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Studies on Peptides. XCVII.[1,2] Synthesis of Porcine Glu^8-Vasoactive Intestinal Polypeptide (VIP)

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The synthesis of Glu^8-vasoactive intestinal polypeptide (porcine VIP) is described. A 1 M solution of trifluoromethanesulfonic acid–thioanisole (1 : 1 equiv.) in TFA was found to cleave all the protecting groups employed, Z, Z(OMe), Bzl and Mts, suppressing the acid-catalyzed aminosuccinimide formation of the Asp residue (position 3) with the free carboxyl group. The activity of Glu^8-VIP was 1/7—1/8 of that of synthetic porcine VIP.

Keywords——synthesis of porcine Glu^8-vasoactive intestinal polypeptide; N^G-mesitylene-2-sulfonylarginine; ring closure of Asp–Ala by acids; trifluoromethanesulfonic acid–thioanisole–*m*-cresol as a deprotecting system; pancreatic secretory activity

During the course of synthetic studies[4,5] on chicken[6] and porcine vasoactive intestinal polypeptides (VIP),[7] we found that the Asp residue (position 8) linked to Asn, even with the free carboxyl group, has a marked tendency to undergo acid-catalyzed aminosuccinimide formation, while the Asp residue (position 3) linked to Ala does so to a certain degree. This side reaction seems to be dependent on the sequence involved, as well as acids employed. We have been acquainted quite recently with the report by Schön and Kisfaludy,[8] who also found this type of side reaction during the treatment of Asp-Phe–NH_2 with 4 N HCl in AcOH or HF.[9]

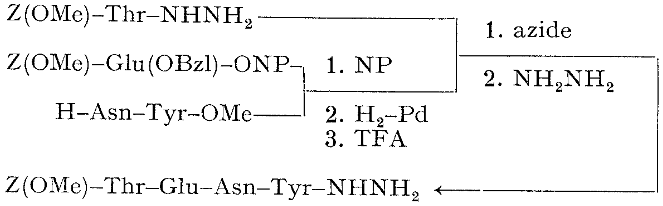

Fig. 1. Synthetic Scheme of the Protected Tetrapeptide Hydrazide, Z(OMe)–(porcine Glu^8–VIP 7—10)–NHNH$_2$

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1) Part XCVI: H, Yajima, K. Akaji, S. Funakoshi, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **28**, 1942 (1980).

2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. The following abbreviations were used: Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl, Bzl=benzyl, Mts=mesitylene-2-sulfonyl, NP=*p*-nitrophenyl, DMF=dimethylformamide, TFA=trifluoroacetic acid.

3) Location: *Sakyo-ku, Kyoto, 606, Japan.*

4) H. Yajima, M. Takeyama, K. Koyama, T. Tobe, K. Inoue, T. Kawano, and H. Adachi, *Chem. Pharm. Bull.*, **27**, 3199 (1979); *idem, Int. J. Peptide Protein Res.*, in press.

5) M. Takeyama, K. Koyama, K. Inoue, T. Kawano, H. Adachi, T. Tobe, and H. Yajima, *Chem. Pharm. Bull.*, **28**, 1873 (1980).

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7) V. Mutt and S.I. Said, *Eur. J. Biochem.*, **42**, 581 (1974).

8) I. Shcön and L. Kisfaludy, *Int. J. Peptide Protein Res.*, **14**, 485 (1979).

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We have replaced the acid-sensitive Asp residue of porcine VIP (position 8) with Glu in order to examine the resulting change in the biological spectrum of VIP. As shown in Fig. 1, a necessary fragment, Z(OMe)–Thr–Glu–Asn–Tyr–NHNH$_2$ [B], was prepared starting with Z(OMe)–Asn–Tyr–OMe.6) After removing the Z(OMe) group with TFA,10) this dipeptide ester was condensed with Z(OMe)–Glu(OBzl)–OH by the NP method11) to afford Z(OMe)–Glu(OBzl)–Asn–Tyr–OMe, from which the Bzl group was removed by hydrogenolysis and the Z(OMe) group by TFA treatment. Subsequently, Z(OMe)–Thr–NHNH$_2$ was condensed with the resulting H–Glu–Asn–Tyr–OMe by means of Rudinger's azide procedure.12) The product, Z(OMe)–Thr–Glu–Asn–Tyr–OMe, was smoothly converted to the corresponding hydrazide [B].

As shown in Fig. 2, the fragment [B] thus obtained was condensed, *via* the azide, with the octadecapeptide amide, H–Thr–Arg(Mts)–Leu–Arg(Mts)–Lys(Z)–Gln–Met(O)–Ala–Val–Lys(Z)–Lys(Z)–Tyr–Leu–Asn–Ser–Ile–Leu–Asn–NH$_2$, an available intermediate for our previous synthesis of porcine VIP.5) The resulting protected docosapeptide amide was purified by gel-filtration on Sephadex LH-20 with DMF as an eluant, and after TFA treatment, was subjected to the final condensation with the N-terminal hexapeptide hydrazide, Z(OMe)–His–Ser(Bzl)–Asp–Ala–Val–Phe–NHNH$_2$, a common fragment for our previous syntheses of chicken and porcine VIPs.4) The desired protected octacosapeptide amide was again purified by gel-filtration on Sephadex LH-60 with DMF as an eluant.

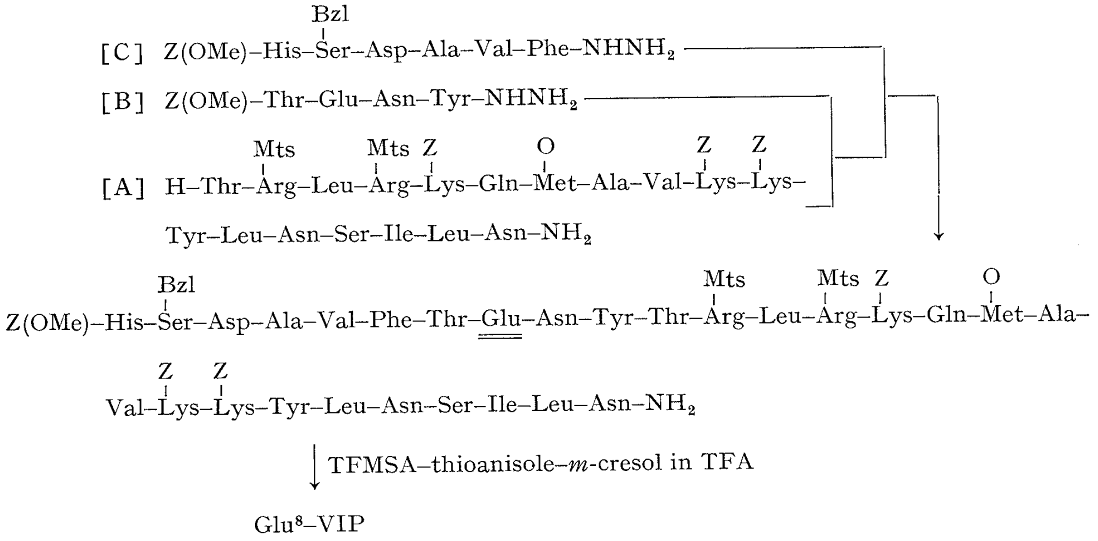

Fig. 2. Synthetic Route for Porcine Glu^8–VIP

Considering the ring closure reaction at the Asp residues mentioned above, the HF-*m*-cresol deprotecting procedure was employed for our previous syntheses of chicken and porcine VIPs, and, in addition, the TFA–thioanisole–*m*-cresol procedure for porcine VIP. In the latter procedure, the side reaction at the Asp residues was greatly suppressed and the acidolytic deprotection was immensely accelerated by thioanisole.13) The Z group was cleaved within 3 to 4 hr, but the complete removal of Mts and Bzl groups required overnight treatment at room temperature. We have found that all the protecting groups now employed, Z, Bzl,

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10) F. Weygand and K. Hunger, *Chem. Ber.*, **95**, 1 (1962).
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and Mts, could be cleaved by a 1 M solution of trifluoromethanesulfonic acid (TFMSA)[14]–thioanisole (1: 1 mol) in TFA containing *m*-cresol (10 equiv. for each protecting group) within 60—90 minutes in an ice-bath, without significant accompanying side reaction at the Asp residues, at least in the case of Z(OMe)–His–Ser(Bzl)–Asp–Ala–Val–Phe–$NHNH_2$ and Z(OMe)–Thr–Asp–Asn–Tyr–$NHNH_2$.[4] When the concentration of TFMSA in TFA was increased to 2 M, the aminosuccinyl derivative was formed at 20° from the former hexapeptide to the extent of 29%, nearly the same as that observed during methanesulfonic acid (MSA)[15] or HBr treatment, as reported previously.[5]

The protected octacosapeptide was treated with thiophenol to reduce the Met(O) residue[16] prior to deprotection, in view of the instability of VIP in solution, as discussed previously.[4] The reduced peptide was then treated with a 1 M solution of TFMSA–thioanisole (1: 1) in TFA containing *m*-cresol in an ice-bath for 60 min and at room temperature for 30 min to remove all the protecting groups as completely as possible. The deprotected peptide was converted to the corresponding acetate on Amberlite CG-4B and purified by gel-filtration on Sephadex G-25, followed by ion-exchange chromatography on CM-cellulose. In the latter step, gradient elution with 0.1 M ammonium bicarbonate buffer, pH 8.0, was employed. The chromatographic pattern obtained here was quite similar to that observed in the purification of porcine VIP, derived by HF deprotection.[5] The product thus obtained exhibited a sharp singlet spot on TLC in two different solvent systems and behaved as a single component in disc electrophoresis at pH 2.3. Its purity was further confirmed by isoelectric focusing[17] in Ampholine, pH 9—11 (LKB). Amino acid ratios in the acid hydrolysate were in good agreement with the theoretical values and satisfactory recovery of Asp, together with other constituent amino acids, was confirmed by aminopeptidase digestion.

When pancreatic secretory effects were examined in the Love-Tachibana rat preparation,[18] the potency of Glu^8–VIP relative to that of synthetic porcine VIP (taken as 1) was 1/7—1/8. The results suggests a significant contribution of the Asp residue (position 8) in the intrinsic potency of porcine VIP.

Experimental

Thin–layer chromatography was performed on silica gel (Kieselgel G, Merck) and *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8: 3: 1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (4: 1: 1: 2), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (30: 6: 20: 24).

Z(OMe)-Glu(OBzl)-Asn-Tyr-OMe——Z(OMe)–Asn–Tyr–OMe (4.73 g, 10 mmol) was treated with TFA–anisole (9.5 ml–2.4 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and dissolved in DMF (30 ml) together with Et_3N (2.76 ml, 20 mmol) and Z(OMe)–Glu(OBzl)–ONP (5.22 g, 10 mmol). After stirring at room temperature for 48 hr, the solution was concentrated and the residue was treated with 10% citric acid and AcOEt. The resulting powder was washed with 10% citric acid, 5% Na_2CO_3 and H_2O and precipitated from DMF with AcOEt; yield 6.0 g (87%), mp 181—183°, $[\alpha]_D^{18}$ +8.0° (c=0.4, DMF), Rf_1 0.52. *Anal.* Calcd for $C_{35}H_{40}N_4O_{11}$: C, 60.68; H, 5.82; N, 8.09. Found: C, 60.91; H, 5.72; N, 7.80.

Z(OMe)-Thr-Glu-Asn-Tyr-OMe——Z(OMe)–Glu(OBzl)–Asn–Tyr–OMe (3.42 g, 4.94 mmol) in DMF (40 ml) was hydrogenated over a Pd catalyst for 3 hr, and the filtered solution was concentrated. The residue was next treated with TFA–anisole (8 ml–2 ml) as usual, then dry ether was added. The resulting powder isolated as described above was dissolved in DMF (8 ml) containing Et_3N (1.37 ml, 9.88 mmol). The azide [prepared from 1.91 g (6.42 mmol) of Z(OMe)–Thr–$NHNH_2$] in DMF (15 ml) and Et_3N (0.89 ml,

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14) *a*) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J.C.S. Chem. Commum.*, **1974**, 107; H. Irie, N. Fujii, H. Ogawa, H. Yajima, M. Fujino, and S. Shinagawa, *ibid.*, **1976**, 922; *b*) Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Kitagawa, T. Akita, and M. Moritoki, *ibid.*, **1979**, 971; Y. Kiso, K. Ito, S. Nakamura, K. Kitagawa, T. Akita, and H. Moritoki, *Chem. Pharm. Bull.*, **27**, 1472 (1979).

15) H. Yajima, Y. Kiso, H. Ogawa, N. Fujii, and H. Irie, *Chem. Pharm. Bull.*, **23**, 1164 (1975).

16) H. Yajima, S. Funakoshi, N. Fujii, K. Akaji, and H. Irie, *Chem. Pharm. Bull.*, **27**, 1060 (1979).

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18) J.W. Love, *Quart. J. Exp. Physiol.*, **42**, 279 (1957); S. Tachibana, *Jpn. J. Pharmacol.*, **21**, 325 (1971).

6.42 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° for 36 hr. The solvent was removed by evaporation and the residue was treated with 5% citric acid and AcOEt. The resulting powder was purified by washing as described above followed by precipitation from DMF with AcOEt; yield 2.44 g (70%), mp 175—178°, $[\alpha]_D^{18}$ $-8.4°$ ($c=0.4$, DMF), Rf_1 0.15. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00, Thr 1.07, Glu 1.06, Tyr 0.97 (recovery of Asp 83%). *Anal.* Calcd for $C_{32}H_{41}N_5O_{13}$: C, 54.62; H, 5.87; N, 9.95. Found: C, 54.54; H, 6.16; N, 9.82.

Z(OMe)-Thr-Glu-Asn-Tyr-NHNH$_2$——Z(OMe)–Thr–Glu–Asn–Tyr–OMe (2.43 g, 3.45 mmol) in DMF (20 ml) was treated with 80% hydrazine hydrate (2.07 ml, 10 equiv.) at room temperature overnight. The solvent was removed by evaporation and the residue was treated with MeOH. The resulting mass was precipitated from DMF with MeOH; yield 1.99 g (82%), mp 205—208°, $[\alpha]_D^{18}$ $-17.3°$ ($c=1.0$, DMF), Rf_1 0.10. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.00, Thr 0.94, Glu 1.07, Tyr 0.98 (recovery of Asp 88%). *Anal.* Calcd for $C_{31}H_{41}N_7O_{12} \cdot 1/2H_2O$: C, 52.24; H, 5.94; N, 13.76. Found: C, 52.11; H, 6.09; N, 14.12.

Z(OMe)-Thr-Glu-Asn-Tyr-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met(O)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH$_2$, Z(OMe)-(porcine Glu^8-VIP 7—28)-NH$_2$——Z(OMe)-(porcine VIP 11-28)-NH$_2$ [A] (1.25 g, 0.40 mmol) was treated with TFA–anisole (3 ml—0.8 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 hr and dissolved in DMF (12 ml) containing Et_3N (0.06 ml, 0.40 mmol). The azide [prepared from 563 mg (0.80 mmol) of Z(OMe)–Thr–Glu–Asn–Tyr–NHNH$_2$ [B]] in DMF (6 ml) and Et_3N (0.22 ml, 1.60 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4° for 48 hr. The solution was concentrated and the residue was treated with 5% citric acid and AcOEt. The resulting powder was washed with 5% citric acid and H_2O and dissolved in a small amount of DMF. The solution was applied to a column of LH-20 (3 × 132 cm), which was eluted with the same solvent. UV absorption at 275 *nm* was determined in each fraction (6.6 ml). The fractions corresponding to the front main peak (tube Nos. 42—49) were combined and the solvent was removed by evaporation. Trituration of the residue with AcOEt afforded a powder, which was precipitated from DMF with AcOEt; yield 871 mg (60%), mp 148—151°, $[\alpha]_D^{18}$ $+6.0°$ ($c=0.7$, DMF), Rf_1 0.12. Amino acid ratios in a 6 N HCl hydrolysate: Asp 3.05, Thr 2.00, Ser 0.78, Glu 2.19, Ala 0.94, Val 0.95, Met+Met(O) 0.95, Ile 1.00, Leu 3.10, Tyr 2.04, Lys 3.12, Arg 2.06 (recovery of Ile 84%). *Anal.* Calcd for $C_{169}H_{246}N_{36}O_{47}S_3 \cdot 6\,H_2O$: C, 54.30; H, 6.96; N, 13.48. Found: C, 54.42; H, 6.75; N, 13.06.

Z(OMe)-His-Ser(Bzl)-Asp-Ala-Val-Phe-Thr-Glu-Asn-Tyr-Thr-Arg(Mts)-Leu-Arg(Mts)-Lys(Z)-Gln-Met-(O)-Ala-Val-Lys(Z)-Lys(Z)-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH$_2$, Z(OMe)-(porcine Glu^8-VIP 1—28)-NH$_2$——The above protected docosapeptide amide (600 mg, 0.165 mmol) was treated with TFA–anisole (2 ml–0.4 ml) and the N$^\alpha$-deprotected peptide isolated as described above was dissolved in DMF (6 ml) containing Et_3N (0.05 ml, 0.33 mmol). The azide [prepared from 311 mg (0.33 mmol) of Z(OMe)–His–Ser(Bzl)–Asp–Ala–Val–Phe–NHNH$_2$ [C]] in DMF (3.5 ml) and Et_3N (0.09 ml, 0.66 mmol) were added to the above ice-chilled solution and the mixture, after stirring at 4° for 48 hr, was concentrated. The residue was treated with 5% citric acid and AcOEt and dissolved in a small amount of DMF. The solution was applied to a column of LH-60 (3 × 132 cm), which was eluted with the same solvent. UV absorption at 275 nm was determined in each fraction (6.6 ml). The fractions corresponding to the front main peak (tube Nos. 51—61) were combined and the solvent was removed by evaporation. Trituration of the residue with AcOEt afforded a powder; yield 404 mg (56%), mp 169—172°, $[\alpha]_D^{18}$ $+12.0°$ ($c=0.3$, DMF), Rf_1 0.12. Amino acid ratios in 6 N HCl hydrolysate: Asp 3.96, Thr 2.03, Ser 1.77, Glu 2.14, Ala 1.91, Val 1.96, Met+Met(O) 0.64, Ile 1.00, Leu 3.05, Tyr 1.91, Phe 1.09, Lys 2.88, His 1.14, Arg 2.08 (recovery of Ile 86%), *Anal.* Calcd for $C_{206}H_{292}N_{44}O_{56}S_3 \cdot 3H_2O$: C, 55.84; H, 6.78; N, 13.91. Found: C, 55.83; H, 6.83; N, 13.93.

H-His-Ser-Asp-Ala-Val-Phe-Thr-Glu-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH$_2$, (porcine Glu^8-VIP)——The above protected octacosapeptide amide (390 mg, 89.1 μmol) in DMF (5 ml) was incubated with thiophenol (0.92 ml, 100 equiv.) at 60° for 24 hr to reduce the Met(O) residue. After removal of the solvent, trituration of the residue with AcOEt afforded a powder; yield 382 mg (98%). The reduced peptide obtained above (87.2 mg, 20 μmol) was treated with a 1 M solution of TFMSA (0.35 ml, 4 mmol)–thioanisole (0.47 ml, 4 mmol) in TFA (total 2 ml) in the presence of *m*-cresol (0.15 ml, 70 equiv.) in an ice-bath for 60 min and at room temperature for 30 min, then dry ether was added. The resulting powder was dissolved in H_2O (20 ml), the solution was treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min and the resin was removed by filtration. The ice-chilled filtrate was adjusted to pH 9 with 5% NH_4OH and after 30 min to 6 with AcOH. After lyophilization, the residue was dissolved in a small amount of 0.1 N AcOH and the solution was applied to a column of Sephadex G-25 (1.8 × 130 cm), which was eluted with the same solvent. UV absorption at 275 um was measured in each fraction (6.8 ml). The fractions corresponding to the front peak (tube Nos. 25—41) were combined and the solvent was removed by lyophilization. The resulting powder was dissolved in H_2O (3 ml) and the solution was applied to a column of CM-cellulose (1.8 × 5.5 cm), which was eluted by gradient concentration with 0.1 M NH_4HCO_3, pH 8.0 (800 ml) through a mixing flask containing H_2O (200 ml). The UV absorption at 275 *nm* was measured in each fraction (4.8 ml). After elution of two minor peaks (tube Nos. 47—53 and 56—70), the main peak (tube Nos. 76—100) with minor tailing was detected. The fractions corresponding to the main peak were combined, and the solvent and NH_4HCO_3 were removed by repeated lyophilization to give a fluffy white powder; yield 25.0 mg (34%), $[\alpha]_{\mathrm{D}}^{18}$ $-60.9°$ (c=0.2, 0.1 N AcOH), Rf_3 0.50, Rf_2 0.24 (on DC-Alufolien Kieselgel, Merck); single band in 15% polyacrylamide gel electrophoresis at pH 2.3 (mobility, 2.9 cm from the origin toward the cathode, after running at 5 mA per tube for 110 min). The purity of the sample was examined by isoelectric focusing in Ampholine pH 9—11 (LKB, 800 vol at 3° for 48 hr). The desired peptide appeared at pH 10.2, and no significant impurity was detected. After gel-filtration on Sephadex G-25, the sample was recovered; 8.03 mg from 9.16 mg (88%). Amino acid ratios in 6 N HCl hydrolysate (numbers in parentheses are theoretical values): Asp 4.23(4), Thr 1.92(2), Ser 1.67(2), Glu 2.10(2), Ala 2.09(2), Val 2.21(2), Met 0.83(1), Ile 1.00(1), Leu 3.15(3), Tyr 2.10(2), Phe 1.11(1), Lys 3.27(3), His 0.96(1), Arg 2.07(2), (recovery of Ile 87%). Amino acid ratios in aminopeptidase (AP-M, Merck, Lot, No. 9652457) digest: Asp 0.98, Glu 1.19, Ala 1.92, Val 2.26, Met 1.01, Ile 1.00, Leu 3.19, Tyr 1.89, Phe 1.02, Lys 3.32, His 1.03, Arg 2.05 (recovery of Ile 85%). *Anal.* Calcd for $C_{148}H_{240}N_{44}O_{42}S \cdot 5CH_3COOH \cdot 4H_2O$: C, 51.12; H, 7.28; N, 16.60. Found: C, 50.92; H, 7.17; N, 16.99.

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