Cooperativity between ligand binding and dimerisation in a derivative of ristocetin A

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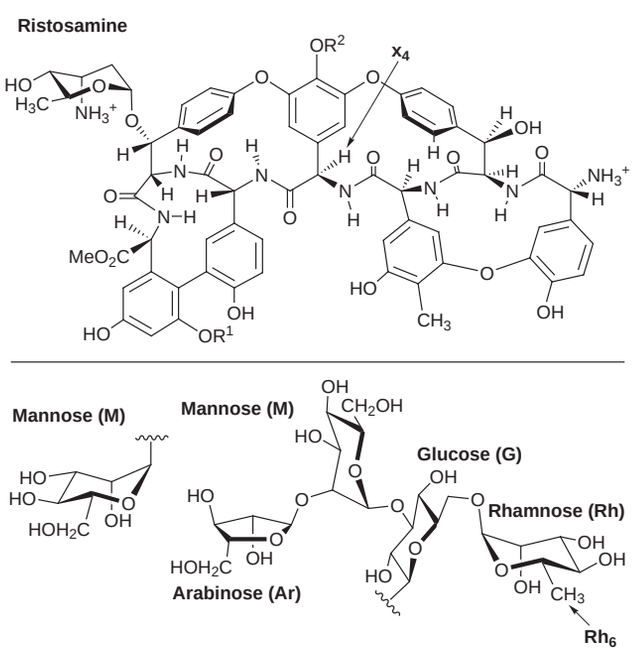
The dimerisation constant of the vancomycin group antibiotic ristocetin A has previously been shown to be lower when it is fully bound by ligand (analogues of bacterial cell wall precursors terminating in –Lys-D-Ala-D-Ala) than in its absence, *i.e.* dimerisation is anticooperative with ligand binding. A derivative of ristocetin A, desrhamno-ristocetin, has now been produced by enzymatic degradation, and the dimerisation constant of this derivative has been measured in the absence and presence of the bacterial cell wall precursor analogue *N*-acetyl-D-Ala-D-Ala. The dimerisation constant is shown to be greater in the presence of the ligand than in its absence, *i.e.* dimerisation is cooperative with ligand binding. This change in behaviour from anticooperativity to cooperativity is postulated to be associated with the partial equalisation of the binding affinities of the two sides of the dimer for ligand. It is therefore energetically more favourable for two ligand molecules to bind to the two halves of a desrhamno-ristocetin dimer than to two monomers.

Introduction

Ristocetin A (Fig. 1) is a member of the vancomycin group of antibiotics which are currently clinically-important in the treatment of infections due to methicillin-resistant *Staphylococcus aureus* (MRSA).[1,2] These antibiotics exert their antibacterial activity by binding to the terminal –Lys-D-Ala-D-Ala sequence of bacterial cell wall precursors, thus inhibiting transglycosylation and ultimately causing cell death.[3,4] In recent work, we have shown that most of the glycopeptide antibiotics form asymmetric homodimers and that dimerisation is, in all cases except that of ristocetin A, cooperative with the binding of bacterial cell wall precursor analogues.[5,6] That is, except for ristocetin A, the dimerisation constants of the antibiotics are greater when ligand is bound into their binding pockets than when the binding pocket is free. The dimerisation constant of the exception, ristocetin A, when not bound by ligand is 500 M^{-1}, but falls to 350 M^{-1} when bound by the bacterial cell wall precursor analogue *N*-α-acetyl-*N*-ε-acetyl-lysyl-D-alanyl-D-alanine (Ac_2-KDADA).[5] A more thorough study of the thermodynamics of the ristocetin A–Ac_2-KDADA system has also recently been provided and these results are considered in the context of the present work.[7]

A degradation product (after acid hydrolysis) of ristocetin A, ristocetin-Ψ (Fig. 1) has also been studied previously and, unlike ristocetin A, this derivative expresses cooperativity between dimerisation and ligand binding.[6,8] The dimerisation constant of ristocetin-Ψ when free is 50 M^{-1}, but rises to 700 M^{-1} when bound to Ac_2-KDADA.[6] The differences in the structures of ristocetin A and ristocetin-Ψ are the loss in ristocetin-Ψ of the mannose of residue 7 and the tetrasaccharide of residue 4 (Fig. 1). Of these two differences, it is the tetrasaccharide which has been implicated in the change from anticooperative behaviour in ristocetin A to cooperative behaviour in ristocetin-Ψ.[6]

To investigate further the molecular origin of the anticooperativity in the system of ristocetin A binding to bacterial cell wall precursor analogues, a partial modification of the tetrasaccharide is described here whereby the rhamnose has been selectively removed. The ligand binding and dimerisation constants of this modified antibiotic have been studied and compared to the equivalent values for ristocetin A and ristocetin-Ψ. The results provide a further insight into the reasons why ristocetin A expresses anticooperativity between these two properties. In addition, an analysis of this complicated system underlines the subtle effects exerted by ligand–antibiotic interactions in influencing the observed macroscopic properties of the antibiotic.



	R^1	R^2
Ristocetin A	M	Ar-M-G-Rh
Desrhamno-ristocetin	M	Ar-M-G
Ristocetin-Ψ	H	H

Fig. 1 Structures of the antibiotics ristocetin A, desrhamno-ristocetin and ristocetin-Ψ. In all cases, the saccharide at R^2 is attached to the peptide portion of the antibiotic *via* glucose. The protons x_4 and Rh_6, referred to in the text, are labelled.

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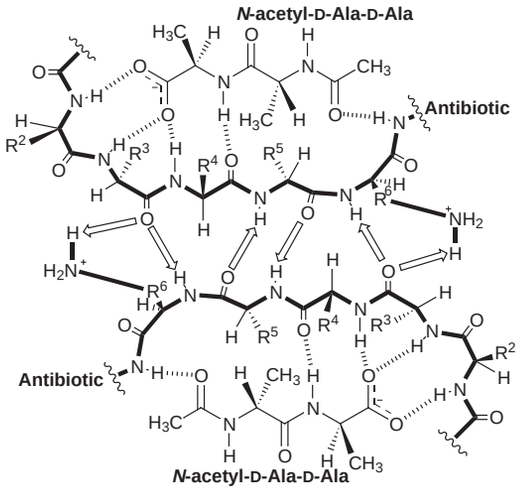

Fig. 2 Backbone structure of the ristocetin A dimer bound to *N*-acetyl-D-Ala-D-Ala. The hydrogen bonds between antibiotic monomers are indicated by the solid arrows and from antibiotic to ligand by the dashed lines.

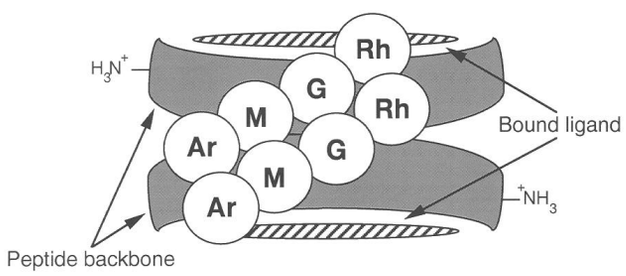

Fig. 3 Schematic diagram illustrating the asymmetry in a dimer of ristocetin A bound to two bacterial cell wall precursor analogues. The tetrasaccharides lie head-to-head but the peptide backbones lie head-to-tail relative to each other. As a result, ligand bound to each side of the dimer interacts with different sugars. The tetrasaccharide sugars are labelled: Rh, rhamnose; G, glucose; M, mannose; Ar, arabinose.

Results and discussion

The anticooperative behaviour of ristocetin A with respect to dimerisation and ligand binding has its origins in the asymmetry of its dimer structure. The dimers are formed by hydrogen bonding between the antiparallel backbones of two antibiotic molecules (Fig. 2) and the C_2-symmetry of this antiparallel arrangement of the backbones is disrupted by the parallel orientation of the tetrasaccharides attached to ring 4 of the two molecules (Fig. 3). A consequence of this asymmetry is that the two halves of the dimer have different affinities for bacterial cell wall precursor analogues. This difference in affinities has been demonstrated for ristocetin A through the observation in ^{1}H, ^{13}C and ^{19}F NMR spectra of the different populations of ligand bound into each half of the dimer after the addition of less than one equivalent of ligand into a solution of the antibiotic.[7,9,10] In one half of the dimer, the ligand can interact with rhamnose and glucose from the tetrasaccharide and in the other half with arabinose and mannose.[11] The interaction of the ligand with glucose and rhamnose is more favourable than that with arabinose and mannose, and consequently ligand binding into this half of the dimer is strongest.[11] The free energies of binding of Ac_2-KDADA to each half of the dimer are -27.9 kJ mol^{-1} and -23.0 kJ mol^{-1} when the opposite half of the dimer is unbound by ligand.[7] When the opposite half of the dimer is already bound by ligand, the binding free energies to each half of the dimer rise to -29.6 kJ mol^{-1} and -24.7 kJ mol^{-1}, respectively.[7] This increase in binding free energy when the other half of the dimer is bound by ligand is indicative of the operation of some cooperativity between ligand binding and dimerisation. However, the free energy of binding of Ac_2-KDADA to ristocetin A monomer is -26.7 kJ mol^{-1}.[7] This binding free energy is similar to that for binding to the more favourable binding site of the dimer (when the other binding site of the dimer is unoccupied), presumably reflecting similar orientations of the tetrasaccharide over the binding pocket in these two cases, *i.e.* with the rhamnose and glucose interacting with the ligand. It is therefore energetically more favourable for ligand to bind to two ristocetin A monomers than to both halves of a dimer. This thermodynamic preference for binding to two monomers is related to the disparity between the binding energies of the ligand to each half of the dimer resulting from the asymmetric arrangement of the tetrasaccharide.

Table 1 Binding constants of Ac-DADA to ristocetin A, desrhamno-ristocetin and ristocetin-Ψ measured by UV difference spectrophotometry at pH 7.0 and 300 K. The value for ristocetin-Ψ was taken from Reference 15 and measured at 301 K

Antibiotic	K_{bind}/M^{-1}
Ristocetin A	$7.1 \pm 0.6 \times 10^4$
Desrhamno-ristocetin	$5.9 \pm 0.5 \times 10^4$
Ristocetin-Ψ	3.3×10^4

The modification to ristocetin A was made by the selective removal of rhamnose from the tetrasaccharide by the enzyme naringinase, which possesses α-L-rhamnosidase activity.[12,13] The reason for removal of this sugar was that it is partly responsible for the greater affinity of Ac_2-KDADA for one side of the dimer over the other through the formation of hydrogen bonds to the ligand and possible burial of hydrophobic surface area from solvent. Its removal was therefore intended to equalise, to some extent, the binding affinities of the ligand to each side of the dimer. If the two binding sites have equal affinity for ligand, as with symmetric dimers such as ristocetin-Ψ, then ligand binding might be expected to be cooperative with dimerisation. Therefore an equalisation of the binding affinities through removal of rhamnose would be expected to reduce the degree of anticooperativity observed, possibly to such an extent that dimerisation would be cooperative with ligand binding.

The ligand binding constants of the cell wall precursor analogue *N*-acetyl-D-Ala-D-Ala (Ac-DADA) to ristocetin A and the modified antibiotic, desrhamno-ristocetin, were measured by UV difference spectrophotometry (Table 1).[14] The binding constant to desrhamno-ristocetin is lower than that to ristocetin A but greater than that to ristocetin-Ψ, demonstrating the positive influence of the sugars on ligand binding, although the magnitudes of the differences are small.[15]

Dimerisation constants of the three antibiotics (ristocetin A, desrhamno-ristocetin and ristocetin-Ψ) when free and when bound by Ac-DADA were measured by one of two different ^{1}H NMR methods depending on whether the two halves of the antibiotic dimer were in slow or fast exchange on the NMR timescale. Where the two halves of the dimer were in slow exchange (ristocetin A free and bound by Ac-DADA), distinct peaks could be observed in the ^{1}H NMR spectrum due to both monomer and dimer species. Distinct dimer peaks could also be observed for the protons in each half of the dimer. The three resonances due to the protons of the rhamnose methyl (Rh_6) group (only present in ristocetin A of the antibiotics studied; Fig. 1) lie in a region of the spectrum relatively unencumbered by other peaks (Fig. 4). These peaks were integrated over a range of ristocetin A concentrations, the resultant data plotted as concentration of monomer *vs.* concentration of dimer and the dimerisation constant calculated by least-squares curve-fitting to the data (Fig. 5).[6,7,16]

For the other two antibiotics, where the two halves of the dimer were in fast exchange on the NMR timescale, dimeris-

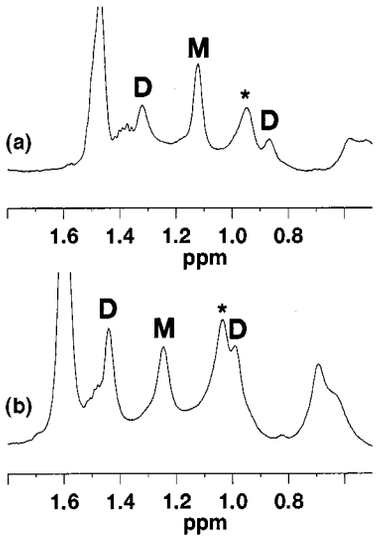

Fig. 4 Portions of the 1-D ^{1}H NMR spectra of ristocetin A in the presence of Ac-DADA showing the resonances due to the protons of the rhamnose methyl (Rh_6) group. The spectra are at ristocetin A concentrations of (a) 1.6 mM and (b) 9.8 mM and at pD 7.0 and 300 K. Resonances due to dimer and monomer are labelled D and M, respectively. It can be seen that at the higher concentration, the proportion of dimer has increased relative to monomer. The asterisked peak is due to the methyl group of the N-terminal alanine of bound Ac-DADA. The non-alignment of the two spectra with respect to chemical shift is a result of the apparent upfield shifting of the reference (TSP) resonance with increasing ristocetin A concentration.

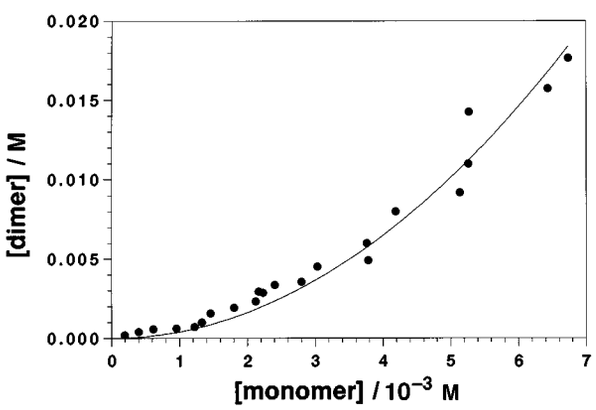

Fig. 5 Dimerisation curve obtained by integration of the Rh_6 (see Fig. 1) resonances of monomeric and dimeric ristocetin A in the presence of Ac-DADA in 1-D ^{1}H NMR spectra at various concentrations of antibiotic and after conversion of the raw data to concentrations of dimer and monomer species. The experiments were performed at pD 7.0 and 300 K.

ation constants were measured by following the chemical shift of a proton (x_4) whose resonances in the dimer and monomer states occurred at distinct positions in the ^{1}H NMR spectrum.[17] The observed resonance in the spectrum in such cases is thus time-averaged and the chemical shift is weighted according to the relative populations of dimer and monomer species. This chemical shift was thus measured at a range of concentrations and the dimerisation constant subsequently determined from a least-squares curve-fit of a plot of chemical shift *vs.* ligand concentration (Fig. 6).

Table 2 shows the dimerisation constants thus measured and those that have been measured previously. It is clear from these values that as sugars are removed from the tetrasaccharide of ristocetin A, the dimerisation constant decreases. This is because the association of the tetrasaccharides in the dimeric species has a positive influence on dimerisation through the formation of sugar–sugar hydrogen bonds and the burial of hydrophobic surface area from solvent. Thus, the removal of

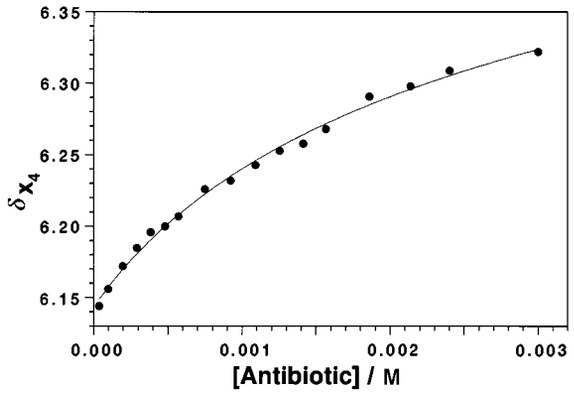

Fig. 6 Dimerisation curve obtained by following the chemical shift of the antibiotic proton x_4 (see Fig. 1) in 1-D ^{1}H NMR spectra during a titration of desrhamno-ristocetin into an NMR tube at pD 7.0 and 300 K

Table 2 Dimerisation constants of ristocetin derivatives when free and when bound to Ac-DADA, measured by ^{1}H NMR spectroscopy. The values were measured at pD 7.0 and 300 K

Antibiotic	K_{dim}^{free}/M^{-1}	K_{dim}^{bound}/M^{-1}	$K_{dim}^{bound}/K_{dim}^{free}$
Ristocetin-Ψ	50[a]	240[a]	4.8
Desrhamno-ristocetin	120 ± 20	320 ± 40	2.7
Ristocetin A	500[b]	400 ± 50	0.8

[a] Taken from Reference 6. [b] Taken from Reference 5.

rhamnose is detrimental to the dimerisation of free antibiotic, and the removal of all four sugars in ristocetin-Ψ is detrimental to a greater extent.

It can also be seen from Table 2 that the dimerisation of desrhamno-ristocetin is, unlike the case with ristocetin A, cooperative with ligand binding. Indeed, dimerisation in the presence of Ac-DADA is almost three times more favourable than in its absence. In thermodynamic terms, the fact that dimerisation is now cooperative with ligand binding means that the binding of ligand into both sides of the desrhamno-ristocetin dimer is more favourable than the binding of two ligands to two monomers. However, as predicted, the degree of cooperativity expressed by desrhamno-ristocetin is not as great as that expressed by ristocetin-Ψ. The lesser cooperativity of desrhamno-ristocetin compared to that exhibited by ristocetin-Ψ suggests that the two binding sites of the desrhamno-ristocetin dimer still possess unequal binding affinities. The partial equalisation of the two binding sites relative to ristocetin A is probably due to a reduction in binding affinity of Ac-DADA for the more favoured binding site of the dimer rather than an increase in binding affinity to the less favoured binding site. This is because the rhamnose which has been removed interacts directly with the more favoured binding site. It is possible that this reduction is enough for the previously less favoured half of the dimer to have become the more favoured binding site, albeit to a small extent, but this could not be confirmed during the current work. There is an additional point to note. Not only will the binding affinities of ligand for each side of the dimer have become more equal, but also the binding constant to monomer will be reduced. This is because the favoured orientation of the saccharide for monomer binding is likely to reflect the orientation of the saccharide in the more favoured binding site of the dimer, *i.e.* the favoured monomer conformation is likely to be that with rhamnose interacting with the ligand. Both these changes will have the effect of increasing the likelihood of cooperativity between ligand binding and dimerisation by increasing the relative favourability of binding to the two halves of a dimer *compared* to binding to two monomers, which is exactly what is observed. This postulate is consistent with the value of the ligand binding constant meas-

ured by UV difference spectrophotometry which does indeed show a reduced binding constant of desrhamno-ristocetin to Ac-DADA relative to ristocetin A. [Due to the concentration of antibiotic used in determining these binding constants (50 μM), virtually all (>98%) of the antibiotic is present as monomer and hence the values obtained by this method can be approximated as the binding constants to monomer.]

In conclusion, ristocetin A, which displays anticooperativity between dimerisation and ligand binding in solution, has been selectively modified to produce a new antibiotic, desrhamno-ristocetin, which displays cooperativity between these two properties. The change from anticooperativity to cooperativity is associated with a reduced difference in the affinities of the two halves of the antibiotic dimer for bacterial cell wall precursor analogues. As a result, due to the favourability of binding ligand to one side of the dimer when the other side is already occupied by ligand, coupled with the intrinsic favourability of ligand binding to antibiotic dimer rather than monomer, it is now more favourable for two ligands to bind to the two halves of the desrhamno-ristocetin dimer rather than to bind to two monomers. Although the concentrations of antibiotic reached *in vivo* are unlikely to reach those required to achieve significant dimerisation, further cooperative effects associated with dimerisation are likely to mean that even weak dimerisation may be important in promoting antibacterial activity.[6] These results provide a further insight into the molecular origins of the anticooperativity between dimerisation and ligand binding observed for ristocetin A and provide an example of the subtle molecular features which can influence the expression of properties of the antibiotic, such as cooperativity.

Materials and methods

Ristocetin A was donated by Abbott Laboratories (Chicago, USA) as the sulfate salt and used without further purification. Naringinase was purchased from Sigma Chemical Co. Desrhamno-ristocetin was prepared according to the procedure described in Reference 13.

^{1}H NMR spectroscopy

Samples were prepared by dissolution in D_2O and the pD adjusted to 7.0 using NaOD and DCl. All pD measurements were with a Corning pH meter equipped with a combination glass electrode, and no corrections were made for isotope effects. One-dimensional spectra were recorded at 300 K on a Bruker DRX500 spectrometer using 32k data points and calibrated with internal 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate, sodium salt (TSP; δ 0.00 ppm). Suppression of the solvent resonance was achieved using presaturation. Data was processed with Bruker XWIN-NMR software.

Dimerisation constants were determined in 100% D_2O and at pD 7.0. To an NMR tube was added 600 μl of TSP (0.1 mM) in 100% D_2O at pD 7.0 (if the dimerisation constant being measured was of antibiotic bound to ligand, then 0.5 mM of the ligand was also added to this tube). Separately, 700 μl of a concentrated solution (*e.g.* 40 mM) of antibiotic in 100% D_2O at pD 7.0 was prepared (if the dimerisation constant being measured was of antibiotic bound to ligand, a sufficient concentration of ligand was added to this solution such that the antibiotic would remain >95% bound by ligand at all points during the titration based on the binding constant for the association). Aliquots of this solution (initially 5 μl and rising to 50 μl by the end of the titration) were added to the NMR tube, which was then shaken, and 1-D NMR spectra were recorded over a range of antibiotic concentrations.

After processing of the spectra, dimerisation constants were determined by one of two methods. If the two halves of the antibiotic dimer were in fast exchange on the NMR timescale, the chemical shift of an antibiotic proton whose resonance shifted as a function of antibiotic concentration was plotted against the total concentration of antibiotic for a range of antibiotic concentrations. Dimerisation constants were calculated by curve-fitting of the plotted data to the theoretical equations for a dimeric association using *Kaleidagraph* version 3.0.5 (Abelbeck Software). If the two halves of the dimer were in slow exchange on the NMR timescale, the monomeric and dimeric resonances of the rhamnose methyl protons (Rh_6, see Fig. 1) were integrated over a range of antibiotic concentrations and the resultant data converted to concentrations of monomer and dimer, respectively. The dimerisation constant was calculated by plotting concentration of monomer *vs.* concentration of dimer and curve-fitting of the plotted data to the theoretical equations for a dimeric association using *Kaleidagraph* version 3.0.5 (Abelbeck Software).

UV Difference spectrophotometry

Experiments were performed using a dual beam Uvikon 940 spectrophotometer equipped with a thermocirculator maintaining a constant temperature of 300 K. To each cell was added 2.5 ml of a solution of antibiotic (50 μM) and sodium dihydrogen phosphate (0.1 M) adjusted to pH 6.0 with sodium hydroxide (1 M). To the front cell was added 1 ml of a solution of Ac-DADA (740 μM) and antibiotic (50 μM) in sodium dihydrogen phosphate (0.1 M) at pH 6.0. The concentration of Ac-DADA was such that after this addition greater than 90% of the antibiotic would be bound by the ligand based on an estimated binding constant for the association. A difference spectrum was then taken, from which the wavelengths to be used in the titration were derived. The wavelengths thus derived from the peaks and troughs of the difference spectrum were 287, 286, 244 and 243 nm. The front cell was then replaced by another containing 2.5 ml of the original antibiotic solution. To this front cell, aliquots (initially 10 μl and rising to 100 μl by the end of the titration) of 1 ml of the same ligand solution as above were then added and the absorbance values measured. The association constant was determined by curve-fitting a plot of change in absorbance (measured as $Abs_{287} + Abs_{286} - Abs_{244} - Abs_{243}$) *vs.* ligand concentration using *Kaleidagraph* 3.0.5 (Abelbeck Software). Determinations were carried out in triplicate, the average result being quoted, with the error being derived from the result with the largest deviation from this average.

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