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Backbone Cyclization of the C-terminal Part of Substance P. Part 1: The Important Role of the Sulphur in Position 11

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Abstract: Novel backbone-to-side chain and backbone-to-backbone cyclic analogues of substance P (SP) were prepared by solid-phase synthesis and screened for biological activity. An analogue containing a thioether-lactam ring between positions 9 and 11 showed an EC_{50} value of 20 nM toward the neurokinin 1 (NK-1) and was inactive toward the NK-2 and NK-3 receptors. On the other hand, in a multiple backbone cyclic peptide library of similar analogues, in which the sulphur was excluded from the ring, very low activity was detected. The activity was re-evaluated and was found to be even lower ($EC_{50} = 0.11$ mM) than the previously published data. These results indicate that the thioether moiety has a crucial role in receptor activation. The results also show tolerance of the NK-1 receptor, but not NK-2 or NK-3, to cyclization of the C-terminal portion of the SP_{6-11} hexapeptide.

Keywords: substance P; agonist; conformational constraint; backbone cyclization

INTRODUCTION

Cyclization is one of the most powerful tools for conferring long-range conformational constraint on peptides. As short linear peptides usually have many orders of conformational freedom, cyclization is used to restrict their flexibility, and hence to provide them with desirable pharmacological features, such as selectivity, metabolic stability and bioavailability. However, a major drawback of this method is the necessity of using the functional groups of the side chains or the terminals, which are often essential for binding and/or receptor activation. The result of using these groups for cyclization is often a decrease or loss of activity [1].

'Backbone Cyclization' (BC) offers a solution to this problem. According to this concept [2, 3], ring closure is effected by joining N^{α} and/or C^{α} atoms in the peptide backbone through appropriate linkers, while the side chains and terminals remain intact.

We have shown that alkyl chains bearing a functional group at the ω position can effectively be attached to the α amino group of practically any amino acid, forming modular building units [4–7] (Figure 1), which are then incorporated into the amino acid sequence and used for cyclization. Following natural cyclization forms, the ω-functional groups used in our building units were normally amine, carboxyl or thiol. Inclusion of such building units in a native peptide sequence enables cyclization

Abbreviations: BC, backbone cyclization; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; Gly(C_n), Fmoc- N^{α}-[ω-(tert-butylcarboxy)alkyl]Gly; Gly(N_n), Fmoc-Nα-[ω-(Boc-amino)alkyl]glycine; GPI, guinea-pig ileum; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; Hcy, homocysteine; MBHA, 4-methylbenzhydrylamine; MePhe, *N*-methylphenylalanine; NKA, neurokinin A; NKB, neurokinin B; PyBroP, bromo-tris-pyrrolidino-phosphonium hexafluorophosphate; RPV, rat portal vein; RVD, rat vas deferens; SMPS, simultaneous multiple peptide synthesis; SP, substance P; SPPS, solid-phase peptide synthesis; Succ, succinyl; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TDW, triply distilled water; TOF, time of flight.

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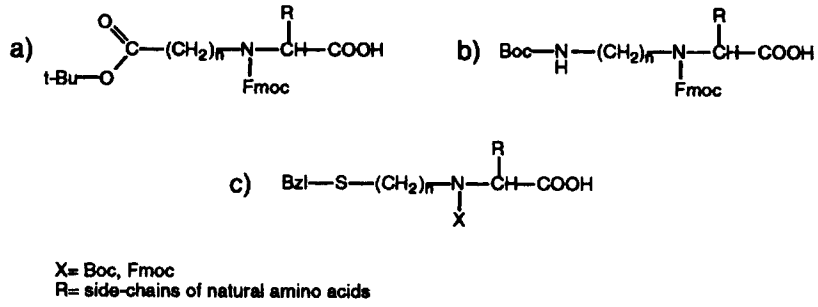

Figure 1 Building units for backbone cyclization containing ω-protected functional groups: (a) ω-carboxy, (b) ω-amino, (c) ω-thio.

through an amide, a disulphide or a thioether bond, without chaning any of the side-chain or terminal groups, and thus without interrupting any of the interactions required for bioactivity.

The first model chosen for demonstration of the feasibility of the BC concept was the mammalian tachykinin substance P. This neuropeptide is a member of the neurokinin family, which also includes neurokinin A and neurokinin B. The neurokinins are the mammalian sub-family of the tachykinins, which all share the common C-terminal sequence Phe-Xaa-Gly-Leu-Met-NH_2 (Xaa is a hydrophobic amino acid). They mediate a large variety of biological activities, such as transmission of pain stimuli, exocrine gland secretions, intestinal motility, vasodilation, neuronally mediated inflammatory skin reactions and behavioural responses (for review see [8] and references therein). Even though the neurokinin receptors are activated preferentially by each of the neurokinins (NK-1 by SP, NK-2 by NKA and NK-3 by NKB), the selectivity of each neurokinin to its receptor is rather poor [8–9]. Thus, for example, NKB activates both the NK-1 and the NK-3 receptors with almost the same EC_{50} (4.2 and 1.3 nM, respectively). Exploration of selective agonists and antagonists for the neurokinin receptors is of considerable importance, and has been the subject of many studies in the past 20 years.

Earlier work in our laboratory yielded two highly selective agonists for the tachykinin receptors NK-1 (WS-Septide, Ac[Arg^6,Pro^9]SP_{6-11}) and NK-3 (Senktide, Succ[Asp^6,$MePhe^8$]SP_{6-11}) [10–12]. Other active peptides and peptidomimetics have been produced in many laboratories around the world (e.g. [13–19]), while recent screening methods promoted the discovery of various non-peptide antagonists (e.g. [20–25]). In spite of the substantial significance of the latter compounds as potential drugs, they could not improve the understanding of agonist–receptor interactions, since they bind to a different site in the receptor [26, 27].

Theoretical energy calculations [28] predicted in the early 1980s, a folded conformation of the C-terminal hexapeptide of SP, stabilized by a hydrogen bond between the C-terminal amide and the γ-amide of Gln^6. These calculations, which were later corroborated by NMR studies [29, 30], promoted preparation of numerous cyclic analogues of SP or its C-terminal fragments, which endeavoured to mimic the bent conformation. However, all the attempts to prepare cyclic analogues of SP or the other tachykinins by classical cyclization methods, led to a decrease or loss of the biological activity [31–35].

These results made SP an interesting target for structure–activity relationship studies by BC. A series of six backbone-to-amino-end cyclic analogues of WS-septide {cyclo–$(CH_2)_m$–NH–CO–$(CH_2)_n$–CO–Arg-Phe-Phe-N-]–CH_2–CO–Leu-Met-NH_2} was prepared with the alkyl chain lengths, m and n, designed to give ring sizes from 17 to 22 atoms. A strong correlation between ring size and activity was found, with the optimum at a 20 atom ring ($m=4$, $n=3$). This analogue, called *cycloseptide*, activated the NK-1 receptor at 5 nM, and was completely inactive toward NK-2 or NK-3. It was also metabolically stable, and maintained 80% of agonist activity after 2 h of incubation with parotid gland slices [6]. This peptide and some other analogues of the series, as well as analogues with the same ring size but with altered chemical nature of the ring, were further subjected to NMR and molecular modelling studies in order to achieve a better understanding of the bioactive conformation of the N- terminal sequence Arg^6-Phe^7-Phe^8 required for activation of the NK-1 receptor [37–39].

In this paper we present analogues containing BC of the C-terminal part of SP_{6-11}, the sequence Gly^9-Leu^{10}-Met^{11}-NH_2. The terminal carboxamide, known to be a crucial component of the pharmacophore, was left intact in the new analogues, while Met^{11} was replaced by several trifunctional amino acids in order to enable backbone-to-side chain cyclization. Alternatively, backbone-to-backbone cyclization was used, closing a ring between the N^{α} amides of Gly building units in positions 9 and 11.

The Met^{11} residue was previously shown to be important for activity, and its replacement by neutral lipophilic residues [40] or charged residues [41] greatly reduced the biological activity. π interactions between the sulphur atom and the aromatic rings of Phe^7 and Phe^8 were hypothesized by Schwyzer to play a key role in activation of NK-1 [42]. This hypothesis was based on distance and angle homology between SP, cycloseptide and the antagonist spantide. However, recent results demonstrated that high activity and selectivity could be achieved when the thioether was replaced by hydrophobic bulky esters of Glu [41, 43] or Asp [44].

In using BC on the Gly^9-Leu^{10}-Met^{11}-NH_2 sequence, we had three goals in mind: (a) to examine whether cyclization of this portion of the peptide could induce improved pharmacological features, or whether it would destroy the activity; (b) in view of the former question, to compare biological activity of cyclic and pre-cyclic analogues; (c) to check analogues with and without sulphur, in order to study its role in receptor activation within the framework of backbone cyclic peptides.

MATERIALS AND METHODS

Protected amino acids, SPPS resins and coupling reagents were purchased from NOVA Biochem, Läufelfingen, Switzerland. Other chemicals were purchased from Sigma, Holon, Israel, or Merck, Darmstadt, Germany. Solvents for peptide synthesis were purchased from Lab-Scan, Dublin, Ireland, and were of Anhydroscan quality. SMPS was performed with 100 mg resin portions sealed in 4 × 5 cm polypropylene fabric bags, which were placed in polypropylene boxes and shaken with a Labotron shaker from INFORS HT, Bottmingen, Germany. HPLC analysis was performed on a Merck Hitachi 655A equipped with a L-6200A gradient pump and a UV–vis detector with tuneable wavelength set at 215 nm. The flow was fixed at 1 ml/min and the eluents were TDW and MeCN (containing 0.1% and 0.085% TFA, respectively). The column was Lichroprep RP-18, 250 × 5 mm i.d. from Merck. Semi-preparative separations were performed on Lichrosorb RP-8 and RP-18, 250 × 10 mm i.d. columns with a fixed flow of 5 ml/min.

Preparation of Glycine Building Units

Fmoc-N^{α}-[ω-(Boc-amino)alkyl]Gly [$Gly(N_n)$, $n=2, 3, 4, 6$] and Fmoc-N^{α}-[ω-(tert-butylcarboxy)alkyl]Gly [$Gly(C_n)$, $n=1, 2, 3, 4, 5$] building units were prepared as described elsewhere [6, 7].

Peptide Synthesis

All analogues were prepared on MBHA resin with 0.57 meq/g substitution level. Analogues **I** and **II** (Figure 2) were prepared in manual SPPS vessels shaken with a MilliGen 504 shaker. The first three amino acids were coupled either as Boc or Fmoc derivatives, while the last three amino acids were Fmoc-protected. In the synthesis of the lactam cyclic library, all of the amino acids were Fmoc-protected. Coupling steps of glycine building units and of the consecutive amino acids were performed with Py-BroP. The coupling to the secondary amino group of the building units was repeated twice. Other couplings were performed with HBTU. Lactam cyclization was performed with TBTU. In all cases we used six-fold excesses of amino acid, six-fold excesses of coupling reagent and twelve-fold excesses of DIEA, and the coupling reactions were performed for 120 min after pre-activation of 10 min. Fmoc deprotection was carried out with 20% piperidine in DMF for 30 min. Boc and *t*-Bu ester groups were cleaved with 55% TFA in DCM for 2 min and then for additional 30 min. Acetylation was performed with a ten-fold excess of acetic anhydride, ten-fold excess of DIEA and one equivalent of DMAP in DMF for 30 min. Reaction with bromoacetyl bromide was performed with ten-fold excess of the reagent and ten-fold excess of DIEA in DMF for 30 min. Blocking of the ω-amino groups for preparation of pre-cyclic analogues was performed by acetylation in the same way used for the N-terminal. Blocking of the carboxyl groups was performed by pre-activation with ten equivalents of DIC (0.5 M in DCM) and of HOBT (0.5 M in DMF) for 30 min, and then addition of methylamine (10 M in MeOH) and reaction for additional 30 min. HF cleavage was performed with 2.5% thioanisol as a scavenger. The reaction took place at 10 °C in the case of **I** and at −5 °C for all other peptides, for 2 h. The crude peptide **I** was dissolved

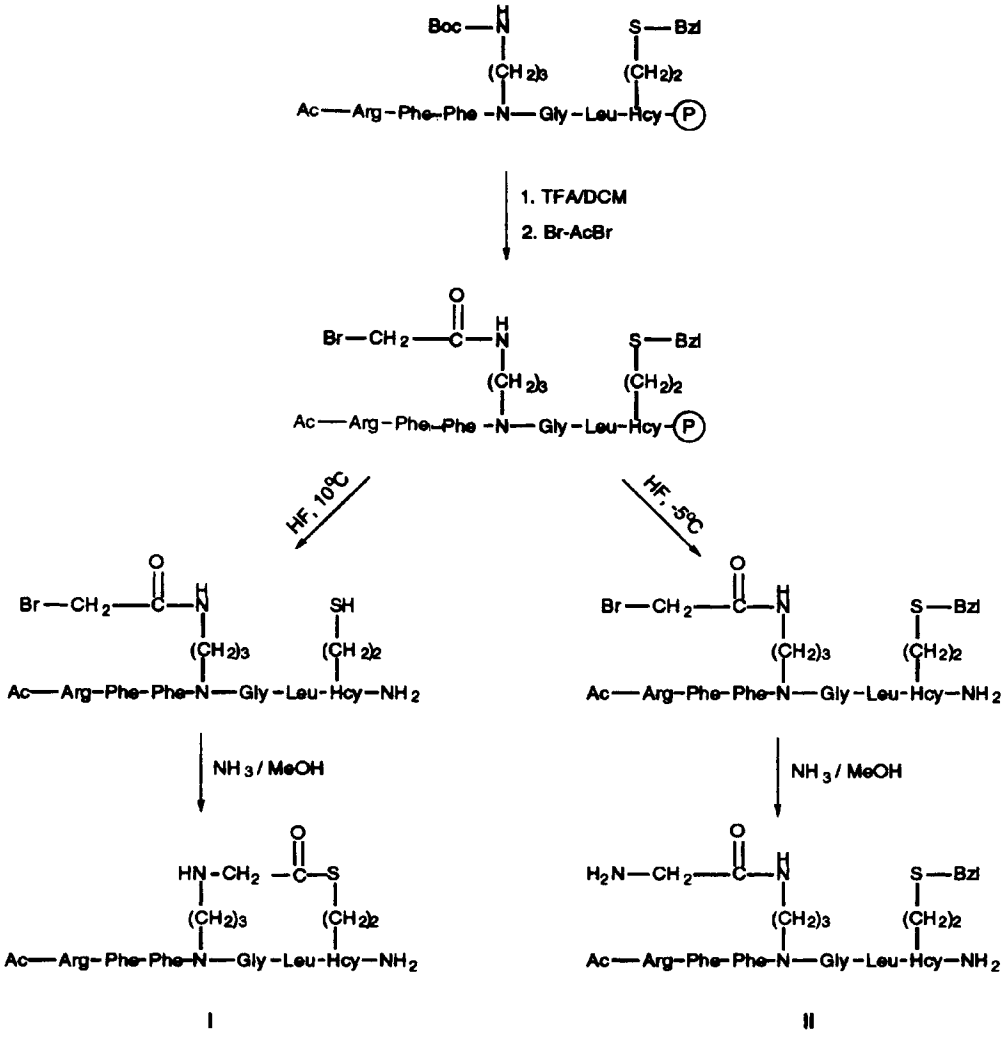

Figure 2 Synthetic scheme for the preparation of the backbone cyclic analogue **I**, containing a thioether-lactam ring, and its pre- cyclic counterpart analogue **II**.

in 2 l of methanol saturated with ammonia immediately after HF cleavage. The solvent was degassed with N_2 prior to saturation with ammonia. The solution was stirred for 72 h at room temperature, and then filtered and the solvent was evaporated *in vacuo*. The crude peptide was purified by HPLC.

Peptide **I**: calculated MW: 893.7 g/mole. FAB-MS (M+H): 894.8. AAA: D,L-Hcy-0.7, Leu-1.0, Phe-1.9, Arg-0.9.

Peptide **II**: calculated MW: 1000.9 g/mole. FAB-MS (M+H): 1001.7. AAA: Leu-1.0, N^{α}-(3-aminopropyl)Gly-1.2, Phe-2.0, Arg-0.8, Gly-0.9.

These peptides were detected by two different equipment types. Detection of the Gly building unit was not possible for **I**, whereas detection of Hcy was not possible for **II**.

Biological Tests

GPI assays with and without atropine for NK-1 and NK-3, RPV assay for NK-2 and RVD assay for NK-3 were performed as described in [9, 12].

RESULTS AND DISCUSSION

In the first analogue synthesized in this study, Met^{11} was replaced by *S*-benzyl-D,L-homocysteine [D,L-Hcy(Bzl)], while an N^{α}-[3-(Boc-amino)propyl]Gly [Gly(N_3)] building unit was incorporated in position 9. Chain elongation was performed by a combination of Boc/Fmoc chemistry on 4-methylbenzhydrylamine (MBHA) resin. (This strategy was recently discussed

in [45].) The amino terminal was acetylated as in WS-septide. The Boc group protecting the ω-amine of the Gly building unit was then removed, and the amine was acetylated with bromoacetyl bromide. HF cleavage yielded the fully deprotected crude peptide, which was immediately dissolved in a large volume of methanolic ammonia. Cyclization was accomplished within 72 h of stirring at room temperature, to give a thioether-lactam ring of 17 atoms (analogue **I**, Figure 2).

This analogue, characterized by FAB-MS and AAA, was submitted to biological activity assay on the three neurokinin receptors. It was found to activate NK-1 (GPI) at 20 nM and was inactive toward NK-2 (RPV, $EC_{50} > 10^{-5}$) and NK-3 (RVD, $EC_{50} > 10^{-5}$). It was hence shown that the bioactive conformation of SP_{6-11}, activating NK-1, could be stabilized by linking the amide nitrogen of Gly^9 to both edges of the peptide.

A pre-cyclic analogue of **I** was fortuitously prepared in another experiment, which was identical to the preparation of **I**, except for the lower temperature used during HF cleavage, in an attempt to improve the peptide quality. The S-benzyl protecting group failed to be cleaved this time, and in the next step of incubation with methanolic ammonia, the bromide was substituted by the ammonia to form a glycine residue coupled to the ω-aminoalkyl chain (Figure 2). This pre-cyclic analogue (**II**) was almost as active as the cyclic analogue **I**. It had an EC_{50} value of 25 nM when tested on free GPI as well as in the presence of the muscarinic blocker atropine, indicating that its activity was mediated through the muscular receptor NK-1 rather than the neuronal receptor NK-3. Although analogue **II** is, perhaps, not the ideal pre-cyclic counterpart of **I**, its activity pattern shows greater tolerance of the NK-1 receptor toward the conformation of the C-terminal tripeptide Gly^9-Leu^{10}-Met^{11} than to the N- terminal tripeptide Arg^6-Phe^7-Phe^8. The pre- cyclic analogue of cycloseptide, in comparison, was two orders of magnitude less active than cycloseptide itself [36]. We therefore concluded that the thioether ring in **I** *allows* the bioactive conformation to be adopted, but does not necessarily *lock* it as in the case of cycloseptide.

After the permissive nature of the receptor toward cyclization of the Gly^9-Leu^{10}-Met^{11} portion was established, we examined the role of the sulphur required for activity in view of Schwyzer's theoretical hypothesis [42]. We decided not to add a hydrophobic moiety, such as benzyl or *t*-butyl as in the active analogues containing Glu [41, 43] and Asp [44] esters in position 11, in order to isolate the influence

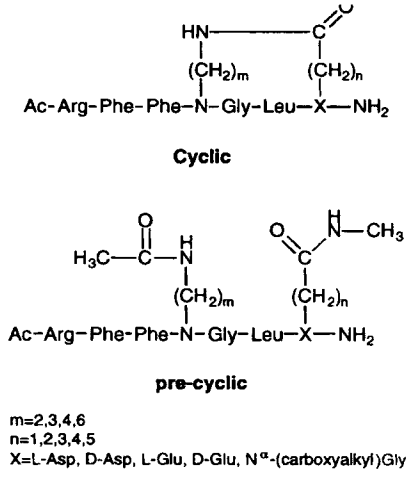

Figure 3 General structure of the cyclic and pre-cyclic analogues composing the new backbone cyclic library with lactam ring between positions 9 and 11.

of the thioether moiety. Instead, we replaced the sulphur by an alkyl chain, and used simple lactam rings for either backbone-to-side chain or backbone-to-backbone cyclization between positions 9 and 11. However, we used *Cycloscan*™ [46] to explore the conformational space available for the C-terminal portion of the peptide, in order to verify that changes in the biological activity are due to structural rather than conformational alterations.

A library of 28 cyclic peptides and their 28 pre-cyclic counterparts was synthesized using the 'Tea-bag' SMPS method [47]. Amino acids containing an ω-carboxyl group, namely L- and D-Asp, L- and D-Glu and N^{α}-(ω-carboxyalkyl)Gly units [Gly(C_n)] replaced Met^{11} this time. The ω-amino group was provided by incorporation of Gly(N_n) units in position 9 (Figure 3). The ω-carboxy and ω-amino groups were protected by *t*-butyl ester and Boc, respectively. Thus, the α-amino groups were protected by Fmoc, but the synthesis was performed on MBHA resin, and the Arg residue was protected by tosyl. The amino terminal was acetylated as before, and cyclization took place on the resin, after the acidolytic removal of the *t*-Bu and Boc groups, by repeated treatments with TBTU/DIEA in DMF [48]. Before the cyclization step each bag was opened and the peptide–resin was divided into two portions. One portion was taken for cyclization, whereas the other portion was used for preparation of the pre-cyclic analogue. The latter analogues were obtained by amidation of the ω-carboxy and ω-amino groups with methylamine and acetic anhydride respectively.

Table 1 Peptides From Mixtures 5C and 5P

Peptide[a]	*m*	n	Ring size	Amino acid analysis[b]	MW Found	MW Calc.
20C	6	2	17	Arg-1.0, Phe-1.7, Leu-1.0, Gly(C2)- 1.2	939	938.14
24C	6	3	18	Arg-1.0, Phe-2.0, Leu-0.9, Gly(C3)- 1.0	953	952.16
26C	3	5	17	Arg-1.0, Phe-2.1, Gly(N3)-1.2, Leu-1.1, Gly(C5)- 0.9	939	938.14
27C	4	5	18	Arg-1.0, Phe-2.1, Gly(N4)-1.1, Leu-0.9, Gly(C5)- 0.9	953	952.16
28C	6	5	20	Arg-1.1, Phe-1.7, Leu-0.8, Gly(C5)- 0.9	981	980.22
20P	6	2	Non-cyclic	Arg-1.0, Phe-1.9, Leu-1.1, Gly(C2)- 1.2	1012	1011.23
24P	6	3	Non-cyclic	Arg-1.0, Phe-2.1, Leu-1.0, Gly(C3)- 1.0	1026	1025.25
26P	3	5	Non-cyclic	Arg-1.1, Phe-1.8, Gly(N3)-1.5, Leu-1.0, Gly(C5)- 1.1	1012	1011.23
27P	4	5	Non-cyclic	Arg-1.0, Phe-1.5, Gly(N4)-1.4, Leu-1.0, Gly(C5)- 1.0	1026	1025.25
28P	6	5	Non-cyclic	Arg-1.0, Phe-1.6, Leu-1.0, Gly(C5)- 1.1	1054	1053.21

[a] C, cyclic; P, pre-cyclic.
[b] Detection of Gly(N6) by the detector of the amino acid analyser was not possible.

Samples of peptide–resin from each bag were then taken and used to form ten mixtures: five of cyclic analogues and five complement mixtures of the pre-cyclic analogues. Thus, mixtures 1C and 1P contained the cyclic and pre-cyclic peptides with L-Asp or L-Glu in position 11, respectively. Mixtures 2C and 2P contained the cyclic and pre-cyclic analogues with the D-amino acids, respectively. The remaining three cyclic mixtures 3C–5C contained peptides with Gly units in position 11 and differed by ring size. Mixtures 3P–5P contained their pre-cyclic counterparts, respectively. The ten mixtures were subjected to HF cleavage, and were then tested, after scavenger removal and lyophilization, for their biological activity as crude mixtures. The GPI test with and without atropine was used for the biological screening of the ten mixtures.

This screening revealed very low activity in all mixtures ranging between 0.1 and 1 mM EC_{50} values. These results confirm the hypothesis of the essential role of the sulphur in activation of the NK-1 receptor. Notably, no significant difference was found between the cyclic and pre-cyclic analogues, in agreement with the behaviour of the sulphur containing analogues **I** and **II**.

We further examined separately the analogues which made up the mixtures with the highest activity (0.1 mM, mixtures 5C and 5P), in order to verify that the activities of the individual peptides were not significantly different from those in the mixtures. The peptides in these two mixtures were, thus, each cleaved separately from the resin, purified by semi-preparative reverse-phase HPLC, characterized by TOF-MS and AAA (Table 1) and tested as pure compounds for their biological activity. None of the individual peptides in these two mixtures was more active than 0.1 mM. Interestingly, even at such a low activity range it was observed that while most of the analogues gave the same EC_{50} values with and without the presence of atropine (characteristic of NK-1 selectivity), for some of the analogues the activity was nullified by atropine (indicating NK-3 selectivity). These results were corroborated in two distinct laboratories. They should, however, be considered with care, since the overall activity was very low.

CONCLUSIONS

Different types of backbone cyclic SP_{6-11} analogues with cyclization from Gly^9 toward the C-terminal were prepared either as individual peptides or as an SMPS library. Analogue **I**, containing a backbone-to-side chain thiother-lactam ring between positions 9 and 11, was found to be a highly active and selective NK-1 agonist. Its pre-cyclic analogue **II** had the same activity and selectivity pattern, unlike the pre-cyclic forms of the backbone-to-amino terminal analogues, which were 100-fold less active than the cyclic peptides.

Very low activity was found in a backbone cyclic peptide library, in which the sulphur atom in residue 11 was replaced by hydrocarbon chains. The library contained 28 backbone-to-side chain and backbone-to-backbone cyclic analogues with a lactam ring between positions 9 and 11, and their 28 pre-cyclic

counterparts. Systematically changing the link of the ring to the backbone, the chirality at the position of cyclization, the ring size and the exact placement of the bond within the ring enabled extensive screening of the conformational space of the C-terminal tripeptide constrained by the ring. The fact that the low activity level was revealed by all of the cyclic and pre-cyclic analogue mixtures strongly indicates that the loss of activity should be attributed to the exclusion of the sulphur and not to conformational constraint. It supports our basic argument that loss of activity resulting from cyclization is likely to be caused by structural modifications rather than from the cyclization itself.

In spite of their low activity, some analogues had different selectivity patterns towards either NK-1 or NK-3, depending on their exact structures.

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