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Stereochemistry and Conformation of Skyllamycin, a Non-Ribosomally Synthesized Peptide from *Streptomyces* sp. Acta 2897

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Abstract: Skyllamycin is a non-ribosomally synthesized cyclic depsipeptide from *Streptomyces* sp. Acta 2897 that inhibits PDGF-signaling. The peptide scaffold contains an N-terminal cinnamoyl moiety, a β-methylation of aspartic acid, three β-hydroxylated amino acids and one rarely occurring α-hydroxy glycine. With the exception of α-hydroxy glycine, the stereochemistry of the amino acids was assigned by comparison to synthetic reference amino acids applying chiral GC-MS and Marfey-HPLC analysis. The stereochemistry of α-hydroxy glycine, which is unstable under basic and acidic conditions, was determined by conformational analysis, employing a combination of data from NOESY-NMR spectroscopy, simulated annealing and free MD simulations. The simulation procedures were applied for both *R*- and *S*-configured α-hydroxy glycine of the skyllamycin structure and compared to the NOESY data. Both methods, simulated annealing and free MD simulations independently support *S*-configured α-hydroxy glycine thus enabling the assignment of all stereocenters in the structure of skyllamycin and devising the role of two-component flavin dependent monooxygenase (Sky39) as *S*-selective.

Introduction

PDGF-signaling plays a crucial role in the development of various diseases including cancer, fibrosis or arteriosclerosis,[1,2] and therefore PDGFs and their receptors constitute important targets for the development of anti-cancer drugs.[3] While the marketed drugs Imatinib and Sorafenib address the ATP-binding pocket of the PDGF receptor and other protein kinases, the cyclic depsipeptide skyllamycin has been found to inhibit the binding of platelet-derived growth factor BB (PDGF BB) to its receptor PDGF β, thus representing an alternative in the inhibition of PDGF signaling.

Skyllamycin A was firstly isolated as antibiotic RP-1776 from *Streptomyces* sp. KY 11784,[4] and subsequently found as a two component mixture together with skyllamycin B, from *Streptomyces* sp. Acta 2897.[5] Skyllamycin A and B (Scheme 1) are non-ribosomally synthesized cyclic 11mer depsipeptides with a 2-[1-(*Z*)-propenyl]-cinnamoyl (PrnCin) moiety attached to the N-terminus. A preliminary structural characterization of the 11mer skyllamycin A peptide scaffold reveals a β-methylation of aspartic acid (β-Me-Asp^3) as well as a remarkably high content of hydroxylated amino acids. Namely, three amino acids are β-hydroxylated (β-OH-Phe^5, β-OH-*O*-Me-Tyr^7 and β-OH-Leu^{11}) and one glycine is hydroxylated (α-OH-Gly^9). β-Hydroxy amino acids are present in various antibacterial substances as for example vancomycin,[6] ramoplanin,[7] or lysobactin,[8] while α-OH-Gly was only found in the antitumor antibiotic spergualin.[9]

In *Streptomyces* sp. Acta 2897, the peptide chain of skyllamycin is assembled by three non-ribosomal peptide synthetases (Sky29, 30, and 31) consisting of 11 modules.[5] The adenylation

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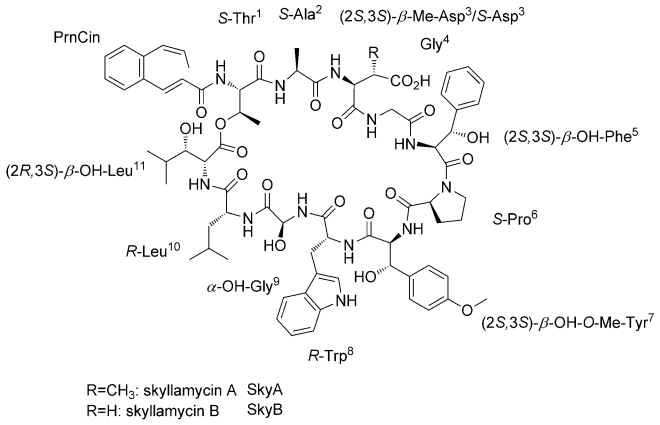

Scheme 1. Structure of cyclodepsipeptides skyllamycin A and B.

domains of each module are specific for the incorporation of each of the 11 amino acids. Furthermore, epimerization domains in module 8 and module 10 indicate that Trp[8] and Leu[10], respectively are *R*-configured in the peptide. A promiscuous P450 monooxygenase introduces the three β-hydroxylations (Sky32) and a two-component flavin dependent monooxygenase (Sky39) is responsible for the α-hydroxylation.

Both the remarkable structural features and the bioactivity of skyllamycin led us to commence a study on a comprehensive assignment of skyllamycin stereochemistry and conformation. This was achieved by combining data from enantiomer analytics of amino acids, with NOESY-NMR spectroscopy, simulated annealing and free molecular dynamic (MD) simulations. The molecular modeling simulations were applied with both *S*- and *R*-configured α-OH-Gly[9], with and without constraints issued from NOESY-NMR data. This joint experimental and in silico procedure provides a clear evidence for the absolute stereochemistry of skyllamycin A.[10]

Results

The considerable tailoring of the skyllamycin peptide namely methylation and hydroxylation of amino acids in skyllamycin (β-OH-Phe[5], β-OH-O-Me-Tyr[7], β-OH-Leu[11], α-OH-Gly[9], and β-Me-Asp[3]) constitutes a significant challenge for a full stereochemical assignment. The main obstacle however concerns α-OH-Gly[9] that is unstable under basic and acidic conditions, thus eluding from conventional amino acid analysis techniques.[5] Unlike previous examples of peptide antibiotics in which crystallization led to a full stereochemical assignment including a 3D model,[11,12] such attempts were not successful for skyllamycin. Therefore, the configuration of all amino acids, except for α-OH-Gly[9], had to be analyzed by amino acid analysis. With regard to the configuration of α-OH-Gly[9], NOESY-NMR spectra were employed to derive constraints for molecular modeling simulations (both simulating annealing and molecular dynamics). To reinforce the robustness and sampling of the conformational analysis, unconstrained simulations were performed and the thereof derived distances were compared to forty NOESY-NMR-derived distances.

Enantiomer analytics of skyllamycin hydrolysates

The stereochemistry of all amino acids except for α-OH-Gly[9] was determined employing the following workflow: 1) hydrolysis of skyllamycin A and B, 2) derivatization of the amino acids followed by 3) chiral GC-MS and/or HPLC-MS analysis, and 4) comparison to reference amino acids. Accordingly, the configuration of the proteinogenic amino acids was determined as *S*-Thr[1], *S*-Ala[2], *S*-Pro[6], *R*-Leu[10] (Table 1). Under the hydrolytic conditions that were applied (6 N HCl,110 °C, 24 h), the degradation of Trp was prevented by addition of dithiothreitol (DTT). Unlike other amino acids, enantioseparation of Trp could not be achieved using a chiral cyclodextrin phase used for GC-MS analytics. Therefore samples were derivatized with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5 L-alanine amide)[10] and analyzed by HPLC-MS to assign *R*-Trp as a constituent of skyllamycin (Table 1). In order to clearly identify the stereoisomers of the non-proteinogenic β-hydroxy amino acids and β-Me-Asp, we synthesized a set of stereoisomers as reference compounds according to previously published procedures.[13–16] Hence, configurations of the non-proteinogenic amino acids were assigned as follows: (2*S*,3*S*)-β-OH-Phe, (2*R*,3*S*)-β-OH-Leu and (2*S*,3*S*)-β-Me-Asp (Table 1). Under acidic hydrolysis conditions the β-OH-*O*-Me-Tyr constituent of skyllamycin was degraded and no remains were detected by GC-MS analysis.[17,18] Skyllamycin A was therefore submitted to ozonolysis which, followed by an oxidative workup, transforms β-OH-*O*-Me-Tyr into the acid-stabile β-OH-Asp.[18] Following these lines, synthetic standards of β-OH-*O*-Me-Tyr were ozonolyzed in the same way. The subsequent GC-MS analysis revealed the presence of (2*S*,3*R*)-β-OH-Asp in the hydrolysate, and thus (2*S*,3*S*)-β-OH-*O*-Me-Tyr in skyllamycin A.

Table 1. Assignment of the amino acid configurations of skyllamycin A by means of chiral GC-MS and HPLC-MS.

Residue	Config.	Chiral GC-MS	Marfey HPLC-MS
Thr[1]	2*S*,3*R*	+	+
Ala[2]	*S*	+	+
β-Me-Asp[3]	2*S*,3*S*	+	–
β-OH-Phe[5]	2*S*,3*S*	+	–
Pro[6]	S	+	+
β-OH-*O*-Me-Tyr[7]	2*S*,3*S*	+	–
Trp[8]	*R*	–	+
α-OHGly[9]	–	–	–
Leu[10]	*R*	+	+
β-OH-Leu[11]	2*R*,3*S*	+	+

Proton–proton distances of skyllamycin from NOESY-NMR data

While NMR spectroscopy is capable to solve the relative stereochemistry, only in combination with molecular modeling and based on the knowledge of at least one remote stereocenter the absolute stereochemistry can be determined. At this stage

Table 2. Proton–proton distances d_{A-B} [Å] determined from NOESY-NMR spectra. Distances in italics were constrained for molecular modeling simulations.

Proton pair A	B	d_{A-B} [Å][a]
PrnCin-H3	β-Me-Asp^3-HMe	3.08
PrnCin-H5	Leu^{10}-H1	3.37
PrnCin-H10	PrnCm-H3	2.80
PrnCin-H10	β-Me-Asp^3-HMe	3.70
Thr^1-NH	Thr^1-Hγ	2.41
Thr^1-Hγ	α-OH-Gly^9-Hα	*2.85* (d_1)
Thr^1-Hγ	α-OH-Gly^9-NH	*3.49* (d_2)
Ala^2-NH	Ala^2-Hα	3.03
Ala^2-NH	Ala^2-Hβ	2.38
Ala^2-Hα	Ala^2-Hβ	2.69
Ala^2-Hβ	β-Me-Asp^3-NH	3.05
Ala^2-Hβ	β-OH-Leu^{11}-Hα	3.66
β-Me-Asp^3-NH	β-Me-Asp^3-Hβ	2.88
β-Me-Asp^3-Hβ	β-Me-Asp^3-HβMe	2.65
β-OH-O-Me-Tyr^7-NH	β-OH-*O*-Me-Tyr^7-Hα	2.85
Trp^8-H1	β-OH-*O*-Me-Tyr^7-Hβ	6.67
Trp^8-H5	Trp^8-H6	2.49
Trp^8-H5	Trp^8-Hα	2.73
Trp^8-H5	Trp^8-Hβ	3.44
Trp^8-H5	Trp^8-Hβ1	2.92
Trp^8-H5	α-OHGly^9-NH	*3.94*
Trp^8-NH	Trp^8-Hβ	2.45
Trp^8-NH	Trp^8-Hβ1	3.10
Trp^8-NH	β-OH-*O*-Me-Tyr^8-NH	3.42
Trp^8-NH	β-OH-*O*-Me-Tyr^8-Hα	2.21
Trp^8-Hα	Trp^8-Hβ1	2.63
Trp^8-Hα	α-OH-Gly^9-NH	*2.06*
Trp^8-Hα	α-OH-Gly^9-Hα	*3.73*
Trp^8-Hβ	α-OH-Gly^9-NH	*3.53*
Trp^8-Hβ1	α-OH-Gly^9-NH	*2.86*
α-OH-Gly^9-NH	α-OH-Gly^9-Hα	*2.82*
α-OH-Gly^9-H	β-OH-Leu^{11}-NH	*3.23*
Leu^{10}-NH	Leu^{10}-Hβ	2.65
Leu^{10}-NH	Leu^{10}-Hβ1	3.31
Leu^{10}-NH	α-OH-Gly^9-Hα	*2.80*
Leu^{10}-Hα	α-OH-Gly^9-Hα	*3.73*
Leu^{10}-Hβ	β-OH-Leu^{11}-NH	3.44
Leu^{10}-Hβ1	β-OH-Leu^{11}-NH	3.76
β-OH-Leu^{11}-Hβ	β-OH-Leu^{11}-Hδ	3.09
β-OH-Leu^{11}-Hβ	β-OH-Leu^{11}-Hδ1	2.79

[a] The distance between Trp^8-H5 and Trp^8-H6 ($d_{Trp5-6} = 2.49$) was used as reference.

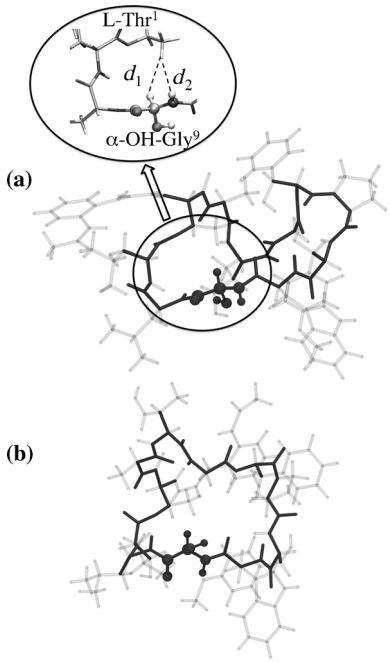

Figure 1. Representative conformations of the peptide backbone with α-OH-Gly (ball model) highlighted for a) *S*-configured (in agreement with NOESY-NMR data; (conformer **3**) and b) *R*-configured skyllamycin (conformer **6**, see text below). The d_1 and d_2 distances were chosen as being characteristic of the intramolecular folding.

of the structure elucidation, α-OH-Gly^9 remained the only amino acid for which the stereocenter was uncertain. Therefore NOESY-NMR spectra of skyllamycin A were recorded in order to identify inter-residue distances characteristic of both conformational and stereochemical features. From the NOESY-NMR-derived signals forty proton–proton distances (d_{A-B}) were obtained corresponding to forty intra- and inter-residue contacts (Table 2 and Supporting Information). Out of these, eleven contacts were assigned to interactions directly involving the α-OH-Gly^9 moiety (Table 2). The eleven corresponding d_{A-B} distances were used when molecular modeling simulations were constrained (see below).

To elaborate the configuration of α-OH-Gly, two distances between both α-OH-Gly^9 and Thr^1 were considered: d_1 (H-γ of (*s*)-Thr^1 to H-α of α-OH-Gly^9) and d_2 (H-γ of (*s*)-Thr^1 to N-H of α-OH-Gly^9) as indicated in Figure 1and Table 2. These two distances, which appear adequately characteristic of the intramolecular folding of skyllamycin A (Figure 1), were followed within the resulting conformers of all simulations. They provide information on the backbone architecture, since protons assigned to Thr^1 and α-OH-Gly^9 show a strong NOE-contact (Table 2 and Supporting Information), while being distant in a sequential context. Hence, only in silico conformers with values of d_1 and d_2 close to the experimental data are likely to resemble the natural conformer in solution.

Simulated annealing of skyllamycin with *S*- and *R*-configured α-OH-Gly^9

The simulated annealing procedure allows a complete exploration of the entire conformational space. It was applied to both *S*- and *R*-configured α-OH-Gly in skyllamycin A, and provided 60 conformers of each stereoisomer. These are grouped into 30 conformers from constrained and 30 from unconstrained simulations (see below). When applied, the force constants of the constraints were low enough to enable sufficient flexibility for d_1 and d_2 in a range from 3 to 13 Å (Figure 2).[19] As a result, the distances d_1 (2.85 Å) and d_2 (3.49 Å) determined from NOESY-NMR spectra could only be achieved for the *S*-configuration at α-OH-Gly^9 (Figure 2 and Table 3). In contrast for the structure with *R*-configuration for all 60 conformers d_1 and d_2 were found in the range >4.5 Å (Figure 2). In addition, when comparing the NOESY-NMR-derived experimental distances to the corresponding distances determined by simulated anneal-

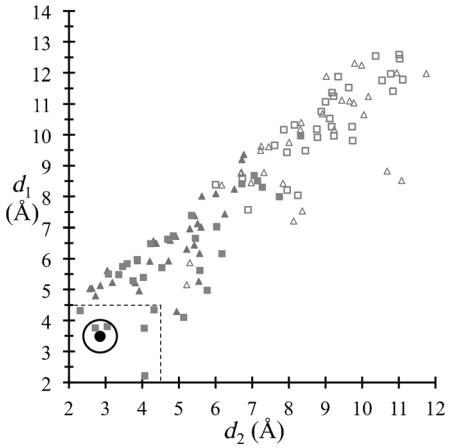

Figure 2. Distribution of intramolecular distances between H-γ of Thr[1] and 1) H-α of α-OH-Gly[9] (d_1, Å) or 2) NH of α-OH-Gly[9] (d_2, Å). Distances d_1 and d_2 were obtained from simulated annealing simulations for skyllamycin with either *R*-configured (triangle, full or empty when obtained from NMR-constrained and unconstrained simulated annealing) or *S*-configured (square, full or empty when obtained from NMR-constrained and unconstrained simulated annealing) α-OH-Gly[9]. Experimental data (black dot) with a standard error of +/- 0.5 (black cycle) are depicted.

Table 3. Comparison of distances d_1–d_2 (Å) of *S*-(α-OH-Gly[9]) skyllamycin conformers displaying the best fit to NOESY-NMR data: Conformer **1** from simulated annealing and conformer **3** from free MD simulations.

	d_1	d_2
simulated annealing (conformer **1**)	3.39	4.66
Free MD simulations (conformer **3**)	3.87	3.74
NOESY-NMR data	2.85	3.49

Table 4. Mean absolute deviation (MAD, Å), root-mean-square deviation ($RMSD_{tot}$, Å) based on all 40 NOESY-NMR-derived distances or on 11 distances in proximity of α-OH-Gly (RMSD, Å) of three conformers each (*R*- and *S*-(α-OH-Gly[9]) skyllamyin A) with the best fit to NOESY-NMR data.

Stereoisomer	Geometry[a]	MAD [Å]	$RMSD_{tot}$ [Å]	RMSD [Å]
S-configured	**1**[b]	0.86	1.85	0.66
	2[b]	0.96	2.30	1.04
	3[c]	0.51	1.40	0.76
R-configured	**4**[b]	1.12	2.32	2.20
	5[b]	1.18	2.43	1.98
	6[c]	1.27	2.59	2.88

[a] The xyz-coordinates of all six conformers are given in Tables S3 and S4 in the Supporting Information. [b] Conformers **1**–**2** and **4**–**5** are obtained from simulated annealing calculations and are considered as having the best agreement with the NOESY-NMR data. [c] Conformers **3** and **6** are obtained after equilibration of the 100 ns-MD simulations (with constrains inspired by NOESY-NMR-distances during the first 10 ns).

ing, the *S*-configured conformers exhibit much lower RMSD (root mean square deviation) compared to those of the *R*-configured skyllamycin (Table 4). From the simulated annealing procedure we could identify two conformers (**1** and **2**) with *S*-configuration, which perfectly fit to the experimental data derived from NOESY-NMR spectra (Figure 2 and Table 4).

Free MD simulations of skyllamycin with *S*- and *R*-configured α-OH-Gly[9]

In addition to simulated annealing, 100 ns free MD simulations were performed with NOESY-NMR-derived distances to protons of α-OH-Gly[9] (Table 2) constrained during the first 10 ns.[20] Interestingly, after the first 10 ns, that is, an unconstrained mode, the *S*-configured structure keeps the expected d_1 and d_2 distances along the rest of the MD simulation (Figure 3a), while

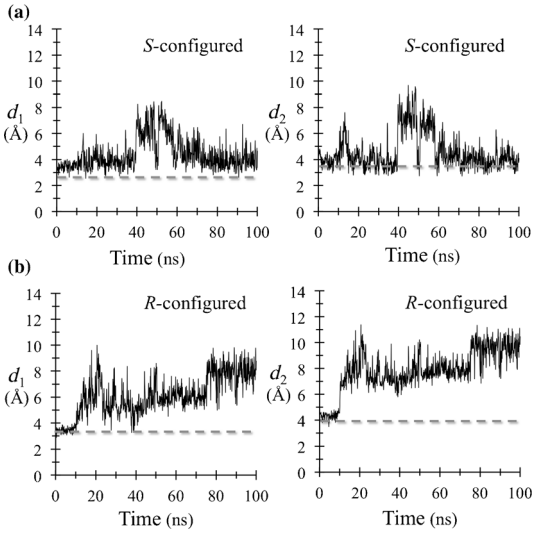

Figure 3. Evolution of interatomic distances d_1 (left) and d_2 (right) (Å) between the protons of Thr[1]-Hγ and α-OHGly[9]-Hα and α-OHGly[9]-NH, respectively, along the MD simulation (NMR-constrained during the first 10 ns) for skyllamycin with a) *S*- and b) *R*-configured α-OHGly[9]. The dashed lines show the d_1 and d_2 distances derived from NOESY-NMR data.

in the *R*-configured structure both distances d_1 and d_2 increase up to 10 Å (Figure 3b). The free MD simulations yield conformers **3** and **6** for *S*- and *R*-configured α-OH-Gly[9] skyllamycin (see Tables S3 and S4 in the Supporting Information), respectively. Conformer **3** has the same geometrical features as conformers **1** and **2** determined by simulated annealing and exhibits a flattened shaped backbone (Figure 1). Likewise conformer **6** shares the same conformational features as conformers **4** and **5** which are of circular shape (Figure 1). Independently from the simulated annealing procedure, this result corroborates that the NOESY-NMR data (Table 4) strongly correlate to an *S*-configured skyllamycin.

Discussion

The cyclic depsipeptide skyllamycin is a highly hydroxylated peptide with enigmatic α- and β-hydroxylations, synthesized by non-ribosomal peptide synthetases in interaction with tailoring enzymes.[5,21] With one exception, the herein presented stereochemical assignment derived from the amino acid analy-

ses is in agreement with those predicted by A domain specificities and the domain arrangement in the non-ribosomal peptide synthetases Sky29-31 of the skyllamycin biosynthesis gene cluster.[5] From the amino acids with one chiral center only Leu[10] and Trp[8] are *R*-configured, while the other amino acids are *S*-configured in the α-position. Modifications in the β-position of non-proteinogenic amino acids, like β-hydroxylation and β-methylation show *S*-configuration. Only β-OH-Leu[11] with a (2*R*,3*S*)-configuration does not fit to the biosynthetic logic, since no epimerization (E) domain in module 11 of the non-ribosomal peptide synthetase Sky31 has been found.[5] However such a case, that is, a non-ribosomal peptide synthetase lacking modules with an epimerization domain while displaying *R*-configuration in the product peptide, is not without precedence as this is also observed for example, for vancomycin[22] or cyclosporine.[23]

The configurational assignment of the above-mentioned amino acids was the basis for subsequent simulated annealing and free MD simulations. Both methods independently favor the *S*-configuration for α-OH-Gly for reasons of steric hindrance and hydrogen-bonding capabilities. Accordingly a view at the architecture of skyllamycin (Figure 4) shows an inter-residue

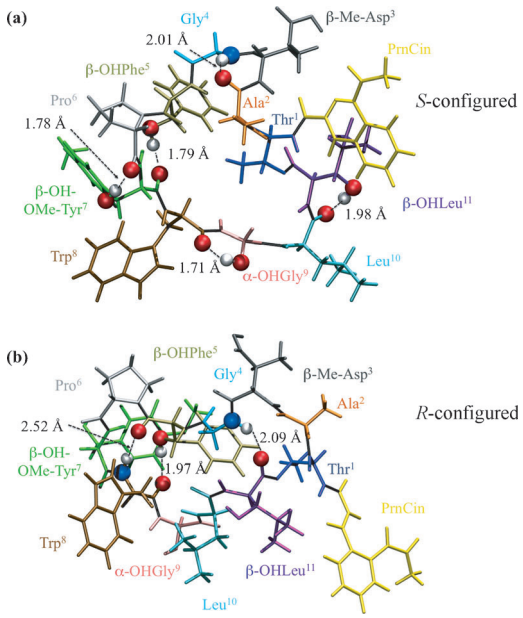

Figure 4. Conformers from MD simulations (**3** and **6**) highlighting the hydrogen bond network for skyllamycin with a) *S*-configured and b) *R*-configured α-OH-Gly[9].

hydrogen-bonding network, which is significantly different in both *S*- and *R*-configured skyllamycin (Figure 4), favoring a flattened-type conformer and a circular-type conformer, respectively (represented by conformers **3** and **6** respectively in Figure 1 and Figure 4). For the *S*-isomer five strong hydrogen bonds with bond distances in the range of 1.78-2.01 Å (see Figure 4a) are observed, contrasted by only three in the range of 1.97-2.52 Å (see Figure 4b) for the *R*-isomer. Interestingly, the hydrogen bond network of the *S*-isomer accommodates all hydroxy groups of β-hydroxy amino acids (β-OH-Phe[5], β-OH-Tyr[7], β-OH-Leu[11]) as well as of α-OH-Gly[9]. Specifically, these interactions include the H-bonds between the β-hydroxyls of β-OH-Phe[5] with the carbonyl (C=O) of β-OH-*O*-Me-Tyr[7]; of β-OH-*O*-Me-Tyr[7] with C=O of Pro[6]; and of β-OH-Leu[11] with C=O of Leu[10]. This is complemented by the interaction of the α-OH (α-OH-Gly[9]) with C=O of Trp[8]. The fifth hydrogen bond is contributed by the interaction between NH of Gly[4] and C=O of Ala[3]. Furthermore, the H-bond network displayed in Figure 4a predominantly favors interactions between the preceding and the subsequent residues of the amino acid sequence. This finding is in stark contrast to the structure of the *R*-isomer, which shows hydrogen bonds between NH of Trp[8] and C=O of β-OH-Phe[5], between β-OH of β-OH-Phe[5] and C=O of Trp[8] and between NH of Gly[4] and C=O of β-OH-Leu[11] and does not display any H-bonds involving α-OH-Gly[9]. The loss of the hydrogen bond between the α-OH (α-OH-Gly[9]) and C=O of Trp[8], which is a main difference between the *S*- to the *R*-configured conformer, appears of particular importance as it leads to a dramatic intramolecular reorganization, which impedes stabilization of the hydrogen-bonding network.

The herein presented findings also have implications for the biosynthesis of skyllamycin: The glutamate mutase (Sky41, Sky 42) that catalyzes the conversion of glutamate to β-methylaspartate is *S*-selective in the β-position. The β-hydroxylations performed by the P450 monooxygenase (Sky32) are all *S*-selective and are introduced while the amino acids are bound to the peptidyl carrier protein before further being coupled to the peptide chain.[21] This could allow for formation of hydrogen bonds between the β-hydroxy groups and neighboring carbonyls already before final macrolactonization by the thioesterase domain of Sky31. The hydrogen bonds could contribute to a preorganization of the peptide chain in order to facilitate the ring closure reaction. Finally, according to our data also the flavin-dependent oxygenase Sky39, introducing the α-hydroxy group into α-OH-Gly, is *S*-selective. The α-hydroxylation is used in primary metabolism for the processing of many peptide hormones, neurotransmitters and growth factors to C-terminal amides. There peptide amidation is catalyzed by the bifunctional enzyme peptidylglycine α-amidating monooxygenase (PAM).[24] The conversion is a two-step process involving the peptidylglycine α-hydroxylating monooxygenase (PHM) and peptidyl α-hydroxyglycine α-amidating lyase (PAL). PHM catalyzes stereoselectively the hydroxylation to *S*-α-OH-Gly, whereas PAL stereoselectively induces the cleavage of the N–C bond in C-terminal *S*-α-OH-Gly.[24,25] PAL does not react with *R*-α-OH-Gly.[25] Likewise, for the secondary metabolite spergualin an *S*-configuration has been determined by use of a serine carboxypeptidase.[26] Interestingly, the *S*-enantiomer of 15-deoxyspergualin showed anti-leucemic activity whereas the *R*-enantiomer is almost inactive.[26] In skyllamycin the stability of this modification and protection from hydrolysis may be supported by its involvement into the extensive H-bond network. With regard to the biosynthesis the time point of α-hydroxylation of Gly by Sky39 is still elusive. Hydroxylation of free or PCP-bound Gly seems highly unlikely due to the required stabilization of an aminal-like structure for subsequent peptide

coupling. Therefore we suggested this reaction to occur either on the NRPS-bound peptide or after NRPS assembly and release from the NPRS assembly line.

Conclusion

In summary, a combination of bioanalytical methods and calculations succeeded in a full stereochemical assignment of the PDGF inhibitor peptide skyllamycin with a particular focus on the configuration of the unusual amino acid α-OH-Gly. These tailoring modifications performed by the biosynthetic assembly line of skyllamycin, consisting of β-methylation of Asp, β-hydroxylations of Phe, Tyr and Leu as well as α-hydroxylation of Gly, are *S*-selective. The full conformational analysis describing intramolecular rearrangements confirms the NOESY-NMR contacts experimentally observed. While this work illustrates gaining data on conformation and the stereochemistry even of flexible molecules by combining MD simulations with NOESY-NMR data this structure elucidation will aid in using skyllamycin as a lead structure for the design of novel PDGF inhibitor molecules.

Experimental Section

Synthesis of β-hydroxy amino acids and of β-methyl aspartic acid

The four stereoisomers of β-hydroxy leucine,[13] β-hydroxy phenylalanine[14] and two stereoisomers of β-methyl aspartic acid[15] were synthesized according to literature procedures. Amino acids (2*S*,3*S*)- and (2*R*,3*R*)-β-hydroxy-*O*-methyl tyrosine were synthesized according to a strategy applying the Sharpless dihydroxylation reaction.[16]

Hydrolysis of skyllamycin A and B

Skyllamycin (0.4 mg) was dissolved in 1 mL of 6 N HCl. The solution was degassed and then heated under nitrogen atmosphere at 110 °C for 24 h. For Trp analytics the same amount of skyllamycin was dissolved in 1 mL of 6 N HCl containing 3% w/v of phenol and 1% w/v of dithioerythritol, degassed and heated under nitrogen at 120 °C for 24 h. Subsequently the hydrolysate was dried at 110 °C under a stream of nitrogen.

Derivatization of α-amino acids for chiral GC-MS analytics

To amino acid or the dry skyllamycin hydrolysate, 200 μL of 2 N HCl in ethanol were added. The mixture was heated at 110 °C for 30 min. Then reagents were removed at 110 °C in a stream of nitrogen. Subsequently 100 μL dichloromethane and 50 μL trifluoroacetic anhydride were added and the mixture was again heated at 110 °C for 10 min. Reagents were removed at room temperature in a stream of nitrogen thus rendering the *N*-trifluoroacetyl ethyl esters for subsequent GC-MS analytics.

Chiral GC-MS analysis

Chiral GC-MS analyses were performed on a Thermo GC 8000 Voyager spectrometer with the chiral stationary phase Lipodex-E (Machery & Nagel, length: 25 m, diameter: 0.25 mm). The dry derivatization mixture was dissolved in 50 μL anhydrous toluene. For GC-MS analytics 1 μL of this solution was applied to the injector. The following temperature program was used: isothermal at 70 °C for 2 min, gradient of 3 °C min^{-1} up to 90 °C, isothermal at 90 °C for 15 min, gradient of 10 °C min^{-1} up to 200 °C, isothermal at 200 °C for 10 min. Retention times of the derivatized amino acids (min): *R*-Ala (13.7), *S*-Ala (15.2), (2*S*,3*R*)-β-OH-Leu (18.0), (2*R*,3*S*)-β-OH-Leu (20.6), *R*-Leu (21.2), *S*-Leu (25.5), *S*-Thr (26.1), (2*R*,3*R*)-β-OH-Leu (26.7), *R*-Thr (27.1), *R*-aThr (27.5), (2*S*,3*S*)-β-OH-Leu (27.8), *S*-aThr (28.5), *S*-Pro (30.0), *R*-Pro (30.7) *R*-Asp (31.0), *S*-Asp (31.3), (2*R*,3*R*)-β-OH-Asp (32.9), (2*S*,3*S*)-β-OH-Asp (33.7), (2*R*,3*S*)-β-OH-Asp (34.9), (2*S*,3*R*)-β-OH-Asp (35.3), (2*S*,3*R*)-β-MeAsp (38.2), (2*S*,3*S*)-β-MeAsp (39.3), (2*S*,3*R*)-β-OH-Phe (43.2), (2*R*,3*S*)- β-OH-Phe (43.2), (2*R*,3*R*)-β-OH-Phe (45.9), (2*S*,3*S*)-β-OH-Phe (46.7).

Ozonolysis of β-hydroxy-*O*-methyl tyrosine and of skyllamycin

Amino acid (1 mg) or skyllamycin (1 mg) was dissolved in MeOH (1 mL) and cooled down to −78 °C. A stream of ozone was bubbled for 75 min through the solution. Subsequently 440 μL of H_2O_2 (30%) were added to the reaction mixture which was then allowed to stand at room temperature for 12 h. The solvent was removed in a stream of nitrogen. As an intermediate step the skyllamycin sample was additionally hydrolyzed (110 °C, 6 N HCl, 24 h). Finally samples were derivatized and analyzed by GC-MS analytics according to above mentioned conditions.

Marfey derivatization and HPLC-MS analysis

100 μL of an aqueous 1% FDAA (1-fluoro-2,4-dinitrophenyl-5 L-alanine amide) solution were added to a 50 μL aliquot of a 0.05 M solution of the amino acids or the skyllamycin hydrolysate in H_2O. Then 20 μL of 1 N $NaHCO_3$-solution were added as a base and the mixture was heated at 40 °C for 80 min. The solution was adjusted to pH 7 adding 10 μL of 2 N HCl. After freeze-drying the residue was dissolved in 1 mL of MeOH. The HPLC-MS analyses were performed on a HPLC 1100 series (Agilent Technologies) hypenated to a QTRAP 2000 ESI-Quadrupol-MS (Applied Biosystems). The samples were separated on a Phenomenex Luna C18 column (1× 50 mm) and eluted with 5% MeCN-0.1% HCOOH in H_2O at a flow rate of 60 μL min^{-1}. The elution program was set as follows: 0–58 min (5–40% MeCN), 58–59 min (40–100% MeCN), 59–62 min (100% MeCN). Retention times of the FDAA derivatives (min): *S*-Thr (25.1), *S*-aThr (26.0), *S*-Asp (26.4), *R*-aThr (28.5), *R*-Asp (29.0), *S*-Ala (30.6), *R*-Thr (31.5), *S*-Pro (32.4), *R*-Pro (34.5), (2*S*,3*S*)-β-OH-Leu (35.2), *R*-Ala (36.3), (2*S*,3*R*)-β-OH-Leu (37.1), (2*R*,3*R*)-β-OH-Leu (41.4), (2*R*,3*S*)-β-OH-Leu (44.7), *S*-Trp (46.9), *S*-Leu (47.5), *R*-Trp (50.8), *R*-Leu (53.8).

NOESY-NMR

NMR spectra were recorded on a Avance III 500 MHz NMR spectrometer (Bruker) equipped with a BBF probe head. NOESY spectra were collected in [D_6]DMSO, the mixing time was varied between 50 and 600 ms. The NMR data were processed using the program TopSpin (Bruker), the cross-peak integrals were integrated using the program Sparky (University of California, San Francisco, CA, USA). Cross-relaxation rates were determined from the initial slope of a polynomial fit (cubic polynomial) of the cross-peak integrals as a function of the mixing time (build-up curves). To calculate the proton–proton distances the distance between the protons 5 and 6 of Trp (d_{Trp5-6} = 2.49 Å) and its cross-relaxation rates were taken as a reference.

Theoretical methods

The conformational analysis of skyllamycin was performed within the molecular mechanic formalism. The topology of the standard amino acids was generated from the Amber library[27] using the ff99SB[28] force field. Concerning the non-standard amino acids, a first optimization was performed at the density function theory (DFT) level with B3LYP/6–31 + G(d,p), using as initial geometries the corresponding standard amino acids (e. g. using the *S*-Asp geometry for (2*S*,3*S*)-β-methyl aspartic acid). The topology was then generated with the Antechamber program, using the ff99SB[28] force field. The corresponding partial atomic charges were assigned within the Antechamber program and with the RESP method from HF/6–31G* single-point calculations.[29,30] An explicitly parameterized dimethylsulfoxide (DMSO) solvation model[31] was used in order to approach the experimental conditions. Periodic boundary conditions were applied in all directions. The molecular systems were first minimized by the steepest descent method as usually achieved to avoid unfavorable atomic contacts. The contacts were then equilibrated during a 100 ps simulation in which the system was heated to 300 K, employing Langevin coupling. The equilibration step was followed by a 10 ns NPT simulation providing basis structures for all other calculations. The pressure was kept constant at 1 bar, using isotropic position scaling.

To ensure a complete potential energy surface analysis of both skyllamycin isomers (*R*- and *S*-configured α-OH-Gly^9), a 30-cycle simulated annealing procedure was performed within the Berendsen temperature coupling algorithm. Each cycle consisted of 1) a 1 ps heating stage up to 2000 K, 2) a 2 ps equilibration stage at 2000 K, and 3) a cooling stage from 2000 K to 0 K for 13 ps. The temperature bath was cooled linearly over this time. Improper and dihedral angle restraints were applied to prevent the interconversion of stereocenters at 2000 K. The explicit solvent was restrained to avoid vaporization artifacts due to high temperature. For both *R*- and *S*-configured isomers of α-OH-Gly^9 of skyllamycin, simulated annealing calculations were carried out either without or with constraints (derived from NOESY-NMR data) providing 60 (30 and 30, respectively) conformations for each isomer. These 60 conformations were systematically minimized following the steepest descent method, before distance analysis.

As a complementary tool to validate the conformational analysis, free 100 ns MD simulations (NPT) were performed for both skyllamycin stereoisomers (*R*-α-OH-Gly^9 and *S*-α-OH-Gly^9). Two types of simulation were achieved for both systems, namely with and without NMR constraints (during the first 10 ns). All molecular mechanic calculations were performed with the AMBER10 package.[27]

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Keywords: conformational analysis · NOESY-NMR spectroscopy · peptide antibiotics · skyllamycin · structure elucidation

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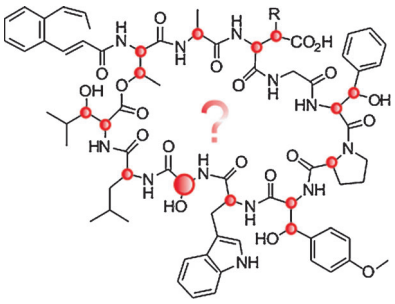
FULL PAPER

Successful assignment: A combination of bioanalytical methods and calculations succeeded in a full stereochemical assignment of the PDGF inhibitor peptide skyllamycin (see figure). While this work illustrates gaining data on conformation and the stereochemistry even of flexible molecules by combining MD simulations with NOESY-NMR data, this structure elucidation will aid in using skyllamycin as a lead structure for the design of novel PDGF inhibitor molecules.



Peptides

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Stereochemistry and Conformation of Skyllamycin, a Non-Ribosomally Synthesized Peptide from *Streptomyces* sp. Acta 2897