Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1195–1200

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

SYNTHESIS OF TOOLS FOR TARGET IDENTIFICATION OF THE ANTI-APOPTOTIC COMPOUND CGP 3466; PART I

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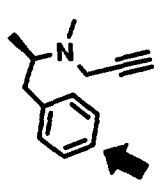
Received 13 January 1998; accepted 7 April 1998

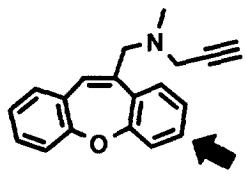
Abstract: Immobilized compounds for BIAcore® studies and affinity precipitation as well as a fluorescent-labeled compound were prepared in order to identify the molecular target of the anti-apoptotic, neurorescuing compound CGP 3466 (N-methyl-N-propargyl-10-aminomethyl-dibenzo[b,f]oxepin).

The anti-parkinsonian drug (-)-deprenyl (1) was reported previously to protect partially-differentiated PC12 cells from cell death induced by trophic withdrawal [1a], and to rescue embryonic mesencephalic dopaminergic neurons from MPP^+-toxicity [1b,c] and glutamate toxicity [1d] *in vitro*. (-)-Deprenyl (1) rescues facial motor neurons [2a] after axotomy and nigral dopaminergic neurons after systemic MPTP treatment *in vivo* [2b]. It also protects hippocampal pyramidal neurons after systemic kainate treatment [2c] or after unilateral carotid occlusion/transient hypoxia [2d]. In the course of a screening program for (-)-deprenyl (1) analogs, CGP 3466 (2) (N-methyl-N-propargyl-10-aminomethyl-dibenzo[b,f]oxepin) was identified as a highly potent, neurorescuing compound. Even though CGP 3466 (2) showed equal effects as (-)-deprenyl (1) in the *in vitro* and *in vivo* paradigms mentioned above, it was generally found to be about 100-fold more potent. In particular, CGP 3466 rescues PAJU-cells from rotenone-induced apoptotic cell death [3a] and it prevents cytosine arabinoside-induced apoptosis in cultures of cerebellar neurons [3b]. *In vivo*, the compound was reported to increase life-span in the progressive motoneuronopathy mouse model [3c] and to prevent neuronal death in models of ischemia and seizure [3d]. The target(s) and mechanism of the neurorescuing effects of CGP 3466 (2) and (-)-deprenyl (1) are not known. For (-)-deprenyl (1) *de novo* gene expression seems to be required [3e], while the monoamine oxidase-B (MAO-B) inhibition is not responsible for its neuroprotective effects.

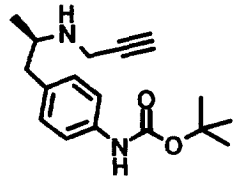
Figure 1



(-)-deprenyl (1)
linker attachment in para-position



CGP 3466 (2)
linker attachment in position 7



3: para-N-BOC-aminodesmethyl deprenyl

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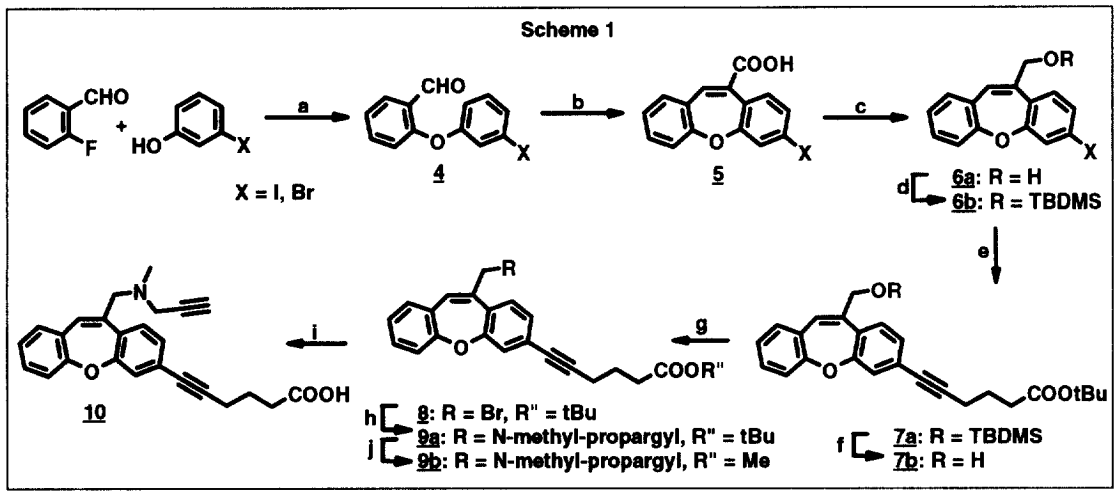
0960-894X/98/$19.00
PII: S0960-894X(98)00188-7

An array of tools for the identification of the target of CGP 3466 (2) was synthesized. The pharmacological and biochemical methods and results of the target-finding investigations are presented elsewhere [3a]. CGP 3466 (2) was found to interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thereby affecting a critical pathway, regulating neural apoptosis [3a]. The syntheses of the tools used for target identification of CGP 3466 (2) are described in the present publication.

Design: All compounds described below required an appropriate linker in a position of the dibenzoxepine moiety not interfering with the biological activity. The structure-activity-relationship of deprenyl-derivatives suggested that the para-position of the aromatic ring would be suitable for the attachment of a linker. This assumption was confirmed by a desmethyl-deprenyl derivative, having a BOC-protected amino function in the para-position (3), see Fig. 1. This molecule was found to have good neurorescuing properties in the PC12 assay [1a] (data not shown). In structural analogy, the position 7 of the dibenzo[b,f]oxepin ring-system of CGP 3466 (2) was therefore chosen as anchor point.

A common precursor **10** was used for (i) immobilization on a resin for affinity precipitation, (ii) immobilization on BIAcore®-chips, and (iii) synthesis of a fluorescent-labeled compound.

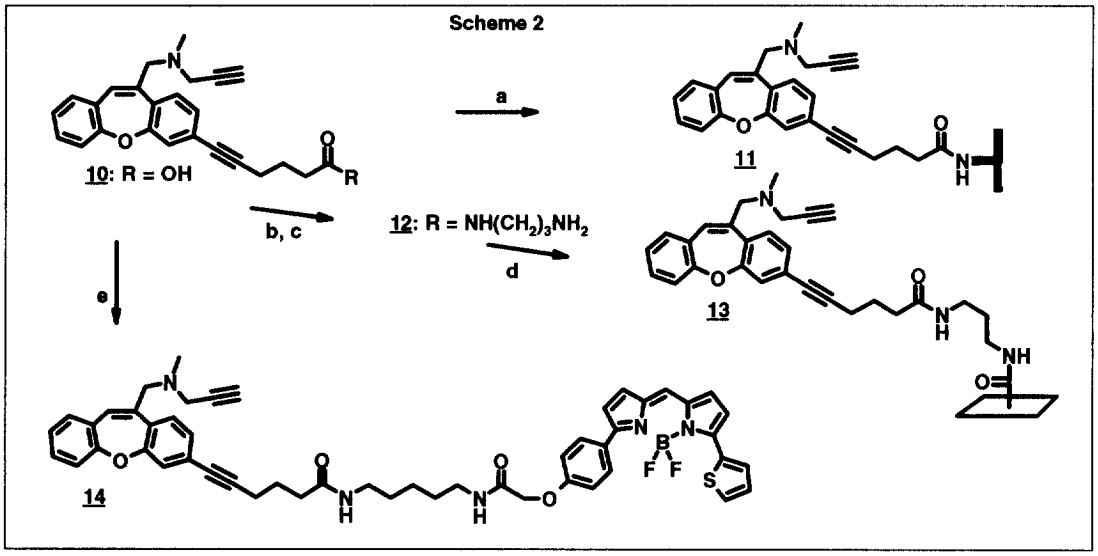


(a) K_2CO_3, N,N-dimethylacetamide (DMA), rflx.; (b) hippuric acid, NaOAc; Ac_2O, H_2SO_4; (c) 1. isobutyl-chloroformiate, N-methylmorpholine (NMM), dimethoxyethane (DME); 2. $NaBH_4$, H_2O; (d) tert.-butyl-dimethylchloro-silane (TBDMSCl), CH_2Cl_2; (e) tert.-butyl hex-5-yn-carboxylate; $(Ph_3P)_2PdCl_2$, CuI, toluene; (f) TBAF, THF, rt.; (g) NBS, PPh_3; (h) N-methylpropargylamine; (i) HCl/dioxane; (j) HCl/MeOH.

Synthesis of precursor 10 (Scheme 1): o-Fluorobenzaldehyde was coupled with 3-iodophenol or 3-bromophenol to the ether **4**. Subsequent azlactonization and ring-closure under strongly acidic conditions led to 7-iodo-dibenzo[b,f]oxepin-10-carboxylic acid **5** (or the 7-bromo-derivative, respectively). Whereas the reduction of the acid **5** with LAH led to unwanted saturation of the 10,11-double bond, formation of a mixed anhydride followed by borohydride-reduction gave the desired alcohol **6a** which was subsequently tert.-butyldimethylsilyl (TBDMS) protected (**6b**). In a Heck-type reaction tert.-butyl hex-5-yn-carboxylate was

introduced in high yield (**7a**). This step gave comparable yields using the 7-bromo-derivative. Removal of the TBDMS-group with fluoride yielded the alcohol **7b**, which was subsequently brominated with NBS/PPh_3 (**8**). Amination with N-methylpropargylamine (**9a**) and hydrolysis of the tert.-butylester gave the precursor molecule **10**. The methyl ester **9b** was obtained by transesterification of the tert.-butylester **9a** with MeOH/HCl. The neurorescuing properties of **9b** were tested *in vitro* in the PC12 assay [1a]. It was found to be active in the same concentration range as the parent compound CGP 3466 (**2**). This result confirmed the good choice of the linker position as well as the nature of the linker chosen for tethering.

Immobilization on resin and BIAcore®-microchips (Scheme 2): The carboxylic acid of the precursor molecule **10** was activated with carbodiimide (EDCI)/hydroxy-benzotriazole (HOBt) and coupled to Toyopearl® AF-Amino 650M resin, yielding **11**. A qualitative Ninhydrin assay (staining of amino groups) demonstrated, that the vast majority of the free amino groups had been amidated. The Raman-spectrum of a dry sample clearly showed the stretch-resonances of both triple bonds: Ar-C≡C-CH_2-linker (2229, 2257cm^{-1}) and N-CH_2-C≡C-H (2103cm^{-1}). In the IR-spectrum only the propargyl resonance was visible. Immobilization on BIAcore® was achieved by elongation of the linker of **10** by 1,3-diaminopropane (**12**) and subsequent coupling to the free carboxy-termini on the surface of a BIAcore®-microchip directly in the BIAcore®-apparatus using a standard coupling kit (**13**).



(a) EDCI, HOBt, Toyopearl® AF-Amino 650M resin; (b) oxalylchloride, DMF, N-BOC-diamino-1,3-propane; (c) HCl/dioxane (d) BIAcore® coupling kit; (e) EDCI, HOBt, BODIPY® TR cadaverine.

Fluorescent-labeling (Scheme 2): In order to obtain the fluorescent labeled CGP 3466-derivative **14**, the precursor **10** was activated with EDCI/HOBt and coupled to the amino group of BODIPY® TR cadaverine. The compound **14** was characterized by ^{1}H-NMR, MS and UV/VIS. It showed an absorption wavelength maximum at 590nm and an emission wavelength maximum at 623nm. Compound **14** proved to have

neuroprotective activity *in vitro* (PC12 assay [1a]) in the same concentration range as the parent molecule CGP 3466 (**2**).

Summary of results from target finding [3a]: Cytosolic and membrane fractions of rat hippocampal lysates were incubated with the immobilized CGP 3466-derivative **11**, followed by extensive washing. Bound proteins were analyzed by SDS-PAGE. Among the most prominent bands, actin, α- and β-tubulin, α- and β-spectrin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were identified. The interaction of purified rabbit muscle GAPDH with the immobilized CGP 3466-derivative **13** was investigated kinetically using BIAcore® technology. In the confocal microscope, the fluorescent labeled derivative **14** was found to co-localize with GAPDH in PC12-cells (not published).

GAPDH has recently been implicated in neuronal apoptosis. Upregulation of GAPDH mRNA and an increase of GAPDH protein in the particulate fraction of cell extracts during age-induced apoptosis of mature cerebellar [4a] and cerebrocortical neurons was reported [4b]. GAPDH mRNA is also upregulated upon cytosine arabinonucleoside-induced apoptosis of cerebellar neurons in culture [4c]. In these systems, apoptosis was delayed significantly by antisense GAPDH oligonucleotides.

Summary and conclusions: In order to identify the target proteins of the highly potent anti-apoptotic compound CGP 3466 (**2**), a set of tool compounds was synthesized. The core molecule CGP 3466 (**2**) was equipped with a linker in a position known to not interfere with the biological activity. One common precursor (**10**) was used for immobilization on BIAcore®-chips and on a resin for affinity precipitation. The same molecule (**10**) was tethered to a fluorescent moiety. The methyl ester of the precursor (**9b**) as well as the fluorescent labeled compound (**14**) were found to exhibit neuroprotective properties in the picomolar range as did the parent molecule CGP 3466 (**2**). GAPDH was identified as a putative target protein of CGP 3466 by using the described tool compounds and employing a variety of biochemical methods [3a].

Experimental Section: Compound 4: A mixture of 2-fluorobenzaldehyde (14.08 g, 113.6 mmol), 3-iodophenol (25 g, 113.6 mmol) and K_2CO_3 (23.5 g, 170.4 mmol) in DMA (140 ml) was heated at 110°C for 4 h. After addition of 300 ml water, the mixture was extracted with tert.-butylmethylether (TBME), the organic phase washed with 2 N $NaOH_{aq}$ and dried over Na_2SO_4. Solvent and volatiles were removed *in vacuo* (0.2 Torr, 150°C), providing aldehyde **4** (26.58 g, 72%). R_f = 0.41 (hexane/AcOEt 4:1); ^{1}H-NMR (200MHz, $CDCl_3$) 6.90-7.96 (m, 8H), 10.48 (s, 1H); MS (ES+) m/e 325 (M+1).

Compound 5: A mixture of aldehyde **4** (26.58 g, 82 mmol), hippuric acid (22.03 g, 82 mmol) and NaOAc (8.06 g, 98.4 mmol) in 90 ml Ac_2O was heated at 80°C for 4h, cooled to rt. Water (40 ml) was added, heated at 60°C for 30 min. then cooled to rt., followed by addition of conc. H_2SO_4 (40 ml). The mixture was refluxed for 105 min., poured into ice-water and extracted with AcOEt. The solvent was dried over Na_2SO_4, removed and the residue chromatographed (hexane/AcOEt 4:1, SiO_2) providing acid **5** (6.74 g, 23%). R_f = 0.15 (AcOEt); ^{1}H-NMR (200MHz, $CDCl_3$) 7.19-7.62 (m, 7H), 8.11 (s, 1H); MS (ES-) m/e 363(M), 319 (M-CO_2).

Compound 6a: To a -15°C DME-solution (35 ml) of acid **5** (6.74 g, 18.5 mmol) NMM (2.05 ml, 18.5 mmol) and isobutylchloroformiate (2.4 g, 18.5 mmol) were added. Precipitates were removed by filtration, then $NaBH_4$ (1.36 g, 37 mmol) in 10 ml H_2O was added at 10°C. Extraction with TBME after addition of 1N HCl_{aq} (24 ml) and H_2O, and removal of solvents *in vacuo* provided crude alcohol **6a** (6.28 g, 97%). R_f = 0.68 (AcOEt); ^{1}H-NMR (200MHz, $CDCl_3$) 4.69 (s, 2H), 6.95 (s, 1H), 7.10-7.40 (m, 6H), 7.52 (dd, 1H), 7.62 (d 1H); MS (ES+) m/e 373(M+Na), 368(M+NH_4^+), 333(M-OH).

Compound 6b: To a CH_2Cl_2-solution (80 ml) of alcohol **6a** (6.28 g, 17.25 mmol) Et_3N (2.65 ml, 18.97 mmol) and TBDMSCl (2.71 g, 18.11 mmol) were added. After 3 d at rt. the solvent was removed *in vacuo*, the residue dissolved in AcOEt and washed with 0.1 N HCl_{aq}, brine, sat. $NaHCO_3$, and brine again. The organic layer was dried over Na_2SO_4, the solvent removed *in vacuo* and the residue chromatographed (hexane/AcOEt 9:1, SiO_2) providing **6b** (5.68 g, 71%). R_f = 0.63 (hexane/AcOEt 4:1); ^{1}H-NMR (200MHz, $CDCl_3$) 0.13 (s, 6H), 0.93 (s, 9H), 4.65 (s, 2H), 6.95 (s, 1H), 7.05-7.32 (m, 6H), 7.46 (dd, 1H), 7.60 (d, 1H); MS (ES+) m/e 482 (M+NH_4^+).

Compound 7a: A mixture of **6b** (5.68 g, 12.23 mmol), tert.-butyl hex-5-yn-carboxylate (7.20 g, 42.81 mmol), bis-(triphenylphosphine)-palladiumchloride 687 mg (0.978 mmol), copper(I)iodide (116.5 mg, 0.612 mmol) and Et_3N (2.22 ml, 15.9 mmol) in DMF (50 ml) was heated at 50°C for 16 h. Volatiles and solvents were removed *in vacuo*. Extraction with AcOEt, washing with H_2O and brine, drying ($MgSO_4$), evaporation of the AcOEt-phase and chromatography (hexane/AcOEt 9:1, SiO_2) yielded **7a** (5.9 g, 96%). R_f = 0.51 (hexane/AcOEt 4:1); ^{1}H-NMR (200MHz, $CDCl_3$) 0.15 (s, 6H), 0.95 (s, 9H), 1.47 (d, 9H), 1.88 (m, 2H), 2.45 (m, 4H), 4.65 (s, 2H), 6.91 (s, 1H), 7.10-7.40 (m, 7H); MS (ES+) m/e 522 (M+NH_4^+).

Compound 7b: A THF-solution (40 ml) of **7a** (5.9 g, 11.7 mmol) and tetrabutylammonium fluoride (1.7 g, 11.7 mmol) was stirred at rt. for 3 h. The mixture was concentrated, washed in AcOEt with H_2O and brine, the solvent dried over $MgSO_4$ and evaporated, yielding crude alcohol **7b** (5.2 g, quant.). R_f = 0.10 (hexane/AcOEt 4:1); ^{1}H-NMR (200MHz, $CDCl_3$) 1.45 (d, 9H), 1.85 (m, 2H), 2.35 (m, 4H), 4.68 (s, 2H), 6.91 (s, 1H), 7.10-7.38 (m, 7H); MS (ES+) m/e 390, 391(M, M+1).

Compound 8: Triphenylphosphine (3.7 g, 14 mmol) and N-bromosuccinimide (2.5 g, 14 mmol) were added to a 0°C THF-solution (40 ml) of **7b** (5.2 g, 11.7 mmol), then stirred overnight at rt. The solvent was evaporated, the residue dissolved in CH_2Cl_2 and washed with H_2O and brine, dried over $MgSO_4$, evaporated and chromatographed (hexane/AcOEt 9:1, SiO_2) providing **8** (3.41 g, 64%). R_f = 0.60 (hexane/AcOEt 4:1); ^{1}H-NMR (200MHz, $CDCl_3$) 1.45 (d, 9H), 1.88 (m, 2H), 2.40 (m, 4H), 4.51 (s, 2H), 7.02-7.48 (m, 8H); MS (ES+) m/e 470, 472 (M+NH_4^+).

Compound 9a: A toluene-solution (10 ml) of **8** (1.19 g, 2.62 mmol) was added to a MeOH-solution (7.5 ml) of N-methylpropargylamine and stirred overnight at rt. After concentration, the residue was washed in AcOEt with H_2O and brine, dried over $MgSO_4$ and evaporated, providing crude **9a** (1.02 g, 88%). R_f = 0.43 (hexane/AcOEt 4:1); ^{1}H-NMR (200MHz, $CDCl_3$) 1.45 (s, 9H), 1.86 (m, 2H), 2.26 (t, 1H), 2.40 (m+s, 5H), 3.41 (d, 2H), 3.58 (s, 2H), 5.28 (s, 2H), 6.85 (s, 1H), 7.05-7.30 (m, 6H), 7.45 (dd, 1H); MS (ES+) m/e 442 (M+1).

Compound 10: Tert.-butylester **9a** (133 mg) was stirred in 6 N HCl/dioxane (10 ml) for 3h. Then all volatiles were evaporated. The crude acid **10** was used immediately for consecutive reactions. MS (ES+) m/e 386 (M+1).

Immobilization on resin (11): Acid **10** (151 mg, 300 µmol), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI, 69 mg, 360 µmol), 1-hydroxybenzotriazole (HOBt, 60.8 mg, 450 µmol) and Et_3N (62.8 µl, 450 µmol) were stirred in DMA/H_2O (3:1) for 30 min., then added to Toyopearl® AF-Amino 650M resin (1 g, ca. 100 µmol free COOH) and shaken 2 h at rt. After washing with DMA/H_2O, a second batch of preactivated acid **10** (see above) was added. In the ninhydrin test supernatant and resin were only slightly greenish. The coated resin **11** was stored in phthalate buffer pH 5, 0.02% NaN_3 at 4°C. IR (KBr): 2103; Raman (powder): 2103, 2229, 2257.

Compound 12: To a CH_2Cl_2-solution (8 ml) of acid **10** (632 mg, 1.59 mmol) and oxalylchloride (143 µl, 1.66 mmol) two drops of DMF were added, and stirred 3 h at rt. Then N-butyloxycarbonyl-1,3-diamino-propane (809 µl, 4.76 mmol) in 10 ml CH_2Cl_2 was added. After stirring overnight, the reaction mixture was washed, ($NaHCO_3$, H_2O, brine), dried over $MgSO_4$, evaporated and chromatographed (SiO_2; CH_2Cl_2-MeOH-NH_3 1000:50:1) yielding the BOC-protected amine (858 mg, 99%). R_f = 0.54 (CH_2Cl_2-MeOH-NH_3 1000:50:1); MS (ES+) m/e 542 (M+1). Treatment with 6N HCl/dioxane (15 ml, rt., 2h) removed the tert.-butyl group and gave the hydrochloride salt of **12** as brown oil (636 mg, 91%). MS (ES+) m/e 442 (M+1).

Immobilization on BIAcore®-chips (13): BIAcore®-chips were activated using the standard activation kit from Pharmacia®. A DMA/H_2O-solution of the free amine **12** was flushed into the cell and kinetics-measurements were immediately started.

Compound 14: Acid **10** (8.2 mg, 18.35 µmol) in CH_2Cl_2 (1 ml) was preactivated 30 min. at rt. with EDCI (4.2 mg, 22.03 µmol), HOBt (3.7 mg, 27.53 µmol) and Et_3N (5.6 µl, 40.38 µmol), then 5-[4-{4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl}phenoxy]-hydrochloride (BODIPY® TR cadaverine, 10 mg, 18.35 µmol) was added and the mixture stirred overnight under light-protection. Preparative TLC (CH_2Cl_2-MeOH 9:1) and lyophilization provided the fluorescent labeled compound **14** (12.8 mg, 80%). R_f = 0.48 (CH_2Cl_2-MeOH 9:1); ^{1}H-NMR (200MHz, $CDCl_3$) 1.25-1.35 (m, 3H), 1.45-1.70 (m, 6H), 1.85-1.93 (m, 2H), 2.25-2.5 (m, 8H), 3.20 (q, 2H), 3.35 (q, 2H), 3.42 (d, 2H), 3.59 (s, 2H), 4.55 (s, 2H), 5.62 (m, 1H), 6.59 (t, 1H), 6.63 (d, 1H), 6.81 (d, 1H), 6.88 (s, 1H), 6.95-7.30 (m, 10H), 7.97 (d, 2H), 8.11 (d, 1H). MS (ES+) m/e 876 (M+1). UV/VIS/fluorescence (CH_3CN-H_2O): λ_{max}(abs) 590nm; λ_{max}(emission) 623nm.

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