Supporting Information

Design, Synthesis and X-ray Structure of Protein-Ligand Complexes: Important Insight into Selectivity of Memapsin 2 (β-Secretase) Inhibitors

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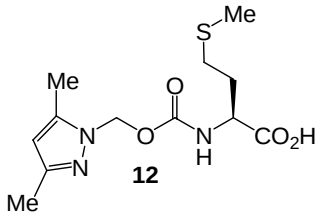
All moisture sensitive reactions were carried out under nitrogen or argon atmosphere. Anhydrous solvents were obtained as follows: THF, diethyl ether and benzene, distilled from sodium and benzophenone; dichloromethane, pyridine, triethylamine, and diisopropylethylamine, distilled from CaH_2. All other solvents were HPLC grade. Column chromatography was performed with Whatman 240-400 mesh silica gel under low pressure of 5-10 psi. TLC was carried out with E. Merck silica gel

60-F-254 plates. ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury 300, Bruker Avance 400 and 500 spectrometers.

Complete reference for 3(a): Vassar, R.; Bennett, B. D.; Babu-Khan, S.; Kahn, S.; Mendiaz, E. A.; Denis, P.; Teplow, D. B.; Ross, S.; Amarante, P.; Loeloff, R.; Luo, Y.; Fisher, S.; Fuller, J.; Edenson, S.; Lile, J.; Jarosinski, M. A.; Biere, A. L.; Curran, E.; Burgess, T.; Louis, J. C.; Collins, F.; Treanor, J.; Rogers, G.; Citron, M. *Science* **1999**, *286*, 735.

Preparation of carboxylic acid 12:



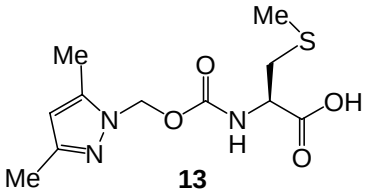
12

A solution of L-methionine methyl ester hydrochloride (242 mg, 1.21 mmol) and triethylamine (0.42 mL, 3.03 mmol) in methylene chloride (4 mL) was added slowly over a period of 30 minutes using a syringe pump to a stirred solution of triphosgene (132 mg, 0.44 mmol) in methylene chloride (2 mL) at 23°C. After a further 5 min of stirring, a solution of (3,5-dimethyl-pyrazol-1-yl)- methanol (152 mg, 1.21 mmol, Aldrich) in methylene chloride was added in one portion. The reaction mixture was stirred for 12 hours, diluted with ethyl acetate, washed with water, brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (50% EtOAC /Hexane) to give 143 mg (36 %) of the corresponding methyl ester. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.94-2.20 (2H, m), 2.0 (3H, s), 2.18 (3H, s), 2.26 (3H, s), 2.41 (2H, m), 3.74 (3H, s), 4.48 (1H, m), 5.50 (1H, br s), 5.82 (1H, s), 5.90 (2H, s).

To a stirred solution of the above ester (140 mg, 0.43 mmol) in a mixture of 10% aqueous THF (3 mL) was added LiOH (27 mg, 0.65 mmol). The mixture was stirred for 3 h at room temperature. After this period, the solvents were removed and the residue was acidified with aqueous 1N HCl to pH~4. The white solid was extracted twice with ethyl acetate and the combined extracts were dried over anhydrous sodium sulfate and

concentrated under reduced pressure to provide compound **12** (134 mg, quantitative) which was carried on to the next step without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 1.94-2.20 (2H, m), 2.0 (3H, s), 2.18 (3H, s), 2.26 (3H, s), 2.48 (2H, m), 3.74 (3H, s), 4.40 (1H, m), 5.50 (1H, br s), 5.82 (1H, s), 5.90 (2H, s).

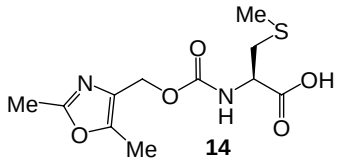
Preparation of carboxylic acid 13:



13

Carboxylic acid **13** was prepared by an analogous procedure to **12** utilizing methylcysteine methyl ester (44% yield). ^{1}H NMR (300 MHz, *d*-MeOH): δ 5.90-5.95 (br, 1H), 5.81 (s, 2H), 4.32 (br, 1H), 2.90-3.05 (br, 2H), 2.29 (s, 3H), 2.17 (s, 3H), 2.02 (s, 3H).

Preparation of carboxylic acid 14:



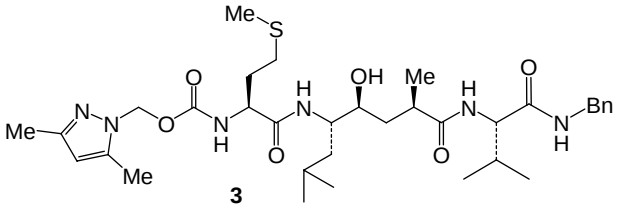
14

Synthesis of dimethyloxazolylmethanol: Sodium nitrite (12.2 g, 0.18 mol) in water was added dropwise to a solution of ethyl acetoacetate (19.5 mL, 0.15 mol) in glacial acetic acid at 23°C over 1h. The resulting mixture was stirred for an additional 1 h at r.t followed by addition of 80 mL of water. After stirring for an additional 2 h, the reaction mixture was extracted with ether (3x), and successively washed with aqueous $NaHCO_3$, water and brine. The organic layer was dried with Na_2SO_4 and concentrated to afford the crude product. Without further purification, the crude product (6.5 g, 40.8 mmol) in a mixture of acetic anhydride (19.3 mL, 0.21 mol), acetic acid (58 mL), and 210 mg of Pd/C (10% w/w) was hydrogenated at 50 psi for 1.5 h. The catalyst and solvent were removed and the residue was triturated with hexanes to give ethyl *N*-acetylacetoacetate as a solid (m.p. 38-40 ºC).

The above solid product (3.3 g, 17.6 mmol) was treated with thionyl chloride (1.3 mL, 17.6 mmol) in dry benzene at 23°C. The mixture was warmed to 30ºC for 1 h, and was placed under vacuum on a rotary evaporator for a further 30 min. The resulting residue was diluted with EtOAc and washed successively with aqueous $NaHCO_3$, water, and brine. The organic layer was dried with Na_2SO_4 and concentrated to give the crude product as a brown oil, which was further reduced by LAH to provide the desired dimethyloxazolylmethanol as a light yellow solid. ^{1}H-NMR: (300 MHz, $CDCl_3$), δ: 4.51 (s, 2 H); 2.58 (s, 3 H); 2.43 (s, 3 H), 2.31 (s, 3 H).

The above alcohol was coupled with methylcysteine methyl ester using the same triphosgene conditions as for the synthesis of compounds **12** and **13** to provide the corresponding methyl ester. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.66 (br, NH, 1 H), 4.94 (s, 2 H), 4.56-4.62 (m, 1 H), 3.76 (s, 3 H), 2.95 (d, *J*=5.4 Hz, 2 H), 2.40 (s, 3 H), 2.31 (s, 3 H), 2.10 (s, 3 H). This ester was hydrolyzed using lithium hydroxide solution in THF/H_2O as for compound **12** and the crude acid **14** was used directly for the next reaction without further purification. ^{1}H-NMR (300 MHz, $CDCl_3$ plus a small amount of CD_3OD), δ: 4.87 (s, 2 H), 4.47 (t, *J*=5.2, 10.5 Hz, 1 H), 3.17 (br, 2 H), 2.92 (dd, *J*= 5.5, 9.0, 1 H), 2.36 (s, 3 H), 2.26 (s, 3 H), 2.086 (s, 3 H); ^{13}H-NMR (75 MHz, $CDCl_3$ plus a small amount of CD_3OD), δ: 172.8, 160.4, 129.6, 101.6, 100.4, 58.2, 53.5, 36.7, 16.3, 13.8, 10.2.

Preparation of Inhibitor 3:



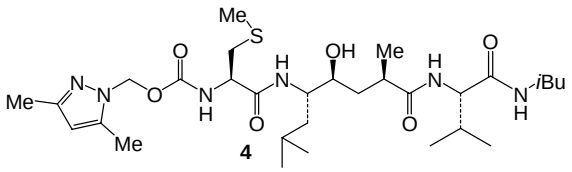
3

Dipeptide isostere **7**[1] (42 mg, 0.1 mmol) and amine **8**[1] (41 mg, 0.2 mmol) were dissolved in DMF (2 mL). To this solution, HOBt (20 mg, 0.15 mmol), EDC (29 mg, 0.15 mmol) and diisopropylethylamine (0.2 mL) were added successively at 0°C. After the addition, the reaction mixture was allowed to warm to 23°C and was stirred overnight. The mixture was poured into aqueous $NaHCO_3$ and the aqueous layer was

extracted with 30% EtOAc/hexane. The combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave a residue which was purified by column chromatography (20% EtOAc/hexane) to give 55 mg (95%) of coupling product **10**. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 0.09 (3H, s), 0.10 (3H, s), 0.91 (9H, s), 0.92-0.98 (12H, m), 1.10 (3H, d, *J* = 6.7 Hz), 1.25 (1H, m), 1.44 (1H, m), 1.46 (9H, s), 1.63 (1H, m), 1.74 (1H, br s), 1.80 (1H, m), 2.18 (1H, m), 2.56 (1H, m), 3.62-3.78 (2H, m), 4.13 (1H, m), 4.48-4.56 (3H, m), 6.35 (1H, br d, *J* = 8.5 Hz), 6.41 (1H, br s), 7.26-7.40 (5H, m).

To a solution of **10** (37 mg, 0.06 mmol) in CH_2Cl_2 (1 mL) was added TFA (0.4 mL) at 23°C. The resulting mixture was stirred at 23°C for 1 h followed by concentration under reduced pressure. The resulting residue was dissolved in DMF (2 mL). To a solution of acid **12** (18 mg, 0.06 mmol) in 2 mL of DMF, HOBt (8 mg, 0.06 mmol), EDC (11 mg, 0.06 mmol) and diisopropylethylamine (0.2 mL) were added successively at 0°C. After the addition, the reaction mixture was allowed to warm to 23°C and was stirred overnight. The mixture was poured into aqueous $NaHCO_3$ and the aqueous layer was extracted with EtOAc. The combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave a residue which was purified by column chromatography (2% MeOH / $CHCl_3$) to provide the inhibitor **3** (16 mg, 40%). ^{1}H-NMR (300 MHz, CD_3OD): δ 0.80-0.97 (12H, m), 1.10 (3H, d, *J* = 6.7 Hz), 1.20-2.38 (8H, m), 2.0 (3H, s), 2.18 (3H, s), 2.24 (3H, s), 2.41 (3H, t, *J* = 6.4 Hz), 2.60 (1H, m), 3.41 (1H, m), 3.80 (1H, m), 4.15 (1H, m), 4.20-4.32 (3H, m), 5.80 (3H, s) 7.17-7.30 (5H, m). MS-ESI (*m/z*): $[M+H]^+$.

Preparation of Inhibitor 4:



4

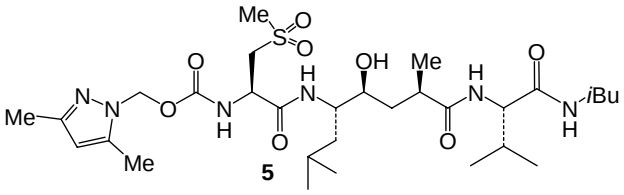
To a stirred solution of **9** (435mg, 1.60 mmol) in CH_2Cl_2 (8 mL) was added TFA (2 mL). The resulting solution was stirred at room temperature for 30 min and was concentrated under reduced pressure. After drying, the amine TFA salt was dissolved in CH_2Cl_2 (10 mL) containing *N*,*N*-diisopropylethylamine (1.16 mL, 6.65 mmol). In a

separate flask, acid **7** (555 mg, 1.33 mmol), HOBt (200 mg, 1.48 mmol), EDC (282 mg, 1.47 mmol) were dissolved in CH_2Cl_2 (20 mL). The above amine solution was transferred to the acid solution. The resulting mixture was stirred at room temperature for 17 h and was quenched with H_2O. The layers were separated and the aqueous layer was extracted with $CHCl_3$ (2 × 20 mL). The combined organic layers were washed with brine, dried with Na_2SO_4 and concentrated under reduced pressure. The resulting oil was purified by column chromatography (2% MeOH in $CHCl_3$) to provide the coupled product **11** (538 mg, 71%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.40 (d, 1H, *J* = 8.7 Hz), 6.14 (t, 1H, *J* = 5.7 Hz). 4.56 (d, 1H, *J* = 9.9 Hz), 4.06 (t, 1H, *J* = 8.4 Hz), 3.76 (dd, 1H, *J* = 5.1, 9.6 Hz), 3.66-3.70 (m, 1H), 3.00-3.09 (m, 1H), 3.11-3.20 (m, 1H), 2.53-2.60 (m, 1H), 2.10-2.20 (m, 1H), 1.72-1.80 (m, 2H), 1.54-1.65 (m, 1H), 1.44 (s, 9H), 1.35-1.48 (m, 2H), 1.20-1.25 (m, 1H), 1.10 (d, 3H, *J* = 6.3 Hz), 0.88-0.95 (m, 18H).

To a stirred solution of **11** (39.8 mg, 0.07 mmol) in CH_2Cl_2 (2 mL) was added TFA (0.4 mL). The resulting solution was stirred at room temperature for 30 min and was concentrated under reduced pressure. The amine TFA salt was dissolved in CH_2Cl_2 (2 mL) containing N,N-diisopropylethylamine (49 μL, 0.3 mmol). In a separate flask, acid **13** (20 mg, 0.07 mmol), HOBt (10.8 mg, 0.08 mmol), EDC (13.4 mg, 0.08 mmol) were dissolved in CH_2Cl_2 (3 mL). The above amine solution was transferred to the acid solution. The resulting mixture was stirred at room temperature for 15 h and was quenched with H_2O. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (2 × 10 mL). The combined organic layer was washed with brine, dried with Na_2SO_4 and concentrated under reduced pressure. The resulting oil was dissolved in THF (2 mL) and aqueous HF (0.5 mL) was added. The resulting mixture was stirred for 45 min and quenched with saturated aqueous $NaHCO_3$ solution. The layers were separated and the aqueous layer was extracted with EtOAc (2 × 10 mL). The combined organic layer was successively washed with brine, dried with Na_2SO_4 and concentrated under reduced pressure. The resulting oil was purified by column chromatography (3% MeOH in $CHCl_3$) to provide the compound **4** (22.2 mg, 64%) as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.87 (d, 2H, *J* = 3.0 Hz), 5.84 (s, 2H), 4.26-4.32 (m, 1H), 4.00-4.06 (m, 1H), 3.80-3.89 (m, 1H), 3.49-3.52 (m, 1H), 3.01-3.14 (m, 1H), 2.92-2.98 (m, 1H), 2.77-2.87 (m, 2H), 2.58-2.64 (m, 1H), 2.31 (s, 3H), 2.19 (s, 3H), 2.11 (s, 3H), 1.98-2.05 (m,

1H), 1.67-1.80 (m, 2H), 1.46-1.56 (m, 2H), 1.29-1.32 (m, 1H), 1.11 (d, 3H, *J* = 7.2 Hz), 0.84-0.92 (m, 18H); ^{13}C-NMR (175 MHz, $CDCl_3$ + CD_3OD): δ 177.7, 172.0, 170.7, 154.8, 150.3, 141.4, 107.0, 70.6, 59.4, 54.4, 52.2, 47.1, 41.0, 38.4, 37.8, 30.7, 30.0, 29.9, 28.5, 26.0, 24.9, 23.3, 22.2, 20.2, 19.5, 18.6, 17.8, 16.0, 13.5, 10.8; MS-ESI (*m/z*): $[M+Na]^+$ calcd. for $C_{30}H_{54}N_6NaO_6S$ = 649.3723, found 649.3726.

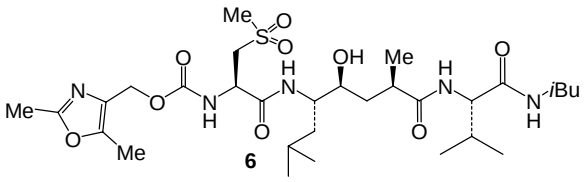
Preparation of Inhibitor 5:



5

To a stirred solution of sulfide **4** (21 mg, 0.034 mmol) in CH_2Cl_2 (3 mL) and MeOH (0.5 mL) at 0°C was added *m*CPBA (17 mg, 0.07 mmol). The solution was slowly warmed to room temperature and the stirring was continued for 3 h. The reaction was quenched with saturated aqueous $NaHCO_3$ solution. The layers were separated and the aqueous layer was extracted with 10 % MeOH in $CHCl_3$ (2 × 10 mL). The combined organic extracts were washed with brine, dried with Na_2SO_4 and concentrated under reduced pressure. The resulting solid was purified by column chromatography (10% MeOH in $CHCl_3$) to provide the compound **5** (18.7 mg, 86%) as a white solid. M.P. 201-206˚C (decomposed). ^{1}H NMR (300 MHz, *d*-MeOH): δ 5.88 (d, 2H, *J* = 4.2 Hz), 5.85 (s, 2H), 4.60-4.63 (m, 1H), 3.94-3.96 (d, 1H, J = 5.7 Hz), 3.58-3.72 (dd, 1H, *J* = 4.5, 14.7 Hz), 3.40-3.47 (m, 2H), 3.00-3.06 (dd, 1H, *J* = 6.9, 13.2 Hz), 2.94 (s, 3H), 2.87-2.89 (dd, 1H, *J* = 6.9, 13.2 Hz), 2.52-2.58 (m, 1H), 2.27 (s, 3H), 2.14 (s, 3H), 1.93-1.99 (m, 1H), 1.60-1.73 (m, 2H), 1.37-1.43 (m, 3H), 1.20-1.27 (m, 2H), 1.05 (d, 3H, *J* = 4.8 Hz), 0.79-0.85 (m, 18H); ^{13}C-NMR (175 MHz, $CDCl_3$ + CD_3OD): δ 177.5, 172.0, 169.1, 155.0, 150.2, 141.7, 107.0, 70.7, 70.4, 59.2, 55.6, 52.3, 50.5, 46.9, 41.9, 40.1, 38.0, 37.6, 30.7, 29.8, 28.4, 24.8, 23.2, 21.9, 20.1, 19.3, 18.5, 17.9, 13.2, 10.6; MS-ESI (*m/z*): $[M+Na]^+$ calcd. for $C_{30}H_{54}N_6NaO_8S$ = 681.3622, found 681.3640.

Inhibitor 6: (Same procedure as **5**)



6

^{1}NMR: (300 MHz, $CDCl_3$), δ: 7.07 (br, 1 H); 4.87 (m, 2 H); 4.24 (m, 1 H); 3.99 (m, 1 H); 3.81 (m, 1 H); 3.44 (m, 1 H); 3.02 (m, 1 H); 2.93 (m, 1 H); 2.79 (m, 2 H); 2.57 (m, 1 H); 2.36 (s, 3 H); 2.26 (s, 3 H); 2.08 (s, 3 H); 1.99 (m, 1 H); 1.59-1.76 (m, 2 H); 1.48 (m, 3H); 1.26 (m, 1 H); 1.08 (d, 3 H); 0.87 (m, 18 H); ^{13}C NMR ($CDCl_3$ + CD_3OD): δ: 177.4, 171.9, 169.3, 160.5, 156.2, 147.5, 129.5, 70.8, 59.3, 58.6, 56.0, 52.4, 50.9, 47.0, 42.5, 40.6, 38.4, 37.7, 30.7, 29.9, 28.5, 24.8, 23.3, 22.1, 20.2, 19.4, 18.7, 17.9, 13.8, 10.2; MS-ESI (*m/z*): $[M+Na]^+$ calcd. for $C_{30}H_{53}N_5NaO_9S$ = 682.3462, found 682.3531.

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Determination of X-ray structure of 5-bound Memapsin 2

Protein purification and crystallization. Recombinant human promemapsin 2 was produced in *Escherichia coli* as inclusion bodies, refolded by rapid dilution and purified on a S-300 gel filtration column as previously described.[2,3] Purified pro-memapsin 2 was activated by clostripain as described by Ermolieff *et al*[4] with minor modifications followed by purification with anion exchange chromatography.[2]

Crystals of free memapsin 2 (APO form) were grown using hanging drop vapor diffusion method as previously described.[5] Monoclinic crystals suitable for structure determination were obtained at 15% PEG 8000, pH 6.5 and 17 ^{o}C with a protein concentration of 18 mg/ml. The crystals grow to full size (0.25 mm x 0.35 mm x 0.05 mm) in 4-5 weeks. The memapsin 2/inhibitor **5** complex crystal was prepared by soaking the free memapsin 2 crystal in concentrated inhibitor solution for two days.

Data collection and processing. Memapsin 2/inhibitor **5** crystal was soaked in the mother liquor plus 20% (v/v) glycerol and quickly frozen under a cryogenic nitrogen gas stream. Diffraction data of a single crystal was recorded on a Mar 345 image plate mounted on a MSC-Rigaku RU-300 X-ray generator with Osmic focusing mirrors. Data were integrated and merged using DENZO and SCALEPACK program package.[6] The crystal

form was determined to be monoclinic with a resolution of 1.86 Å. The unit cell parameters are a=86.6, b=131.0, c=88.2, β=97.4°.

Structure determination. The structure was determined by molecular replacement implemented with the program AmoRe[7] using the previously determined memapsin 2 structure[8] (PDB ID: 1M4H) as a search model. Rotation and translation functions followed by the rigid-body refinement with data from 15 Å to 3.5 Å resolution in space group $P2_1$ gave unambiguous solutions for the four memapsin 2 molecules in the asymmetric unit. Refinement procedures including simulated annealing were carried out with CNS[9] and iterative cycles of model building used graphics program O.[10] A random selection of 8% of reflections was set aside as the test set for cross-validation during the refinement. The refined model has well defined electron density for the inhibitor and its corresponding structure was built into the active site. The four molecules in the crystallographic asymmetric unit have essentially identical structures.

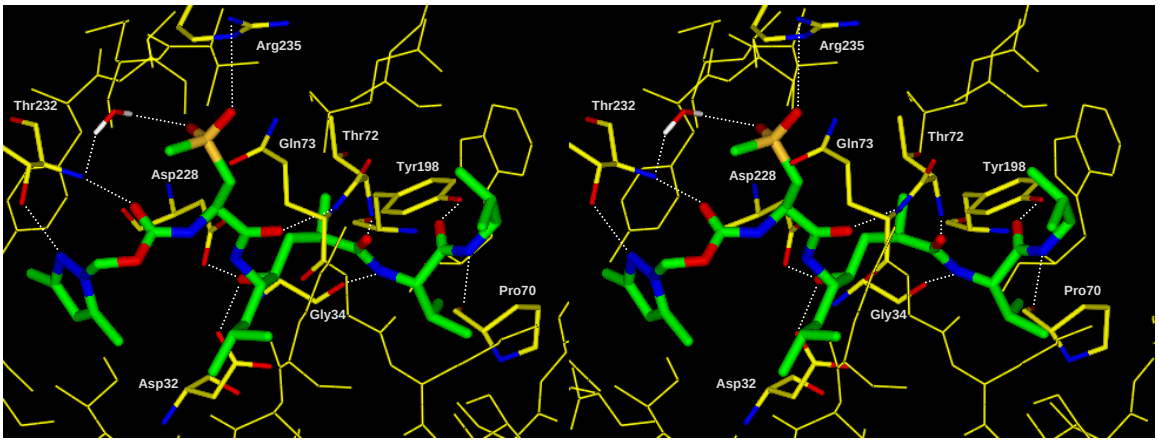

Figure 1. Stereoview of Inhibitor-**5**-bound X-ray structure of memapsin 2

References:

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