscopy. JASCO-710 spectropolarimeter with a N_2-flow of 2 L/min. Spectrum acquisition at 25°C, 2 mm cuvette. Seven accumulations, background correction and FFT-based smoothing were performed using the JASCO software package.

MALDI-TOF mass spectrometry. (1) Sample preparation: sample (10–100 μM in MeOH, 10 μL) and matrix solutions (50 mM 2,5-dihydroxybenzoic acid or 1,8,9-trihydroxy-anthracene in $CHCl_3$, 10 μL) were thoroughly mixed. Then 1 μL of the solution was applied on a stainless-steel target plate and air dried. (2) Mass determination: Bruker Reflex™ with delayed extraction in the reflectron mode using external calibration.

6.2. General procedures

The phrase 'aqueous work-up' refers to an extraction of the organic layer with sat. $NaHCO_3$-, 1 M $NaHSO_4$-, deionised H_2O and sat. NaCl ('brine') solutions consecutively (a third of the volume of the organic layer each), followed by drying with Na_2SO_4 and evaporation.

Thin layer chromatography (TLC) and flash column chromatography (FCC) were performed on silica gel (Merck Si60, 40–60 μm). Columns for FCC were slurry-packed. For difficult separations, the silca-gel slurry was sonicated (1 min) before packing. TLC-spots were visualised by UV-light and by staining with 2% molybdophosphoric acid in EtOH, 1% ninhydrine in EtOH or 2% *p*-methoxy-benzaldehyde in EtOH followed by 20% H_2SO_4, and heating.

Methylester deprotection (GP 1). The respective ester was dissolved in THF (0.05 M) and cooled to 0°C. 2.5 equiv. $LiOH{\cdot}H_2O$ was added as an aqueous solution (1:4 v/v) and stirred until conversion was complete. The mixture was then neutralised with 1 M $NaHSO_4$-solution (pH-paper), the THF was evaporated and the residue partitioned between EtOAc (0.02 M) and 1 M $NaHSO_4$ (2:1 v/v). The aqueous layer was extracted twice with EtOAc (equal volume), then all organic fractions were pooled and washed with brine (1:3 v/v), dried with Na_2SO_4, evaporated and dried to constant weight (0.01 Torr). The crude carboxylic acid was found (TLC, NMR, HPLC) to be sufficiently pure in all cases.

Z-Group cleavage (GP 2). The *Z*-protected compound was dissolved in the indicated solvent (0.02 M), 15 wt% Pd/C (5%, Degussa E101 NO/W) were added and the mixture was degassed and purged with H_2 (3×). The flask was warmed to the temperature given and hydrogenated (1 bar) with vigorous stirring until conversion ceased. The catalyst was filtered off using a pad of Celite® 512 (2 cm), the pad rinsed with MeOH, and the combined fractions evaporated, coevaporated with toluene/EtOH 10:1 and dried to constant

weight (0.01 Torr). In several cases the crude amines such obtained had to be further purified.

HOBt-EDC-mediated couplings (GP 3). The materials were dissolved in CH_2Cl_2 (indicated concentration) and cooled to 0°C. HOBt (1.5 equiv.) and NEt_3 were added (1.5, 3 equiv. if an amine-hydrochloride was used), followed by EDC (1.25 equiv.) after 15 min. After 15 min at 0°C the mixture was stirred at the indicated temperature until conversion was complete.

HBTU-mediated couplings (GP 4). The materials were dissolved in CH_2Cl_2 (0.03 M) and cooled to −5°C, HOBt (2.5 equiv.) dissolved in DMF (1:10 v/v) and DIEA (2.5 equiv.) were added to give a clear solution, followed after 5 min by HBTU (1.5 equiv.). The mixture was stirred at the indicated temperature.

Formamide formation (GP 5). The amine was dissolved in $CHCl_3$ (0.01 M), the solution cooled to 0°C and treated with HCOOH (10 equiv.), DIEA (10 equiv.), HOBt (cat. crystal) and EDC (6 equiv.) successively and stirred for 1 h at 0°C and 2 h at 20°C.

HATU-mediated couplings (GP 6). The materials were dissolved in CH_2Cl_2 (0.03 M) and cooled to 0°C, HOAt (4 equiv.) dissolved in DMF (1:3 v/v) and DIEA (3 equiv.) were added to give a clear yellow solution, followed after 5 min by HATU (2.5 equiv.). The mixture was stirred at the indicated temperature.

6.3. Preparations

6.3.1. *Z*-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide (24). 5.40 g (16.0 mmol) *Z*-Trp-OH **19** and 4.79 g (16.0 mmol) 2-(*t*-butyl-diphenylsilyloxy)-ethylamine **20** were coupled according to GP 3 (0.4 M, 20°C, 3 h). The mixture was then diluted with $CHCl_3$ (200 mL), and after aqueous work-up, FCC (300 g, PE/EtOAc 3:1→2:1→1:1) gave 8.50 g (13.7 mmol, 86%) of amide **24** as a colourless foam. R_f=0.14 (*n*-Hex/EtOAc 2:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.89 (s, 9H), 3.04 (dd, *J*=14.5, 7.7 Hz, 1H), 3.12–3.33 (m, 3H), 3.34–3.53 (m, 2H), 4.28–4.45 (m, 1H), 5.00 (d, *J*=12.2 Hz, 1H), 5.05 (d, *J*=12.2 Hz, 1H), 5.33–5.47 (m, 1H, NH), 5.75–5.90 (m, 1H, NH), 6.80–6.90 (m, 1H, ar.), 6.96–7.14 (m, 2H, ar.), 7.15–7.41 (m, 12H, ar.), 7.41–7.51 (m, 4H, ar.), 7.53–7.62 (m, 1H, ar.), 7.72 (s, 1H, NH). α_D=−3.1, α_{365}=−17.1 (*c*=1.00, $CHCl_3$, 20°C). HRMS (EI): for $C_{37}H_{41}N_3O_4Si$ (M^+) calcd 619.2866, found 619.2870. $C_{37}H_{41}N_3O_4Si$ (619.84) calcd C 71.70, H 6.76, N 6.78; found C 71.59, H 6.75, N 6.69.

6.3.2. *Z*-Trp-D-Leu-OMe (23). 8.46 g (25.0 mmol) *Z*-Trp-OH **19** and 4.78 g (26.3 mmol, 1.05 equiv.) H-D-Leu-OMe·HCl **20** were coupled according to GP 3 (0.3 M, 20°C, 2 h). The mixture was diluted with $CHCl_3$ (200 mL), and after aqueous work-up, recrystallisation from boiling cyclohexane/EtOAc (1:1) gave 10.1 g (21.8 mmol, 87%) of dipeptide **23** as colourless needles, mp=154.5–155°C. R_f=0.26 (*n*-Hex/EtOAc 1:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ=0.70 (d, *J*=1.5 Hz, 3H), 0.72 (d, *J*=1.5 Hz, 3H), 1.07–1.25 (m, 2H), 1.26–1.40 (m, 1H), 3.11 (dd, *J*=14.5, 7.7 Hz, 1H), 3.24 (dd, *J*=14.5, 5.1 Hz, 1H), 3.55 (s, 3H), 4.33–4.57 (m, 2H), 4.94–5.06 (m, 2H), 5.41 (d, *J*=5.7 Hz, 1H, NH), 6.07 (d, *J*=8.3 Hz, 1H, NH), 6.93 (d, *J*=2.3 Hz, 1H, ar.), 7.02 (t, *J*=7.4 Hz, 1H, ar.), 6.93 (m, 1H, ar.), 7.18–7.30 (m, 6H, ar.), 7.50–7.58 (m, 1H, ar.), 8.17 (s, 1H, NH). α_D=−0.5, α_{365}=−11.3 (*c*=1.00, $CHCl_3$, 25°C). HRMS (FAB): for $C_{26}H_{32}N_3O_5$ ($M+H^+$) calcd 466.2342, found 466.2352. $C_{26}H_{31}N_3O_5$ (465.55) calcd C 67.08, H 6.71, N 9.03; found C 67.10, H 6.70, N 9.09.

6.3.3. *Z*-Trp-D-Leu-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide (14). 1.58 g (3.4 mmol) of dipeptide **23** was C-deprotected according to GP 1 (R_f=0.09 in $CHCl_3$/MeOH 10:1) as well as 1.42 g (2.3 mmol) of amide **24** was N-deprotected according to GP 2 (MeOH, 20°C, 2 h, R_f=0.40 in $CHCl_3$/MeOH/aq. NH_3 10:1:0.1). The combined crude materials were coupled following GP 3 (0.1 M, −10→20°C, 3 h). After dilution with $CHCl_3$ (200 mL), aqueous work-up, FCC (250 g, $CHCl_3$/acetone 5:1→4:1) gave 1.90 g (2.0 mmol, 87%) of tripeptide **14** as a colourless foam. R_f=0.16 ($CHCl_3$/acetone 4:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.61 (d, *J*=6.4 Hz, 3H), 0.65 (d, *J*=6.4 Hz, 3H), 0.97 (s, 9H), 1.14–1.19 (m, 1H), 2.86–2.96 (m, 2H), 3.02–3.08 (m, 2H), 3.19–3.30 (m, 2H), 3.59–3.63 (m, 2H), 4.14–4.26 (m, 1H), 4.27–4.38 (m, 1H), 4.41–4.52 (m, 1H), 4.88 (d, *J*=12.4 Hz, 1H), 4.90 (d, *J*=12.8 Hz, 1H), 6.89–6.97 (m, 2H, ar.), 6.90–7.22 (m, 6H, ar.), 7.24–7.45 (m, 12H, ar.), 7.54–7.65 (m, 5H, ar.), 7.98–8.06 (m, 1H, NH), 8.03–8.19 (m, 2H, NH), 10.78 (d, *J*=1.8 Hz, 1H, NH), 10.82 (d, *J*=2.2 Hz, 1H, NH). α_D=24.0, α_{365}=91.5 (*c*=1.05, MeOH, 30°C). HPLC: 15.7 min (M1). HRMS (FAB): for $C_{54}H_{63}N_6O_6Si$ ($M-OH^-$) calcd 919.4578; found 919.4591. $C_{54}H_{64}N_6O_7Si$ (**14**·H_2O, 937.23) calcd C 69.20, H 6.88, N 8.97; found C 69.33, H 6.80, N 8.73.

6.3.4. *Z*-(Trp-D-Leu)$_2$-OMe (13). 1.17 g (2.50 mmol) dipeptide **23** were C-deprotected following GP 1 (R_f=0.09 in $CHCl_3$/MeOH 10:1) as well as 1.24 g (2.65 mmol, 1.05 equiv.) of **23** were *N*-deprotected following GP 2 (MeOH/THF 5:1, 25°C, 1 h; R_f=0.26 in $CHCl_3$/MeOH 10:1). The crude materials were combined and coupled following GP 4 (5°C, 2 h) and the mixture diluted with EtOAc (200 mL). After aqueous work-up, FCC (200 g, $CHCl_3$/acetone 5:1→4:1→3:1) provided 1.84 g (2.41 mmol, 96 %) of tetrapeptide **13** as a colourless foam. R_f=0.12 ($CHCl_3$/acetone 4:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.62 (d, *J*=5.7 Hz, 3H), 0.67 (d, *J*=5.7 Hz, 3H), 0.73 (d, *J*=5.3 Hz, 3H), 0.78 (d, *J*=5.7 Hz, 3H), 1.12–1.26 (m, 3H), 1.33–1.61 (m, 3H), 2.90 (dd, *J*=14.3, 9.4 Hz, 2H), 3.09 (ddd, *J*=21.0, 14.7, 5.8 Hz, 2H), 3.60 (s, 3H), 4.15–4.41 (m, 3H), 4.49–4.63 (m, 1H), 4.88 (d, *J*=12.8 Hz, 1H), 4.93 (d, *J*=12.8 Hz, 1H), 6.94 (t, *J*=7.35 Hz, 2H, ar.), 7.04 (dt, *J*=7.3, 3.5 Hz, 2H, ar.), 7.12 (d, *J*=4.9 Hz, 2H, ar.), 7.20 (m, 2H, ar.) 7.24–7.37 (m, 5H, ar.), 7.59 (m, *J*=3.0 Hz, 1H), 7.61 (d, *J*=3.4 Hz, 1H), 8.09 (d, *J*=7.9 Hz, 1H, NH), 8.18 (d, *J*=8.2 Hz, 1H, NH), 8.26 (d, *J*=7.5 Hz, 1H, NH), 10.80 (s, 2H, NH). α_D=−13.8, α_{365}=−60.0 (*c*=1.07, CH_2Cl_2, 25°C). HPLC: 8.8 min (M1). HRMS (FAB): for $C_{43}H_{53}N_6O_7$ ($M+H^+$) calcd 765.3976, found 765.3968. $C_{43}H_{52}N_6O_7$ (764.93) calcd C 67.52, H 6.85, N 10.99; found C 67.15, H 6.77, N 10.67.

6.3.5. *Z*-(Trp-D-Leu)$_3$-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide (25). 640 mg (696 μmol) of tripeptide **14**

were *N*-deprotected following GP 2 (MeOH/THF 5:1, 30°C, 12 h) and purified by FCC (30 g, $CHCl_3$/MeOH 100:5→100:7) to give 432 mg (550 μmol, 79%) of the pure amine. 535 mg (700 μmol, 1.3 equiv.) of tetrapeptide **13** were *C*-deprotected following GP 1. The combined materials were coupled following GP 4 (5°C, 2 h) and the mixture was diluted with $CHCl_3$ (50 mL). After aqueous work-up, FCC (120 g, $CHCl_3$/acetone 3:1→2:1→3:2) gave 698 mg (460 μmol, 84%) of heptapeptide **25** as a colourless foam. R_f=0.07 ($CHCl_3$/acetone 3:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.25–0.47 (m, 18H), 0.71–0.85 (m, 12H), 0.86–1.01 (m, 6H), 2.60–2.78 (m, 4H), 2.79–3.08 (m, 6H), 3.42 (t, *J*=6.2 Hz, 2H), 3.89–4.06 (m, 3H), 4.07–4.19 (m, 1H), 4.20–4.40 (m, 3H), 4.63 (d, *J*=12.4 Hz, 1H), 4.71 (d, *J*=12.8 Hz, 1H), 6.66–6.76 (m, 4H, ar.), 6.77–6.93 (m, 8H, ar.), 6.94–7.00 (m, 1H, ar.), 7.02–7.14 (m, 7H, ar.), 7.15–7.26 (m, 6H, ar.), 7.30–7.46 (m, 9H, ar.), 7.69 (d, *J*=7.2 Hz, 1H, NH), 7.79 (d, *J*=7.5 Hz, 1H, NH), 7.85–7.96 (m, 3H, NH), 7.99 (d, *J*=7.5 Hz, 1H, NH), 8.05 (d, *J*=8.7 Hz, 1H, NH), 10.55 (s, 2H, NH), 10.58 (s, 1H, NH), 10.60 (s, 1H, NH). α_D=−11.3, α_{365}=−51.6 (*c*=1.64, $CHCl_3$, 25°C). HPLC: 17.0 min (M1). MS (ESI) calcd for $C_{88}H_{104}N_{12}O_{10}SiNa$ ($M+Na^+$) 1539.8; found 1539.9. $C_{88}H_{106}N_{12}O_{11}Si$ (**25**·H_2O, 1535.98) calcd C 68.81, H 6.96, N 10.94; found C 68.82, H 6.93, N 10.97.

6.3.6. *Z*-Ala-D-Val-OMe (21). 2.33 g (10.0 mmol) *Z*-Ala-OH **15** and 1.67 g (10.0 mmol) H-D-Val-OMe·HCl **16** were coupled according to GP 3 (0.2 M, 0→20°C, 12 h). After aqueous work-up, FCC (200 g PE/MTBE 1:1→MTBE) gave 3.14 g (9.30 mmol, 93%) of dipeptide **21** as a colourless powder. R_f=0.18 (PE/MTBE 1:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.83 (dd, *J*=6.8, 1.9 Hz, 6H), 1.20 (d, *J*=7.2 Hz, 3H), 2.02 (dq, *J*=20.0, 6.8 Hz, 1H), 3.63 (s, 3H), 4.13–4.22 (m, 2H), 5.00 (s, 2H), 7.26–7.35 (m, 5H, ar.), 7.40 (d, *J*=7.9 Hz, 1H, NH), 8.12 (d, *J*=8.7 Hz, 1H, NH). α_D=−26.9, α_{365}=−106 (*c*=0.99, $CHCl_3$, 25°C). HRMS (EI): for $C_{17}H_{24}N_2O_5$ (M^+) calcd 336.1685 found 336.1688. $C_{17}H_{24}N_2O_5$ (336.39) calcd C 60.70, H 7.19, N 8.33; found C 60.93, H 7.17, N 8.27.

6.3.7. *Z*-Val-D-Val-OMe (22). 2.51 g (10.0 mmol) *Z*-Val-OH **17** and 1.67 g (10.0 mmol) H-D-Val-OMe·HCl **16** were coupled following GP 3 (0.1 M, 5 equiv. NEt_3, 20°C, 18 h). After aqeous work-up, the crude product was crystallised from $CHCl_3$/Et_2O to give 3.36 g (8.99 mmol, 90%) of dipeptide **22** as a colourless powder, mp=161–163°C. R_f=0.25 (PE/MTBE 1:1). α_D=−11.1, α_{365}=−48.0 (*c*=0.96, $CHCl_3$, 25°C). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.81–0.86 (m, 12H), 1.89–2.07 (m, 2H), 3.62 (s, 3H), 4.04 (dd, *J*=9.0, 6.8 Hz, 1H), 4.17 (dd, *J*=7.9, 6.4 Hz, 1H), 5.02 (s, 2H), 7.21 (d, *J*=9.4 Hz, 1H, NH), 7.23–7.35 (m, 5H, ar.), 8.17 (d, *J*=8.3 Hz, 1H, NH). HRMS (EI): for $C_{19}H_{28}N_2O_5$ (M^+) calcd 364.1998, found 364.2001. $C_{19}H_{28}N_2O_5$ (364.44) calcd C 62.62, H 7.74, N 7.69; found C 62.63, H 7.69, N 7.66.

6.3.8. *Z*-Ala-D-Val-Val-D-Val-OMe (12). 1.87 g (5.56 mmol) dipeptide **21** were C-deprotected following GP 1 (R_f=0.31 in $CHCl_3$/MeOH/HOAc 100:10:1). *N*-deprotection of 1.85 g (5.08 mmol) dipeptide **22** was performed according to GP 2 (R_f=0.54 in $CHCl_3$/MeOH/aq. NH_3 100:10:1). The combined materials were coupled following GP 4 (CH_2Cl_2/DMF 3:1, 0°C, 1 h). The mixture was acidified to pH 4 with 2 M HCl, partitioned between EtOAc/H_2O/brine (100:30:10 mL) and extracted with EtOAc (3×50 mL) and $CHCl_3$/*i*-PrOH (3:1, 50 mL). The combined organic layers were washed with brine (50 mL), dried with Na_2SO_4 and concentrated. FCC (150 g, $CHCl_3$/MeOH 100:1) gave 2.44 g (4.57 mmol, 90%) of the tetrapeptide **12** as a colourless solid, mp=212–214°C. R_f=0.66 ($CHCl_3$/MeOH 20:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.82 (m, 18H), 1.20 (d, *J*=6.8 Hz, 3H), 2.00 (m, 3H), 3.60 (s, 3H), 4.16 (m, 2H), 4.34 (m, 2H), 5.00 (s, 2H), 7.31 (m, 5H), 7.38 (d, *J*=7.4 Hz, 1H), 7.78 (d, *J*=9.0 Hz, 1H), 7.94 (d, *J*=9.0 Hz, 1H), 8.16 (d, *J*=8.3 Hz, 1H). α_D=+3.0, α_{365}=+8.1 (*c*=1.00, MeOH, 23°C). HPLC: 6.87 min (M1). HRMS (FAB): for $C_{27}H_{43}N_4O_7$ ($M+H^+$) calcd 535.3132; found 535.3149. $C_{27}H_{42}N_4O_7$ (534.66) calcd C 60.65, H 7.92, N 10.48; found C 60.60, H 7.90, N 10.41.

6.3.9. *Z*-Ala-D-Val-Val-D-Val-(Trp-D-Leu)$_3$-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide (11). 568 mg (374 μmol) of heptapeptide **25** were *N*-deprotected following GP 2 (MeOH/THF 5:1, 40°C, 8 h) and purified by FCC (30 g, $CHCl_3$/MeOH 40:1→20:1→10:1) to give 80 mg (53 μmol, 14%) of unchanged **25** and 445 mg (321 μmol, 86%) of pure amine. 257 mg (480 μmol, 1.5 equiv.) of tetrapeptide **12** were C-deprotected following GP 1 (THF/MeOH/H_2O 3:1:1, 20°C, 8 h). The combined amine and carboxylic acid were coupled following GP 4 (30 mM, 0→10°C, 8 h) and the mixture was diluted with $CHCl_3$ (50 mL). After aqueous work-up, FCC (50 g, toluene/EtOH 100:5→100:6→100:7) provided 445 mg (236 μmol, 74%) of HPLC-pure undecapeptide **11**. 47 mg (34 μmol, 11%) of unchanged heptamer-amine were eluted with CH_2Cl_2/MeOH 10:1. R_f=0.20 (toluene/EtOH/HCOOH 100:7:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.43–0.59 (m, 21H), 0.63 (d, *J*=6.4 Hz, 3H), 0.71–0.82 (m, 12H), 0.83–0.92 (m, 3H), 0.96 (s, 9H), 1.03–1.24 (m, 9H), 1.69–1.84 (m, 1H), 1.89–2.05 (m, 2H), 2.78–2.95 (m, 4H), 3.02–3.26 (m, 6H), 3.62 (t, *J*=6.4 Hz, 2H), 4.04–4.23 (m, 5H), 4.24–4.36 (m, 2H), 4.40–4.56 (m, 4H), 4.96 (s, 2H), 6.85–6.95 (m, 4H, ar.), 6.96–7.04 (m, 4H, ar.), 7.05–7.08 (s, 4H, ar.), 7.23–7.32 (m, 9H, ar.), 7.34–7.45 (m, 7H, ar.), 7.48–7.56 (m, 4H, ar.), 7.57–7.64 (m, 4H, ar.), 7.69–7.80 (m, 2H, NH), 7.86–7.97 (m, 4H, NH), 8.02–8.33 (m, 6H, NH), 10.68 (s, 1H, NH), 10.73 (s, 3H, NH). α_D=36.4, α_{365}=136 (*c*=0.42, MeOH, 30°C). HPLC: 18.7 min. (M1). MS (ESI): for $C_{106}H_{136}N_{16}O_{14}SiNa$ ($M+Na^+$) calcd 1908.0; found 1908.1.

6.3.10. H-Ala-D-Val-Val-D-Val-(Trp-D-Leu)$_3$-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide (26). 400 mg (212 μmol) of undecapeptide **11** were deprotected following GP 2 (MeOH/DMF 10:1, 25 wt% of Pd/C, 40°C, 4 h), dissolved in Et_2O (70 mL), washed with H_2O (5 mL), sat. $NaHCO_3$ (20 mL) and brine (20 mL), dried with Na_2SO_4 and evaporated to give 356 mg (203 μmol, 96%) of amine **26** as a colourless gum, which was stored at −30°C. R_f=0.06 (CH_2Cl_2/MeOH 10:1). HPLC: 23.6 min (M4+ 0.1% TFA, 65→75% B in 25 min, 0.5 mL/min). MS (ESI): for $C_{98}H_{131}N_{16}O_{12}Si$ ($M+H^+$) calcd 1752.0, found 1752.1.

6.3.11. TBDPS-protected minigramicidin (27). 27.2 mg (15.5 μmol) of amine **26** were converted to the formamide

using GP 5, the mixture was then diluted with $CHCl_3$ (50 mL) and purified after aqueous work-up by FCC (10 g, 5.5→6→6.5→7.5% EtOH in $CHCl_3$) to provide 23.9 mg (13.4 μmol, 87%) of N^1-formyl undecapeptide **27** as a colourless powder. R_f=0.45 ($CHCl_3$/MeOH 10:1). ^{1}H NMR (600 MHz, DMSO-d_6): δ=0.48 (app. d, *J*=6.5 Hz, 6H), 0.524 (d, *J*=6.5 Hz, 3H), 0.532 (d, *J*=6.3 Hz, 3H), 0.55 (app. d, *J*=6.4 Hz, 9H), 0.62 (d, *J*=6.5 Hz, 3H), 0.77 (d, *J*=6.8 Hz, 3H), 0.78 (app. d, *J*=7.1 Hz, 6H), 0.80 (d, *J*=6.8 Hz, 3H), 0.81–0.93 (m, 3H), 0.96 (s, 9H), 1.05–1.10 (m, 2H), 1.10–1.17 (m, 4H), 1.18 (d, *J*=7.0 Hz, 3H), 1.76 (m, 1H), 1.98 (m, 2H), 2.82–2.92 (m, 4H), 3.06–3.19 (m, 4H), 3.24 (m, 1H), 3.33 (m, 1H), 3.62 (t, *J*=6.8 Hz, 2H), 4.09–4.15 (m, 3H), 4.19 (m, 1H), 4.28 (dd, *J*=7.9, 6.5 Hz, 1H), 4.32 (dd, *J*=8.7, 6.4 Hz, 1H), 4.44–4.57 (m, 5H), 6.88–6.92 (m, 4H), 6.97–7.02 (m, 4H), 7.06 (m, 4H), 7.26 (m, 4H), 7.36–7.44 (m, 6H), 7.50–7.55 (m, 4H), 7.58–7.61 (m, 4H), 7.73 (d, *J*=8.1 Hz, 1H), 7.91 (s, 1H, –CHO), 7.88–8.00 (m, 5H), 8.08 (t, *J*=5.8 Hz, 1H), 8.12 (d, *J*=7.3 Hz, 1H), 8.17–8.20 (m, 3H), 8.25 (d, *J*=8.0 Hz, 1H), 10.69 (s, 1H), 10.74 (m, 3H). HPLC: 18.9 min (M4, 65→75% B in 25 min, 0.7 mL/min). MS (ESI): for $C_{99}H_{130}N_{16}O_{13}SiNa$ ($M+Na^+$) calcd 1802.0, found 1801.8.

6.3.12. Minigramicidin (28). 20.0 mg (11.2 μmol) of TBDPS-protected **27** was dissolved in $CHCl_3$ (2 mL), cooled to 0°C and HF was added (500 μL, 5 vol% in CH_3CN). The mixture was stirred for 2 h at 0°C and for 1 h at 20°C and became pink during that time. The mixture was then partitioned between $CHCl_3$ and sat. $NaHCO_3$-solution (10 mL each) and extracted with $CHCl_3$/*i*-PrOH (5:1, 3×10 mL). The combined organic layers were dried with Na_2SO_4 and evaporated. FCC (3 g, $CHCl_3$/MeOH/HCOOH 100:8:1→100:10:1) delivered 10.5 mg (6.8 μmol, 59%) of Minigramicidin **28** as an off-white powder. R_f=0.27 ($CHCl_3$/MeOH/HCOOH 100:10:1). ^{1}H NMR (600 MHz, DMSO-d_6): δ=0.49 (d, *J*=6.4 Hz, 3H), 0.50 (d, *J*=6.5 Hz, 3H), 0.53 (d, *J*=6.4 Hz, 3H), 0.54 (d, *J*=7 Hz, 3H), 0.55 (d, *J*=6.9 Hz, 3H), 0.56 (d, *J*=6.9 Hz, 3H), 0.57 (d, *J*=6.9 Hz, 3H), 0.63 (d, *J*=6.6 Hz, 3H), 0.77 (d, *J*=6.7 Hz, 3H), 0.78 (d, *J*=6.9 Hz, 3H), 0.79 (d, *J*=7.0 Hz, 3H), 0.80 (d, *J*=7.0 Hz, 3H), 0.82–0.90 (m, 2H), 0.91–0.99 (m, 1H), 1.04–1.19 (m, 6H), 1.18 (d, *J*=6.9 Hz, 3H), 1.77 (m, 1H), 1.98 (m, 2H), 2.83–2.93 (m, 4H), 3.06–3.21 (m, 6H), 3.40 (m, 2H), 4.09–4.17 (m, 4H), 4.27 (dd, *J*=8.5, 5.9 Hz, 1H), 4.31 (dd, *J*=8.6, 6.4 Hz, 1H), 4.43–4.57 (m, 5H), 4.70 (t, *J*=5.5 Hz, 1H, –OH), 6.89–6.95 (m, 4H), 6.98–7.03 (m, 4H), 7.05–7.08 (m, 4H), 7.25–7.29 (m, 4H), 7.52 (m, 2H), 7.55 (app. d, *J*=8.0 Hz, 2H), 7.75 (d, *J*=8.7 Hz, 1H), 7.90–7.97 (m, 3H), 7.91 (s, 1H, –CHO), 7.98–8.05 (m, 3H), 8.15 (d, *J*=7.4 Hz, 1H), 8.18–8.22 (m, 3H), 8.30 (d, *J*=8.3 Hz, 1H), 10.69 (m, 1H), 10.73–10.76 (m, 3H). HPLC: 9.5 min (M4, 65→75% B in 25 min, 0.5 mL/min). MS (MALDI-TOF): for $C_{83}H_{112}N_{16}O_{13}Na$ ($M+Na^+$) calcd 1563.8, found 1563.9.

6.3.13. TBDPS-protected succinyl-linked minigramicidin (2). 89 mg (51 μmol) of amine **26** were dissolved in CH_2Cl_2 (3 mL) and treated with pyridine (30 μL) and succinic anhydride (50 mg dissolved in 1 mL DMF, 0.5 mmol, 10 eq). After the complete conversion (1 h), sat. $NaHCO_3$-solution (1 mL) was added and the mixture stirred vigorously for 1 h at 20°C. The biphasic mixture was partitioned between $CHCl_3$ (20 mL) and 2 M $NaHSO_4$ (10 mL, Caution!), and the aqueous layer was extracted with $CHCl_3$ (2×5 mL). The combined organic layers were washed with brine (10 mL), dried with Na_2SO_4, and evaporated. FCC (10 g, $CHCl_3$/MeOH/HCOOH 100:4:1→100:6:1) gave 82.4 mg (44.5 μmol, 88%) of the N^1-succinyl undecapeptide as a colourless solid. R_f=0.16 ($CHCl_3$/MeOH/HCOOH 100:5:1). HPLC: 10.4 min (M5, 70→100% B).

This carboxylic acid was combined with 79 mg (45 μmol, 1.0 equiv.) of amine **26** and coupled according to GP 6 (0°C, 14 h). After dilution with $CHCl_3$ (50 mL) and aqueous work-up, FCC (15 g, $CHCl_3$/MeOH/HCOOH 100:2.5:7→100:4:7) provided 142 mg (39.6 μmol, 89%) of TBDPS-protected minigramicidin **2** as a colourless foam. The pooled column fractions containing **2** have been washed with sat. $NaHCO_3$-solution (equal volume, Caution!) and dried with Na_2SO_4 to remove the HCOOH before evaporation. R_f=0.25 ($CHCl_3$/MeOH/HCOOH 100:3:7). ^{1}H NMR (600 MHz, DMSO-d_6): δ=0.49 (app. d, *J*=6.0 Hz, 12H), 0.53–0.58 (m, 30H), 0.63 (d, *J*=6.6 Hz, 6H), 0.76–0.81 (m, 24H), 0.82–0.91 (m, 6H), 0.96 (s, 18H), 1.07–1.20 (m, 12H), 1.16 (d, *J*=7.0 Hz, 6H), 1.79 (m, 2H), 2.00 (m, 4H), 2.24 (m, 2H, succ.), 2.38 (m, 2H, succ.) 2.85–2.95 (m, 8H), 3.09–3.21 (m, 8H), 3.26 (m, 2H), 3.33 (m, 2H), 3.63 (t, *J*=6.7 Hz, 2H), 4.08–4.16 (m, 6H), 4.19 (m, 2H), 4.24–4.29 (m, 4H), 4.33 (m, 2H), 4.44–4.58 (m, 8H), 6.88–6.93 (m, 8H), 6.98–7.02 (m, 8H), 7.06–7.08 (m, 8H), 7.25–7.29 (m, 8H), 7.36–7.43 (m, 12H), 7.51–7.56 (m, 8H), 7.59–7.62 (m, 8H), 7.77 (m, 4H), 7.90–7.98 (m, 8H), 8.01 (d, *J*=7.1 Hz, 2H), 8.08 (t, *J*=5.7 Hz, 2H), 8.13 (d, *J*=6.3 Hz, 2H), 8.21 (m, 4H), 8.26 (d, *J*=8.0 Hz, 2H), 10.69 (s, 2H), 10.74 (m, 6H). HPLC: 21.3 min (M5). MS (MALDI-TOF): for $C_{200}H_{262}N_{32}O_{26}Si_2Na$ ($M+Na^+$) calcd 3607.0; found 3607.0.

6.3.14. Succinyl-linked minigramicidin (3) and its mono-TBDPS-protected-form (4). 25.0 mg (6.97 μmol) of bis-TBDPS-ether **2** were dissolved in MeOH (2 mL), HF (5 vol% in CH_3CN, 2 mL) was added and the solution stirred for 24 h at 20°C (HPLC control). The mixture was partitioned between $CHCl_3$ (20 mL) and sat. $NaHCO_3$ solution (20 mL, CAUTION!) and the aqueous layer extracted with $CHCl_3$/*i*-PrOH (5:1, 5×10 mL). The combined organic layers were washed with brine (15 mL), dried with Na_2SO_4 and evaporated. FCC (2.5 g, $CHCl_3$/HCOOH/MeOH 100:7:4→100:7:7→*i*-PrOH/H_2O 99:1) followed by GPC (Sephadex LH20, $CHCl_3$/MeOH 1:1) provided 19.6 mg (6.30 μmol, 90%) of minigramicidin **3** as an off-white solid. R_f=0.25 ($CHCl_3$/MeOH/HCOOH 100:7:7). ^{1}H NMR (600 MHz, DMSO-d_6): δ=0.49–0.54 (m, 18H), 0.55–0.59 (m, 24H), 0.64 (d, *J*=6.4 Hz, 6H), 0.74–0.79 (m, 24H), 0.88–0.94 (m, 4H), 0.94–1.00 (m, 2H), 1.12–1.22 (m, 12H), 1.17 (d, *J*=6.9 Hz, 6H), 1.80 (m, 2H), 1.97–2.02 (m, 4H), 2.22–2.28 (m, 2H, succ.), 2.34–2.40 (m, 2H, succ.), 2.85–2.93 (m, 8H), 3.06–3.21 (m, 12H), 3.40 (m, 4H), 4.04–4.16 (m, 8H), 4.17–4.23 (m, 4H), 4.30 (m, 2H), 4.42–4.54 (m, 8H), 4.78 (bs, 2H, –OH), 6.88–6.95 (m, 8H), 6.97–7.02 (m, 8H), 7.07 (app. d, *J*=8.6 Hz, 8H), 7.26–7.29 (m, 8H), 7.49 (app. d, *J*=7.8 Hz, 4H), 7.54 (d, *J*=6.9 Hz, 2H), 7.55 (d, *J*=7.0 Hz, 2H), 7.94 (t, *J*=5.2 Hz, 2H),

7.96–8.01 (m, 4H), 8.01–8.06 (m, 4H), 8.06–8.12 (m, 4H), 8.25 (d, J=7.0 Hz, 2H), 8.27–8.34 (m, 8H), 10.71 (bs, 1H), 10.77 (bs, 1H), 10.78 (bs, 1H), 10.79 (bs, 1H). HPLC 12.4 min (M5). MS (MALDI-TOF): for $C_{168}H_{226}N_{32}O_{26}Na$ ($M+Na^+$) calcd 3130.7; found 3130.9.

The mono-TBDPS-protected **4** was obtained on quenching the reaction at an earlier stage of conversion (HPLC control). In a typical experiment employing 36 mg (10.1 μmol) of **2**, FCC (5 g, $CHCl_3$/HCOOH/*i*-PrOH/MeOH 100:10:2.7:1.3→100:10:4:2→*i*-PrOH/H_2O 99:1) gave 8.1 mg (2.3 μmol, 23%) **2**, 7.8 mg (2.3 μmol, 23%) **4** and 12.4 mg (4.0 μmol, 39%) **3**. Data for **4**: R_f=0.23 ($CHCl_3$/HCOOH/*i*-PrOH/MeOH 100:10:4:2). ^{1}H NMR (600 MHz, DMSO-d_6): δ=0.47–0.51 (m, 12H), 0.52–0.58 (m, 24H), 0.61 (d, J=7.1 Hz, 3H), 0.63 (d, J=7.1 Hz, 3H), 0.75–0.80 (m, 24H), 0.83 (d, J=7.1 Hz, 3H), 0.85 (d, J=6.8 Hz, 3H), 0.81–0.90 (m, 4H), 0.96 (s, 9H), 0.91–0.99 (m, 2H), 1.05–1.19 (m, 12H), 1.15 (d, J=6.9 Hz, 6H), 1.76–1.81 (m, 2H), 1.95–2.02 (m, 4H), 2.19–2.27 (m, 2H, succ.), 2.33–2.40 (m, 2H, succ.), 2.85–2.92 (m, 8H), 3.07–3.21 (m, 10H), 3.23–3.30 (m, 4H), 3.62 (t, J=6.8 Hz, 2H), 4.04–4.20 (m, 8H), 4.23–4.27 (m, 4H), 4.32 (m, 2H), 4.43–4.56 (m, 8H), 4.70 (t, J=5.7 Hz, 1H, –OH), 6.88–6.95 (m, 8H), 6.97–7.03 (m, 8H), 7.05–7.09 (m, 8H), 7.25–7.29 (m, 8H), 7.36–7.43 (m, 6H, TBDPS), 7.49–7.56 (m, 8H), 7.58–7.61 (m, 4H, TBDPS), 7.74–7.78 (m, 4H), 7.89–7.93 (m, 7H), 7.94 (d, J=6.5 Hz, 1H), 8.00 (app. d, J=7.3 Hz, 2H), 8.02 (app. d, J=8 Hz, 1H), 8.07 (t, J=5.8 Hz, 1H), 8.12 (m, 2H), 8.18–8.22 (m, 4H), 8.25 (d, J=7.7 Hz, 1H), 8.29 (d, J=9.0 Hz, 1H), 10.69 (m, 2H), 10.74 (m, 6H). HPLC 15.1 min (M5). MS (MALDI-TOF): for $C_{184}H_{244}N_{32}O_{26}SiNa$ ($M+Na^+$) calcd 3368.8; found 3368.9.

6.3.15. Succinic-1-(Val-Gly-OMe)-4-(Val-Gly-OBn)-diamide (9). 550 mg (1.51 mmol) Boc-Val-Gly-OBn[21] was dissolved in CH_2Cl_2 (40 mL) and treated with TFA (10 mL). After 15 min the mixture was diluted with toluene (50 mL) and the crude ammonium salt was recovered by evaporation (R_f=0.30, $CHCl_3$/MeOH/aq. NH_3 200:10:1). This material was dissolved in CH_2Cl_2/DMF/pyridine 5:1:1 (7 mL) and succinic anhydride (140 mg, 1.4 mmol) was added. After stirring for 3 h the mixture was diluted with EtOAc (50 mL) and extracted with sat. Na_2CO_3 solution (3×10 mL). The combined aqueous layers were acidified with 4 N HCl to pH 2 and extracted with EtOAc (4×20 mL). Evaporation and FCC of the residue (30 g, $CHCl_3$/MeOH/HCO_2H 20:1:0.1) gave 474 mg (1.37 mmol, 81%) of succinyl-Val-Gly-OBn as a colourless solid. This was coupled with 502 mg (1.56 mmol) H-Val-Gly-OMe[22] according to GP 3 (10 mM, 18 h, 20°C). After dilution with CH_2Cl_2 (100 mL), the insoluble product was filtered off and recrystallised from boiling *i*-PrOH to give 500 mg (0.93 mmol, 70%) of diamide **9** as a colourless powder, mp=238°C. R_f=0.35 ($CHCl_3$/MeOH/HCO_2H 100:10:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.72–0.95 (m, 12H), 1.87–2.05 (m, 2H), 2.39 (s, 4H), 3.59 (s, 3H), 3.68–3.96 (m, 4H), 4.14 (t, J=7.5 Hz, 2H), 5.09 (s, 2H), 7.29–7.42 (m, 5H), 7.89 (d, J=8.3 Hz, 2H), 8.31–8.49 (m, 2H). HRMS (EI): for $C_{26}H_{38}N_4O_8$ (M^+) calcd 535.2768; found 535.2759. $C_{52}H_{78}N_8O_{17}$ ((**9**)$_2$·H_2O, 1087.24) calcd C 57.45, H 7.23, N 10.31; found C 57.67, H 7.13, N 10.43.

6.3.16. *Z*-Ala-D-Leu-Ala-D-Val-Val-D-Val-(Trp-D-Leu)$_3$-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide (32). 200 mg (0.110 mmol) amine **26** was coupled with 40 mg (0.12 mmol, 1.1 equiv.) *Z*-Ala-D-Leu-OH **10**[20] according to GP 4 (5 mM, 3 h, 0→20°C). After aqueous work-up, FCC (40 g, 2→4% MeOH in $CHCl_3$) gave 200 mg (0.096 mmol, 87%) of tridecapeptide **32** as a colourless solid. R_f=0.55 ($CHCl_3$/MeOH/HCO_2H 100:10:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.42–0.65 (m, 24H), 0.69–0.90 (m, 21H), 0.96 (s, 9H), 1.04–1.25 (m, 12H), 1.37–1.61 (m, 3H), 1.70–1.85 (m, 1H), 1.87–2.06 (m, 2H), 2.78–2.95 (m, 4H), 3.04–3.30 (m, 6H), 3.57–3.66 (m, 2H), 4.00–4.38 (m, 9H), 4.40–4.60 (m, 4H), 4.93–5.06 (m, 2H), 6.85–6.95 (m, 4H, ar.), 6.96–7.10 (m, 7H, ar.), 7.23–7.46 (m, 16H, ar.), 7.47–7.57 (m, 4H, ar.), 7.58–7.63 (m, 4H, ar.), 7.66–7.82 (m, 2H, NH), 7.85–8.00 (m, 5H), 8.03–8.32 (m, 6H), 10.69 (s, 1H, NH), 10.74 (s, 3H, NH). HPLC: 12.2 min (M2). MS (MALDI-TOF) calcd for $C_{115}H_{152}N_{18}O_{16}SiNa$ ($M+Na^+$) calcd 2092.1, found 2092.2.

6.3.17. Succinic-1-(Val-Gly-OMe)-4-(Val-Gly-Ala-D-Leu-Ala-D-Val-Val-D-Val-(Trp-D-Leu)$_3$-Trp-2-(*t*-butyl-diphenylsilyloxy)-ethylamide)-diamide (33). 19 mg (40 μmol) Benzylester **9** was debenzylated according to GP 2 (MeOH, R_f=0.30 in $CHCl_3$/MeOH/HCO_2H 100:10:1) as well as 41 mg (20 μmol) tridecapeptide **32** was N-deprotected following the same procedure (R_f=0.40 in $CHCl_3$/MeOH/aq. NH_3 100:10:1, HPLC: 19.6 min (M2)). The crude products were combined and coupled without further purification according to GP 4 (1 mM, 20°C, 4 h). After aqueous work-up, FCC (2 g, 2→4% MeOH in $CHCl_3$) furnished 30 mg (12.7 μmol, 63%) of octadecamer **33** as an off-white solid. (R_f=0.45 in $CHCl_3$/MeOH/HCO_2H 100:10:1). ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.43–0.66 (m, 24H), 0.72–0.91 (m, 33H), 0.96 (s, 9H), 1.02–1.25 (m, 12H), 1.39–1.64 (m, 3H), 1.69–1.86 (m, 1H), 1.89–2.08 (m, 4H), 2.35–2.46 (m, 4H), 2.79–2.97 (m, 4H), 3.04–3.29 (m, 6H), 3.56–3.91 (m, 10H), 4.03–4.36 (m, 11H), 4.40–4.60 (m, 3H), 6.84–6.95 (m, 4H, ar.), 6.96–7.10 (m, 8H, ar.), 7.22–7.31 (m, 4H, ar.), 7.32–7.46 (m, 6H, ar.), 7.47–7.65 (m, 8H, ar.) 7.67–8.30 (m, 16H, NH), 8.37 (t, J=5.7 Hz, 1H, NH), 10.70 (s, 1H, NH), 10.74 (s, 3H, NH). HPLC: 9.1 min (M2). MS (MALDI-TOF) for $C_{126}H_{176}N_{22}O_{21}SiNa$ ($M^+ + Na^+$): calcd 2384.3, found 2384.5.

6.3.18. TBDPS-protected, succinyl-linked gramicidin (1). 20 mg (8.5 μmol, 1.1 equiv.) of octadecamer **33** were C-deprotected according to GP 1 (R_f=0.30 in $CHCl_3$/MeOH/HCO_2H 100:10:1), as well as 16 mg (7.8 μmol) of tridecapeptide **32** were N-deprotected following GP 2 (MeOH, R_f=0.33 in $CHCl_3$/MeOH/aq. NH_3 100:10:1; HPLC 15.33 min (M2)). The crude materials were combined and coupled according to GP 6 (1 mM, 20°C), until conversion of the amine was complete (4 h, HPLC monitoring, M2). The reaction mixture was diluted with $CHCl_3$ (40 mL) and after aqueous work-up, preparative TLC (20 cm×20 cm×1 mm, $CHCl_3$/MeOH 10:2, 20 mg runs) followed by GPC (Sephadex LH 20, 1.5×9.0 cm^2 column, $CHCl_3$/MeOH 1:1) provided 13 mg (3.0 μmol, 40%) of compound **1** as a colourless solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ=0.35–0.69 (m, 48H), 0.70–0.90 (m, 60H), 0.96 (s, 18H), 1.04–1.31 (m, 30H), 1.40–1.60 (m,

6H), 1.73–1.84 (m, 2H), 1.89–2.05 (m, 6H), 2.33–2.47 (m, 4H), 2.80–3.01 (m, 8H), 3.04–3.28 (m, 12H), 3.55–3.76 (m, 8H), 4.02–4.32 (m, 20H), 4.41–4.60 (m, 8H), 6.84–6.93 (m, 7H, ar.), 6.95–7.03 (m, 8H, ar.) 7.04–7.13 (m, 8H, ar.), 7.23–7.31 (m, 8H, ar.), 7.33–7.45 (m, 13H, ar.), 7.47–7.66 (m, 16H, ar.), 7.68–8.32 (m, 32H, NH), 10.68 (s, 2H, NH), 10.74 (m, 6H, NH). HPLC: 9.3 min (M3). MS (ESI) for $C_{232}H_{318}N_{40}O_{34}Si_2$: ($M+2Na^+$) calcd 2156.7, found 2156.1.

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