SYNTHESIS OF TRITIUM LABELLED CHOLECYSTOKININ DERIVATIVE : [^{3}H]-Boc-[Nle^{28,31}] - CCK$_{27-33}$

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SUMMARY :

The synthesis of a new acetylenic analogue of the C-terminal heptapeptide segment of cholecystokinin (CCK) in which the Met^{28} and Met^{31} residues are replaced by two acetylenic precursors of norleucine (Nle), L-2-amino-4-hexynoic acid (Aha), is described. Reductive tritiation of this acetylenic heptapeptide Boc-[Aha^{28,31}]-CCK$_{27,33}$ led to the labelled [^{3}H]-Boc-[Nle^{28,31}]-CCK$_{27-33}$ which displays a specific activity of about 150 Ci/mmol. According to its full biological potency, this CCK analogue can be used for various biological assays including binding studies.

KEY WORDS : peptide synthesis, tritium labelling, cholecystokinin heptapeptide analogue, CCK receptor, L-2-amino-4-hexynoic acid.

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INTRODUCTION :

The gastrointestinal peptide hormone cholecystokinin, originally isolated in the gut, exists in multiple molecular forms[1,2]. The predominant fragment in the nervous system, CCK_{26-33} (or CCK-8), Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2[3] is very likely the physiological effectors of CCK receptors in the brain. Therefore, a tritiated CCK-8 analogue appeared to be the most suitable ligand for characterization of CCK binding sites, as well as study on possible receptor heterogenecity. However, owing to the low concentration of CCK binding sites, the radioactivity of the CCK effector must be as high as possible. [^{3}H]-CCK_{26-33} with specific radioactivity of 30-40 Ci/mmol has been proposed as ligand for receptor studies[4]. Nevertheless, in addition to its insufficient radioactivity, the synthesis of this tritiated probe is rather difficult. This method requires the sulfatation with pyridine-SO_3 complex of desulfated CCK-8 with tritium on the aromatic ring of the tyrosine. Moreover, the presence of the two methionine residues makes the final product highly susceptible to oxydation. Recent findings in our laboratories of fully potent Boc-[$Nle^{28,31}$]-CCK-8[5] and of efficient procedure of converting L-2-amino-4-hexynoic acid (Aha) to tritium labelled norleucine[6] prompted us to prepare a fully radioactive CCK-7 analogue to be used for receptor binding study.

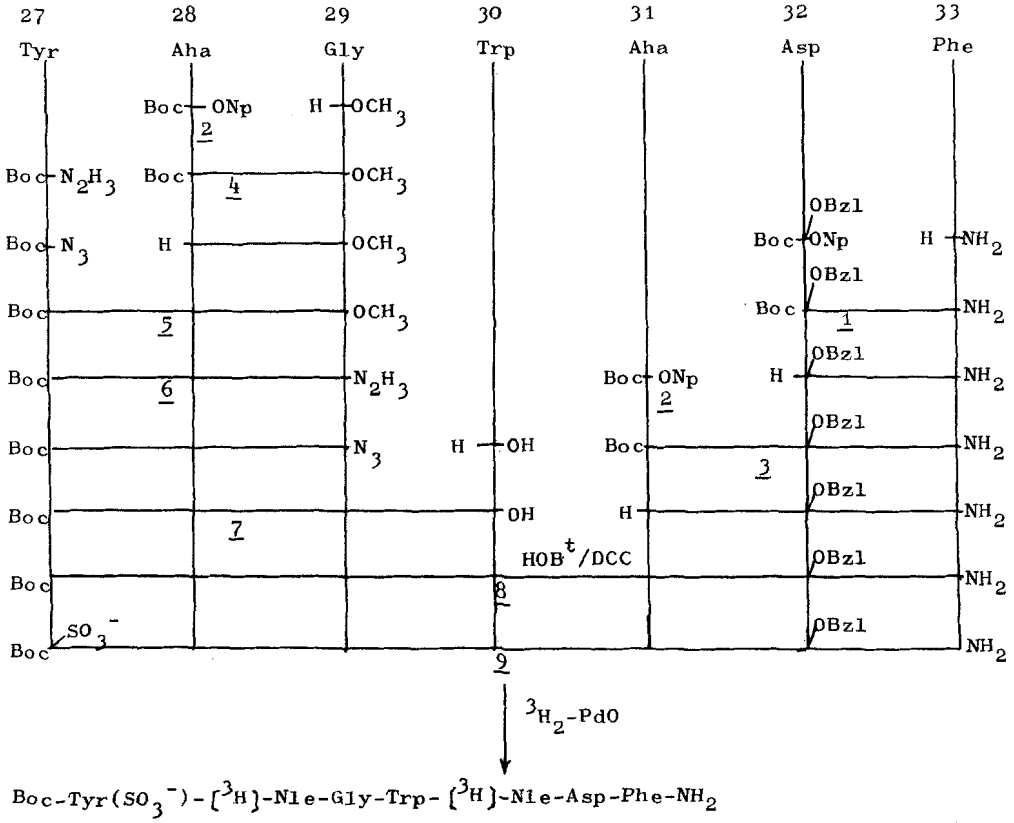
In this paper, we report the synthesis of an acetylenic precursor of a fully potent analogue of CCK-8, Boc-[$Aha^{28,31}$] - CCK-7 and tritium labelling of thus obtained peptide. The synthesis of Boc-Tyr(SO_3^-)-Aha-Gly-Trp-Aha-Asp-Phe-NH_2 was performed by solution method as outlined in Scheme 1.

EXPERIMENTAL PART :

Material and procedures.

Protected amino acid derivatives were prepared according to the methods known in the literature[7-9]. All optically active amino acids had (L)-configuration. All other chemicals and solvents were of analytical grade from Prolabo (France), Fluka (Switzerland) or E. Merck (W. Germany).

Leucine aminopeptidase was purchased from Sigma Chemical Co. (U.S.A.). Analytical thin layer chromatography was carried out on E. Merck silica gel 60 F254 precoated plates in the following solvent systems (v/v). A : chloroform-methanol (9:1). B : chloroform-methanol-H_2O (8:3:1 lower phase). C : n-butanol-pyridine-acetic acid-H_2O (4:1:1:2). D : ethylacetate-pyridine-acetic acid-H_2O (30:20:6:11). Melting points were taken on a Büchi apparatus and are uncorrected. Optical rotations were determined with a Perkin-Elmer Model 141 polarimeter. ^1H NMR spectra were performed with Bruker WP 200 MHz and Bruker WH 270 MHz instruments in $CDCl_3$ or DMSO d_6 using tetramethylsilane as an internal standard. Amino acid analysis was carried



Scheme 1. Synthetic route for the preparation of

$[^3H]$-Boc-[$Nle^{28,31}$]-CCK_{27-33}

out on a LKB Biochrom 4400 Amino Acid Analyser. Elemental analyses were performed at the Institut de Chimie des Substances Naturelles. Catalytic tritiation was conducted at the Commissariat à l'Energie Atomique (France). ^{3}H-Scannings of TLC plates were performed with a Berthols Scanner. Peptide weight determinations were carried out after acid hydrolysis with a LKB **Biochrom 4400** Amino Acid **Autoanalyser**. Radioactive countings (^{3}H) were determined with a liquid scintillation counter SL 3000 Intertechnique.

The following abbreviations are used : Aha, 2-amino-4-hexynoic acid ; Boc, tert-butyloxycarbonyl ; ONp p-nitrophenyloxy ; OBzl, benzyloxy ; HOBt, t-hydroxybenzotriazole ; DCC, dicyclohexylcarbodiimide ; DMF, dimethylformamide ; TFA, trifluoroacetic acid ; EtOAc, ethylacetate;DMSO,dimethylsulfoxide.

I. Peptide synthesis.

Boc-Asp(OBzl)-Phe-NH_2 1

To a solution of phenylalanine amide (as TFA salt obtained from 1.071 g (4.05 mmol) of N-Boc-Phe-NH_2) in DMF (10 ml) and triethylamine (0.6 ml, 4.3 mmol) was added N-Boc-asparatic acid β-benzyl-α-p-nitrophenyl ester (1.86 g, 4.05 mmol) at room temperature. The mixture was stirred for 24 h at room temperature. After evaporation in vacuo, the residue was dissolved in EtOAc and washed with 5% $NaHCO_3$, 10% citric acid and water. The solution was dried over Na_2SO_4. After evaporation the residue was washed with ether and recrystallized from EtOAc-ether to afford 1 (1.47 g, 77%) m.p. 137-138°C (Lit[10] 134-135°C) : $[\alpha]_D^{20}$ - 32.3° (C 1.0, DMF) : Rf 0.38 (A), 0.73 (B).
^{1}H NMR ($CDCl_3$) δ 1.43 (s, 9H, Boc), 2.90 (m, 2H, CH-β), 3.14 (m, 2H, CH-β), 4.47 (m, 1H, CH-α), 4.71 (m, 1H, CH-α), 5.16 (s, 2H, Ø-CH_2), 5.58 (d, 2H, -$CONH_2$), 6.18 (broad s, 1H), 6.95 (d, 1H, NH-Boc), 7.30 (s, 5H, ArH), 7.37 (s, 5H, ArH).
Anal. calc. for $C_{25}H_{31}N_3O_6$ C 63.95, H 6.65, N 8.95. Found C 63.46, H 6.72, N 9.33.

Boc-Aha-Asp(OBzl)-Phe-NH_2 3

A solution of 1 (470 mg, 1.0 mmol) in TFA (1.3 ml) and anisol (0.35 ml) was stirred at 0°C for 1 h. After complete evaporation in vacuo, the residue was triturated with ether, filtered off and dissolved in DMF (5 ml). To this solution were added 2-tert-butyloxycarbonylamino-4-hexynoic acid p-nitrophenyl ester 2[6] (350 mg, 1.0 mmol) and triethylamine (0.28 ml, 2 mmol) at room temperature. The mixture was stirred for 48 h at room temperature. After evaporation in vacuo, the crude product was purified by flash chromatography on Kieselgel 60 by eluting first with chloroform then with the lower phase of the solvent mixture of chloroform-methanol-water (8:3:1). Fractions containing the product were concentrated. Crystallization from ethanol-diisopropylether afforded 3 (444 mg, 85%). m.p. 159-161°C ; $[\alpha]_D^{20}$ - 111.5° (C 1.0, DMF) ; Rf 0.47 (A), 0.75 (B) ; ^{1}H NMR (DMSOd_6) δ 1.43 (s, 9H, Boc), 1.75 (s, 3H, CH_3-C≡C-), 2.53 (m, 2H, -C≡C-CH_2-), 2.65 (q, 1H, CH-β), 2.85 (m, 1H, CH-β), 2.90 (m, 1H, CH-β), 3.07 (q, 1H, CH-β), 4.13 (m, 1H, CH-α), 4.45 (m, 1H, CH-α), 4.68 (m, 1H, CH-α), 5.13 (s, 2H, Ø-CH_2-), 7.00 (d, 1H, BocNH), 7.28 (m, 5H, ArH), 7.40 (s, 5H, ArH).
Anal. calc. for $C_{31}H_{38}N_4O_7$ C 64.34, H 6.62, N 9.68. Found C 64.25, H 6.58, N 9.66.

Boc-Aha-Gly-OCH_3 4

To a solution of glycine methylester (314 mg, as HCl salt, 2.5 mmol) in DMF (8 ml) and triethylamine (0.35 ml, 2.5 mmol) were added 2-tert-butyloxycarbonyl-amino-4-hexynoic acid p-nitrophenyl ester 2 (870 mg, 2.5 mmol) and triethylamine (0.35 ml, 2.5 mmol). The mixture was stirred for 48 h at room temperature. After evaporation in vacuo, the residue was dissolved in EtOAc and was washed with 5% $NaHCO_3$, 10% citric acid and water. After drying over $MgSO_4$, the solution was concentrated. The residue was flash chromatographed on Kieselgel 60 by eluting first with chloroform then with the solvent mixture of chloroform-methanol (9:1). After evapo-

ration, the residue was recrystallized with EtOAc-ether to yield 4 (650 mg, 86%). m.p. 76-77°C ; $[\alpha]_D^{20}$ - 5.5° (C 1.0, MeOH) : Rf 0.53 (A), 0.75 (B). ^{1}H NMR($CDCl_3$) δ 1.47 (s, 9H, Boc), 1.75 (t, J = 2 Hz, 3H, CH_3-C≡C-), 2.62 (m, 2H, -C≡C-CH_2-), 3.75 (s, 3H, OCH_3), 4.00 (d, 2H, CH-α), 4.23 (m, 1H, CH-α), 6.87 (broad s, 1H, Boc NH).
Anal. calc. for $C_{14}H_{22}N_2O_5$ C 56.36, H 7.43, N 9.40. Found C 56.50, H 7.50, N 9.40.

Boc-Tyr-Aha-Gly-OCH_3 5

A solution of Boc-Aha-Gly-OCH_3 4 (550 mg, 1.84 mmol) in TFA (2.4 ml) and anisol (0.6 ml) was stirred for 1 h at 0°C. After evaporation in vacuo, the residue was triturated three times with dry ether and dissolved in DMF (3.0 ml) and added with triethylamine (0.28 ml, 2.0 mmol).

To a solution of N-tert-butyloxycarbonyl tyrosine hydrazide (344 mg, 1.84 mmol) in DMF (1.5 ml) were added 3.85 N HCl/DMF (1.15 ml, 4.42 mmol) and isopentylnitrite (0.3 ml, 2.21 mmol) at -5°C. After stirring for 10 min. at -5°C, the mixture was neutralized with triethylamine (0.62 ml) at 0°C and was poured into the amine component prepared as described above.
After further addition of triethylamine (0.3 ml) the reaction mixture was stirred at 4°C for 48 h. The solvent was removed in vacuo. The resulting residue was dissolved in EtOAc and washed with 10% citric acid and with water. After evaporation the residue solid was recrystallized from EtOAc-n-hexane to afford 5 (512 mg, 61%). m.p. 167-168°C ; $[\alpha]_D^{20}$ -13.4° (C 1.0, MeOH) ; Rf 0.56 (A), 0.64 (B). ^{1}H NMR($CDCl_3$) δ 1.33 (s, 9H, Boc), 1.75 (s, 3H, CH_3-C≡C-), 2.58 (m, 2H, -C≡C-CH_2-), 2.71 (d, 1H, CH-β), 2.93 (d, 1H, CH-β), 3.70 (s, 3H, OCH_3), 3.96 (d, 2H, CH-α), 4.20 (m, 1H, CH-α), 4.48 (m, 1H, CH-α), 6.73 (d, J = 6 Hz, 2H, ArH), 7.00 (d, 1H, BocNH), 7.11 (d, J = 6 Hz, 2H, ArH).
Anal. calc. for $C_{23}H_{31}N_3O_7$ C 59.86, H 6.77, N 9.10. Found C 59.80, H 6.66, N 9.10.

Boc-Tyr-Aha-Gly-N_2H_3 6

To a solution of Boc-Tyr-Aha-Gly-OCH_3 5 (462 mg, 1 mmol) in methanol (10 ml) was added dropwise hydrazine hydrate (0.06 ml, 5 mmol). The mixture was allowed to stand at room temperature for 48 h. After concentration in vacuo, the residue was dissolved in methanol and evaporated. This procedure was repeated twice. The crude product was recrystallized from methanol to yield 6 (438 mg, 95%). m.p. 194-195°C ; $[\alpha]_D^{20}$ - 13.9° (C 5.0, DMF) ; Rf 0.38 (A). Anal. calc. for $C_{22}H_{31}N_5O_6$ C 57.25, H 6.77, N 15.18. Found C 56.96, H 6.71, N 15.17.

Boc-Tyr-Aha-Gly-Trp-OH 7

To a solution of Boc-Tyr-Aha-Gly-N_2H_3 6 (240 mg, 0.52 mmol) in DMF (2.0 ml) were added at -5°C 3.85 N HCl/DMF (0.33 ml, 1.26 mmol) and isopentylnitrite (0.085 ml, 0.62 mmol). After stirring for 15 min at -5°C, the mixture was neutralized with triethylamine (0.18 ml, 1.28 mmol) and poured into a solution of tryptophane(107 mg, 0.53 mmol) in water (1.2 ml) and triethylamine (0.16 ml, 1.14 mmol). The reaction mixture was stirred at 4°C for 48 h. After evaporation in vacuo, the residue was purified on preparative thin layer chromatography by eluting with the organic phase of the solvent mixture of chloroform-methanol-water (8:3:1). The product was extracted from silica gel with DMF which was then filtered through Millipore and evaporated in vacuo. The residue was taken in small amount of EtOAc and added ether to afford 7 (235 mg, 71%). m.p. 192-194°C. $[\alpha]_D^{20}$ - 4.0° (C 1.0, DMF). Rf 0.03(A), 0.27(B), 0.73(C) ; ^1H NMR(DMSO d_6) δ 1.33 (s, 9H, Boc), 1.72 (s, 3H, CH_3-C≡C-), 2.52 (s, 2H, -C≡C-CH_2-), 2.54 (m, 1H, CH-β), 2.66 (m, 2H, CH-β), 3.10 (m, 1H, CH-β), 3.95 (s, 2H, CH-α), 4.16 (broad s, 1H, CH-α), 4.43-4.45 (broad s, 2H, CH-α), 6.61 (s, 2H, ArH), 6.98 (s, 3H, ArH + BocNH), 7.0 (m, 1H, ArH), 7.25 (d, 1H, ArH), 7.56 (m, 1H, ArH), 7.96-8.10 (m, 2H, ArH), 10.50 (s, 1H, indol NH).

Anal. calc. for $C_{33}H_{39}N_5O_8.2.5\ H_2O$ C 58.39, H 6.53, N 10.31. Found C 58.23, H 6.42, N 10.00.

Boc-Tyr-Aha-Gly-Trp-Aha-Asp(OBzl)-Phe-NH_2 8

A solution of Boc-Aha-Asp(OBzl)-Phe-NH_2 3 (478 mg, 0.83 mmol) in TFA (1.1 ml) and anisol (0.22 ml) was stirred for 1 h at 0°C. After complete evaporation in vacuo, the residue was triturated with ether. The precipitates were treated with 1 HCl (0.83 ml) and completely dried. The residual powder was washed with 5% $NaHCO_3$, filtered off and dried in vacuo over KOH to yield H-Aha-Asp(OBzl)-Phe-NH_2 (376 mg, 95% yield). To a cooled solution (0°C) of Boc-Tyr-Aha-Gly-Trp-OH 7 (500 mg, 0.78 mmol) in DMF (20 ml) were added HOBt (111 mg, 0.78 mmol), the amine component prepared as described above (376 mg, 0.78 mmol) and finally DCC (165 mg, 0.78 mmol). The reaction mixture was stirred at room temperature for 24 h. After the removal of dicyclohexylurea and solvent, the residue was purified on Sephadex LH-20 using DMF as eluent. After evaporation in vacuo, the residue was triturated with ether and filtered to yield 8 (613 mg, 71%). m.p. 187-188° : $[\alpha]_D^{20}$ - 26.5° (C 1.0, DMF) ; Rf 0.37(A), 0.50(B), 0.96(C). ^1H NMR (DMSO d_6) δ 1.33 (s, 9H, Boc), 1.72 (s, 6H, CH_3-C≡C-), 2.50 (m, 4H, -C≡C-CH_2-), 2.66 (m, 2H, CH-β), 2.73-3.20 (m, 6H, CH-β), 3.73 (m, 2H, CH-α), 4.16 (m, 2H, CH-α), 4.36-4.50 (m, 2H, CH-α), 4.60-4.76 (m, 2H, CH-α), 5.15 (s, 2H, Ar-CH_2-), 6.71 (d, J = 6.6 Hz, 2H, ArH), 6.99 (d, 1H, BocNH), 7.00-7.43 (m, 16H, ArH), 7.70 (d, 1H, ArH), 10.90 (s, 1H, indol NH). Anal. calc. for $C_{59}H_{67}N_9O_{12}.H_2O$ C 63.71, H 6.25, N 11.33. Found C 63.71, H 6.28, N 11.22. Amino acid analysis (after catalytic hydrogenation with 10% Pd/C followed by the hydrolysis with leucine aminopeptidase). Asp 1.00(1), Gly 1.00(1), Nle 2.02(2), Tyr 0.90(1), Phe 0.96(1), Trp 0.89(1).

Boc-Tyr(SO_3Na)-Aha-Gly-Trp-Aha-Asp(OBzl)-Phe-NH_2 9

To a solution of Boc-Tyr-Aha-Gly-Trp-Aha-Asp(OBzl)-Phe-NH_2 8 (267 mg, 0.24 mmol) in dry DMF (5 ml) and dry pyridine (2.5 ml) was added pyridine-SO_3 complex (900 mg).

The reaction mixture was allowed to stand for 48 h at room temperature. After evaporation in vacuo, the residue was taken in cold water (10 ml) and the pH of the solution was adjusted at 7.0 with 10% Na_2CO_3. The solution was concentrated in vacuo and was added with DMF to precipitate inorganic salts. The solution was filtered and evaporated in vacuo. The crude product was purified by preparative thin layer chromatography with the solvent mixture of chloroform-methanol-water (8:3:1, lower phase) as eluent. The product was extracted from silica gel with DMF. After concentration of the DMF solution, the residue was triturated with ether to yield 9 (730 mg, 45%). m.p. 176-179°C ; $[\alpha]_D^{20}$ - 32.7° (C 1.0, DMF) ; Rf 0.08(A), 0.29(B), 0.74(C). ^{1}H NMR (DMSO d_6) δ 1.28 (s, 9H, Boc), 1.66 (s, 6H, CH_3-C C-), 2.45 (m, 4H, -C C-CH_2-), 2.55-3.15 (m, 8H, CH-β), 3.63 (m, 2H, CH-α), 4.08 (m, 1H, CH-α), 4.30 (m, 3H, CH-α), 4.55 (m, 2H, CH-α), 5.00 (s, 2H, -CH_2-Ar), 6.80-7.25 (m, 18H, ArH, BocNH), 7.50 (d, 1H, ArH), 7.73 (d, 1H, ArH), 10.68 (s, 1H, indol H).
Anal. calc. for $C_{59}H_{66}N_9O_{15}SNa.3\ H_2O$ C 56.64, H 5.80, N 10.07, S 2.56. Found C 56.30, H 5.85, N 9.89, S 2.70.

Catalytic hydrogenation of 9 in a solvent mixture of methanol-pyridine-acetic acid (4:1:1) in the presence of 10% Pd/C at room temperature and pressure for 1 h afforded in quantitative yield Boc-Tyr(SO_3Na)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2, of which ^{1}H NMR spectra and Rf were identical with those of Boc-Tyr(SO_3Na)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 prepared separately starting from norleucine[5].

II. Tritium labelling.

A solution of Boc-Tyr(SO_3Na)-Aha-Gly-Trp-Aha-Asp(OBzl)-Phe-NH_2 (2 mg, 1.67 μmol) in a solvent mixture (0.9 ml) of methanol-pyridine-acetic acid (4:1:1) was taken into a tritiation reactor[11] and was frozen. The catalyst (21 mg of 10% Pd/C) was then dispersed on the surface and the reaction vial was connected to the automatic tritium gas transfer unit[12]. After a vacuum of 10^{-4} Torr was reached, pure tritium gas (80 Curies) was introduced and compressed to 1 bar and the catalyst

was flushed for 15 min into the still frozen solution. The reaction mixture was brought to 20°C and magnetically stirred for 2.5 h. The absorption of tritium gas produced a reduction of pressure of 0.88 bar. The reaction mixture was frozen again and unreacted tritium gas was removed. The catalyst was filtered through Millipore GS and labile tritium atoms were eliminated by evaporation in a rotavapor with a large volume of 50% aqueous methanol (70 ml). The peptide was purified on TLC (solvent system EtOAc-pyridine-acetic acid-water : 30:20:6:11) and analysed on HPLC with isocratic solvent system (TEAP, pH 6.5, 0.1 M, 32% CH_3CN, flow rate 1.2 ml/min).

The autoradiochromatogram and ^3H-scanning revealed a single peak commigrating with Boc-Tyr(SO_3H)-Nle-Gly-Trp-Nle-Asp-Phe-NH_2 and corresponding to the spot detectable with 4,4'-tetramethyldiamino-diphenyl methane (TDM). The peptide was extracted from silica gel powder with 6 x 10 ml of pure methanol. After acid hydrolysis (6 N HCl, 110°C, 15 h), the specific radioactivity was found to be close to 144 Ci/mmol.

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