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Discovery of neurokinin antagonists

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Abstract: Neurokinins (substance P, neurokinin A, neurokinin B) play an important role in pain transmission, smooth muscle contraction, bronchoconstriction, activation of the immune system, and neurogenic inflammation. A neurokinin antagonist would thus be expected to have clinical potential for a variety of diseases. We have discovered two new neurokinin antagonists by the following two different strategies; 1) Screening of fermentation products of microorganisms. 2) Rational drug design of a low-molecular-weight antagonist from a known octapeptide lead.
Antagonist WS9326A was isolated from *Streptomyces violaceoniger* and was identified as a cyclic heptapeptide lactone, which was confirmed by total synthesis. Hydrogenation of two of the double bonds afforded FK224, an antagonist of both substance P (SP) and neurokinin A.
A search of the essential parts of the octapeptide lead, (D-Pro^4,D-Trp^{7,9,10},Phe^{11})SP(4-11), resulted in the discovery of the tripeptide SP antagonist, Boc-Gln-D-Trp(CHO)-Phe-OBzl. Chemical modification of the metabolically fragile benzyl ester and subsequent structural optimization led to Ac-Thr-D-Trp(CHO)-Phe-NMeBzl, which is highly potent and stable against metabolic degradation. By reconstructing the skeleton so that the spatial orientation of the essential pharmacophores was preserved, new branched tripeptides such as (1*H*-indol-3-ylcarbonyl)-Lys(Ac-Thr)-Phe-NMeBzl were designed. Further studies on the structure-activity relationship disclosed that even a dipeptide structure had potent activity. This finding culminated in the discovery of FK888 which is a potent and specific SP antagonist.

Introduction

Drug discovery and its progress can be accomplished by application of a wide variety of advanced chemistry. In particular, heteroatom chemistry is one of the main contributors, because pharmacophores to elicit biological responses usually contain heteroatom(s). I describe herein the discovery processes of new neurokinin antagonists as an example of recent drug discoveries.

The neurokinins are a family of neuropeptides which includes substance P (SP) and the two structurally related peptides, neurokinin A (NKA) and neurokinin B (NKB). In 1931 von Euler and Gaddum (Ref. 1) suggested the existence of SP in the extract of mammalian guts. The structure was finally confirmed and sequenced by Chang et al. (Ref. 2) in 1971 as Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2. Subsequently NKA and NKB were both isolated from porcine spinal cord in 1983. The structures were elucidated by the independent contributions of Kimura et al. (Ref. 3) and Kangawa et al. (Ref. 4); NKA is His-Lys-Thr-Asp-Ser-Phe-Val-Gly-Leu-Met-NH_2, whereas NKB is Asp-Met-His-Asp-Phe-Phe-Val-Gly-Leu-Met-NH_2. The neurokinins are characterized by a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH_2, where X is a phenylalanine or a valine. The receptors of the neurokinins have been intensively studied in pharmacological as well as genetic aspects. They are now classified into three subtypes, NK_1, NK_2 and NK_3, which have high affinity to SP, NKA and NKB, respectively (Ref. 5). These receptors are widely distributed in the central nervous system (CNS) and/or the peripheral system.

A number of physiological and pathophysiological studies indicate that the neurokinins exert quite diverse functions in the whole body (Ref. 6). For instance, SP acts in the CNS as a pain transmitter and also as a regulator of dopaminergic and adrenergic neurons. In the peripheral system, SP and/or NKA are involved in activation of the immune system, vasodilation, smooth muscle contraction, bronchoconstriction, stimulation of salivary secretion, neurogenic inflammation, and so on. The role of NKB has remained unclear, although involvement in the CNS function has been implied. In this regard neurokinin

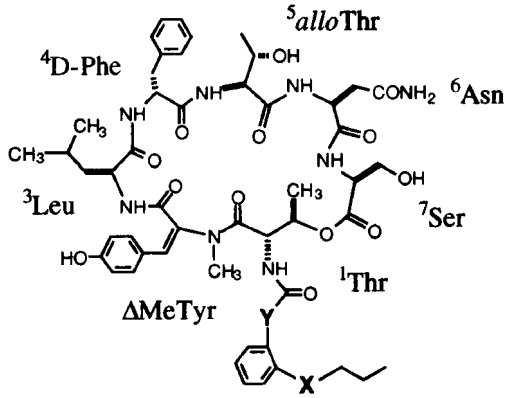
antagonists might be useful for treating pain, psychosis, possibly Alzheimer's disease, inflammation, rheumatoid arthritis, gastrointestinal disorders, migraine, and respiratory diseases such as asthma and bronchitis. Among these therapeutic applications, we have been primarily interested in the etiological participation of neurokinins in asthma via neurogenic inflammation. Neurogenic inflammation in the airways is now ascribed to the pathological phenomena caused by SP and NKA (Ref.7). In the asthmatic state, the sensory nerve c-fiber endings located in submucosal gland, blood vessel and smooth muscle are exposed as a result of epithelial damage, and thereby become sensitive to various stimuli. Once these nerve endings are stimulated by chemical mediators from inflammatory cells or non-specific stimuli such as smoke and cold air, neurokinins are released from the c-fiber endings, causing the pathological features of asthma such as contraction of smooth muscle, airway edema and mucus hypersecretion. Therefore a suitable antagonist which blocks these functions of neurokinins would become a new type of drug for respiratory diseases such as asthma and bronchitis.

Being motivated by this hypothesis, we started our research for discovering a new neurokinin antagonist, particularly an SP antagonist, according to two different strategies; 1) Screening of fermentation products of microorganisms. 2) Rational drug design of a low-molecular-weight antagonist from a known octapeptide lead.

Discovery of WS9326A from *Streptomyces violaceoniger*

Isolation and Structure Elucidation of WS9326A

Fermentation products from more than twenty thousand soil samples were screened to discover a new SP antagonist. The receptor binding assay using guinea-pig lung membranes and tritium-labelled SP was employed for this purpose. A novel compound, named WS9326A (Fig. 1), was isolated from a soil sample of Suwa city, Nagano, Japan. The microorganism producing this compound was identified as *Streptomyces violaceoniger* through the taxonomic studies (Ref.8). The detailed pharmacological studies indicated that WS9326A had the dual antagonistic activities to SP as well as NKA (Ref.9).



	WS9326A	FK224
X	-CH=CH-(Z)	-CH_2CH_2-
Y	-CH=CH-(E)	-CH_2CH_2-

Fig. 1. Structures of WS9326A and FK224

Structure elucidation of WS9326A was performed on the basis of chemical and physical evidence (Ref. 10). The molecular formula was established to be C54H68N8O13 by elemental analysis and high-resolution FABMS. Acetylation afforded the triacetyl derivative, whereas methylation with CH2N2 and subsequent acetylation provided the diacetyl monomethyl derivative, suggesting that three hydroxyl groups exist and one of them would be phenolic. The IR spectrum implied the presence of a peptide linkage (1650 cm-1) and an ester or lactone (1730 cm-1). The amino acid analysis of the acid hydrolysate revealed one residue each of Asp, Ser, Leu, Phe, methylamine and NH3, and two residues of Thr. One of the two Thr residues was observed to be *allo*Thr by chiral column GC-MS. Formation of methylamine could be ascribed to the presence of a dehydro *N*-methyl amino acid, and NH3 was presumably derived from either Asn or C-terminal carboxamide. Indeed, the amino acid analysis of the hexahydro derivative, obtained by hydrogenation (Pd-black, 4 atm) of WS9326A, indicated no formation of methylamine, instead a new peak, which coeluted with authentic (*DL*)*N*-methyltyrosine, suggesting that dehydro-*N*-methyltyrosine (ΔMeTyr) exists in the structure of WS9326A. The stereochemistry of the amino acids was determined by chiral column GC-MS of the *N,O*-trifluoroacetyl *n*-butyl ester derivatives, indicating L-configuration for the Asp, Ser, Leu, Thr, and *allo*Thr, and D-configuration for the Phe. Alkaline hydrolysis of WS9326A afforded

an acid fragment, which was identified as 3-(2-(1(*Z*)-pentenyl)phenyl)-2(*E*)-propenoic acid by NMR studies and confirmed by comparison with an authentic synthetic sample. Seven amino acids and the acyl component account for all the elements present in WS9326A. The molecular formula of WA9326A shows 25 degrees of unsaturation; however, the above fragments lead to 24 degrees. The remaining unsaturation is then due to the cyclic nature of the compound. Mild alkaline hydrolysis of WS9326A afforded the acid compound having molecular formula $C_{54}H_{70}N_8O_{14}$, indicating the presence of a lactone ring.

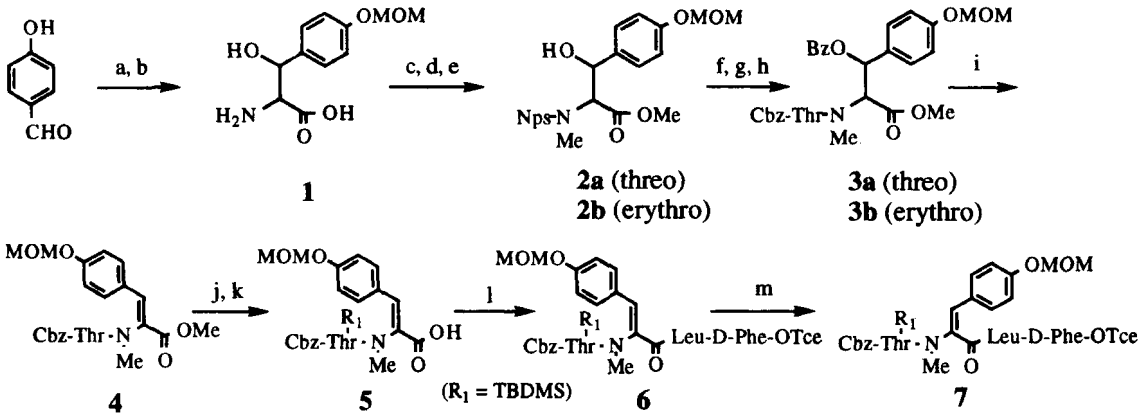
To elucidate the planar structure, extensive NMR analyses were carried out on triacetyl-WS9326A. NMR signals for individual amino acids ^{1}Thr, ^{3}Leu, ^{4}Phe, ^{5}Thr, ^{6}Asn, ^{7}Ser and the acyl component attached to ^{1}Thr were readily assigned by 2D NMR experiments including ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY, and COLOC (data not shown). The gross structure for WS9326A was then assembled by connecting the individual amino acids on the basis of connectivities observed in the COLOC and the ROESY analyses. The ΔMeTyr unit showed ROE between β-olefin proton and *N*-methyl proton, indicating *E*-configuration. The configuration of the two double bonds in the acyl component was assigned on the basis of vicinal ^{1}H-^{1}H coupling constants of the double bonds. Thus, the planar structure of WS9326A was identified as that depicted in Fig. 1.

The only remaining problem, the positions of Thr and *allo*Thr, was solved by chemical methods. Hydrogenation (Pd-Black, 1atm) of WS9326A produced the tetrahydro derivative FK224 (Fig. 1), whose activities against SP and NKA are increased by 10-fold (*vide infra*). Alkaline hydrolysis of FK224, followed by mild acid hydrolysis, afforded two degradation products. The structure of one product, which is negative to ninhydrin, was identified to be Thr having a pendant 3-(2-pentylphenyl)-propanoic acid. The amino acid analysis of the other product revealed the presence of *allo*Thr. These results confirmed that ^{5}Thr is *allo*Thr.

Total Synthesis of WS9326A

The key intermediate **3** for the synthesis of the (*E*)-ΔMeTyr unit was synthesized from 4-hydroxybenzaldehyde (Scheme 1). Coupling of MOM-protected 4-hydroxybenzaldehyde with Gly provided a 1:1 mixture of *threo*- and *erythro*-isomers of **1**. The mixture was *N*-methylated, the amino group protected with a 2-nitrophenylsulfenyl (Nps) group, and converted to the methyl ester. The *threo*- and *erythro*-isomers of **2** were separated by a silica gel chromatography. Each isomer was then separately converted to intermediate **3a** or **3b**. Both isomers **3a** and **3b**, when treated with DBU, produced exclusively the *Z*-isomer **4**. Photochemical isomerization of **4** did not give the desired *E*-isomer. However, treating the tetrapeptide **6** with a high pressure Hg lamp afforded a mixture of the starting **6** and *E*-isomer **7** in a 1:1 ratio. Each isomer was isolated and purified by a silica gel chromatography.

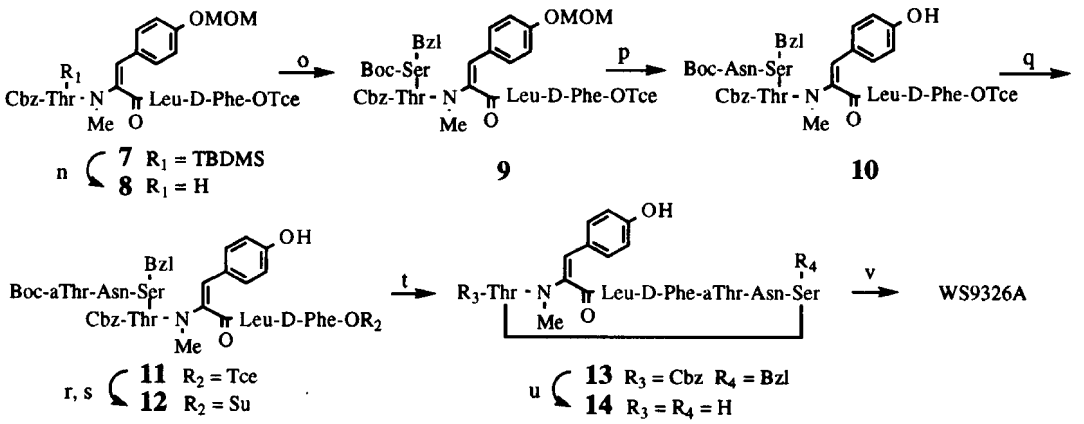
Scheme 1. Synthetic approach to (*E*)-dehydro-*N*-methyltyrosine unit.



(a) CH_3OCH_2Cl, TEA,THF,rt,1h; (b) Gly,KOH,EtOH,rt,19h; (c) $(MeO)_2SO_2$,1N NaOH,90°C,20min;
(d) NpsCl,BSA,CH_2Cl_2,0°C,2h;(e) CH_2N_2/ether; (f) BzCl,DMAP,TEA,CH_2Cl_2,rt,2d;
(g) PhSH,TFA,CH_2Cl_2,0°C,30min; (h) Cbz-Thr,EEDQ,CH_2Cl_2,rt,20min;(i) DBU,toluene,rt,30min;
(j) TBDMSCl,imidazole,DMF,rt,16h; (k) 1N NaOH,30°C,2d; (l) Leu-D-Phe-OTce,EEDQ,CH_2Cl_2,rt,15h;
(m) toluene:acetone=10:1,hv(100W),0°C,1.5h;

After removal of the TBDMS group in **7** (Scheme 2), the hydroxy of the Thr was reacted with Boc-Ser(Bzl) to give the ester **9**. Elongation of the peptide chain afforded the linear protected peptide **11**.

Scheme 2. Synthesis of WS9326A.



(n) 67%AcOH,25°C,28h; (o) Boc-Ser(Bzl),EDC,DMAP,CH_2Cl_2,rt,12h; (p) 4N HCl/dioxane,rt,30min,then Boc-Asn, TEA,HOBT,EDC,CH_2Cl_2,rt,1h; (q) 4N HCl/dioxane,rt,30min,then Boc-aThr,TEA,HOBT,EDC,CH_2Cl_2,rt,8h; (r) 90%AcOH,Zn,rt,9h; (s) HOSu,WSCD,CH_2Cl_2,rt,15h; (t) TFA,rt,30min,then DMF pyridine,rt,16h; (u) HF pyridine,rt,1h; (v) 2-(1(*Z*)-pentenyl)-cinnamoyl chloride,CH_2Cl_2,BSA,DMF,rt,1h.

Deprotection of the trichloroethyl group and subsequent reaction with *N*-hydroxysuccinimide gave the active ester **12**. The cyclization reaction proceeded in a 40% yield by deprotection of the Boc group in the *allo*Thr with TFA and subsequent treatment with pyridine in a highly diluted solution. The Cbz and Bzl groups were removed with HF-pyridine to give the free cyclic peptide lactone **14**. The final step, acylation with 2-(1(*Z*)-pentenyl)-cinnamoyl chloride, was carried out to afford WS9326A. The synthetic product was identical with the natural WS9326A in terms of behavior on chromatogram and spectral data.

Discovery of FK888 by Rational Drug Design

Design of Tripeptide SP Antagonist (Ref.11)

We selected the octapeptide **15** (Scheme 3) as a lead for rational drug design to discover a low-molecular-weight SP antagonist. The reason for the selection was based on the report (Ref. 12) that this peptide was the most potent and specific SP antagonist in guinea-pig trachea, when we initiated this study in 1986. We presumed at the beginning that the essential domain to bind to the receptor might be as small as a few amino acids. Based on this working hypothesis, we synthesized the six fragment tripeptides (Scheme 3), by which the whole amino acid sequence of **15** is covered. The fragment tripeptides along with the intermediates bearing protecting group(s) were tested in the receptor binding assay using guinea-pig lung membranes and tritium-labeled SP. We found that the protected tripeptide benzyl ester **16** (Scheme 4), which corresponds to the glutamine-D-tryptophan-phenylalanine region in **15**, exhibited a potent binding affinity to the receptor with IC50 of 90nM. The activity was about 7-fold improved in comparison with the lead octapeptide **15** having IC50 of 600nM.

Scheme 3. Octapeptide lead and fragment tripeptides.

D-Pro-Gln-Gln-D-Trp-Phe-D-Trp-D-Trp-Phe-NH_2

⇩ **15**

D-Pro-Gln-Gln

Gln-Gln-D-Trp + Protected tripeptides

Gln-D-Trp-Phe

D-Trp-Phe-D-Trp

Phe-D-Trp-D-Trp

D-Trp-D-Trp-Phe-NH_2

Scheme 4. Metabolically stable tripeptide.

Boc-Gln-D-Trp(CHO)-Phe-OBzl

⇩ **16**

Ac-Thr-D-Trp(CHO)-Phe-NMeBzl

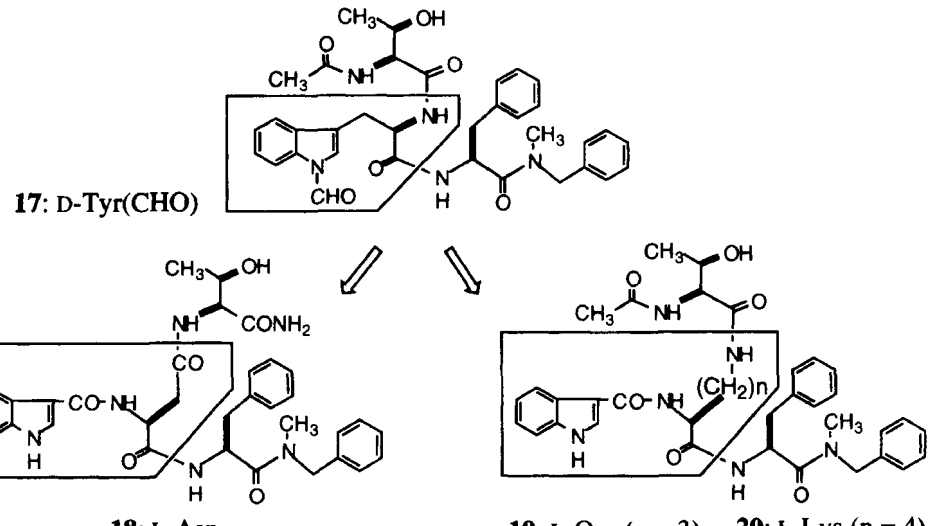
17

The tripeptide benzyl ester **16**, however, did not show any antagonistic activity against SP in *in vivo* experiments, because the benzyl ester part was readily degraded to the corresponding acid, which has no SP antagonistic activity. Thus we modified the benzyl ester part of **16** into a *N*-methyl-*N*-benzylamide structure which was stable against enzymatic metabolism (Scheme 4). Subsequently we optimized the amino terminal part into Ac-Thr to reach the potent tripeptide amide **17**. The IC50 value was 5.8nM, being about a hundred times more potent than the lead octapeptide **15**. Compound **17** is structurally quite novel, and was shown to be a specific and potent SP antagonist in *in vivo* models. However, this compound lacked solubility in water, and was poorly absorbed when administered orally. To overcome these problems we designed another class of compounds.

Design of Branched Tripeptide SP Antagonist (Ref.13)

The structure-activity relationship studies on **17** revealed that the D-Trp(CHO)-Phe-NMeBzl structure including the stereochemistry is essential for receptor recognition and also that the Ac-Thr part is necessary but variable (Ref.11b). We thus designed the novel branched tripeptides (Scheme 5) which mimic the spatial orientation of the essential components in **17**, presumably the indole nucleus and the two benzene rings. We intended in this design to keep the Phe-NMeBzl part unchanged and to introduce another α-amino acid such as L-Asp (**18**), L-Orn (**19**) or L-Lys (**20**), which has an 1*H*-indol-3-ylcarbonyl at the α-amino group. The newly introduced α-amino acids would necessarily have an L-configuration, when compared to **17**. An appendage which corresponds to the Ac-Thr part in **17** was introduced using the side chain functional groups. However the formyl group of the indole nitrogen was omited because of synthetic problems. As expected, these newly designed tripeptides were found to exhibit potent activity in the binding assay, although the IC50 values were 10–20nM, being somewhat reduced in comparison with **17**.

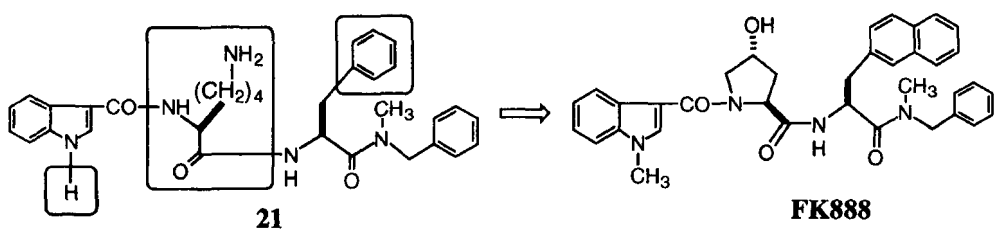
Scheme 5. Design of branched tripeptide SP antagonists.



Design of FK888 (Ref.14)

As the next step, we carried out further modification of the Ac-Thr moiety of the most active branched tripeptide **20**. We found in the course of modification studies that a variety of amino acids were able to exhibit potent activity and also that even the dipeptide compound **21** (Scheme 6), which lacks the Ac-Thr,

Scheme 6. Discovery of a potent and specific SP antagonist, FK888.



had almost equal activity to the parent tripeptide **20**. Subsequent modification of the L-Lys part into various amino acids turned out that (2*S*,4*R*)-hydroxyproline is the best. We finally optimized the other parts, the substituent on the indole nitrogen and the phenylalanine part, to culminate in the most potent compound FK888.

Pharmacological Properties of FK224 and FK888

The receptor binding properties and potencies of FK224 and FK888 were investigated using human neurokinin receptor subtypes expressed in transfected COS-7 cells (Ref. 15). The IC50 values of FK224 are 190, 190 and >3200nM for NK_1, NK_2 and NK_3, respectively, and those of FK888 are 0.72, 810 and 2200nM. These results indicate that FK224 has dual actions to NK_1 as well as NK_2 but FK888 is highly selective to NK_1.

The *in vivo* activities of FK224 and FK888 were evaluated in the experimental model for asthma (Ref. 16), the airway edema in guinea-pigs induced by SP or capsaicin, which is the pungent ingredient of pepper and is known to release neurokinins from the nerve endings. When administered intravenously, FK224 suppressed the edema induced by SP and capsaicin with ED50's of 0.14 and 0.30mg/kg, respectively, whereas FK888 exhibited more potent activities with 0.011 and 0.019mg/kg. FK888 was also effective after oral administration, although the ED50 values were 4.2mg/kg for SP and 9.5mg/kg for capsaicin.

Conclusion

Both FK224 and FK888 are now being developed in clinical stage. The discoveries of these neurokinin antagonists will hopefully contribute to provide a new remedy for respiratory diseases such as asthma and bronchitis and also to serve as a powerful tool for investigation of the biological functions of neurokinins.

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