Bromobalhimycin and Chlorobromobalhimycins—Illuminating the Potential of Halogenases in Glycopeptide Antibiotic Biosyntheses

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Glycopeptide antibiotics are of considerable clinical importance as last-resort antibiotics against staphylococcal and enterococcal infection. Intensive research has focussed on chemical, biological, and medicinal aspects of glycopeptide antibiotics.[1] Only recently, details of central features of their biosynthesis have been revealed.[2]

Structure – activity relationship (SAR) studies on glycopeptide antibiotics and semisynthetic derivatives show that the three characteristic biaryl/biarylether rings formed by cross-linking of the aromatic sidechains are essential features for an efficient binding to the D-Ala-D-Ala peptide motif of the bacterial cell wall.[3, 4] Minor changes in the structure can lead to dramatic losses of antibiotic activity.[3, 4] While linear and differently mono- and dibridged heptapeptides proved to be antibiotically inactive,[2] variation of the carbohydrate moieties as "peripheral features" has a much less pronounced effect on D-Ala-D-Ala-binding. Comparative binding studies and minimal inhibitory concentration (MIC) tests performed on a limited number of structurally related glycopeptides have shown that chlorine atoms attached to β-hydroxytyrosine in amino acid positions 2 and 6 strongly enhance the antibiotic activity; the 2-position is of particular importance in this respect.[3, 5] Contributions by the Williams group[1a, 6] delivered a mechanistic hypothesis for the enhancing effect of chlorine substituents on the mechanism of dimerization and thus on D-Ala-D-Ala binding with its implications for antibiotic activity.

Herein, we describe novel, hitherto unknown vancomycin-type bromobalhimycins, chlorobromobalhimycins, and other brominated glycopeptide antibiotics of subtypes I – III.[1b] This work gives insight into the role of halogen substitutions in the antibiotic activity of this family of compounds and complements previous contributions on the role of halogenases in glycopeptide antibiotic biosyntheses.[7]

Our experiments are based on the total substitution of chloride by the other halogenide salts fluoride, bromide,[8] and iodide in the culture media of *Amycolatopsis balhimycina*[9] (formerly assigned as *Amycolatopsis mediterranei*[10]), the strain producing vancomycin-type balhimycin (**1**; Scheme 1). Bromide supplementation was originally described for griseofulvin,[11] tetracyclin,[12] and bromothricin.[13] While fluoride and iodide supplementation proved toxic to *A. balhimycina* cultures, fermentation with bromide salts yielded an antibiotically active compound. Purification of this compound from bromide-containing media was performed according to previously published procedures.[2, 14] A doubly charged ion $[M+2H]^{2+}$ with a mass to charge ratio of = 767.6643 amu was found with high resolution FTICR-MS that corresponds to $C_{66}H_{73}Br_2N_9O_{24}$ ($[M+2H]^{2+}$ = 767.6640 amu) with a relative mass error (Δ) of 0.3 ppm, and displays the expected characteristic isotopic pattern of two-fold brominated balhimycin (Figure 1 d). Structural elucidation by means of 2-dimensional NMR experiments (COSY, TOCSY, NOESY, HSQC, HMBC) revealed a structure analogous to that of balhimycin but with both chlorine atoms substituted by bromine (Table 1). NOESY experiments showed that the positions of the bromine atoms correspond to those of chlorine in balhimycin. The compound was therefore named bromobalhimycin (**2**).

The fact that *A. balhimycina* normally produces balhimycin (**1**) with two chlorine atoms prompted us to investigate whether the biosynthetic enzymes display selectivity for chlorine as compared to bromine. Culture media were supplemented with equimolar amounts of bromide and chloride salts. Mass spectrometric analysis of the resulting culture filtrates revealed a mixture of balhimycin (**1**), bromobalhimycin (**2**), and surprisingly a balhimycin derivative with one bromine and one chlorine atom (Figure 1 c), obtained in an approximately 1:1:2 ratio, consistent with statistical incorporation of Br or Cl atoms. Chlorobromobalhimycins carrying one bromine and one chlorine atom can theoretically exist as two positional isomers. Attempts to further separate these two possible isomers failed and mass spectrometric fragmentation of the tricyclic aglycon

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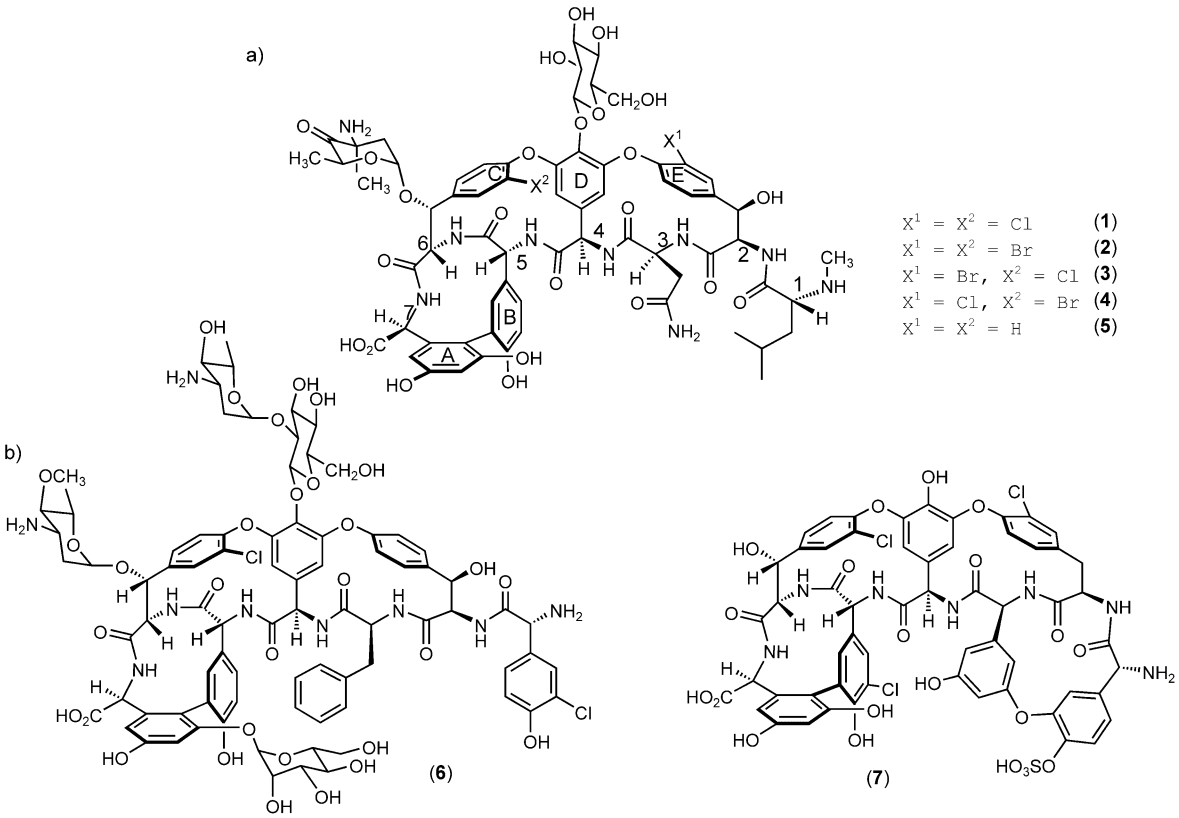

Scheme 1. *a) Structures of the glycopeptide antibiotics balhimycin (**1**), bromobalhimycin **2**, chlorobromobalhimycins **3**, and **4**, and dechlorobalhimycin (**5**). b) Glycopeptide antibiotics actinoidin B (Type II; **6**), and A47934 (Type III; **7**), amenable to bromide supplementation.*

with ESI-MS/MS experiments rendered no utilizable fragments. In order to prove the existence of two chlorobromobalhimycin isomers, a previously characterized balhimycin biosynthesis mutant[2b] that produced a monocyclic peptide precursor amenable to mass spectrometric fragmentation was grown under conditions of bromide/chloride supplementation. Mass spectrometric fragmentation of this peptide and unambiguous assignment of the fragments (Figure 2) revealed no preference for bromide substitution in amino acid positions 2 and 6 of the monocyclic peptide. As a consequence, we concluded that the observed chlorobromobalhimycin compounds do indeed consist of two isomers, with no positional selectivity for the introduction of a bromine or chlorine atom (**3**, **4**; Figure 1c).

In order to study the effects of halogen substitution on antibiotic activity we performed agar diffusion tests against *Bacillus subtilis* with balhimycin (**1**), bromobalhimycin (**2**), and dechlorobalhimycin (**5**); the last compound bears no halogen substitution. In these tests, the dechlorinated glycopeptide (**5**) clearly showed reduced antibiotic activity, bromobalhimycin and balhimycin behaved similarly to one another. Subsequently, MIC tests were performed on selected Gram-positive strains. While dechlorobalhimycin was consistently less active than the other two compounds, bromobalhimycin and chlorobromobalhimycin showed antibiotic activity comparable to that of balhimycin against all enterococcal, staphylococcal, and streptococcal strains tested, with the exception of *Staphylococcus aureus* Smith, against which bromobalhimycin displayed diminished activity. (Table 2). All compounds, however, were equally inactive against vancomycin-resistant Enterococci.

Finally, the general applicability of the bromide supplementation approach was tested with other glycopeptide antibiotics. Producer strains of representative Type-I – IV glycopeptide antibiotics were cultivated in bromide- or chloride/bromide-containing culture media. For vancomycin (Type I; *Amycolatopsis orientalis*), actinoidin B (**6**; Type II; *Amycolatopsis keratinophila* subsp. *nogabecina*), and antibiotic A47934 (**7**; Type III; *Streptomyces toyocaensis* NRRL15009) the corresponding bromo- and bromo/chloroderivatives were detected by HPLC-ESI-MS and ESI-FTICR-MS. ESI-FTICR mass spectra of representative examples for actinoidin B and A47934 (Scheme 1) bearing various bromine substitution patterns are shown in Figure 1. With the teicoplanin producer (Type IV; *Actinoplanes teichomyceticus*) however, bromination has not yet been achieved, possibly as a result of fermentation problems.

We have used the approach described above to exploit the biosynthetic flexibility of the glycopeptide antibiotic producer strains to generate novel structural diversity. The possibility of bromine substitution by media supplementation seems to be valid for the halogens in the C-*O*-D and D-*O*-E rings of chlorine-containing glycopeptide antibiotics, that is, the halogenation of β-hydroxytyrosine and tyrosine (A47934) residues. Interestingly, glycopeptides actinoidin B and A47934 both contain a hydroxyphenylglycine moiety that is amenable to bromination. From the biosynthesis gene cluster of A47934 from *S. toyocaensis*

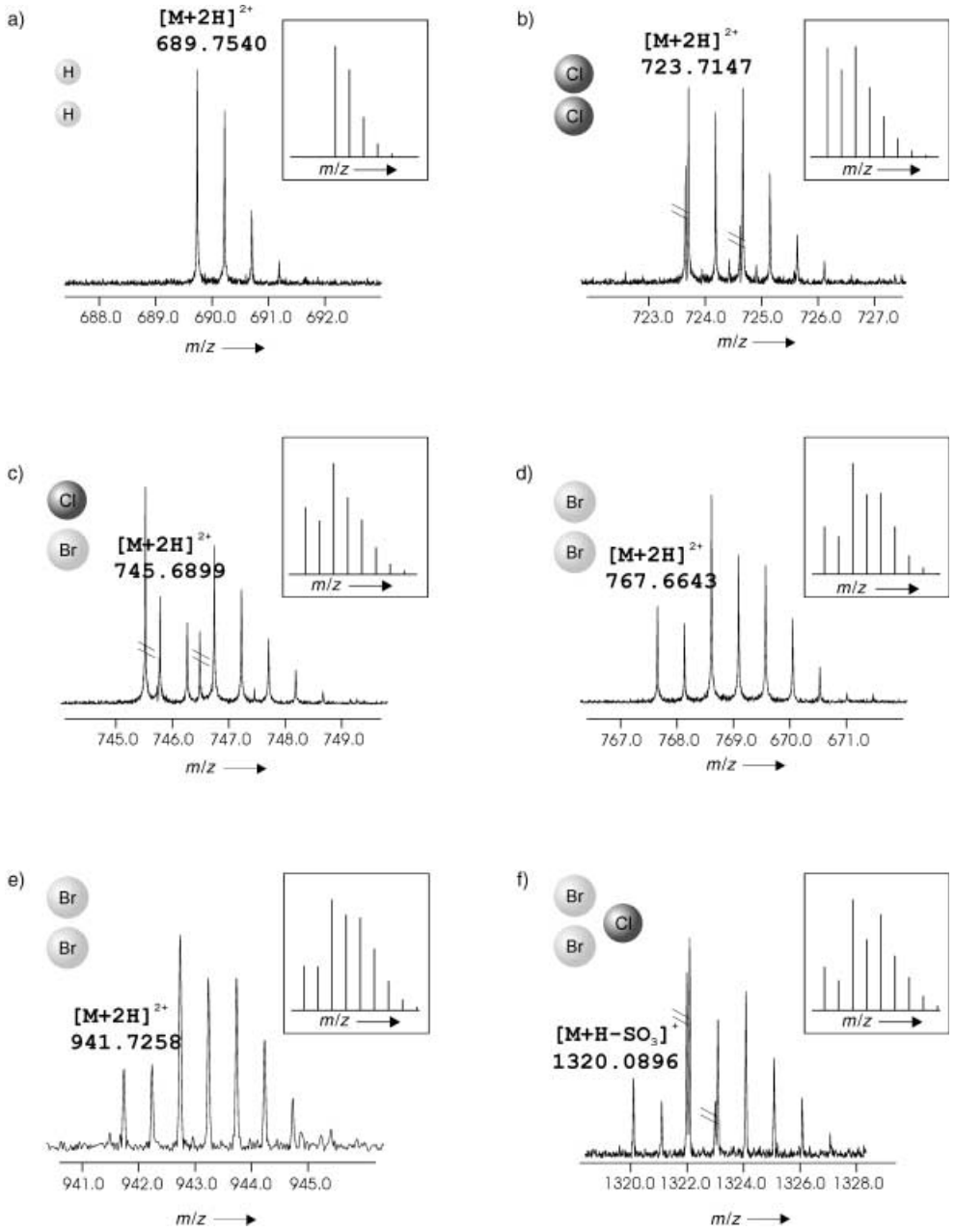

Figure 1. *ESI-FTICR mass spectra of a) dechlorobalhimycin (**5**), b) balhimycin (**1**), c) chlorobromobalhimycins **3** and **4**, and d) bromobalhimycin (**2**) showing the doubly protonated molecular ions $[M+2H]^{2+}$. Representative examples for Type II and III glycopeptide antibiotics: e) $[M+2H]^{2+}$ ion of bromoactinoidin B (**6**; 2 × Br) and f) $[M+H-SO_3]^{+}$ ion of A47934 (**7**; 1 × Cl, 2 × Br). The isotopic patterns observed are compared with those calculated for the proposed structure (inserts). Crossed peaks originate from the internal mass calibration standards (polyethylene glycol 600 and polypropylene glycol 1020).*

NRRL15009 two halogenase genes are known,[15] which, in accordance with our results both accept bromide as a substrate. Our results indicate that most of the halogenases involved in glycopeptide biosynthesis can equally well use bromine or chlorine atoms and that the natural occurrence of chlorine-modified glycopeptides is thus due to the predominance of chloride over bromide in common fermentation media. This situation clearly contrasts with the halogenases from marine organisms, where the ratio of chlorine to bromine of approximately 300:1 in seawater necessitates high bromine specificity for those enzymes involved in the synthesis of brominated compounds.[16] Thus, glycopeptides are an interesting model for studying halogenases and their substrate specificities. It is noteworthy that bromobalhimycin maintains full antibiotic activity despite the differences in atomic radii between bromine, chlorine and hydrogen (H: 120 pm; Cl: 181 pm; Br: 195 pm).[17] According to X-ray structures of balhimycin dimers, it was suggested by Sheldrick,[18] that the chlorine substituent of the 2-position projects into a pocket of the other dimer partner and thus stabilizes the dimerization mechanism. Our results imply similar effects for the dimerization of bromobalhimycins.

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

	N/C′ δ [ppm]	α δ [ppm]	β δ [ppm]	others δ [ppm]
[1] Leu	9.00	4.16	1.61/1.61	γ: 1.59; δ: 0.84/0.86; N–CH_3: 2.63
	169.7	58.9	38.8	γ: 23.5; δ: 22.2; N–CH_3: 31.0
[2]Bht	8.38	5.08	5.22	OH^{γ}: 5.71; 2: 7.37;
	168.4	59.0	74.2	1: 139.3; 2: 131.7; 3: 116.1; 4: 150.8
				5: 7.43; 6: 7.95
				5: 124.6; 6: 132.3
[3]Asn	7.65	4.88	2.31/2.31	δ: 7.39/6.56
	168.8	51.6	38.1	γ: 169.9
[4]Hpg	8.08	6.37	-	2: 5.41
	169.4	53.5	-	1: 131.9; 2: 104.6; 3: 151.8; 4: 132.6
				6: 5.25
				5: 152.1; 6: 103.9
[5]Hpg	8.64	4.46	-	2: 7.12; OH^{4}: 9.46
	166.9	53.7	-	1: 135.7; 2: 135.6; 3: 121.7; 4: 154.9
				5: 6.70; 6: 6.77
				5: 116.0; 6: 125.6
[6]Bht	8.48	4.92	5.18	2: 7.62
	166.3	58.7	70.1	1: 139.7; 2: 131.2; 3: 116.5; 4: 151.7
				5: 7.27; 6: 7.57
				5: 124.2; 6: 128.7
[7]Dpg	8.57	4.49	-	$OH^{3,5}$: 9.28; 4: 6.42
	172.1	56.4	-	1: 135.8; 2: 117.4; 3: 157.3; 4: 102.1
				6: 6.21
				5: 157.3; 6: 105.1

	1′	2′	3′	4′	5′	6′
Glc[b]	5.36	3.39	3.26	3.28	3.43	3.70/3.46
	102.7	74.3	77.1	69.3	76.1	60.6

	1′	2′	3′	4′	5′	6′	3′-Me
Ovn[c]	4.97	2.32/2.53	-	-	4.56	1.29	1.22
	92.2	40.6	49.5	207.9	66.0	14.0	28.5

[a] [bromobalhimycin] = 20.5 mg mL^{-1}, [D_6]DMSO, 298 K; Bht = bromo-β-hydroxytyrosine. [b] Glucose chemical shifts in ppm. [c] 4-Oxovancosamine chemical shifts in ppm.

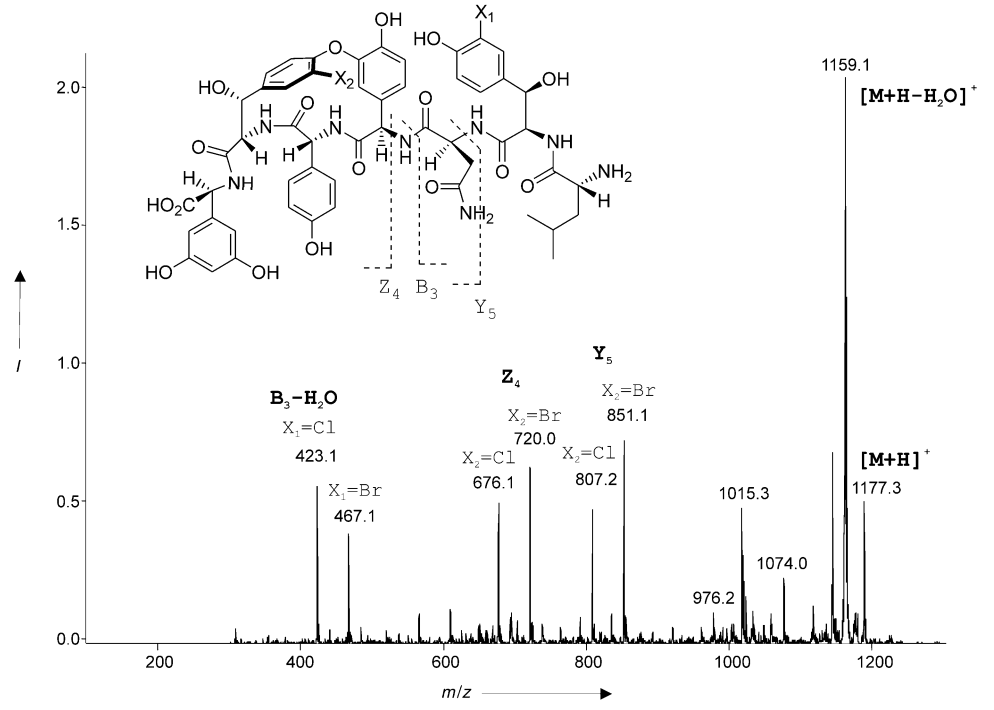

Figure 2. *ESI-MS/MS mass spectrum of monocyclic chlorobromobalhimycin heptapeptide.[2b] The occurrence of fragment pairs ($\Delta m_{Br-Cl} = 44$ amu) suggests the presence of two regioisomers for chlorobromobalhimycin.*

Table 2. Results of MIC test against Gram-positive microorganisms.

Microorganism	MIC [μg mL^{-1}] Balhimycin (**1**)	MIC [μg mL^{-1}] Bromobalhimycin (**2**)	MIC [μg mL^{-1}] Dechlorobalhiycin (**5**)	MIC [μg mL^{-1}] Vancomycin
S. aureus Smith	0.5	4	4	2
S. pyogenes[a]	0.5	0.5	8	0.5
E. faecalis[b]	0.5	0.5	4	1
E. faecium[b]	1	0.5	8	2

[a] *Streptococcus pyogenes*. [b] Enterococci.

Together with the previously presented mutasynthetically generated fluorobalhimycin,[14] we have a number of new halogen-substituted glycopeptides at hand to further systematically investigate the effects of size and polarity of the halogen substituents on glycopeptide conformation, D-Ala-D-Ala-binding, and the dimerization mechanism. More detailed investigations by means of NMR spectroscopy, X-ray crystallography, and thermocalorimetry are in progress. Furthermore, in order to increase diversity of glycopeptide structures and function, the brominated derivatives might be suitable for triggering chemical modifications. A prime example is the Suzuki reaction, for which mild conditions have been developed suitable for the synthesis of *ortho*-substituted bromoarenes.[19, 20] The feasablity of this approach is currently being investigated.

Experimental Section

The following bacterial strains were used: *Amycolatopsis balhimycina* DSM 44591 (Balhimycin), *A. orientalis* (Vancomycin), *A. keratinophila* subsp. *nogabecina* DSM 44589 (Actinoidin B), *Streptomyces toyocaensis* NRRL 15009 (A47934), *Actinoplanes teichomyceticus* DSM 43866 (Teicoplanin). Bacteria were grown in liquid culture according to protocols described previously.[2, 7, 14] The inorganic chlorides $CaCl_2$ and $MgCl_2$ were substituted by the corresponding bromides in equimolar ratios. Bromobalhimycin was isolated from 6 L culture filtrate (yield: 30.4 mg) by using a prepLCMS Merck – Hitachi HTP-MS System (Merck, Darmstadt, Germany) and analyzed according to reported procedures.[14] LC-MS experiments were peformed on a Bruker Esquire 3000 plus instrument (Bruker Daltonics, Bremen, Germany) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). FTICR-ESI-MS spectra were recorded on an APEX II FTICR mass spectrometer (Bruker Daltonics). NMR experiments were recorded on an AMX 600 NMR spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5-mm triple-resonance probehead with z-gradients. The bacterial strains used for MIC determinations were obtained from Biosearch Italia (Gerenzano, Italy). MICs were determined by the broth microdilution method according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997) in Mueller – Hinton broth (Difco Laboratories), adjusted to contain 20 mg L^{-1} $CaCl_2$ and 10 mg L^{-1} $MgCl_2$. The starting inoculum was 5×10^5 CFU mL^{-1} for all bacteria. Cultures were incubated at 37 °C for 24 h.

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