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(Hydroxymethyl)acylfulvene: An Illudin Derivative with Superior Antitumor Properties

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Reaction of the fungal sesquiterpene illudin S with excess paraformaldehyde in dilute H_2SO_4 gives (hydroxymethyl)acylfulvene. The primary allylic hydroxyl thus formed can undergo very facile replacement by a variety of nucleophiles. (Hydroxymethyl)acylfulvene (MGI.114) was more toxic than a precursor, acylfulvene, but less toxic than the parent compound illudin S to HL 60 cells.

Natural products from plants and microorganisms have proven to be a major source of important anticancer agents and lead compounds for cancer chemotherapy.[1] Mushrooms of the class Basidiomycetes are an exception. Although they occur widely and some are well known to contain a variety of highly poisonous substances,[2] only *Omphalotus illudens* (jack o'lantern mushroom) is known to produce promising anticancer compounds. These are the sesquiterpenes illudin S (**1**) and illudin M (**2**).[3] The illudins are extremely cytotoxic compounds but have a low therapeutic index particularly in solid-tumor systems.[4] However, modification of their structures has yielded several analogues that possess a greatly improved therapeutic index. Remarkable efficacy has been observed in tests on mouse xenografts of leukemias and various solid tumors. Previous reports from our laboratories have described investigations on first- and second-generation analogues, dehydroilludin M (**3**)[4−7] and acylfulvene (**4**).[8,9] In this report the synthesis and properties of a third-generation analogue designated 6-(hydroxymethyl)acylfulvene (HMAF, **5**) are disclosed.

There is evidence that illudins and certain derivatives can behave as alkylating agents. Thus, they react spontaneously with sulfur nucleophiles, such as glutathione or cysteine, at an optimum pH of about 6.[10] At physiological pH, however, they do not react with oxygen or nitrogen nucleophiles. Illudins can also undergo enzymatic reduction with NADPH as cofactor.[11] This reduction, like the reaction with thiols, involves Michael-type addition of nucleophile to the α,β-unsaturated carbonyl group giving a cyclohexadiene intermediate that is rapidly converted to a stable aromatic structure (Scheme 1). The cyclopropane is opened concurrently by reaction with any neighboring nucleophile.

Reaction of illudins with vital thiols in the cell would partly explain the toxicity of the compounds. Dehydroilludin M (**3**) and acylfulvene (**4**) have been found to be less reactive to thiols and correspondingly less toxic to human leukemia (HL-60) cells. Their improved selective cytotoxicity is consistent with lowered reactivity to thiols.

We hypothesize that the compounds **3** and **4** can also be activated by reduction with NADPH. Although bioreductive activation has not been demonstrated experimentally, it should be noted that these compounds are readily reduced to aromatic products, in the same way as illudin S, on catalytic hydrogenation or by treatment with zinc and dilute acid.[3]

As reported earlier, acylfulvene (**4**) is formed by reverse Prins reaction when illudin S is dissolved in dilute H_2SO_4.[9] The other major product is the bisacylfulvene (**6**). The intermediate in the conversion of the former to the latter compound appears to be (hydroxymethyl)acylfulvene (HMAF, **5**) as indicated in Scheme 2; this derivative was detected in trace amounts. We investigated ways for obtaining more of the compound because we expected it to be more H_2O-soluble than acylfulvene and therefore more suitable for biological tests. Also the allylic hydroxyl appeared to be easily displaced, albeit under strongly acidic conditions. Thus, an additional site is present in the molecule at which nucleophilic attack can occur.

Moderate yields of HMAF can be obtained by reacting illudin S with a large excess of paraformaldehyde in Me_2CO-H_2O solution containing H_2SO_4 (1 N) at room temperature for 72 h. Isolation of HMAF by chromatography gives a 40% yield of crystalline material. HMAF can also be obtained by reaction of acylfulvene with paraformaldehyde in dilute H_2SO_4. The structure has been confirmed by X-ray crystallographic analysis (Figure 1).

Many derivatives of HMAF have been prepared. Acetylation of the primary hydroxyl group occurs on treatment of HMAF with $Ac_2O-NaOAc$ at room temperature. Other derivatives are formed by remarkably facile displacement of the primary hydroxyl group. Thus, HMAF reacts with phenol in dry CH_2Cl_2 with BF_3-etherate at −78 °C to give (*p*-hydroxybenzyl)-acylfulvene (**7**) in quantitative yield. The structure was confirmed by X-ray crystallographic analysis (Figure 2). The (*p*-methoxybenzyl)acylfulvene (**8**) was prepared in the same way from anisole.

HMAF reacts very slowly with thiols at pH 6 compared to illudin S.[9] Products are formed indicating that the primary hydroxyl is replaced by thiol. The main product from reaction of **5** with *p*-thiocresol was obtained more readily by carrying out the reaction in dilute H_2SO_4; it has been identified as the derivative **9**.

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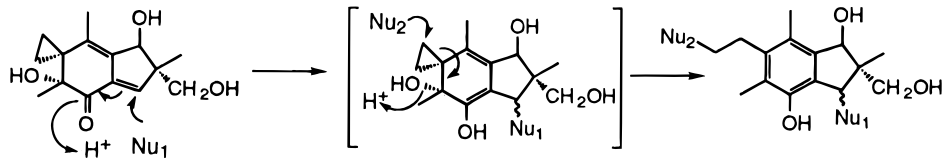
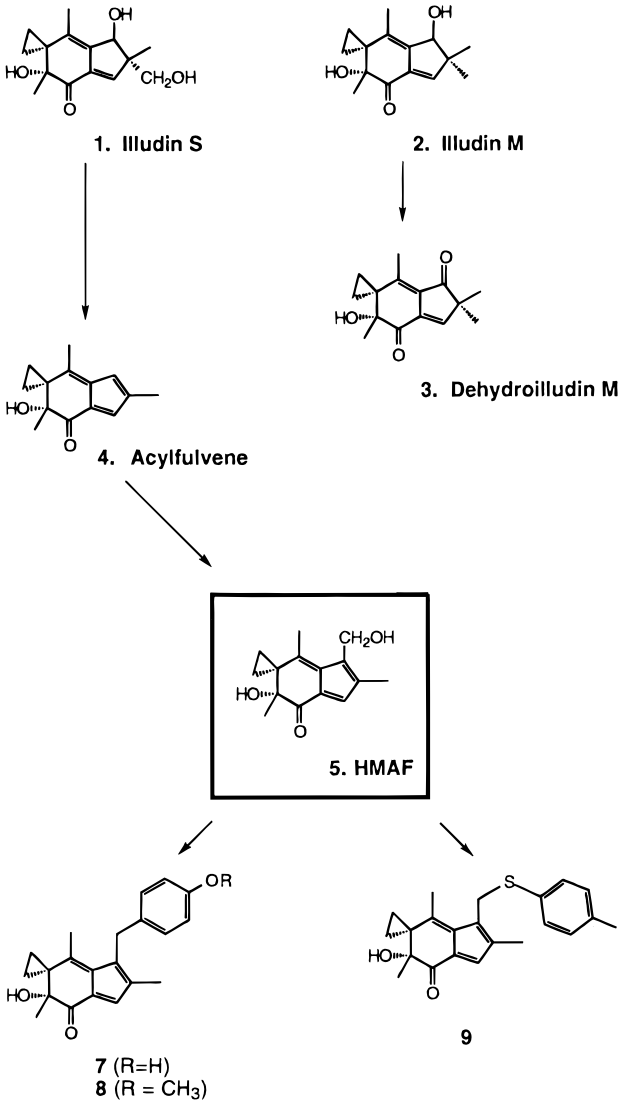
Scheme 1



Nu_1 = GS^- or NADPH Nu_2 = H_2O, DNA, protein



Like the parent illudin S, dehydroilludin M, and acylfulvene, HMAF was found to exhibit selective toxicity to certain cells on short exposure.[12] Thus it was more toxic to human leukemia (HL 60) cells and metastatic lung carcinoma (MV 522) cells in a 2-h exposure but relatively nontoxic (less than one tenth) to leukemia/lymphoma 8392 B cells. HMAF was toxic to all three cell lines after 48-h exposure. This selective toxicity is associated with active transport of these compounds into sensitive cells.[4] Comparison of toxicity of HMAF with that of acylfulvene and illudin S when tested in HL60 cells is given in Table 1.

Other important properties of illudin analogues should be noted. They are cytotoxic in vitro to a variety of multidrug resistant (mdr) cell lines.[7] Also, repair of illudin-induced DNA damage requires the action of ERCC2 and ERCC3 DNA repair enzymes (also known as DNA helicases of the TFIIH complex), before repair of damaged DNA can proceed.[13] This contrasts with DNA damage induced by other DNA-reactive chemotherapeutic agents such as mitomycin C. It has recently been found that cytotoxic effects of HMAF in human CEM leukemia cells involve inhibition of DNA synthesis, blockage in the S phase, and a potent induction of apoptotic death.[14]

In in vivo studies the first-generation analogue, dehydroilludin M, showed better efficacy against the MV 522 lung carcinoma xenografts (established in 4-wk-old athymic Balb/c nu/nu mice) than nine known anticancer agents including cisplatin, cytoxan, and paclitaxel.[6,7] Its efficacy was comparable to that of mitomycin C. Illudin S at a dose of 2.5 mg/Kg (ip) three times per week was extremely toxic, and all the animals died after three doses. No increase in life span was observed with sublethal doses (0.25 mg/Kg or 0.5 mg/Kg three times per week).[8] The efficacy of the second-generation analogue, acylfulvene, exceeded that of dehydroilludin M and mitomycin C.[8] Although these compounds prolonged life span in the MV 522 model, they did not induce regression of the primary tumor implants.

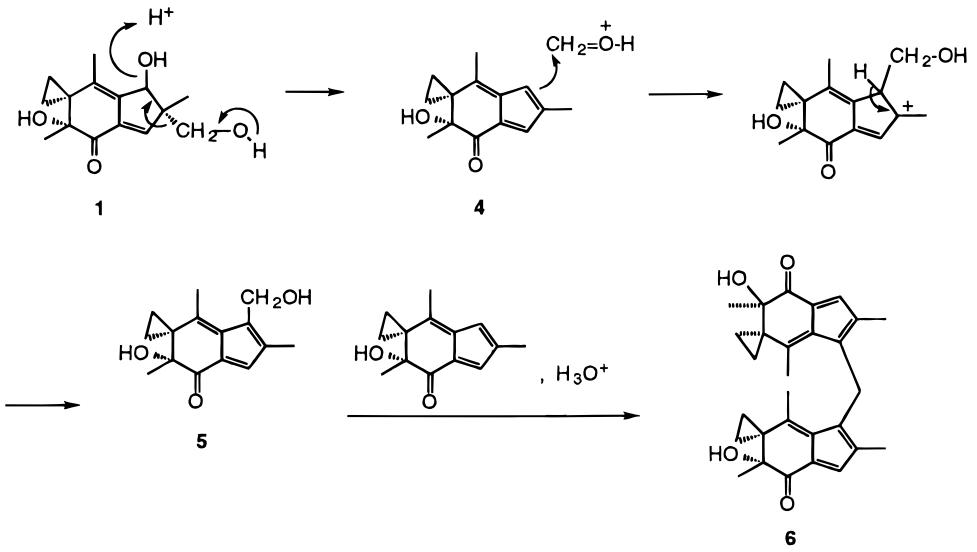
However, in tests with MV 522 xenografts, HMAF caused complete tumor regression in all animals at the maximum tolerated dose of 10 mg/Kg (iv) three times per week for 3 weeks. This resulted in increased life span of more than 150%. In comparison, treatment of mice with paclitaxel, doxorubicin, or cisplatin failed to prolong the life span of tumor-bearing animals. Mitomycin C caused a mild increase in median life span. Details of these experiments are reported elsewhere.[12] HMAF has also been found to exhibit outstanding activity against breast, colon, and skin cancers.[15] The compound entered Phase 1 clinical trials in December 1995.

Experimental Section

General Experimental Procedures. ^1H- and ^{13}C-NMR spectra were obtained at 300 or 500 MHz and 75 or 125 MHz, respectively. Spectra were recorded of solutions in $CDCl_3$ with Me_4Si as internal standard. HRMS were determined at the University of Minnesota Mass Spectrometry Service Laboratory. Column chromatography was carried out with Si gel (Davisil 100−200 mesh and 230−425 mesh, Fisher Scientific). Analytical TLC was carried out on Whatman 4410 222 Si gel plates. Reactions were routinely monitored by TLC. Single-crystal X-ray diffraction measurements were made with a Siemens P3/V diffractometer using Wyckoff scans, $\lambda = 0.710\ 73$ Å from HOP graphite monochromator, and SHELXTL PLUS for structure solution and refinement.

(Hydroxymethyl)acylfulvene (5). Paraformaldehyde (5 g) was added to a solution of H_2O (250 mL), Me_2CO (400 mL), and dilute H_2SO_4 (2 M, 250 mL), and

Scheme 2



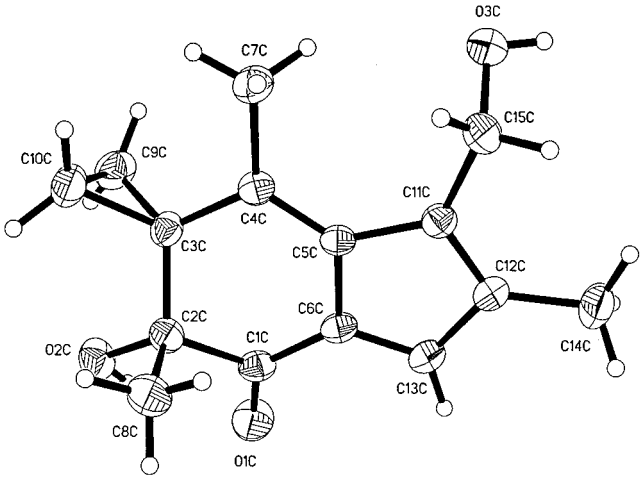


Figure 1. ORTEP view of X-ray molecular structure of HMAF.

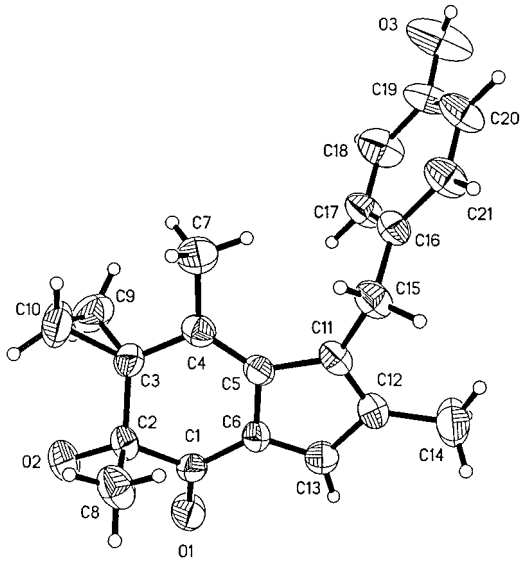


Figure 2. ORTEP view of X-ray molecular structure of compound **7**.

the mixture was heated to dissolve all the solid. To the cooled (0 °C) solution illudin S[3] (1 g, 3.78 mmol) was added, and the resulting solution was stirred and allowed to warm to room temperature. After 72 h, the

Table 1. IC_{50} Values for Illudin Analogues When Tested in HL-60 Cells[a]

compounds	nM
illudin S or M (**1**, **2**)	3 ± 1 (0.8 ng/mL)
dehydroilludin M (**3**)	296 ± 8 (73 ng/mL)
acylfulvene (**4**)	415 ± 31 (90 ng/mL)
HMAF (**5**)	73 ± 8 (18 ng/mL)
compound **7**	380 ± 40 (122 ng/mL)
compound **8**	1050 ± 100 (353 ng/mL)
compound **9** (not tested because of insolubility)	

[a] For cytotoxicity tests the compounds were dissolved in DMSO (1 mg/mL stock solution) and the solutions diluted in 20% DMSO/phosphate-buffered saline just prior to addition to cultures of HL 60 cells. Control cells received equal amounts of the DMSO/phosphate-buffered saline. After incubation for 48 h the cells were washed, trypan blue was added, and the cells were counted. These values correlate closely with those determined by colony forming assay.[8]

orange-yellow solution was extracted with EtOAc (3 × 200 mL) and the combined extracts washed with saturated $NaHCO_3$ (100 mL), followed by saturated brine. The extracts were concentrated under reduced pressure (~40 °C) to ca. 150 mL, then dried over $MgSO_4$. Removal of solvent and chromatography of the residue on Si gel with EtOAc−hexane afforded acylfulvene (136 mg), bisacylfulvene (53 mg), HMAF (353 mg, 38%), and unchanged illudin S (139 mg). There were small amounts of more polar products resulting from opening of the cyclopropane ring with aromatization. HMAF crystallized on adding a little ether to the orange gum; mp 127−129 °C; $[\alpha]^{25}_D = -639°$, (*c* 0.096 EtOH); ^{1}H NMR δ 0.73 (ddd, 1H), 1.09 (ddd, 1H), 1.38 (ddd, 1H), 1.38 (s, 3H), 1.48 (ddd, 1H), 2.15 (s, 3H), 2.19 (s, 3H), 3.91 (s, 1H), 4.66 (br s, 2H), 7.10 (s, 1H); ^{13}C NMR δ 9.2, 12.7, 14.0, 16.0, 27.4, 37.4, 55.7, 76.1, 126.3, 132.8, 134.9, 138.5, 141.7, 159.9, 197.9; MS *m*/*z* 246 (M^+), 228, 218, 186, 185; UV λ_{max} (EtOH) 235 nm (ϵ 15 100), 330 (ϵ 9200) with tailing to 480 nm; IR (KBr) ν_{max} 3470, 3430, 1633, 1495 cm^{-1}. HMAF was also obtained in similar yield from illudin S by first converting it to acylfulvene in dilute H_2SO_4 and then reacting acylfulvene with excess paraformaldehyde in dilute H_2SO_4−Me_2CO solution.

(*p*-Hydroxybenzyl)acylfulvene (7). Phenol (40 mg, 0.4 mmol) was added to a solution of HMAF (70 mg,

0.28 mmol) in dry CH_2Cl_2 (25 mL). The reaction mixture was cooled to −78 °C and BF_3−etherate (0.3 mL, 2.7 mmol) was added dropwise. The reaction mixture was stirred at that temperature for 1 h, then H_2O was added. The organic layer was separated and first washed with $NaHCO_3$ solution and then with brine and dried over $MgSO_4$. The solvent was removed leaving a red residue that was purified by chromatography on Si gel with hexane−EtOAc to yield (*p*-hydroxybenzyl)acylfulvene (**7**) as red crystals (90 mg, 98%), mp 143−144 °C; ^{1}H NMR δ 0.59−1.43 (m, 4H), 1.36 (s, 3 H), 1.76 (s, 3H), 2.07 (s, 3H), 3.95 (s, 1H), 3.93 (d, J = 16.8 Hz, 1H), 4.01 (d, J = 16.8 Hz, 1H), 4.83 (s, 1H), 6.74 (d, J = 8.4 Hz, 2H), 6.91 (d, J = 8.4 Hz, 2H), 7.22 (s, 1H). MS m/z 322 (M^+), 294 ($M^+ - 2\ CH_2$), 279 ($M^+ - 2\ CH_2 - CH_3$), 251 ($M^+ - 2\ CH_2 - CH_3 - CO$), 215, 107. UV (EtOH) λ_{max} 228 nm (ϵ 12 000) with inflections at 243 and 262 nm, 325 (ϵ 7100), 410 nm (ϵ 2600).

(*p*-Methoxybenzyl)acylfulvene (8). Anisole (0.04 mL, 0.37 mmol) was added to a solution of HMAF (10 mg, 0.04 mmol) in dry CH_2Cl_2 (5 mL). The mixture was cooled to −78 °C and BF_3−etherate (0.04 mL, 0.36 mmol) was added dropwise. The reaction mixture was stirred at that temperature for 1 h, then H_2O was added. The organic layer was separated and washed with H_2O, saturated $NaHCO_3$ solution, and brine and dried over $MgSO_4$. Concentration of the solution gave a red residue that was dried *in vacuo*, to yield (*p*-methoxybenzyl)acylfulvene (**8**) as a red gum in quantitative yield (14 mg). ^{1}H NMR δ 0.59−1.40 (m, 4H), 1.36 (s, 3H), 1.76 (s, 3H), 2.07 (s, 3H), 3.78 (s, 3H), 3.95 (s, 1H), 3.96 (d, J = 12, Hz, 1H), 4.02 (d, J = 12 Hz, 1H), 6.81 (d, J = 8.5 Hz, 2H), 6.96 (d, J = 8.5 Hz, 2H), 7.22 (s, 1H); MS m/z 336 (M^+), 308 ($M^+ - CH_2CH_2$), 215, 121. UV (EtOH) λ_{max} 410 nm (ϵ 2700), 325 nm (ϵ 7000), 267 nm (ϵ 10 000), 245 nm (inflection), 226 nm (ϵ 19 000).

p-Thiocresol Derivative of HMAF (9). HMAF (125 mg, 0.51 mmol) was added to a mixture of diluted H_2SO_4 (10 mL, 1 M) and Me_2CO (10 mL). To the resulting solution *p*-thiocresol (59 mg, 0.48 mmol) was added, and the mixture was stirred for 5 h. It was extracted with EtOAc and the extract washed with saturated $NaHCO_3$, then brine. After drying ($MgSO_4$), the solution was concentrated and chromatographed with EtOAc−hexane, yielding the derivative **9** as an orange oil (127 mg, 76%); ^{1}H NMR δ 0.78 (m, 1H), 1.07 (m, 1H), 1.32 (m, 1H), 1.38 (s, 3H), 1.50 (m, 1H), 1.82 (s, 3H), 2.14 (s, 3H), 2.31 (s, 3H), 3.95 (s, 1H), 4.04 (br s, 2 H), 7.05 (s, 1H), 7.07 (d, J = 8.0 Hz, 2H), 7.23 (d, J = 8.0 Hz, 2H); ^{13}C NMR δ 9.4, 12.6, 14.1, 16.2, 21.0, 27.6, 33.1, 37.6, 76.0, 126.1, 129.5, 129.8, 131.3, 132.2, 135.0, 137.3, 138.4, 142.3, 159.2, 193.3; HRMS m/z 352.1499 (M^+, calcd for $C_{22}H_{24}SO_2$, 352.1498), 229.1232 ($M^+ - C_7H_7S$, base peak); UV (EtOH) λ_{max} 333 nm (ϵ 6600); IR (KBr) ν_{max} 3460, 1663, 1596 cm^{-1}.

X-ray crystal structure analysis of 5, $C_{15}H_{18}O_3$, at 199 K: *P*1, a = 9.511 (3) Å, b = 9.556 (2) Å, c = 12.447 (4) Å, α = 98.03 (2)°, β = 103.09 (2)°, γ = 113.99 (2)°, Z = 3, calcd density = 1.262 g/cm^3, crystal size 0.3 × 0.6 × 0.8 mm, 10°/min 0.6° Ω scan range, 4547 observed ($F > 4\sigma F$) from 3 to 55° 2θ, R = 3.5%, residual electron density 0.31 and −0.22 e/A^3, 8.8 data/variable.

X-ray crystal structure analysis of 7, $C_{21}H_{22}O_3$, at 298 K: *P*2(1)2(1)2(1), a = 6.607 (3) Å, b = 10.435 (3) Å, c = 25.705(9) Å, Z = 4, calcd density = 1.208 g/cm^3, crystal size 0.4 × 0.4 × 0.35 mm, 5°/min 0.6° Ω scan range, 1617 observed ($F > 4\sigma F$) from 3 to 50° 2θ, R = 4.3%, residual electron density 0.19 and −0.22 e/A^3, 7.4 data/variable.

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Supporting Information Available: X-ray crystallographic data for compounds **5** and **7** (25 pages); a table of observed and calculated structure factors (5 pages). Ordering information is given on any current masthead page. Atomic coordinates including tables of bond distances and angles from the X-ray results have also been deposited with the Cambridge Crystallographic Data Centre.

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(15) Unpublished data provided by MGI Pharma Inc., Minneapolis. This company has been licensed by the University of California to develop illudin analogues for treating tumors. U.S. Patents 5,439,936 and 5,439,942 on these compounds were issued to the University of California, Aug 8, 1995.

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