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Synthesis, and Investigation of the Reactive Oxygen Species of a Novel Cyclicpeptide-2,6-dimethoxyhydroquinone-3-mercaptoacetic Acid Conjugate

Yu Fei SONG[1], Hui Min ZHEN[2]

[1]Institute of Molecular Science, Shanxi University, Taiyuan 030006
[2]Taiyuan Teacher's College, Taiyuan 030006

Abstract: In this paper, we report the synthesis of a potential anti-cancer agent: Cyclo[Val-Lys(DMQ-MA)-Gaba]. The cytotoxic agent conjugated to the N-terminal and the ξ-amino group of lysine of the tripeptide is 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid (DMQ-MA). By employing the rhodamine B degradation method and ESR spectra, we testify the reactive oxygen species---hydroxyl radicals produced by the drug.

Keywords: 2, 6-Dimethoxyhydroquinone-3-mercaptoacetic acid, cyclicpeptide.

In the previous paper, we had reported the synthesis of a novel series of linear peptide conjugates of the cytotoxic agent 2,6-dimethoxyhydroquinone-3-mercaptoacetic acid[1] (DMQ-MA). In this paper, we choose the cyclic peptides over linear peptides as drug carriers. Our preference for this study is based on the following reason: cyclic peptides, with their N- and C- terminals blocked, are more hydrophobic and would pass through the cell membrane more readily than liner peptides[2]. Also cyclic peptides are more resistant to endogenous proteolytic enzymes than linear peptides[3], and have the increased specificity for a particular cell membrane. So they are excellent potential cytotoxic drug carriers. Among the peptide chains, the Gaba was chosen as a spacer group of the peptide and would increase the ring size, reducing the strain during the cyclization reaction. The lysyl residue was used for anchorage of DMQ-MA *via* the ξ–amino function.

Experimental

All of the protected amino acids were purchased from Sigma Chemical Co. Medium pressure column chromatography was performed using Merck 230-400 mesh silica gel. TLC system was performed on Merck silica gel 60 on aluminum sheets. Low-resolution mass spectra were taken from MA SPEC System operating in the FAB mode. Elemental analysis was performed at Institute of Coal Chemistry, Chinese Academy of Sciences. Florescence measurements were made with Perkin-Elmer LS50-B Fluorescence spectrophotometer. Ultra-Violet spectra were determined on the Hewlett

Packard 8453 spectrophotometer. ESR spectra were carried out on Bruker 200D EPR spectrometer.

DMQ, Boc-Lys-(Boc) and DMQ-MA were synthesized in Prof L.Sheh's Lab according to the ref 1, 4 and his US Patent.

Boc-Lys(Cbz)-Gaba-Opa **1b**: Boc-Gaba-Opa **1a** (8.0 g, 25 mmol), where Boc is *t*-butyloxycarbonyl and Opa is phenacyl, in 15 mL of CH_2Cl_2 was stirred with 17 mL of trifluoroacetic acid (TFA) for 50 min and the solvents were removed in *vacuo*. The residue was the TFA salt of Gaba-Opa. N_{α}-Boc-N_{ε}-Cbz-Lysine hydrochloride (9.5 g, 25 mmol) in 25 mL of CH_2Cl_2 was treated with 1-hydroxybenzotriazole (HOBt 4.1 g, 30.1 mmol) and 1,3-dicyclohexylcarbodiimide (DCC 6.2 g, 30.1 mmol) at 0°C for 15 min, and then at room temperature for 70 min. After that, it was added to the TFA salt of Gaba-Opa prepared above in 10 mL of CH_2Cl_2, and N,N -diisopropylethylamine (DIEA) was added to adjust the pH to 7.0. The reaction mixture was filtered after 90 min, diluted with 200 mL of CH_2Cl_2 and washed successively with citric acid (10%, 60 mL), saturated $NaHCO_3$ (60 mL) and saturated NaCl (60 mL), dried over $MgSO_4$ and evaporated the solvents to give a crude solid. The product was purified with silica gel chromatography and eluted stepwise with CH_2Cl_2, 1%, 2%, 3%-12% CH_3OH/CH_2Cl_2 respectively, and then the solvents were evaporated to get a white solid **1b**. Rf = 0.60 (CH_3OH : CH_2Cl_2 = 15:85). Yield 84%. LRMS [MH^+] = 584. Analytical for $C_{31}H_{41}N_3O_8$, C,63.79, H, 7.80, N, 7.20. Found C, 64.04, H, 7.34, N, 7.37.

Boc-Val-Lys(Cbz)-Gaba-Opa **1c**: **1b** (4.18 g, 7.16 mmol) was deblocked with 12 mL of TFA and 10 mL of CH_2Cl_2. Boc-Val (1.56 g, 7.16 mmol) in 20 mL of CH_2Cl_2 was treated with HOBt (1.16 g, 8.6 mmol) in ice bath for 15 min, DCC (1.78 g, 8.6 mmol) was added, continued to react at 0 °C for 15 min and then at room temperature for 60 min, whereupon it was added to the TFA salt of Lys(Cbz)-Gaba-Opa. DIEA was used to adjust the pH to 7.0. After 90 min, the reaction mixture was disposed in a similar way as that of **1b**, and evaporated in *vacuo* to give the crude solid. It was purified by silica gel chromatography and eluted stepwise with CH_2Cl_2, 1%, 2-12%, 15% CH_3OH/CH_2Cl_2 to afford the tripeptide as a white solid **1c**. Rf = 0.52 (CH_3OH : CH_2Cl_2 = 20:80). Yield 79%. LRMS [MH^+] = 683. Analytical for $C_{36}H_{50}N_4O_9$, C, 63.33, H, 7.38, N, 8.21. Found C, 63.78, H, 7.79, N, 8.03.

Boc-Val-Lys(Cbz)-Gaba-OH **1d**: The tripeptide **1c** (2.0 g, 2.9 mmol) was dissolved in glacial acetic acid (100 mL) and water (15 mL). Zn powder (12 g) was added in small portions. After stirring overnight, the mixture was filtered and the solvents were removed in *vacuo*. The residue was washed successively with EDTA-Na_2 (2.5%, 70 mL), water (20 mL) and dired over P_2O_5 in *vacuo* to afford a white solid **1d**, which was used in subsequent steps without further purification.

Cyclo[Val-Lys(Cbz)-Gaba] **1e**: The free acid **1d** (1.0 g, 1.78 mmol) was dissolved in 70 mL of CH_2Cl_2 and esterified with pentafluorophenol (pfp; 0.98 g, 5.31 mmol) and DCC (0.55 g, 2.67 mmol) at 0℃ for 20 min and then reacted at room temperature for 4.0 hours. The reaction mixture was filtered, diluted with 250 mL of CH_2Cl_2 and 50 mL of EtOAc, and washed successively with 20 mL of saturated $NaHCO_3$ and 20 mL of water, dried over $MgSO_4$. The solid obtained was treated with TFA/CH_2Cl_2 (1.2:1) for 50 min and the solvents were removed in *vacuo*. The TFA salt obtained was dissolved in

dioxane and injected *via* a syringe pump into a stirred solution of dioxane (400 mL), pyridine (200 mL), and ethanol (15 mL) maintained at 85°C for 8 hours. The solvents were removed in *vacuo* and the residue redissolved in CH_2Cl_2 (350 mL), then the solution was washed successively with 50 mL 10% citric acid, 50 mL of saturated $NaHCO_3$, 30 mL water and dried over $MgSO_4$, evaporated to give a crude solid. It was purified by silica gel chromatography by stepwise elution with CH_2Cl_2, 1%, 2-12% CH_3OH/CH_2Cl_2 respectively to give a white solid **1e**. **1e** (20 mg, 44.8 μmol) was dissolved in a solution of dioxane (3 mL) and MeOH (1.5 mL) to which a drop of concentrated HCl was added. The mixture was hydrogenated over 10% Pd-C for 90 min, filtered over celite and the solvents were removed in *vacuo*. DMQ-MA (13 mg, 50 μmol) in 1 mL DMF was stirred with Pfp (13 mg, 180 μmol) and DCC (12 mg, 58 μmol) was added, stirred at 0°C for another 20 min and at room temperature for 6 hours. The reaction mixture was added to the deblocked cyclic peptide prepared above and triethylamine (TEA) was used to adjust the pH to 9.0. The reaction was allowed to proceed for 18 hours and the solvents were removed in *vacuo*. The crude product was purified by silica gel chromatography and eluted stepwise with CH_2Cl_2, 1%, 2-12%, 15% $CH_3OH/CHCl_3$ to afford a purple solid Cyclo[Val-Lys(DMQ-MA)-Gaba] **1f**. Rf = 0.53 (CH_3OH : CH_2Cl_2 = 20:80). Yield 31%. LRMS [M+23] = 576 (Determination with FAB Mass)

Hydroxyl radical assay by rhodamine B degradation

Hydroxyl radicals were quantitated by following degradation of the dye, rhodamine B, in the presence of Cyclo[Val-Lys(DMQ-MA)-Gaba]. The reaction was monitored at 554 nm in phosphate buffer (10 mmol/L, pH = 7.0). The change in concentration of rhodamine B ($\varepsilon = 10.7\times10^4\ M^{-1}\ cm^{-1}$) at varying times is a direct measure of the concentration of hydroxyl radicals produced in the reaction mixture. 20 μL Cyclo[Val-Lys(DMQ-MA)-Gaba] were added to 2 mL rhodamine B each time, there was a sudden decrease of the absorbance of rhodamine B, which indicated that hydroxyl radical was produced quickly (within 10 s). Hydroxyl radicals were detected over the time of the experiment in the presence of drugs indicating that such reactive oxygen species (ROS) were formed under these conditions.

The effect of the drugs on the fluorescence spectra of the rhodamine B was determined. The variation of the spectra was evaluated by monitoring the changes on the intensity of 584 nm of the rhodamine B. The decrease of fluorescence spectra of the rhodamine B was very quick. The result indicated that there was interaction between the dye and the produced hydroxyl radicals, which led to the degradation of the dye.

Investigation of the hydroxyl radical by ESR spectra

In order to testify that the action mechanism of the potential anti-cancer drug, we investigated if the hydroxyl radical was generated when the drug was mixed with ascorbic acid by ESR spectra. From the results of the spectra, we found the characteristic 1:2:2:1 peak of DMPO-OH spin adduct. There were also strong ascorbyl

radical signals in the spectra. The ascorbic acid competed the hydroxyl radicals with DMPO to form spin adduct. The results showed that when the concentration of the AH_2 was lower than 150 μmol/L, the signal of the DMPO-OH adduct increased with the increase of the concentration of AH_2, while it was over 150 μmol/L, the signal decreased.

Results and Discussion

The C-terminal carboxyl group was protected by the phenacyl group (Opa) since it was stable to TFA used in the cleavage of the Boc group prior to the coupling reactions[5]. The Opa group could be readily removed by Zn/acetic acid before the esterification of the C-terminal carboxyl group with pentafluorophenol without affecting all other protecting groups. The non-chiral γ–aminobutyric acid residue was chosen as the C-terminal residue in order to minimize racemization during the cyclization process. Cyclization using the elevated temperature procedure in dioxane-pyridine-ethanol afforded the protected cyclic peptide cyclo[Val-Lys(Cbz)-Gaba]. After removing Cbz group by catalytic hydrogenation in the presence of Pd-C 10%/HCl, the cyclic tripeptide hydrochloride salt was alkylated *in situ* with the pentafluorophenyl ester of DMQ-MA to give the conjugate. The synthesis of the cyclicpeptide-DMQ-MA conjugate appeared to be rather involved since the use of DCC/HOBt method, direct DCC coupling, or the succinimide method failed to give the desired products. FAB MS determined the cyclic peptide-DMQ-MA conjugate. Maintenance of the temperature at 85°C was crucial and little or no racemization occured under these cyclization conditions[6].

We also used the rhodamine B degradation method and ESR spectra employing the spin trap DMPO to confirm the reactive oxygen species hydroxyl radicals. This work provides the proof of the action mechanism of the potential anti-cancer drugs DMQ-MA-peptides.

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