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Synthesis of Haptens for Use in Immunoassays of Δ^4-3-Ketosteroids[1)]

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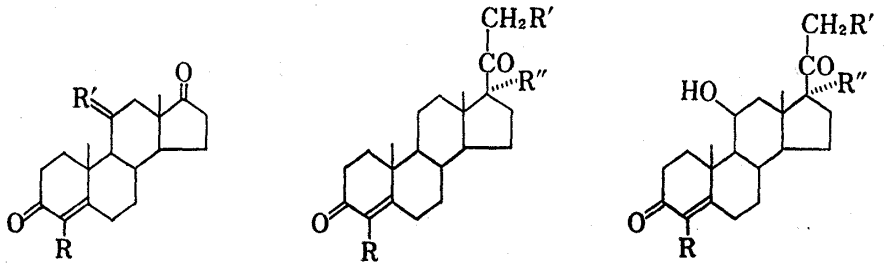
In order to develop specific and sensitive immunoassays, new carboxylated derivatives of androstenedione, 11β-hydroxyandrostenedione, adrenosterone, progesterone, 17α-hydroxyprogesterone, corticosterone, 11-deoxycorticosterone and 21-deoxycortisol were synthesized. The preparation of the 4-carboxymethylthio and 4-carboxyethylthio derivatives of the Δ^4-3-ketosteroids was carried out by base-catalyzed ring opening of the 4,5-epoxides with mercaptoacetic acid and mercaptopropionic acid, respectively. The *N*-succinimidyl esters of these carboxylated steroids were also prepared.

Keywords——Δ^4-3-ketosteroid; hapten for immunoassay; 4,5-epoxide ring opening; 4-carboxymethyl thioether; 4-carboxyethyl thioether; *N*-succinimidyl ester

Immunoassays of Δ^4-3-ketosteroids are frequently required in clinical chemistry.[2)] Antisera for use in the immunoassays have been elicited in animals by immunization with hapten molecules linked to a carrier protein. The specificity of antibodies is significantly influenced by the position on the steroid molecule used for conjugation to the carrier. In radioimmunoassay using ^{125}I-radioligands and enzyme immunoassay, the combination of antibody and labeled antigen is an important factor determining the sensitivity, because the antibody recognizes the bridge between the label and antigen. It is often found that satisfactory sensitivity cannot be obtained in a homologous system, where the same haptenic derivative is used for the immunogen as well as for the labeled antigen. For the purpose of improving sensitivity, heterologous systems are employed. The position C-4 in the Δ^4-3-ketosteroid molecule appears to be an attractive site for attachment of the carrier. In the previous papers of this series, we reported on the specificity and sensitivity in the enzyme immunoassays for cortisol[3)] and testosterone.[4)] The satisfactory results prompted us to develop immunoassay systems for other Δ^4-3-ketosteroids as well. This paper deals with the synthesis of derivatives having a thioether bridge at the C-4 position of androstenedione, 11β-hydroxyandrostenedione, adrenosterone, progesterone, 17α-hydroxyprogesterone, corticosterone, 11-deoxycorticosterone and 21-deoxycortisol.



1: R′=H$_2$
2: R′=OH, H
3: R′=O

4: R′=R″=H
5: R′=H, R″=OH
7: R′=OH, R″=H

6: R′=OH, R″=H
8: R′=H, R″=OH

a: R=H, b: R=SCH$_2$COOH, c: R=SCH$_2$CH$_2$COOH

d: R=SCH$_2$COON(succinimidyl), e: R=SCH$_2$CH$_2$COON(succinimidyl)

Chart 1

The Δ^4-3-ketosteroids (**1a**—**8a**) were converted into the epimeric 4,5-epoxides upon exposure to alkaline hydrogen peroxide. When the 4,5-epoxides were treated with mercapotacetic acid in alkaline media, ring opening took place to give the carboxymethyl thioethers (**1b**—**8b**). In the proton nuclear magnetic resonance (^{1}H-NMR) spectra of these compounds, a characteristic signal including a geminal coupling (J=*ca.* 14 Hz) was observed at an average value of 3.67±0.08 ppm, which was assigned to the 6α-proton. The methylene protons of the carboxymethylthio group resonate in the range of 3.3—3.5 ppm as a singlet or a four-line AB pattern. The 4-carboxyethyl thioethers (**1c**—**8c**) were also obtained in good yields by reaction with mercaptopropionic acid as a nucleophile.

Condensation of the carboxyl group of a hapten with the amino groups of a carrier protein, enzyme and ^{125}I-radioligand has been performed by the mixed anhydride and carbodiimide methods. However, these are not always satisfactory with respect to reproducibility. The use of *N*-succinimidyl ester for the labeling appears to be convenient and capable of overcoming the above problem by simplifying the reaction procedure.[5] Treatment of the carboxylated steroids with *N*-hydroxysuccinimide in 95% dioxane in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride afforded the desired *N*-succinimidyl esters (**1d,e**—**8d, e**) in yields of 50—80%. In the ^{1}H-NMR spectra, the signal for the protons of the succinimidyl group was observed at an average value of 2.84±0.04 ppm.

We described previously that a "bridge" heterologous system rather than "site" heterology is preferred in enzyme immunoassay[4] and the use of enzyme-labeled steroid prepared from a hapten having a bridge shorter than that used for antibody production is advantageous for obtaining increased assay sensitivity.[3a] Therefore, it is desirable to have available various haptens possessing different bridges at the same position. An ester type of bridge, such as a hemisuccinoyl or hemiglutaroyl function, can also be introduced into the C-4 position *via* the 4-hydroxy-Δ^4-3-ketosteroids, which are prepared by the acid-catalyzed ring opening of a 4,5-epoxide,[6] oxidation of a 3-keto-5β-steroid with molecular oxygen in the presence of potassium *tert*-butoxide,[7] or rearrangement of an oxime derivative.[8] The haptenic compounds obtained in this study may be useful in the development of practical immunoassays for the corresponding Δ^4-3-ketosteroids.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were determined in $CHCl_3$ unless otherwise specified. ^{1}H-NMR spectra were measured with a JEOL FX-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard.

Epoxidation of Δ^4-3-Ketosteroids——A mixture of the Δ^4-3-ketosteroid (**1a**—**8a**) (1 mmol), 30% H_2O_2 (0.4 ml), and 10% NaOH (1 ml) in MeOH (10 ml) was stirred at 0°C for 3 h. In some cases, additional amounts of the reagents and solvent were required. After neutralization with AcOH followed by removal of the MeOH under reduced pressure, the resulting mixture was extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and passed through an Al_2O_3 (10 g) layer on a sintered-glass funnel. The filtrate was evaporated down under reduced pressure. The product was purified, if necessary, by chromatography on silica gel, and used for the subsequent reaction without separation of 4,5-epoxide epimers.

General Procedures for the Preparation of 4-Thioether and *N*-Succinimidyl Ester——A solution of the 4,5-epoxide (1.7 mmol) in EtOH (2 ml)–dioxane (3 ml) was added to mercaptoacetic acid or β-mercaptopropionic acid (2.8 mmol) in 25% KOH (0.8 ml), and the resulting solution was stirred at room temperature under an N_2 gas stream for 2 h. After addition of H_2O followed by extraction with ether, the aqueous layer was acidified with 1 N HCl and extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. Recrystallization of the crude product from an appropriate solvent gave the 4-thioethers (**1b, c**—**8b, c**).

N-Hydroxysuccinimide (1.4 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (1.4 mmol) were added to a solution of the thioether (1 mmol) in 95% dioxane (10 ml), and the mixture was stirred at room temperature for 2 h. The resulting solution was diluted with AcOEt, washed with H_2O, and dried over anhydrous Na_2SO_4. The solution was passed quickly through an Al_2O_3 (10 g) layer on a sintered-glass funnel, and the filtrate was evaporated down. The product was purified, if necessary, by chromatography on silica gel, and recrystallized from an appropriate solvent.

4-(Carboxymethylthio)androstenedione (1b)——Colorless plates from aqueous MeOH. mp 181—182°C. $[\alpha]_D^{21}$ +173° (c=0.34). *Anal.* Calcd for $C_{21}H_{28}O_4S$: C, 66.99; H, 7.54. Found: C, 66.66; H, 7.60. ^{1}H-NMR ($CDCl_3$) δ: 0.94 (3H, s, 18-CH_3), 1.27 (3H, s, 19-CH_3), 3.39 (2H, s, SCH_2CO), 3.72 (1H, m, 6α-H). The *N*-succinimidyl ester **1d**: Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.92 (3H, s, 18-CH_3), 1.25 (3H, s, 19-CH_3), 2.80 (4H, s, succinimidyl), 3.50—3.92 (3H, 6α-H and SCH_2CO).

4-(2-Carboxyethylthio)androstenedione (1c)——Colorless leaflets from aqueous MeOH. mp 154—155°C. $[\alpha]_D^{20}$ +189° (c=0.35). *Anal.* Calcd for $C_{22}H_{30}O_4S$: C, 67.66; H, 7.74. Found: C, 67.38; H, 7.50. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, s, 18-CH_3), 1.26 (3H, s, 19-CH_3), 2.54 and 2.92 (each 2H, m, SCH_2CH_2CO), 3.72 (1H, m, 6α-H). The *N*-succinimidyl ester **1e**: Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.93 (3H, s, 18-CH_3), 1.26 (3H, s, 19-CH_3), 2.84 (4H, s, succinimidyl), 3.77 (1H, m, 6α-H).

4-(Carboxymethylthio)-11β-hydroxyandrostenedione (2b)——Colorless leaflets from acetone–hexane. mp 184—185°C. $[\alpha]_D^{19}$ +162° (c=0.28, EtOH). *Anal.* Calcd for $C_{21}H_{28}O_5S$: C, 64.26; H, 7.19. Found: C, 64.12; H, 7.20. ^{1}H-NMR ($CDCl_3$–CD_3OD (5: 1)) δ: 1.18 (3H, s, 18-CH_3), 1.51 (3H, s, 19-CH_3), 3.32 (2H, s, SCH_2CO), 3.67 (1H, m, 6α-H), 4.36 (1H, m, 11α-H). The *N*-succinimidyl ester **2d**: Colorless leaflets from CH_2Cl_2–hexane. mp 225—227°C. $[\alpha]_D^{19}$ +152° (c=0.10). *Anal.* Calcd for $C_{25}H_{31}NO_7S\cdot 1/2H_2O$: C, 60.22; H, 6.47; N, 2.81. Found: C, 60.34; H, 6.19; N, 3.06.

4-(2-Carboxyethylthio)-11β-hydroxyandrostenedione (2c)——Colorless plates from acetone–hexane. mp 192—193°C. $[\alpha]_D^{21}$ +198° (c=0.31). *Anal.* Calcd for $C_{22}H_{30}O_5S$: C, 64.99; H, 7.44. Found: C, 64.73; H, 7.21. ^{1}H-NMR ($CDCl_3$–CD_3OD (4: 1)) δ: 1.17 (3H, s, 18-CH_3), 1.49 (3H, s, 19-CH_3), 2.49 and 2.89 (each 2H, m, SCH_2CH_2CO), 3.63 (1H, m, 6α-H), 4.40 (1H, m, 11α-H). The *N*-succinimidyl ester **2e**: Colorless leaflets from CH_2Cl_2–hexane. mp 142—144°C. $[\alpha]_D^{19}$ +156° (c=0.10). *Anal.* Calcd for $C_{26}H_{33}NO_7S\cdot 1/2H_2O$: C, 60.92; H, 6.69; N, 2.73. Found: C, 61.04; H, 6.67; N, 2.82.

4-(Carboxymethylthio)adrenosterone (3b)——Colorless prisms from acetone–hexane. mp 187—189°C. $[\alpha]_D^{14}$ +276° (c=0.28). *Anal.* Calcd for $C_{21}H_{26}O_5S\cdot H_2O$: C, 61.74; H, 6.91. Found: C, 62.08; H, 6.69. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, s, 18-CH_3), 1.47 (3H, s, 19-CH_3), 3.39 (2H, s, SCH_2CO), 3.75 (1H, m, 6α-H). The *N*-succinimidyl ester **3d**: Colorless leaflets from CH_2Cl_2–hexane. mp 164—166°C. $[\alpha]_D^{18}$ +225° (c=0.08). *Anal.* Calcd for $C_{25}H_{29}NO_7S$: C, 61.58; H, 6.00; N, 2.87. Found: C, 61.36; H, 5.97; N, 2.97.

4-(2-Carboxyethylthio)adrenosterone (3c)——Colorless leaflets from acetone–hexane. mp 180—181°C. $[\alpha]_D^{12}$ +266° (c=0.32). *Anal.* Calcd for $C_{22}H_{28}O_5S\cdot 1/2H_2O$: C, 63.90; H, 7.07. Found: C, 64.21; H, 7.00. ^{1}H-NMR ($CDCl_3$) δ: 0.89 (3H, s, 18-CH_3), 1.48 (3H, s, 19-CH_3), 2.55 and 2.94 (each 2H, m, SCH_2CH_2CO), 3.75 (1H, m, 6α-H). The *N*-succinimidyl ester **3e**: Colorless leaflets from CH_2Cl_2–hexane. mp 184—186°C. $[\alpha]_D^{20}$ +216° (c=0.13). *Anal.* Calcd for $C_{26}H_{31}NO_7S$: C, 62.26; H, 6.23; N, 2.79. Found: C, 61.93; H, 6.19; N, 2.91.

4-(Carboxymethylthio)progesterone (4b)——Colorless needles from aqueous EtOH. mp 160—161°C. $[\alpha]_D^{21}$ +179° (c=0.32). *Anal.* Calcd for $C_{23}H_{32}O_4S\cdot 3/4H_2O$: C, 66.07; H, 8.08. Found: C, 66.16; C, 7.88. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.13 (3H, s, 21-H), 3.37 (2H, s, SCH_2CO), 3.67 (1H, m, 6α-H). The *N*-succinimidyl ester **4d**: Colorless leaflets from MeOH. mp 162—164°C. $[\alpha]_D^{23}$ +183° (c=0.07). *Anal.* Calcd for $C_{27}H_{35}NO_6S$: C, 64.64; H, 7.03; N, 2.79. Found: C, 64.41; H, 7.04; N, 2.82.

4-(2-Carboxyethylthio)progesterone (4c)——Colorless needles from aqueous EtOH. mp 176—177°C. $[\alpha]_D^{24}$ +171° (c=0.36). *Anal.* Calcd for $C_{24}H_{34}O_4S\cdot 1/4H_2O$: C, 68.13; H, 8.22. Found: C, 68.17; H, 8.13. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.13 (3H, s, 21-H), 2.54 and 2.92 (each 2H, m, SCH_2CH_2CO), 3.66 (1H, m, 6α-H). The *N*-succinimidyl ester **4e**: Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 1.24 (3H, s, 19-CH_3), 2.12 (3H, s, 21-H), 2.83 (4H, s, succinimidyl), 3.72 (1H, m, 6α-H).

4-(Carboxymethylthio)-17α-hydroxyprogesterone (5b)——Colorless needles from aqueous EtOH. mp 197—198°C. $[\alpha]_D^{21}$ +93° (c=0.34). *Anal.* Calcd for $C_{23}H_{32}O_5S\cdot 1/4H_2O$: C, 64.99; H, 7.71. Found: C, 64.71; H, 7.84. ^{1}H-NMR ($CDCl_3$) δ: 0.74 (3H, s, 18-CH_3), 1.22 (3H, s, 19-CH_3), 2.27 (3H, s, 21-H), 3.36 and 3.44 (each 1H, d, J=15 Hz, SCH_2CO), 3.66 (1H, m, 6α-H). The *N*-succinimidyl ester **5d**: Colorless leaflets from CH_2Cl_2–hexane. mp 207—209°C. $[\alpha]_D^{13}$ +100° (c=0.08). *Anal.* Calcd for $C_{27}H_{35}NO_7S$: C, 62.65; H, 6.82; N, 2.71. Found: C, 62.40; H, 6.80; N, 2.53.

4-(2-Carboxyethylthio)-17α-hydroxyprogesterone (5c)——Colorless needles from aqueous EtOH. mp 199—202°C. $[\alpha]_D^{21}$ +81° (c=0.27). *Anal.* Calcd for $C_{24}H_{34}O_5S\cdot 1/4H_2O$: C, 65.65; H, 7.92. Found: C, 65.35; H, 8.10. ^{1}H-NMR ($CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.25 (3H, s, 21-H), 2.50 and 2.91 (each 2H, m, SCH_2CH_2CO), 3.67 (1H, m, 6α-H). The *N*-succinimidyl ester **5e**: Colorless leaflets from AcOEt–hexane. mp 160—162°C. $[\alpha]_D^{16}$ +104° (c=0.12). *Anal.* Calcd for $C_{28}H_{37}NO_7S$: C, 63.25; H, 7.02; N, 2.63. Found: C, 63.30; H, 6.93; N, 2.49.

4-(Carboxymethylthio)corticosterone (6b)——Colorless needles from acetone–hexane. mp 176—178°C. $[\alpha]_D^{21}$ +159° (c=0.20, EtOH). *Anal.* Calcd for $C_{23}H_{32}O_6S\cdot 1/4H_2O$: C, 62.63; H, 7.43. Found: C, 62.53; H, 7.31. ^{1}H-NMR ($CDCl_3$–CD_3OD (10: 1)) δ: 0.92 (3H, s, 18-CH_3), 1.48 (3H, s, 19-CH_3), 3.32 (2H, s, SCH_2CO), 3.63 (1H, m, 6α-H), 4.17 (2H, s, 21-H), 4.33 (1H, m, 11α-H). The *N*-succinimidyl ester **6d**: Colorless leaflets from CH_2Cl_2–hexane. mp 187—189°C. $[\alpha]_D^{19}$ +148° (c=0.11). *Anal.* Calcd for $C_{27}H_{35}NO_8S\cdot 5/4H_2O$: C, 58.31; H, 6.80; N, 2.52. Found: C, 58.24; H, 6.50; N, 2.80.

4-(2-Carboxyethylthio)corticosterone (6c)——Colorless plates from acetone–hexane. mp 168—171°C.

$[\alpha]_D^{21}$ +164° (c=0.28, EtOH). *Anal.* Calcd for $C_{24}H_{34}O_6S$: C, 63.97; H, 7.61. Found: C, 63.76; H, 7.84. ^{1}H-NMR ($CDCl_3$–CD_3OD (10: 1)) δ: 0.93 (3H, s, 18-CH_3), 1.49 (3H, s, 19-CH_3), 2.50 and 2.88 (each 2H, m, SCH_2CH_2CO), 3.60 (1H, m, 6α-H), 4.18 (2H, s, 21-H), 4.34 (1H, m, 11α-H). The *N*-succinimidyl ester **6e**: Colorless leaflets from MeOH. mp 104—106°C. $[\alpha]_D^{20}$ +123° (c=0.12). *Anal.* Calcd for $C_{28}H_{37}NO_8S \cdot 5/4H_2O$: C, 58.98; H, 6.98; N, 2.46. Found: C, 58.87; H, 6.41; N, 2.67.

4-(Carboxymethylthio)-11-deoxycorticosterone (7b)——Colorless leaflets from aqueous EtOH. mp 160—163°C. $[\alpha]_D^{15}$ +179° (c=0.26). *Anal.* Calcd for $C_{23}H_{32}O_5S$: C, 65.68; H, 7.67. Found: C, 65.59; H, 7.75. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 3.38 (2H, s, SCH_2CO), 3.67 (1H, m, 6α-H), 4.19 (2H, s, 21-H). The *N*-succinimidyl ester **7d**: Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 1.22 (3H, s, 19-CH_3), 2.80 (4H, s, succinimidyl), 3.50—3.92 (3H, 6α-H and SCH_2CO), 4.18 (2H, s, 21-H).

4-(2-Carboxyethylthio)-11-deoxycorticosterone (7c)——Colorless leaflets from aqueous EtOH. mp 140—143°C. $[\alpha]_D^{14}$ +175°C (c=0.30). *Anal.* Calcd for $C_{24}H_{34}O_5S \cdot 1/2H_2O$: C, 64.98; H, 7.95. Found: C, 65.23; H, 7.91. ^{1}H-NMR ($CDCl_3$) δ: 0.71 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.54 and 2.93 (each 2H, m, SCH_2CH_2CO), 3.68 (1H, m, 6α-H), 4.19 (2H, s, 21-H). The *N*-succinimidyl ester **7e**: Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 1.23 (3H, s, 19-CH_3), 2.82 (4H, s, succinimidyl), 3.72 (1H, m, 6α-H), 4.19 (2H, s, 21-H).

4-(Carboxymethylthio)-21-deoxycortisol (8b)——Colorless leaflets from aqueous EtOH. mp 207—208°C. $[\alpha]_D^{15}$ +152° (c=0.27, EtOH). *Anal.* Calcd for $C_{23}H_{32}O_6S$: C, 63.28; H, 7.39. Found: C, 63.13; H, 7.52. ^{1}H-NMR ($CDCl_3$–CD_3OD (4: 1)) δ: 0.91 (3H, s, 18-CH_3), 1.47 (3H, s, 19-CH_3), 2.21 (3H, s, 21-H), 3.31 (2H, s, SCH_2CO), 3.60 (1H, m, 6α-H), 4.40 (1H, m, 11α-H). The *N*-succinimidyl ester **8d**: Colorless semi-crystals. ^{1}H-NMR ($CDCl_3$) δ: 1.02 (3H, s, 18-CH_3), 1.45 (3H, s, 19-CH_3), 2.28 (3H, s, 21-H), 2.82 (4H, s, succinimidyl), 3.58 (1H, m, 6α-H), 3.74 (2H, s, SCH_2CO), 4.44 (1H, m, 11α-H).

4-(2-Carboxyethylthio)-21-deoxycortisol (8c)——Colorless prisms from MeOH. mp 231—234°C. $[\alpha]_D^{23}$ +159° (c=0.28, EtOH). *Anal.* Calcd for $C_{24}H_{34}O_6S$: C, 63.97; H, 7.61. Found: C, 63.71; H, 7.62. ^{1}H-NMR ($CDCl_3$–CD_3OD (4: 1)) δ: 0.88 (3H, s, 18-CH_3), 1.48 (3H, s, 19-CH_3), 2.19 (3H, s, 21-H), 2.47 and 2.86 (each 2H, m, SCH_2CH_2CO), 3.63 (1H, m, 6α-H), 4.40 (1H, m, 11α-H). The *N*-succinimidyl ester **8e**: Colorless leaflets from CH_2Cl_2–hexane. mp 173—175°C. $[\alpha]_D^{21}$ +98° (c=0.09). *Anal.* Calcd for $C_{28}H_{37}NO_8S$: C, 61.40; H, 6.81; N, 2.56. Found: C, 61.11; H, 6.99; N, 2.43.

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