Fluorobalhimycin—A New Chapter in Glycopeptide Antibiotic Research**

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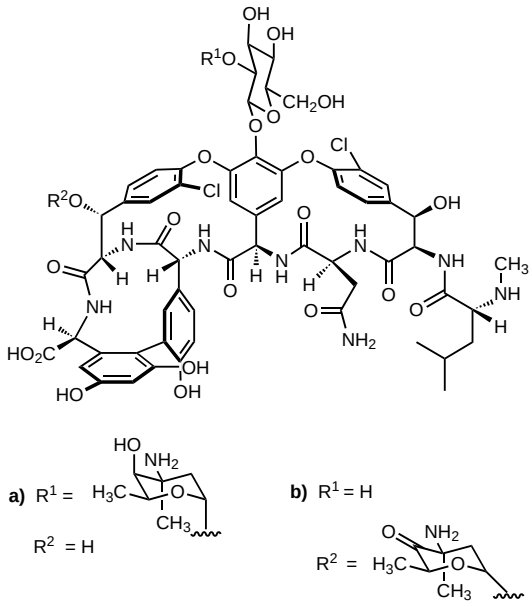
Dedicated to Professor Anton Rieker on the occasion of his 70th birthday

The glycopeptide antibiotic vancomycin (Scheme 1) has been used for more than 30 years against enterococcal and staphylococcal infections and meanwhile has the status of an antibiotic of last resort.[1] The significance of vancomycin together with its unique properties have attracted many researchers to perform work on chemical, biological, and medicinal aspects.[2] Over the past decade the increasing number of vancomycin-resistant bacterial strains has led scientists to investigate resistance mechanisms and to seek new approaches to overcome vancomycin-resistance problems.[2,3] As a consequence, a vast number of natural and semisynthetic glycopeptide derivatives have been characterized and tested. Considerable success was obtained with the semisynthetic derivative LY333328, a potent glycopeptide, which displays antibiotic activity against both methicillin-resistant *Staphylococcus aureus* strains (MRSA) and vancomycin-resistant enterococci (VRE) and which is meanwhile being tested in clinical studies.[4] However, these variations relate primarily to peripheral features of vancomycin and the characteristic structure of the aglycon has been left mainly unchanged because of the highly demanding chemical and biochemical accessibility of the tricyclic aglycon.



Scheme 1. Structures of the glycopeptide antibiotics a) vancomycin and b) balhimycin.

Herein we present the first in vivo modified glycopeptides with non-natural fluorine substituents. In previous contributions,[5,6] we used the balhimycin-producing[7] (Scheme 1) strain *Amycolatopsis mediterranei* to obtain insights into the biosynthesis through gene disruption mutants. Based on these results we focused on the two diastereomeric 3-chloro-β-hydroxytyrosine moieties (Scheme 1) as an attractive target for the introduction of structural variations, since they represent a basic element of the tricyclic aglycon structure. The presence of a chlorine substituent in each of the two β-hydroxytyrosine moieties in natural balhimycin plays a significant role for enhancing antibiotic activity.[8] From the *A. mediterranei* wild-type strain (Figure 1, 1a) we generated a deletion null-mutant in the *bhp* gene (Figure 1, 1b), OP696,[6] which is deficient in β-hydroxytyrosine biosynthesis and thus is unable to produce balhimycin (Figure 1, 3a). In mutasynthesis experiments,[9] we supplemented OP696 with several analogues of this amino acid. β-Hydroxytyrosine and derivatives were easily obtained as a racemic mixture of four stereoisomers in good yields by using a three-step synthesis.[10]

Supplementing a culture of OP696 with 3-*f*luoro-β-*h*ydroxy*t*yrosine (3-Fht) resulted in antibiotic activity of the culture filtrate against the indicator strain *Bacillus subtilis* (Figure 1, 3b). The amino acid 3-Fht did not show any antibiotic activity. Isolation from culture filtrates revealed an antibiotically active compound, and subsequent analysis with ESI-FTICR mass spectrometry revealed a mass of $[M+2H]^{2+} =$ 707.7439 Da ($\Delta m = 0.3$ ppm; Figure 2) that corresponds to the molecular mass of 1413.4878 Da and to the elemental composition $C_{66}H_{73}F_2N_9O_{24}$ of a twofold fluorinated balhimycin, which we named fluorobalhimycin (Figure 1).

One- and two-dimensional NMR experiments (TOCSY, NOESY, HSQC, HMBC; Tables 1 and 2) allowed the structure to be assigned as shown in Figure 1. The presence of two fluorine substituents in fluorobalhimycin was verified independently by ^{19}F NMR spectroscopy with $\delta(^{19}F) = -131$ and -134 ppm compared to $\delta(^{19}F) = -139$ ppm for the racemic amino acid 3-Fht. The positioning of the fluorine substituents in the biaryl ether ring systems is analogous to that of the chlorine atoms in balhimycin,[8,11] as confirmed by the observed NOE contacts.

In further experiments we evaluated the substrate specificity of the mutant OP696. To this end, we synthesized three sets of β-hydroxytyrosines (Scheme 2). The first set comprised β-hydroxytyrosines with the phenolic hydroxy group in 2-, 3-,

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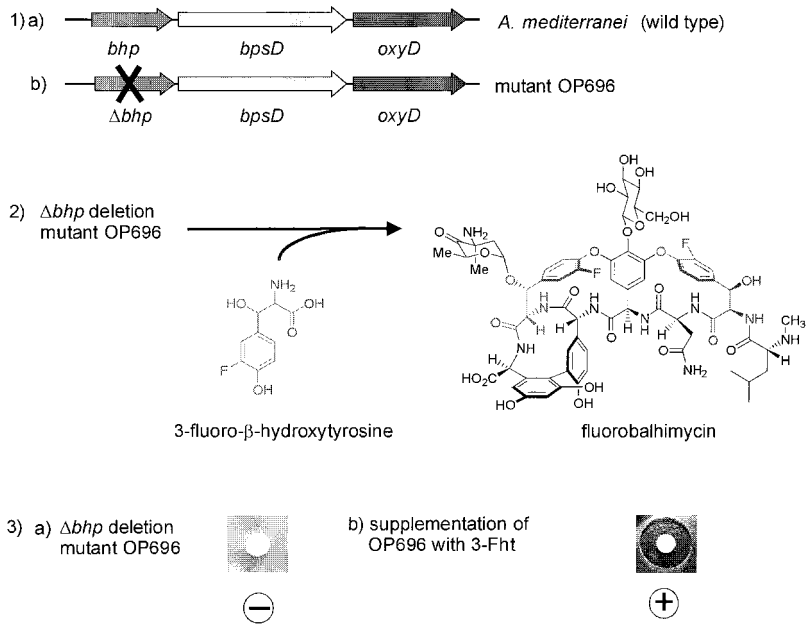


Figure 1. 1) Schematic representation of the part of the gene organization of *A. mediterranei* strains involved in β-hydroxytyrosine biosynthesis: a) wild type, b) mutant OP696; *bhp*: balhimycin perhydrolase; *bpsD*: balhimycin peptide synthetase D; *oxyD*: oxygenase D.[8] 2) Pathway to fluorobalhimycin by supplementation of mutant OP696. 3) Bioassays of culture filtrates tested against *B. subtilis*: a) mutant OP696 (no antibiotic activity), b) mutant OP696 after supplementation with 3-fluoro-β-hydroxytyrosine (shows antibiotic activity).

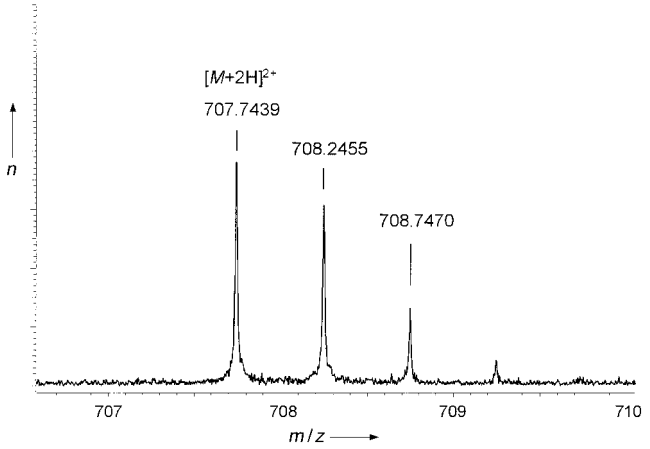


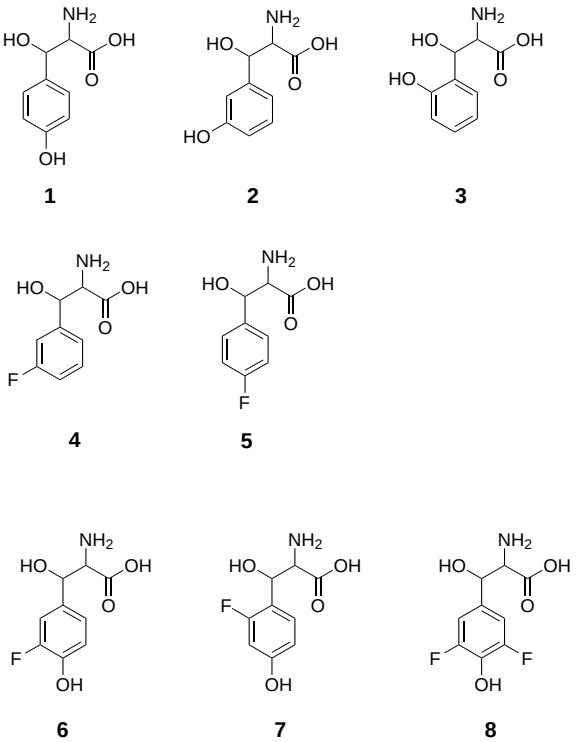
Figure 2. ESI-FTICR mass spectrum of fluorobalhimycin, $[M+2\,H]^{2+}$ = 707.7439 Da.

and 4-position of the aromatic ring (hydroxyphenylserines **1**–**3**). The second and third set was composed of fluorophenylserines (**4** and **5**) and differently fluorine-substituted β-hydroxytyrosines (**6**–**8**) (Scheme 2). From the tested derivatives **1**–**3,** only feeding with β-hydroxytyrosine (**1**) resulted in antibiotic activity of the culture filtrate.[6] Shifting of the hydroxy function into the 2- and 3-position of the aromatic ring (**2** and **3**) led to no antibiotic activity of thus supplemented culture filtrates and no corresponding metabolites could be detected by LC-ESI-MS. The same negative results as for **2** and **3** were also obtained for supplementation with fluorophenylserines **4** or **5** and D- or L-tyrosine, respectively.

Interestingly, feeding mutant OP696 with amino acids **7** or **8** led to antibiotic activity of the culture filtrates against *B. subtilis* and to detection of corresponding molecular masses of balhimycin derivatives with LC-ESI-MS (data not shown).

Our results show that the β-hydroxytyrosine motif (**1**) is essential for acceptance as a substrate in the subsequent interplay of various glycopeptide biosynthesis enzymes with peptide synthethases and oxygenases[12] as the most directly affected ones. This cascade of interacting enzymes differentiates on the one hand between β-hydroxylated (**1**–**8**) and non-β-hydroxylated tyrosines (D- and L-Tyr) and between derivatives bearing (**1, 6**–**8**) or missing (**2**–**5**) the phenolic hydroxy group in the 4-position of the arene. However, substitution of aromatic hydrogen atoms with fluorine in position 3 or 2 of the arene (**6** or **7**) or even addition of a second fluorine substituent in position 5 of the arene (**8**) is tolerated.

The aim of our current studies is the structure elucidation of all detected fluorobalhimycins, and the determination of the configuration of the β-hydroxytyrosine stereoisomers that are accepted as substrates. We are further investigating the



Scheme 2. Structural formulas of synthetic β-hydroxyamino acids used for supplementation assays: β-hydroxytyrosine (**1**), (3-hydroxyphenyl)serine (**2**), (2-hydroxyphenyl)serine (**3**), (3-fluorophenyl)serine (**4**), (4-fluorophenyl)serine (**5**), 3-fluoro-β-hydroxytyrosine (**6**), 2-fluoro-β-hydroxytyrosine (**7**), 3,5-difluoro-β-hydroxytyrosine (**8**).

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Table 1. ^{1}H and ^{13}C NMR shifts of fluorobalhimycin.[a]

	N	α	β	Others
[1]Leu	[b]	3.14	1.45/1.39	γ: 1.69; δ: 0.84/0.88, N-CH_3: 2.32
		62.1	40.3	γ: 24.1; δ: 22.4/22.9, N-CH_3: 33.3
[2]Fht	7.92	4.79	5.14	OH^{γ}: 5.83; 2: 7.17;
		58.3	71.3	1: 137.4; 2: 115.4; 3: 140.9; 4: 154.0;
				5: 7.34; 6: 6.92
				5: 124.3; 6: 115.3
[3]Asn	[b]	4.34	2.13/2.13	δ: 7.36/6.96
		51.1	37.0	
[4]Hpg[c]	8.29	5.79	–	2: 5.76;
		54.6	–	1: 132.6; 2: 107.8; 3: 151.7; 4: [b];
				6: 5.39
				5: 151.9; 6: 104.6
[5]Hpg[c]	8.83	4.59	–	2: 7.15; OH^{4}: 9.45
		53.7	–	1: 135.8; 2: 136.9; 3: 122.1; 4: 155.4
				5: 6.70; 6: 6.76
				5: 116.4; 6: 125.9
[6]Fht	6.63	4.34	5.26	2: 7.63;
		60.6	74.7	1: 134.7; 2: 115.6; 3: 140.7; 4: 154.2;
				5: 6.67; 6: 7.32
				5: 125.8; 6: 124.3
[7]Dpg[d]	8.52	4.42	–	4: 6.39; $OH^{3,5}$: 9.14
		56.7	–	1: 136.3; 2: 117.9; 3: 157.4; 4: 102.5;
				6: 6.22;
				5: 157.2; 6: 105.9

[a] in ppm, $c = 1.2$ mg mL^{-1}, [D_6]DMSO, 298 K. [b] assignment not determined. [c] Hpg = hydroxyphenylglycine, [d] Dpg = dihydroxyphenylglycine.

Table 2. ^{1}H and ^{13}C chemical shifts of the sugar residues of fluorobalhimycin.[a]

Sugar	1′	2′	3′	4′	5′	6′	3′-Me
Glc[b]	5.32	3.33	3.26	3.20	3.22	3.65/3.47	–
	102.7	74.3	76.8	70.1	77.8	61.2	–
Ovn[c]	4.95	2.24/2.34	–	4.43	–	1.23	1.18
	93.3	39.3	57.8	68.7	208.7	14.9	20.6

[a] In ppm, $c = 1.2$ mg mL^{-1}, [D]$_6$DMSO, 298 K. [b] Glucose. [c] 4-Oxovancosamine.

scope of the *A. mediterranei* OP696 mutant to elucidate whether other substituted β-hydroxytyrosines and fluoro-β-hydroxytyrosines are accepted as substrates. Our results show that mutasynthesis can be used for creating a set of new glycopeptide antibiotics using building blocks that should be readily accessible in combination with genetically engineered mutants. This approach may also be successful in modifying other parts of the glycopeptide molecule, for example the two other aromatic amino acids 4-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine. In summary, we have a tool at hand to generate novel, hitherto unknown glycopeptide antibiotics, with a perspective towards antimicrobial activity also against vancomycin-resistant organisms.

Experimental Section

The mutant OP696 of *Amycolatopsis mediterranei DSM5908* was grown in liquid culture according to protocols described previously.[5,6] Before inoculation with the mutant, amino acids were added to the medium in a final concentration of 0.4 g L^{-1}. Fluorobalhimycin was isolated by preparative LC-MS from 6 L of culture filtrate supplemented with 3-Fht (yield: 0.7 mg; HTP-MS System from Merck-Hitachi) and analyzed according to reported procedures.[7,8] NMR experiments were recorded on an Avance 600 NMR spectrometer (Bruker) equipped with a 2.5 mm DUAL ^{13}C-^{1}H-D Z-GRAD microsample head and on an AMX 600 NMR spectrometer (Bruker) equipped with a 5 mm Z-GRAD triple resonance probehead. ^{19}F NMR measurements ([D_6]DMSO, ^{1}H–^{19}F decoupled experiment) were performed on an Avance DRX 250 NMR spectrometer (Bruker). Mutasynthesis experiments of amino acids were repeated three times, and the culture filtrates obtained were tested against *B. subtilis*[6] and investigated with LC-ESI-MS on an Agilent 1100 HPLC System (Hewlett–Packard) coupled with an Esquire 3000plus ESI-ion trap mass spectrometer (Bruker Daltronics). FTICR-ESI mass spectra were recorded on an APEX II FTICR Mass Spectrometer (Bruker-Franzen).

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