Properties of an Extract from Canada Thistle Roots That Stimulates Germination of Dormant Teliospores of Canada Thistle Rust (*Puccinia punctiformis*)

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A stimulator of teliospore germination was concentrated from thistle roots (*Cirsium arvense*) by steam distillation and extraction by hexane. Concentrations of 25 $\mu L/L$ stimulated teliospore germination 50% in 7 days. Teliospores exposed to volatiles from crude extract at time intervals ranging from 1 min to 24 h reached a maximum germination plateau with 1 h or longer exposures when counted at 7 days. TLC, NMR, and GC–mass spectrometric analysis of the hexane extract indicated the presence of C_{17} unsaturated hydrocarbons with four, three, two, and one double bonds. 1-Pentadecene was also found. Most of the stimulatory activity occurred with the compound separated by TLC at R_f 0.26, shown to be (*Z,Z,Z*)-1,8,11,14-heptadecatetraene, or aplotaxene. The spot at R_f 0.60 contained 1-heptadecene and 1-pentadecene. These compounds were analyzed quantitatively by gas chromatography. Aplotaxene was found in thistle roots at concentrations of 0.032 $\mu g/g$ of fresh roots. Synthetic aplotaxene was found to possess no stimulatory activity on Canada thistle rust teliospore germination.

Canada thistle [*Cirsium arvense* (L.) Scop.] is a widely dispersed noxious weed that is difficult and expensive to control by herbicides. This plant is susceptible to a rust, *Puccinia punctiformis*, that is particularly devastating in the aecial stage. Infection is initiated from basidiospores produced by germinating teliospores. Turner et al. (1982) reported a biologically active substance from Canada thistle roots that stimulated teliospore germination. French (1985) has reported stimulation of germination of urediniospores of a number of rust species by volatile aroma or flavor compounds. For example, urediniospores of *P. punctiformis* were stimulated by 5-methyl-2-hexanone (French, 1983). Stimulatory compounds such as nonanal and 6-methyl-5-hepten-2-one were identified in urediniospores of *Puccinia graminis* var. tritici, *Uromyces phaseoli*, and other species (French et al., 1977; Rines et al., 1974).

Teliospores are the dormant, overwintering stage of Canada thistle rust, and they germinate very slowly and irregularly. We are interested in inducing germination in these teliospores at will to facilitate Canada thistle infection with this systemic, highly damaging aecial phase of the fungus. Our ultimate goal is to develop an effective biocontrol procedure for this noxious, spiny, perennial weed that is becoming an increasingly important problem

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as it spreads by wind-borne seeds and by root fragmentation resulting from certain agricultural practices. This research summarizes some of the physical, chemical, and biological properties of a stimulant to teliospore germination from Canada thistle roots.

MATERIALS AND METHODS

Rust Collection and Bioassay. Teliospores of *P. punctiformis* (Strauss) Roehl. were collected from field stands of Canada thistle [*C. arvense* (L.) Scop.] plants in Great Falls, MT. The teliospores were collected from air-dried leaves on a vacuum cyclone harvesting device (Tervet and Cassell, 1951). Spores were sieved through 44-μm-mesh screen and stored at 4 °C until use. Alternatively, thistle leaf tissue bearing teliospores was soaked overnight in distilled water and then ground in a Waring blender. This material was filtered through four layers of cheesecloth to remove debris, and the teliospores were collected on Whatman No. 1 filter paper by vacuum filtration. The spores were air-dried on the filter paper and stored at 4 °C until use.

Hexane extracts of roots were bioassayed by incorporating microliter amounts, measured by syringe, into 1% water agar (5.0 mL) and placing them in 5.0-cm plastic Petri plates. The usual concentration range was 0, 10, 25, 50, 100, 250, 500, and 1000 μL/L of concentrated hexane extract of steam-distilled roots. Teliospores were transferred from the dried filter paper by a cotton swab from which they were tapped to the agar surface. Alternatively the teliospores were suspended in isopentane and transferred by pipet to the agar surface. The solvent evaporated rapidly, distributing the spores on the agar. The plates were held at 18 °C in a dark incubator. Eight counts of 100 spores were made microscopically at 100× at 7 and 10 days. Statistical significance of the results was obtained by the Student's t test at $P = 0.01$. Control spores usually had 0% germination.

Extraction and Analysis. Canada thistle plants were grown in pots of soil in the greenhouse or collected from field stands during the normal growing season. Approximately 1000 g of roots was collected from plants, washed in tap water, and ground in distilled water in a Waring blender. The ground material was placed in a 5-L Pyrex flask and steam-distilled. The distillate was collected in a cooled 2-L Pyrex flask containing 40 mL of HPLC-or pesticide-grade hexane. Two distillations were made and the hexane layers pooled and evaporated to small volume (0.2 mL) under a stream of dry nitrogen gas in a fume hood.

Stimulator extracts were chromatographed on 0.5-mm layers of silica gel on 20 × 20 cm glass plates (prepared in the laboratory) with pesticide-grade petroleum ether as the mobile phase. Spots were detected by placing the plates in a covered glass tank containing iodine crystals. Exposure to the iodine vapor turned the separated spots yellow to dark brown, depending on the concentration and the length of time the plates remained in the tank. Fractions separated by preparative thin-layer chromatography on glass plates were individually scraped off, extracted with hexane, and evaporated to low volume under a dry nitrogen stream in the fume hood.

Gas chromatographic (GC) characterization of compounds was performed with a Perkin-Elmer 3920 gas chromatograph using nitrogen as carrier gas, with a column of 10% SP-1000 on 80/100-mesh Supelcoport, 10 ft × 1/8 in., and a Shimadzu C-R-1-B integrating recorder. Chromatographic temperature parameters: injector, 220 °C; interface–flame ionization detector, 240 °C; analyses oven programmed from 150 to 200 °C at a rate of 1 °C/min. Quantitative analyses were made against known standards of 1-heptadecene (Aldrich), 1,8,11-heptadecatriene, and 1,8,11,14-heptadecatetraene, and an internal standard of hexadecane at 2000 μL/L.

Mass spectrometric (MS) data were obtained on a Hewlett-Packard HP 5995-C bench-top GC–MS. Spectra were acquired with use of the solid probe, which was programmed from 25 to 290 °C at 32 °C/min. The MS operating conditions covered a mass range of 40 to 800 amu, with an ion source temperature of 150 °C and a multiplier voltage of 2000 V. Proton NMR data were obtained on a JEOL JMN-400Hz high-resolution spectrometer. Spectra were obtained in $CDCl_3$ with a 5 nM probe at 21 °C, pulse width of 3.0 μs (50 °C), 16–64 transients, and a pulse delay of 2.0 s. All peaks were referenced to the $CHCl_3$ peak at 7.24 ppm.

Polyene Synthesis. The required *cis*-polyenes were synthesized by the general procedure of Bacha and Kochi (1968) and as exemplified by Boland and Jaenicke (1981). The following procedure for (*Z,Z,Z*)-1,8,11,14-heptadecatetraene, aplotaxene, is typical. Linolenic acid (Sigma, 99%) (2.0 g, 7.1 mmol) was added to a solution of pyridine (0.7 mL) in benzene (70 mL) containing cupric acetate (0.2 g of the dihydrate). This mixture was stirred under nitrogen at ambient temperature for 0.25 h. Lead tetraacetate (4.7 g, 10.6 mmol) was added, and the reaction mixture was heated to reflux for 1 h. The mixture was cooled in ice, hexane (170 mL) was added, and the resulting mixture was then suction-filtered through a bed of Celite. The filtrate was washed with 2 N nitric acid (2 × 70 mL), 2 N ammonium hydroxide (3 × 70 mL), and water (2 × 100 mL). The organic phase was dried (magnesium sulfate) and then concentrated by flash evaporation. The residue was chromatographed on silica gel (20 g), eluting with hexane (60 mL) and then 10% ethyl ether (50 mL). The solvent was removed to produce aplotaxene (0.54 g, 32.7%) that was 97% pure by GLC analysis. Spectral and chromatographic data were as described (Bacha and Kochi, 1968; Boland and Jaenicke, 1981). Similarly, linoleic acid was employed to obtain (*Z,Z*)-1,8,11-heptadecatriene (0.63 g, 38.7%).

Vapor and Temperature Effects. The effect of exposure time of spores to stimulator vapors was tested in 5-cm Petri plates. Teliospores were placed on a 5-cm Millipore filter disk resting on a saturated 5-cm filter pad. A sample of hexane extract (10 μL) was placed on lids of the Petri plates. Teliospores were exposed for times ranging from 1 to 1440 min (24 h). At the end of each exposure time, teliospores were washed into a millipore funnel and collected on 8.0-μm filter disks. Spores were washed under vacuum twice with 100 mL of distilled water and transferred to the surface of 1% water agar in 5-cm plastic Petri plates for germination as described above.

The fugacity of the active stimulatory component was tested by placing 1.0 μL of the hexane extract on small aluminum foil squares and placing them on the hot stage of a Fisher-Johns melting point apparatus for 5 min. Residue was rinsed off the foil with 0.1 mL of ethyl ether into 5 mL of warm 1% water agar. The foil was also placed in the agar, which was thoroughly agitated, and then poured into 5-cm plastic Petri plates—minus the foil—for bioassay.

EXPERIMENTAL RESULTS

After 7-day incubation in darkness at 18 °C, as many as 50% of the teliospores germinated when exposed to a range from 0 to 1000 μL/L of the concentrated hexane extract of steam-distilled thistle roots. Maximum activity (Figure 1) was 50% at 25 μL/L, or 0.028 μg of extract/g

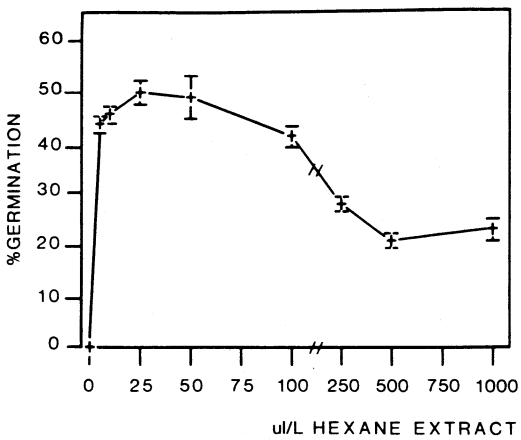

Figure 1. Effect of concentrated hexane extract of steam-distilled Canada thistle roots on germination of teliospores of *P. punctiformis*, in darkness at 18 °C for 7 days. Vertical bars are standard error of means. 25 μL/L = 0.028 μg of concentrated hexane extract/g fresh weight of roots.

Table I. Bioassay of TLC Fractions of the Hexane Extract of the Steam Distillate of Canada Thistle Roots

R_f[a]		bioactivity	
hexane extract	sep components rechromatographed[b]	concn of max act,[c] μL/L	% germin (10 days, 18 °C, dark)
0.60	0.61	500	3
0.50	0.50	100	6
0.38	0.39	500	5
0.26	0.28	5000	59[d]
0	0	500	14[d]

[a] Developed on 20 × 20 cm glass plates of 0.25 mm silica gel with petroleum ether. [b] Components of hexane extract were extracted from silica gel and rechromatographed on silica gel, developed with petroleum ether. [c] Maximum germination value over concentration ranges of 100, 500, 1000, 2500, and 5000 μL/L. Control germination 5%. [d] Significant over controls at $P = 0.01$.

fresh weight of the roots. No activity was found in distillates of leaves and stems (data not shown).

The hexane extract separated into five primary spots when developed on thin-layer plates of silica gel with petroleum ether (Table I). Most of the stimulatory activity was at R_f 0.26 with lesser amounts at the origin and in spots at R_f 0.38, 0.50, and 0.60. The material at R_f 0.26–0.28 stimulated germination up to 50% at 5000 μL/L (Table I). In other experiments, concentrations of 10 and 100 μL/L of R_f 0.26 stimulated germination 60% and 80%, respectively, at 12 days. The spots separated by TLC were analyzed individually by GLC. Gas chromatographic analysis detected the same main components as TLC, plus others. These peaks were collected in glass capillaries cooled in dry ice from a stream splitter on the GC. Mass spectrometric and NMR analyses indicated the component with R_f 0.28 (RT 16.54 on GC) to be (*Z,Z,Z*)-1,8,11,14-heptadecatetraene, or aplotaxene, as reported previously (French and Turner, 1984). This component (R_f 0.28) gave the proper integration and ^{1}H resonance positions corresponding to those previously reported (Boland and Jaenicke, 1981) for the compound (*Z,Z,Z*)-1,8,11,14-heptadecatetraene. The compound at R_f 0.39 gave an ^{1}H spectrum almost identical with the above tetraene, except its low-field section integrated for three double bonds associated with the 1,8,11-heptadecatriene, which has been previously reported (Boland and Jaenicke, 1981). In addition, two other resonances at δ 0.82 and 1.68 were also observed in this spectrum. These probably represent the presence of an impurity (some branched-chain analogues) contaminating the heptadecatriene. The components at R_f 0.61

Table II. Gas–Liquid Chromatography and Mass Spectrometric Analysis of the Fractionated Hexane Extract of the Steam Distillate of Canada Thistle Root Extract

R_f[a]	RT[b]	MS identificn: no. C (dbl bonds) name	μg/g root
0.61	5.51	15 (1) 1-pentadecene	0.0058
	11.04	17 (1) 1-heptadecene	0.0013
0.50	11.87	17 (2)	
0.39	13.78	17 (3) 1,8,11-heptadecatriene[c]	0.0037
0.28	16.54	17 (4) 1,8,11,14-heptadecatetraene (aplotaxene)[c]	0.0308

[a] Developed on 20 × 20 cm glass plates of 0.25 mm silica gel with petroleum ether. [b] Retention time. Run on 10% SP1000, 80/100 Supelcoport, 10 ft × $^1/_8$ in. [c] Double bonds confirmed by NMR.

Table III. Effect of Injector and Interface Temperatures on Bioactivity Recovered from Chromatography on GLC Column[a]

temp, °C		% germination[b]		
injector	interface	2.5 μL	7.5 μL	10 μL
150	153	7.1[c]	13.1[c]	7.1[c]
180	198	2.0[c]	1.6[c]	2.0[c]
250	250	0.1	0.3	0.1

[a] 1.0 μL of hexane extract injected. 10% SP2100, 80/100 Supelcoport, 6 ft × $^1/_8$ in.; 150–250 °C, 2 °C/min, attenuation 20×, 1:9 stream splitter. [b] GC effluent was collected from 100 to 250 °C in a dry ice chilled capillary. Capillary was rinsed with 20 μL of hexane, of which 2.5, 7.5, and 10.0 μL was incorporated into agar for bioassay. Water control, 0% germination; stimulated control (0.075 μL of hexane extract), 46.3% germination. [c] Significant over water control at $P = 0.01$.

were found to be 1-heptadecene (RT 11.04) and 1-pentadecene (RT 5.51) (Table II). The components at R_f 0.39 and 0.50 also were found to be C_{17} unsaturated hydrocarbons with three and two double bonds, respectively. Bioassay of all the fractions collected from GC, however, showed little biological activity.

Synthetic aplotaxene was tested directly in agar, and as a volatile compound, and was found to be inactive. Linolenic acid and methyl linolenate, possible precursors of aplotaxene, also were inactive. These data are not shown.

Samples of the hexane extract (1 μL) injected into the gas chromatograph and collected from a 1:9 stream splitter gave maximum germination of 13% at 150 °C injector temperature (Table III). Bioassay of a 0.075-μL sample of this extract gave 46% germination; 0% germinated in the blank control. The maximum germination values at injector temperatures of 180 and 250 °C were 2% and 0.3%, respectively. Over 90% of the sample was lost at 150 °C, and more at the higher temperatures. At 150 °C, resolution of peaks on the GLC was extremely poor. These data indicate that the stimulatory compound cannot be isolated by GC, at least under these conditions. Since aplotaxene can be isolated by GC, the stimulatory compound obviously is not aplotaxene.

Since the active component of the hexane extract appeared to be heat sensitive in the GC, a fugacity test was run to determine volatility or heat stability. Hexane extract (1 μL) was subjected to temperatures ranging in 10 °C intervals from 100 to 180 °C for 5 min (Figure 2). At temperatures above 150 °C, activity rapidly disappeared. At 180 °C very little stimulatory activity remained. These results agreed well with the activity tests run on GC. The loss in activity from heating could result from the volatility of the stimulator, or from its thermal destruction.

A volatility test of the concentrated hexane extract indicated that exposure of teliospores to volatiles from 10 μL of extract for 1 h was sufficient to trigger maximum germination percentage at the end of 7 days at 18 °C

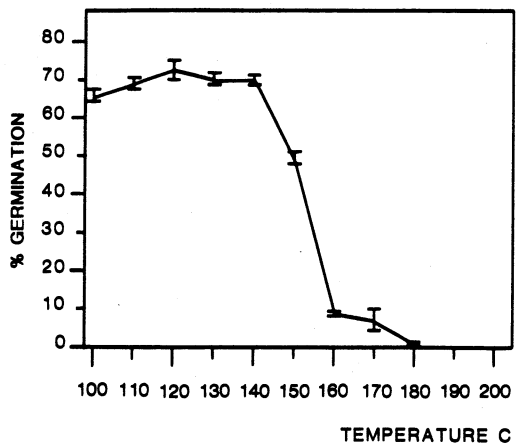

Figure 2. Effect of 5-min heating times on stimulatory activity of 1.0 μL of hexane extract at 7 days, 18 °C, in darkness. Vertical bars are standard error of means.

Table IV. Concentration of Aplotaxene in Several Samples of Roots of Canada Thistle

sample date	quantity, g	total μg	μg/g root fr wt
2/27/85	1014	23.5	0.023
5/1/85[a]	1270	40.9	0.032
5/2/85	900	41.0	0.045
5/7/85	950	11.6	0.011
7/2/85[a]	790	37.7	0.047
7/9/85	1000	24.5	0.024
			0.030[b]

[a] Roots from male plants; all others female. [b] Average.

(Figure 3). Unexposed teliospores did not germinate. Exposure time intervals of 1 min to 30 min increased germination from 7 to 58%. Exposures over 1 h, up to 24 h, gave germination values not much greater than for the 1-h exposure; maximum germination was 65%.

The aplotaxene content of the hexane extract was determined, with a known amount of hexadecane as internal standard. Hexadecane did not appear to be present naturally in the extract. Six samples of roots, ranging from 790 to 1270 g, averaged 0.03 μg of aplotaxene/g fresh weight of root (Table IV). The thistle plant is dioecious, but no obvious differences in aplotaxene between male and female plants were detected.

DISCUSSION

The first endogenous stimulators of teliospore germination reported appear to be a group of C_{13} hydrocarbons, with double and triple bonds, particularly *trans*-1,11-tridecadiene-3,5,7,9-tetrayne, which were identified from leaves of safflower (*Carthamus tinctorius*) (Binder et al., 1978). These compounds stimulated germination of teliospores of *Puccinia carthami*. Aplotaxene, a C_{17} hydrocarbon with four double bonds, appeared to be a reasonably similar compound capable of stimulating teliosospores of Canada thistle rust (*P. punctiformis*) (French and Turner, 1984). Synthetic aplotaxene, however, was inactive as a teliospore stimulator. Aplotaxene is known to occur in roots of a number of thistle species, including *Cirsium hypoleucum* (Bohlmann and Abraham, 1981), *Cirsium canum* (L.) (Bohlmann and Abraham, 1981), *Cirsium japonicum* (Yano, 1977), *Centaurea incana* (Aclinou et al., 1982), *Arctium lappa* Linn. (Washino et al., 1985), *C. tinctorius* (Binder et al., 1975), *Saussurea lappa* Clarke (Klein and Thomel, 1976), and *Inula racemosa* (Bokadia et al., 1986).

Binder et al. (1975) found aplotaxene in seedlings of safflower. It is also found in costus root oil, from *S. lappa* (Gove, 1968; Klein and Thomel, 1976), which is used as

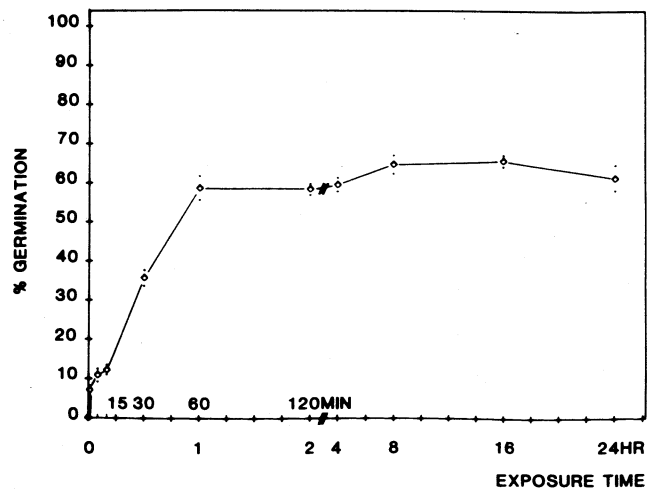

Figure 3. Effect of time of exposure to 10 μL (≃50 g of fresh weight of roots) of concentrated hexane extract of steam-distilled thistle roots on germination of teliospores of *P. punctiformis* at 7 days, 18 °C, in darkness.

an ingredient of perfume. Yano (1977) found aplotaxene in *C. japonicum*. None of these authors reported any biological activity for aplotaxene. Yano also found three other C_{17} unsaturated hydrocarbons, including dihydroaplotaxene (C_{17}, three double bonds), tetrahydroaplotaxene (C_{17}, two double bonds), and hexahydroaplotaxene (C_{17}, one double bond), or 1-heptadecene. Yano also found that the quantity of aplotaxene in *C. japonicum* roots remained constant throughout the growing season, but the amount of the other C_{17} unsaturated hydrocarbons decreased in the roots just before the plant bolted and flowered (Yano et al., 1983). While this may have little bearing on the stimulation of teliospore germination, it should be noted that the most devastating stage of the Canada thistle rust is the systemic orange spermagonial/black aecial stage beginning in the early bolting stage. In addition to aplotaxene and the related less unsaturated C_{17} compounds, aplotaxene epoxide, 11,12-epoxyheptadeca-1,8,14-triene, and *cis*-8,9-epoxyheptadeca-1-ene-11,13-diyn-10-ol also have been reported in thistle roots (Bohlmann and Abraham, 1981; Yano, 1980). We have found no reports of thermal instability or volatility, or of biological activity of these compounds.

The fact that the unknown active compound cochromatographs on TLC with aplotaxene suggests the two compounds share similar chemical properties. In other experiments we have shown that the active compound is volatile or is destroyed by heat. Volatility tests indicated that the active material readily diffused to the teliospores and that a 1-h exposure to the compound at the beginning of a 7-day germination period was sufficient to stimulate germination. In other words, a 1-h exposure was enough to completely activate the germination process, and although the teliospores were thoroughly washed after exposure, this did not remove the stimulus. Obviously the compound does not have to be present in the spore's environment throughout the germination period to be effective. In this regard, the physical exposure mechanism of germination is similar to that we have previously reported for nonanal and urediniospores of *P. graminis* var. tritici and *Puccinia coronata*, for β-ionone and urediniospores of *U. phaseoli* (French et al., 1977), for benzonitrile and urediniospores of *Puccinia helianthi* (French, 1984), and for methyl salicylate and seeds of *Rumex crispus* (curly dock) (French et al., 1986).

In summary, we have shown that the unidentified teliospore stimulator of Canada thistle rust is steam distill-

able, volatile, and soluble in organic solvents. Aplotaxene was isolated and identified in the most stimulatory fraction from thistle root distillates, but was inactive. The active compound has physical properties similar to those of the other volatile flavor or aroma compounds that we have reported as stimulatory to urediniospores of over 25 rust species and to several species of weed seed (French, 1985). The diffusion mechanism of this compound in triggering germination is similar to that of the previously reported compounds and suggests that a brief exposure of teliospores to the volatile stimulator would be sufficient to activate the germination process of these dormant spores. If the synthesis of a prospective active compound is found to be too expensive or difficult, we may not be able to supply absolute proof of identity by demonstrating biological activity. However, we are continuing our efforts toward ultimate chemical identification of this apparently labile stimulator, hoping it may prove helpful in adapting Canada thistle rust for use as a biocontrol agent of this noxious weed.

ACKNOWLEDGMENT

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Registry No. 1-Pentadecene, 13360-61-7; (*Z,Z,Z*)-1,8,11,14-heptadecatetraene, 10482-53-8; 1-heptadecene, 6765-39-5; 1,8,11-heptadecatriene, 93812-47-6.

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