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Conversion of gibberellin A_{20} to gibberellins A_1 and A_5 in a cell-free system from *Phaseolus vulgaris*

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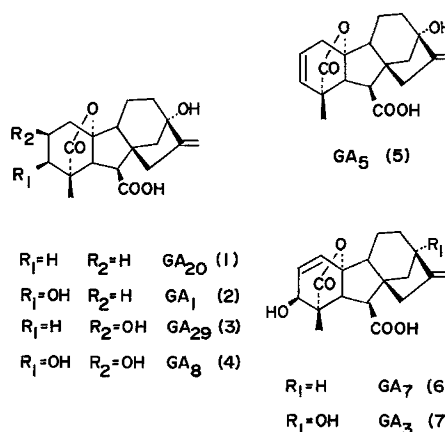
Abstract. The soluble fraction of a cell-free system from immature seeds of *Phaseolus vulgaris* L. converts gibberellin A_{20} (GA_{20}) to GA_1 and GA_5. It does however not metabolize GA_1 and GA_{29} to GA_5, showing that in this system GA_{20} is converted directly to GA_5. The steps from GA_{20} to GA_1 (3-hydroxylation) and from GA_{20} to GA_5 (Δ^2 double-bond formation) require oxygen, Fe^{2+} and α-ketoglutarate, and are stimulated by ascorbate. The enzymes catalyzing these conversions have properties similar to those of GA oxidases found in *Cucurbita maxima* and *Pisum sativum*.

Key words: Gibberellin biosynthesis – α-Ketoglutarate – Oxidase – *Phaseolus* (GA synthesis).

Introduction

Cell-free systems that catalyze steps in gibberellin (GA) biosynthesis have been mainly prepared from immature seeds of Cucurbitaceae and Leguminosae (see Graebe 1982, Hedden 1983 for recent reviews).

In the Fabaceae (Leguminosae), most of the endogenous GAs are 13-hydroxylated GAs which also are found in many other higher plants studied but do not occur in the Cucurbitaceae (Takahashi 1974; Graebe and Ropers 1978). Sponsel et al. (1979) suggested that from a chemotaxonomic consideration of endogenous GAs the Fabaceae could be divided into two groups. The major endogenous GAs of *Pisum sativum* (Frydman et al. 1974) and



Vicia faba (Sponsel et al. 1979), both in the tribe Vicieae, are hydroxylated only in the 13-position (e.g. GA_{20}; structure 1) but those of *Phaseolus vulgaris* (Hiraga et al. 1974) and *Phaseolus coccineus* (MacMillan et al. 1960), in the tribe *Phaseoleae*, are 3,13-dihydroxylated (e.g. GA_1; structure 2). Recently, we studied the metabolism of GAs in a cell-free system from *Pisum sativum*; we elucidated the biosynthetic pathway of all major endogenous GAs of this plant and concluded that the system was favorable for studying the conversion of C_{20}-GAs to C_{19}-GAs. The immediate precursor of C_{19}-GAs was confirmed to be C-20 aldehyde GAs (Kamiya and Graebe 1983).

We now have prepared a cell-free system from *Phaseolus vulgaris* to study 3-hydroxylation and the introduction of a double bond in GAs. The mode of introduction of the Δ^2 double bond to form GA_5 (5) has not been studied before. A cell-free system from suspensors of *P. coccineus* prepared by Ceccarelli et al. (1981) also produces GA_1, GA_5 and GA_8 (4) but these authors did not study the sequence in which these GAs are formed. The biosynthetic pathway of GA_5 has so far been unknown.

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Abbreviations: GA_n = gibberellin A_n; HPLC = high-performance liquid chromatography; GC–MS = combined gas chromatography-mass spectrometry; TLC = thin-layer chromatography; TMSi/TMSi = trimethylsilyl ether/trimethylsilyl ester

In this paper, we describe the metabolism of GA_{20} (1) to GA_1 (2) and GA_5 (5) in the cell-free system from *P. vulgaris*.

Materials and methods

Preparation of cell-free extracts. Phaseolus vulgaris L. cv. Kentucky Wonder (from Kaneko Seeds, Tokyo, Japan) was grown in the summer of 1983. Immature seeds were harvested 17 d after anthesis and the seed coats removed. The embryos were homogenized in potassium-phosphate buffer (0.05 M, pH 8.0; 1:1, v/w) with a mortar and pestle on ice for 5 min. The homogenate was filtered through cheesecloth and centrifuged at 2000 *g* for 10 min at 4° C. The supernatant was concentrated with dry Sephadex (G-25 fine, 3:1, v/w; Pharmacia Fine Chemicals, Uppsala, Sweden), then centrifuged at 1000 rpm for 3 min to separate the concentrate from the Sephadex. The concentrate (two to three-fold) was centrifuged at 200000 *g* for 1 h at 4° C. The supernatant, referred to as S-200, was stored in liquid N_2.

Gel filtration of S-200. Lyophilized S-200 (10 ml) was dissolved in H_2O (5 ml) and filtered through a Sephadex G-25 column (1.6 cm diameter, 44 cm long) equilibrated and eluted with potassium-phosphate buffer (0.05 M, pH 8.0). The effluent was monitored at 280 nm and 254 nm. The protein-containing fraction (25 ml) eluting immediately after the void volume and absorbing at 280 nm was collected and lyophilized. Before use, the protein fraction was dissolved in H_2O (8 ml).

Preparation of $[^{14}C]GAs$ and $[^{18}O]GA_{20}$. $[^{14}C]$Gibberellin A_{12} was prepared from $[2\text{-}^{14}C]$mevalonic acid using a cell-free system from the endosperm of *Cucurbita maxima* (Graebe et al. 1974); $[^{14}C]GA_{20}$ and $[^{14}C]GA_{29}$ were prepared from $[^{14}C]GA_{12}$ using a cell-free system from the embryo of *Pisum sativum* (Kamiya and Graebe 1983); $[^{14}C]GA_1$ was prepared from $[^{14}C]GA_{20}$ using the *P. vulgaris* cell-free system described in this paper. The specific activities of the $[^{14}C]$GAs were as follows: $[^{14}C]GA_{20}$, 1.67 GBq $mmol^{-1}$; $[^{14}C]GA_{29}$, 0.59 GBq $mmol^{-1}$; $[^{14}C]GA_1$, 0.67 GBq $mmol^{-1}$. The radioactivity was measured with a liquid scintillation counter (Packard Instruments, Downers Grove, Ill., USA; model 3300) using a toluene scintillator (efficiency approx. 88%) and converted to Bq by using an internal standard.

Gibberellin A_{20} (100 µg) was heated with 2 N $K^{18}OH$ (90A% ^{18}O) (20 µl) at 100° C for 3 h in a sealed tube, then acidified with 5 N HCl, and extracted with ethyl acetate. The ethyl-acetate extracts were dried under an N_2 flow to give $[^{18}O]GA_{20}$. The proportion of $[^{18}O]$ in the $[^{18}O]GA_{20}$ was estimated to be 60 A% by combined gas-chromatography-mass spectrometry (GS-MS).

Incubation of $[^{14}C]GAs$ and $[^{18}O]GA_{20}$ under normal conditions. $[^{14}C]$Gibberellin A_{20}, $[^{14}C]GA_1$ and $[^{14}C]GA_{29}$ (1000 Bq) were incubated for 4 h at 30° C with S-200 (1 ml) containing 0.5 mM $FeSO_4$, 5 mM ascorbate and 5 mM α-ketoglutarate at pH 8.0. $[^{18}O]GA_{20}$ (5 µg) was incubated with S-200 (5 ml, containing the same cofactors and under the same conditions.

Incubation under N_2 or with different cofactors. Three 1-ml portions of S-200 containing 0.5 mM $FeSO_4$, 5 mM ascorbate and 5 mM α-ketoglutarate were lyophilized in test tubes (1 cm diameter, 10 cm long) with rubber caps. Two syringe needles were put through the cap and N_2 gas was passed through the tubes to replace air. One portion was dissolved in H_2O (1 ml) and was incubated with $[^{14}C]GA_{20}$ (1000 Bq) in air at 30° C for 2 h. The second portion was dissolved in de-gassed H_2O and was incubated with $[^{14}C]GA_{20}$ first under N_2 for 1 h and then in air also for 1 h. The third portion was incubated for 2 h under N_2 after addition of de-gassed H_2O and $[^{14}C]GA_{20}$

For the study of cofactor requirements, $[^{14}C]GA_{20}$ (530 Bq) was incubated with the gel-filtered protein fraction (1 ml) for 4 h at 30° C and cofactors as in Table 2.

Extraction of GAs from the incubation mixtures. After incubation, the mixtures were acidified to pH 2 with 5 N HCl and acetone was added (1:1, v/v). The products were extracted with ethyl acetate (same volume as acetone) three times and the combined ethyl-acetate extracts were washed with a small amount of water and dried under N_2 flow.

Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Silica-gel plates (20·20 cm^2, 0.2 mm thickness; DC-Alufolien Kieselgel 60, Art. 5553, E. Merck, Darmstadt, FRG) were developed with ethyl acetate: acetic acid (100:1, v/v). Rf values of the GAs studied were as follows; GA_5 and GA_{20}, 0.63; GA_1 0.48; GA_{29}, 0.36. Preparative TLC was performed on 0.2-mm layers which had been washed with methanol:acetone (1:1, v/v) before use. The plates were scanned for radioactivity with a radiochromatogram scanner (Aloka TLC-101; Aloka Co., Tokyo, Japan), and the radioactive zones were eluted with methanol:acetone (1:1, v/v) for further analysis.

For HPLC, a Nucleosil 5 N $(CH_3)_2$ column (4.6 mm diameter, 50 mm long) eluted with methanol containing 0.05% acetic acid was used. Flow rate was 1 ml min^{-1}. The eluant was collected in 1-ml fractions, by means of a Spectra Physic (San Jose, Calif., USA) SP8770 pump.

Combined gas chromatography-mass spectrometry (GC-MS) and GC-high-resolution MS. The mass spectrometers used were Hitachi (Tokyo, Japan) M80A (GC-MS) and JEOL (Tokyo) DX-300 (GC-high-resolution MS) equipped with a glass column (3 mm diameter, 1 m long) packed with 2% OV-1 on Chromosorb W (Gaschro Kogyo, Tokyo). The ionizing voltage was 15–20 eV and the oven and injection temperatures were 210° C. The carrier gas was helium at a flow rate of 50 ml min^{-1}.

The ^{14}C-specific activities were determined as described in Kamiya and Graebe (1983). Samples for GC-MS and GC-high-resolution MS were trimethylsililated with N-methyl-N-(trimethylsilyl)trifluoroacetamide at 80° C for 30 min.

Results

Conversion of GA_{20} to GA_1 and GA_5 by soluble enzymes. $[^{14}C]$Gibberellin A_{20} was incubated with S-200 in the presence of Fe^{2+}, α-ketoglutarate and ascorbate. As shown in Fig. 1, GA_{20} (1) was metabolized to a more polar compound with an Rf value on TLC identical to that of authentic GA_1 (2). Since GA_5 (5) and GA_{20} (1) were not separated by TLC with this solvent system, the radioactive zone corresponding to GA_{20} was eluted from the Silica-gel plate and further purified by HPLC to give two radioactive fractions (Fig. 2). The retention time of the radioactive compound first eluting was identical with that of GA_{20}, and the retention

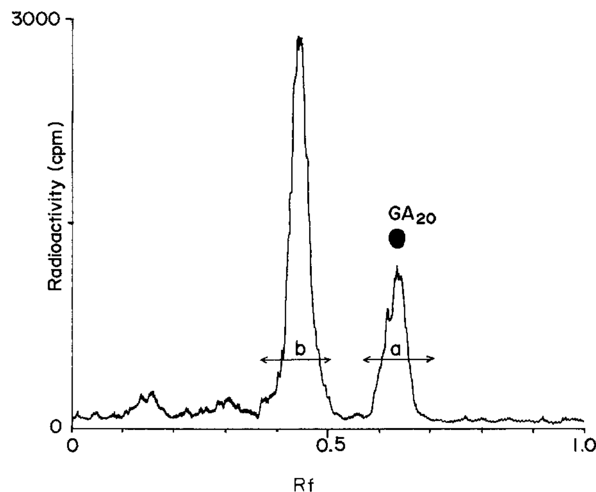

Fig. 1. Separation of radioactive products obtained by incubation of $[^{14}C]GA_{20}$ with a cell-free preparation from *P. vulgaris* (S-200) and cofactors. Separation by Silica-gel TLC. *a* GA_{20} and GA_5-like fraction; *b* GA_1-like fraction. *Black circle* shows the position of GA_{20}

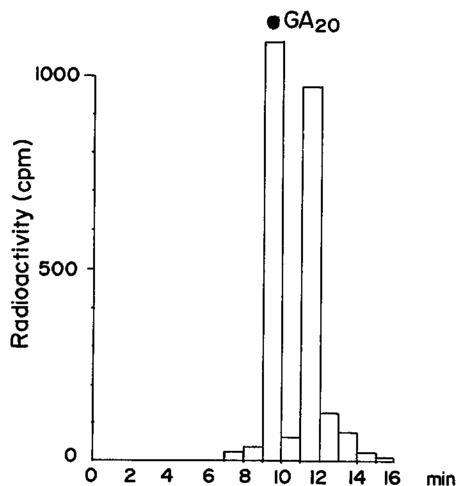

Fig. 2. High-performance liquid chromatography of the $[^{14}C]GA_{20}$ and GA_5-like fraction eluted from the Silica-gel TLC plate shown in Fig. 1. *Black circle* shows the retention time of GA_{20}

time of the second fraction identical with that of GA_5 (Yamaguchi et al. 1982). The fraction resembling GA_1 was the main product while the GA_5-like fraction was about 40% of it. This ratio remained almost constant in different incubations. Conclusive identification was obtained by using $[^{18}O]GA_{20}$ as a substrate and identifying the products by GC-MS. Alkaline treatment of GA_{20} in 2 N $K^{18}OH$ afforded $[^{18}O]GA_{20}$ with 60 A% ^{18}O. $[^{18}O]$Gibberellin A_{20} was incubated with S-200, Fe^{2+}, α-ketoglutarate and ascorbate. After incubation, the ethyl acetate-soluble fraction was purified

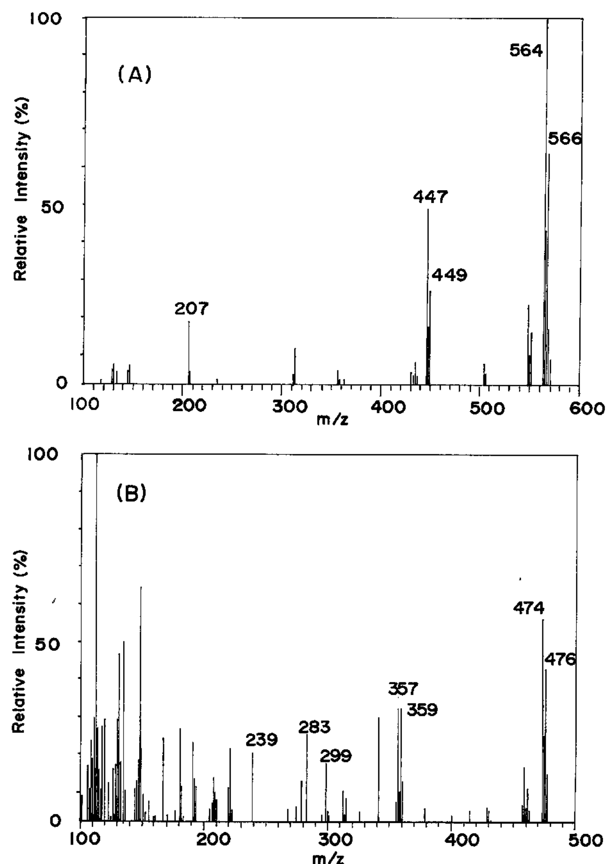

Fig. 3A, B. Mass spectra of trimethylsilyl derivatives of GA_1 and GA_5 obtained by incubation of $[^{18}O]GA_{20}$ with a cell-free preparation of *P. vulgaris* (S-200) and cofactors. **A** Mass spectrum of TMSi/TMSi$[^{18}O]GA_1$; **B** mass spectrum of TMSi/TMSi$[^{18}O]GA_5$

by TLC and HPLC as for the ^{14}C experiments. The fractions corresponding to GA_1, GA_5 and GA_{20} were derivatized and analyzed by GC-MS. Trimethylsilyl derivatives of the GA_1 and GA_5 fractions gave clear spectra with strong $(M+2)^+$ ions shwoing the presence of one ^{18}O atom in each compound (Fig. 3). The presence of ^{18}O was confirmed by GC-high-resolution MS; M^+ of $[^{18}O]GA_1$ trimethylsilyl ether/trimethylsilyl ester (TMSi/TMSi) (C_{28} H_{48} $^{16}O_5$ $^{18}O_1$ Si_3; calculated 566.2701, observed 566.2737), M^+ of $[^{18}O]GA_5$ TMSi/TMSi (C_{25} H_{38} $^{16}O_4$ $^{18}O_1$ Si_2, calculated 476.2201 observed 476.2257).

The ^{18}O atom in the lactone ring was not exchanged with ^{16}O of water during the incubation and purification since the $[^{18}O]GA_{20}$ recovered after incubation showed almost the same A% ^{18}O as the starting material. On the other hand, GA_1 was diluted with endogeneous GA_1 (approximately two-fold). This result is in agreement with the amounts of endogeneous GAs in *P. vulgaris* reported by Yamane et al. (1977).

Table 1. Incubation of [^{14}C]GA_1, [^{14}C]GA_{20} and [^{14}C]GA_{29} with a cell-free preparation from *P. vulgaris* (S-200) and cofactors[a]

Substrate	Radioactivity in products (Bq)			
	GA_1	GA_5	GA_{20}	GA_{29}
GA_1	727	0	0	0
GA_{20}	435	152	100	22[b]
GA_{29}	0	0	0	587

[a] 0.5 mM $FeSO_4$, 5 mM α-ketoglutarate, 5 mM ascorbate
[b] GA_{29}-like compound, not identified conclusively by GC-MS

Table 2. Cofactor requirements for the conversion of [^{14}C]GA_{20} to GA_1 and GA_5 by a gel-filtered preparation from *P. vulgaris* (S-200)

Cofactors[a]				Products (Bq)[b]	
Fe^{2+}	α-Keto-glutarate	Ascorbate	NADPH	GA_1	GA_5
+	+	+	−	70.1	30.8
−	+	+	−	24.5	16.8
+	−	+	−	8.3	8.8
+	+	−	−	9.8	9.0
+	+	−	+	14.6	10.5

[a] 0.5 mM $FeSO_4$, 5 mM α-ketoglutarate, 5 mM ascorbate, 1 mM NADPH
[b] Mean values of two experiments. The ratios of GA_1 and GA_5 were identical in both experiments

Table 3. Requirement for air in the conversion of [^{14}C]GA_{20} to GA_1 and GA_5 by S-200

Conditions	Radioactivity in products (Bq)[a]	
	GA_1	GA_5
2 h in air	165.0	72.7
1 h in N_2, then 1 h in air	165.0	80.1
2 h in N_2	61.7	24.3

[a] Mean values of three experiments

There are two other possible precursors for GA_5 (5), namely GA_1 (2) and GA_{29} (3). Gibberellin A_5 could conceivably be formed via GA_1 by dehydration of the C-3 hydroxyl group or, less likely, via GA_{29} by dehydration of the C-2 hydroxyl group. [^{14}C]Gibberellin A_{29} and [^{14}C]GA_1 were therefore incubated with S-200 in the presence of Fe^{2+}, α-ketoglutarate and ascorbate. As shown in Table 1, GA_1 and GA_{29} were not converted to GA_5. Thus GA_{20} is directly converted to GA_5.

Cofactor and oxygen requirements for the conversion of GA_{20} to GA_1 and GA_5. The protein fraction of S-200 was separated from the small-molecule fraction by Sephadex G-25 column chromatography and incubated with [^{14}C]GA_{20} as the substrate to define the cofactor requirements for its conversion to GA_1 and GA_5. Table 2 shows that the conversion of GA_{20} (1) to GA_1 (2) and GA_5 (5) requires α-ketoglutarate, Fe^{2+} and ascorbate. Ascorbate could not be replaced by NADPH.

When GA_{20} was incubated under aerobic and anaerobic conditions with S-200 and various cofactors, the conversion of GA_1 and GA_5 was found to be dependent on the presence of oxygen for full activity, but that about one third of the activity remained even under a nitrogen atmosphere. This may have been a consequence of incomplete replacement of adsorbed oxygen with nitrogen, but the possibility remains that the requirement for oxygen is less than absolute.

Discussion

The cell-free system prepared from immature seeds of *Phaseolus vulgaris* very actively converted GA_{20} (1) to GA_1 (2) and GA_5 (5). [^{14}C]Gibberellin A_{53}, the precursor of 13-hydroxylated GAs in peas (Kamiya and Graebe 1983), was also converted by the *P. vulgaris* system to several, more polar products, but these were not identified and the data are not shown here. ^{18}O was used as a convenient stable isotope label for conclusive identification of products by GC-MS. [^{2}H]Gibberellins are more commonly used but their preparation is often time-consuming (Hedden 1979). In comparison, it is easy to label GAs having a γ- or δ-lactone with ^{18}O by treatment with 2 N K^{18}OH; ^{18}O-labeled GA_9, GA_{15}, GA_{20} and GA_{44} were prepared by this method. Since the ^{18}O atom in the lactone ring was shown not to exchange with ^{16}O of water during the incubation and purification, [^{18}O]GAs should be good substrates for metabolic studies.

Gibberellin A_3 (7), GA_5 (5) and GA_7 (6) contain double bonds at Δ^1 or Δ^2 in ring A but the mechanism of the introduction of a double bond is unknown. The chemical synthesis of GA_5 from GA_1 by dehydration of C-3 hydroxyl group (Murofushi et al. 1977) indicated that GA_5 might arise from GA_1 or GA_{29} (3) in the metabolic pathway. In our experiments, GA_1 and GA_{29} were not converted to GA_5, so GA_{20} must have been directly converted to GA_5, thus eliminating the route by dehydration of the C-2 or C-3 hydroxyl group. This result is in agreement with those from the in-vivo experiments of Yamane et al. (1977). These authors reported that GA_1 was metabolized to GA_8 (4), and GA_{20} was metabolized to GA_1, GA_{29} and GA_8. Their chromatographic systems

did not separate GA_{20} and GA_5, so any GA_5 formed would have remained undetected.

The amount of GA_1 produced without adding Fe^{2+} was about one third and the amount of GA_5 about one half of the amount produced when all cofactors were present (Table 2). This was probably because of incomplete removal of Fe^{2+} from the enzyme solution by Sephadex gel filtration. Hedden and Graebe (1983) reported the same cofactor requirements for the soluble GA oxidases in the endosperm of *Cucurbita maxima* and also found residual activity in the absence of Fe^{2+} after Sephadex gel filtration; this residual activity was strongly reduced by addition of bipyridine to bind Fe^{2+}. Removal of α-ketoglutarate or ascorbate strongly reduced the conversion of GA_{20} to GA_1 and GA_5; the ascorbate could not be replaced by NADPH. Although there is no incorporation of oxygen into the GA molecule when GA_{20} is converted to GA_5, the depression of both conversions by over 60% under nitrogen shows that oxygen is required nevertheless. This resembles the case of the conversion of GA_{19} to GA_{20} in the pea system, which also requires oxygen (Kamiya and Graebe 1983) although the two oxygen atoms of the lactone ring come from C-19 carboxylic acid and not from molecular oxygen (Bearder et al. 1976).

The mechanism of the Δ^2 double-bond formation could be explained by an Fe–O enzyme complex (Visser 1980) attacking GA_{20} (1) at the C-3 position. If the Fe–O bond is cleaved, GA_1 (2) will be formed. If the O–C bond is cleaved, GA_5 (5) may be formed with elimination of the hydrogen at C-2. However, our data do not allow conclusions with respect to a common enzyme-substrate complex as an intermediate in the formation of the two products.

Although a 2-hydroxylating enzyme has been isolated and characterized from mature seeds of *P. vulgaris* (Hoad et al. 1982), 2-hydroxylation activity in our preparation was weak. The immature seeds we used may have at the wrong stage of development for strong 2-hydroxylating activity. In peas, 2β-hydroxylation activity is typical for later stages of seed development (Frydman et al. 1974; Sponsel 1983).

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