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In Vitro Activity and Selectivity of Glucosidic $SP_{(6-11)}$ Analogues

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HARO, I., P. RUIZ, G. VALENCIA, J. M. GARCÍA-ANTÓN, F. REIG AND R. E. RODRIGUEZ. *In vitro activity and selectivity of glucosidic $SP_{(6-11)}$ analogues.* PHARMACOL BIOCHEM BEHAV **34**(3) 527–532, 1989.—The relative potencies of a series of substance P (6–11) analogues have been determined for spasmogenic activity in the guinea pig ileum in vitro and for potentiation of electrically evoked contractions in the rat vas deferens in vitro. ED_{50} values were determined for the new analogues. Substance P and its methyl ester were used as standard agonists. Substitution of Gly^9 by Pro on $[Glu^6]SP_{(6-11)}$ increased four times the activity on the NK-1 receptor. The glycosilation of $[Glu^6]SP_{(6-11)}$ by the incorporation of a β-D-glucopyranosyl amide residue on the γ-carboxyl group of Glu^6 reduced both the activity and selectivity. The simultaneous substitution of Gly^9 by Pro and the incorporation of a monosaccharide moiety on the γ-carboxyl of Glu^6 on $[Glu^6]SP_{(6-11)}$ yielded an analogue with 60-fold enhanced selectivity relative to substance P for the NK-1 receptor. These results may indicate that the critical factor providing potency to $SP_{(6-11)}$ analogues is mostly related to conformational rather than hydrophilicity aspects of the molecular structure.

Substance P Structure-activity relationship Substance P receptors Guinea pig ileum assay Rat vas deferens assay

SUBSTANCE P is an undecapeptide that belongs to the tachykinin family. It acts as a potent spasmogen in smooth muscle and as a neurotransmitter in the mammalian peripheral and central nervous systems (7, 15, 21, 22).

It is now accepted that the actions of different tachykinins are mediated by multiple receptors. According to the recently adopted nomenclature (13) there are three receptor subtypes, namely, NK-1, NK-2 and NK-3 whose most selective endogenous substrates are SP, neurokinin A and neurokinin B, respectively (16, 18, 19, 25, 30).

As far as the in vitro activity is concerned, it has been found that the various tissue preparations present different receptor populations. In the longitudinal muscle of the guinea pig ileum, SP induces muscle cell contractions either by a direct action through the NK-1 receptor (2,23) or by an action on the NK-3 receptor which may be mediated by the release of acetylcholine from the myenteric nerves involving the NK-3 receptors (14). Moreover, in the rat vas deferens, SP acts by activation of the NK-2 receptor (18).

Conformation-activity relationship studies of substance P have suggested the existence of a folded conformation stabilized by a hydrogen bond between the carbonyl group of Met^{11} and the amide of Gln^6 (28). Conformational energy calculations (20) and data obtained from nuclear magnetic resonance (NMR), infrared spectroscopy (IR) and circular dichroism (CD) have been proved consistent with a model structure having an extended region corresponding to the first five residues and a hair-pin folding in the second segment of the molecule (6).

Moreover, structure-activity relationship studies have shown that the last six residues are mainly responsible for biological activity mostly related to muscle contraction in GPI (27). Thus, $SP_{(5-11)}$ and $SP_{(6-11)}$ seem to be equipotent or even more potent than $SP_{(1-11)}$ for inducing contractions of the GPI (3, 4, 32). For this reason it has been suggested that the C-terminal moiety of the molecule might be regarded as the "message sequence" and the N-terminal portion as the "adress" (6). These assumptions are in agreement with results of model membrane-substance P interaction studies, in the sense that the C-terminal part of the molecule inserts perpendicularly into the membrane as an α-helix, whereas the N-terminal segment remains in the aqueous phase (10, 26, 29).

Accordingly, we have conducted the substitution of Gly^9 by Pro on the $[Glu^6]SP_{(6-11)}$ sequence in order to find out if a rotation restrainment induced by the presence of Pro could afford an analogue presenting a more stable folded conformation. Moreover, as a hydrophilic character inducing water solubility has proved to be an activity requirement for $SP_{(6-11)}$ analogues (24), and since sugar moieties have been claimed to play an important role in membrane recognition processes (1,8), we have examined the influence of a change of the hydrophobic-hydrophilic balance of the molecule by the introduction of a monosaccharide residue on the side chain of Glu^6. In addition, the influence of the above simultaneous modifications on the $[Glu^6]SP_{(6-11)}$ activity has been studied.

METHOD

Spasmogenic Test on the Isolated Guinea Pig Myenteric Plexus

Segments of ileum, always taken from an approximate 20 cm distance from the caecum, were obtained from male albino guinea pigs (250–300 g) purchased from Biocenter, Spain. One single ileum preparation from each animal was used. The segments, 3–3.5 cm in length, were cut open along the mesenteric attachment and mounted in a 13 ml glass organ bath containing oxygenated (95% O_2–5% CO_2) Krebs solution (mM composition: NaCl 141; KCl 5.6; $CaCl_2$ 2.5; KH_2PO_4 14; $MgSO_4 \cdot 6H_2O$ 14; $NaHCO_3$ 12 and glucose 30) maintained at 37°C.

The preparations were kept under a resting load of 0.4 g and longitudinal contractions were recorded isotonically by a Harvard transducer connected onto an Omniscribe (Houston Instruments) recorder specially modified by Panlab (Barcelona). Before starting the experiments, tissues were equilibrated for 20–30 min. Peptides were applied at 10-min intervals with 30 sec of contact time. Peptide doses were not randomized. Thus, increasing doses of each analogue were applied until a maximum of contraction was obtained. Maximum responses were determined by assaying increasing amounts of drugs until two successive doses showed equal response. Following this protocol the tissues used never became tachyphylactic to the actions of either SP or the analogues since analogous responses were obtained by equal doses applied before and after the preparations have reached maximal responses. Every preparation has been used to assay an average of ten doses.

Selective assay of the muscular NK-1 (SP-P) receptor in guinea pig ileum was performed in the presence of 1 μM atropine, a muscarinic blocker which prevents the action of neuronally released acetylcholine and, thereby, eliminates the neurogenic component of the contraction response (16).

Selective assay of the neuronal NK-3 (SP-N) receptor was performed following inactivation of the muscular receptor by desensitization with the selective NK-1 agonist substance P methyl ester (0.1 μM) as described by Laufer *et al.* (16). Dose-response curves were obtained (Table 2).

Potentiation of Electrically Evoked Contractions of Rat Vas Deferens Assay

The activity on NK-2 (SP-E) receptors was measured following the procedure described by Lee *et al.* (18), with some modifications. Male rats (Wistar, Interfauna, Spain) weighing 200–300 g were killed by decapitation and segments of the vas deferens (3 cm) were removed and suspended in a 13 ml organ bath containing oxygenated Krebs solution of the same composition as in GPI experiments. The bath was maintained at 37°C. After an equilibration period of 30 min, the vas deferens was stimulated transmurally (40 V; 0.1 Hz; 1 msec) by a Harvard electrical stimulator (model 50-5107) and the contractions were recorded isotonically by the equipment described in the GPI experiments under a resting load of 1 g. Similar protocol to the one described for the GPI preparation was followed. Agonists were applied at 10-min intervals and were allowed a contact time of 2–3 min. Dose-response curves were obtained and resulting ED_{50} are indicated on Table 2. On Fig. 2 contraction response to analogue I on this preparation is shown.

Materials

SP and SP methyl ester (SPOMe) were obtained from Bachem, atropine sulphate from Fluka and $[Glu^6]SP_{(6-11)}$ (I), $[Glu^6, Pro^9]SP_{(6-11)}$ (II), $[Glu^6\ \gamma\text{-}\beta\text{-D-Glcp}]SP_{(6-11)}$ (III) and $[Glu^6\gamma\text{-}\beta\text{-D-Glcp}, Pro^9]SP_{(6-11)}$ (IV) were synthesized in our laboratory by classical methodologies (11,12). The purity of peptides checked by HPLC was >95%.

All peptides were dissolved in water, divided into small samples and stored (−20°C) until required. Under these conditions, no deterioration of peptides, as measured by bioassays and HPLC techniques, was observed over a period of several months.

Treatment of Data

The results for each agonist are expressed as the percentage of the maximum of contraction that could be obtained with that particular compound. Relative potencies were calculated from ED_{50} values (concentration of agonist producing 50% of the maximum of contraction) obtained from the Hill plots [log (effect/1-effect) vs. log concentration] (5).

Potencies given as ED_{50} values were expressed as the mean ± S.E.M. of at least 3 independent sets of experiments in three different animals. In each experiment, triplicate assays were performed. The statistical significance of the experiment was analysed by Student's *t*-test ($p<0.05$).

RESULTS

Following the procedure described for the GPI assay typical recordings such as the one presented for compound II in Fig. 1 have been obtained. Plotting the relative contraction (% maximum contraction) against log molar concentration parallel S-shaped dose-response curves for SP and SP analogues I to IV have been obtained. Each pair of dose-response values represents the NK-1 + NK-3 activity of SP and SP analogues. ED_{50} values extrapolated from the linear representation of log (effect/1-effect) vs. log concentration of these sigmoid curves are shown in Table 1. As can be seen, only analogue IV shows similar potency to SP on the GPI preparation.

A second set of experiments were performed to systematically study the selectivity of peptides I, II, III and IV for the tachykinin receptor subtypes. As far as the muscular receptor activity is concerned, selective assays were performed in the presence of 1 μM atropine. Moreover, selective tests of neuronal receptor were performed following inactivation of muscular receptor by desensitization with SPOMe 0.1 μM. Following these protocols, two new sets of dose-response curves were obtained. Again, by the linear representation of those sigmoidal graphs the ED_{50} values presented in Table 2 have been calculated. From the data presented, compound IV is more potent than SP and SPOMe on the NK-1 receptor, whereas on the NK-3 only the activity of compound III is of the same order of magnitude as SP. Due to the protocol followed to assess activity on the NK-3 receptor, the actual value of ED_{50} for SPOMe has not been determined. The figure presented in Table 2 ($\sim 10^{-6}$ M) is the minimum concentration which shows response after the desensitization treatment.

Biological activity on the rat vas deferens preparation (NK-2 receptors) was also determined for analogues I to IV and reference compounds SP and SPOMe. An original recording obtained for analogue I is shown in Fig. 2. Initial dose-response curves were lineated by log (effect/1-effect) vs. log (concentration) plots in order to calculate the ED_{50} values for NK-2 receptor, which are shown in Table 2. The activity on the NK-2 receptor of the analogues prepared is well under the one shown by SP.

The selectivity of SP, SPOMe and I to IV analogues for NK-1, NK-2 and NK-3 receptors has been examined by means of the ED_{50} ratios given in Table 3. Taking the ratios NK-3/NK-1 and NK-2/NK-1 as a measure of selectivity for the NK-1 receptor, it can be seen that the most NK-1 selective analogues on this series are compounds II and IV.

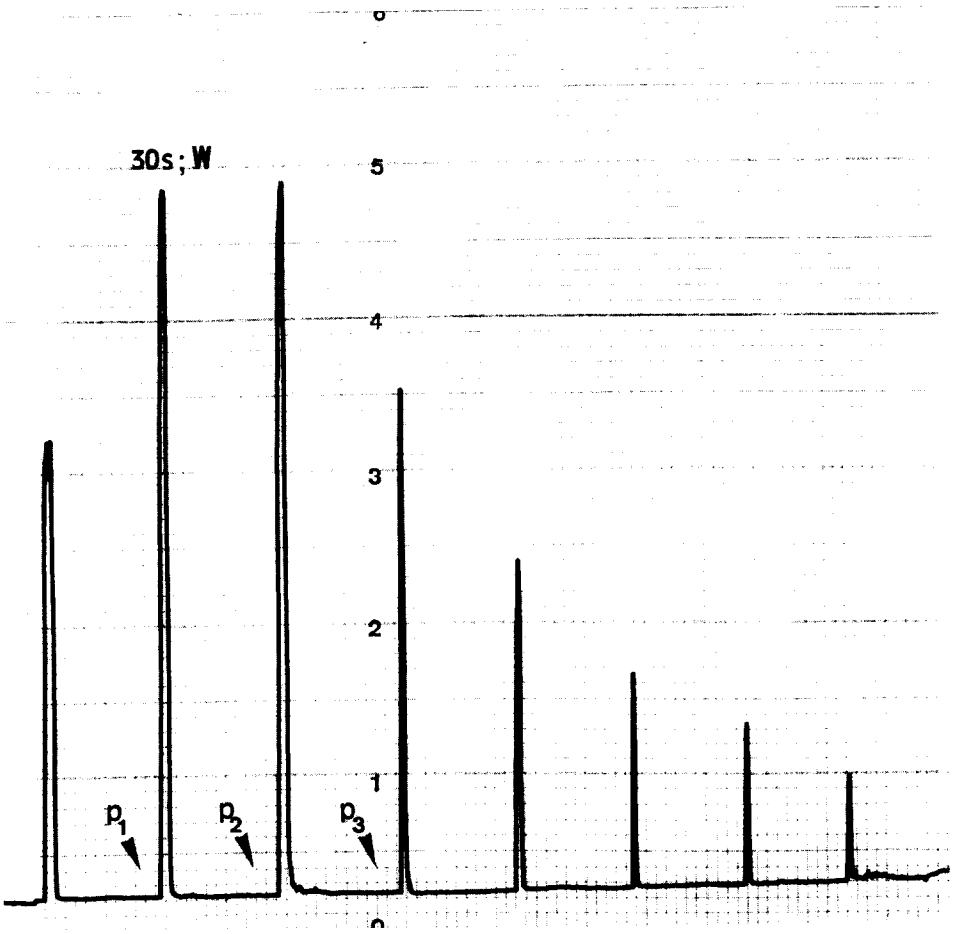

FIG. 1. Original recording of contractions of a guinea pig ileum preparation induced by analogue III. P_1: $6.2 \cdot 10^{-5}$ mg; P_2: $7.8 \cdot 10^{-5}$ mg; P_3: $4.7 \cdot 10^{-5}$ mg of [Glu^6, Pro^9]SP$_{(6-11)}$. Since $P_1 < P_2$ gave identical responses P_1 was considered to be the concentration which determined 100% of contraction. The following twitches were produced by decreasing concentrations of analogue III. Several doses of peptides were applied at 10-min intervals with 30 seconds of contact time. Washing cycles of 30 sec (W) were applied throughout the experiment.

TABLE 1

ACTIVITIES EXPRESSED AS ED_{50} (M) VALUES FOR SP AND SP ANALOGUES I TO IV OBTAINED ON GPI PREPARATIONS

Product	ED_{50} (NK-1 + NK-3)
SP	$(3.2 \pm 0.2) \cdot 10^{-9}$
(I)	$(7.2 \pm 0.9) \cdot 10^{-8}$
(II)	$(2.14 \pm 0.05) \cdot 10^{-8}$
(III)	$(1.9 \pm 0.9) \cdot 10^{-7}$
(IV)	$(2.3 \pm 0.2) \cdot 10^{-9}$

(I): [Glu^6]SP$_{(6-11)}$; (II): [Glu^6,Pro^9]SP$_{(6-11)}$; (III): [Glu^{6}γ-β-D-Glcp]SP$_{(6-11)}$; (IV): [Glu^{6}γ-β-D-Glcp,Pro^9]SP$_{(6-11)}$.

These values have been calculated from Hill plots obtained by the linear representation of dose-response curves.

TABLE 2

ED_{50} (M)$\cdot 10^7$ VALUES OF SP STANDARD AND ANALOGUES I TO IV FOR NK-1, NK-2 AND NK-3 RECEPTORS

Product	NK-1	NK-2	NK-3
SP	(0.070 ± 0.005)	(2.3 ± 0.7)	(0.30 ± 0.02)
SPOMe	(0.083 ± 0.008)	(260 ± 50)	~10
(I)	(1.15 ± 0.03)	(80 ± 9)	(1.90 ± 0.05)
(II)	(0.316 ± 0.009)	(110 ± 20)	(2.82 ± 0.07)
(III)	(4.7 ± 0.1)	(12 ± 8)	(0.87 ± 0.02)
(IV)	(0.023 ± 0.001)	(47 ± 7)	(2.4 ± 0.1)

(I): [Glu^6]SP$_{(6-11)}$; (II): [Glu^6,Pro^9]SP$_{(6-11)}$; (III): [Glu^{6}γ-β-D-Glcp]SP$_{(6-11)}$; (IV): [Glu^{6}γ-β-D-Glcp,Pro^9]SP$_{(6-11)}$.

ED_{50} (M) values were determined in the presence of 1 μM atropine (muscular receptor, NK-1) or SPOMe 0.1 μM preincubation (neuronal receptor, NK-3), using the guinea pig ileum as biological preparation. The ED_{50} values for NK-2 receptors were obtained on rat vas deferens preparations.

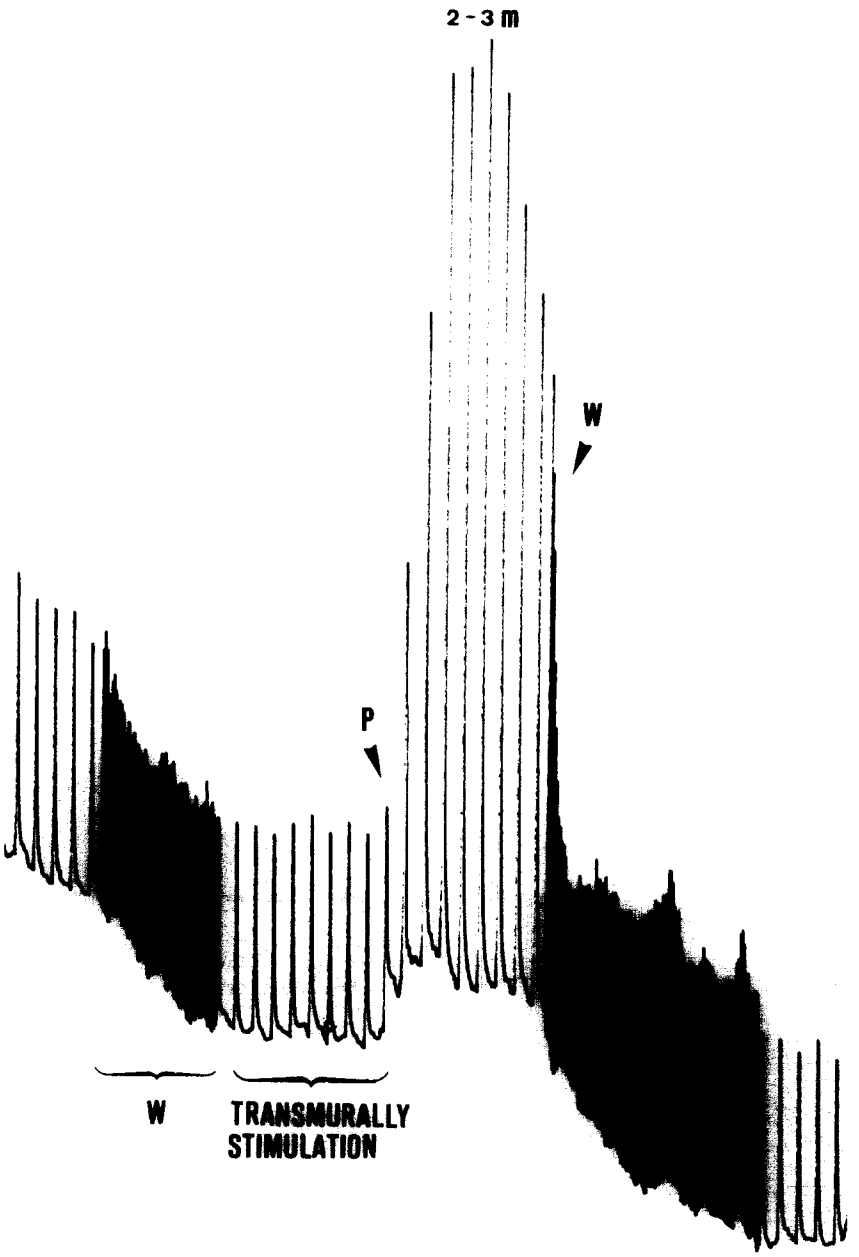

FIG. 2. Contraction response to 0.74 mg of $[Glu^6]SP_{(6-11)}$ on the rat vas deferens preparation transmurally stimulated (40 V, 0.1 Hz, 1 msec). Agonists were applied at 10-min intervals with 2–3 min of contact time. Washing cycle is represented as W.

DISCUSSION

The development of selective agonists for a particular tachykinin receptor subtype is an important tool for applications ranging from basic pharmacological studies of the role of tachykinins to more therapeutically oriented ones that would require selective activation or inhibition of a specific receptor subtype. Looking for more selective $SP_{(6-11)}$ analogues we have previously designed and synthesized four C-terminal hexapeptide SP analogues. In the present paper, we report a systematic study of activity and selectivity for the three peripheral tachykinin receptors.

Following the hypothesis that a folded conformation could be mostly responsible for SP activity (6, 28, 31), any attempt to stabilize this conformation could result in activity enhancement. Disregarding the effect of sugar incorporation, the substitution of Gly^9 by Pro showed, in our case, that the resulting analogue II is more active on the NK-1 receptor than the analogues I (four times) and III (fifteen times) which contain Gly in position nine (Table 2). We have also confirmed this assumption by a theoretical conformation study of this analogue performed with a molecular modelling program (Chem-X). It has been found that an intramolecular hydrogen bond between the oxygen atom of the carbonyl function of Met^{11} and one of the α-amino hydrogen atoms of Glu^6 was possible since their atomic distance is 2.6 A (9).

In an effort to prepare SP analogues with improved selectivity by favouring this folded active conformation, Laufer *et al.* (17)

TABLE 3

COMPARISON OF RELATIVE POTENCIES OF SP, SPOMe AND I TO IV SP ANALOGUES ON GUINEA PIG ILEUM (NK-1 AND NK-3 RECEPTORS) AND RAT VAS DEFERENS (NK-2 RECEPTOR) IN VITRO PREPARATIONS

	Ratio ED_{50}	
Product	NK-3/NK-1	NK-2/NK-1
SP	4.3	33
SPOMe	130	3150
(I)	1.6	70
(II)	9	350
(III)	0.2	2.5
(IV)	104	2034

(I): $[Glu^6]SP_{(6-11)}$; (II): $[Glu^6,Pro^9]SP_{(6-11)}$; (III): $[Glu^6\gamma\text{-}\beta\text{-D-Glcp}]SP_{(6-11)}$; (IV): $[Glu^6\gamma\text{-}\beta\text{-D-Glcp},Pro^9]SP_{(6-11)}$.

replaced Gly^9 by a proline residue on the hexapeptide $[pGlu^6]SP_{(6-11)}$. This substitution had no effect on potency measured on the NK-1 receptor, but lowered the activity on the NK-3 receptor two orders of magnitude. It was suggested that the high hydrophobicity of this derivative reduced its availability as a receptor ligand substrate leading to a potency loss.

To better confirm if this suggestion could help to improve the activity of the analogue containing proline, a reduction of the hydrophobic character of the molecule was carried out by attaching a monosaccharide moeity to analogue II. Thus, although derivative IV resulted to be as potent as standard SP on the GPI preparation (Table 1), its potency was not significatively increased. From these findings it seems that the critical factor providing potency to $SP_{(6-11)}$ analogues is mostly related to conformational rather than hydrophilicity aspects of the molecule. This has also been confirmed by the synthesis of analogue III which by lacking the enhancing conformation factor was solely modified on its hydrophilic character. Such an analogue has proved to be even less potent than $SP_{(6-11)}$ (Table 2).

As far as the selectivity is concerned, analogues II and IV have resulted to be the most selective analogues of this series for the NK-1 receptor. As expected for selective NK-1 agonists, the contraction effect is not affected by the muscarinic blocker atropine, but it is reduced following desensitization of the NK-1 receptor with a selective NK-1 agonist such as SPOMe. Thus, NK-1 potencies of analogues II and IV became 9- and 100-fold respectively reduced when compared to NK-3 potencies (Table 2). This is more properly seen when comparing selectivity ratios. As Table 3 shows, NK-1 selectivity of analogues II and IV as expressed by the NK-2/NK-1 ratio was approximately 10 and 60 times superior to standard SP respectively. Moreover, the specificity calculated by this ratio of analogue IV is of the same order of magnitude as the one obtained for SPOMe. Such SPOMe potency is in good agreement with literature values (30) which makes this a remarkable result since SPOMe is one of the most selective agonists for the NK-1 receptor.

Therefore, although the sugar moiety incorporation does not favour $SP_{(6-11)}$ potency, it greatly enhances selectivity. This could be possibly related with the already mentioned involvement of sugar residues in recognition processes.

In summary, we have studied the effects produced on GPI and RVD of the one amino acid substitution and a hydrophobicity modification on the $SP_{(6-11)}$ basic structure. The substitution of Gly^9 by proline has produced an analogue (analogue II) four times more potent on the NK-1 receptor than the parent compound (I). This is in good agreement with the enhancing effect of a folded conformation (6). The modification of the hydrophobic character by the incorporation of a β-D-glucopyranosyl residue on the 6th position of $SP_{(6-11)}$ sequence has yielded a less potent analogue than the parent compound on the NK-1 receptor. However, the simultaneous substitution of Gly^9 by Pro and the incorporation of the sugar moiety has led to an analogue with 100-fold enhanced selectivity through the NK-1 as compared to standard SP, while maintaining a NK-1 potency of the same order as SP. From these findings it seems likely that the most critical factor providing NK-1 potency to $SP_{(6-11)}$ analogues is related to conformational rather than hydrophilicity aspects of this structure.

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