Kinetics and Thermodynamics of Binding of a Model Tripeptide to Teicoplanin and Analogous Semisynthetic Antibiotics

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The thermodynamics and kinetics of binding of model tripeptides ϵ-N-acetyl-α-*N*-dansyl-L-Lys-D-Ala-D-Ala (ADLAA) or α-*N*,ϵ-*N*-diacetyl-L-Lys-D-Ala-D-Ala (AALAA) to teicoplanin (**1a**) and a series of semisynthetic derivatives with (**1b**−**f**) or devoid of (**2a**−**g**) the glycidic side arms and modified at the terminal amino acids of the peptide backbone have been studied by fluorescence or UV spectroscopy. The binding process is suggested to occur via a two-step mechanism. The first, fast process is likely governed by an electrostatic interaction between the C- and N-termini of the peptide chain of the substrate and of the antibiotic, respectively, while the second slower one, accounts for the formation of the hydrogen bonds responsible of the major contribution to the overall binding energy. The binding constants with all modified derivatives are smaller than that with native teicoplanin. Larger modification of the overall binding constant are observed when the sugar residues are removed and, to a lower extent, when the N-terminus of the peptide chain is acylated. The kinetic process is very little affected by the modifications introduced.

Introduction

Resistance of bacteria to physicochemical stress is linked to the presence of the peptidoglycan polymer in the structure of their cell walls. Accordingly, interference in peptidoglycan biosynthesis may result in the death of the bacteria.[1] Several antibiotics, including β-lactams,[2] interfere with peptidoglycan biosynthesis. Glycopeptide antibiotics,[3] such as vancomycin and teicoplanin, specifically recognize the C-terminal D-Ala-D-Ala residue of the precursor peptide forming a tight complex held together by five hydrogen bonds. As a consequence, the enzymatic process that leads to peptidoglycan synthesis is greatly inhibited.

Much work has been devoted to the understanding of the principles that govern the activity of these antibiotics[4] and the structural parameters that affect their action including modification of selected sites known to modify their hydrophobic/lipophilic balance, their net charge or the pK_a of some groups.[5] In fact, though it is completely accepted that activity is strictly connected to binding, the strength of association does not correlate directly with the efficiency of the antibiotic. As a notable example[6] a vancomycin derivative modified by alkylation of an amino sugar proved 1 order of magnitude more active than parent vancomycin vis-à-vis a 23-fold lower binding constant with model peptide diacetyl-L-Lys-D-Ala-D-Ala.

It appears that a better understanding of the process of binding both from the kinetic and thermodynamic point of view is preliminary to any successful correlation of structure with reactivity. Although the interaction of the antibiotic with the C-terminus of the peptide precursor to peptidoglycan should be influenced by the presence of the cell membrane and, accordingly, should be correctly studied in such an environment, relevant information may also be acquired from the study of the bimolecular process occurring with model peptides in homogeneous solutions. Recently, Pratt and his associates[7] introduced a fluorescent peptide, ϵ-N-acetyl-α-N-dansyl-L-Lys-D-Ala-D-Ala (ADLAA) useful for the determination of the quite large rate and binding constants of glycopeptide antibiotics. Binding constants larger than *ca.* 1×10^6 M^{-1}, when measured by absorbance, are affected by large errors. Using this fluorescent-labeled peptide they studied the kinetics and mechanism of binding of vancomycin and a few related antibiotics and concluded that the binding process occurs in two steps, the slowest one involving a solvent (water) rearrangement prior to the final complex formation.

Though structurally similar to vancomycin, teicoplanin[8] (**1a**) presents some peculiarities: a lipophilic hydrocarbon chain on one of the amino sugars and a fourth

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macrocycle that imparts further rigidity to the seven-amino-acid backbone. This last structural feature is present in another antibiotic of this class, ristocetin A.

Following our interest in teicoplanin antibiotics we undertook an investigation on several teicoplanin derivatives aimed at evaluating the kinetics and thermodynamics of binding using Pratt's fluorescent substrate as a model of the peptidoglycan precursor. Native teicoplanin was modified by one or more of the following: (i) removal of the sugar side chains (aglycons **2a**−**g**); (ii) conversion of C38-bound carboxylate into ester or amide (**1b**, **2b,c**); (iii) conversion of C15-bound primary amine into amide (**1c,e,f**, **2e**−**g**); (iv) introduction of one or more amino group on the C15 or C38 side (**1e,f 2d**−**g**). The idea behind these modifications was to assess the role of the "side arms" in the recognition process while leaving unchanged the rigid peptide backbone which is responsible of the hydrogen bonds involved in the complex formation. Evidence concerning the involvement of these "side arms" in the binding process were presented recently by Williams for vancomycin derivatives.[5a,d] The results of this thermodynamic and kinetic investigation are reported in this paper.

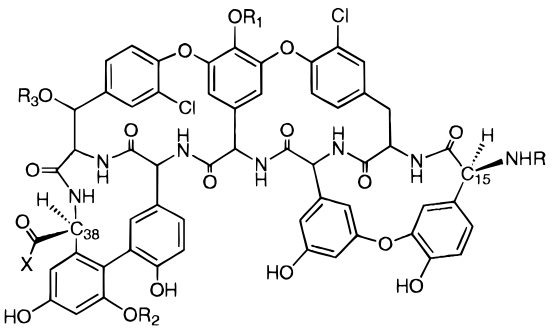
Results and Discussion

Antibiotics and Their Behavior in Aqueous Solution. All compounds studied in this work have been already described.[9] They can be divided in two major groups: those with the three glycidic residues still present (**1a**−**f**) and those devoid of them, aglycons **2a**−**g**. As expected, the removal of the sugar side chains makes the aglycons less soluble in neutral aqueous solutions than compounds **1**. Nevertheless, because of the very low concentrations used in the present study, solubility proved never to be a major problem with these compounds. However, it is well known that teicoplanin **1a** shows amphiphilic properties and formation of aggregates above a critical concentration (cac, critical aggregate concentration) has been reported.[10] A similar behavior for derivative **1c** has been described by ourselves.[11] We also showed that the aggregation state of the antibiotic does influence both the binding isotherm and the kinetic of binding. For this reason we investigated the possibility that some of the antibiotics could form aggregates in the conditions used for the experiments. The most useful technique proved the measurement of surface tension against concentration. Typically, these plots with amphiphilic molecules show a discontinuity taken as the concentration at which aggregates start to form (cac).[12] For five out of six glycopeptides we observed formation of aggregates. The cac values determined in water at pH = 7.0 (0.1 M phosphate buffer) and 25 °C are reported in Table 1. The cac obtained from the binding profiles (see below), when observed, have been also reported within brackets.

Table 1. Critical Aggregate Concentrations (cac) Determined for Some of the Antibiotics Studied at 25 °C and pH = 7.0 (0.1 M phosphate buffer)

antibiotic	10^4 cac,[a] M
1a	2.0
1b	0.42
1c	0.14 (0.10)[b]
1d	0.68
1f	0.29 (0.20)[b]
2a	− (0.02)[b]
2d	− (0.015)[b]

[a] Determined by surface tension measurements. [b] Determined from discontinuities in the binding profiles.



1 : R_1 = N-Acyl-β-D-glucosaminyl (acyl = $C(=O)(CH_2)_6CH(CH_3)_2$; R_2 = α-D-Mannosyl; R_3 = N-Acetyl-β-D-glucosaminyl

1a : X= OH; R = H **1b** : X= OCH_3; R = H
1c : X= OH; R = Cbz **1d** : X= $NH(CH_2)_3N(CH_3)_2$; R = H
1e : X= OH; R = Gly **1f** : X= OH; R = L-Lys

2 : $R_1 = R_2 = R_3$ = H

2a : X= OH; R = H **2b** : X= $NH(CH_2)_2NH(CH_2)_3NH(CH_2)_2NH_2$; R = H
2c : X= $NH(CH_2)_3NH(CH_2)_3NH(CH_2)_3NH_2$; R = H
2d : X= OH; R = $(CH_2)_3CONHCH_3$ **2e** : X= OH; R = Gly
2f : X= OH; R = D-Lys **2g** : X= OH; R = L-Lys

All derivatives have cac lower than parent teicoplanin. The failure to observe aggregation tensiometrically in the case of **1e** and all aglycons does not mean that aggregates are not formed with these compounds. It is possible that (a) cac occurs at a concentration too low to be detected; (b) the effect of these molecules on surface tension is negligible. From our experience, we take a concentration close to 5×10^{-6} M as the lowest cac that can be determined with this technique. However, occurrence of aggregation phenomena below this concentration is suggested by the binding profiles of aglycons **2a** and **2d** which show discontinuities with respect to a well behaved curve. A similar behavior was observed for **1c** and **1f** at a concentration very close to the cac determined tensiometrically. By analogy, the concentrations at which these discontinuities occur for **2a** and **2d** may indicate aggregation for these compounds, too. These two concentrations are also reported in Table 1.

Binding. The overall binding constants of the antibiotics studied (equilibrium 1) were determined in aqueous phosphate buffer (0.1 M) at pH = 7.0 and 25 °C following the increase of fluorescence of the tripeptide ADLAA upon increasing the antibiotic concentration.

$$\text{antibiotic} + \text{peptide} \underset{k_d}{\overset{k_f}{\rightleftharpoons}} \text{complex} \qquad K_b = k_f/k_d \quad (1)$$

In two cases (compounds **2b** and **2c**) the binding constants had to be determined by UV-vis following the change of absorbance of the antibiotic upon increasing the concentration of tripeptide α-*N*,ε-*N*-diacetyl-L-Lys-D-Ala-D-Ala (AALAA) because, at the concentration used,

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Table 2. Thermodynamic (K_b, M^{-1}) and Kinetic (k_f, M^{-1} s^{-1}) Binding Constants[a] of the Antibiotics To Model Peptide ADLAA at 25 °C and pH = 7.0 (0.1 M phosphate buffer)

antibiotic	$10^{-5}K_b$, M^{-1}	$10^{-6}k_f$, M^{-1} s^{-1}
1a	253 ± 45 (370 ± 73)[b]	17.2
1b	78 ± 7	21.9
1c	15 ± 1	3.8
1d	37 ± 5 (278 ± 93)[b]	10.9
1e	55 ± 6	4.05
1f	205 ± 25	9.7
2a	27 ± 5	25.8
2b	30 ± 11[c]	12.8
2c	14 ± 9[c]	14.9
2d	21 ± 6	14.2
2e	6.1 ± 0.4	10.1
2f	4.6 ± 0.2	7.4
2g	4.2 ± 0.3	2.5
eremomycin	0.42 ± 0.02 (0.35 ± 0.08)[c]	15.4
vancomycin[d]	3.0	9.3
ristocetin A[d]	3.0	7.2

[a] Refers to eq 1. [b] pH = 5.3. [c] Binding to AALAA, determined by absorbance spectroscopy, see text. [d] Taken from ref 7b.

a significant photochemical decomposition of the antibiotic occurred. This is highlighted by the formation of an emission band at 420 nm that interferes with the emission band of the complex (555 nm). The formation of this band is not related to the substrate while it depends on the concentration of the antibiotic and the time of exposure to UV light. The effect is maximized when the sample is irradiated at 280 nm (instead of 330 nm), the maximum of the absorbance of the phenolic residues of the peptide backbones. It is conceivable that in the excited state, the amino groups introduced at C38 act as electron acceptors leading to facilitated oxidation of the phenolic groups.

In order to rule out any interference of the dansyl moiety of the peptide in the binding process, control experiments were also performed by UV-vis with the antibiotic eremomycin using the tripeptide AALAA. The binding constant, identical within the limit of the experimental error with that determined by fluorescence,[13] is reported in Table 2. The control was made with this antibiotic since its low binding constant allows an accurate determination also by UV-vis spectroscopy.

For native teicoplanin (**1a**) and C38-functionalized amino derivative **1d**, binding constants were also determined at a lower pH (5.3). In both cases, particularly for **1d**, this leads to an increase of the binding constant.

Because of the aggregation phenomena discussed above, the concentration interval of the antibiotic explored for binding constant determination was, whenever possible, below the cac. Aggregation, in fact, as we have already shown,[11] may artificially increase fluorescence as a consequence of a change toward a more hydrophobic environment leading to an apparently lower binding constant. However, for the aglycons, if aggregation occurs (see above), cac should be low and well within the range of concentration used. If this is the case and a nonspecific binding (driven by hydrophobic interactions) of the tripeptide to the aggregate occurs,[14] it is possible that the binding constants are overestimated (by up to a factor of 2). All the binding constants are reported in Table 2.

From the analysis of Table2, we see that the aglycons have affinity constants for the model peptide at least 1 order of magnitude lower than the glycopeptides. If we take into account the effect of aggregation (see above) the binding constants are probably even smaller. This means that the sugar residues do play a role in the binding process though they are not indispensable for its occurrence. Similar observations have been made by Williams[5d] and Pratt.[7b] Another group influencing binding is the amine at the N-terminus:[5d] its acylation leads to a decrease of the affinity constant by a factor of 16 (compare **1a** with **1c**). However, if this amine is acylated and a new one is introduced slightly away from the binding site, only little of the binding strength is lost (compare **1a** with **1e**, and **2a** with **2e**). Similarly the introduction of more than one amino group on this side of the antibiotic, after acylation of the N-terminus, has little relevance (compare **1e** with **1f**, and **2e** with **2f** and **2g**). Esterification of the C38-bound carboxylate only slightly decreases the binding (compare **1a** with **1b**). However, the introduction of a positive charge on that side arm seems to compensate, at least in part, this effect (compare the binding constant of **1d** at pH 7.0 and 5.3; **2b** has a slightly higher binding constant than **2a**). Clearly it appears that the carboxylate side arm is less involved in the binding process than the C15-bound N-terminus though it may affect the solvation of the antibiotic and hence influence its availability at the level of the bacterial membrane. Noteworthy is the fact that these compounds appear more efficient than native teicoplanin in tests *in vitro* against Gram-positive bacteria (see below).

Overall, putting together all the above contributions, the difference in binding constant between teicoplanin (**1a**) which has the highest affinity for the substrate and the least effective aglycons (**2f** and **2g**) is slightly less than 2 orders of magnitude. However, these binding constants are still 1 order of magnitude higher than that measured for eremomycin and similar to that of vancomycin. The clear conclusion is that modification of the side arms of the antibiotic, while leaving intact the peptide backbone and hence leaving mostly unaffected the hydrogen bonding units, accounts for a maximum ΔG of binding of −2.7 kcal/mol. The five hydrogen bonds are responsible of a ΔG of at least −6.8 kcal/mol.

Kinetics. Kinetics of binding were followed by fluorescence spectroscopy using the stopped-flow technique. Pseudo-first-order conditions were maintained with [antibiotic] ≥ 8[peptide]. As for the binding constants determination, ADLAA was used as the substrate. However, the concomitant photochemical decomposition mentioned in the previous section led, for substrates **2b** and **2c**, to the appearance of a second, slower kinetic process which, in the case of **2b**, was hampering a reliable determination of the rate constant. In this case rate constants were determined by absorbance using AALAA as the substrate and kinetic measurements at the lower antibiotic concentrations could not be run under strictly pseudo-first-order conditions. For all antibiotics studied the dependence of the observed rate constant *vs* [antibiotic] was linear in the interval examined. The major limitation to explore higher concentrations (in order to appreciate possible curvatures) was in the maximum rate constant that could be measured with confidence with our stopped-flow instrument ($k_{obs} \approx 1 \times 10^3$ s^{-1}). From

(13) Substantial agreement has been found by Pratt, too; see: Popieniek, P. H.; Pratt, R. F. *Anal. Biochem*. **1987**, *165*, 108.

(14) However it has been recently reported that teicoplanin exists as two conformers in aqueous solution and it has been suggested that one of them is preferred for ligand binding and the other for aggregate formation. Upon binding with model dipeptide *N*-acetyl-D-Ala-D-Ala, aggregates disappear and this is in agreement with the above suggestion. See: Westwell, M. S.; Gerhard, U.; Williams, D. H. *J. Antibiot*. **1995**, *48*, 1292.

the slopes of these linear plots the second order rate constant (k_f) for the equilibrium of eq 1 can be obtained. These values are also reported in Table 2. The aggregation of the antibiotic may lead to a less pronounced slope. This could be clearly highlighted in the case of compound **1c**,[11] due to the fortunate value of its cac: with this compound the slope, above cac, is smaller by a factor of three and it is quite possible that for the aglycons and glycopeptide **1e** we are underestimating by a similar factor the second order rate constant.[15] However, for the general considerations we will draw later on, this is not a major problem since the trend at large is not greatly affected by this phenomenon. The second order rate constants reported in Table 2 are based on the very simple bimolecular mechanism of binding represented in eq 1. As already pointed out by Pratt[7] this simplistic mechanism does not seem to hold true for these antibiotics. In fact the rate constants are too slow for a diffusion-controlled process even taking into account the formation, in some cases, of small aggregates. For this reason Pratt has suggested a two step mechanism involving the fast formation of the precomplex AP* which eventually evolves, slowly, to the final complex AP:

$$A + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AP^* \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} AP \qquad (2)$$

where A and P stand for the antibiotic and the peptide, respectively. The kinetic equation for such a mechanism is shown in eq 3:

$$k_{obs} = k_2 K_1 [A]_o + k_{-2} \qquad (3)$$

where K_1 is the equilibrium constant for the first, fast binding process and $[A]_o$ indicates the total concentration of antibiotic. Eq 3 holds true providing $[A]_o \gg [P]_o$ and assuming $1/K_1 \gg [A]_o$, conditions experimentally verified because the linearity of the plots k_{obs} *vs* $[A]_o$ requires $K_1 < 10^4$ M^{-1}. Based on this analysis, the following relationship exists between k_f (Table 2) and k_2: $k_f = k_2 K_1$. Accordingly, in order to get k_2 we must divide k_f by K_1 which is experimentally not accessible, and a change of k_f may be due to a change of k_2, K_1, or both of them. This point will be discussed further below. The kinetic values in Table 2 are strikingly similar one to the other and the difference between the fastest antibiotic, aglycon **2a**, and the slowest, **2g**, is only a factor of 10. Among the slowest compounds we find **1c**, **1e**, **2f**, and **2g**, all having the C15 side arms modified, regardless of whether the glycidic side chains are present or not. This implies that modification of the C15 side of the antibiotics has not only implications on the overall thermodynamic of binding but also on the kinetics. On the contrary, the sugars do not affect the kinetics but only the thermodynamics of the process.

Mechanism of Binding. The graph of Figure 1 summarizes, in a pictorial way, the features pertinent to the thermodynamics and kinetics of binding discussed in the previous sections.

In Figure 1 the antibiotics have been sorted in order of decreasing binding strength. As already pointed out, the kinetic process is very little affected by the modifications we have introduced in native teicoplanin. Pratt has suggested[7b] that the rate determining step (k_2 in eq 2) is

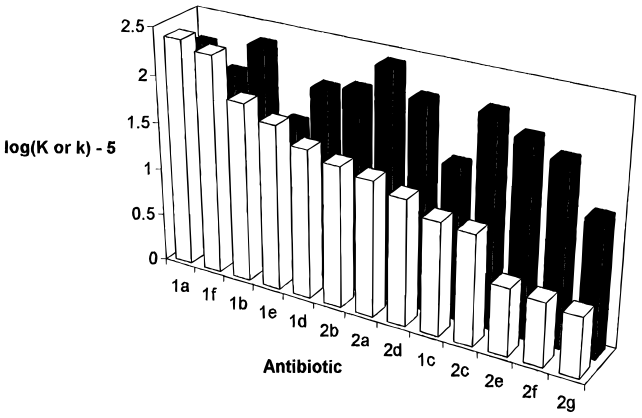

Figure 1. Istogram representation of the log K_b (foreground) and log k_f (background) for the antibiotics studied sorted in order of decreasing K_b.

associated with desolvation (or solvent rearrangement) of the peptide and/or antibiotic rather than with a change of conformation of the peptide backbone. His conclusion comes from observations related to the solvent effect (on changing the solvent from water to acetonitrile the rate becomes faster) and the little dependence of the rate on the structure of the complex. These conclusions may be true in our case, too: the compounds showing a significant decrease of rate are those having the C15-bound amine acylated. Since this group is close to the site involved in the formation of the H-bonds with the carboxylate terminus of the incoming peptide, it is conceivable that a change of solvation in that region influences the rate of binding.

On the other hand, an alternative possibility, also consistent with the observed effect of the substituents, is that the preassociation equilibrium (K_1) is governed by an electrostatic interaction between the antibiotic protonated N-terminus and the carboxylate, imposing the correct orientation for the formation of the final complex. If this is the case, the rate effect would be, in fact, an effect on K_1. Since purely electrostatic binding to vancomycin, like that of acetate, has been recently estimated[16] to be 30 M^{-1}, we may take this value as the maximum effect due to the perturbation of the electrostatic contribution to the overall binding constant. The fact that our k_f values do not differ more than 1 order of magnitude is indeed consistent with a modification of K_1 due to perturbation of the electrostatic interactions. It is interesting to note that the binding of fatty acids to bovine serum albumin has been reported[17] to be a two-step process, the first of which involves an electrostatic interaction. We must admit that the apparent rate changes are relatively small so that speculation on the matter should be taken with caution and leave open the possibility that the observed trend is a result of a change of K_1 or k_2.

We note, on the contrary, that changing the rigidity of the peptide backbone of the antibiotic does not affect the rate of the process as can be seen comparing the data of teicoplanin and ristocetin A with those of vancomycin and eremomycin (see Table 2). A rate-determining conformational rearrangement of the antibiotic would imply a significant difference in rate among these compounds because of the difference in the rigidity of the backbones. This conclusion is in agreement with what recently

(15) The most simple explanation for this behavior is that since the interaction, above cac, occurs with the aggregate and not with the single monomer, the antibiotic concentration should be divided by the number of monomers in each aggregate. However other explanations are possible as discussed in reference 11.

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(17) Scheider, W. *J. Phys. Chem.* **1980**, *84*, 925.

reported by Pastore[18] as a result of parallel NMR and molecular dynamics studies: "the binding of vancomycin to Ac-D-Ala-D-Ala dipeptide does not involve major conformational changes within vancomycin".

From the point of view of the thermodynamics, the side arms (the sugar residues and the peptide N-terminus) do play a role in stabilizing the complex. This appears to be very similar to that which occurs with vancomycin.[5d] In that case, the modification of the structure of the flexible amino acid at the N-terminus results in up to a 37-fold decrease of the binding constant with respect to the native antibiotic.[5a]

Biological Activity. If we now compare available data[9] on *in vitro* activity of the antibiotics of the present study against Gram-positive bacteria strains, we do not discern any relevant difference related to the modification of the structure but for the removal of the glycidic residues. Aglycons are, in fact, more active than the corresponding antibiotic with the glycidic side arms still present. A very simple explanation could be the more favorable partition of the aglycons in the membrane due to their lower hydrophilicity. It has been pointed out recently by Williams[14] that teicoplanin binds quite effectively to a liposomal membrane with its hydrocarbon chain, and this could explain its enhanced activity with respect to other glycopeptide antibiotics.[19] It is conceivable that the actual concentration of the aglycons in the membrane is even higher because of their enhanced hydrophobic character. However, their interaction with the membrane itself should be quite aspecific and, hence, not necessarily with the correct orientation which probably is attained with teicoplanin derivatives still bearing the hydrocarbon side chain. This argument is in accord with the higher hydrophobicity found for the aglycons in solvent partition experiments.[9e] However the partition[20] in the C_8-stationary phase of HPLC column appears to favor the antibiotic still having its long hydrocarbon chain on the glycidic R_1 residue (see formula). This fact, though apparently contradictory, may reveal a specific interaction of the hydrocarbon chain of the native antibiotic with the hydrophobic phase of the column (mimicking the biological membrane) which is obviously not attainable with the aglycons. If these speculations are correct we must admit that the comparison between physicochemical and biological data is biased by the actual availability of the antibiotic in the bacterial membrane where its interaction with nascent peptidoglycan occurs in a pseudo-intramolecular fashion.

Conclusion

We have investigated the mechanism of binding of a series of antibiotics related to teicoplanin to a model peptide by analyzing the thermodynamics and kinetics of the process. In accord with previous findings, the binding process appears to occur in two steps. The first, very fast step is probably driven by a weak electrostatic interaction of the peptide with the antibiotic while the second one is slower and accounts for the formation of the hydrogen bonds, the major energetic contribution to the formation of the final complex. None of the modifications introduced in native teicoplanin increases the affinity of the semisynthetic derivatives to the model substrates. However, the loss of binding strength is no higher than 2.7 kcal/mol (with respect to a ΔG of *ca.* -9.5 kcal/mol for the overall binding), suggesting that the rigid peptide backbone of the antibiotic is not much affected in its interaction with the substrates by these modifications.

A possible correlation between biological activity *in vitro* and hydrophobicity of the antibiotics suggest that their availability on the membrane greatly affects their activity. This effect is somehow similar to what is observed in the case of the reactivity of functional micellar or vesicular aggregates with hydrophobic substrates.[21] In that case large rate accelerations of chemical reactivity are typically explained by taking into account higher concentrations at the reaction loci, *i.e.* at the interface aggregate/water solution. In this regard, Williams[22] has quite recently shown that when the binding process occurs in a micelle as a model of a biological membrane, larger binding contants are observed.

Experimental Section

Materials. Teicoplanin (component A2), **1a**, and semisynthetic derivatives **1b**−**f** and **2a**−**g** were obtained from Dr. A. Malabarba of the Lepetit Research Center and have been already described.[9] ADLAA (Backem Switzerland) and AALAA (Sigma) were used as received. Milli-Q water was used for the preparation of all solutions. Buffer solutions were prepared immediately before their use to avoid the formation of biological contaminants.

Equilibrium and Rate Constants Determination. Antibiotic stock solutions were prepared by suspending the proper amount of material in 5 mL of 0.1 M (pH = 7.0) phosphate buffer. The solution was sonicated for 15min in a bath sonicator and filtered through a 0.22 μm Millipore filter and the concentration of the solution checked by measuring the absorbance at 280 nm at pH = 1 (HCl buffer). ADLAA or AALAA solutions in 0.1 M phosphate buffer (pH = 7.0) were prepared by proper dilution of a 2.0×10^{-4} M stock solutions. Fluorescence intensity was measured at 555 nm with excitation wavelength of 330 nm in samples thermostated at 25.0 ± 0.1 °C; [ADLAA] = 5.0×10^{-7} M. For absorbance measurements (substrate = AALAA), the concentration of antibiotic was held constant, [antibiotic] = 5×10^{-6}-2×10^{-5} M, and the absorbance determined at 250 and 280 nm upon addition of increasing concentration of peptide. The binding isotherm was fitted using the program HOSTEST II.[23] Kinetics were followed (a) by monitoring the change with time of the fluorescence intensity at $\lambda > 420$ nm (using a proper filter) with excitation $\lambda = 330$ nm upon mixing antibiotic and ADLAA solutions; [ADLAA] = 2.0×10^{-6} M or (b) by determining the change of absorbance at 250 nm with time of 2×10^{-5} M solutions of antibiotic upon mixing this latter with a solution of AALAA of the proper concentration. Curve fitting was performed by nonlinear regression analysis using the software program provided with the stopped-flow instrument.

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