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Alanine Scan of the Peptide Antibiotic Feglymycin: Assessment of Amino Acid Side Chains Contributing to Antimicrobial Activity

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The antibiotic feglymycin is a linear 13-mer peptide synthesized by the bacterium *Streptomyces* sp. DSM 11171. It mainly consists of the nonproteinogenic amino acids 4-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine. An alanine scan of feglymycin was performed by solution-phase peptide synthesis in order to assess the significance of individual amino acid side chains for biological activity. Hence, 13 peptides were synthesized from di- and tripeptide building blocks, and subsequently tested for antibacterial activity against *Staphylococcus aureus* strains. Furthermore we tested the inhibition of peptidoglycan biosynthesis enzymes MurA and MurC, which are inhibited by feglymycin. Whereas the antibacterial activity is significantly based on the three amino acids D-Hpg1, L-Hpg5, and L-Phe12, the inhibitory activity against MurA and MurC depends mainly on L-Asp13. The difference in the position dependence for antibacterial activity and enzyme inhibition suggests multiple molecular targets in the modes of action of feglymycin.

Introduction

Peptide antibiotics synthesized by bacteria and fungi show remarkable structural variety and involve sophisticated mechanisms of action.[1] In particular, nonribosomally synthesized peptide antibiotics often contain nonproteinogenic amino acids and display various unusual structural features, such as N-methylation, β-hydroxylation, and biaryl crosslinking.[2] Among these there exists a number of antibacterial peptides that contain the nonproteinogenic amino acids 4-hydroxyphenylglycine (Hpg) and 3,5-dihydroxyphenylglycine (Dpg), or both. Examples are the type I–IV glycopeptide antibiotics (e.g., vancomycin, teicoplanin, and complestatin),[3] ramoplanin,[4] and arylomycin,[5] which mostly display antibacterial activity and address different targets of the bacterial cell wall or membrane.

Recently, the peptide antibiotic feglymycin, which is synthesized by the bacterium *Streptomyces* sp. DSM 11171, attracted our attention.[6] This 13-mer peptide (**1**, Scheme 1) mainly consists of the amino acids Hpg and Dpg. Furthermore, structural analysis reveals an alternation of D- and L-configurations for the peptide stretch between D-Dpg2 and L-Hpg11. Sheldrick and co-workers revealed an antiparallel β-helical dimer[7] by X-ray structure analysis; this is reminiscent of gramicidin A[8] and polytheonamide B.[9] The first total synthesis of feglymycin was recently developed by our group and is based on a [6+7] fragment coupling strategy (Scheme 1).[10] One of the main challenges of the synthesis was establishing suitable coupling conditions to suppress racemization and epimerization of phenylglycines and phenylglycine-containing peptides. Furthermore, phenolic protecting groups caused severe solubility problems in the later stages of the peptide assembly, thus requiring an appropriate condensation strategy and suitable protecting groups for mild final deprotection.

In subsequent studies, and unlike in previous reports,[6,7] we found that feglymycin displays significant inhibition of three selected *S. aureus* strains, with minimal inhibitory concentrations (MICs) of 0.25–0.5 μM, in addition to a broad-spectrum inhibitory activity against HIV replication, with an IC_{50} of 0.8–3.2 μM.[11] The remarkable antibacterial activity initiated subsequent studies and a more detailed search for a molecular target. The relatively high molecular mass of feglymycin implicated peptidoglycan biosynthesis as a reasonable target.[12] However, an intermediate of the late stages of this biosynthesis pathway, that is, lipid I, was experimentally excluded.[13] Further assays with the cytoplasmic enzymes MurA–F from *E. coli* and MurA–D from *S. aureus* (assembly of muramyl pentapeptide) finally revealed noncompetitive inhibition of MurA (UDP-*N*-acetylglucosamine enolpyruvyl transferase) and MurC (UDP-*N*-acetylmuramyl-L-alanine ligase) of both *S. aureus* (IC_{50} = 3.5 and 1.0 μM, respectively) and *E. coli* (IC_{50} = 3.4 and 0.3 μM, respectively).[13] Hence, feglymycin is the first natural product inhibitor with MurC as a main target. Unlike other synthetic compounds, such as substrate or transition-state analogues

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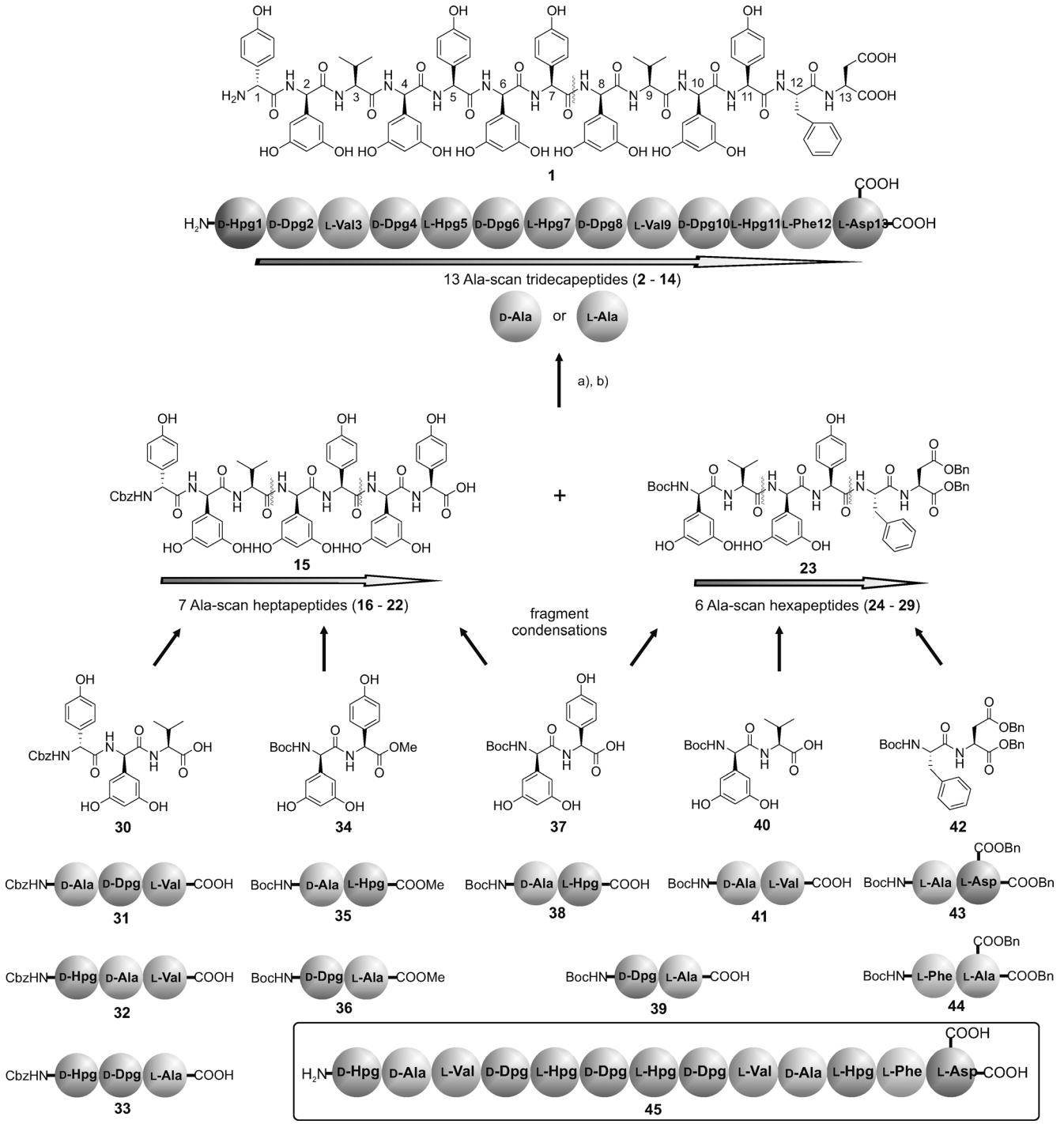


Scheme 1. Structures of feglymycin (**1**) and key peptide fragments. The flexible strategy for the total synthesis from four dipeptides and one tripeptide was applied to the design of a straightforward synthesis concept for an alanine scan of feglymycin, by exchanging amino acids in these building blocks. Seven new Ala-containing dipeptides (**35**, **36**, **38**, **39**, **41**, **43**, **44**) and three new tripeptides (**31**–**33**) were synthesized for assembly into 13 alanine scan peptides (**2**–**14**) and one double-alanine derivative [D-Ala2,10]-feglymycin (**45**). a) N-terminal deprotection of hexapeptides; b) fragment condensation.

with competitive binding,[14] feglymycin represents an unusual case of a noncompetitive inhibitor.

We sought to establish further details on the mode of action of feglymycin. In this context, the "alanine scan" technique proved as a useful and valuable tool for identifying the importance of specific residues for antibacterial and other bioactivities.[15] Boger and co-workers were able to relate distinct influences of amino acid side chains with antibacterial activity when using the 17-mer peptide ramoplanin.[15b,c] Therefore we designed a strategy to successively replace each amino acid position by Ala and thereby yielded 13 new feglymycin derivatives. Their antibacterial properties against *S. aureus* and inhibition of the peptidoglycan biosynthesis enzymes MurA and MurC were tested. This initial structure–activity relationship

study on feglymycin gives insights into contributions of single amino acid side chains to the biological activity.

Results

Synthesis of N-terminal alanine scan heptapeptides (D-Hpg1-L-Hpg7) and of C-terminal alanine scan hexapeptides (D-Dpg8-L-Asp13)

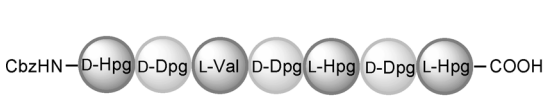
Conceptually, we were aiming to exchange every position of the 13-mer peptide feglymycin, while retaining the wild-type D/L configuration. Thus the direct influence of Ala-exchange on the secondary structure of the peptide would be minimized. The synthetic strategy, which was based on the total synthesis published previously by our group,[10] was adapted to the requirements of the alanine scan. Accordingly, the [6+7] fragment coupling of the 13-mer peptide was further partitioned into coupling of Ala-containing di- and tripeptides to readily available building blocks. Hence, for each alanine scan peptide, the preparation of only one new dipeptide was necessary. Overall, the synthetic concept afforded the preparation of ten new Ala-containing di- and tripeptides (Scheme 1). This allowed a manageable level of synthetic effort, as similar procedures could be applied for the assembly of these peptides. In summary, we obtained a straightforward synthesis of feglymycin Ala-analogues by using a convergent strategy with newly synthesized building blocks and known fragments for feglymycin total synthesis.

In general, all peptide couplings were performed with 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT) and $NaHCO_3$ in *N*,*N*-dimethylformamide (DMF). For small oligopeptides, the use of tetrahydrofuran (THF) was also feasible.[10,16] These conditions ensured the epimerization-free assembly of all arylglycine-containing oligopeptide fragments, with good to high overall yield (60–90%). Standard peptide coupling protocols (e.g., TBTU, DIPEA) were applied to generate dipeptide building blocks that did not contain either Hpg or Dpg.[17] The benzylic side-chain-protecting groups were generally maintained in the first coupling step for the synthesis of dipeptides. However, as the use of DEPBT as a coupling reagent does not necessarily require the protection of phenolic hydroxy groups, these were quantitatively removed by catalytic hydrogenolysis at the dipeptide stage. For rapid removal of the temporary Boc group from di- and tetrapeptides, 4 N HCl in dioxane was used.[18] To realize the alanine scan, seven N-terminal heptapeptides and six C-terminal hexapeptides were prepared in quantities that allowed us to perform multiple fragment condensations (Figure 1).

As examples, the overall synthesis strategy for the preparation of peptides Cbz-[D-Ala4]-hepta-OH (**19**) and Boc-[L-Ala4]-hexa-OBn (**27**) are outlined in Schemes 2 and 3, respectively. For enhanced purification, hexa- or heptapeptides were precipitated from the reaction mixtures with water, and purified by silica gel column chromatography. Prior to assembly of the final 13-mer peptide, the methylester protecting group of the N-terminal heptapeptide was cleaved under mild basic conditions with trimethyltin hydroxide in 1,2-dichloroethane at 85 °C (Scheme 2).[19]

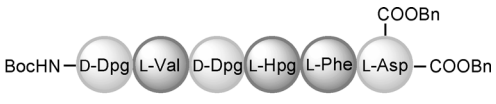
Synthesis and analytical characterization of Ala-exchange peptides of feglymycin

In the preparation of the penultimate [6+7] fragment coupling of Ala-substituted hepta- (**16**–**22**) or hexapeptides (**24**–**29**) with the corresponding wild-type peptide fragments **15** and **23**, we employed Boc deprotection of sensitive hexapeptides with 25% trifluoroacetic acid (TFA) in CH_2Cl_2 with triethylsilane (TES) as scavenger. The deprotections were quantitative after 30 min at ambient temperature, and the obtained peptide TFA salts could be used in the subsequent coupling step after simple precipitation with Et_2O. Fragment condensations of heptapeptides **16**–**22** with hexapeptide **23** and of hexapeptides **24**–**29** with heptapeptide **15** were performed by using DEPBT as the coupling reagent with $NaHCO_3$ as a mild base, in DMF. The best results (in terms of coupling efficiency and yield) were obtained after a short stirring time (1 h) at 0 °C, followed by a reaction time of ~2 d at ambient temperature. In a similar fashion, the peptide [D-Ala2,10]-feglymycin (**45**) bearing a double exchange of D-Dpg against D-Ala was assembled by coupling **17** to **26**. The resulting target peptides were precipitated by addition of water, and separated from water-soluble byproducts by centrifugation. As an intermediate purification step, we used Sephadex LH20 size-exclusion chromatography with MeOH. To ensure high purity of the final alanine scan



15

Heptapeptide fragments	
Cbz-[D-Ala1]-hepta-OH (**16**)	78 mg
Cbz-[D-Ala2]-hepta-OH (**17**)	71 mg
Cbz-[L-Ala3]-hepta-OH (**18**)	82 mg
Cbz-[D-Ala4]-hepta-OH (**19**)	86 mg
Cbz-[L-Ala5]-hepta-OH (**20**)	66 mg
Cbz-[D-Ala6]-hepta-OH (**21**)	67 mg
Cbz-[L-Ala7]-hepta-OH (**22**)	45 mg



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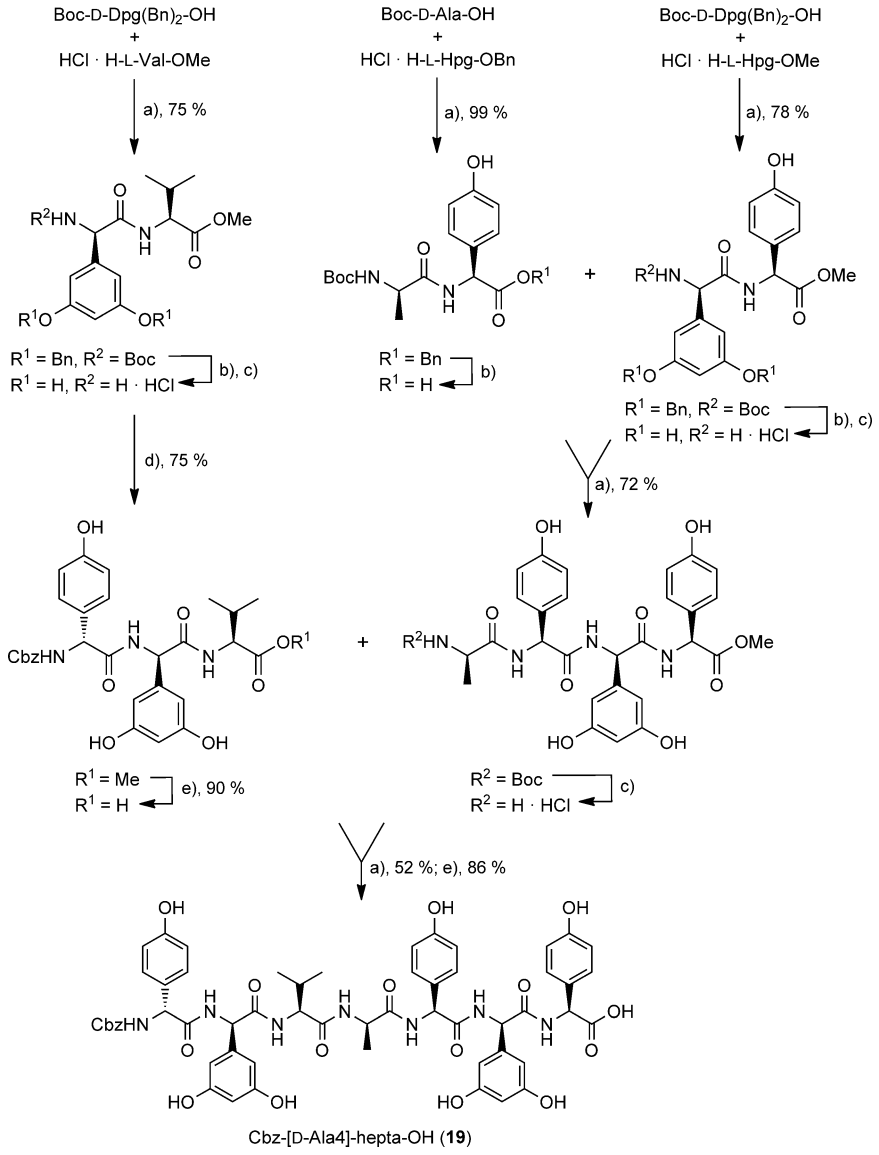
Hexapeptide fragments	
Boc-[D-Ala1]-hexa-OBn (**24**)	124 mg
Boc-[L-Ala2]-hexa-OBn (**25**)	129 mg
Boc-[D-Ala3]-hexa-OBn (**26**)	130 mg
Boc-[L-Ala4]-hexa-OBn (**27**)	116 mg
Boc-[L-Ala5]-hexa-OBn (**28**)	146 mg
Boc-[L-Ala6]-hexa-OBn (**29**)	139 mg

Figure 1. Series of alanine exchange peptides of the N-terminal heptapeptide fragment **15** and of the C-terminal hexapeptide fragment **23**.



Scheme 2. Synthesis of the N-terminal peptide fragment Cbz-[D-Ala4]-hepta-OH (**19**). a) DEPBT, $NaHCO_3$; b) H_2, Pd/C; c) 4 N HCl/dioxane; d) Cbz-D-Hpg-OH, DEPBT, $NaHCO_3$; e) Me_3SnOH.

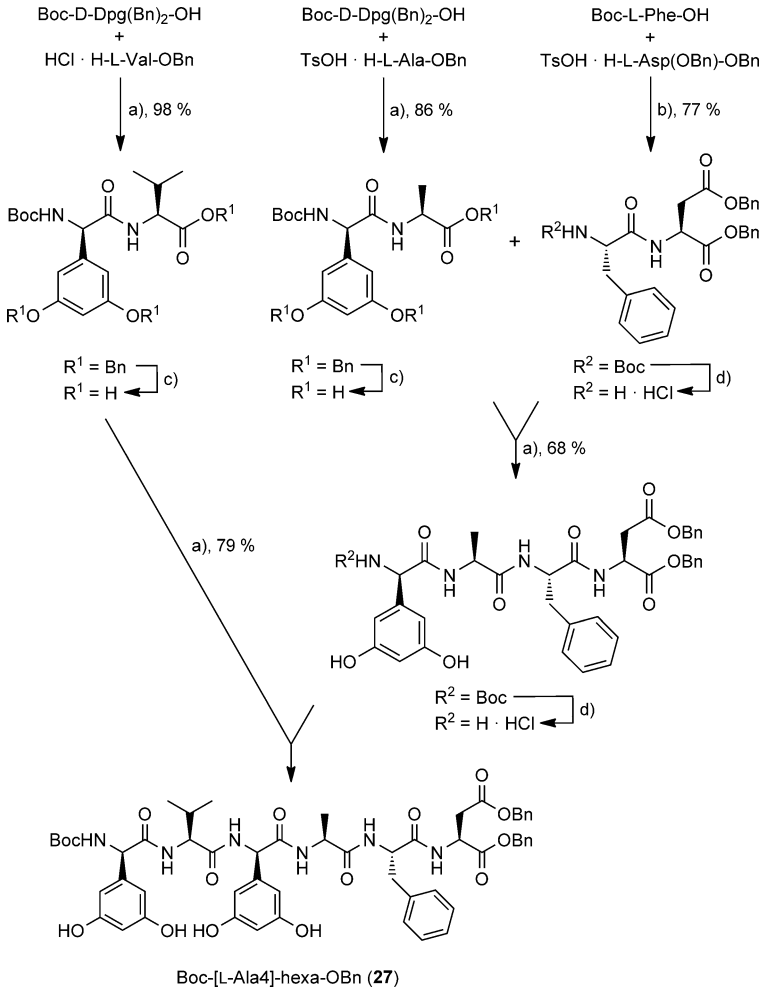
peptides, the protected tridecapeptides were additionally purified by reversed-phase HPLC prior to final quantitative deprotection with H_2 and Pd/C (10%) in MeOH. The peptides thus obtained could be directly used for biological assays.

The identities of all Ala-feglymycin derivatives (compounds **2**–**14**, **45**) were confirmed by high resolution mass spectrometry, ESI-MS/MS, and 2D NMR spectroscopy (see the Supporting Information). All peptides showed solubility in water and MeOH similar to that of feglymycin. An exception was [D-Ala1]-feglymycin (**2**), which showed significantly lower solubility and eluted only as a broad peak from the analytical reversed-phase column. To examine whether the exchange of each individual amino acid against Ala had an influence on the secondary structure of the corresponding peptides, CD spectra were recorded in water and trifluoroethanol (TFE; 20% aqueous). Except for [L-Ala13]-feglymycin (**14**, substitution of the C-terminal L-Asp), all alanine scan analogues showed comparable CD spectra (see the Supporting Information), and therefore likely assemble as similar antiparallel β-helical dimers, as suggested for wild-type feglymycin.[7]

Antibacterial activity of feglymycin alanine scan peptides

The antibacterial testing was performed with three *S. aureus* standard reference strains: one MRSA (ATCC33592, resistant to gentamicin and methicillin) and two MSSA (ATCC29213 and ATCC13709, sensitive to methicillin and oxacillin). The antibacterial activities (Figure 2A) were determined as MICs in order to assess the influence of Ala-exchanges on inhibition of bacterial growth. Of the series of peptides tested, feglymycin derivatives with the mutations Val3Ala (**4**), Hpg7Ala (**8**), Val9Ala (**10**), Hpg11Ala (**12**), and Asp13Ala (**14**) showed MICs between 0.25 and 1 μM (feglymycin **1**, 0.25–0.5 μM). Replacements of L-Val by the sterically less demanding but comparably nonpolar L-Ala apparently had no effect on activity. Likewise, substitutions Hpg7Ala (**8**) and Hpg11Ala (**12**) had an almost negligible effect, although a more pronounced contribution from aromatic side chains and from hydrogen bonding capabilities of the phenolic groups had been expected. Surprisingly, the Asp13Ala mutant (**14**, lacking the acidic side chain functionality) had no negative impact on antimicrobial activity (MIC = 0.5 μM).

All feglymycin peptides with exchange of the amino acid D-Dpg→D-Ala at positions 2, 4, 6, 8, and 10 (peptides **3**, **5**, **7**, **9,** and **11**) showed MICs (0.5–4 μM) higher than for the wild-type peptide (four- to 16-fold reduction in antimicrobial activity). This finding points to a significant contribution of D-Dpg to the antimicrobial effects. However, the most pronounced impact was found for [D-Ala1]-feglymycin (**2,** MIC = 8 μM), and [L-Ala5]-feglymycin (**6**) and [L-Ala12]-feglymycin (**13**), which showed MICs greater than 32 μM. Hence, residues D-Hpg1, L-Hpg5, and L-Phe12 are crucial for antibacterial activity, as other exchanges seem to be fairly well tolerated. The control "double-mutant" peptide (dual D-Dpg→D-Ala mutations, [D-Ala2,10]-feglymycin (**45**), MIC = 4–8 μM) showed accumulating



Scheme 3. Synthesis of the C-terminal peptide fragment Boc-[L-Ala4]-hexa-OBn (**27**). a) DEPBT, $NaHCO_3$; b) EDC, HOAt, $NaHCO_3$; c) H_2, Pd/C; d) 4 N HCl/dioxane.

effects upon removal of natural D-Dpg amino acids, although this was less pronounced than for **2**, **6**, and **13**.

Inhibition of peptidoglycan biosynthesis enzymes MurA and MurC

These Ala-exchange peptides were then tested for inhibitory activity against enzymes MurA and MurC (early peptidoglycan biosynthesis). These enzymes have been identified to have been inhibited by feglymycin, whereas subsequent stages (e.g., lipid I biosynthesis) remained unaffected.[13] The IC_{50} values were determined according to protocols established previously for feglymycin.[13] Apart from [L-Ala5]-feglymycin (**6**, $IC_{50} = 11.2$ μm) and [L-Ala13]-feglymycin (**14**, $IC_{50} = 16.9$ μm), all Ala-exchange peptides (Figure 2B) inhibited MurA at concentrations similar to that for feglymycin (**1**, $IC_{50} = 2.5$ μm). The effect observed for [L-Ala13]-feglymycin (**14**) was paralleled in the series testing MurC (feglymycin $IC_{50} = 0.3$ μm): more than tenfold reduction in inhibitory activity ($IC_{50} = 3.4$ μm; Figure 2C).

Discussion and Conclusion

A full alanine scan of feglymycin was conducted to provide insights into the contribution of each amino acid side chain of feglymycin (**1**) to its antimicrobial activity against *S. aureus* strains. The most important residues were D-Hpg1, L-Hpg5, and L-Phe12. With MICs of 8 μM (**2**) and >32 μM (**6** and **13**), Ala-exchange at these positions yielded the most significant effects. This implies significant position-dependent contributions from the aromatic side chains, which might be attributable to π–π interactions. In the case of Hpg, the additional possibility of H-bond formation should be considered. However, only a negligible influence on antibacterial activity was found for the L-Hpg→L-Ala peptides [L-Ala7]- and [L-Ala11]-feglymycin (**8** and **12**). This implies that the importance of all four Hpg's of feglymycin for antimicrobial activity is not equal. The least effects on antibacterial activity were found for the two L-Val→L-Ala peptides, **4** and **10**, possibly because of comparable polarity and less-pronounced steric effects of the side chains. The MIC values of the D-Dpg→D-Ala mutant peptides **2**, **4**, **6**, **8**, and **10** were mostly between 2–4 μM (four- to 16-fold decrease in antibacterial activity compared to feglymycin). Unlike D-Hpg1, L-Hpg5, and L-Phe12, the aromatic amino acid D-Dpg in general seems to exert a less significant influence on antibacterial activity. In addition, the intermediate MIC of the control "double mutant" peptide [D-Ala2,10]-feglymycin (**45**, MIC 4–8 μM) suggests, rather, an additive effect of Ala substitutions of this amino acid on antimicrobial activity.

The results of the antibacterial assays contrast with those from investigations of the Ala-exchange peptides for their inhibitory activity against MurA and MurC (enzymes of early peptidoglycan biosynthesis). The inhibition assays against MurA and MurC consistently identified L-Asp13 as the only residue substitution to significantly contribute to a drop in inhibition (seven- to 12-fold). The activity of MurC was also reduced in the presence of the [D-Ala2,10] peptide (**45**, eightfold), whereas MurA activity was not affected. In addition, for [L-Ala5]feglymycin (**6**), an inhibitory effect of L-Hpg5 for MurA was determined (greater than fivefold reduction in inhibition).

Remarkably, comparison of the anti-staphylococcal assays and the enzyme inhibition assays draws a picture of different positional dependence between antibacterial activity in the *S. aureus* system and MurA/MurC inhibition. Although three residues, D-Hpg1, L-Hpg5, and L-Phe12, were identified as crucial for antibacterial activity in vitro, only L-Asp13 contributed to inhibition of MurA and MurC. In this context, the outcome of the anti-HIV assays performed for the Ala-exchange peptides deserves closer attention.[11] Previous investigations of feglymycin by our groups allowed us to assign the HIV surface protein gp120 as the molecular target. The data showed that the residue dependence of the alanine scan in the anti-HIV assay

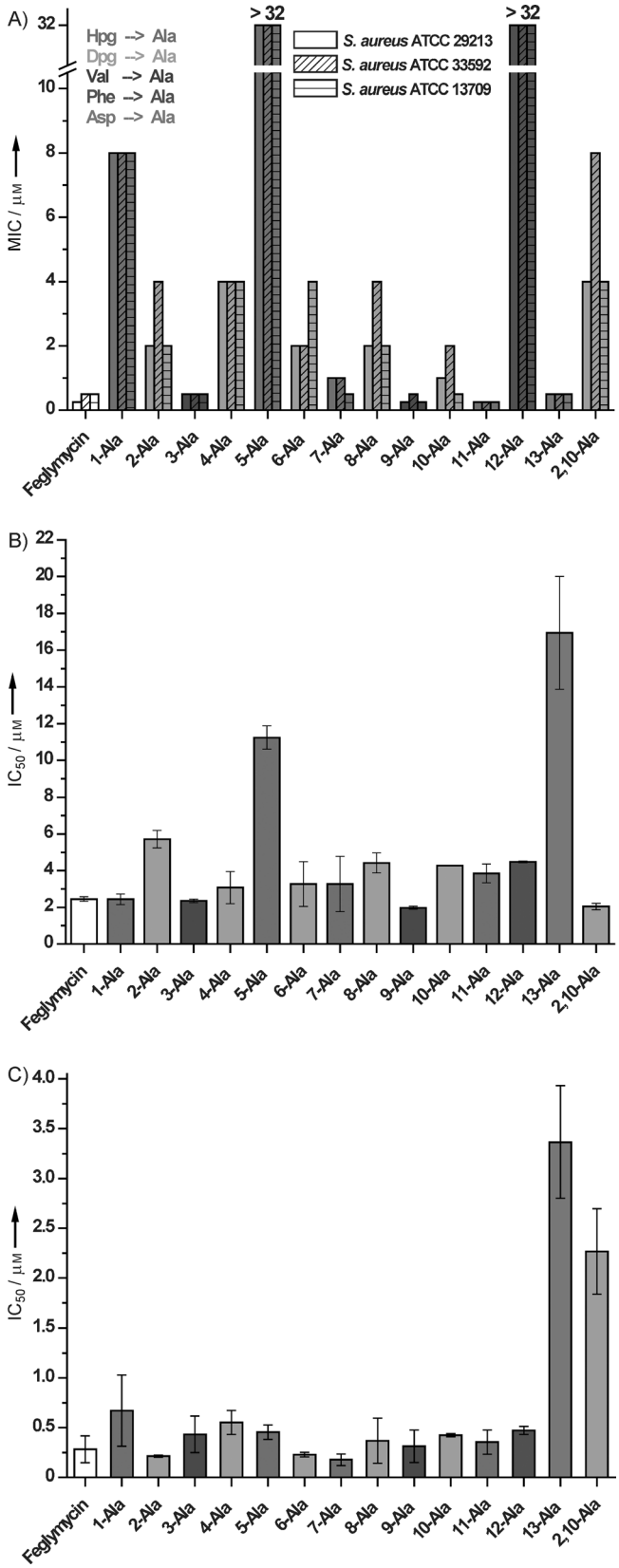


Figure 2. A) Antibacterial activity of feglymycin alanine scan peptides (MIC, minimum inhibitory concentration: no visible growth after incubation at 37 °C for 20 h). IC_{50} values for the inhibition of B) MurA and C) MurC (IC_{50}, inhibitor concentration that decreases enzyme activity by 50%).

largely reflected that of the MurA and MurC inhibition testing. Accordingly, [L-Ala13]-feglymycin (**14**) showed minor potency in inhibition of HIV-1, whereas [L-Ala5]-feglymycin (**6**) might suggest a more specific interaction by L-Hpg5 in the inhibition of the target protein MurA (i.e., not required for MurC or gp120).

The divergence in the outcome of the assays with *S. aureus* and MurA/MurC enzymes appears indicative of two independent pharmacophoric regions in feglymycin, addressing molecular targets at different interaction sites. Naturally, one would expect similar patterns of inhibition for in vitro and enzyme inhibitory data. Therefore, it is reasonable to assume that although feglymycin exhibits pronounced effects on the peptidoglycan biosynthesis enzymes MurA and MurC, additional or other molecules are antibacterial targets. As we exclude surface-exposed late peptidoglycan biosynthesis steps as feglymycin targets, other targets or mechanisms of action have to be considered. This is corroborated by the physical properties of feglymycin (e.g., $M_W \approx 1.9$ kDa), which are not characteristic of cell-permeable molecules. Therefore, apart from molecular targets on the surface of the bacterial cell, dedicated uptake systems might be involved in the mode of action. Our observation is reminiscent of previous studies performed on the antibacterial compound ramoplanin ($M_W \approx 2.5$ kDa). Initially, inhibition of intracellular MurG[20] and components of the surface-located trans-glycosylation step[21] were suggested as molecular targets, but more recently it appears that the trans-glycosylation step of lipid II synthesis is inhibited.[22]

The differences between in vitro and enzyme inhibition results for the alanine scan peptides clearly require further work on the conformation and on target-dependent conformational changes of feglymycin. Our future attempts will be directed at elucidating the mode of action and identification of the dedicated molecular targets of feglymycin. In combination with new data, this study could be useful for the design of simplified analogues of feglymycin that require less-demanding efforts for their synthesis. Therefore, this study constitutes an important step towards a more stable and structurally less complex peptidomimetic with improved biological activity.

Experimental Section

Reagents and analytical methods: Chemicals were obtained from the following commercial sources and used without further purification: ABCR (Karlsruhe, Germany), Alfa Aesar (Karlsruhe, Germany), Bachem, Fisher Scientific, Iris Biotech (Marktredwitz, Germany), Merck, Orpegen Pharma (Heidelberg, Germany), and Sigma–Aldrich. Deuterated solvents for NMR spectroscopy were obtained from Euriso-Top (Saint-Aubin, France). Flash chromatography was carried out with Davisil silica gel (40–63 μm; Grace, Deerfield, IL) or the Combi*Flash Rf* system with prepacked silica gel columns (Teledyne Isco, Lincoln, NE). Analytical thin layer chromatography was performed with precoated aluminium sheets (silica gel 60 F254; Merck). An analytical HPLC system 1100 (Agilent Technologies) with DAD detector and a Luna C18(2) 100Å column (5 μm, 4.6× 100 mm; Phenomenex, Torrence, CA) was used for reaction and purity control. Preparative RP-HPLC was performed with an 1100 HPLC system with DAD detector (Agilent Technologies) and an

ODS-5 ST RP-C18 column (Grom-Sil 120, 10 μm, 20×250 mm; Alltech Grom, Worms, Germany). Size exclusion chromatography was performed with Sephadex LH20 (GE Healthcare) packed into a glass column (ECO25 999 VOE, 25×1000 mm; Kronlab/YMC Europe, Dinslaken, Germany). Separations were carried out on an ÄKTApurifier 10 FPLC system (GE Healthcare). ^{1}H and ^{13}C NMR spectra were recorded with an Avance 400 MHz or a DRX 500 MHz NMR spectrometer (Bruker). 2D NMR experiments were performed on a DRX 500 MHz NMR spectrometer. EI-MS and EI-HRMS spectra were recorded on a Finnigan MAT 95 S (Thermo Scientific). HPLC ESI-MS and ESI-HRMS measurements were performed with an Orbitrap LTQ XL (Thermo Scientific) in combination with a 1200 HPLC system (Agilent Technologies) and Hypersil-100 C18 column (5 μm, 3×50 mm; Thermo Scientific). HPLC ESI-MS/MS spectra were obtained with a Qtrap 2000 mass spectrometer (Applied Biosystems) in combination with an 1100 HPLC system (Agilent Technologies) with a Luna 3u C18(2) column (100 Å, 3 μm, 1×50 mm; Phenomenex). HPLC ESI-MS/MS spectra for all deprotected feglymycin derivatives were recorded on an ESI-Triple-Quadrupole mass spectrometer 6460 (Agilent Technologies) in combination with a 1290 Infinity LC system (column: Eclipse Plus C18 1.8 μm, 2.1×50 mm; Agilent Technologies). IR spectra were recorded on a Nicolet Magna-IR 750 FTIR spectrometer (Thermo Scientific) or a Nicolet Avatar 360 E.S.P. FTIR spectrometer (Thermo Scientific). CD spectra were recorded with a J715 CD spectrometer (JASCO Research). Optical rotations were determined with a P-2000 digital polarimeter (JASCO Research).

General procedure for peptide couplings with EDC/HOAt: $NaHCO_3$ (3.0 equiv), HOAt (1.1 equiv) and EDC·HCl (1.1 equiv) were added to a solution of the N- and C-terminal-protected amino acid (ratio 1:1) in dry DMF (5 mL per mmol amino acid) at 0 °C. The mixture was stirred at this temperature for 2 h. After a further 24 h at RT, the reaction mixture was diluted with water (20 mL per mmol amino acid) and extracted with EtOAc (3×20 mL per mmol amino acid). Afterwards, the combined organic phases were washed with saturated aqueous $NaHCO_3$ (3×), aqueous $KHSO_4$ (5%, 3×) and brine (1×), dried over Na_2SO_4, and filtered. The solvent was removed in vacuum, and the residue was purified by flash chromatography on silica gel.

General procedure for peptide couplings with TBTU: DIPEA (3.0 equiv) was added to a solution of the N- and C-terminal-protected amino acid (ratio 1:1) in dry CH_2Cl_2 (10 mL per mmol amino acid). After stirring for 10 min, TBTU (2.0 equiv) was added to the solution. After 24 h at RT, the mixture was reduced to one fourth of the original volume under reduced pressure. The residue was diluted with water and extracted with EtOAc (3×20 mL per mmol amino acid). Then, the combined organic phases were washed with saturated aqueous $NaHCO_3$ (3×), HCl (1 N, 3×) and brine (1×), dried over Na_2SO_4, and filtered. The volatiles were removed by rotary evaporation, and the crude product was purified by flash chromatography on silica gel.

General procedure for peptide couplings with DEPBT: If the C-terminal amino acid or peptide was still *N*-Boc-protected, the Boc-group was removed directly before peptide coupling. Therefore, the amino acid or peptide was treated with 4 N HCl/dioxane (10 mL per mmol starting material) at RT under Ar, and stirred until complete conversion was detected by analytical TLC. Afterwards, the solvent was removed under vacuum, and the residue was taken up in Et_2O and evaporated again. This procedure was repeated twice and the resulting solid was dried under reduced pressure for a further 30 min. The corresponding amino acid or peptide hydrochloride was obtained in quantitative yield, and subsequently dissolved in dry DMF (10 mL per mmol amino acid or peptide) together with the N-terminal-protected coupling partner (1.0 equiv amino acid or peptide). After cooling to 0 °C, $NaHCO_3$ (2.0 equiv) and DEPBT (2.0 equiv) were added. The mixture was stirred for 1 h at this temperature and for a further 24 h at RT. The turbid reaction mixture was diluted with water (50 mL per mmol amino acid or peptide) and extracted with EtOAc (4×30 mL per mmol amino acid or peptide). The combined organic layers were successively washed with water (2×), saturated aqueous $NaHCO_3$ (3×), and brine (1×), dried over Na_2SO_4, and filtered. After removal of volatiles under reduced pressure the residue was purified by flash chromatography on silica gel. Because of the low solubility of protected hexa- and heptapeptides in EtOAc, a modified procedure was use for work-up of the corresponding reaction mixtures. After addition of an excess of water, the precipitate, which contained most of the desired peptide, was centrifuged. Then, the aqueous supernatant was extracted with EtOAc as described above. After the washing step the organic layer was dried over Na_2SO_4 and combined with the pellet of the centrifugation step dissolved in MeOH. The solvent was subsequently removed by rotary evaporation and the resulting crude product was purified by flash chromatography on silica gel.

General procedure for fragment couplings with DEBPT for the synthesis of N- and C-terminal-protected tridecapeptides: Directly before the fragment condensation step, the C-terminal coupling partner (hexapeptide) was *N*-Boc-deprotected. Therefore, the hexapeptide was suspended in CH_2Cl_2 (16 mL per mmol hexapeptide) and treated with Et_3SiH (15.0 equiv) and TFA (4 mL per mmol hexapeptide) under Ar. After 30 min at RT, the deprotected peptide trifluoroacetate was precipitated by addition of Et_2O (200 mL per mmol hexapeptide), centrifuged and dried under vacuum for 1 h. The resulting colorless solid was dissolved in dry DMF (10 mL per mmol hexapeptide) at 0 °C under Ar together with the N-terminal-protected heptapeptide (0.9 equiv), $NaHCO_3$ (5.0 equiv), and DEPBT (2.5 equiv). After 1 h at 0 °C and 40 h at RT, the reaction mixture was diluted with water. The precipitated product was centrifuged, washed once with water and dried under reduced pressure. The residue was dissolved in MeOH (1 mL per 100 mg of crude product) and purified by Sephadex LH20 size-exclusion chromatography. The purest fractions were pooled and concentrated by rotary evaporation. The obtained protected tridecapeptide was further purified by reversed-phase preparative HPLC.

General procedure for cleavage of benzylic protecting groups of dipeptides by catalytical hydrogenolysis: Pd/C (10%, 200 mg per mmol peptide) was added to a solution of the protected peptide in THF (10 mL per mmol peptide), and the flask was successively flushed with Ar and H_2. The reaction progress was monitored with analytical TLC. After complete consumption of the starting material, the reaction mixture was filtered (0.45 μm Rotilabo PTFE syringe filter; Carl Roth), and the filter was washed thoroughly with MeOH. After removing the solvent under reduced pressure, the resulting solid was dissolved in a small amount of MeOH, treated with water, and lyophilized. Unless otherwise stated, the corresponding deprotected peptide was obtained in quantitative yield.

General procedure for cleavage of benzylic protecting groups of tridecapeptides by catalytic hydrogenolysis: Water (1–2 drops) and Pd/C (10%; 0.5 mg per mg of peptide) were added to a solution of the N- and C-terminal-protected tridecapeptide in MeOH (0.1 mL per mg of peptide). The flask was flushed with, successively, Ar and H_2. After 8 h at RT, the reaction mixture was filtered (0.45 μm Rotilabo PTFE syringe filter; Carl Roth) and the filter was washed with MeOH. The volatiles were removed by rotary evapora-

tion. The obtained product was dissolved in a minimum quantity of MeOH, treated with water, and lyophilized. Unless otherwise stated, the corresponding deprotected tridecapeptide was obtained in quantitative yield.

General procedure for methyl ester cleavage with trimethyltin hydroxide: Peptide methyl ester and Me_3SnOH (20.0 equiv) were suspended in 1,2-dichloroethane (90 mL per mmol peptide) under Ar and heated to reflux (85 °C). After a reaction time of 8 h, the solvent was removed under reduced pressure, and the residue was suspended in saturated aqueous $NaHCO_3$ (100 mL per mmol peptide). Then, the slightly turbid solution was acidified to pH 4–5 with solid citric acid. After extraction with EtOAc (4×50 mL per mmol peptide), the combined organic layers were washed with aqueous citric acid (10%, 1×), water (2×), and brine (1×), dried over Na_2SO_4, and filtered. The volatiles were removed by rotary evaporation. The obtained product was dissolved in a small quantity of MeOH, treated with water, and lyophilized.

Antimicrobial testing: All synthesized feglymycin derivatives were tested for their antimicrobial activity in collaboration with Sanofi–Aventis. All experiments were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI).[23]

Inhibition of the enzymes MurA and MurC: The inhibition of the enzymes MurA and MurC from *E. coli* was determined in coupling assays as described previously.[13]

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