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A Nuclear Magnetic Resonance Study of the Interaction between Vanomycin and Acetyl-*D*-alanyl-*D*-alanine in Aqueous Solution

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SUMMARY

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The association between vancomycin and acetyl-D-alanyl-D-alanine has been studied by nuclear magnetic resonance spectroscopy. The proton resonances of the three methyl groups in the peptide are all shifted upfield in the complex, probably because the methyl groups are bound in the proximity of aromatic rings in vancomycin. An analysis of binding as a function of pH showed that while the most stable complex is formed between the peptide anion and the vancomycin cation, weaker complexes are formed between uncharged peptide and vancomycin cation and between peptide anion and uncharged vancomycin. The interacting cationic group in vancomycin was identified as the NH_2-terminal *N*-methylleucine. The peptide proton chemical shifts are unaffected by temperature, but the acetyl shift is decreased at high pH by ionization of the *N*-methylleucine or vancosamine. In aglucovancomycin, the binding constant is weaker and all the chemical shifts in the bound complex are reduced.

INTRODUCTION

Vancomycin, an antibiotic isolated from *Streptomyces orientalis*, n. sp., is active against Gram-positive bacteria and has been shown to interfere with the biosynthesis of bacterial cell walls. Perkins (1) found that mucopeptide precursor molecules containing the terminal -D-Ala-D-Ala fragment bind strongly to vancomycin. In fact, acetyl-D-Ala-D-Ala itself forms a complex with vancomycin, with an association constant of 1.4×10^4 M^{-1}. The attachment of the bulky vancomycin molecule to precursor molecules terminating in D-Ala-D-Ala would prevent their incorporation into the cell wall structure, and Perkins concluded that the ability of vancomycin to form complexes with these peptides which are unique to bacteria was linked to the mechanism of action of the antibiotic. Nieto and Perkins (2) investigated the extent to which vancomycin-peptide interactions depend on the length and composition of the peptide chain, by measuring the association constants of a wide range of synthetic peptides. They found that in order to form a stable complex, (a) three amide linkages are required; (b) the terminal carboxyl group must be free; (c) the two COOH-terminal peptides must be either glycine or have a D configuration for favorable interaction with vancomycin, a D-Ala residue being especially favorable in both posi-

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tions; and (d) the third residue from the COOH terminus should have an L configuration for optimal binding.

We have used nuclear magnetic resonance spectroscopy to study the complex formation *in vitro* between vancomycin and acetyl-D-Ala-D-Ala in an attempt to define detailed interactions between specific groups of nuclei in both molecules.

EXPERIMENTAL PROCEDURE

The peptide D-Ala-D-Ala was obtained commercially (International Chemical and Nuclear Corporation or Cyclo Chemical Corporation). It was acetylated using the method described by Perkins (1) and purified chromatographically. Vancomycin hydrochloride was kindly supplied by Lilly Research Centre, Ltd., England, and was purified chromatographically using sulfoethyl Sephadex C-25 at pH 4, eluting with a linear salt gradient of 0–0.3 M NaCl in 5 mM sodium acetate buffer solution. There was no difference between the results obtained in the binding experiments with vancomycin and acetyl-D-Ala-D-Ala using purified and nonpurified vancomycin; in most of the experiments commercial vancomycin was used without further purification. Aglucovancomyin was prepared by mild acid hydrolysis of vancomycin, which removes glucose and vancosamine; about three-quarters of the original biological activity is retained (3).

Low-concentration solutions in D_2O were used in the binding studies because of problems of aggregation (vancomycin) and solubility (complex). Spectra were run on a Varian XL-100-15 spectrometer. Fourier transform techniques combined with block-averaging of the transformed spectra overcame the sensitivity and, to a certain extent, the dynamic range problems, respectively. However, it was also necessary to eliminate partially the very large solvent signal (residual HOD in the D_2O solvent), using the method of rapid pulsing described by Benz *et al.* (4). Dioxane was used as an internal reference for measuring chemical shifts. When a controlled temperature was required, a Varian variable-temperature accessory was employed, the dial settings being calibrated with a thermocouple. The pH measurements were made with a glass electrode, using a Radiometer model 26 pH meter; the values given are meter readings, and no allowance has been made for any deuterium isotope effects. The NMR samples were made up in the following appropriate buffer solutions: pH 1.1–3.5, glycine-NaCl-DCl or DCl-KCl; pH 3.5–5.8, CD_3COOD-NaOD; pH 5.9–8.0, KH_2PO_4-NaOD; pH 8.0–11.5, H_3BO_3-KCl-NaOD or glycine-NaCl-NaOD.

RESULTS AND DISCUSSION

To investigate the interaction between vancomycin and acetyl-D-Ala-D-Ala using NMR techniques it is necessary to assign unambiguously the proton resonance signals under consideration. This is relatively straightforward for acetyl-D-Ala-D-Ala (see Fig. 1a), where the assignments of all the signals can be made from chemical shift comparisons with other compounds and from the ionization shifts observed when the carboxylate group is protonated. For vancomycin a complete assignment of the proton spectrum is not possible at the present time, mainly because the full structural elucidation has not been completed. However, it is well established that vancomycin (mol wt 1560) contains an NH_2-terminal *N*-methylleucine, and the assignments for the leucyl γ-CH_3 and *N*-CH_3 protons could be made with ease. These were the only vancomycin signals used to monitor the interactions. Concentrated solutions of vancomycin (30 mg/ml) gave proton NMR spectra with very broad lines as a result of aggregation; well-resolved spectra were obtained by examining dilute solutions (0.5 mg/ml) or by raising the temperature. Nieto and Perkins (5) had previously observed aggregation of vancomycin using spectrophotometric measurements, and their estimated value for the self-association constant is similar to the value obtained from the NMR experiments if a monomer-dimer model is assumed ($K = 0.7 \pm 0.1$ mM^{-1}). In our studies vancomycin concentrations of less than 0.5 mg/ml were used, and at these dilutions we could expect no complications from aggregation phenomena.

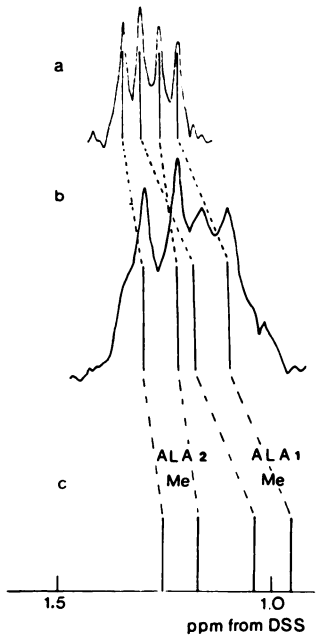

FIG. 1. *^1H resonance spectra at 100 MHz of α-methyl protons of acetyl-D-Ala-D-Ala*

a. Free solution (0.8 mM, pH 5.5, 100 transients of 1-sec acquisition time). b. In the presence of vancomycin (peptide concentration, 0.4 mM; vancomycin, 0.125 mM; pH 5.5; 13 × 150 transients at 1-sec acquisition time). c. Calculated bound shifts. [DSS is the reference, sodium 4,4-dimethyl-4-silapentane sulphonate.]

To obtain quantitative binding data, solutions containing a fixed ratio of peptide to vancomycin were examined at different dilutions. Complex formation results both in broadening and in upfield chemical shifts of all methyl resonances of acetyl-D-Ala-D-Ala (see Fig. 1b). These effects are greatest for the $C_1(\alpha)CH_3$ doublet band and smallest for the acetylmethyl signal. Figure 2 shows the binding curves obtained by plotting the observed chemical shifts of the acetyl-D-Ala-D-Ala and vancomycin peaks against concentration. Separate NMR signals for the bound and free species are not observed; instead, a single spectrum results from the species being in fast exchange, and the observed chemical shifts are the weighted mean positions of the free and bound species.

The binding curves were fitted to theoretical curves, from which binding constants and the bound chemical shifts could be obtained. If δ_f and δ_b are the chemical shifts of the free and bound species, respectively, then with δ_o as the observed averaged chemical shift and K as the equilibrium constant

$$\frac{1}{K} = \left[\delta_b - \delta_o\right]\left[\frac{V_0}{(\delta_o - \delta_f)} - \frac{P_0}{(\delta_b - \delta_f)}\right]$$

for peptide signals and

$$\frac{1}{K} = \left[\delta_b - \delta_o\right]\left[\frac{P_0}{(\delta_o - \delta_f)} - \frac{V_0}{(\delta_b - \delta_f)}\right]$$

for vancomycin signals, where P_0 and V_0 are the total peptide and vancomycin concentrations, respectively.

Reasonable agreement is found between the association constants obtained in this study (Table 1) and those quoted by Nieto and Perkins (2); the discrepancies are due to the higher molecular weight (1800 rather than 1560) of vancomycin used by those workers in their calculation of K and also to the differences in ionic strength and temperature between the two studies (25° and 0.02 M compared with 35° and 0.05 M in our experiments).

Effect of pH on Association Constants

Dilution experiments were repeated in the pH range 2.2–10.5, and the association constants and bound shifts were obtained.

The results for the association constants (Fig. 3) are similar to the results obtained by Nieto and Perkins (5) for binding of diacetyl-L-Lys-D-Ala-D-Ala using optical rotatory dispersion and ultraviolet difference spectra. The curve shows four turning points, at pH 2.9, 3.9, 7.7, and 8.6.

Using the general rules outlined by Dixon (6), we can interpret the ionization changes involved as follows.

pH < 3.5. In this pH range there is association of the vancomycin cation with uncharged peptide to form a cationic complex

$$V^+ + PH \rightleftharpoons V^+PH$$

The pK of 2.9 corresponds to the equilibrium

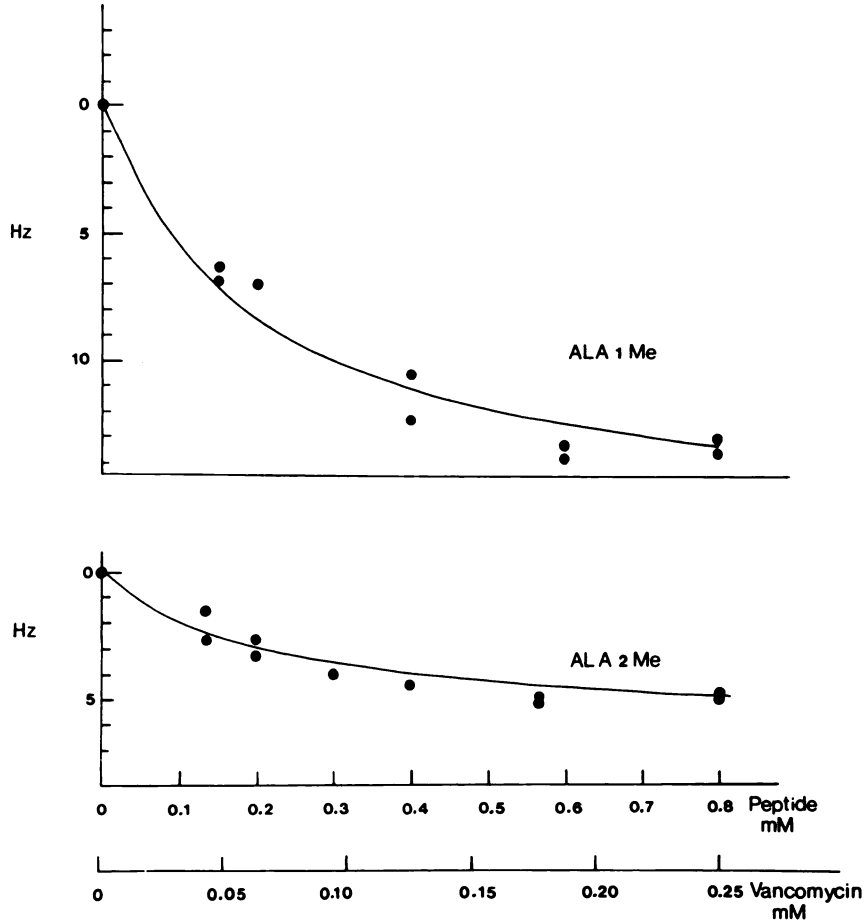

FIG. 2. *Shifts (at 100 MHz) of α-methyl resonances in a 3.2:1 mixture of acetyl-D-Ala-D-Ala and vancomycin*

TABLE 1

Association constant and bound shifts[a] (^1H chemical shifts at 100 MHz) for acetyl-D-Ala-D-Ala and vancomycin at pD 5.5 and 35°

K (M^{-1})	$1.4 \pm 0.1 \times 10^4$
$\Delta C_1(\alpha)Me$	57 ± 3 Hz
$\Delta C_2(\alpha)Me$	20 ± 1 Hz
$\Delta C_2(-Ac)Me$	8.6 ± 1 Hz

[a] $\Delta = \Delta_{free} - \Delta_{bound}$ (Hertz). Upfield shifts are positive.

$$VP + H^+ \rightleftharpoons V^+PH$$

and thus to the perturbed ionization of the peptide when complexed.

pH 3.5–4.5. In this pH range there is association of the vancomycin cation with the anionic peptide to form a neutral complex. The pK of 3.9 corresponds to the pK of the free peptide.

The equilibria concerned are

$$P^- + H^+ \rightleftharpoons PH$$

$$P^- + V^+ \rightleftharpoons PV$$

pH 4.5–7.0. The pH-independent binding in the biologically relevant pH region 4.5–7.0 corresponds to a major association interaction:

$$P^- + V^+ \rightleftharpoons PV$$

pH 7.2–8.2. In this range there is again association of the vancomycin cation with the peptide anion to form a neutral complex:

$$P^- + V^+ \rightleftharpoons PV$$

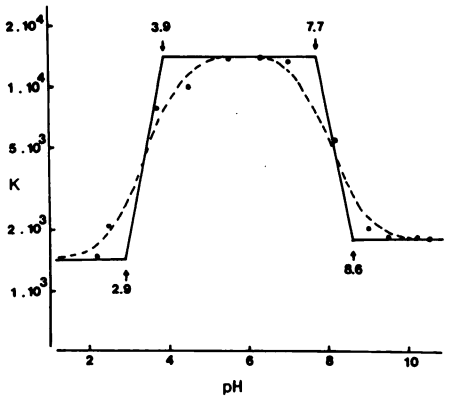

FIG. 3. *Plot of affinity constant of acetyl-D-Ala–D-Ala for vancomycin as a function of pH*

The segments have slopes of +1 (pH 2.9 → 3.9) and −1 (pH 7.7 → 8.6). The broken line was calculated from the ionization equations, using the pK values indicated by the turning points.

The pK of 7.7 measures the ionization of the interacting cationic group in free vancomycin:

$$V^+ \rightleftharpoons V + H^+$$

pH > 8.2. In this range uncharged vancomycin associates with the peptide anion to form an anionic complex. The pK of 8.6 corresponds to the perturbed ionization of vancomycin in the complex, i.e.,

$$PV \rightleftharpoons P^-V + H^+$$

A study of the pH-dependent chemical shifts gives excellent confirmation of this scheme.

1. The chemical shift of the $C_1(\alpha)$ methyl peak in the spectrum of free acetylated peptide displays a pH dependence which gives a pK of 3.8 ± 0.1 for the peptide carboxyl group. This value is in agreement with the pK of 3.9 deduced above.

2. The pH dependence of the proton chemical shift of the leucine *N*-methyl group in free vancomycin gives a pK value of 7.7 ± 0.2 (Fig. 4), identical with the value found in the analysis of the association constant data. There is only one other basic group in vancomycin, the amino group in the amino sugar, vancosamine, but this has a pK of 9.2; it is thus established that the ionization of the *N*-methylleucine is important in the formation of the complex.

3. In the complex, the *N*-methylleucine can still be titrated (Fig. 4), and its pK is shifted to 8.7 ± 0.2, again in good agreement with the value of 8.6 deduced from the analysis.

It was not possible to complete the analysis and establish directly that the apparent transition at pH 2.9 was due to a perturbed ionization of the peptide carboxyl group, because the ionization shift is small compared with the errors involved in determining the shift in the complex.

The scheme is thus self-consistent and shows that the increased stability of the complex in the middle pH range is due to the electrostatic interaction between the peptide carboxylate anion and the *N*-methylleucine cation. The magnitude of this contribution to the free energy of the complex can be estimated to be $\Delta F^\circ = -1.4$ kcal/mole. It is interesting that both the high-pH anionic complex and the low-pH cationic complex give approximately the same value of binding constant. It should be borne in mind that these two groups are capable of forming hydrogen-bonded associations in either of the latter situations, and these probably contribute appreciably to the stability of the complex. The failure of binding of the methyl ester of *N*-acetyl-D-Ala-D-Ala is probably sterically determined by the inability of the site to accommodate the increased bulk of the methyl group.

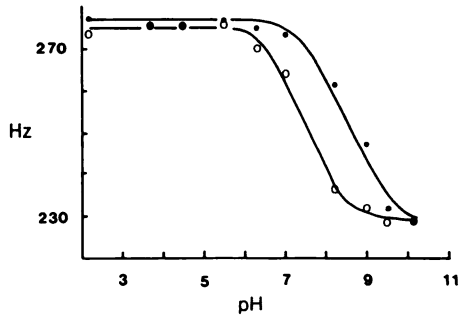

FIG. 4. *Shifts (at 100 MHz) of leucine N-methyl resonance of vancomycin as a function of pH in the absence of ligand* (O——O) *and in the presence of a large excess of acetyl-D-Ala-D-Ala* (●——●).

Effects of pH on Bound Chemical Shifts

Examination of Fig. 5 indicates that generally the bound shifts are remarkably insensitive to pH variation. The only peptide bound shift to display any variation with pH is that of the acetylmethyl group protons, which decreases above pH 8. This change could arise from either the ionization of the leucine *N*-methyl group (pK 8.7 ± 0.2 in the complex) or that of the NH_2 group in vancrosamine (pK 9.2 ± 0.1 in free vancomycin). The invariance of the other bound shifts with pH suggests that there are no detectable changes in conformation of the bound peptide and the groups in the vancomycin binding site over the pH range studied. The alanine C_1-(αMe) and C_2(αMe) groups are clearly not in close proximity to ionizable groups on vancomycin, which would cause changes in the bound chemical shifts by through-space electric field effects.

The large bound shifts found for both alanine methyl groups (0.57 and 0.2 ppm) are upfield and are most probably due to the shielding effects of ring currents from neighboring aromatic groups. (It is known that vancomycin contains five aromatic rings.) Shielding contributions of these magnitudes can be experienced over a wide range of positions above the plane of an aromatic ring system. For example, a shift of 0.57 ppm could arise from the methyl protons being within the range 2–4.2 A above a single aromatic ring (7). It is not possible to treat the data in a quantitative manner because, even if all the shielding contributions in the bound shifts of the alanine CH_3 protons arose from ring current effects, one could not exclude the possibility that more than one aromatic ring is involved. In the absence of the molecular structural details for vancomycin it is not prudent to speculate further on this matter.

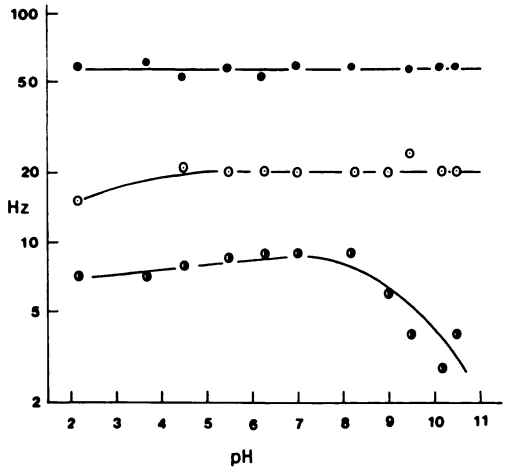

FIG. 5. *Bound shifts (at 100 MHz) of residues in acetyl-D-Ala-D-Ala as a function of pH*
●—●, $\Delta C_1(\alpha)Me$; ⊙—⊙, $\Delta C_2(\alpha)Me$; ◑—◑, $\Delta C_3(Ac)Me$.

In the complex the doublet due to the C(γ)-methyls of leucine is shifted upfield by 13 Hz. This bound shift is pH-independent in the range 3.7–10.5 and is therefore apparently unaffected by the ionization state of the leucine or the vancosamine. At pH 2.2, however, the shift is reduced to 3 Hz, suggesting that these groups in the leucine are in close proximity to the peptide carboxylate group.

Effects of Temperature

Table 2 contains the results of dilution experiments carried out at pH 5.5 and temperatures of 35°, 42°, and 54°; the association constants decrease with temperature. The thermodynamic parameters of complex formation are: $\Delta F°$ (25°) = −6.04 kcal/mole; $\Delta H°$ = −13.9 kcal/mole; $\Delta S°$ = −24 e.u.

Similar results were obtained by Nieto and Perkins (5) for the temperature dependence of the affinity of diacetyl-L-Lys-D-Ala-D-Ala for vancomycin.[2] There is no change in any of the bound shifts with temperature, indicating that no detectable conformational changes occurred within the binding site. Furthermore, the factors contributing to the bound shifts must be insensitive to temperature changes, as would be expected if the shielding arises from ring current effects.

Effects of Urea

Dilution experiments in 4 M urea-d_4 at pH 5.5 indicate a decrease in the associa-

[2] The sign of the $\Delta S°$ contributions is incorrect in the paper by Nieto and Perkins (5).

TABLE 2

Association constants and ^1H chemical shifts[a] at 100 MHz of acetyl-D-Ala–D-Ala bound to vancomycin and aglucovancomycin under various physical conditions

Sample	K	$\Delta C_3(-Ac)Me$	Ala ΔC_2 (α)Me	Ala ΔC_1 (α)Me
	mM^{-1}	*Hz*	*Hz*	*Hz*
Acetyl-D-Ala-D-Ala + vancomycin (35°)	14 ± 2	8.6 ± 1	20 ± 1	57 ± 3
Acetyl-D-Ala-D-Ala + vancomycin (42°)	6 ± 1	7.8 ± 0.5	20	57
Acetyl-D-Ala-D-Ala + vancomycin (54°)	4 ± 0.5	7.3	22	58 ± 1
Acetyl-D-Ala-D-Ala + vancomycin in 4 M urea (35°)	4 ± 1	6.5 ± 1	17 ± 2	55 ± 5
Acetyl-D-Ala-D-Ala + aglucovancomycin	1.8 ± 0.2	6.6	15	36

[a] $\Delta = \Delta_{free} - \Delta_{bound}$ (Hertz). Upfield shifts are positive.

tion constant to 4 mM^{-1} (see Table 2), a result similar to that found by Nieto and Perkins (5). However, no significant differences in the calculated bound shifts were detected. Thus, although the affinity of acetyl-D-Ala–D-Ala for vancomycin is lowered in urea, the mode of binding remains unaltered and suggests that urea interacts with free peptide or vancomycin.

Complex Formation Between Aglucovancomycin and Acetyl-D-Ala-D-Ala

The results of the experiment on aglucovancomycin and acetyl-D-Ala–D-Ala are shown in Table 2. Not only is the association constant an order of magnitude lower than for the vancomycin-peptide complex, but the bound peptide methyl shifts are reduced to about three-quarters of their previous values. Obviously, some modification of the binding site has taken place, but since the over-all pattern of observed bound shifts for the peptide is similar to that for the complex with vancomycin itself, it seems that no major change in bound peptide conformation has occurred.

It is not possible to decide whether the shift changes are directly attributable to the absence of shielding interactions from the sugars or to secondary effects caused by conformational changes in the binding site of vancomycin which change the disposition of the aromatic groups with respect to the peptide methyl groups.

Solubility limitations prevented dilution studies of aglucovancomycin and peptide over a wide pH range. Such measurements would have been useful in deciding the role of the vancosamine NH_2 ionization (pK_a = 9.2 ± 0.1) in the binding process.

CONCLUSIONS

The association constants measured using NMR methods for the complex formation involving vancomycin and acetyl-D-Ala–D-Ala under conditions of different pH, temperature, and solvent are shown to be broadly in agreement with the findings of Nieto and Perkins. An important contribution to the stability of the complex is provided by the electrostatic interaction between the carboxylate ion of the peptide and the protonated leucine *N*-methyl group of vancomycin. The NMR method allows us to monitor directly the change in pK of the leucine *N*-methyl protonation, from 7.7 ± 0.2 in vancomycin to 8.7 ± 0.2 in the vancomycin-peptide complex, which clearly implicates the protonated *N*-methyl group in the binding process.

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