Backbone dynamics and amide proton exchange at the two sides of the eremomycin dimer by ^{15}N NMR†

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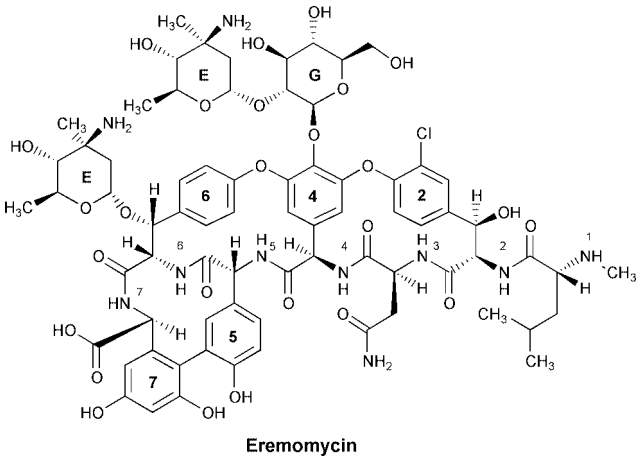
^{15}N-labelling of the glycopeptide antibiotic eremomycin has made possible the dynamic characterization of the two sides of the dimer; in the presence of acetate anion the backbone amides are dynamically equivalent and the fastest amide proton exchange rate was found at the heart of the binding pocket.

For quite a long time vancomycin antibiotics were potentially considered as a last line of defence against *methicillin resistant Staphylococcus aureus* (MRSA). However, vancomycin resistance of Gram-positive bacteria is an increasingly emerging health problem worldwide. The mode of action of glycopeptide antibiotics is the binding of cell wall fragments terminating in the D-Ala-D-Ala sequence to the carboxylate anion binding pocket of the antibiotic. This essential interaction is diminished by the development of a lactate type terminus in the resistant bacteria. Many members of the glycopeptide antibiotics family have been shown to form asymmetric dimers in water solution and also in the crystal form. Dimerisation cooperatively enhances binding to the cell wall, while a lipophilic sidechain was shown to be a useful membrane anchoring device in teicoplanin. Eremomycin[1] is known as the strongest homodimer among the vancomycin antibiotics. Its ^{1}H/^{13}C NMR assignment has been presented either under fast exchange conditions[2] or as partial ^{1}H assignment for the two halves of the dimer in the presence of unnatural ligands.[3] Though potentially useful for monitoring H-bonds, ^{15}N NMR assignment was reported in an early report only for vancomycin in DMSO solution,[4] where dimers are not formed.



Eremomycin

Here we demonstrate that ^{15}N labelling allows the detection of all amides in the dimeric eremomycin,‡ allowing thereby more insight into the dynamics and amide proton exchange of the heptapeptide aglycone. In order to enhance the differences between the chemical shifts of the two sides it is useful to load the antibiotic with a weakly binding ligand. In the present case the acetate buffer itself will do for this purpose. We used 2 mM acetate buffer, pH = 3.9 at 280 K. Under these conditions both binding sites of the more abundant dimer (> 95%) are partially occupied by the acetate anion. According to the published[5] binding constant K_{bind}(w2*) = 19.5 dm^3 mol^{-1}, K_{bind} (w2) = 8.8 dm^3 mol^{-1}, we can calculate that w2* and w2 sites are 49% and 30% loaded with acetate, respectively. Indeed, using the w2*, w2 limiting proton chemical shifts[6] we obtain 0.52 ppm difference between the two signals in complete agreement with our HSQC experiment (Fig. 1). On the other hand, acetate exchange must be fast since we observe only two signals for each amide in the HSQC spectrum. Though the water flip-back experiment[7] is not too sensitive to water saturation transfer effects, we see that the w2 signals is a bit smaller when compared to w2* (Fig. 1), according to a higher solvent access probability at the less loaded site. If in a twin HSQC experiment we selectively presaturate (3s) the water resonance intentionally, in the difference of the two spectra we map the amide exchange rates at the two sides of the eremomycin dimer. There is a slight difference between w2 and w2* as shown in Fig. 2, but more importantly this figure clearly demonstrates that the very heart of the binding pocket is saturated most effectively. This might suggest that w2 is the most solvent exposed site. However, efficient saturation of a buried amide could happen indirectly as well. Going from the N-terminus to the C-terminus saturation is gradually reduced, and the two halves of the dimer seem to be equivalent. Excluding solvent from the dimer interface is in accordance with the H-bonding network proposed

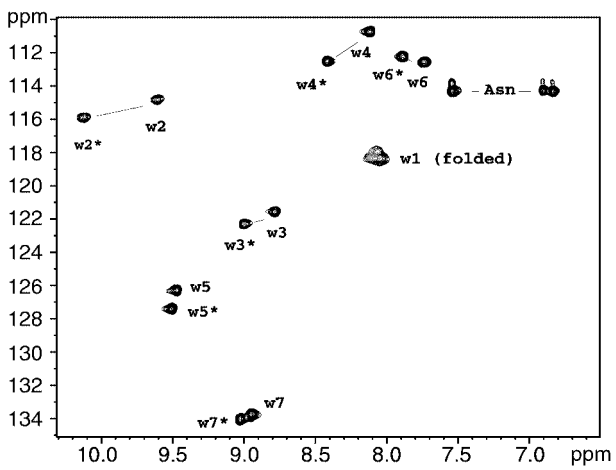


Fig. 1 ^{15}N-^{1}H HSQC spectrum (Bruker DRX-500) of the eremomycin dimer in acetate buffer. Assignments were obtained from ^{15}N-TOCSY-HSQC, ^{13}C-HSQC and homonuclear NOESY spectra. ^{1}H assignments of w2, w3 and w4 are in agreement with Bardsley and Williams.[5] w2* identifies the epi-vancosamine capped side of the dimer.

† Electronic supplementary information (ESI) is available: eremomycin production and isolation of ^{15}N eremomycin. See http://www.rsc.org/suppdata/cc/b0/b010144n/

Table 1 S^2 order parameters of the backbone amides as determined at two temperatures from the model-free analysis of ^{15}N relaxation data. Typical errors of S^2 are ±0.02 as obtained from Monte-Carlo error estimation. For the Asn3 sidechain NH_2 groups $S^2 = 0.33 \pm 0.04$. Average deviation between measured and calculated T_1,T_2 and NOE values are less than 3, 4 and 6%, respectively

Temp.	2*	2	3*	3	4*	4	5*	5	6*	6	7*	7
280 K	0.84	0.86	0.93	0.91	0.82	0.84	0.89	0.92	0.87	0.87	0.86	0.86
275 K	0.88	0.86	0.92	0.93	0.83	0.83	0.91	0.88	0.87	0.88	0.88	0.90

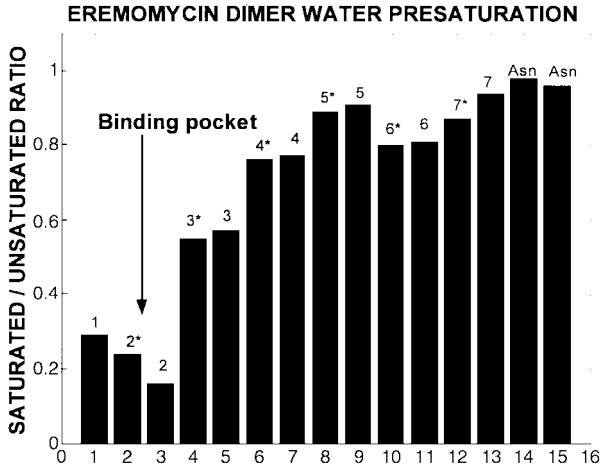


Fig. 2 Efficacy of water saturation transfer measured as a ratio of HSQC experiments with and without 3 s on-resonance selective pre-irradiation of the water protons. The lowest percentage value indicates the highest saturation effect. A similar pattern was observed in a NOESY experiment for the resolved signals (not shown).

to explain the head to tail dimer. There are some interesting points worth mentioning on the NH_2 group of the asparagine sidechain. It appears to be completely hidden for proton exchange with water, suggesting a strongly hydrophobic shielding at the back of the binding pocket. Furthermore, in the HSQC spectrum it exhibits an additional, low intensity (15% of the main signal) set of resonances. For the vancomycin dimer Sheldrick[8] showed that in the crystal, one of the binding pockets is occupied by an acetate ion and the other one is closed by the asparagine sidechain, which occupies the place of a ligand. He also suggested that the acetate loaded binding pocket exhibits high flexibility but the closed binding pocket is relatively rigid and the asparagine sidechain may hold the binding pocket in a suitable conformation for peptide docking. For the water solution of dimeric eremomycin now we see that both binding pockets are accessible at least for the acetate. In our ROESY experiments we failed to observe longer residence time structural water close to the amides. In order to test the rigidity of the dimer we measured the usual ^{15}N relaxation parameters (T_1,T_2 and NOE) and applied the standard model-free[9] evaluation generally used for proteins. Table 1 shows the S^2 order parameters from these experiments. All order parameters are above 0.8 (1 stands for completely restricted motion) and the differences between the exchanging sites are within experimental error. Evaluation of some resolved exchange peaks of an off-resonance ROESY eperiment (τ_{mix} = 50 ms) using the initial rate approach yields $k = 1 \pm 0.1\ s^{-1}$ for the flip-flop rate of the disaccharide moiety. Repeating the measurements at 275 K decreases the apparent exchange rate to $k = 0.5\ s^{-1}$. The control relaxation study at the lower temperature gives essentially similar S^2 pattern with a global correlation time increased from 3.7 to 4.1 ns. It appears, that the most rigid part of the dimeric backbone is around the asparagine (w3,w3*) and w5,w5*. This may be a sign of stabilizing H-bonding interactions either to the ligand or within the homo-dimer. At the same time the Asn sidechain is rather floppy ($S^2 = 0.33$). It was hypothized theoretically, that another binding site may be in action;[10] however, at least for the acetate, this could not be observed.

In conclusion our NMR data on ^{15}N labelled eremomycin gave the full backbone assignment for the two sides of the dimer. The fastest amide proton exchange is detected at the heart of the binding pocket at the exterior of the dimer. We found that the heptapeptide backbone is equally rigid at the two sides, and probably other factors explain the observed differences in binding affinity.

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Notes and references

‡ The strain *Amycolatopsis orientalis* subsp. *eremomycini* INA-238 was used for the eremomycin production. Approximately 40% random incorporation of the ^{15}N isotope was detected in ^1H NMR spectra. Supplementary information is provided in electronic form by the ESI.†

1 G. F. Gause, M. G. Brazhnikova, N. N. Lomakina, T. F. Berdnikova, G. B. Fedorova, N. L. Tokareva, V. N. Borisova and G. Batta, *J. Antibiot.*, 1989, **42**, 1790.
2 G. Batta, F. Sztaricskai, K. E. Kövér, C. Rudel and T. F. Berdnikova, *J. Antibiot.*, 1991, **44**, 1208.
3 P. Groves, M. S. Searle, J. P. Mackay and D. H. Williams, *Structure*, 1994, **2**, 747.
4 G. E. Hawkes, H. Molinari, S. Singh and L. Lian, *J. Magn. Reson.*, 1987, **74**, 188.
5 B. Bardsley and D. H. Wiliams, *Chem. Commun.*, 1997, 1049.
6 P. Groves, M. S. Searle, M. S. Westwell and D. H. Williams, *J. Chem. Soc., Chem. Commun.*, 1994, 1519.
7 M. Sattler, J. Schleucher and C. Griesinger, *Prog. Nucl. Magn. Reson. Spectrosc.*, 1999, **34**, 93.
8 M. Schafer, T. R. Schneider and G. M. Sheldrick, *Structure*, 1996, **4**, 1509.
9 G. Lipari and A. Szabo, *J. Am. Chem. Soc.*, 1982, **104**, 4546.
10 W. G. Prowse, A. D. Kline, M. A. Skelton and R. J. Loncharich, *Biochemistry*, 1995, **34**, 9632.