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Original Article

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Selective tyrosine conjugation with a newly synthesized PCB -TE2A-luminol bifunctional chelator

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ABSTRACT

Selective amino acid conjugation of bulky antibodies is a valuable asset for real-time diagnosis and therapy. However, selective conjugation incorporating a chelate-bearing radioactive atom into an antibody without affecting its immunoreactivity is a challenging task. A bifunctional chelator (BFC), a selective amino acid-targeting probe, and a linker have been developed to overcome this problem. Here, we report the synthesis of a novel propylene cross-bridged chelator (PCB)–1,8-N,N′-bis-(carboxymethyl)-1,4,8,11-tetraazacyclotetradecane (TE2A)–luminol BFC via a click reaction and radiolabel it with a ^{64}Cu ion for tyrosine-selective conjugation of trastuzumab. In the initial optimization study, we tried different oxidative addition conditions such as electro-oxidation, hemin, horseradish peroxidase, iodogen tube, chloramine-T, and iodo beads. In this study, up to 82% of ^{64}Cu-PCB-TE2A-luminol was conjugated with the antibody in an iodo bead-catalyzed oxidative addition reaction with an isolated yield of 24.4%.

Key Word: Bifunctional chelator, PCB-TE2A-luminol, trastuzumab, tyrosine-selective conjugation.

Introduction

Radiolabeling of antibodies provides valuable, real-time information to treat various diseases and monitor in vivo processes. However, direct ionic radiometalation of antibodies is not possible. Instead, bifunctional chelators (BFCs) are used as supports to connect radiometals and antibodies (1). Generally, antibodies are highly sensitive and are easily inactivated or aggregate due to strong acidic or basic conditions, elevated reaction time, temperature, radioactivity, and organic solvents (2, 3). Therefore, the development of stable radiolabeling techniques and selective amino acid conjugation of antibodies, while preserving their immunoreactivity, is important for the construction of antibody-drug conjugates (4).

Although genetic engineering is the easiest method to incorporate reactive functional groups in antibodies for homogenous selective conjugation (5, 6), direct modification of native proteins is another approach. Most of the conjugation sites in antibodies are lysine (Lys) and cysteine (Cys) residues that are highly distributed over the antibody surface and consequently lead to heteroge-

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neity in the conjugation process with simultaneous loss of immunoreactivity. Recently, there have been tremendous advances in site-selective conjugation via naturally occurring amino acids (7). However, tyrosine-selective modification of antibodies has not been explored extensively. Because of their amphiphilic nature, few tyrosine residues are exposed on the protein surface. This makes tyrosine a good target for site-selective conjugation of antibodies. Recently, palladium-catalyzed phenol alkylation, diazonium salt–Mannich reaction, Nickel(II), Ceric(IV) ammonium molybdate-oxidative coupling, and a Ru $(bpy)_3$ photocatalyst have been used for tyrosine modification.

In 2009, Barbas et al. identified 4-phenyl 3H-1,2,4-triazole-3,5(4H)-diones (PTADs) as potential candidates for tyrosine-selective conjugation through a click-like, ene-type reaction for the functionalization of peptides, carbohydrates, and other bioactive small molecules (8, 9). However, PTADs are unstable under physiological conditions and generate isocyanate as a by-product upon decomposition, which is highly electrophilic and reacts with N-terminal amine groups resulting in relatively low selectivity. Recently, Nakamura et al. utilized luminol as an alternative probe for tyrosine conjugation in the presence of a hemin/H_2O_2 catalyst (10) because the oxidized form of luminol shows analogs structure of PTAD. To our knowledge, no radiolabeling methods using luminol have been reported for tyrosine-selective conjugation to date. Therefore, we synthesized a propylene cross-bridge (PCB) BFC connected with a luminol probe via a copper-catalyzed click reaction. The PCB allows ^{64}Cu radiolabeling and conjugation of luminol to trastuzumab using this oxidative addition methodology.

Materials and Methods

1. General

All ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 on a 500 MHz AVANCE III Bruker Bio Spin instrument and δ (chemical shift) presented as parts per million (ppm), with multiplicities denoted as singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), and multiplet (m). The coupling constant *J* was measured in Hz (Hertz). A mass spectrometer (JEOL JMS700, Jeol, Tokyo, Japan) was used to perform high-resolution mass spectrometric (HRMS) analyses. All chemicals and solvents purchased from Sigma-Aldrich (USA) and Thermo Fisher Scientific (USA) were used without further purification. Trastuzumab was procured from Roche (Basel, Switzerland) and phosphate-buffered saline (PBS) from Hyclone. Copper-64 was produced using an MC50 cyclotron (Scanditronix, Vislanda, Sweden) at the KIRAMS (Korea Institute of Radiological and Medical Sciences) in Seoul, South Korea, via a ^{64}Ni (*p,n*) ^{64}Cu nuclear reaction. Waters 600 high-performance liquid chromatography (HPLC) series (USA) and Agilent HPLC systems were used for purification. Radio-thin layer chromatography (TLC) data were analyzed using a Bioscan 2000 imaging scanner (USA).

Synthesis of 6 (and 7)-((6-azidohexyl)oxy)-2-methyl-2,3-dihydrophthalazine-1,4-dione (isomer mixture) (4)

Compound **4** was synthesized using a reported procedure (10). Compound **3** (720 mg, 2.35 mmol) was dissolved in dry THF (50 mL) in 100 mL of round bottom flask , acetic anhydride (1100 mg, 10.78 mmol) added dropwise to the reaction mixture, and the solution stirred overnight at ~ 80 °C. The solvent was removed under reduced pressure using a rotary evaporator. The residual viscous liquid was dissolved in dry ethanol (30 mL), and methylhydrazine hydrochloride (322.5 mg, 7.0 mmol) was added to the reaction mixture. The mixture was stirred for 6 h

under reflux conditions and the reaction quenched with water. Subsequently, the reaction mixture was extracted (3 x 50 mL) with ethyl acetate and washed with brine (100 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using hexane and ethyl acetate (1:1) to afford compound 4 as a white solid (625 mg, yield 85%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.38 - 1.48 (m, 4H), 1.54 - 1.59 (m, 2H), 1.75 - 1.79 (m, 2H), 3.32 - 3.35 (m, 3H), 3.53 - 3.55 (m, 3H), 4.13 - 4.15 (t, J = 7 Hz, 2H), 7.42 - 7.44 (dd, J = 8 Hz, 1H), 7.87 - 7.88 (d, J = 8.5 Hz,1H), 11.48 (s, 1H) ppm.

Synthesis of PCB-TE2A-luminol (5) via click reaction

PCB-TE2A-alkyne (10 mg, 0.026 mmol) was taken in a small glass vial with a magnetic stir bar, and azide-tethered luminol probe compound **4** (9.64 mg, 0.032 mmol) dissolved in H_2O/t-butanol/DMSO 1:1:1 ratio (0.6 mL) was added to the vial. Subsequently, sodium ascorbate (7.6 mg, 0.039 mmol) and $CuSO_4$ · $5H_2O$ (15.8 mg, 0.01 mmol) were added to the reaction mixture at room temperature. The reaction mixture was stirred at room temperature for another 48 h and then lyophilized. The crude reaction mixture was mixed with MeOH and centrifuged. The supernatant solution was transferred to another vial and purified using a Waters HPLC reverse-phase C18 column (10 μm, 10 mm x 250 mm). The flow rate was kept constant at 1.8 mL/min, and a gradient consisting of water (A) and acetonitrile (B) was used for the purification of the compound. The proportion of B was increased from 20% to 40% (0 - 10 min), then to 60% (25 min) and 80% (30 min) before decreasing to 20% (40 min). Compound **5** was obtained by lyophilizing the collected compound fractions. The isolated yield was 4.46 mg (22.5%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 1.23 - 1.31 (m, 2H), 1.45 - 1.46 (m, 3H), 1.74 - 1.86 (m, 6H), 2.12 - 2.24 (m, 3H), 2.51 (m, 1H), 2.71 - 2.74 (m, 7H), 3.02 - 3.15 (m, 5H), 3.33 - 3.53 (m, 3H), 3.55 (s, 3H), 4.11- 4.14 (m, 5H), 4.33 (t, J = 7 Hz, 3H), 7.41 - 7.43 (dd, J = 2.5 Hz, J = 6 Hz 1H), 7.57 (d, J = 2.5 Hz,1H), 7.88 - 7.89 (d, J = 9.0 Hz,1H), 7.96 (s, 1H), 10.35 (s, 1H) ppm. ^{13}C NMR (125 MHz, DMSO-d_6): δ 22.55, 22.82, 25.28, 26.00, 26.60, 28.70, 29.40, 29.70, 30.08, 38.10, 40.43, 49.70, 50.37, 60.01, 68.51, 108.46, 122.43, 123.01, 126.90, 131.23, 144.21, 157.54, 162.02, 173.20 ppm. HRMS (ESI) Expected mass $C_{35}H_{53}N_9O_7$ $[M + H]^+$: 712.4141; observed mass: 712.4142.

Radiolabeling of PCB-TE2A-luminol

The PCB-TE2A-luminol (compound **5**) chelate (1 mg) was disolved in 1 mL of DMSO to prepare a stock solution (1 μg/μL). The PCB-TE2A-luminol was complexed with ^{64}Cu by mixing $^{64}CuCl_2$ (37 - 185 MBq) in 0.01 M HCl (5 - 10 μL) with the chelator solution (10 - 20 μg) in 0.1 M NH_4OAc buffer (pH 8.0, 100 μL). The solution was incubated at 80 °C for 1 h in a thermomixer (Eppendorf) with agitation at 800 rpm. The radiolabeling was confirmed by radio-TLC using Merck C18 TLC plates (1:1) ACN/H_2O (R_f = 0.17). Purification was performed using a Waters HPLC reverse-phase C18 column (5 μm, 4.6 mm x 150 mm) The flow rate was kept constant at 1 mL/min. The mobile phase consisted of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B). A gradient was used for the purification, with a gradual increment in 0.1% TFA acetonitrile (B) from 0 - 20% (5 min) to 20 - 90% (5 - 25 min) and then decreasing to 90 - 0% (30 - 40 min). The purity of the isolated radioactive ^{64}Cu-PCB-TE2A-luminol complex was checked using the Agilent HPLC reverse-phase C18 column (5 μm, 4.6 mm x 150 mm) The flow rate was kept constant at 1 mL/min. The mobile phase consisted of 0.1% TFA in water (A) and 0.1% TFA acetonitrile (B), with a gradient involving

a gradual increment in 0.1% TFA acetonitrile (B) from 0 - 20% (15 min) to 20 - 90% (15 - 30 min) and decreasing to 90 - 0% (30 - 40 min).

Tyrosine-selective conjugation

Trastuzumab (35 μg in 10 μL of PBS) was added to the ^{64}Cu-PCB-TE2A-luminol (27.75 MBq) in PBS (pH 7.4, 100 μL); the oxidant was then added to the reaction mixture and incubated at 25°C for 30 - 60 min. The progress of conjugation was monitored by using instant radio-TLC on silica gel (SG) medium developed in (1:3) 0.1 M NH_4OAc/MeOH (target R_f = 0) with a conjugation yield of 82%. The radiolabeled antibody was loaded (25.0 MBq) onto a centrifugal filter unit with a molecular cutoff (MWCO) of 50,000 (Sartorius Vivacon 500 μL, Regenerated Cellulose, Millipore Corp., Bedford, MA, USA) and centrifuged at 12,000 rpm for 7 min. The filter unit was washed with 200 μL of PBS three times, and 6.64 MBq of radiolabeled antibody conjugate was collected in PBS. The radiochemical purity of the conjugate was confirmed to be 100% by instant radio-TLC on silica gel (SG) medium developed in the (1:3) 0.1 M NH_4OAc/MeOH solvent system. ^{64}Cu-PCB-TE2A-luminol-trastuzumab remained at the base line (R_f = 0), whereas ^{64}Cu-PCB-TE2A-luminol eluted with the solvent front with R_f = 1.0 (Figure 6).

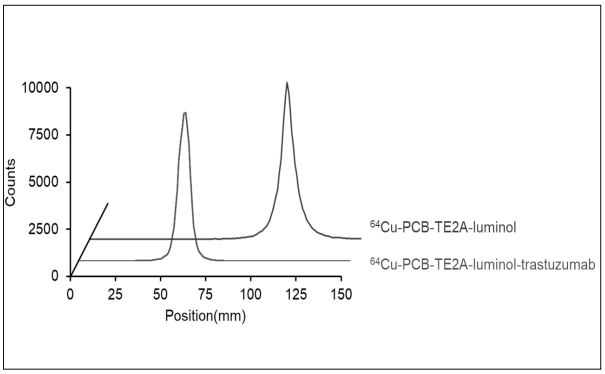


Figure 6. Radio-TLC profile of ^{64}Cu-PCB-TE2A-luminol and ^{64}Cu-PCB-luminol-trastuzumab. All iTLC plates developed using mobile phase consisting of (1:3) 0.1 M NH_4OAc/MeOH; ^{64}Cu-PCB-luminol-trastuzumab remained at the base line (R_f = 0), whereas ^{64}Cu-PCB-TE2A-luminol showed R_f = 1.0.

Results and Discussion

Synthesis of azide conjugate probe for click reaction (4)

The designed azide conjugate luminol probe was synthesized using a 3-step process (Scheme 1A) with an overall yield of 79%. In the first step, the hydroxy functional group in 4-hydroxy-dimethyl phthalate compound 1 was functionalized with 1-azido-6-bromohexane. Then, compound **2** was hydrolyzed with alkaline MeOH solution to form compound **3**. Compound **3** was refluxed with acetic anhydride overnight, after which all acetic anhydride was removed under reduced pressure. The residue was reacted with methyl hydrazine to form the azide-conjugated luminol probe 4 with 85% yield. The synthesized compound was characterized by ^{1}H NMR and its spectrum was found to match reported data (Figure 1).

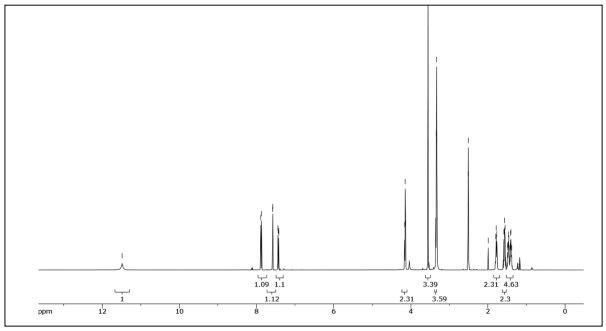
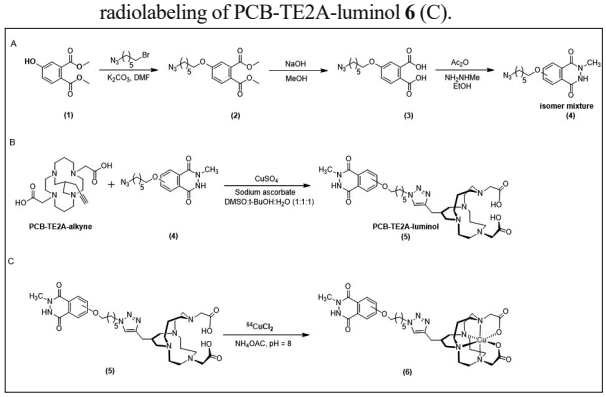


Figure 1. ^{1}H NMR of azide conjugate luminol probe (4).

Scheme 1. Synthetic route of azide conjugate probe **4** for click reaction (A), PCB-TE2A-luminol **5** via click reaction (B), and radiolabeling of PCB-TE2A-luminol **6** (C).



Preparation of the chelator tether with luminol probe

The synthesized compound **4** was readily linked to the PCB-TE2A-alkyne chelator via a copper-catalyzed (CuAAC) click reaction with the reactive terminal alkyne (Scheme 1B). As copper complex formation was only observed with the PCB chelate at higher temperature, the copper-catalyzed reaction was not expected to affect the click reaction conjugate formation. The newly isolated compound **5** (PCB-TE2A-luminol) was confirmed via spectroscopy analysis using ^{1}H NMR (Figure 2), ^{13}C NMR (Figure 3), and HRMS (Figure 4).

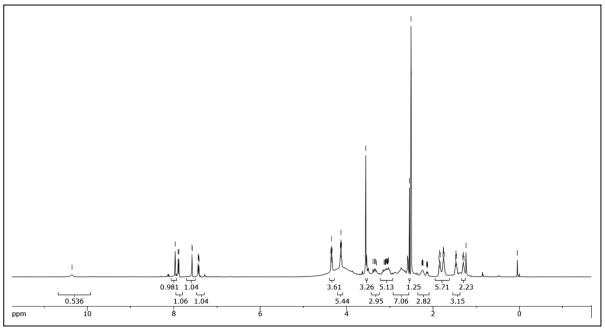


Figure 2. ^{1}H NMR of PCB-TE2A-luminol (5).

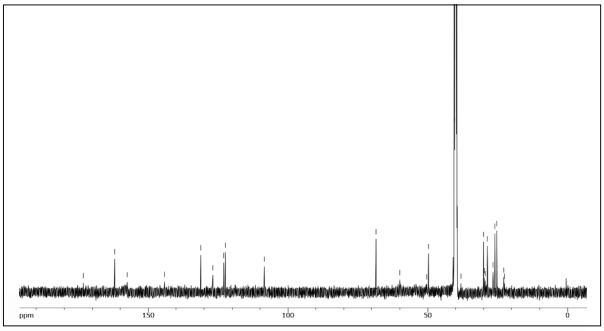


Figure 3. ^{13}C NMR of PCB-TE2A-luminol (5)

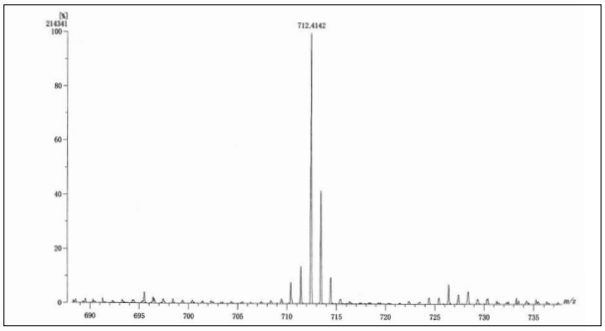


Figure 4. HRMS spectrum of PCB-TE2A-luminol (5)

Preparation of ^{64}Cu-Radiolabeled PCB-TE2A-luminol

After successful synthesis of PCB-TE2A-luminol, compound **5** was radiolabeled with $^{64}CuCl_2$ at 80°C using 0.1 M NH_4OAc (pH 8.0, 100 μL) (Scheme 1C), resulting in 79.24% radiolabeling conversion. The pure radiolabeled chelator was isolated using Waters HPLC with a yield of 49.47% and radiochemical purity of 97.4% and used further for antibody-selective conjugation (Figure 5).

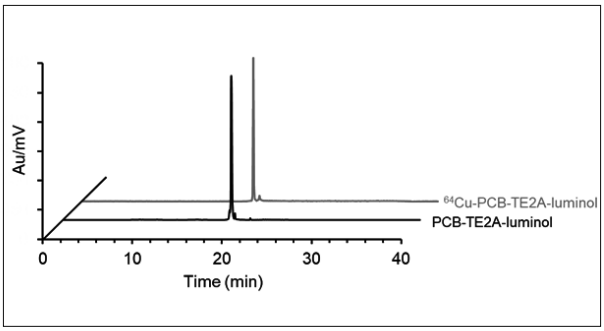


Figure 5. HPLC profile of PCB-TE2A-luminol and radio-HPLC profile of ^{64}Cu-PCB-TE2A-luminol under 254 nm. C18 column (5 μm, 4.6 mm x 150 mm) The flow rate is kept constant at 1 mL/min. Mobile phase consisting of 0.1% TFA in water (A) and 0.1% TFA acetonitrile (B) used for checking the purity, with slow increment of 0.1% TFA acetonitrile (B). Method: increase in B from 0–20% (15 min) to B 20–90% (15–30 min) and then decrease to 90–0% (30–40 min).

Preparation of selective tyrosine conjugation

Luminol is a chemiluminescence reagent used to detect trace amounts of blood in criminal investigations based on its oxidation of luminol in the presence of hemoglobin(catalyzed)/H_2O_2 (oxidant). The oxidized form of luminol shares structural features with PTADs. Based on this observation, Nakamura et al. used various oxidation conditions for the selective conjugation of radiolabeled ^{64}Cu-PCB-TE2A-luminol to trastuzumab (Herceptin) on the tyrosine moiety (Scheme 2). Initially, we started our investigation using an electro-oxidation method using a 1.5 V battery in Tris buffer, pH 7.4, at 0 °C [CAUTION: as this reaction generates a high amount of heat during the reaction, start this at 0 °C]. The progress of the reaction was monitored by instant radio-TLC on silica gel (SG) medium developed in a (1:3) 0.1 M NH_4OAc/MeOH solvent system.

However, no conjugation was observed with the antibody in this method. We then used hemin (9) catalyst (1 mM, 20 μL) with H_2O_2 (1 mM, 10 μL) in PBS; 22.15% conjugation was observed. To improve the conjugation further, we used horseradish peroxidase (11) (1 mM, 4 μL)/H_2O_2 (250 μM, 1 μL) in Tris buffer. However, no conjugation was observed. Therefore, we tried oxidative conjugation with iodinating oxidants, such as iodogen tube, chloramine-T (25 μg), and iodo beads (2 beads), in PBS. Interestingly, we observed that iodo beads resulted in 80.65% conjugation conversion; iodogen tube led to 1.32% conjugation; and chloaramine-T to 7.23% conjugation (Table 1). After successfully identifying the optimal conditions, we repeated this reaction and purified this reaction mixture using a Sartorious Vivacon-50 kDa MWCO membrane. The immunoconjugate was loaded onto the membrane, and the membrane washed three times with 200 μL PBS and centrifuged at 12,000 rpm for 7 min. The isolated yield was 24.4% with purity of 100%. Most of the conjugate was stuck on the filtration vial, which could be due to aggregation of the antibody or overoxidation.

Scheme. 2 Schematic representation of selective tyrosine conjugation.

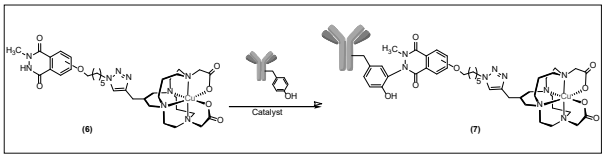


Table 1. Optimization of $_{64}$Cu-PCB-TE2A-luminol tyrosine-selective conjugation with trastuzumab.

Trail	Catalyst	Oxidant	Buffer	Radio TLC conversion
1	Electro oxidation	-	Tris-Buffer	ND
2	Hemin	H_2O_2	PBS	22.1
3	Horseradish peroxidase	H_2O_2	Tris-Buffer	ND
4	Iodogen tube	-	PBS	1.3
5	Chloramine-T	-	PBS	7.2
6	Iodo bead	-	PBS	80.6

Unless otherwise mentioned, all the reactions were carried out using ^{64}Cu-PCB-TE2A-luminol (100μCi) and trastuzumab (35 μg) at 25°C, 750 rpm for 30 min. All iTLC plates were developed using mobile phase consisting of 1:3 0.1 M NH_4OAc/MeOH. ND-Not detected

Conclusion

We have successfully synthesized a new, luminol-tethered, propylene cross-bridge, bifunctional chelator for selective tyrosine conjugation. In our initial oxidative addition trials, we found that using iodo beads resulted in a promising conjugation yield, although the isolated yield was very low, and most of the conjugate was adherent to the filter membrane. Currently, we are focusing on improving the conjugation yield, reducing the adhesion of the radioimmune conjugate to the filter membrane, and identifying the number of activated tyrosine residues in the trastuzumab via matrix-assisted laser desorption/ionization mass spectrometry. Further, we plan to utilize this radiolabeled conjugate for use in a tumor model for diagnostic and therapeutic purposes in the future.

Acknowledgments

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