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ARENOBUFAGIN AND GAMABUFOTALIN FROM THE VENOM OF THE CENTRAL ASIAN GREEN TOAD *Bufo viridis*. INTRODUCTION, STRUCTURAL–FUNCTIONAL FEATURES

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Two bufodienolides have been isolated in the individual form from the Central Asian green toad Bufo viridis *for the first time. It has been established by ^1H NMR, mass spectrometry, and x-ray structural analysis that these compounds are gamabufotalin (3β,11α,14-trihydroxy-5β,14β-bufa-20,22-dienolide) and a crystalline modification of arenobufagin (3β,11α,14-trihydroxy-12-oxo-5β,14β-bufa-20,22-dienolide). The biological activity of Bakagin — the total bufadienolides of the venom of* B. viridis, *consisting predominantly of the compounds named — has been studied. The promising nature of a further study of Bakagin as a cardiotonic drug has been shown.*

Among the enormous number of biologically active substances of natural origin one of the central positions is occupied by animal venoms or zootoxins, which are unique in their chemical nature and physiological action. The animal venoms include the secretions of the parotid (suprascapular) gland of toads, called toad venom.

Toad venom has long been considered an important medicinal agent with an unusually high biological activity and a broad range of pharmacological action [1]. It possesses cardiotropic [2, 3], antibiotic [4], and neurotropic [5, 6] activities, and also antitumoral [7], radioprotective [8, 9], immunomodeling [10], and many other properties [9, 11]. Advances in the experimental study of toad venom and clinical observation have led to the situation that medicinal preparations based on it have been developed in a number of countries (Japan, India, etc.) [12, 13]. The venom of the Central Asian green toad *Bufo viridis* has scarcely been studied in this respect.

In view of the chemical peculiarity of the toad venom (relatively high content of highly hydrophobic compounds — bufadienolides and sterols), primary fractionation was conducted on Sephadex LH-20 (Fig. 1). Fraction 1 contained mainly protein components, and from it we had previously isolated a new phospholipase A_2 [14]. According to the results of testing, of the other fractions fraction 6 alone contained practically all the paralytic activity of the venom.

Attention is attracted by the fact that although the toxic fraction had the highest absorption at 280 nm it amounted to only 1-2% of the weight of the material deposited on the column. To all appearance, this fraction was enriched with aromatic compounds or contained a chromophoric group absorbing strongly in this region of the spectrum. This agrees well with literature reports that the main toxic components of toad venoms are indole derivatives (bufotenin, serotonin, tryptamine, etc.) and also bufadienolides (bound and free genins).

The toxic fraction was separated on a column with the hydrophobic support Polikhrom P-1. According to the results of testing, paralytic activity was possessed by the fraction eluted at a 10% concentration of ethyl alcohol. The further separation of this fraction was achieved by reversed-phase chromatography in a high-performance liquid chromatograph on an Octadecyl Si-100 column with a concentration gradient of acetonitrile (Fig. 2).

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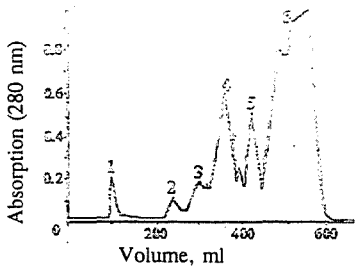


Fig. 1

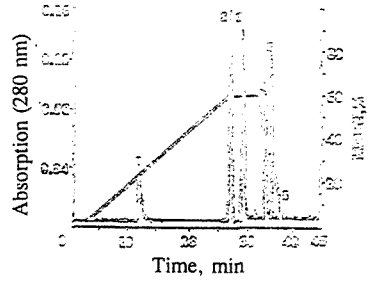


Fig. 2

Fig. 1. Gel chromatography of the whole venom of the green toad *B. viridis*.

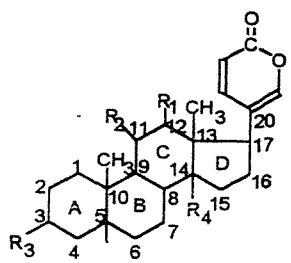
Fig. 2. Separation of the toxic fraction in HPLC.

The high mechanical strength and resolving power of this resin enabled us to isolate in the pure form two toxic compounds (fractions 2 and 3), which were called IT-1 and IT-2. The yields of these components were, respectively, 0.1 and 0.2% of the initial dry weight of the whole venom.

The administration of IT-1 in a dose of 5 μg to an individual cockroach led to pronounced paralysis in 5-10 min, and the action continued for 5-7 h. The administration of IT-2 in an equivalent dose caused paralysis (in 2-3 min) and a subsequent lethal outcome.

In order to determine the chemical structures of the compounds obtained, we developed a preparative variant of their isolation. In view of the fact that the main toxic components of toad venoms are bufotoxins and bufodienolides, which are extracted well by ethyl alcohol [15], for the primary fractionation of the whole venom in the preparative variant we included a stage of extracting comparatively large amounts of the venom with ethanol. The subsequent separation of the extract by reversed-phase chromatography gave a pattern identical with that obtained before under analogous chromatographic conditions (see Fig. 2). To accumulate the toxic components we used reversed-phase chromatography on a LiChrosorb RP-8 10 μm semipreparative column (10 mm $\times$ 25 cm).

^{1}H NMR spectroscopy gave a PMR spectrum of IT-2 (200 MHz, CD_3OD) characterized by the presence of the singlets of two methyl groups (0.93 and 1.21 ppm), a complex multiproton signal in the "methylene hump" region (1.0-2.2 ppm), and three one-proton signals in the weak-field region at 6.31 (J = 10.0 Hz), 7.53 (J = 2.5 Hz) and 7.92 ppm (J = 10.0 and 2.5 Hz). The parameters of the spectrum permitted the assumption of the presence in the IT-2 molecule of a steroid skeleton with angular methyl groups at C_{18} and C_{19} and also a coumalin ring. A comparison with the spectra of other compounds [16-19] permitted the conclusion that IT-2 belonged to the bufadienolide series:



The definitive positions of the functional groups and the spatial structrue of the IT-2 molecule followed from the results of x-ray structural analysis.

These showed that rings *A*, *B*, and *C* have the $_6C^9$-, $_1C^4$-, and $_8C^{12}$-chair conformations, respectively. The ring linkages are *cis-A/B* and *-C/D*, and *trans-B/C*, which is characteristic for the bufadienolide skeleton. In ring *A* of the molecule the O(3)—H hydroxyl occupies the axial position, and in ring *C* the O(11)—H and O(14)—H groups are present in the equatorial positions. The Cambridge Crystallographic Data Center includes a substance with such a structure — arenobufagin, isolated from Ch'an Su, a Chinese preparation of toad venom, in the form of a diacetyl derivative [20]. The structure of arenobufagin was established after the hydrolysis of the acetyl groups of diacetylarenobufagin [21]. Arenobufagin was first isolated in the individual form from the South-American toad *Bufo arenarum*, which explains its name [22].

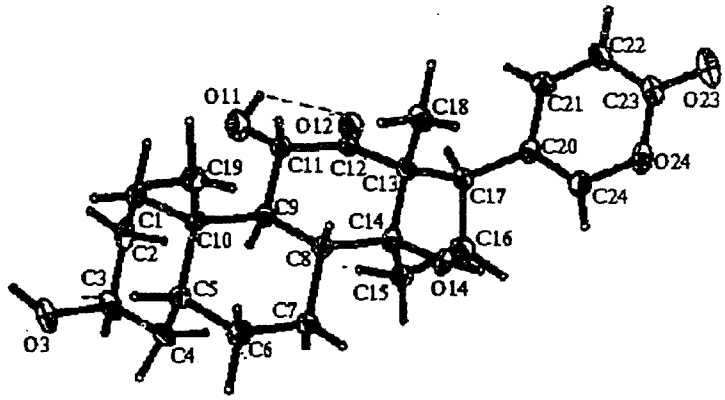


Fig. 3. Conformation of the arenobufagin molecule (polymorph B).

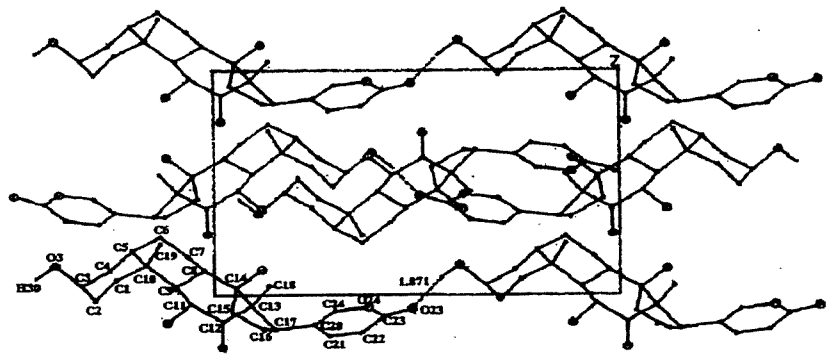


Fig. 4. Crystal structure of arenobufagin (polymorph B), projection on *ac*.

The crystallographic parameters of the IT-2 that we had isolated differed from the analogous parameters of arenobufagin, which shows the existence of polymorphism in this steroid (the arenobufagenin crystals were obtained from solutions in methanol—ethyl acetate (1:1), and single crystals of IT-2 were grown from solutions in ethyl alcohol). In future, the modification included in the Data Centre will be called polymorph A, and the crystal variety the structure of which we have deciphered polymorph B.

In polymorph A, the five-membered ring *D* has a conformation intermediate between an E^{14} envelope and a ${}_{13}T^{14}$ half-chair. In polymorph B, this ring acquires the conformation of a distorted ${}_{14}T^{15}$ half-chair (Fig. 3). In the two crystal modifications of arenobufagin, an appreciable difference is also observed in the position of the δ-lactone ring relative to the steroid backbone of the molecule. Calculations have shown [23] that for the free rotation of the lactone ring about the ordinary C(17)−C(20) bond the molcule must overcome a fairly high energy barrier. Nevertheless, the position of this ring differs in the two polymorphs (the C(13)C(17)C(20)C(21) torsional angles are −89.1 and 81.0° in polymorphs A and B, respectively). In polymorph B the position of the lactone ring is the same as in bufalin and its precursors [24].

The bond lengths and the valence angles in the molecule have the usual values for such compounds [24] and scarcely differ from the analogous values in polymorph A [21].

Of the three hydroxy and two carbonyl groups capable of participating in intermolecular hydrogen bonds, only the O(3)-H and C=O(23) groups realize their H-bonding possibilities: the O(3)-H group of one arenobufagin molecule is H-bound to the carbonyl oxygen atom of the δ-lactone ring of a neighboring molecule obtained from the first symmetric transformation (x, y, z + 1), which leads to the formation of infinite chains along the z axis (Fig. 4). The length and angle of this bond amount to 2.93 Å and 156.2° (H···O 1.87 Å) respectively. However, the other carbonyl oxygen atom and the O(11)-H group take part in an intramolecular H-bond O(11)-H···O(12) with a length of 2.54 Å and an angle of 107.8° (H···O(12) 2.07 Å).

Polymorph A is characterized by a richer system of intermolecular H-bonds, since three H-bonds with the participation of the O(3)-H, O(11)-H, O(14)-H, and C=O(23) groups form a three-dimensional lattice. In the polymorph B structure of arenobufagin, only the O(14) oxygen atom is not involved in H-bonds.

TABLE 1. Coordinates of the Atoms ($\times 10^4$; for H atoms, $\times 10^3$) and Equivalent Temperature Factors U_{eq} ($Å^2 \times 10^3$) in the Structure of Arenobufagin (polymorph B)

Atom	x	y	z	U eq
C(1)	702(7)	11377(6)	7431(4)	49(2)
C(2)	−194(7)	10693(7)	7936(4)	60(3)
C(3)	459(7)	9697(7)	8275(4)	60(3)
C(4)	1116(8)	9108(6)	7582(4)	54(3)
C(5)	2006(7)	9801(5)	7039(4)	46(2)
C(6)	2631(7)	9130(6)	6337(5)	53(3)
C(7)	1735(7)	8821(5)	5640(4)	49(2)
C(8)	1105(6)	9835(5)	5258(4)	36(2)
C(9)	393(7)	10457(5)	5947(4)	42(2)
C(10)	1353(7)	10797(5)	6673(4)	39(2)
C(11)	−364(7)	11391(6)	5582(4)	52(2)
C(12)	−1133(7)	11016(5)	4810(4)	44(2)
C(13)	−453(7)	10473(5)	4095(4)	40(2)
C(14)	306(6)	9519(5)	4468(4)	39(2)
C(15)	−709(7)	8700(6)	4596(4)	49(2)
C(16)	−1495(8)	8745(6)	3788(5)	58(3)
C(17)	−1486(7)	9919(6)	3509(4)	45(2)
C(18)	389(7)	11316(6)	3651(4)	53(2)
C(19)	2322(7)	11578(6)	6338(5)	54(3)
C(20)	−1292(6)	10110(6)	2554(4)	44(2)
C(21)	−1785(8)	11066(6)	2166(5)	60(3)
C(22)	−1602(8)	11279(7)	1347(5)	66(3)
C(23)	−906(8)	10591(7)	831(5)	62(3)
C(24)	−687(7)	9447(6)	2040(5)	56(3)
O(3)	1359(5)	9977(5)	8918(3)	68(2)
O(11)	−1183(6)	11850(5)	6174(3)	94(3)
O(12)	−2255(5)	11209(5)	4828(3)	72(2)
O(14)	1140(4)	9119(4)	3833(3)	50(2)
O(23)	−569(6)	10742(6)	99(3)	92(3)
O(24)	−507(5)	9632(5)	1189(3)	69(2)
H(11)	24(7)	1211(6)	716(4)	11(3)
H(12)	135(7)	1160(6)	781(4)	6(2)
H(21)	−98(6)	1030(5)	762(4)	6(2)
H(22)	−46(6)	1120(5)	834(4)	6(2)
H(31)	−21(6)	917(5)	864(4)	8(2)
H(41)	167(6)	865(5)	795(4)	6(2)
H(42)	43(7)	883(6)	720(4)	9(2)
H(51)	277(5)	1013(5)	741(3)	4(2)
H(61)	334(7)	964(6)	614(4)	12(3)
H(62)	306(7)	841(6)	660(4)	7(2)
H(71)	97(6)	832(5)	587(4)	6(2)
H(72)	195(7)	838(6)	527(5)	9(3)
H(81)	186(5)	1026(4)	499(3)	3(1)
H(91)	−24(7)	996(6)	637(4)	7(2)
H(111)	33(6)	1189(5)	541(4)	6(2)
H(151)	−32(5)	804(4)	471(3)	2(2)
H(152)	−118(5)	890(4)	519(3)	4(2)
H(161)	−226(7)	840(6)	384(4)	6(2)
H(162)	−99(6)	820(5)	335(4)	7(2)
H(171)	−242(6)	1031(5)	366(4)	4(2)
H(181)	115(6)	1150(6)	407(4)	6(2)
H(182)	−3(6)	1206(6)	335(4)	6(2)
H(183)	99(6)	1112(5)	323(4)	5(2)
H(191)	207(7)	1242(6)	621(5)	9(2)
H(192)	273(5)	1134(5)	585(3)	4(2)
H(193)	289(7)	1175(5)	688(4)	7(2)
H(211)	−236(8)	1144(6)	257(5)	11(3)
H(221)	−182(7)	1196(6)	109(5)	8(2)
H(241)	−36(7)	880(6)	212(4)	10(3)
H(3)o	78(7)	1046(6)	937(5)	9(3)
H(11)o	−186(7)	1218(6)	586(5)	11(3)
H(14)o	86(7)	878(6)	346(5)	11(3)

Thus, from the venom of the Central Asian green toad *Bufo viridis* we have isolated arenobufagin differing from that known previously not only in crystal structure but also in the conformation of the molecule. In contrast to the known polymorph A, in which intermolecular H-bonds unite the molecules into a three-dimensional skeleton, the new polymorph B is characterized by the presence of a one-dimensional H-associate of arenobufagin molecules.

The PMR spectrum of IT-1, like that of IT-2, contains signals characteristic for bufadienolides, with slight variations in their parameters. The most appreciable is the absence of a sharp doublet at 4.41 ppm (H-11) and a doublet of doublets for H-17 at 4.15 ppm. These facts and the mass and composition of the molecular ion, 402 ($C_{24}H_{34}O_3$), permitted the assumption that there is no keto group at C_{12} in the IT-1 molecule. The definitive structure of IT-1 was again determined by x-ray structural analysis.

From solutions of IT-1 in ethyl alcohol were grown single crystals of two forms, both belonging to the space group $P2_12_12_1$. Acicular crystals had the following crystallographic parameters: a = 7.892(1); b = 14.753(1); c = 17.883(2) Å; V = 2076.9(1.4) Å^3, Z = 4, d_{calc} = 1.23 g/cm^3, while for crystals with a rhombic habitus: a = 10.166(3); b = 13.360(5); c = 17.523(18) Å; V = 2390(2) Å^3. Analysis of the Cambridge Crystallographic Data Center showed that it contained a substance having parameters identical with those of the acicular crystals, this being the known gamabufotalin. On this basis, it may be assumed that the rhombic crystals were a solvate of gamabufotalin with ethanol (most probable ratio 1:1 at a calculated density d_{calc} of 1.19 g/cm^3) since the phenomenon of inclusion (the formation of inclusion compounds with solvent molecules) is very characteristic for steroids and is encountered fairly frequently [25].

Gamabufotalin was first isolated by Kotake et al. [26] from the skin of the Japanese toad *Bufo vulgaris formosus*, and later from the preparation Ch'an Su [17]. We are the first to have isolated this compound from the venom of the green toad *Bufo viridis*.

Gamabufotalin and arenobufagin made up 80% of the total bufadienolide fraction of the *B. viridis* venom. The residual 20% contained another four, unidentified, bufadienolides (see Fig. 2). K. Shimada et al. have previously isolated from the venom of the green toad *B. viridis* Laur such bufadienolides as marinobufagin, hellebrigenin, telocinobufagin, and bufotalinin [27]. It is not excluded that these unidentified compounds are also present in the Central Asian subspecies *B. viridis*.

In view of the fact that a cardiotonic action is characteristic for this series of compounds [13, 28, 29], we have conducted a preliminary investigation of the biological activity of this total mixture of bufadienolides, which has been called Bakagin. In *in vitro* experiments, the perfusion of isolated papillary muscles of the left ventricle of the cat with a nutrient solution containing Bakagin in a concentration of 5×10^{-8}-5×10^{-6} g/ml was accompanied by a dose-dependent increase in the amplitude of the isometric contraction of these muscles. The greatest increase in the amplitude of the contractions was observed for concentrations of 2.5-5 $\times 10^{-7}$ g/ml. The perfusion of Bakagin in such concentrations had practically no influence on the frequency of the contractions of the right auricle. An increase in the concentration to 1×10^{-6} g/ml was accompanied by a 20-30% increase in the frequency of the contractions of an isolated auricle. The results of a determination of the selectivity of the positive inotropic action of Bakagin as in [30] showed that it is of interest as a potential cardiotonic compound.

EXPERIMENTAL

The whole venom of the green toad *Bufo viridis* was obtained from the Central Asian Zonal Combine. It consisted of a pale yellow vitreous mass sparingly soluble in aqueous salt solutions. The venom preparation was extracted with 0.05 M NH_4HCO_3 buffer containing 10% of ethyl alcohol. The extract was freed from insoluble components by centrifugation and was freeze-dried. Th dry venom was stored at −4°C for several months before use.

The paralytic activities of the whole venom and of the fractions obtained were determined on cockroaches, *Pereplaneta americana*, weighing about 500 mg. Their introperitoneal injection in a dose of 100 μg caused pronounced paralysis in 100% of the insects. A lethal outcome required considerably higher doses.

The gel permeation chromatography of the whole venom of the green toad *Bufo viridis* was carried out on a 2.6 × 100 cm column of Sephadex LH-20 in 0.05 M ammonium bicarbonate buffer containing 60% of ethanol with pH 8.2. Rate of elution 45 ml/h.

Detection was conducted at 280 nm on an ultraviolet detector of the Uvicord S type (LKB, Sweden). Fractions were collected on an Ultrorac collector (LKB, Sweden).

The hydrophobic chromatography of fraction 6 was conducted on a 1 × 10 cm column of Polikhrom P-1. Elution was begun with deionized water, followed by a stepwise concentration gradient of ethanol: 10, 20, 30, 40, 50, and 60%. The

toxic fraction was isolated by reversed-phase chromatography in an HPLC instrument (Du Pont, USA) using a 4.6 × 25 cm Octadecyl Si-100 column. Buffer A: 0.1% TFAA; pH 2.00; buffer B: acetonitrile. Gradient of acetonitrile, B: 5%, 3 min; 5-60%, 25 min; 60%, 5 min; 60-5%, 5 min. Rate of elution 1 ml/min.

In the preparative variant: 10 mm × 25 cm LiChrosorb RP-8 10 μm column. Buffer A: deionized water; buffer B: acetonitrile. Gradient of acetonitrile, B: 5%, 5 min; 5-30%, 15 min; 30-60%, 20 min; 5%, 5 min. Rate of elution 2.5 ml/min.

^{1}H NMR spectra were recorded on a XL-200 spectroemter (Varian, USA) with a working frequency of 200 MHz in the regime of pulse accumulation followed by Fourier transformation of the free induction signal. The interval between pulses, D1, was 1 sec, the time of recording the free induction signal, AT, 1.5 sec, and the number of accumulations, NT, 5000.

The samples used were 1% solution of the substances under investigation in methanol-d_4, with TMS as internal standard.

Mass spectra were taken on a MKh-1310 instrument (Leningrad), using a CPV-5 system for direct injection, at 170°C and an ionizing voltage of 50 V.

Gamabufotalin. Mass spectrum (*m/z*): 402, 341, 191. PMR spectrum (200 MHz, CD_3OD): 0.76 (3H, s, CH_3-18), 1.10 (3H, s, CH_3-19), 4.05 (1H, br.s, $W_{1/2}$ = 8, H−3), 6.27 (1H, d, J = 10.0 Hz, H−23), 7.44 (1H, d, J = 2.5 Hz, H−21), 7.96 (1H, dd, J = 10.0 Hz and 2.5 Hz, H−22).

Arenobufagin. Mass spectrum (*m/z*): 416, 370, 355, 204, 188, 175. PMR spectrum (200 MHz, CD_3OD): 0.93 (3H, s, CH_3-18), 1.21 (3H, s, CH_3-19), 4.06 (1H, br.s, $W_{1/2}$ = 8, H−3), 4.15 (1H, dd, J = 10.0 Hz and 7.2 Hz, H−17), 4.41 (1H, d, J = 10.9 Hz, H−11), 6.31 (1H, d, J = 10.0 Hz, H−23), 7.53 (1H, d, J = 2.5 Hz, H−21), 7.92 (1H, dd, J = 10.0 Hz and 2.5 Hz, H−22).

Single crystals suitable for x-ray structural analysis were obtained from solutions in ethanol by slow evaporation of the solvent at room temperature. The parameters of the unit cell of the crystal were found and refined on a Syntex-$P2_1$ diffractometer using 15 reflections ($20° < 2\theta < 40°$). Crystallographic characteristics of arenobufagin, $C_{24}H_{32}O_6$: a = 10.594(3), b = 12.518(2), c = 15.776(3) Å, V = 2092.1(1.3) Å^3, d_{calc} = 1.22 g/cm^3, Z = 4. Space group $P2_12_12_1$. Experimental results (integral intensities of the reflections) were collected on the diffractometer mentioned above by the $\theta/2\theta$ scanning method using CuK_α radiation monochromatized by reflection from a graphite crystal. A total of 1927 reflections were recorded, of which 1365 remained after rejection according to the criterion $F < 4\sigma$ (F). The group of reflections was corrected for polarization and Lorentz factors.

The structure was determined by the direct method with the aid of the SHELX-86 program on an AT-386 PC [31] and was refined by the MLS using programs of the SHELX-76 package [32]. The hydrogen atoms were found from Fourier difference syntheses and their positional and temperature parameters were refined.

The final R-factor was 0.055. The coordinates of the atoms corresponding to this value of the R-factor are given in Table 1.

The influence of Bakagin on the parameters of the isometric contraction of preparations of the cardic muscles of guinea pigs and cats was studied by the method of Chekman and Tkachuk [30].

The mass spectra of the compounds investigated were taken by Ya. V. Rashkes (dec.) of Institute of the Chemistry of Plant Substances, Academy of Sciences of the Republic of Uzbekistan.

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