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Phosphinyl- and Phosphinothioylamino Acids and Peptides. VI. The Protection of the Hydroxyl Function in the Tyrosine Side-chain by the Dimethylphosphinothioyl Group

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The use of the dimethylphosphinothioyl(Mpt) group for the protection of the hydroxyl function in the tyrosine side-chain was studied. *N,O*-Bis(Mpt)tyrosine was obtained directly by a Schotten-Baumann-type reaction of Mpt–Cl with tyrosine. The *O*-Mpt group was stable under acidic conditions, and it could be removed easily by alkaline hydrolysis or ester-exchange reaction. The usefulness of this new protecting group for the peptide synthesis was shown in the solid-phase synthesis of [D-Ala^2, L-Leu^5]-enkephalin(H–L-Tyr–D-Ala–Gly–L-Phe–L-Leu–OH) and its *N*-allyl derivative.

In the previous papers from our laboratory it was reported that a series of phosphinothioyl groups, such as diphenylphosphinothioyl(Ppt) and dimethylphosphinothioyl(Mpt), was useful as acid-labile amino-protecting groups for amino acids.[1–5] In accordance with the well-known acid lability of the phosphorus-nitrogen bond, these protecting groups could be removed by such mild HCl reagents as 0.2 M(1 M= 1 mol dm^{-3}) HCl in dichloromethane containing 0.2 M of triphenylphosphine(TPP). Since phosphinothioyl chlorides produced by cleavage do not react with the aromatic rings of tryptophan and tyrosine, the phosphinothioylamino acids have been successfully utilized for the synthesis of tryptophan homooligomers[6] and enkephalins.[7] When these groups are introduced into such other functional groups as hydroxyl and mercapto groups, they can be expected to become alkali-labile protecting groups according to the nature of the P–O and P–S bonds. As the first example of this line of investigation, the protection of the hydroxyl function in the tyrosine side-chain was attempted.

Tyrosine is a key amino acid in many biologically active peptides, such as enkephalin,[8,9] angiotensin II,[10] and gastrin.[11] A strategy which uses no side-chain protecting group for the tyrosine OH function has been recommended for the synthesis of a luteinizing hormone-releasing hormone,[12] and our previous syntheses of [L-Leu^5]-enkephalin and its analog were performed according to this strategy. However, the OH function of tyrosine should be protected for the synthesis of larger peptides. Benzyl ether-type protecting groups have been widely used, although the undesirable rearrangement to 3-benzyltyrosine during deprotection by acid[13] is one of the most hazardous side-reactions in peptide synthesis. In this report we would like to propose a new type of protecting group which would enable the direct alkylation of tyrosyl peptides.

Results and Discussion

A reaction of dimethylphosphinothioyl chloride (Mpt–Cl) with ethyl L-tyrosinate(H–L-Tyr–OEt) in the presence of triethylamine (TEA) gave a mixture of *N*-mono- and *N,O*-bis(Mpt)-L-tyrosine ethyl esters (**1** and **2**), which could then be separated by silica-gel column chromatography. When **2** was treated for 3 h with 0.2 M HCl in dichloromethane containing 0.1 M of TPP at room temperature, the *N*-Mpt group was removed selectively to give ethyl *O*-Mpt-L-tyrosinate hydrochloride(**3**) quantitatively. On the other hand, when the mixture of **1** and **2** was hydrolyzed by 1 M aqueous sodium hydroxide solution, *N*-Mpt-L-tyrosine was obtained in a 65% yield.[5] These results clearly show that the *O*-Mpt group was, unlike the acid-labile *N*-Mpt group, stable under acidic conditions and removable under basic conditions.

$$\text{H-L-Tyr-OEt} \xrightarrow[\text{CHCl}_3]{\text{2Mpt-Cl+2TEA}} \underset{(\mathbf{1})}{\text{Mpt-L-Tyr(Mpt)-OEt}} + \underset{(\mathbf{2})}{\text{Mpt-L-Tyr(Mpt)-OEt}}$$

$$\mathbf{2} \xrightarrow{\text{HCl}} \underset{(\mathbf{3})}{\text{HCl·H-L-Tyr(Mpt)-OEt}}$$

In order to determine the utility of the *O*-Mpt derivative of tyrosine, the alkali-lability of the *O*-Mpt group was examined more precisely by means of the pH-controlled alkaline hydrolysis of **2**. At pH 10.3, the cleavage of the *O*-Mpt group was still slow, and 90% of the **2** was recovered after a reaction period of 8 h at room temperature. This result was utilized for the preparation of *N,O*-bis(Mpt)tyrosine(**4**), a key derivative for the synthesis of tyrosine peptides, as will be described below.

With an increase in the pH value, the hydrolysis rate of the *O*-Mpt group became much faster. In these experiments, the reaction products were analyzed by means of high-performance liquid chromatography (HPLC); the results are shown in Fig. 1. At pH 11.9, the *O*-Mpt was removed rapidly, while the carboxylate ester bond was almost unaffected.

$$\mathbf{2} \xrightarrow[\text{rapid}]{\text{OH}^-} \text{Mpt-L-Tyr-OEt} \xrightarrow[\text{slow}]{\text{OH}^-} \text{Mpt-L-Tyr-O}^- \quad (\text{pH } 11.9)$$

Then, various kinds of basic reagents were examined in order to determine the most proper conditions for the removal of the *O*-Mpt group. The results are summarized in Table 1. It should be noted that the reagents with nucleophilic oxygen were clearly

Table 1. Time required for the removal of the *N*-Mpt or *O*-Mpt group from **2**

Reagent[a]	Time[b]	
	N-Mpt of **2**[c]	*O*-Mpt of **2**[c]
0.2 M HCl/0.2 M TPP/CH_2Cl_2	15 min	Stable for 24 h
1 M HCl/CH_3CO_2H	15 min	Stable for 24 h
HBr/CH_3CO_2H	5 min	Stable for 24 h
CF_3CO_2H	60 min	Stable for 24 h
1 M NaOH:CH_3OH(1:1)	Stable for 24 h	5 min
Tesser's base[14]	Stable for 24 h[d]	5 min[d]
1 M TEA/CH_3OH(RT)	Stable for 48 h	Incomplete for 48 h (66%)[e]
1 M TEA/CH_3OH(reflux)	Stable for 12 h	12 h
1 M TEA/C_2H_5OH(RT)	Stable for 48 h	Incomplete for 48 h (7%)[e]
1 M TEA/C_2H_5OH(reflux)	Stable for 12 h	Incomplete for 12 h (13%)[e]
20% NH_2NH_2/CH_3OH[f]	Stable for 48 h	24 h

a) The reagents were used in 4 cm^3 portions. b) Detected by TLC. c) The **2** was used in 0.2 mmol portions. d) The **2** was used in a 0.1 mmol portion. e) Isolated yield. f) The reagent was used in a 2 cm^3 portion.

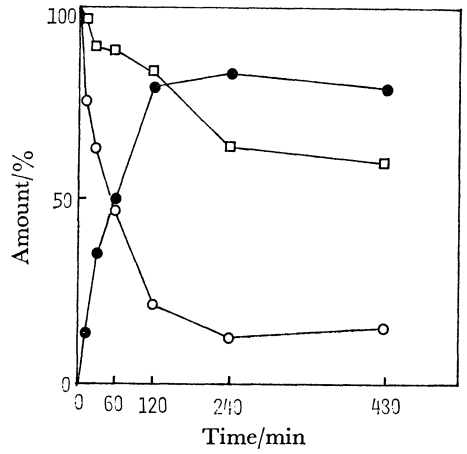
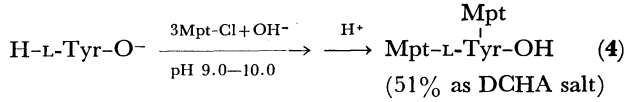


Fig. 1. Alkaline hydrolysis of **2** at constant pH. □: Decrease of **2** at pH 10.9, ○: decrease of **2** at pH 11.9, ●: increase of **1** at pH 11.9.

preferrable to those with nucleophilic nitrogen.

In order to confirm the usefulness of the *O*-Mpt group for the synthesis of tyrosine-containing peptides, the solid-phase syntheses of [D-Ala^2, L-Leu^5]-enkephalin(**5**) and its *N*-allyl derivative were attempted. The synthetic scheme is outlined in Fig. 2.

The procedures of solid-phase synthesis were the same as those previously described.[5] All the amino acids were incorporated as Mpt derivatives except for glycine, which was coupled as its diethylphosphinothioyl (Ept) derivative. *N,O*-Bis(Mpt)-L-tyrosine(**4**) was obtained directly from tyrosine by a Schotten-Baumann-type reaction. When tyrosine was allowed to react with 3 equiv. of Mpt–Cl with special care to keep the pH of the reaction mixture in the range between 9.0 and 10.0, **4** was obtained in a 51% yield



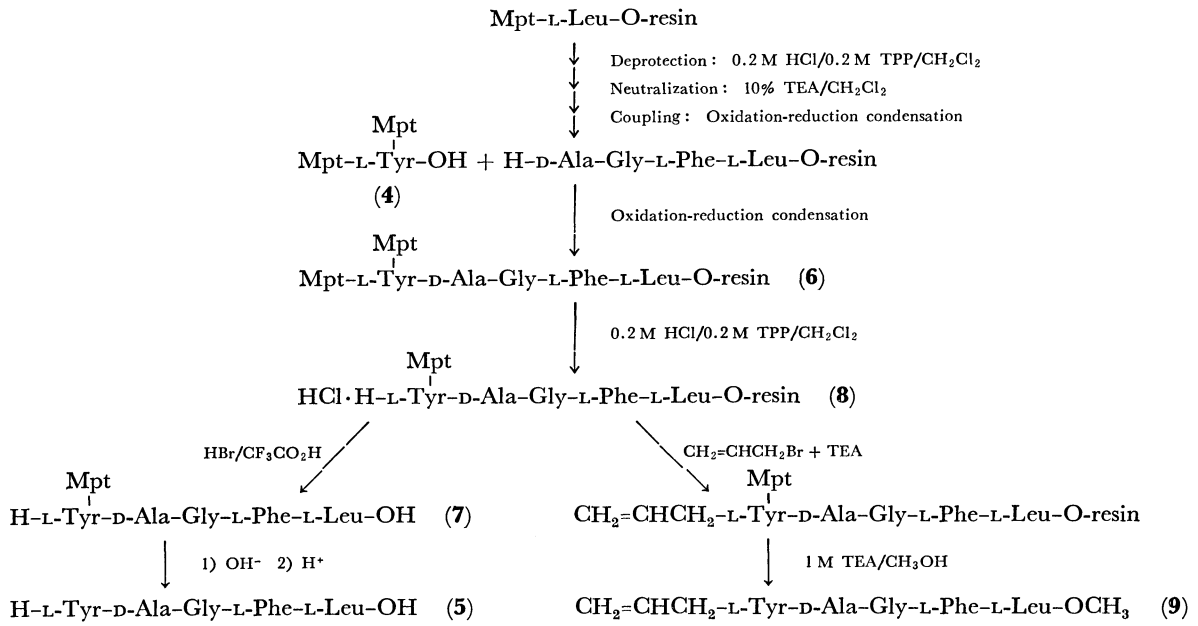


Fig. 2. Synthetic scheme for [D-Ala^2, L-Leu^5]-enkephalin and its *N*-allyl derivative.

as its dicyclohexylamine(DCHA) salt. All the couplings were mediated with the oxidation-reduction condensation method,[15] using tris(*p*-methoxyphenyl)-phosphine and 2,2'-dithiodipyridine. After the coupling of **4**, the *N*-Mpt group of *N,O*-bis(Mpt)pentapeptide resin(**6**) was selectively removed by the use of 0.2 M HCl solution in dichloromethane containing 0.2 M of TPP. Then, *O*-Mpt-[D-Ala^2, L-Leu^5]-enkephalin(**7**) was cleaved from the resin support by the use of HBr in trifluoroacetic acid. Pure **7** was obtained in a 53% yield as colorless crystals by purifying it by means of preparative thin-layer chromatography and successive gel chromatography on Sephadex LH-20. The removal of the *O*-Mpt group was performed by treating it with 1 M aqueous sodium hydroxide solution(1.1 equiv.) in methanol at room temperature overnight. Purification in a manner similar to that in **7** gave pure **5** in a 94% yield. Both **5** and **7** were homogeneous on thin-layer chromatography, and **7** gave satisfactory amino-acid ratios.

The biological activity of the **5** synthesized was measured by Professor Tetsuo Oka of Tokai University and found to be identical with that of an authentic sample by Mouse vas Deference assay and Guinea Pig Ileum assay.

For *N*-allylation, **8** was neutralized and further treated with 50 equiv. of allyl bromide in chloroform in the presence of TEA at room temperature for 12 h and then washed. The same treatments were repeated twice more. After thorough washing, the crude *N*-allylpentapeptide methyl ester(**9**) was cleaved from the resin support by transesterification with 1 M TEA in methanol, accompanied by the simultaneous removal of the *O*-Mpt group. After usual purification, pure **9** was obtained in a 38% yield, calculated from Mpt-L-Leu-resin. The structure of **9** was ascertained by NMR, MS, and amino-acid analysis. The acid hydrolyzate of **9** gave appropriate amino-acid ratios except for *N*-allyltyrosine, the amount of which could not be determined because of its low color value.[16]

N-Allyl-[L-Leu^5]-enkephalin is an interesting derivative of enkephalin because it shows partial agonistic and antagonistic activities,[17] but the synthesis of selectively alkylated tyrosyl peptides was not easy. This new method will be a useful route for these compounds. The syntheses of various kinds of alkylated derivatives of enkephalins are now in progress. The biological activity of these compounds will be reported by Professor Oka elsewhere.

Experimental

Thin-layer chromatography (TLC) was performed on silica-gel plates (Merck $60F_{254}$) in the following solvent systems: diethyl ether (R_f^1), chloroform–methanol–acetic acid (95:5:3, R_f^2), 1-butanol–acetic acid–water (4:1:1, R_f^3), chloroform–methanol–aqueous ammonia (60:30:5, R_f^4) and chloroform–methanol (9:1, R_f^5). The products were detected on TLC plates using ultraviolet light, iodine vapor, ninhydrin, and the Pauly reagent.

*N,O-Bis(dimethylphosphinothioyl)-L-tyrosine Ethyl Ester (**2**) and N-Dimethylphosphinothioyl-L-tyrosine Ethyl Ester (**1**) (Mpt-L-Tyr(Mpt)-OEt and Mpt-L-Tyr-OEt).* A solution of Mpt–Cl (25.71 g, 0.2 mol) in 50 cm^3 of chloroform was added to a suspension of H-L-Tyr-OEt·HCl (24.57 g, 0.1 mol) in 150 cm^3 of chloroform and 42 cm^3 (0.3 mol) of TEA at 0 °C. After having been stirred overnight at room temperature, the solution was evaporated *in vacuo*. The residue was dissolved in ethyl acetate. The solution was washed successively with water, an ice-cold 5% citric acid solution, water, a 5% sodium hydrogencarbonate solution, water, and a saturated sodium chloride solution, dried over anhydrous sodium sulfate, and evaporated. The oily residue was dissolved in dichloromethane and placed in a silica-gel column (3 cm × 50 cm). Elution with 600 cm^3 of dichloromethane gave **2** as colorless crystals; 26.11 g (66%). Mp 79—82 °C; $[\alpha]_D^{25}$ −12.5° (*c* 1, ethanol); R_f^1 0.37, R_f^2 0.69. Found: C, 45.43; H, 6.41; N, 3.69; P, 16.06%. Calcd for $C_{15}H_{25}NO_3P_2S_2$: C, 45.79; H, 6.40; N, 3.56; P, 15.75%. Further elution with the same solvent (about 3 dm^3) gave **1** as colorless crystals; 2.82 g (9%). Mp 68—69 °C; $[\alpha]_D^{25}$ −15.0° (*c* 1, ethanol); R_f^1 0.55, R_f^2 0.48. Found: C, 51.76; H, 6.77; N, 4.47; P, 10.32%. Calcd for $C_{13}H_{20}NO_3PS$: C, 51.81; H, 6.69; N, 4.65; P, 10.28%.

*O-Dimethylphosphinothioyl-L-tyrosine Ethyl Ester Hydrochloride (H-L-Tyr(Mpt)-OEt·HCl) (**3**).* Compound **2** (0.74 g, 2 mmol) was treated with 0.2 M HCl in dichloromethane containing 0.1 M of TPP. After having been kept standing at room temperature overnight, the solution was evaporated. The residue was distributed between water and diethyl ether, and the organic layer was extracted with 1 M hydrochloric acid several times. The combined aqueous layer and extracts were evaporated and dried over sodium hydroxide pellets *in vacuo*. The solid residue was washed with diethyl ether to give the product, 0.68 g (100%), which was then recrystallized from ethanol–diethyl ether to give pure **3** as colorless crystals; 0.56 g (83%). Mp 154—156.5 °C; $[\alpha]_D^{25}$ −15.0° (*c* 1, ethanol); R_f^2 0.17, R_f^3 0.62. Found: C, 45.40; H, 6.37; N, 4.25; P, 8.51%. Calcd for $C_{13}H_{21}NO_3PSCl\cdot 1/2H_2O$: C, 45.02; H, 6.68; N, 4.04; P, 8.93%.

*N,O-Bis(dimethylphosphinothioyl)-L-tyrosine Dicyclohexylamine Salt (Mpt-L-Tyr(Mpt)-OH·DCHA) (**4**).* L-Tyr (5.44 g, 30 mmol) was dissolved in 1 M sodium hydroxide solution (60 cm^3), and then Mpt–Cl (11.56 g, 90 mmol) in diethyl ether (30 cm^3) was added, drop by drop, at 0 °C. While the mixture was being stirred vigorously, 1 M sodium hydroxide solution was added carefully at a rate chosen so as to maintain the pH of the solution between 9.0 and 10.0 until no drop in the pH was observed. Then the solution was extracted twice with ethyl acetate. The aqueous layer was acidified with solid citric acid to pH 3—4 at 0 °C and was then extracted with ethyl acetate several times. The organic layer was washed with water, dried over anhydrous sodium sulfate, and evaporated *in vacuo*. The oily residue was dissolved in 40 cm^3 of ethyl acetate and treated with DCHA (6 cm^3, 30 mmol). DCHA salt was collected by filtration, washed with ethyl acetate and diethyl ether, and dried. Recrystallization from ethanol gave **4** as colorless crystals; 8.32 g (51%). Mp 169—170 °C; $[\alpha]_D^{25}$ −15.0° (*c* 1, ethanol); R_f^2 0.44, R_f^3 0.81. Found: C, 55.09; H, 8.00; N, 4.66%. Calcd for $C_{25}H_{44}N_2O_3P_2S_2$: C, 54.92; H, 8.11; N, 5.11%.

*Solid-phase Synthesis of H-L-Tyr(Mpt)-D-Ala-Gly-L-Phe-L-Leu-OH (**7**).* Mpt-L-Leu-resin was prepared from chloromethyl resin by the method of Gisin,[18] and the amount of esterified Leu was obtained as 0.33 mmol/g by means of amino-acid analysis. Mpt-L-Leu-resin (1 g) was placed in a reaction vessel of a Beckman Model 990 peptide synthesizer, after which synthesis was carried out according to the previously reported schedule,[5] using **4** at the final acylating step. After the final deprotection of the *N*-ter-

minal Mpt group, the *O*-Mpt-pentapeptide resin (**8**) was washed thoroughly with dichloromethane, ethanol, and diethyl ether, and dried *in vacuo*. The *O*-Mpt-pentapeptide was removed from the resin by anhydrous hydrogen bromide in trifluoroacetic acid (TFA) in the presence of 50 equiv. of anisole. The filtrate and TFA washings were combined and evaporated *in vacuo*. The residue was dried over sodium hydroxide pellets *in vacuo* and triturated with diethyl ether to yield the white solid. This crude peptide was then submitted to preparative silica-gel thin-layer chromatography using the chloroform–methanol–acetic acid (85:25:20) solvent system. The desired band (R_f=0.7—0.8) was eluted with methanol and evaporated *in vacuo*. The residue was again dissolved in a small volume of methanol and placed in a Sephadex LH-20 column (2×100 cm) monitored by means of UV absorbancy at 260 nm. The methanol eluate (flow rate 0.8 cm³/min) was collected as fractions of 4 cm³ each. The fractions (Nos. 34—43) containing a single component were collected and evaporated *in vacuo* to give **7** as colorless crystals; 0.116 g (53%). Mp 159—163 °C (dec); $[\alpha]_D^{25}$ −10° (*c* 1, methanol); R_f^3 0.68, R_f^4 0.66. Found: C, 55.15; H, 6.77; N, 10.24%. Calcd for $C_{31}H_{44}N_5O_7PS\cdot CH_3OH$: C, 55.40; H, 6.97; N, 10.09%. Amino-acid ratios in hydrolyzate obtained by the use of 6 M HCl at 110 °C for 24 h: $Tyr_{0.70}$, $Ala_{0.99}$, $Gly_{1.02}$, $Phe_{0.99}$, $Leu_{1.00}$. The low value for Tyr is attributable to the difficulty of the acid hydrolysis of *O*-Mpt–Tyr.

[D-*Ala*², L-*Leu*⁵]-*enkephalin (5)*. Compound **7** (0.020 g, 0.03 mmol) was dissolved in 1 cm³ of methanol. To this solution, 0.1 cm³ of a 1 M aqueous sodium hydroxide solution was added at 0 °C. After having been stirred overnight, the solution was neutralized and evaporated *in vacuo*. The residue was placed in a Sephadex LH-20 column (2× 100 cm), and the desired fractions were collected to yield **5** as colorless crystals (0.0179 g, 94%). Mp 182—184 °C (lit,[5] 182—184 °C); $[\alpha]_D^{25}$ −20° (*c* 0.1, methanol) (lit,[5] −25.0° (*c* 0.1, methanol)); R_f^4 0.50 (lit,[5] 0.49). Amino-acid ratios in hydrolyzate obtained by the use of 6 M HCl at 110 °C for 24 h: $Tyr_{0.97}$, $Ala_{1.02}$, $Gly_{0.99}$, $Phe_{0.99}$, $Leu_{1.02}$.

N-*Allyl*-[D-*Ala*², L-*Leu*⁵]-*enkephalin Methyl Ester (9)*. Compound **8** synthesized from Mpt-L-Leu-resin (0.5 g, Leu content: 0.30 mmol/g) as has been described above, was used. After neutralization, the resin was treated by the following steps: (1) washing three times with chloroform, (2) allylation with 50 equiv. of allyl bromide and TEA in chloroform for 12 h, (3) washing three times with chloroform, (4) repetition of step (2) and (3) twice more, (5) washing two times with *N*,*N*-dimethylformamide, (6) washing two times with ethanol, followed by drying *in vacuo* over phosphorus pentaoxide. For the removal from resin, the peptide resin was refluxed in methanol containing 1 M of TEA for 12 h and then filtered. The same procedure was repeated, after which all the filtrates and methanol washings were collected and evaporated. The residue was distributed between water and ethyl acetate, and the organic layer was extracted with 1 M hydrochloric acid three times. The aqueous layer was neutralized with solid sodium hydrogencarbonate at 0 °C and then extracted with ethyl acetate three times. The collected extracts were washed with water and dried over anhydrous sodium sulfate. The solution was evaporated *in vacuo*, and the residue was purified by means of preparative thin-layer chromatography using the chloroform–methanol (95:5) solvent system. The desired band (R_f=0.2—0.3) was eluted with methanol and evaporated *in vacuo*. The residue was dissolved in methanol again, placed in a Sephadex LH-20 column (2×100 cm), and eluted with methanol (flow rate 0.75 cm³/min). The desired fractions (Nos. 37—42; 4.5 cm³/fraction) were collected to yield **9** as colorless crystals (0.0723 g, 38% from Mpt-L-Leu-resin): Mp 155—156 °C; NMR (CD_3OD) δ=0.97 (6H, d), 1.27 (3H, d), 3.70 (3H, s), 4.97—5.77 (3H, m), 6.73, 7.04 (4H, m), and 7.28 (5H, s); MS (70 eV), *m/e* (relative intensity), 623 (M^+, 2), 515 (22), 176 (100), 120 (65), 107 (19), 91 (13), and 77 (5). R_f^3 0.75, R_f^5 0.52. Amino-acid ratios in hydrolyzate obtained by the use of 12 M HCl–propionic acid at 130 °C for 2 h: $Ala_{0.94}$, $Gly_{1.05}$, $Phe_{0.97}$, $Leu_{1.04}$. *N*-Allyl-Tyr was detected, but not measured because of its low color value.

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