Microbiological Transformation of Terpenes

II. Transformations of α-Pinene

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ABSTRACT

PREMA, B. R. (National Chemical Laboratory, Poona, India) AND P. K. BHATTACHARYYA. Microbiological transformation of terpenes. II. Transformations of α-pinene. Appl. Microbiol. **10**:524–528. 1962.—Several strains of fungi were tested for their ability to metabolize α-pinene in shake cultures. A strain of *Aspergillus niger* showing marked efficiency in this respect was selected for further studies. The optimal conditions for fermentation were established with respect to substrate concentration, time, and temperature. From the fermentation products three major metabolites of α-pinene were isolated: a ketone, $C_{10}H_{14}O$, identified as *d*-verbenone; an alcohol, $C_{10}H_{16}O$, identified as *d-cis*-verbenol; and a crystalline diol, $C_{10}H_{18}O_2$, characterized as *d-trans*-sobrerol.

The yields of some essential oils from plant origin are affected by the microorganisms infecting the plant. A case in point is that of *Aquilaria agallocha*, commonly known in India as "agarwood" (Sadgopal and Varma, 1952). Wood from the green healthy tree is odorless; odoriferous wood is found in comparatively mature trees, and has widespread fungal infection associated with irregular patches and dark streaks highly impregnated with an oleoresin.

The microorganisms associated with the production of this oleoresin, commonly called "agar" or "aguru," were isolated by earlier workers (Tunstall, quoted by Sadgopal and Varma, 1952; Bose, 1938) and identified as an *Aspergillus* sp. believed to be primarily responsible for the production of "agar," a *Penicillium* sp., and a *Fusarium* sp.

One of the main odoriferous principles in agar has been identified as a monohydroxy compound, agarol (Bhattacharyya and Jain, 1959), the hydroxylation probably being a direct effect of microbial action on a suitable hydrocarbon precursor of the eudesmane system. A clear-cut experimental proof for this biogenetic formulation of agarol is not, however, available.

Among the few isolated reports on the biological conversion of terpenoids by microorganisms are the oxidation of camphor (Bradshaw et al., 1959; Conrad, DeBus, and Gunsalus, 1961) and the degradation of citronellol (Seubert, 1960) by soil pseudomonads. The fungi extensively used in the hydroxylation of steroids do not appear to have been applied to transformations of mono- or sesquiterpenes.

Since it was not possible to obtain agarwood hydrocarbon in sufficient quantities, studies were undertaken to investigate the capability of microorganisms, particularly fungi, to convert some easily available mono- and sesquiterpene hydrocarbons to oxygenated products which might be of potential interest in the perfumery industry. This communication deals mainly with the selection of microorganisms and studies on the transformation of α-pinene (Fig. 1) by a selected strain of *A. niger*. A preliminary note on these studies has appeared elsewhere (Bhattacharyya et al., 1960).

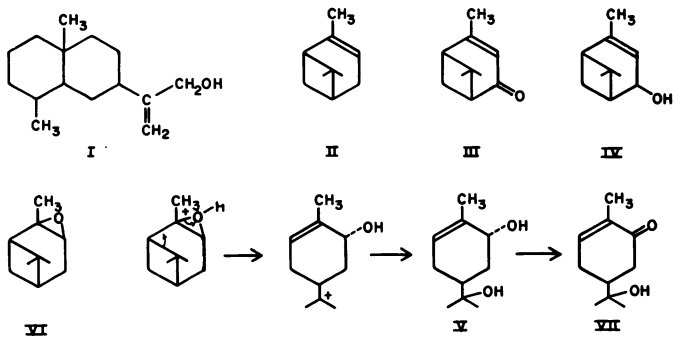

FIG. 1. *Compounds investigated:* I, *agarol*; II, *α-pinene*; III, *d-verbenone*; IV, *d-cis-verbenol*; V, *d-transobrerol*; VI, *α-pinene epoxide*; VII, *hydroxycarvotanacetone.*

MATERIALS AND METHODS

All melting points and boiling points are uncorrected. Optical rotation was determined in ethanol solution, unless otherwise stated, using a 1-dm tube. The ultraviolet spectra were recorded in 95% ethanol. The infrared spectra were recorded either in Nujol mull or as a liquid film of the pure sample, using a Grubb-Parson's double-beam spectrophotometer or a Perkin-Elmer Infracord with sodium chloride optics. α-Pinene (redistilled), $[\alpha]_D = +24.5°$, was obtained from the British Drug House.

Six organisms were isolated from scrapings of the infected part of the bark by dilution and streak plate techniques (Prescott and Dunn, 1959). The isolated cultures were grown for 7 days at 28 C in Sabouraud's agar and identified as *Penicillium steckii*, *Geotrichum* sp., *Aspergil-*

lus versicolor (three strains differing from each other in morphological details and pigment production), and *Cladosporium* sp.

Results

Selection of strains. The ability of the six fungi isolated from the infected bark and some of the common strains available in the culture collection of this laboratory to utilize α-pinene was determined. The fungi were grown on a rotary shaker in 100 ml of Czapek-Dox medium (Prescott and Dunn, 1959) containing corn steep solids (0.5%) and yeast extract (0.05%) in 500-ml Erlenmeyer flasks (six flasks for each mold) for 24-hr at 28 C. After the addition of 0.5 ml of α-pinene, the fermentation was continued for an additional 24 hr. The contents of the flasks containing each strain were pooled and filtered. The mycelia were extracted with acetone, and the acetone extracts were added to the filtrate. Both the acetone-treated mycelia and the filtrate were extracted three times with ether, using one-sixth volume of the solvent each time. The combined ether extract was washed with distilled water, dried over sodium sulfate, and evaporated. The residue was weighed. The results are presented in Table 1 for 12 strains of fungi (6 isolates from infected agarwood and 6 from the culture collection). The strain of *A. niger* (612) exhibiting the maximal utilization of α-pinene was selected for further studies.

Optimal concentration of α-pinene. Six graded doses of α-pinene (0.2, 0.4, 0.6, 0.8, and 2.0%, v/v) were added to six sets of 24-hr cultures, each set consisting of ten flasks. After 8 hr of incubation at 28 C, the cultures were extracted in the manner described above. The ether extract from each set was separated into unreacted α-pinene and the oxygenated products by a modified four-transfer Craig distribution between *n*-hexane and 90% ethanol (20 ml of each phase). The four hexane layers were equilibrated successively with the four alcoholic layers and pooled. The alcoholic layers were also combined separately. The combined hexane layers were evaporated, and the residue weighed as α-pinene. The alcoholic layers were evaporated carefully to one-fifth of the original volume, and the oxygenated products were extracted with ether. The ether extracts were dried, evaporated, and weighed. The oxygenated products increased up to 0.6% α-pinene, and then dropped sharply with further increases in concentration (Fig. 2).

Optimal time of incubation. The optimal period of incubation leading to maximal yields of the conversion products was determined by running six sets of experiments (with ten flasks per set) with 0.5% α-pinene; each set was allowed to run for a different length of time. The results (Fig. 3) indicate that maximal conversion was attained in 8 hr, a period up to which a reasonable material balance was obtained. After 20 hr, there was very little product or substrate remaining in the incubation mixture, In control experiments run without the mold for 24 hr.

TABLE 1. *Utilization of α-pinene by different strains of fungi*

Organism	α-Pinene added	Extracts recovered after 24 hr
	g	*g*
Control	2.15	1.677
Aspergillus niger (612)	2.15	0.302
Penicillium steckii (1)	2.15	0.351
Aspergillus versicolor (5)	2.15	0.408
Geotrichum sp. (2)	2.15	0.416
Cladosporium sp. (6)	2.15	0.438
Aspergillus niger (621)	2.15	0.622
Rhizopus nigricans	2.15	0.742
Aspergillus versicolor (4)	2.15	0.846
Aspergillus niger (517)	2.15	0.921
Aspergillus niger (516)	2.15	0.932
Rhizopus arrhizus	2.15	1.278
Aspergillus versicolor (3)	2.15	1.658

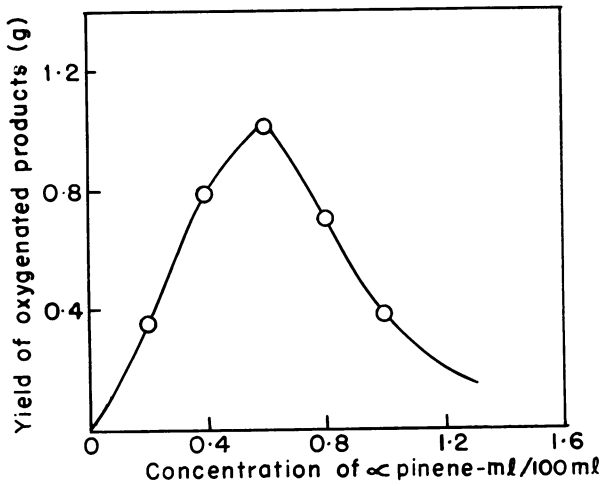

FIG. 2. *Effect of concentration of α-pinene on the yield of oxygenated products.*

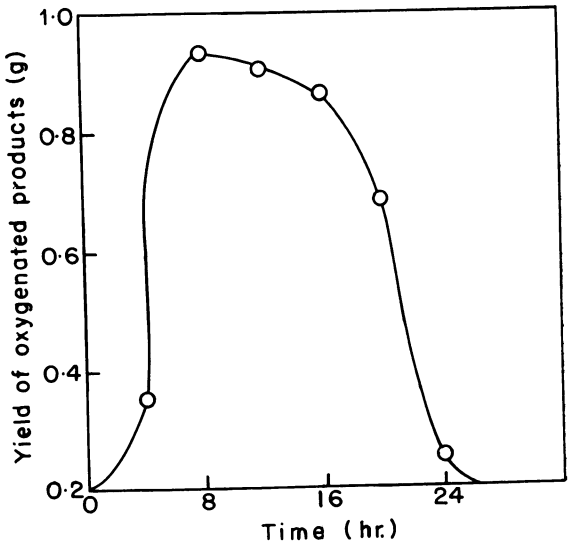

FIG. 3. *Effect of time on the yields of oxygenated products.*

20 to 30% of the initial α-pinene was lost, presumably owing to evaporation. No oxygenated products could be detected in these flasks.

Optimal temperature. The conversion of α-pinene to oxygenated products was studied at two different temperatures, 27 to 28 and 30 to 31 C. The results (Table 2) indicate that the transformation was virtually abolished at higher temperatures.

Fermentation. In a typical fermentation, α-pinene (0.5%, v/v) was added to each of 40 shake flasks containing 1-day-old fungal pellets. The fermentation was allowed to continue for 4 hr. Another lot of α-pinene was added, and the fermentation was continued for a further period of 4 hr.

Along with the experimental flasks, two sets of controls, each consisting of five flasks, were incubated. The first set contained medium and α-pinene without inoculum, and the second contained inoculated medium without α-pinene.

After the fermentation, the cultures were filtered. The pooled mycelia were extracted three times with acetone, using just enough acetone to cover the pellets each time. The acetone extracts were added to the medium. The mycelial pellets and the filtrates were extracted three times with ether and butanol, using a total of about one-fourth volume of each solvent.

The pooled ether extract was washed with 5% sodium carbonate and distilled water, dried, and then evaporated to yield an oily residue, A (20 to 22 g). The sodium carbonate extract, after acidification and ether extraction, yielded very small amounts of acidic metabolites.

The butanol extract was also worked up in an identical manner to yield a crystalline semisolid mass, C (500 to 600 mg).

The first set of controls (without α-pinene) yielded only small amounts of mold lipids, and the second set (without inoculum) yielded unreacted α-pinene after processing.

The oily residue, A, obtained from the ether extracts, was partitioned between *n*-hexane and 90% ethanol in a four-transfer modified Craig distribution (75 ml of each phase per tube) in the manner described above. The hexane layers yielded mostly unreacted α-pinene (14 to 16 g), with traces of mold lipids. The pooled alcoholic layers were worked up in the usual manner, to yield a yellow oily residue, B (3 to 5 g). On fractional distillation under reduced pressure, B yielded two clear-cut fractions: (i) a colorless oil, D (2.5 to 4 g), distilling at 120 to 125 C (bath temperature) 16 mm, and (ii) a highly viscous oil, E (500 to 600 mg), bp 145 to 150 C (bath temperature)/0.6 mm, which crystallized on standing at room temperature. This fraction was pooled with the butanol fraction, C.

TABLE 2. *Effect of temperature on the transformation*

No. of flasks	Time	Amount per flask	Temp	Unreacted α-pinene	Oxygenated products
	hr	*ml*	*C*	*g*	*g*
10	8	0.5	28.5	3.15	0.878
10	8	0.5	31.0	3.9	0.087

Separation of a ketone, and an alcohol from fraction D. A sample of fraction D (2.5 g) was added with vigorous stirring to a saturated solution of sodium bisulfite (15 ml). The stirring was continued for 30 min, and the reaction mixture was allowed to deposit crystals of the bisulfite complex for 24 hr in the cold.

The mixture was filtered, and a ketone (Fig. 1, III) was liberated from the complex with 30 ml of 0.1 N acetic acid; the ketone was extracted in ether. The ether extract was dried, evaporated, and distilled to yield the ketone (500 mg): bp 110 to 115 C/16 mm; $[\alpha]_D = +223°$ (c, 0.92); $n_D^{25.5} = 1.4960$; λ_{max} 253 mμ (log ε 3.81). Found: C, 79.74; H, 9.47. Calculated for $C_{10}H_{14}O$: C, 79.95; H, 9.39. Semicarbazone mp 203 to 205 C; $[\alpha]_D = +77°$ (c, 0.72). Literature (DuPont, Zacharewicz, and Dulou, 1934): mp 206 to 207 C, $[\alpha]_D = +77.6°$ for *d*-verbenone semicarbazone.

The opalescent filtrate from the bisulfite complex was diluted with water and extracted with ether. The ether extract after drying and evaporation yielded an alcohol (1.5 to 3.0 g). The product (Fig. 1, IV) was purified by distillation under reduced pressure: bp 112 to 115 C/14 mm; $[\alpha]_D = +64°$ (c, 1.7, 4) $n_D^{25.5} = 1.4929$. Literature (Schulz and Doll, 1940): for *d-cis*-verbenol, $[\alpha] = +67°$; infrared $_{max}$ 3,360 cm^{-1} (OH). Found: C, 78.99; H, 10.41. Calculated for $C_{10}H_{16}O$: C, 78.89; H, 10.59. *p*-Nitrobenzoate (crystallized from ethyl acetate): mp 93 to 95 C; $[\alpha]_D = +17°$ (c, 2.01). Literature (Schulz and Doll, 1940): mp 98 to 99 C for *d-cis*-verbenol-*p*-nitrobenzoate, $[\alpha]_D = +14°$.

A sample of fraction D (2 g) was chromatographed over 50 g of neutral grade II alumina. The column was eluted successively with 100-ml portions of *n*-hexane, *n*-hexane + 5% benzene, *n*-hexane + 10% benzene, *n*-hexane + 50% benzene, benzene, benzene + 5% ether, benzene + 10% ether, benzene + 50% ether, ether + 10% ethanol, ether + 50% ethanol, and ethanol. This procedure resolved fraction D into compounds III and IV (Fig. 1). The fractions were combined into two pools on the basis of the weight curve (Fig. 4).

Manganese dioxide oxidation of alcohol (IV) to d-verbenone (III). A solution of alcohol IV (500 mg) in chloroform (75 ml) was vigorously stirred with active manganese dioxide (30 g) at room temperature (27 C) for 24 hr. The mixture was then filtered, and the solid manganese dioxide was repeatedly washed with hot chloroform. The combined chloroform extracts were evaporated to yield an oily residue to *d*-verbenone (400 mg), which was purified by distillation under reduced pressure: $[\alpha]_D + 224°$ (c, 0.90); λ_{max} 252.5 mμ (log ε 3.825). Semicarbazone: mp 203 to 205 C.

Chromium trioxide oxidation. Alcohol IV (100 mg) was added to a complex of 50 mg of chromium trioxide in five drops of pyridine. After keeping overnight at 28 C, the mixture was decomposed with water and extracted with ether. The ether extract was worked up in the usual manner to yield 80 mg of *d*-verbenone.

Diol V. The solid fractions C and E were pooled and chromatographed over 30 g of neutral alumina (grade II). The column was developed with 100-ml portions of solvents in the sequence previously described for the separation of compounds III and IV, excepting for the fact that chloroform was used in place of ether. Benzene + 50% chloroform eluted a crystalline solid (800 mg). Recrystallized from benzene: mp 145 to 147 C; $[\alpha]_D = +152°$ (c, 12.8 in benzene). Reported for *d-trans*-sobrerol (Schmidt, 1953): mp 147 to 149 C; $[\alpha]_D = +150°$; $\tilde{\nu}_{max}$ 3,240 cm^{-1} (OH), 829 cm^{-1} (trisubstituted double bond). Found: C, 70.66; H, 10.7. Calculated for $C_{10}H_{18}O_2$: C, 70.54; H, 10.66. The compound gave a very feeble tetranitromethane test. Mixed mp with an authentic sample of *trans*-sobrerol synthesized according to Moore, Golumbic, and Fisher (1956): 146 to 147 C. The infrared spectra of the two samples were identical.

Hydroxy carvotanacetone from Diol V. Diol V (200 mg) was shaken for 24 hr with 20 g of manganese dioxide in 50 ml of chloroform. The reaction mixture was filtered and the filtrate worked up in the usual manner to yield hydroxycarvotanacetone (Simonsen, 1949): λ_{max} 235 mμ (log ε 3.97); semicarbazone, mp 164 to 165 C (Schmidt, 1953). The synthetic sobrerol also yielded the identical product under the same conditions.

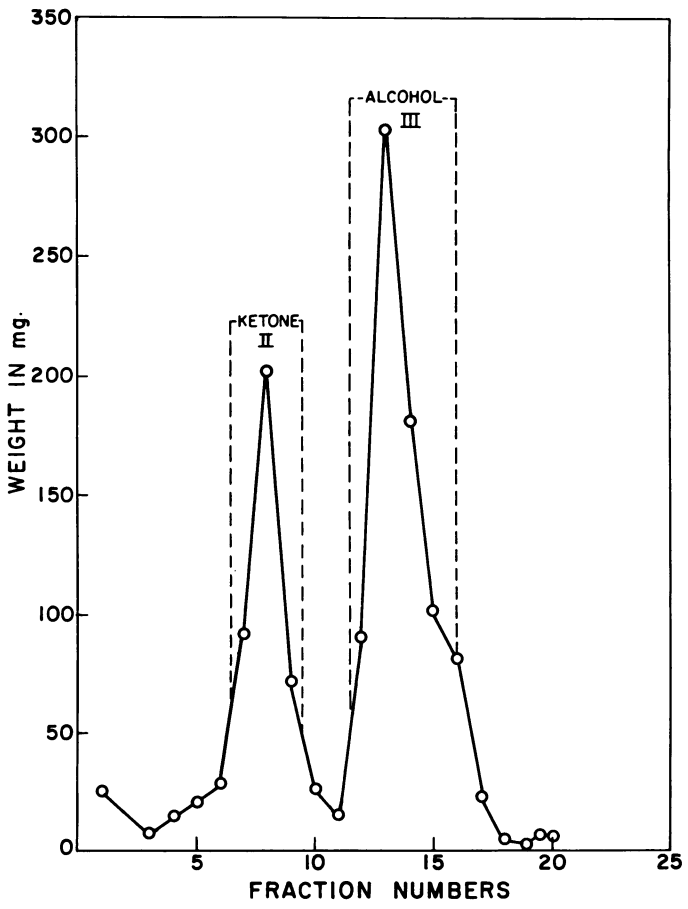

FIG. 4. *Column chromatography of fraction D.*

Discussion

It was found that shake cultures (24 to 48 hr) of each of the 12 fungal strains tested could bring about a partial to complete disappearance of 0.5% of added α-pinene in 24 hr (Table 1). In most cases, extraction of the cultures with ether or chloroform resulted in the recovery of small amounts of high-boiling and sweet-smelling oils. These findings would indicate that a variety of microbial flora may have the ability to metabolize the hydrocarbon. *A. niger* 612 showed a remarkable efficiency in this respect, metabolizing α-pinene in the test system in 8 to 24 hr under different experimental conditions, and was selected for further studies.

It was established that the accumulation of oxygenated products increased with increases in concentration of α-pinene up to a level of 0.6% (Fig. 2). Higher concentrations were inhibitory. An incubation period of 6 to 8 hr was optimum, and both α-pinene and the oxygenated products disappeared with longer incubation periods (Fig. 3). The transformation of α-pinene by the experimental strain was surprisingly sensitive to changes in incubation temperature. Temperatures above 30 C almost completely inhibited the utilization of α-pinene, without materially affecting the growth of the mold (Table 2).

In large-scale fermentations, a total of 1% α-pinene was added in two lots of 0.5% at 4-hr intervals. This procedure, although not satisfactory for the total utilization of α-pinene, gave better over-all yields of the oxygenated conversion products.

The procedure for extraction of the unreacted substrate and the oxygenated transformation products, present both in the medium and in the mycelium, was based on that used for steroids (Peterson et al., 1952).

The lower boiling fractions from the ether extracts yielded a mixture of the ketone (III) and alcohol (IV). Ketone III was separated from alcohol IV, either through (i) the crystalline bisulfite complex of III or by (ii) chromatography over alumina. The recovery of both the compounds was better by the bisulfite method, although the degree of separation obtained by chromatography was somewhat better.

From a comparison of the physicochemical data of ketone III and its semicarbazone with those reported in the literature for *d*-verbenone and its corresponding derivative (DuPont et al., 1934), it appeared that the compounds may be identical. The identity was fully established by the synthesis of verbenone from α-pinene according to the method of Badoche (1953). Alcohol IV

yielded *d*-verbenone (III) by oxidation with manganese dioxide (Turner, 1954) or with chromium trioxide in pyridine, indicating that it may be a *cis*- or *trans*-verbenol. From the physical constants of IV and those of its *p*-nitrobenzoate, IV was identified as *d-cis*-verbenol. The physical data of Diol V correspond to those reported for *d-trans*-sobrerol in the literature (Schmidt, 1953); this compound was synthesized by an acid-catalyzed rearrangement of α-pinene epoxide (Moore et al., 1956), and the identity of Diol V with *d-trans*-sobrerol was conclusively established through comparative infrared spectra and mixed melting point determinations. Both were converted to the identical ketone, hydroxy-carvotanacetone (VII), by oxidation with manganese dioxide.

Verbenol, verbenone, and sobrerol are also found among the autoxidation products from α-pinene (Moore et al., 1956). The major product of autoxidation is, however, *trans*-verbenol, whereas *cis*-verbenol appears to be the major metabolite of α-pinene by *A. niger*.

No appreciable amount of autoxidation products could be detected in the control experiments conducted without the mold. Furthermore, the observed inhibition of the transformation at higher temperatures strongly indicates that verbenol and verbenone are true transformation products of the mold.

The α-pinene employed in these studies had an optical rotation corresponding to a mixture of 70% *d*- and 30% *l*-α-pinene. All the metabolites, *d*-verbenol, *d*-verbenone, and *d-trans*-sobrerol, on the other hand, were obtained optically pure (in contrast with the autoxidation products, which were mixtures) and probably were derived from *d*-α-pinene by stereospecific oxygenation processes. The unreacted pinene, $[\alpha]_D = +23.2°$, recovered after the fermentation, however, did not show any significant difference in rotation from that of the starting material, $[\alpha]_D = +24.6°$. This would indicate that *l*-α-pinene was also metabolized simultaneously with the *d* isomer by a different pathway and probably broken down to smaller fragments.

The accumulation of oxygenated products for a brief period may be due to their slower rate of further oxidation compared with that of their formation from α-pinene. On prolonged incubation, both α-pinene and the oxygenated products disappear from the medium, and are presumably broken down to carbon dioxide and water. Deep-tank fermentations with a certified stainless steel (40 gal) fermentor and with more vigorous aeration and agitation have not been very successful so far, the yields of verbenol and verbenone being considerably reduced under these conditions.

Since the same strain of *A. niger* converts progesterone to 11α-hydroxyprogesterone (Peterson et al., 1952) in about 70% yield (*unpublished data*) under essentially similar experimental conditions, it is logical to presume that the oxygenation process in the case of steroids and terpenoids may be similar.

The formation of sobrerol can be explained by a postulated attack of the reactive oxygenating species on the double bond leading to α-pinene epoxide (VI) or its equivalent, which may give rise to sobrerol (V) by a proton-catalyzed (probably a nonenzymatic) process (Fig. 1). It should be mentioned here that epoxidation of unsaturated steroids by microorganisms is not unknown (Bloom and Shull, 1955).

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