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Interaction between 2′,5′-A Core Analogues and 2′,5′-Specific Phosphodiesterase

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Analogues of (2′,5′-)triadenylate (2′,5′-A core) were synthesized and their stabilities against 2′-phosphodiesterase (2′PDi) purified from Ehrlich ascites cells were determined. Replacement of the ribose group with an arabinose in different positions of the trimer resulted in dramatic changes in the rate of hydrolysis by 2′PDi. Analogues with an arabinose at the 2′ terminal or in the middle position or both were stable against 2′PDi. These stable analogues did not inhibit the degradation of 2′,5′-A core by 2′PDi indicating that they did not have the same affinity for the enxyme. Modifications at the 3′ hydroxyls also resulted in structures resistent to degradation by 2′PDi. Replacement of the 6-aminopurine moiety of adenosine by 2-aminopurine resulted in only small changes in stability against 2′PDi. These results show that the configuration of the sugar at 2′ and 3′ carbons are important for the recognition by 2′-phosphodiesterase.

Interferon exerts on cells a number of effects, one of which is the formation of a mediator of antiviral activity, ppp A(2′−5′)A_n, where n=1−14 (referred to collectively as 2′,5′-A). (For a review, see Ref. 1). The induction of 2′,5′-A synthetase by interferon results in the synthesis of 2′,5′-A,[2] which is capable of activating a latent endoribonuclease, RNase L,[3–6] possessing the potential of degrading mRNA, rRNA and viral genome RNA. The concentration of 2′,5′-A, which thus influences the rate of RNA degradation, depends both on its rate of synthesis and degradation. The degradation of 2,5-A is mediated by a phosphodiesterase (2′PDi) which presumably is also induced by interferon.[5,6,7] Several reports[8,9,10,12,14,15] describe the synthesis and evaluation of analogous of 2′,5′-A. Increased stability against degradation by 2′PDi, or other nucleases, and at the same time an ability to activate RNase L or interfering with the binding of 2′,5′-A to 2′PDi could result in antiviral effects.

In what follows, we describe the synthesis of some analogues to A(2′−5′)A_2 (2′,5′-A core) and the determination of their stacking – unstacking properties by circular dichroism spectroscopy and their degradation by a partly purified 2′PDi as well as their inhibition of 2′PDi activity against 2′,5′-A.

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EXPERIMENTAL

Materials

The dinucleoside monophosphates 2',5'-ApA, 3',5'-ApA, 2',5'-UpU, 3',5'-UpU, 2',5'-CpA and 3',5'-CpA were from Sigma Chemical Corporation. St. Louis, Miss. USA. The concentrations of the oligonucleotides are given in adenosine 5'-monophosphate (AMP)-equivalents and calculated from the absorbance on the basis of an A_{260} of 15.6×10^3 M^{-1} cm^{-1} for AMP. DEAE cellulose, DE 52, and phosphocellulose, P 11, were from Whatman.

The chemical synthesis of 2',5'-A core was accomplished using two approaches. In the first one[18] 9-phenylxanthen-9-yl and 2,2-dibromomethylbenzoyl were used for the protection of the 3' and 5' hydroxyl functions, respectively, and in the second one[19] the 1,1,3,3-tetraisopropyldisiloxane group was used for the simultaneous protection of the 3' and 5' hydroxyl functions, followed by a two-step phosphorylation procedure that gave the internucleotide 2'→5' linkage. The fully protected dimer was then unmasked at the 5'-end in a quantitative yield and after a condensation reaction[20] gave the fully protected 2',5'-A core. It is the versatility of the second approach that we have exploited in synthesizing all analogues used in this work. The chemical synthesis of the 9-β-D-arabinofuranosyl (*ara*) $A(2'-5')A_2$ and other analogues constituting all combinations of 9-β-D-arabinofuranosyl (*ara*) and 9-β-D-ribofuranosyl (*ribo*) sugars and the conformational analyses of these analogues using circular dichroism spectroscopy have been reported previously.[20,21] The chemical synthesis of the 2-aminopurine analogues has also been achieved using the procedure reported for the arabino analogues.[21] However, the physico-chemical properties of the 2',5'-A analogues comprising the 2- aminopurine moiety, as shown in Table 1, have not been reported previously.

Preparation of 2',5'-phosphodiesterase (2'PDi). Erlich ascites tumor (EAT) cells grown intraperitoneally in NMRI mice were harvested 10 days after inoculation. The cells were lysed with gentle sonification in 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 5 mM $MgCl_2$, 120 mM KCl, 1 mM dithiothreitol (DTT), 10 % glycerol, 0.5 % NP 40, and 10 μM phenylmethanesulfonyl fluoride (PMSF). After centrifugation for 1.5 h at 39 K rpm in T865 (Sorvall), the supernatant was fractionated by differential precipitation with $(NH_4)_2SO_4$ at pH 8.2.[22] The fraction precipitating between 30 and 47.5 % $(NH_4)_2SO_4$ was dialysed over-night against 25 mM KCl in 20 mM HEPES of pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 10 % glycerol, and 10 μM PMSF. Further purification (DEAE-cellulose, phosphocellulose) was made according to Schmidt *et al.*[5] Fractions of phosphodiesterase activity, purified 200-fold compared to cell-lysate, (2'PDi) were stored in aliquots at −70 °C.

Methods

Physico-chemical determinations. Circular dichroism (CD) spectra were recorded using a JASCO spectrometer as described previously.[21] UV spectra were determined using a Cary 2240 spectrophotometer. ^1H-NMR spectra were recorded using a Jeol FX 90 Q spectrometer at 89.99 MHz at 70 °C and pD 6.8 using 10–20 mg of the compounds in 0.3–0.4 ml D_2O. Sodium 3-trimethylsilyl propanoate was used as an internal standard. The NMR spectrometer had a resolution of 0.25 Hz.

Enzyme assay. Ten μl of 2'PDi was incubated with 900 pmol oligonucleotide in a 30 μl reaction mixture containing 25 mM KCl, 20 mM HEPES pH 7.5, 5 mM $Mg(OAc)_2$, 1 mM DTT, and 10 % glycerol at 30 °C for 30 min. The reaction was stopped by boiling the tubes for 3 min and 10 μl of each reaction mixture was analysed by HPLC (Waters pump model 6000 A, UV detector model 444, injector U6K and a data module). The solvent used was 0.1 M KH_2PO_4, pH 3.2 and the anion-exchange column was a Whatman partisil 10 SAX (8 mm ∅×100 mm).

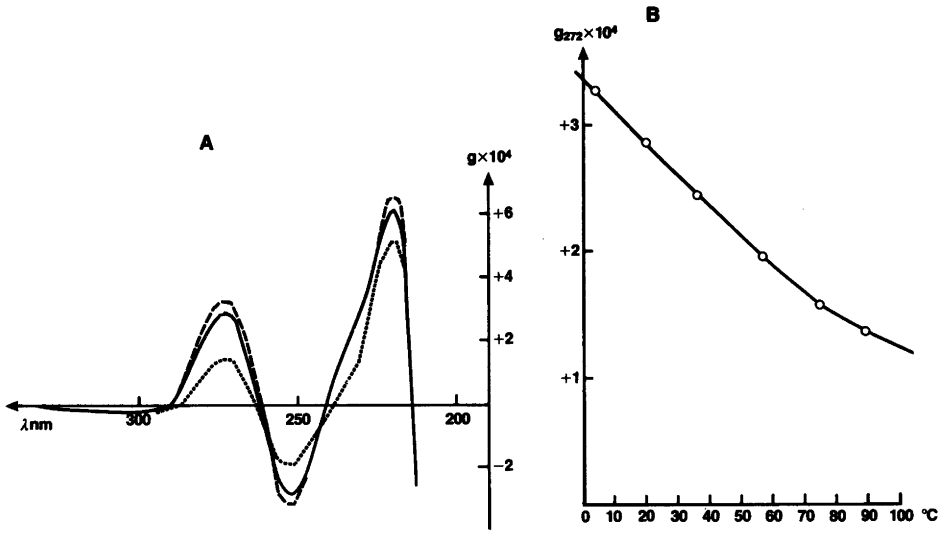

Fig. 1. Spectroscopial properties of A2′p5′A2′p5′A^{N2}. A. The CD spectrum in 0.01 M Na phosphate, pH 7.4 at various temperatures: ——, +20 °C; – – –, +4 °C; ●●●, +89 °C. B. Plot of dissymmetry *g*-factor as a function of temperature at 272 nm in 0.01 M Na phosphate pH 7.4.

RESULTS

Determination of stacking properties of modified oligonucleotides by circular dichroism spectroscopy. Plots of *g*-factors *vs.* temperature have been generated for the trimers A2′p5′A2′p5′A^{N^2}, A^{N^2}2′p5′A2′p5′A and A2′p5′A^{N^2}2′p5′A. The *g*-factor plotted was that

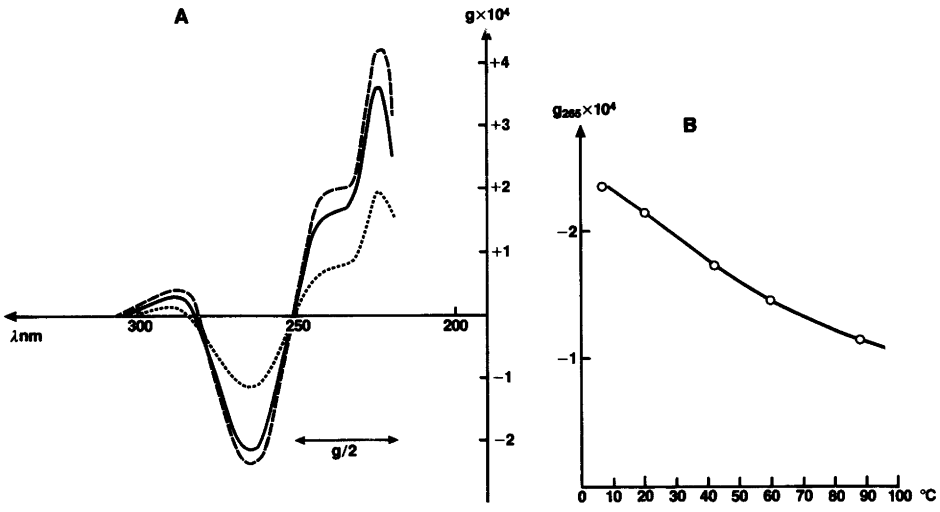

Fig. 2. Spectroscopial properties of A2′p5′A^{N^2}2′p5′A. A. The CD spectrum in 0.01 M Na phosphate, pH 7.4 at various temperatures: ——, +20 °C; – – –, 6 °C; ●●●, +88 °C. B. Plot of dissymmetry *g*-factor as a function of temperature at 265 nm in 0.01 M Na phosphate pH 7.4.

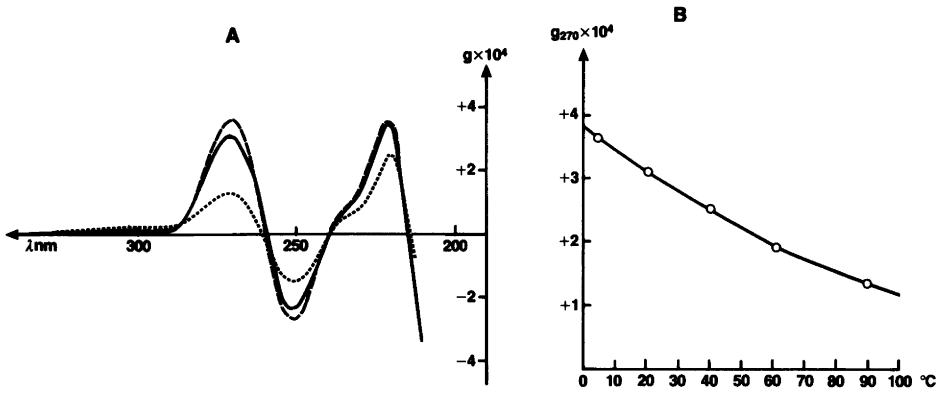

Fig. 3. Spectroscopial properties of $A^{n^2}2'p5'A2'p5'A$. A. The CD spectrum in 0.01 M Na phosphate, pH 7.4 at various temperatures: ——, +21 °C; – – –, +5 °C; ●●●, +90 °C. B. Plot of dissymmetry *g*-factor as a function of temperature at 270 nm in 0.01 M Na phosphate pH 7.4.

corresponding to a suitable CP peak between 260 and 280 nm be it positive or negative, as shown in Figs. 1–3.

Setting aside the problem of intensities the shapes of the *g* versus *I* curves are similar for all three trimers at high temperatures (50–80 °C), a shown in Figs. 1–3. Thus there is no clear difference in the stacking ability of these trimers except that the geometry of the stacked states may be different.

Changing the position of the NH_2-group on the adenine from the 6-to the 2- position changes the electronic spectrum and hence the coupling scheme responsible for the observed CD spectrum will alter.

$A^{N^2}2'p5'A2'p5'A$ and $A2'p5pA2'p5'A^{N^2}$ both show a bisignate curve around 260 nm that is similar to that of the neutral right-handed oligoadenosine. On the other hand

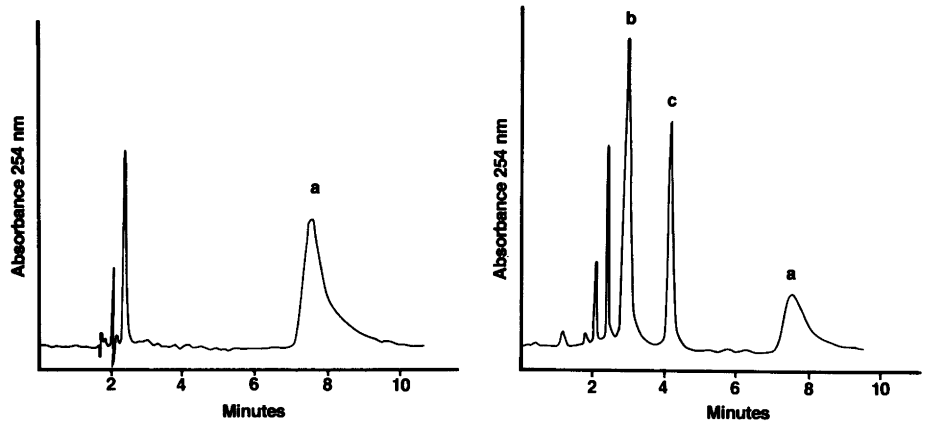

Fig. 4. Hydrolysis of 2',5'-A core by 2'PDi. A. 2',5'-A core, 300 pmoles, was applied to an anion-exchange column and eluted. B. 2',5'-A core, 900 pmoles, was incubated with 2'PDi and separated on an anion-exchange column. The incubation conditions and HPLC procedure were as described in Methods. (a) A2'p5'A2'p5'A; (b) A2'p5'A; (c) 5'AMP.

Table 1. The physico-chemical properties of the 2-aminopurine analogues of ribo-(2′,5′-A).

Substrates	^{1}H NMR (D_2O at 70 °C) absorptions at pD 6.8		TLC: 1 M NH_4OAc–ethanol; 2:8 v/v Merck silica gel 60 F_{254} precoated plates	Hypochromicity at 259 nm calculated by the enzymatic hydrolysis; ε is assumed to be 19,000 for 5′-AMP	Yield %	
	H-2 and H-8 protons	δ H-1′ (coupling constants in Hz)			Overall yield for fully properted material	Fully deprotected material
ribo-(A^{N^6}2′p5′A^{N^6}2′p5′A^{N^2})	8.32(*s*,1H);8.29(*s*,1H); 8.21(*s*,1H);8.17(*s*,2H); 8.11(*s*,1H);	6.4(*d*,1H,1.8); 6.1(*d*,1H,2.1) 5.93(*d*,1H,3.1)	0.32	24 %	58	85
ribo-(A^{N^6}2′p5′A^{N^2}2′p5′A^{N^6})	8.27(*s*,1H);8.22(*s*,1H); 8.15(*s*,2H);8.07(*s*,2H)	6.33(*s*,1H,2.1);6.21(*s*,1H,1.9) 6.08(*s*,1H,2.8)	0.37	19 %	63	93
ribo-(A^{N^2}2′p5′A^{N^6}2′p5′A^{N^6})	8.23(*s*,2H);8.19(*s*,1H); 8.12(*s*,2H);8.09(*s*,1H)	6.38(*s*,1H,3.1);6.22(*s*,1H,2.2) 6.12(*s*,1H,1.8)	0.29	22 %	47	74

A2′p5′A^{N^2}2′p′5′A does not have a A2′p5′A pair and the CD spectrum is entirely due to A2′p5′A^{N^2} couplings; its CD spectrum is therefore quite different. The 6-aminopurine moiety has strong absorption at $\lambda > 300$ nm unlike the 2-aminopurine (A^{N^2}) bases and spectra in this region should be characteristic of only the 6-amino purine moiety (A). Unfortunately, CD in this region is exceedingly weak and it is not clear that a "stacking – unstacking" process is occurring. However, the A^{N^2} base unlike the A base readily fluorescence and the temperature variation of this fluorescence should offer a means of assessing the stacking ability of this base alone. Accordingly, the relative fluorescence of these trimers at various temperatures was measured and indeed it is observed that upon heating to 85 °C, the fluorescence at 367 nm increases to about 130 % of its value at 20 °C. Therefore, it is possible that all the bases in these trimers are involved in a stacking – unstacking equilibrium. It should be added that this interpretation of data on fluorescence spectroscopy is based upon a lack of effect of temperature on the 2-aminopurine riboside used as a control.

Characterization of 2′PDi activity. The partly purified 2′PDi was characterized by using different dinucleoside monophosphates as substrates and determining by HPLC the disappearance of the substrate peak. Table 2 shows that the phosphodiesterase had a specificity against 2′→5′-linkages and the activity was considered to be due to 2′PDi. The 2′,5′-UpU was the most sensitive and 3′,5′-ApA was the only 3′,5′-dinucleoside monophosphate hydrolyzed in accordance with the pattern of activity reported by Schmidt *et al.*[6] The hydrolysis products were idientified on HPLC as nucleoside 5′-monophosphate and the respective nucleoside.

Degradation of arabino analogues of 2′,5′-A by 2′PDi. The 2′,5′-A analogues with free 5′-hydroxyls and ribose replaced by arabinose in all possible combinations, were used as substrates for 2′PDi. The degradation of the trimers was followed by HPLC as exemplified in Fig. 4 where the decrease in trimer concentration and increase in dimer concentration were used to quantify the hydrolysis. As shown in Table 3 only the trimers with ribo-ribo at the 3′ terminal were degraded by 2′PDi. All combinations with arabino in the middle position or at the 3′ end or both were stable against 2′PDi. The presence of an arabino moiety at the 5′ end only marginally changed the rate of hydrolysis as compared to that of 2′,5′-A.

Stability of base and sugar modifications in 2′5′-A. Replacement of the 6-aminopurine moiety by the 2-aminopurine resulted in very small changes in the rate of hydrolysis (Table 4), indicating only a marginal interaction by the exocyclic amino function. On the other hand, methylation of the 3′-hydroxyl groups in 2′,5′-A gave a compound which was stable against degradation by 2′PDi. A similar result was also obtained with trimers blocked with

Table 2. Characterization of 2′PDi activity (conditions, see Methods).

Dinucleoside monophosphate	Hydrolysis/%[a]
2′,5′-UpU	37
3′,5′-UpU	0
2′,5′-CpA	30
3′,5′-CpA	0
2′,5′-ApA	20
3′,5′-ApA	10

[a] The hydrolysis was calculated as the decrease in substrate analyzed by HPLC.

Table 3. Degradation of arabino analogues of 2′,5′-A by partly purified 2′PDi.

Compound	Hydrolysis/%
A2′p5′A2′p5′A (2,5-A core)	42
A2′p5′A2′p5′araA	<1
A2′p5′araA2′p5′araA	<1
araA2′p5′araA2′p5′araA	<1
araA2′p5′araA2′p5′A	<1
araA2′p5′A2′p5′A	33
A2′p5′araA2′p5′A	<1
araA2′p5′A2′p5′araA	<1

Table 4. Degradation of 2′,5′-A analogues with base and sugar modifications by partly purified 2′PDi (see Methods).

	Hydrolysis/%
A2′p5′A2′p5′A (2′,5′-A)	56
A2′p5′A2′p5′A^{N2}	42
A2′p5′A^{N2}2′p5′A	33
A^{N2}2′p5′A2′p5′A	56
(3′dA)2′p5′ (3′dA)2′p5′ (3′dA) (cordycepin)	<1
3′-O-methyl A2′p5′3′-O-methyl A2′p5′3′-O-methyl A	<1
A2′p5′A2′p5′A2′ pixyl	<1

the pixyl group at the free 2′hydroxyl function. A 2′,5′-trimer of cordycepin was also resistant against degradation by 2′PDi.

Competition experiments. To determine if the stable trimers could bind to 2′PDi and thus prevent the degradation of 2′,5′-A, mixtures of stable trimers (See Tables 3 and 4) and 2′,5′-A were incubated with 2′PDi. The presence of the stable trimers araA2′p5′araA2′p5′araA, araA2′p5′A2′p5′araA and A2′p5′A2′p5′araA did not affect the degradation of equimolar concentrations (900 or 450 pmoles/assay of each compound) of 2′,5′-A or araA2′p5′A2′p5′A, indicating a low or no binding of these 2′,5′-A analogues to 2′PDi (Not shown).

DISCUSSION

Analogues to 2′,5′-A and its core are interesting compounds both from the point of view of elucidating the intracellular function of 2′,5′-A and as being potential antiviral drugs. It is thus important to analyse the stability of these analogues, their ability to activate RNase L and their ability to compete with 2′,5′-A at the active site of different enzymes.

The stability of 2′,5′-A, 2′,5′-A core and some analogues in cell extracts have been described previously.[8–11,13–15,21] However, an investigation of the structure-activity relationships for degradation of 2′,5′-A analogues by 2′PDi has not been reported. The 2′PDi activity isolated from EAT cells, purified on DEAE and phosphocellulose columns and used in this study had the specificity for 2′→5′-linkages and a pattern of activity (Table 2) against dinucleoside monophosphates in agreement with that described by Schmidt *et al.*[6]

The presence in a trimer of an araA residue at the 3′-terminal or in the middle position or both resulted in molecules which were resistant to the phosphodiesterase activity of 2′PDi. (Table 3). The compounds in Table 3 which were resistant to 2′PDi activity have earlier been found to be resistant to an unfractionated cell extract.[21]

The stable araA analogues of 2′,5′-A core seemed to have a low affinity to the active site of 2′PDi since their presence did not influence the degradation of 2′,5′-A. The conformation of the trimers with arabinose in the second and/or third position differs considerably from that of 2′,5′-A[21] and this might explain a lack of competition, which otherwise should be expected if they interacted at the same site of the enzyme.

The absence of a 3′-hydroxyl group and the presence of methylated phosphates in the phosphodiester bonds likewise resulted in 2′,5′-A core analogues resistant to 2′PDi. A cordycepin analogue of 2′,5′-A has previously been found to be resistant to degradation by a cell extract and it was also unable to activate RNase L[9,14] but can be hydrolyzed by esterases in serum complemented medium.[16] A similar observation was made by Epstein *et al.*[13] who found xylo analogues of 2′,5′-A core to be resistant to degradation by L-cell extracts but not to be degraded in a serum-containing medium.[17] Methylation of the 3′-hydroxyl groups resulted in a structure resistant to 2′PDi (Table 4). This is in accordance with the results of den Hartog *et al.*[24] and Baglioni *et al.*[8] using crude cell extracts and the results further show the importance of the 3′-hydroxyl for the stability to 2′PDi degradation.

The previously determined stacking properties of 2′,5′-A trimers containing araA showed[21] that the sensitivity to exonucleolytic enzymes was directly dependent upon the degree of the stacking – unstacking state. Changes in the adenosine base in the 2′,5′-A core analogues, as shown in Table 4, did not appreciably affect the degradation by 2′PDi. This is in agreement with the observation that no clear difference in the relative stacking properties of the A^{N2} containing analogues was apparent by circular dichroism spectroscopy.

This is a first step to analyse the interaction between 2′,5′-A and purified enzymes involved in its function. Further analysis of the interaction with the present and new compounds will be required to delineate the structures able to interact at the active sites on different enzymes involved in the function of 2′,5′-A.

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