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Mutasynthesis of Glycopeptide Antibiotics: Variations of Vancomycin's AB-Ring Amino Acid 3,5-Dihydroxyphenylglycine

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Vancomycin (Figure 1a) is an antibiotic of last resort for treatment of life-threatening infections with methicillin-resistant *Staphylococcus aureus* (MRSA).[1] Emerging enterococcal and staphylococcal resistances against vancomycin require the evaluation of approaches to novel vancomycin-related glycopeptides. However, due to their structural complexity, generation of molecular diversity in glycopeptides, in particular in the D-Ala-D-Ala peptide-binding region of the heptapeptide backbone (aglycon), via total synthesis poses inherent problems.

Alternative biotechnological approaches would constitute attractive options, and considerable success has been achieved in the enzymatic attachment of various carbohydrate residues to glycopeptide aglycons.[2] Recently, Boger and co-workers demonstrated that permethylation of vancomycin-type aglycons resulted in improved activity against VanB-resistant bacteria.[3] These results indicate that aglycon modifications still are a promising target for the development of novel glycopeptide compounds. Here we report on the generation of a series of modified glycopeptide antibiotics by mutasynthesis in which the C-terminal amino acid (*S*)-3,5-dihydroxyphenylglycine (DPg) has been substituted, employing a number of phenylglycines or their mandelic and phenylacetic acid analogues, respectively.

Balhimycin (Figure 1b) is a vancomycin-type glycopeptide antibiotic produced by the actinomycete *Amycolatopsis balhimycina*.[4] Recent advances in the investigation of balhimycin biosynthesis by means of gene inactivation and analysis of the metabolites have provided an understanding of the central steps of the assembly of the aglycon from a linear peptide precursor.[5] Combined with the knowledge of the biosynthesis of the aromatic amino acid β-hydroxytyrosine,[6] 4-hydroxyphenylglycine (HPg),[7] and 3,5-dihydroxyphenylglycine (DPg),[8−10] these findings constitute the basis for the further biotechnological generation of altered glycopeptide aglycons. Thus, in previous work, structural variations at the 3-chloro-β-hydroxytyrosine (Cht) moieties by means of mutational biosynthesis led to the first fluorinated vancomycin-type glycopeptide antibiotic.[11] Subsequently, we directed our attention toward the atropisomeric AB macrocycle formed between ^{5}HPg and ^{7}DPg, which is of crucial importance for the antibiotic activity of this compound family.[12] Inactivation of the *dpgA* gene, which is involved in the first step of DPg biosynthesis, rendered a glycopeptide-deficient Δ*dpgA* mutant, the antibiotic activity of which could be restored by supplementation of the culture medium with DPg.[8] To elucidate structural and steric requirements for the mutasynthetic assembly of DPg analogues and their putative precursors into glycopeptides, a variety of substrates were synthesized, including 3-hydroxy- (3-HPg), 3-methoxy- (3-MeOPg), 3-hydroxy-5-methoxy- (HMeOPg), and 3,5-dimethoxyphenylglycine (DMeOPg), as well as the corresponding mandelic acid and phenylacetic acid analogues. After supplementation of *A. balhimycina* Δ*dpgA* cultures, the presence of glycopeptides in the culture filtrates was proved by HPLC-ESI-MS (Table 1) with the characteristic chlorine isotope pattern as a marker as well as via plate diffusion tests, the latter indicating restored antibiotic activity which was comparable to that of balhimycin. The balhimycin derivatives from the 3-MeOPg supplementation were characterized by high-resolution ESI-FTICR-MS (Figure 2 and Supporting Information).

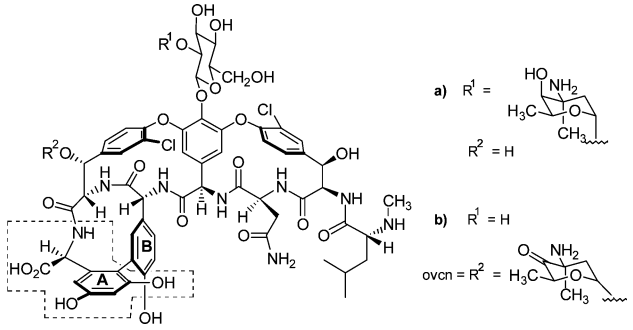

Figure 1. Structure formulas of glycopeptide antibiotics vancomycin (a) and balhimycin (b). The highlighted amino acid is (*S*)-3,5-dihydroxyphenylglycine (DPg, AA-7); ovcn = 4-oxovancosamine.

Feeding of 4-hydroxy-substituted (*R*)- and (*S*)-HPg, the latter also being the product of the HPg biosynthesis pathway, did not compensate for the lack of the DPg moiety, and no glycopeptides were detected with HPLC-MS. Supplementation with the synthetic amino acids HMeOPg and DMeOPg resulted in tricyclic glycopeptides, the majority of which were glucosylated at ^{4}HPg. In contrast to the products obtained by supplementation with the natural substrate DPg, no glycosylation with 4-oxovancosamine (ovcn) was detected (Table 1). Employment of the monosubstituted 3-HPg and its methoxy analogue 3-MeOPg as substrates also led to antibiotically active tricyclic derivatives. Glycosylation patterns comprised modifications with glucose, 4-oxovancosamine, and *ureido*-vancosamine (urvcn) in analogy to wild-type metabolites.[4] However, using 3-HPg and 3-MeOPg, bicyclic glycopeptides lacking the AB-ring biaryl were also detected. The absence of the AB-ring in the bicyclic glycopeptides was confirmed by LC-MS/MS experiments.

In a second set of feeding experiments, mandelic acid (MA) and phenylacetic acid (PA) analogues of HMeOPg, DMeOPg, 3-HPg, and 3-MeOPg were tested, since 3,5-dihydroxyphenylacetic acid (DPA)[9,10] and 3,5-dihydroxymandelic acid (DMA)[10] derivatives have both been postulated, albeit controversially, as precursors in the biosynthesis of DPg. Except for DPA, none of the phenylacetic acid analogues succeeded in being accepted as a substrate. Of the mandelic acid derivatives, both DMA and 3-HMA yielded balhi-

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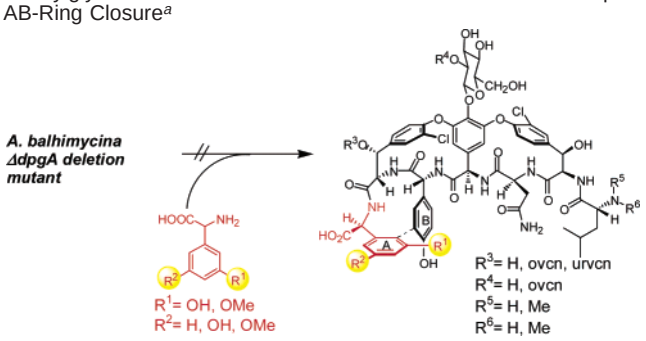
Table 1. Structures of Mutasynthetically Generated Balhimycins from Supplementation of Δ*dpgA* Mutant with Substituted Phenylglycines and the Influence of This Substitution Pattern upon AB-Ring Closure[a]



[M + H]+ m/z	AA[b]	R1	R2	R3	R4	R5/R6	AB ring[c]
1291.3*	3-HPg	OH	H	H	H	H/Me	−
1305.3*	3-HPg	OH	H	H	H	Me/Me	−
1129.2*,[d]	3-HPg	OH	H	H[d]	H	H/Me	−
1143.2*,[d]	3-HPg	OH	H	H[d]	H	Me/Me	−
1430.3	3-HPg	OH	H	ovcn[e]	H	H/Me	+
1303.2	3-HPg	OH	H	H	H	Me/Me	+
1628.5	3-MeOPg	OMe	H	urvcn[f]	ovcn[e]	H/Me	+
1487.4	3-MeOPg	OMe	H	urvcn[f]	H	H/Me	+
1430.2	3-MeOPg	OMe	H	ovcn[e]	H	H/H	+
1289.1	3-MeOPg	OMe	H	H	H	H/H	+
1303.1	3-MeOPg	OMe	H	H	H	H/Me	+
1291.2*	3-MeOPg	OMe	H	H	H	H/H	−
1305.2*	3-MeOPg	OMe	H	H	H	H/Me	−
1305.1	HMeOPg	OH	OMe	H	H	H/H	+
1319.1*	HMeOPg	OH	OMe	H	H	H/Me	+
1319.2*	DMeOPg	OMe	OMe	H	H	H/H	+

[a] According to LC-ESI-MS experiments. Major metabolites are indicated with an asterisk. The most probable orientation of substituents at the DPg moiety in the metabolites is shown. [b] Supplemented amino acid. [c] Indicates whether the AB ring (AA 5−7) is closed (+) or open (−). [d] Non-glucosylated (aglycon). [e] ovcn = 4-oxovancosamine (Figure 1). [f] urvcn = *ureido*-vancosamine (Figure 2).

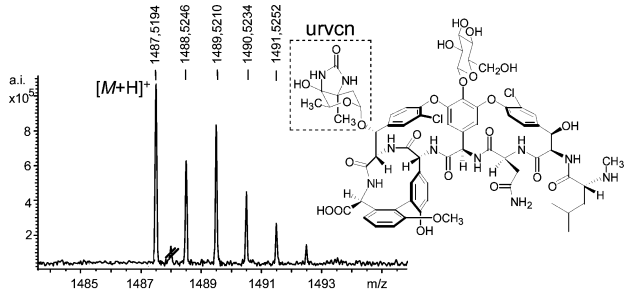


Figure 2. ESI-FTICR-MS (internal calibration) of two-fold chlorinated *ureido*-[7][3-MeOPg]-balhimycin; urvcn = *ureido*-vancosamine.

mycin derivatives. The acceptance of both mandelic and phenylacetic acids would favor the proposed DPA biosynthesis pathways.[9,10]

According to our results, the presence of at least one hydroxy or methoxy substituent in the 3-position at the phenylglycine is of crucial importance for its acceptance as a substrate for the formation of bicyclic and tricyclic aglycons. A 3,5-disubstitution pattern with either hydroxy or methoxy groups as well as a "mixed" substitution pattern (HMeOPg) is tolerated and leads to completely cyclized aglycons. Apparently, steric and electronic requirements for substrate recognition by the peptide synthetases to form the heptapeptide are met by the monosubstituted substrates 3-HPg and 3-MeOPg. The additional detection of bicyclic glycopeptides with 3-HPg/3-MeOPg suggests that narrow constraints on the orientation of the ring of this amino acid are posed by the oxygenase OxyC[12] in order to achieve AB-ring formation and thus the production of antibiotically active products. Consequently, the AB-ring bridge is expected to be formed exclusively in the position ortho to the electron-donating hydroxy or methoxy substituent. Interestingly, substrate specifity of the Δ*dpgA* mutant is more discriminating toward mandelic and phenylacetic acids in comparison to their amino acid analogues. This increased discrimination could be associated with their intracellular uptake and/or degradation, or preferably with an additional number of biosynthetic steps required before their loading onto the peptide synthetase.

In summary, we have shown that the mutasynthesis approach can be extended to the AB macrocycle, allowing the generation of a variety of antibiotically active vancomycin-type derivatives selectively modified at the DPg residue. Access to such compounds by synthetic routes is extremely laborious, and our approach is a first step toward a biotechnological generation of modified glycopeptide antibiotics.

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Supporting Information Available: General experimental procedures and characterization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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