# Trichorzins HA and MA, Antibiotic Peptides from *Trichoderma harzianum*

## I. Fermentation, Isolation and Biological Properties

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Trichorzins HA and MA, original 18-residue peptides, were isolated from two strains of the widespread soil fungus *Trichoderma harzianum* which have been shown to exhibit antibiotic activity against phytopathogenic fungi. These linear peptides belonging to the peptaibol class are biosynthesized as a complex of closely related analogues. Nine major pure peptides, six trichorzins HA and three trichorzins MA, were isolated by reversed-phase HPLC. The isolated peptides exhibited antibacterial activity against *S. aureus* and increased the membrane permeability of egg phosphatidylcholine/cholesterol (7/3) liposomes, as measured by monitoring leakage kinetics of a fluorescent probe. Structure-activity relationships were deduced from the antibiotic and membrane-modifying properties.

Linear hydrophobic peptides containing a high proportion of the α,α-dialkylated amino acids, α-amino isobutyric acid (Aib, U) and isovaline (Iva, J), an acetylated (or acylated) N-terminus and a C-terminal amino alcohol have been defined as peptaibols. They form a unique class of antibiotic peptides containing seven to twenty residues and are mostly biosynthesized by soil fungi of the genus *Trichoderma*[1~7]. Based on their chain lengths, different subclasses of peptaibols are distinguished: long-sequence peptaibols (18- to 20-residue peptides)[1~5], short-sequence peptaibols (11- to 16-residue peptides)[6] and lipopeptaibols (7 or 11-residue peptides with an N-terminal amino acid acylated by a short lipidic chain)[7].

The main interest in peptaibols stems from their ability to form amphipathic helices which interact with phospholipid bilayers, perturbing their permeability properties by forming voltage-gated ion channels[8~11]. A broad range of bioactivities related to the membrane-perturbing properties characterizes the peptaibols; haemolysis[12], catecholamine-secretion activity in adrenal chromaffin cells[13], uncoupling of rat liver mitochondria[14] and inhibition of the multiplication of different types of cells[10,15] were described in addition to the general antifungal properties.

In our course of investigation on original sequences of peptaibols, we examined the peptaibol content of several *T. harzianum* strains exhibiting antibiotic activity against phytopathogenic fungi and isolated different groups of peptaibols. Among them, we characterized two distinct groups of 18-residue peptides, trichorzins HA and MA (Fig. 1). Details on the sequence determination

Fig. 1. Sequences of trichorzins HA and MA and of selected peptaibols of different chain lengths.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HA I | Ac | Aib | Gly | Ala | Aib | Aib | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | Leuol |
| HA II | Ac | Aib | Gly | Ala | Aib | Aib | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Iva | Gln | Leuol |
| HA III | Ac | Aib | Gly | Ala | Aib | Iva | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | Leuol |
| HA V | Ac | Aib | Gly | Ala | Aib | Iva | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Iva | Gln | Leuol |
| HA VI | Ac | Aib | Gly | Ala | Aib | Iva | Gln | Iva | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Iva | Gln | Leuol |
| HA VII | Ac | Aib | Gly | Ala | Aib | Iva | Gln | Val | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Iva | Gln | Leuol |
| MA I | Ac | Aib | Ser | Ala | Aib | Aib | Gln | Aib | Leu | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | Valol |
| MA II | Ac | Aib | Ser | Ala | Aib | Iva | Gln | Aib | Leu | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | Valol |
| MA III | Ac | Aib | Ser | Ala | Aib | Iva | Gln | Iva | Leu | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | Valol |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SA II | Ac | Aib | Ala | Aib | Ala | Aib | Ala | Gln | Aib | Leu | Aib | Gly | Aib | Aib | Pro | Val | Aib | Iva | Gln | Gln | Pheol |
| TA IIIc | Ac | Aib | Gly | Phe | Aib | Aib | Gln | Aib | Aib | Aib | Ser | Leu | Aib | Pro | Val | Aib | Iva | Gln | Gln | Leuol | |
| KA V | Ac | Aib | Gly | Ala | Aib | Ile | Gln | Aib | Aib | Aib | Ser | Leu | Aib | Pro | Val | Aib | Ile | Gln | Gln | Leuol | |
| HC VIII | Ac | Aib | Asn | Leu | Aib | Pro | Ala | Val | Aib | Pro | Iva | Leu | Aib | Pro | Leuol | | | | | | |
| HC XV | Ac | Aib | Gln | Leu | Aib | Pro | Ala | Ile | Aib | Pro | Iva | Leu | Aib | Pro | Leuol | | | | | | |

Leuol: leucinol; Valol: valinol; Pheol: phenylalaninol.

of trichorzins are reported in the following paper[16]. We report here on the isolation of the main trichorzins and describe their antimicrobial, antifungal and membrane-modifying properties.

## Materials and Methods

### Fermentation

Two *T. harzianum* strains isolated from soil samples collected in Uruguay (M-903602) and in Malaysia (M-922835) have been selected for their antibiotic activity against phytopathogenic fungi. They were stocked as lyophilized samples in the "Collection de souches fongiques du Muséum National d'Histoire Naturelle" and obtained as agar slants containing 2% w/v malt-agar at 27°C for 5 days. A spore suspension as inoculum was obtained. The fermentation medium (20 liters) was disposed into 120 Roux flasks, each containing 160~170 ml of the sterilized synthetic medium (glucose 0.5%; $KH_2PO_4$ 0.08%; $KNO_3$ 0.07%; $Ca(H_2PO_4)_2$ 0.02%; $MgSO_4 \cdot 7H_2O$ 0.05%; $MnSO_4 \cdot 5H_2O$ 0.001%; $CuSO_4 \cdot 5H_2O$ 0.0005%; $FeSO_4 \cdot 7H_2O$ 0.0001%). The flasks were inoculated with 2 ml of spore suspension each and were incubated in the stationary mode, for 10 to 15 days, at 27°C. Sporulation of the fungi was considered as complete when the originally colorless mycelium had uniformely turned into a green colour.

### Isolation

The filtered fermentation broths of the two *T. harzianum* strains were independently extracted three times with *n*-butanol, giving after removing the solvent under reduced pressure, 1.1 g and 2.0 g of crude extracts for strains M-903602 and M-922835, respectively.

The residues were submitted to gel filtration on Sephadex LH 20 with methanol as eluent. Crude peptide mixtures (M-903602: 493 mg; M-922835: 164 mg) were then chromatographed over silica gel (Kieselgel 60 H Merck, Darmstadt) with $CH_2Cl_2$ - MeOH (9:1→1:1) as eluent. Harzianins HC (142 mg; strain M-903602) or harzianins MB (28 mg; strain M-922835) eluted first with $CH_2Cl_2$ - MeOH (8:2) followed by trichorzins HA (197 mg; strain M-903602) or trichorzins MA (53 mg; strain M-922835) with $CH_2Cl_2$ - MeOH (7:3).

Chromatographies of the extracts and of the fractions ($SiO_2$, Merck 60 $F_{254}$) were visualized by spraying with anisaldehyde reagent: *p*-anisaldehyde - sulfuric acid - acetic acid (1:1:50) and heated. Rf values ($CH_2Cl_2$ - MeOH, 7:3) were: HA = 0.5; HC = 0.7; MA = 0.4; MB = 0.6. TLC was performed on the mycelium crude methanolic extracts and did not reveal the presence of peptides.

### HPLC Separations

A Waters liquid chromatograph (6000 A and M45 pumps, a 680 automated solvent programmer, a WISP 701 automatic injector and a 481 UV-vis. detector) was used with a semi-preparative C18 column (Spherisorb ODS2, 5 μ, 7.5 × 300 mm; AIT France).

*Trichorzins* HA: eluent: MeOH - $H_2O$ (88:12); flow rate, 2 ml/minute. Rt (minute): HA I = 23, HA II = 26, HA III = 28, HA V = 32, HA VI = 37, HA VII = 39. Purity of the isolated peptides was checked on an analytical column (3.5 × 250 mm); eluent MeOH - $H_2O$ (86:14), flow rate 1 ml/minute.

*Trichorzins* MA: eluent: MeOH - $H_2O$ (86:14); flow rate 2 ml/minute. Rt (minute): MA I = 37, MA II = 42, MA III = 49. Purity of each isolated peptide was checked on an analytical column (3.5 × 250 mm); eluent MeOH - $H_2O$ (86:14), flow rate 1 ml/minute.

### Biological Properties

The antibacterial properties of trichorzins HA and MA were examined against *S. aureus* (strain 209P) and *E. coli* (strain RL 65), by the agar diffusion test using 6 mm diameter pits. They were compared to those exhibited by other peptaibols of different chain lengths, such as harzianins HC[6], trikoningin KA V[17] and saturnisporins SA[5]. The peptide samples were dissolved in DMSO at a concentration of 4 mg/ml. Other concentrations were obtained by dilutions. 50 μl of each solution were deposited into the pits (200 to 1.5 μg per pit). Inhibition zones were measured after 24 hours of incubation at 37°C.

The antifungal properties of trichorzins HA were examined against *Sclerotium cepivorum* grown on malt-agar medium in micro-plates of 4.5 cm diameter and compared to those of harzianins HC isolated from the same *Trichoderma* strains. The appropriate amounts of peptide mixtures were dissolved in MeOH in order to give a final peptide concentration of 100 μg/ml of culture medium and a 0.5% amount of MeOH. As inoculum, one *S. cepivorum* sclerotium was deposited in the middle of the plate which was incubated at 25°C for 72 hours; the control plates without peptides were then completely spread over. Four plates were prepared for each peptide mixture. Growth diameters were measured and percent inhibition determined as regards to the control plates.

### Membrane-modifying Activity

Liposome permeabilization was measured by fluorescence spectroscopy at 20°C on an Aminco SPF 500 spectrofluorometer. The peptide-induced release of intravesicular content was monitored by the Weinstein method[18], using the property of quenching relief upon dilution of an encapsulated fluorescent probe, carboxyfluorescein (CF). Egg phosphatidylcholine (ePC) type V E and cholesterol (Chol) were purchased from Sigma; ePC was used without further purification and Chol was recrystallized from methanol. CF from Eastman Kodak was separated from hydrophobic contaminants and recrystallized from ethanol as previously described[9].

CF-entrapped small unilamellar vesicles were prepared by sonication of an ePC - Chol (7:3) mixture ([lipid] = 0.6 mM), as previously described[9~11]. The liposomes

obtained by sonication were separated from unencapsulated CF by gel filtration (Sephadex G75). Leakage kinetics were monitored for different $R_i^{-1}$ = [peptide]/[lipid] molar ratios obtained by adding aliquots of methanolic solutions of peptides (methanol concentration kept below 0.5% by volume). The time-course of fluorescence change corresponding to CF efflux was recorded ($\lambda_{exc}$ = 488 nm, 1 nm band pass, $\lambda_{em}$ = 520 nm, 1 nm band pass) after rapid and vigourous stirring. Percentage of released CF at time t was determined as $\%CF = (F_t - F_0)/(F_T - F_0) \times 100$, where $F_0$ is the fluorescence intensity of the vesicle suspension in the absence of peptide, $F_t$ is the fluorescence intensity measured at time t = 20 minutes in the presence of peptide, $F_T$ is the total fluorescence intensity determined by disrupting the vesicles by addition of 50 $\mu$l of a 10% solution of Triton X100. 50% CF leakage was induced for trichorzin concentrations ranging between $10^{-6}$ and $2.5 \times 10^{-5}$ M.

## Results and Discussion

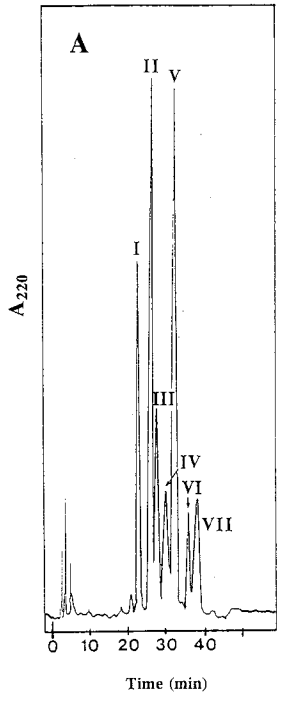
### Isolation and HPLC Separations of Trichorzins HA and MA

Peptaibol mixtures were obtained by fermentation of *T. harzianum* strains M-903602 and M-922835. Each culture broth extract was analyzed by TLC ($CH_2Cl_2$-MeOH, 7 : 3) and submitted to two successive chromatography steps including exclusion chromatography over Sephadex LH 20 followed by silica gel chromatography in order to separate the peptide groups of different polarity. The M-903602 strain led to trichorzins HA (197 mg, Rf: 0.5) and harzianins HC (142 mg, Rf: 0.7)[6], whereas the M-922835 strain provided trichorzins MA (53 mg, Rf: 0.4) and a highly complex mixture of short-sequence peptaibols, harzianins MB (28 mg, Rf: 0.6), which are not described here.

When analyzed by C18 reversed-phase HPLC, the HA mixture appeared to be composed of at least seven components (Fig. 2-A), whereas the trichorzin MA mixture contained mainly three peptides (Fig. 2-B).

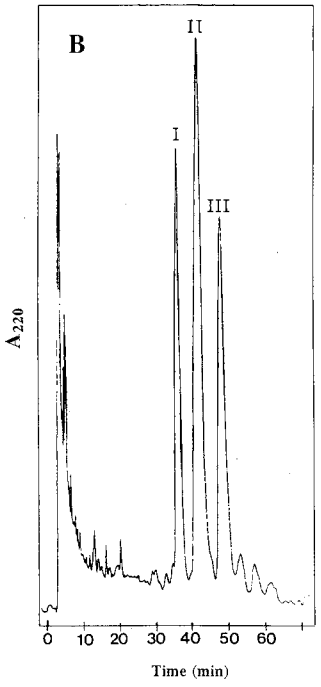
The major peptides of the HA and MA mixtures were isolated by repetitive semi-preparative HPLC. Six trichorzins HA (HA I~III and HA V~VII) and three trichorzins MA (MA I~III), labelled on Figs. 2-A and 2-B respectively, were shown to be homogeneous by further MS and NMR spectroscopy. They were submitted to structural analysis[16] and their antibacterial, antifungal and membrane-modifying properties were measured.

Fig. 2-A. HPLC chromatogram of trichorzins HA.




Spherisorb ODS2 (5 $\mu$), 7.5 mm × 30 cm, MeOH-$H_2O$ (88 : 12), flow rate 2 ml/minute, absorption monitored at 220 nm.

Fig. 2-B. HPLC chromatogram of trichorzins MA.




Spherisorb ODS2 (5 $\mu$), 7.5 mm × 30 cm, MeOH-$H_2O$ (86 : 14), flow rate 2 ml/minute, absorption monitored at 220 nm.

Table 1. Growth inhibition of *S. aureus* induced by different concentrations of peptaibols of different chain lengths.

| Peptide | μg/pit | Growth inhibition (diameter, mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 200 | 100 | 50 | 25 | 12.5 | 6.2 | 3.1 | 1.5 |
| SA II | | 19 | 18 | 18 | 15 | 15 | 12 | 10 | — |
| KA V | | 17 | 16 | 15 | 15 | 14 | 12 | 11 | — |
| HA | | 20 | 19 | 18 | 17 | 13 | 9 | — | — |
| HA V | | 15 | 14 | 14 | 13 | 13 | 12 | — | — |
| MA II | | 15 | 15 | 13 | 12 | 10 | 9 | — | — |
| HC | | 17 | 15 | 12 | — | — | — | — | — |

## Biological Properties

The antibacterial activity of trichorzins HA and MA was examined against *S. aureus* and *E. coli* and compared to that exhibited by other peptaibols of different chain lengths. Saturnisporin SA II[5] and trikoningin KA V[17] were chosen as 20-residue and 19-residue peptaibols respectively, the natural mixture of harzianins HC[6] as 14-residue peptaibols (Fig. 1).

All of the examined peptaibols were inactive against *E. coli*, being in agreement with previous observations[19~21]. The results obtained against *S. aureus* showed that the activity of the natural peptide mixture HA was approximately the same as that of pure trichorzins HA V and MA II (Table 1). The antimicrobial activity of the different trichorzins appeared thus of the same magnitude, as previously observed for other long-sequence peptaibols[5].

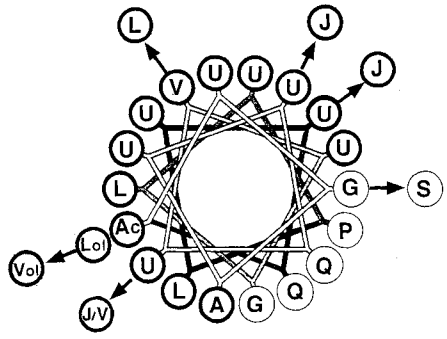
Among them, the 20-residue saturnisporin SA II and the 19-residue KA V appeared as the most potent antibiotics in this series (activity observed up to 3 μg/pit). It was followed by the 18-residue trichorzins (activity up to 6 μg/pit), whereas the 14-residue harzianins HC were inactive for concentrations below 50 μg/pit, pointing to a relationship between the peptide chain length and the antibiotic activity.

When examining the antifungal activity of trichorzins and harzianins on the phytopathogenic fungus *Sclerotium cepivorum*, the same conclusion arose, as trichorzins were much more potent (75% inhibition at 100 μg/ml) than harzianins (40% inhibition at 100 μg/ml).

## Membrane-modifying Activity

Previous studies have shown long-sequence peptaibols to promote voltage-gated ion channels, when incorporated into neutral planar lipid bilayers at concentrations around $10^{-7}$ M[11]. In the absence of voltage, the 19- and 20-residue peptaibols, alamethicin[8] and trichorzianins[8~10] bind to phospholipid bilayers and modify membrane permeability at concentrations as low as $10^{-6}$ M. Optimal membrane activity is observed for a hydrophobic neutral α-helical peptide of 19~20 residues. Introduction of a negative charge in the C-terminal extremity considerably reduces the activity, which is completely suppressed by shortening the α-helix length to 12-residue. In organic solvents and in micellar media, long-sequence peptaibols are predominantly α-helical[5,22] and exhibit more or less pronounced amphipathicity due to sequestration of the less hydrophobic residues on one side of the helix. Such a structure is involved in the permeabilization process of liposomes by peptaibols[8]. Preliminary results suggest that trichorzins would adopt a similar conformation as shown on Fig. 3.

Fig. 3. Helical wheel of trichorzins exhibiting the hydrophobic and hydrophilic sectors.




Hydrophobic residues are indicated by bold circles; U: Aib, J: Iva, Lol: Leuol, Vol: Valol.

Trichorzin HA V has been shown previously to exhibit membrane-modifying properties by increasing the permeability of liposomes[11]. The liposome permeabilization by the series of isolated trichorzins was thus examined in order to i) deduce the structure-activity relationship arising in the trichorzin series, ii) compare the permeabilization induced by 18-residue peptaibols to that exhibited by longer or shorter sequences, iii) correlate the membrane permeability modifications to the antibiotic properties.

Leakage kinetics of the CF fluorescent probe, previously entrapped at a self-quenched concentration in

small unilamellar vesicles composed of ePC-Chol (7:3)[18] were monitored by fluorescence spectroscopy, for different peptide to lipid ratios ($R_i^{-1}$). The percentage of escaped CF at 20 minutes as a function of different $R_i^{-1}$ ratios allowed to determine for each peptide, the ratios allowing 50% leakage in 20 minutes of the entrapped probe on the linear part of the resulting curves (Fig. 4); the [lipid]/[peptide] $R_i$ ratios for 50% leakage were used to compare more easily the efficiency of the different peptides (Table 2).

Trichorzins HA and MA exerted similar permeabilization of vesicles in the same concentration range, HA I being the least potent peptide and HA VII the most efficient. Increasing the peptide hydrophobicity by successive replacements of Aib residues for isovaline (or valine) resulted in an increase in the activity. Similar results arose from the trichorzin MA series, pointing to the major role played by hydrophobicity in the interaction of trichorzins with phospholipids and the subsequent permeabilization process. Nevertheless, replacement of the Gly residue at position 2 in the sequences of trichorzins HA for the more hydrophilic Ser did not decrease the efficiency of the MA peptides, pointing to the role of the helix amphipathicity.

Comparison of the trichorzin activity with that previously exhibited by other peptaibols with different chain lengths and hydrophobicity (Table 2), showed that the peptide efficiency on liposomes was optimal for a 18~19 residue chain length. The present results point to the major role of the hydrophobicity of the 18~20 residue helix, the activity being modulated by the helix amphipathicity. The permeability modification data, obtained on liposomes composed of zwitterionic phospholipids and cholesterol parallel, although not strictly, the antibiotic properties exhibited by peptaibols (Tables 1, 2). The peptide/membrane interaction and the subsequent permeability modifications look to account for a great part of the antibiotic activity by perturbing the ionic balance of the cell, as the peptide chain length and neat hydrophobicity play a key-role in the two processes. The lipid composition of the bacteria membranes which differs from that of the liposomes may be one of the parameters responsible for the observed difference.

Fig. 4. Trichorzin-induced CF leakage from ePC-Chol (7:3) vesicles at t=20 minutes for different $R_i^{-1}$ = [peptide]/[lipid] ratios.

(■) HA I, (□) HA II, (△) HA III, (▲) HA V, (◇) HA VI, (◆) HA VII, (○) MA I, (●) MA II, (+) MA III; the percentages of leaked CF at time t were determined as described under materials and methods; [lipid] = 0.6 mM.

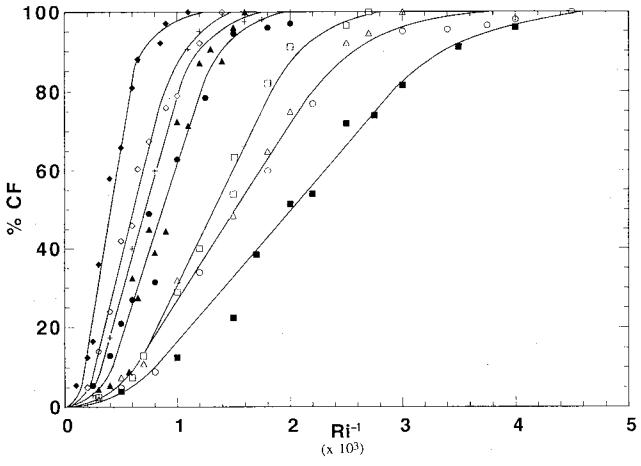



Table 2. [Lipid]/[peptide] ratios allowing 50% leakage in 20 minutes of the entrapped CF ($R_{i50}$) for peptaibols of different chain lengths.

| Number of residues | Peptide | $R_{i50}$ ($\times 10^{-2}$) |
|---|---|---|
| 20 | SA II | 9.1 |
| 19 | TA IIIc | 22.2 |
| 19 | KA V | 7.7 |
| 18 | HA I | 5.0 |
| 18 | HA II | 7.7 |
| 18 | HA III | 6.7 |
| 18 | HA V | 11.1 |
| 18 | HA VI | 16.7 |
| 18 | HA VII | 25.0 |
| 18 | MA I | 6.7 |
| 18 | MA II | 11.1 |
| 18 | MA III | 14.3 |
| 14 | HC VIII | 0.4 |
| 14 | HC XV | 2.5 |

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