# Identification in the NK1 tachykinin receptor of a domain involved in recognition of neurokinin A and septide but not of substance P

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**Abstract The three mammalian tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), exert their physiological effects through specific receptors, NK1, NK2 and NK3, respectively. However, homologous binding studies have recently demonstrated that, contrary to the generally accepted belief, NKA could bind NK1 receptor with high affinity (Hastrup and Schwartz, 1996). Using COS-7 cells expressing the human NK1 receptor, we show that two simultaneous point mutations (E193L and V195R) in a restricted five amino acid sequence (the (193–197) region), selected because of its hydropathic complementarity with the common C-terminal extremity of tachykinins, abolish both the high-affinity binding and highly potent biological activity of NKA, without affecting those of SP. In addition, the same mutations also suppressed the high functional activity of septide, a synthetic SP atypical agonist ([pGlu$^6$-Pro$^9$] SP 6–11). These results suggest that the (193–197) region, located at the end of the second extracellular loop of the receptor, could be part of a common high-affinity binding domain for both NKA and septide, distinct from the SP binding site.**

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*Key words:* NK1 receptor; Substance P; Neurokinin A; Septide; Tachykinin; Hydropathic complementarity

## 1. Introduction

The three main mammalian tachykinins, substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), exert their various biological effects (e.g. nociception, control of smooth muscle activity and exocrine secretion, inflammation and immunomodulation) through interactions with three different receptors, named NK1, NK2 and NK3 [1], which belong to the G protein-coupled seven transmembrane domain receptor family [2]. It has been generally admitted for more than ten years that, despite some degree of cross-reactivity, SP is the natural endogenous ligand for the NK1 receptor, NKA for the NK2 receptor, and NKB for the NK3 receptor. However, as recently reviewed [3], it has also been recognized for a long time that some observations did not fit with this classical 'three-peptide-three-receptor dogma'. Examples include the finding that NKA can behave as a potent agonist in functional assays considered to be specific for the NK1 receptor [4,5], or the observation that NKA can exert in vivo physiological actions without identified NK2 receptors on target cells, especially in the central nervous system [6]. In fact, it has been clearly demonstrated in CHO cells expressing only the recombinant NK1 receptor [7], that NKA could indeed induce a potent pharmacological effect at nanomolar concentrations, although surprisingly, its apparent affinity estimated through its ability to displace radiolabeled SP in competition binding experiments was rather low (in the micromolar range). Recently, however, Hastrup and Schwartz [8], also working on recombinant receptor-expressing cells, reported for the first time that NKA is actually a high-affinity ligand for the NK1 receptor. This was established from homologous binding analysis, i.e. by using radioactive NKA directly, instead of studying the indirect effect of this tachykinin on the binding of labeled SP. So, the question now under debate concerns the precise location of this site for NKA on the NK1 receptor, namely do NKA and SP bind to the same or distinct sites? If the latter hypothesis is correct, it might in theory be possible to find region(s) in the NK1 recombinant receptor in which mutations would affect the binding of one tachykinin without changing the binding of the other one. The aim of this paper was to investigate this possibility.

In order to orient the search for such a region, we decided to follow a molecular recognition theory stating that peptides or proteins can interact through amino acid sequences displaying inverse hydropathic profiles [9]. More than 40 examples of ligand characterization based on complementary hydropathy have been reported in the literature [10]. In the field of antigen-antibody interactions, we recently illustrated this theory by demonstrating, using surface plasmon resonance technology, the importance of hydropathic complementarity for the kinetic parameters of peptide-antibody binding [11]. In terms of biological receptors, the search for hydropathically opposed sequences by means of computer programs has already been used successfully to identify the putative binding sites for a dozen ligands (reviewed in [12]) and, very recently, complementary hydropathy has even served to identify a novel prion protein receptor [13].

In the present study, we report the occurrence in the human NK1 receptor of four sequences whose hydropathic profiles are opposed to that of the common C-terminal sequence of tachykinins, this C-terminal extremity having long been known to be directly involved in receptor binding. We show that two point mutations, simultaneously performed in one of these sequences (the (193–197) region) in order to suppress the hydropathic complementarity with tachykinin agonists, resulted in a nearly complete loss in the direct binding of radiolabeled NKA without significantly affecting that of labeled SP. Accordingly, these mutations largely impaired the highly potent pharmacological effect of NKA without changing that

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of SP. In addition, we observed that the same mutations in the (193–197) region also abolished the pharmacological action of septide, a synthetic C-terminal SP analog ([pGlu$^6$-Pro$^9$] SP 6–11). Although this compound has long been considered as a potent and selective agonist of the NK1 receptor [14], its atypical pharmacological and binding properties [7,15] have led some authors to postulate more recently the existence of a specific 'septide-sensitive' receptor (review in [16]).

Taken together, our results support the hypothesis of the existence of two distinct high-affinity binding sites on the NK1 receptor, one for SP and the other for both NKA and septide, this latter site being at least in part located at the distal extremity of the second extracellular loop.

## 2. Materials and methods

### 2.1. Materials

$^{125}$I-substance P (74 TBq/mmol), $^{125}$I-NKA (74 Tbq/mmol) and myo-[2-$^3$H]inositol (651 Gbq/mmol) were from Amersham France. Non-labeled peptides were from Neosystem (Strasbourg, France). Cell culture medium Ham F-12, fetal calf serum, HEPES, penicillin, streptomycin were from GIBCO (Eregny, France). Bacitracin, lithium chloride (LiCl) and bovine serum albumin (BSA) were from Sigma. Cell culture plastic dishes were from NUNC (Roskilde, Denmark).

### 2.2. Determination of hydropathically opposed sequences in the human NK1 receptor and tachykinin agonists

In order to identify in the human NK1 receptor putative interaction sites with SP, NKA and septide, we used a computer program which we described recently [17]. This software selects amino acid sequences of at least five amino acids whose hydropathic profiles are opposed in the two molecules to be compared, i.e. here the NK1 receptor on the one hand and the C-terminal extremities of SP, NKA and septide, on the other hand. This opposition is quantitatively estimated through the calculation of linear regression analysis of hydropathic values (taken from the Kyte and Doolittle scale) for a given position in each sequence: a correlation coefficient ($r$) between $-0.8$ and $-1$ was imposed as reflecting a high degree of opposition.

### 2.3. Human NK1 receptor mutagenesis

The cDNA clone of the human NK1 receptor in pCDM8 was a generous gift from Dr. N.P. Gerard (Harvard Medical School, Boston, USA). In order to abolish the hydropathic complementarity between the (193–197) NK1 sequence and tachykinin agonists, we chose to replace at the same time two amino acids by residues with opposite hydropathic values, i.e. Glu$^{193}$ ($-3.5$) by Leu (+3.7) and Val$^{195}$ (+4.2) by Arg ($-4.5$). Mutagenesis was realized using the PCR site-directed mutagenesis system (BRL/Life Technologies) according to the specifications of the supplier. Sequences of mutagenic oligonucleotides used for NK1 receptor mutant, i.e. E193L and V195R, are AUU UAU UUG AAA CGU UAC CAC AUC UGT GTG ACT GTG CTG ATC TAC TTC CTC CCC and GAU GUG GUA ACG TTT CAA AUA AAU CTT GTT CGG (the modified amino acids are encoded by the underlined sequences). Wild-type or mutant receptors were subcloned at *Xho*I-*Eco*RI in pcDNA3 (in Vitrogen). Sequences were checked using the dideoxy termination method.

### 2.4. Cell culture and transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% $CO_2$. The pcDNA3 construct harboring the human NK1 receptor cDNA modified and non-modified sequence was transfected into COS-7 cells by electroporation. Control sham transfections were performed with the same expression vector alone.

### 2.5. Binding assays

Direct binding analysis was performed as described previously [7]. In brief, COS-7 cells ($10^5$/well) were incubated for 100 min in the presence of 0.01 to 10 nM of isotopic dilutions of [$^{125}$I]-SP or [$^{125}$I]-NKA. Non-specific binding was determined in the presence of 1 μM SP or NKA. Experimental data were analyzed using the computer program LIGAND.

### 2.6. Measurements of phosphatidylinositol hydrolysis

Experiments were carried out following a standard protocol already described in details [7]. Briefly, after labeling with [$^3$H]inositol for 24 h, COS-7 cells ($10^5$/well) were incubated for 30 min at 37°C in the presence of tachykinin agonists. Incubations were terminated by lysing cells with Triton X-100 and [$^3$H]inositol phosphates were separated by chromatography of the aqueous phase recovered after addition of a chloroform/methanol (1:2) mixture.

## 3. Results

### 3.1. Occurrence of hydropathically opposed sequences in the human NK1 receptor and tachykinin agonists

Applying the restrictive parameters of the computer program described in Section 2, four sequences were identified in the NK1 receptor on the basis of hydropathic complementarity with the C-terminal extremity of tachykinins: the (13–17) and (20–26) sequences, located in the N-terminal extracellular extremity, the (136–140) sequence in the second intracytoplasmic loop, and the (193–197) sequence at the extremity of the second extracellular loop. For example, the (20–26) sequence (Fig. 1) was best hydropathically opposed (not shown) with the C-terminal extremity of SP ($r = -0.94$ over a stretch of seven amino acids), while the (193–197) sequence appeared best opposed (Fig. 1) to those of NKA and septide ($r = -0.81$ and $-0.84$, respectively). We chose this latter re-

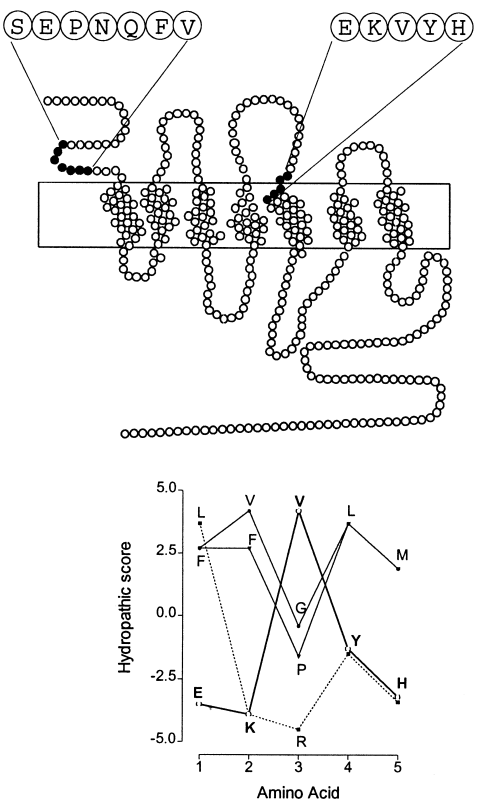



Fig. 1. Structure of the human NK1 receptor for substance P with two sequences identified on the basis of hydropathic complementarity with the common C-terminal extremity of tachykinins: the SEPNQFV (20–26) sequence and the EKVYH (193–197) sequence (upper panel). The hydropathic profile of the EKVYH (193–197) receptor sequence is compared with those of NKA (6–10): FVGLM and septide (2–6): FFPLM (lower panel). The hydropathic profile of the (E193L, V195R) mutant (193–197) sequence is also indicated (dotted line).

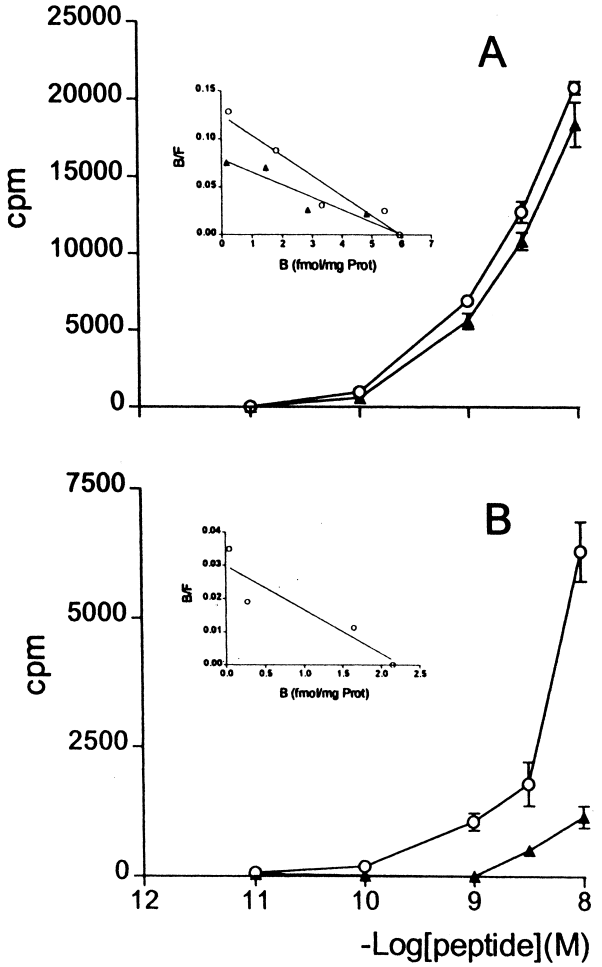



Fig. 2. Saturation analysis of the binding of labeled SP (A) and labeled NKA (B) to COS-7 cells expressing wild-type (○) or (E193L, V195R) mutant (▲) NK1 receptors. Cells were incubated in the presence of increasing concentrations of isotopic dilutions of $^{125}$I-SP or $^{125}$I-NKA (10 to 10 000 pM). Non-specific binding was determined in the presence of 1 μM unlabeled SP or NKA, respectively. Results are the mean ± S.D. of two independent experiments done in triplicate. Inset: Scatchard analysis of the data.

gion for further analysis by means of site-directed mutagenesis since it was located in the second extracellular loop, i.e. a region shown to be often involved in the binding of various ligands on 7TM receptors [18]. As shown in Fig. 1, the two simultaneous mutations E193L and V195R introduced in this region abolished the hydropathic complementarity with the C-terminal extremity of NKA and septide ($r$ = +0.28 and +0.61, respectively, in the double mutant receptor).

### 3.2. *Effects of point mutations in the (193–197) sequence of the human NK1 receptor on the direct binding of radiolabeled SP and NKA*

Saturation binding experiments showed that labeled SP (Fig. 2A) was able to bind both wild-type and mutant NK1 receptor with a similar high affinity ($K_d$ = 0.12 ± 0.02 versus 0.18 ± 0.02 nM, respectively). Moreover, identical maximal binding values were found for SP on wild-type and mutant receptors ($B_{max}$ = 5.98 ± 0.76 and 6.00 ± 0.18 fmole/mg protein, respectively, which corresponds to about 22 000 binding sites/cell). These results indicate that mutations in the (193–197) region of the NK1 receptor did not significantly affect either the expression level of recombinant receptors on cell membranes, or the binding affinity of SP for these receptors. By contrast, mutations largely impaired the binding of labeled NKA (Fig. 2B). Indeed, whereas NKA appeared to bind wild-type NK1 receptor with a high affinity ($K_d$ = 0.16 ± 0.02 nM), its binding on mutant receptor was almost totally abolished, at least for concentrations up to $10^{-8}$ M. On wild-type receptor, the maximal binding capacity of labeled NKA was estimated as 2.21 ± 0.06 fmole/mg protein (8100 binding sites/cell), a $B_{max}$ value amounting to about 37.0 ± 1.0% of that found for SP.

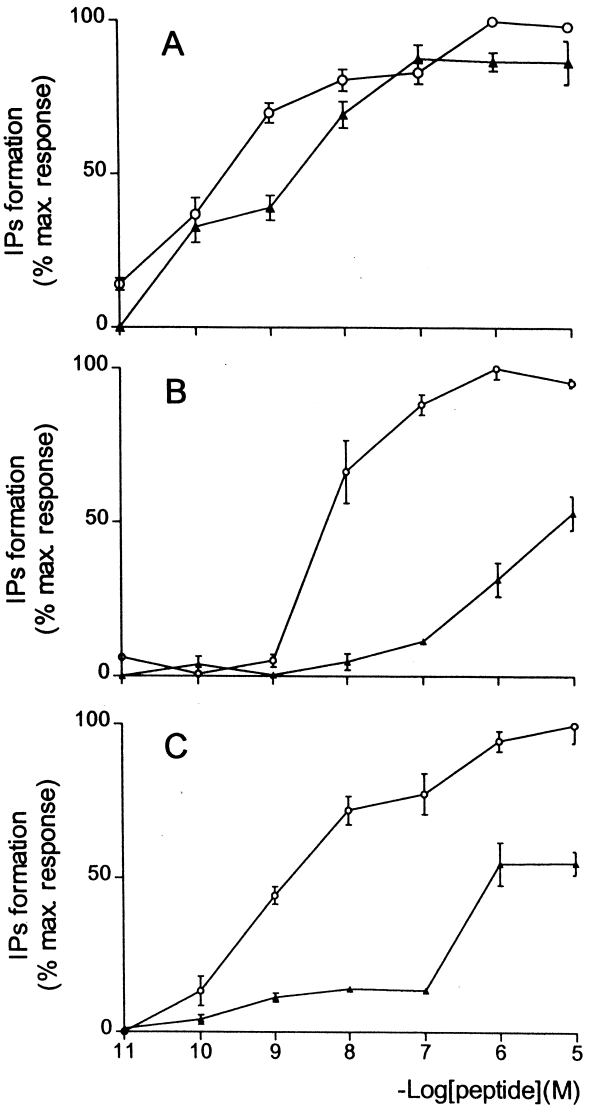



Fig. 3. Inositol phosphate (IP) production as a function of the concentration of tachykinin ligands in COS-7 cells expressing wild-type (○) or (E193L, V195R) mutant (▲) NK1 receptors. Cells were incubated in the presence of increasing concentrations of SP (A), NKA (B) or septide (C). Basal level of IP production was determined in wells to which no agonist was added. Results are the mean ± S.D. of four independent experiments done in triplicate. They are expressed as percent of maximal response for each agonist on wild-type receptor-expressing cells. Similar absolute values were obtained using saturating concentrations of SP, NKA and septide on wild-type receptor-expressing cells.

### 3.3. Effects of point mutations in the (193–197) sequence of the human NK1 receptor on biological activity of SP, NKA and septide

As shown in Fig. 3, SP, NKA and septide were highly potent in inducing inositol phosphate formation in COS-7 cells expressing the wild-type receptor, with $EC_{50}$ values of $0.47 \pm 0.22$, $4.78 \pm 1.70$ and $1.85 \pm 0.78$ nM, respectively, confirming earlier reports [7]. In contrast, in COS-7 cells bearing NK1 receptors with mutations in the (193–197) region (expressed at a level similar to that found in the wild-type receptor-bearing cells, see above), we found that the high potency of both NKA (Fig. 3B) and septide (Fig. 3C) in inducing a biological response was abolished ($EC_{50} > 1$ μM), whereas that of SP (Fig. 3A) was little affected ($EC_{50} = 2.30 \pm 1.20$ versus $0.47 \pm 0.22$ nM for the wild-type receptor-expressing cells). In addition, we checked in control experiments that the differences in the amount of inositol phosphate production induced by NKA or septide in wild-type and mutant NK1 receptor-expressing cells were not related to experimental conditions such as the incubation time (30 min) in the presence of agonists, since similar values were obtained when using longer incubation periods, up to 2 h (results not shown).

## 4. Discussion

The data presented here point to the importance of a short sequence, the (193–197) region in the human NK1 receptor (located at the end of the second extracellular loop), in the high-affinity binding of NKA, but not of SP, and in the specific triggering of its biological response. They constitute the first report, to our knowledge, of point mutations which affect differently the true affinities (measured in homologous binding experiments) of two different endogenous tachykinins for the same receptor.

The hydropathic complementarity methodology used here to guide the search for putative interactive sequences in the NK1 receptor has been successfully employed in recent years to identify ligand binding domains in a large variety of proteins, including enzymes, antibodies, MHC proteins and biological receptors (see [19] for a review). Although our present study was focused on the (193–197) region of the NK1 receptor, it might be no coincidence that the (20–26) extracellular sequence (Fig. 1), also identified here on the basis of hydropathic complementarity with the common C-terminal extremity of tachykinins, very precisely corresponds to a region which has been already shown by Fong et al. [20,21] to play a major role in the high-affinity binding of the C-terminal part of SP. Together with our present results concerning the (193–197) region, these data from other groups could appear to lend additional support to this methodology based on hydropathic complementarity.

The finding that the same mutations in the (193–197) region of the NK1 receptor abolish the potent pharmacological effects of both NKA and a tachykinin-like peptide, the septide, strongly suggests that this region would be part of a common high-affinity binding site for both agonists. This would be in close agreement with the recent study by Hastrup and Schwartz [8] showing, for the first time, that both radiolabeled peptides bound to the NK1 receptor with similar high affinity in homologous binding assays as well as in competition against each other. The existence of a common NKA/septide binding site would also readily explain why both peptides exert identical pharmacological effects on cell lines expressing the recombinant NK1 receptor, and have been said to belong to the 'septide-like' peptide family, triggering the phospholipase C transduction pathway, as opposed to SP and other 'SP family' peptides, inducing both the phospholipase C and the adenylyl cyclase pathways [7].

More importantly, our results strengthen the hypothesis that this NKA/septide site, involving the (193–197) region, is distinct from the SP binding site, possibly located at least in part around the (20–26) region, as discussed above. Alternatively, it could be inferred from our present results that the mutations in the second extracellular loop would alter the conformation of a unique tachykinin site, rendering this area unable to accommodate NKA and septide, but preserving its SP binding capability. However, to our mind, this explanation seems rather unlikely when considering the results of numerous directed mutagenesis experiments [20–23] involving hybrid, chimeric or locally mutated recombinant receptors, which indicate that the binding site of SP is at least partially located on the extracellular N-terminal part of the receptor, while that of septide is indeed extending rather in the C-terminal domain. The actual presence of two distinct SP and NKA/septide binding sites on the NK1 receptor would also fit well with the observations that some non-peptide SP antagonists (like RP 67580) behave competitively for SP but non-competitively for NKA and/or septide [7,15,24], whereas conversely, other non-peptide antagonists (like CP 96345) are more potent in antagonizing septide- than SP-induced responses [25]. In this context, it is noteworthy that it has been clearly shown by mutational analysis [26] that CP 96345 (unlike SP and RP 67580) binds in the vicinity of the E193 and H197 amino acids, i.e. precisely in the (193–197) region that we suggest here to be part of the NKA/septide binding site. Since this region has been also proposed by the same authors [27] to play a major role in regulating receptor conformation, it would be of great interest to re-examine the homologous as well as the heterologous binding properties of tachykinin agonists on the several mutant receptors that have been already engineered.

As regards the current receptor models, it seems likely that the NK1 receptor could assume at least two conformations in equilibrium in the membrane, an 'SP-preferring' and a 'general tachykinin' conformer [3]. Our finding that the $B_{max}$ value for NKA was almost three times lower than that for SP confirms previous data from other groups [8,28] and is in good agreement with this model, stating in addition that the interconversion between the conformers occurs readily upon SP binding, but with much more difficulty upon NKA or septide binding [3]. Consequently, a model in which the NK1 receptor has two active conformers, each one bearing two distinct binding sites for SP and NKA/septide, appears highly consistent with our present data and also with most of the pharmacological and biochemical properties of tachykinin peptides, including septide.

Finally, we should emphasize that, as discussed recently by Colquhoun [29], the interpretation of the effects of mutations on G protein-coupled receptors is never unambiguous. For instance, our results do not exclude that mutations in the (193–197) region might affect the allosteric transition or distribution between the two receptor conformers, as described for other mutations in the NK1 receptor [30]. In this context, photoaffinity labeling experiments could afford novel insights

into the location of tachykinin binding domain(s). However, since ligand binding sites very probably correspond to different receptor areas scattered all along the primary sequence, it is not surprising that amino acids located outside the (193–197) or (20–26) regions have already been implicated in the high-affinity binding of agonists [31,32].

In conclusion, regardless of whether or not it was chance that allowed us to identify the (193–197) sequence, the finding on the NK1 receptor of a restricted region specifically involved in the in vitro pharmacological action of NKA strengthens the physiological importance of this endogenous tachykinin, co-released in vivo with SP, in triggering specific responses through NK1 receptors and/or in modulating the SP-induced responses. In addition, this newly identified extracellular region in the NK1 receptor may now appear as an original pharmacological target for development of novel selective bioactive compounds.

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