Isolation and characterization of calmodulin-inactivating cholesterol hydroperoxides

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Abstract A series of cholesterol hydroperoxides has been prepared and tested as inactivators of calmodulin. Two previously undescribed compounds, tentatively identified as 20-(R)-25-dihydroperoxy-5-cholesten-3β-ol and its 20-(S) isomer inactivate calmodulin with 50% loss of activity at 5–10 μM. Cholesterol derivatives with a single hydroperoxy group at C-20 or C-25 are less effective, while 7α-hydroperoxy-cholesterol and 25-hydroxy-cholesterol are inactive. The side-chain hydroperoxide compounds were isolated from a mixture shown earlier to suppress formation of fatty streaks in aortas of rabbits fed a diet supplemented with cholesterol.—**Tipton, C. L., M. Shih, and W. J. Magat.** Isolation and characterization of calmodulin-inactivating cholesterol hydroperoxides. *J. Lipid Res.* 1991. **32:** 1403–1408.

Supplementary key words 25-hydroxycholesterol • atherosclerosis • oxysterol

Cholesterol autoxidation proceeds via the initial formation of hydroperoxides, which then break down to form a very large number of products, including alcohols, carbonyl compounds, and epoxides (1). Evidence for and against roles for these products in atherogenesis has been reviewed by Smith and Johnson (2). Oxidatively modified low density lipoprotein is thought to be involved in the development of atherosclerosis (3) but there is little evidence that this involves oxysterols. A mixture of hydroperoxides isolated from autoxidized cholesterol is a potent inactivator of calmodulin and suppresses formation of fatty streaks in the aortas of rabbits fed an atherogenic diet (4). We now report the isolation and tentative identification of two new isomeric cholesterol hydroperoxides that inhibit calmodulin at low concentrations and compare them with previously known cholesterol hydroperoxides. Portions of this work have been disclosed previously in a patent (5) and in abstract form (6).

EXPERIMENTAL

The starting material for isolation of cholesterol hydroperoxides was a large sample of cholesterol (Nutritional Biochemical Corporation, Cleveland, OH) that had been stored at room temperature for more than 20 years. Silica gel (230–400 mesh), hematoporphyrin free base, N,N-dimethyl-*p*-phenylene diamine dihydrochloride, bovine brain calmodulin, and 5'-nucleotidase were purchased from Sigma Chemical Co., St. Louis, MO. Calmodulin-deficient calmodulin-dependent cyclic nucleotide phosphodiesterase was partly purified by the method described by Sharma, Taylor, and Wang (7) with the omission of the calmodulin-Sepharose 4B affinity chromatography. 25-Hydroxycholesterol was purchased from Steraloids, Inc., Wilton, NH. Silica gel (60–200 mesh) and 20 × 20 cm glass-backed thin-layer chromatography (TLC) plates were from E. Merck, Darmstadt, Germany. Thin-layer chromatography was also carried out on 4 × 8 cm plastic-backed silica gel sheets (Macherey-Nagel Polygram Sil G) obtained from Brinkman Instruments, Inc., Westbury, NY. Except when otherwise stated, HPLC was done by using an assembly of components from Beckman Instrument Co., Fullerton, CA. The column, from Rainin Instrument Co., Inc., Woburn, MA, was a 10 mm ID × 25 cm Microsorb 5 μm C18 reverse-phase column with a 5-cm guard column of the same material. NMR spectra were obtained by using a Nicolet NT-300 instrument at 300 MHz. Samples were dissolved in $CDCl_3$. Mass spectra were obtained with a Finnegan 4000 mass spectrometer.

20-(R)-20-Hydroperoxycholest-5-ene-3β-ol and the 20-(S) stereoisomer were synthesized and supplied by Dr. M. G. Ranasinghe (Dept. of Chemistry, Iowa State University, unpublished results).

Inactivation of calmodulin was assayed by using a modification of the method described by Leung et al. (8). Calmodulin was diluted so that a final volume of 4.875 ml contained 100 μg calmodulin, 180 μmol Tris buffer

Abbreviations: cAMP, adenosine-3',5'-(hydrogen phosphate); EGTA, ethyleneglycol-*bis*-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; HPLC, high performance liquid chromatography; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TLC, thin-layer chromatography.

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(pH 7.5), 18 μmol imidazole, 22.5 μmol $Mg(OAc)_2$, and 50 μmol $CaCl_2$. To each one-tenth aliquot of this solution was added 12.5 μl of a methanolic solution of a sterol derivative. After 30 min of incubation at 30°C, 10-μl portions were removed for assay. The assay mixture contained, in a final volume of 90 μl, 3.6 μmol Tris buffer (pH 7.5), 3.6 μmol imidazole, 450 nmol $Mg(OAc)_2$, 54 nmol $CaCl_2$, 108 nmol cAMP, 0.09 units of 5'-nucleotidase, and 1–2 $\times 10^{-3}$ units of cAMP-phosphodiesterase. Tubes containing 22.5 nmol EGTA in place of the $CaCl_2$ were included to measure calmodulin-independent activity. After a 30-min incubation at 30°C, the reaction was terminated by dilution with 0.91 ml of cold water, and the P_i produced was determined by the malachite green method (9). The difference in activity between tubes with $CaCl_2$ and those with EGTA was taken to be the calmodulin-dependent activity.

Purification of calmodulin-inactivating cholesterol hydroperoxides

Aged cholesterol (50 g) was dissolved in hot methanol (1.5 l) and cooled to 4°C before the crystals were separated by filtration. The supernatant was reduced to about 200 ml on a rotary evaporator and cooled, and a second crop of cholesterol was removed by filtration. The filtrate was extracted three times with hexane, and the methanol-rich layer was evaporated to dryness to yield, typically, 5 g. This residue was fractionated by two successive SiO_2 chromatography steps according to Still, Kahn, and Mitra (10). The first column contained silica gel, 60–200 mesh, packed to a depth of 15 cm in a 50-mm i.d. column. The sample was eluted with hexane–2-propanol 8:1 (v/v) and 50-ml fractions were collected. The fractionation was monitored by TLC by using benzene–ethyl acetate 3:2 (v/v) as the developing solvent and 0.1% N,N-dimethyl-*p*-phenylenediamine in 50% methanol containing 1% acetic acid as the spray reagent for detection of hydroperoxides (11). The compounds of interest eluted in fractions 4 to 9. These fractions were evaporated to dryness and applied to a second column consisting of silica gel, 230–400 mesh, packed to a depth of 20 cm in a 50-mm i.d. column. This column was eluted with hexane–2-propanol 8.5:1 (v/v). Fractions of 50 ml each were collected, and the compounds of interest were found in fractions 16–21. The final fractionation was by HPLC with acetonitrile–water 63:37 (v/v) as the mobile phase at a flow rate of 4.0 ml/min; absorbance of the eluate was monitored at 210 nm. Two major components, designated HP-A and HP-B, retention times 16 and 19 min, respectively, were found; the yields were about 10 mg each from 50 g of starting material. Mass spectra (desorption/chemical ionization of isobutane): HP-A. *M/z* 433 ($M+1-H_2O$) 3; 417 ($M+1-H_2O_2$) 4; 399 ($M+1-H_2O-H_2O_2$) 9; 383, 9; 371, 8; 365 ($M+1-H_2O-2\ H_2O_2$) 8; 317 ($M+1-OH-C_{22}$ to C_{27}) 66; 299 ($317-H_2O$) 74; 273 ($M-C_{20}$ to C_{27}) 74; 257, 68; 255 ($273-H_2O$) base peak. HP-B. Same peaks with the following intensities. *M/z* 433, 3; 417, 3; 399, 7; 383, 7; 381, 7; 371, 6; 365, 6; 317, 39; 299, 41; 273, 83; 257, 70; 255, base peak.

Fractions eluting from the second SiO_2 column just ahead of those containing HP-A and HP-B were combined and used for the isolation of 25-hydroperoxycholesterol by TLC on a 20 × 20 cm silica gel TLC plate. After development with benzene–ethylacetate 3:2 (v/v), a hydroperoxide was found at R_f 0.30–0.31. The band was scraped from the plate and eluted with ethyl acetate. ^{1}H-NMR (key resonances, ppm) ($CDCl_3$): δ 0.68 (3H,s,C-18); 0.93 (3H,d, J=6.3Hz, C-21); 1.00 (3H,s,C-19); 1.21 (6H, s,C26,27), indicating substitution at C-25. Mass spectrum (desorption/chemical ionization in NH_4^+): *m/z* 436 ($M+NH_4^+$) 52; 420, 69; 418 ($M+NH_4^+-H_2O$) base peak; 402 ($M+NH_4^+-H_2O_2$) 75; 401 ($M+NH_4^+-H_2O-OH$) 85; 385, 72; 384 ($M+NH_4^+-H_2O-H_2O_2$) 62; 343, 94; 273 (M^+–sidechain) 22; 255 (M^+–sidechain–H_2O) 13. We concluded that this material was 25-hydroperoxycholesterol and identified it as such in studies of calmodulin inactivation.

Thermally accelerated cholesterol autoxidation

Cholesterol that had been recrystallized repeatedly from methanol was placed in a glass tray to form a shallow layer and heated at 50°–60°C in the dark for several weeks. At intervals of 7 days, 10-g samples were removed, and the cholesterol hydroperoxides were purified as described before. The eluate from the second silica gel chromatography column, which was enriched in the hydroperoxides of interest as indicated by TLC, was examined by HPLC under the same conditions as described except that an analytical scale (4.6 mm × 25 cm) Beckman Ultrasphere 5 μm C18 reverse-phase column was used.

Synthesis of 7α-hydroperoxy-cholest-5-ene-3β-ol

Three g of cholesterol that had been purified by repeated crystallization from methanol was photo-oxidized in 100 ml chloroform containing 50 mg hematoporphyrin with continuous stirring under fluorescent light (12). After 2 days, the reaction was terminated by the addition of diethyl ether and active carbon. After filtration with Celite, the clear yellow filtrate was taken to dryness and chromatographed on a silica gel column with benzene–ethyl acetate 8:3 (v/v). The hydroperoxide was located by TLC of the fractions and recrystallized from methanol. $[\alpha]_d$ = −124 ($CHCl_3$); lit. −139; m.p. 158-160°; lit. 154–156.5° (12). ^{1}H NMR ($CDCl_3$); δ 0.649 (3H,s,C-18); 0.854 (6H,dd, J=6.5Hz, C26,C27); 0.908 (3H,d, J=6.6Hz,C-21); 0.983 (3H,s,C-19); 4.149 (1H,dd, $J_{7,8}$=3.5Hz, $J_{6,7}$=4.7Hz, C-7); 5.710 (1H,dd, $J_{6,4}$=1.5Hz, $J_{6,7}$=4.8Hz,C-6).

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TABLE 1. ¹H-NMR spectra of compounds HP-A and HP-B, their reduction products, and reference compounds

Compound	Position			
	C-18	C-19	C-21	C-26,27
	δ, ppm			
Cholesterol	0.674s	1.003s	0.810d, J = 6.6Hz	0.858d, J = 6.9Hz
HP-A	0.783s	1.008s	1.248s	1.237s
HP-B	0.820s	1.010s	1.249s	1.237s
S-20-OOH[a]	0.79s	1.02s	1.25s	0.88d
R-20-OOH[a]	0.83s	1.02s	1.25s	0.88d
Reduced HP-A	0.866s		1.293s	
Reduced HP-B	0.866s		1.147s	
S-20-OH[b]	0.87s		1.28s	
R-20-OH[b]	0.87s		1.13s	

[a]Isomers of 20-hydroperoxy-3β-hydroxycholest-5-ene, reported by van Lier and Rousseau (14).
[b]Isomers of cholest-5-ene-3β,20-diol, reported by Honda and Komori (15) and Nes and Varkey (16).

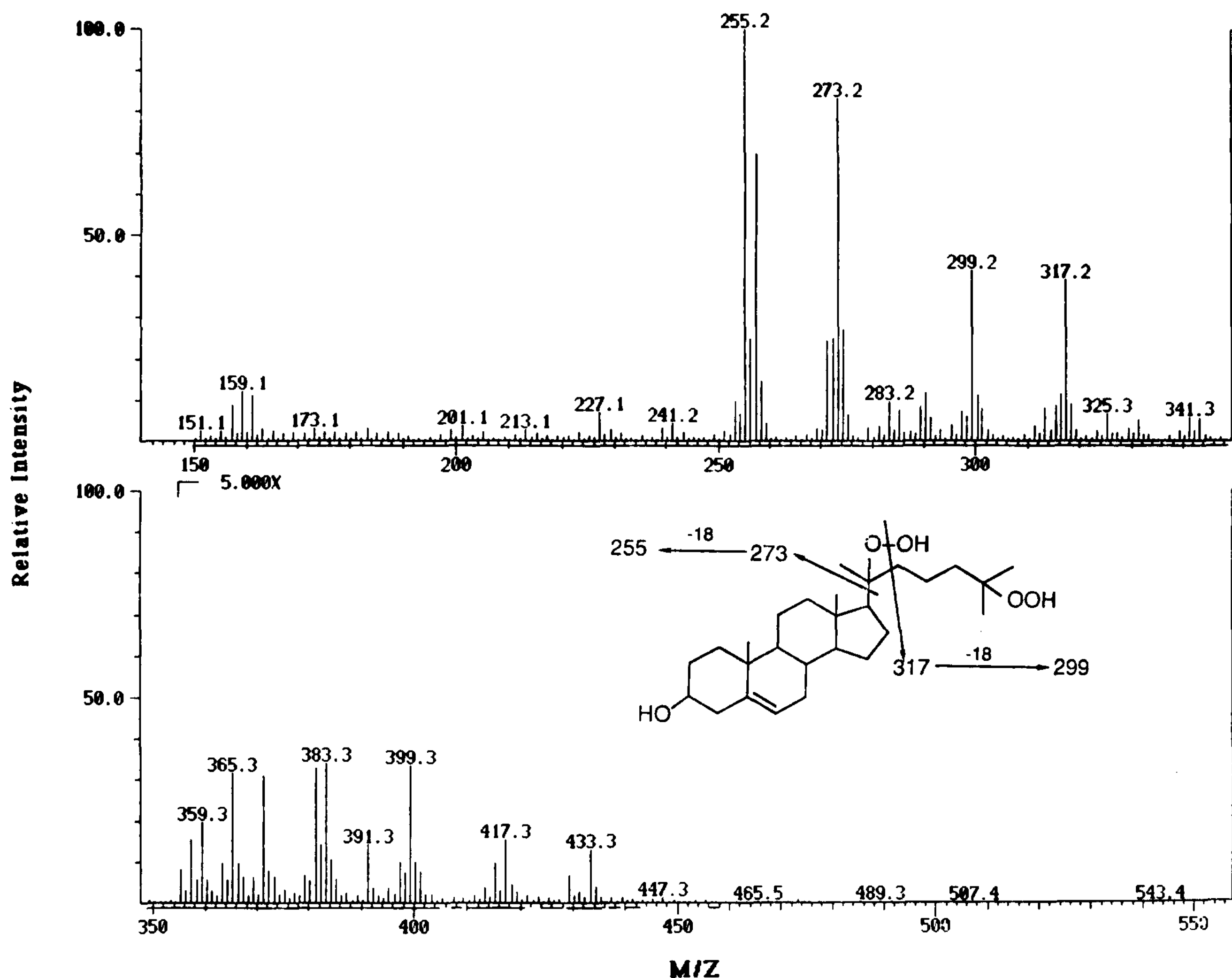

Fig. 1. Mass spectrum of compound B. Major fragments resulting from C-C bond cleavage are illustrated. Those resulting from losses of water and hydrogen peroxide are discussed in the text.

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RESULTS

Characterization of isolated hydroperoxides

The NMR spectra of HP-A and HP-B clearly show oxygen substitution at C-20 and C-25 (**Table 1**). The doublets in the spectrum of cholesterol for the methyl groups, C-21 and C-26,27, are replaced in the spectra of HP-A and HP-B by singlets. The mass spectrum of HP-B is shown in **Fig. 1**; that of HP-A is virtually identical. Fragmentation occurs predominantly from $(M+1)^+$ ions (13). The mass spectra suggest that the substituents at C-20 and C-25 are hydroperoxy groups and that there is no modification of the cholesterol molecule except on the sidechain. The stereochemistry of the oxy substituent at C-20 was deduced from the 300 MHz ^{1}H-NMR spectra (Table 1). Comparison of chemical shifts at C-18 and C-21 of HP-A and HP-B and their reduction products with the previously reported stereoisomers of 20-hydroxycholesterol and 20-hydroperoxycholesterol (13–15) suggests that HP-A is the α (S) isomer and HP-B the β (R) isomer. The other hydroperoxides have been described previously, and our preparations have the expected NMR spectra.

The conclusion that there are two hydroperoxy groups is further supported by the observation that an intermediate with properties of a hydroperoxide is formed during reduction with dilute $NaBH_4$ (**Table 2**). Hydroperoxides are selectively reduced in the electrochemical detector used in this experiment; alcohols do not react. A new product, with a shorter retention time than the starting material, was observed with both the electrochemical detector and the absorbance detector when 50 mM borohydride was used. When 500 mM borohydride was used, another product, with longer retention time than the starting material, was seen only with the absorbance detector. This is presumably the triol. The intermediate hydroperoxide has the same retention time as authentic 20-R-20-hydroperoxy-cholest-5-ene-3β,25-diol but it could be either that compound or the 25-hydroperoxy, 20-hydroxy isomer.

TABLE 2. HPLC of products of reduction of HP-B by $NaBH_4$

Compound	Retention Time	Reduction at Au-Hg Electrode
	min	
Experiment 1		
HP-B	12.76	yes
Ch-20-OOH, 25-OH[a]	10.97	yes
Products from		
2 min reaction	11.07	yes
	12.79	yes
40 min reaction	10.99	yes
	12.77	yes
Experiment 2		
HP-B	11.14	yes
Reduction product	11.26	no

In experiment 1, 10 μl of HP-B (10 mM in ethanol) was mixed with an equal volume of $NaBH_4$ (100 mM in methanol). Samples were removed after 2 min and 40 min for HPLC. In experiment 2, the starting concentration of $NaBH_4$ was 1 M. HPLC utilized a Lichrosorb RP-18 (5 μ) 4.6 mm × 50 mm reverse phase column (E. Merck, Darmstadt, Germany). The effluent was monitored with a V4 absorbance detector (ISCO, Lincoln, NE) at 210 nm in tandem with a LC-3A amperometric detector (Bioanalytical Systems Inc., West Lafayette, INI). The working electrode was a Au-Hg amalgam, the reference electrode was Ag-AgCl, and the applied potential was 40 mV. The mobile phase was acetonitrile–water 65:35 (v/v) containing 0.25 mM sodium perchlorate. The solvent was degassed under vacuum and purged continuously with a stream of He. Flow rate was 1.0 ml/min.

[a]20-(R)-20-Hydroperoxy-cholest-5-ene-3β,25-diol. Gift from Dr. M. Ranasinghe, Dept. of Chemistry, Iowa State University.

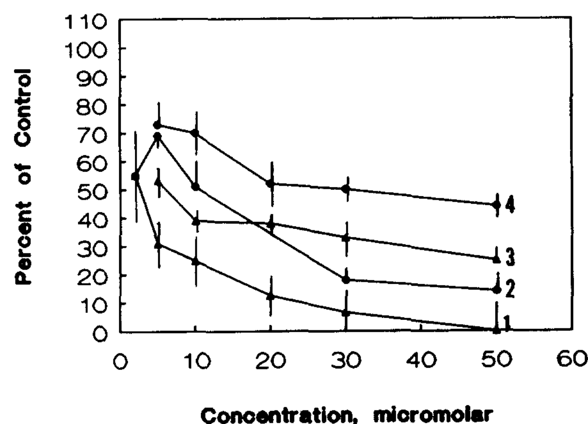

Fig. 2. Calmodulin inactivation by cholesterol hydroperoxides. 1: compound A; 2: compound B; 3: 20-(R)-hydroperoxycholesterol; 4: 20-(S)-hydroperoxycholesterol. Bars indicate standard deviations, based on two to six determinations. Control values (100%) are 26.3 to 48.3 nmoles P_i.

Calmodulin inactivation

The effects of two isomeric pairs of 20-hydroperoxycholesterols on the activity of calmodulin in the cAMP-phosphodiesterase assay are shown in **Fig. 2.** At 30 and 50 μM, the effectiveness of these compounds increases in the order 20-S-hydroperoxycholesterol, 20-R-hydroperoxycholesterol, HP-B, HP-A. **Table 3** compares these compounds with some related oxysterols and cumene hydroperoxide at 30 μM. The compounds with a single hydroperoxy group on the sterol sidechain have about the same activity as cumene hydroperoxide. The greater activity of HP-A and HP-B may simply be a concentration effect because they have two hydroperoxy groups on the sidechains. The most striking comparison is with 7α-hydroperoxycholesterol, which has no inhibitory activity. Whether this reflects some specific orientation in the binding of the oxysterols that does not allow the 7α-hydroperoxy group access to oxidizable groups on the protein or a difference in reactivity between secondary and tertiary hydroperoxides is not yet clear.

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TABLE 3. Inactivation of calmodulin by some cholesterol autoxidation products

Compound	Control Activity	Percent of Control Activity
	nmoles P_i	%
HP-A	48.3 ± 6.7	8 ± 1
HP-B	36.6 ± 6.2	18 ± 3
20(R)-Hydroperoxide	26.3 ± 0.2	33 ± 4
20(S)-Hydroperoxide	31.5 ± 2.4	50 ± 6
25-Hydroperoxide	51.0 ± 2.4	51 ± 5
Cumene hydroperoxide	48.9 ± 4.9	36 ± 5
7α-Hydroperoxide	66.1 ± 6.6	100 ± 12
25-Hydroxycholesterol	41.7 ± 1.6	92 ± 4

Control activity determined in an 30-min assay as described in Experimental; the second column is the activity of calmodulin, as percent of control, after a 30-min incubation with 30 μM oxysterol. The values are means of two to five determinations ± standard deviation.

Thermal acceleration of autoxidation

HPLC analysis of partly purified hydroperoxides from cholesterol that had been allowed to autoxidize for 2 weeks at 50°-60°C showed that HP-A and HP-B were present at about the same concentrations as in similar preparations from aged cholesterol (data not shown).

DISCUSSION

In an effort to identify compounds responsible for the ability of a mixture of hydroperoxides to inhibit the initiation of atheroma formation in rabbits (4), we adopted calmodulin inhibition as a convenient assay to guide the purification of individual hydroperoxides. Although this allowed us to isolate two compounds that are highly active as calmodulin inhibitors, we have no direct evidence that calmodulin inhibition is involved in the effects of cholesterol hydroperoxides on atherogenesis in rabbits eating a cholesterol-supplemented diet. The structural analysis of these compounds is tentative until more material is available for further study. Experiments that support the idea that HP-A and HP-B may be effective in suppressing atherogenesis are being reported separately (Shih, M., S. N. Mathur, F. J. Field, and C. L. Tipton, manuscript in preparation). The yields of these compounds by the procedures reported here for isolation from autoxidized cholesterol are low (about 0.02% each), so that we have not been able to obtain enough material to do the feeding experiments necessary to determine their effects on atherogenesis.

Many calmodulin inhibitors are hydrophobic substances and calmodulin has a hydrophobic binding site that is important in inhibition (17). Oxidation of methionine residues in calmodulin has been shown to inactivate the protein (18). The combination of a hydroperoxide functional group, capable of facile oxidation of thioethers, with a generally hydrophobic structure in HP-A and HP-B may explain the ability of these compounds to inactivate calmodulin.

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