# Synthesis and biological activity of novel backbone-bicyclic Substance-P analogs containing lactam and disulfide bridges

GAL BITAN [1], INNA SUKHOTINSKY [2], YAFFA MASHRIKI [3], MENACHEM HANANI [3], ZVI SELINGER [2] and CHAIM GILON [1]

*Departments of [1] Organic and [2] Biological Chemistry. The Hebrew University of Jerusalem. Jerusalem. Israel and [3] Laboratory of Experimental Surgery. Hadassah University Hospital. Jerusalem. Israel*

A biased library of 60 novel backbone-bicyclic Substance P analogs was prepared by the simultaneous multiple peptide synthesis method. The peptides, containing both a lactam and a disulfide ring, were synthesized by combined Boc and Fmoc chemistries, and were cyclized on the resin. Cleavage of the *S*-benzyl group and oxidation of the sulfhydryl groups was enabled by adaptation of the diphenylsulfoxide–trichloromethylsilane method to solid-phase synthesis. The peptides were screened for NK-1 and NK-3 activity, and were found to be weak agonists. © Munksgaard 1997

*Key words* backbone-cyclic peptides; peptide libraries; Substance P analog

A few years ago we introduced a concept for imposing conformational restrictions on peptides called backbone-cyclization (1). After the first demonstrations of the feasibility of this concept as a powerful tool for turning natural peptides into highly active, selective and biologically stable peptidomimetics (2–4) and for their SAR studies (5–7), we dedicated much effort to the improvement of the synthetic methods required for the preparation of backbone-cyclic peptides. We took two parallel paths in this effort: the first one was preparation of 'building units', namely, $N^\alpha$-($\omega$-Y-alkyl)amino acids, where Y is a protected amino (8–10), carboxy (9) or thiol (10) group. The second one was study of the incorporation of these units into peptides and of new cyclization types. Currently backbone-cyclization is routinely excercized in our laboratory for conformational resriction of various peptides. However, all of the synthetic methodology has been developed using Substance P (SP) as a model, as this peptide has been the subject of many

of our previous studies. We have also developed a novel methodology for screening of small biased backbone-cyclic peptide libraries (11). This methodology, called Cycloscan®, involves in an iterative manner synthesis, biological screening and molecular modeling, and enables systematic exploration of the conformational space available for a given bioactive sequence.

In the course of our SAR studies of backbone cyclic analogs of SP, we found that high activity and selectivity toward the neurokinin NK-1 receptor was obtained by cyclizing the *C*-terminal hexapeptide of SP ([Arg$^6$]SP$_{6-11}$) from the peptide bond between Phe$^8$ and Gly$^9$ either to the *N*-terminus (4), or to the side-chain of residue 11 (12) (Fig. 1). Thus, peptides 1 and 2 gave EC$_{50}$ values of 5 and 20 nM, respectively,
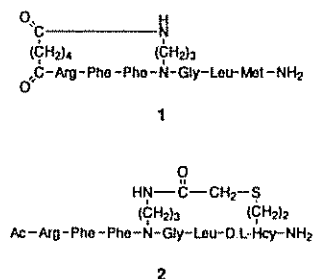
FIGURE 1

Structure of backbone-cyclic SP$_{6-11}$ analogs which are NK-1 selective agonists containing backbone-to-amino-end (1) and Backbone-to-side-chain (2) rings

421

on the NK-1 receptor, and were inactive toward the NK-2 and NK-3 receptors.

We further used Cycloscan® to substantiate the theory of Schwyzer, regarding the crucial role of the sulfur atom, located in the side-chain of Met[11] in SP and 1, and within the ring of analog 2 (13). This theory, assuming π-interactions between the sulfur atom and the aromatic rings of Phe[7] and Phe[8] play a key role in activation of NK-1, was based on distance and angle homology between 1, the antagonist spantide and SP itself. To investigate the role of the sulfur we prepared a library of backbone-cyclic analogs with cyclization from the backbone nitrogen of Gly[9] to the side-chain or the backbone nitrogen of the residue on position 11, similar to analog 2. In this library, however, the sulfur atom was replaced by different alkyl chains and the ring was closed only by an amide bond, without the thioether moiety. Consequently the biological activity was greatly reduced (11).

In the current study we introduce the second phase of Cycloscan®: a library of bicyclic analogs of $SP_{6-11}$, which approximately merges peptides 1 and 2 into one system. The new analogs which were prepared contained a backbone-to-amino-terminus lactam ring as in 1, and a second, semi-fused ring linking the backbone nitrogen of Gly[9] with either the backbone or the side-chain of the residue in position 11. The ring with residue 11 in the new library was closed, however, by a disulfide bond, rather than a thioether, demonstrating for the first time the utilization of disulfide type bonds in the frame of backbone-cyclization.

In the design of the new bicyclic library we could foresee either of two possible results: 1) bicyclization would constrain the pharmacophoric groups of the peptide in the bioactive conformation, giving highly active analogs, or 2) as both parts of the peptide are constrained, the necessary interactions between pharmacophoric groups will be denied and consequently the activity will be suppressed. Although we screened the conformational space available to the bicyclic peptides by systematically changing the spacer lengths, and hence the size of the rings, over-rigidification and loss of activity due to the bicyclization could not be over-ruled, and in fact took place as a result of the bicyclization.

## RESULTS

### Synthesis of the library

The new bicyclic biased library, containing 60 peptides based on the $[Arg^6]SP_{6-11}$ sequence (Fig. 2), was manually synthesized by the 'tea bag' SMPS method (14) using a combination of Boc and Fmoc methodologies (15). As in peptides 1 and 2 Gly[9] of SP was replaced by $Gly(N_n)$ building units (Fig. 3). In addition, Met[11] of SP was replaced by a sulfhydryl
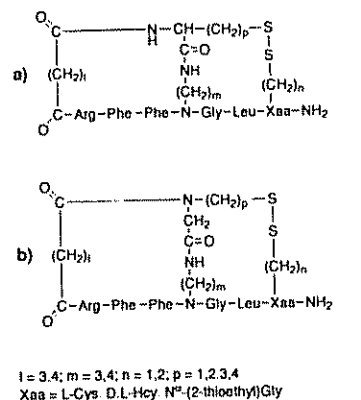


$l = 3,4; m = 3,4; n = 1,2; p = 1,2,3,4$
Xaa = L-Cys, D,L-Hcy, N$^\alpha$-(2-thioethyl)Gly

**FIGURE 2**

Primary structures of the new backbone-bicyclic $SP_{6-11}$ analog library. Two types of peptides were prepared with: (a) amino-acids and (b) building units at the bridgehead
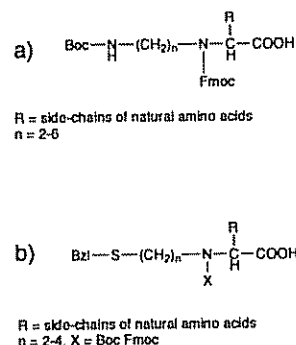


**FIGURE 3**

Gly building units used for construction of the backbone-bicyclic library. (a) ω-Amine units [Gly($N_n$)]; (b) ω-thiol units [Gly($S_n$), X = Boc, Fmoc)]

containing amino acid (Cys or D,L-Hcy) or by a $Gly(S_2)$ building unit. The lengths of the alkyl spacers $l$, $m$, $n$ and $p$ (Fig. 2) were designed to give ring sizes of 21–23 and 18–23 atoms for the amide and disulfide rings, respectively.

The linear peptide chain was synthesized until the point of the coupling of the $Gly(N_n)$ building units by Fmoc chemistry on MBHA resin. The first amino acid coupled to the resin was either L-Cys or D,L-Hcy or a $Gly(S_2)$ building unit. Then the Fmoc protecting group was removed and Fmoc-Leu was coupled, followed by Fmoc removal. The coupling reagent was HBTU, except for the coupling of Fmoc-Leu to the secondary amine of the $Gly(S_2)$ unit. For this coupling PyBroP was used, and the reaction was repeated twice. The next step was coupling of $Gly(N_3)$ or $Gly(N_4)$ building units to the Leu[10] residue. The ω-amino groups of these units were protected by Boc (Fig. 3). After the coupling of the units the ω-Boc group was removed with TFA, while the Fmoc group on the α-amine was retained. A Boc

protected trifunctional amino acid was then coupled to the ω-amine to form a bridgehead for bicyclization. These trifunctional amino acids were L-Cys, D,L-Hcy, Gly(S₂), Gly(S₃) and Gly(S₄). Only after construction of the bridgehead was the Fmoc group removed from the α-amine of the Gly(N$_n$) building units (Fig. 4).

Coupling of Fmoc-Phe to the secondary amino group of the Gly(N$_n$) units was accomplished again with PyBroP and two repetitions of the coupling reaction. The C-terminal hexapeptide sequence synthesis was then completed with consecutive couplings of Fmoc-Phe and Fmoc-Arg(Tos). After completion of the synthesis of the linear peptide chains with the bridgeheads, dicarboxylic acid spacers were coupled (as the corresponding anhydrides) to the N-terminal amino group. Then the Boc group was removed from the α-amine of the trifunctional group at the bridgehead, and a lactam ring was closed between this

amine and the spacer (Fig. 4) to yield monocyclic analogs.

Cyclization of the disulfide ring on the resin, in the bags, was not possible by any known method at the time of performing this study, since the thiol protecting group was benzyl. This group was chosen since it enabled facile synthesis of the building units, but its removal required HF cleavage or sodium in liquid ammonia, both inadequate for the designed library. We overcame this reluctance by adaptation of the method developed by Kiso et al. for removal of the protecting group and in situ oxidation to disulfide (16). However, this method, based on a combination of DPSO and methyltrichlorosilane in TFA, was only used previously in solution. Thus, it was necessary to adjust the reaction conditions for solid-phase cyclization. We used small portions of peptide-resin from bags no. 59 and 60 (containing long alkylthiol chains) as models for studying the
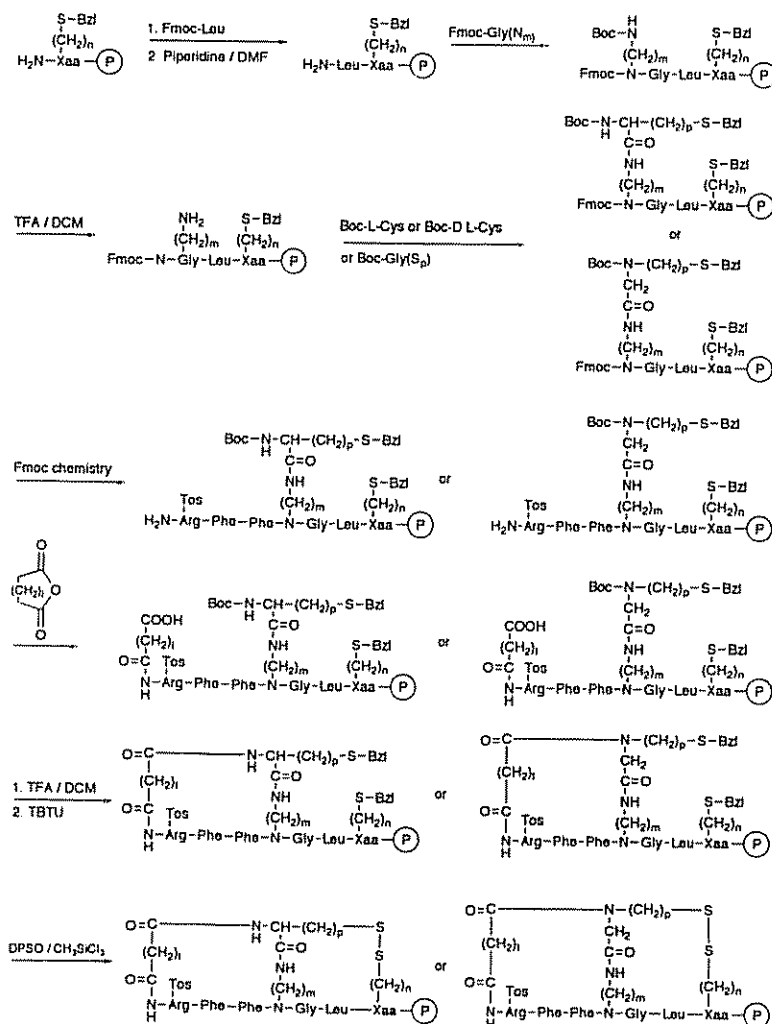


FIGURE 4
Synthetic scheme for the bicyclic library.

solid-phase conditions for this reaction. Maintaining the quantities as in the original procedure (i.e. 250 equiv of $CH_3SiCl_3$, 10 equiv. of DPSO and 100 equiv. of anisol in TFA), the reaction was performed for 6 h at room temperature in the first experiment. Then the model peptides were cleaved from the resin by HF HPLC separation and MS analysis revealed only partial oxidation, while large amounts of di-protected and mono-protected thiol containing peptides were still present in the crude mixture. A second experiment, in which the reaction was performed under similar conditions at 50 °C for 6 h, caused total destruction of the peptide. Only in the third experiment was complete oxidation obtained by turning back to room temperature and extending the reaction time to 18 h. The rest of the library underwent disulfide cyclization for 18 h in three groups of 20 bags each (in order to avoid very large volumes of TFA). Later on it has been found that by changing the reaction medium from neat TFA to a 1:1 mixture of TFA and DCM the reaction time could be shortened again to 6 h, thanks to improved swelling of the resin beads. The peptides were cleaved from the resin by HF and submitted to biological screening (see below). The overall yield of the bicyclic analogs was quite low, 5–20%, yet they were obtained in relatively high purity (Fig. 5), which was sufficient for biological screening. Further exploration of the DPSO–$CH_3SiCl_3$ system revealed that the low yield resulted from partial cleavage of the peptides from the resin due to the strong acidic conditions during the cyclization step. After this work had been performed an example of using the method of Kiso for disulfide cyclization on the resin was published by

Andreu *et al* (17), who also obtained a low yield of the synthesized cyclic peptide

*Biological screening*

After completion of the synthesis and both lactam and disulfide cyclizations, 20 mg peptide-resin samples from each bag were taken and used to form eight mixtures. The mixtures were composed of 5–9 peptides, based on ring size similarity. The mixtures were cleaved from the resin by HF, and were then subjected to biological screening by GPI contraction assay after scavenger removal and lyophilization. The GPI smooth muscle contraction assay was performed with and without the presence of atropine, in order to distinguish between direct activation of the muscular NK-1 receptor and indirect action through the neuronal NK-3 receptor. The results of the screening are summarized in Table 1. Unfortunately it was found that over-rigidification, caused by bicyclization, imposed too high a conformational constraint on the peptides, and therefore the activity was 2–3 orders of magnitude lower than the native agonist.

In order to verify that the activity of the individual peptides was not significantly different than that of the crude mixtures we examined the nine analogs which composed mixture 4, as separate purified compounds. Since the yield of the first synthesis was relatively low, these nine analogs were re-synthesized, and the disulfide cyclization was carried out this time by the method of Kiso *et al* in solution, after HF cleavage. It was evident that although partial cleavage of the benzyl groups took place during the cleavage, giving rise to a complex mixture of products, only one major product of high purity was obtained after



**FIGURE 5**

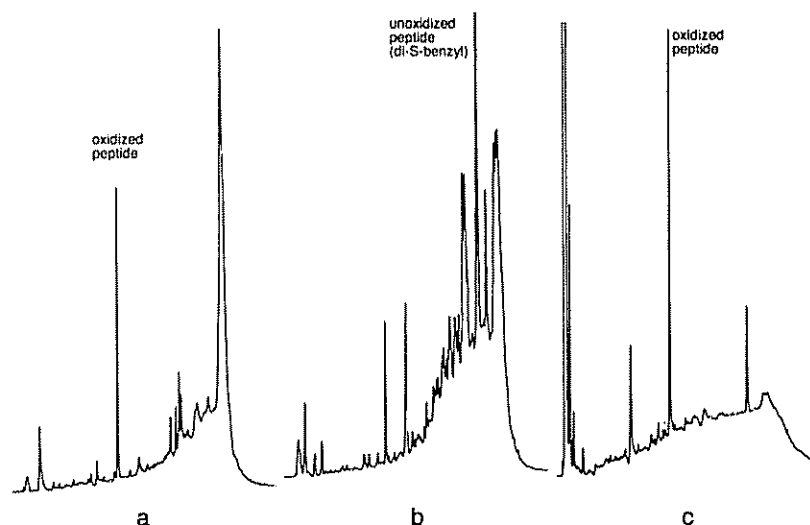HPLC chromatograms of analog 28 (Xaa = Hcy, $l = 4$, $m = 3$, $n = 2$, $p = 2$): (a) after oxidation on the resin; (b) after cleavage from the resin before oxidation; (c) after oxidation of the crude mixture from (b) in solution (before purification).

TABLE 1

*Preliminary biological results*

| Mixture | No of peptides | Ring-size | | $EC_{50}$ ($\mu$M) | |
|---|---|---|---|---|---|
| | | Disulfide | Amide | Without atropine | With atropine |
| 1 | 6 | 18–19 | 21 | 5 3 | 150 |
| 2 | 6 | 18–19 | 22[a] | 7 9 | 1300 |
| 3 | 5 | 18–19 | 22–23[b] | 0 85 | 1 1 |
| 4 | 9 | 20 | 22 | 3 6 | 7 2 |
| 5 | 7 | 20–21 | 21 | 1 5 | 3 0 |
| 6 | 7 | 21 | 22 | 2 1 | 6 3 |
| 7 | 8 | 20–21 | 23 | 7 0 | 7 2 |
| 8 | 9 | 22–23 | 21–23 | 1 6 | 1 8 |

[a] Xaa = Cys, HCys.

[b] For peptides with lactam ring of 22 atoms, Xaa = Gly(S2)

the cyclization reaction (Fig. 5). Nevertheless, the yield of the disulfide ring formation reaction was still quite low owing to excessive oligomerization, even at high dilution conditions (0.1 $\mu$M concentration of crude peptide). In some cases the oxidized peptides were pure enough for the biological assay. In other cases the products were purified by preparative HPLC. The activity of the nine individual peptides, which were assayed separately was not higher than that of the mixture (ca. 1 $\mu$M).

## DISCUSSION

Our assumption, based on Schwyzer's theory and on our previous experimental findings, was that in both analogs 1 and 2, the activity resulted from a certain pattern of folding of the peptide. Earlier theoretic and NMR studies had shown that the C-terminal hexapeptide of SP adopts a bent conformation (18, 19). This conformation is believed to allow for essential interactions between the sulfur atom of Met[11] and the aromatic rings of Phe[7] and Phe[8]. In both analogs 1 and 2 one part of the molecule is constrained by cyclization while the other part is conformationally free. We hypothesized that in 1 the linear C-terminal portion folds to enable interaction of the sulfur with the constrained phenyl rings, whereas in 2 it is the linear N-terminal part which folds to bring the phenyls to the right place for interaction with the sulfur. The new bicyclic library was designed to resemble structurally both 1 and 2. Thus, the N-terminal part was cyclized by a lactam ring as in 1, whereas the in the C-terminal part we used disulfide cyclization instead of thioether cyclization, for synthetic reasons. Evidently, however, since both parts of the peptide were constrained this time, the necessary interactions between the sulfur and the phenyl rings could not take place, and the result was

a decrease in the biological activity In principle it is possible that some biological activity was overlooked owing to the screening method. Synergism, uneven quantities and/or counteraction may confuse the results when assaying 5–9 peptide mixtures as has been done here. However, our growing experience with Cycloscan® of Substance P and other systems has shown that these experiments can be carried out with a certain level of confidence. In none of the cases were any synergistic effects found when individual peptides activities were compared to peptide mixtures. Interestingly in this case, in mixtures 1 and 2, which contained peptides with smaller ring sizes, the activity seemed to be exerted, at least partly, through the NK-3 receptor, while in the other mixtures, in which the ring sizes were bigger and hence the constraint was smaller, the NK-1 receptor was responsible for activity. These results should be regarded with care since the overall activity was relatively low, yet they may indicate a new pattern of selectivity, which is based on ring size and location of the bond in the ring, while the overall structure remains the same.

## EXPERIMENTAL PROCEDURES

Protected amino acids, SPPS resins and coupling reagents were purchased from NOVA Biochem, Läufelfingen, Switzerland. Other chemicals were purchased from Aldrich, Milwaukee, WI, USA 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 analyser equipped with a L-6200A gradient pump and a UV–VIS detector with tunable 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-$N_\alpha$-[$\omega$-(Boc-amino)alkyl]Gly [Gly($N_n$), $n$ = 3, 4] and Fmoc-$N_\alpha$-[$\omega$-(benzylthio)alkyl]Gly [Gly($S_n$), $n$ = 2, 3, 4] building units were prepared as described elsewhere (9, 10).

*Peptide synthesis.* The library was prepared on 100 mg portions of MBHA resin with 0.57 mequiv./g substitution level. In general coupling steps were performed

with HBTU. Couplings of Gly($N_n$) and Gly($S_n$) building units and of the consecutive amino acids were performed with PyBroP. The coupling to the secondary amino group of the building units was repeated twice. In all cases a three-fold excess of amino acid, a three-fold excess of coupling reagent and a seven-fold excess of DIEA were used, and the coupling reactions were performed for 120 min after pre-activation of 10 min. Capping after acylation of the resin was performed with a solution of 0.5 M acetic anhydride, 0.125 M DIEA and 0.05 M HOBT in DMF for 60 min. Fmoc deprotection was carried out with 20% piperidine in DMF for 30 min. Boc groups were cleaved with 55% TFA in DCM for 2 min and then for an additional 30 min. Acylation of the *N*-terminus with glutaric anhydride was performed with 10 equiv. of the anhydride, 1 equiv. of DMAP and 10 equiv. of DIEA for 60 min. Adipic acid (10 equiv.) was first activated with 10 equiv. of DIC for 30 min in DMF, and then the acylation reaction took place in similar conditions as with glutaric anhydride. Lactam cyclization was performed with 3 equiv. of TBTU and 4 equiv. of DIEA. Cleavage of *S*-benzyl groups and cyclization were performed by addition of a solution of 250 equiv. $CH_3SiCl_3$, 10 equiv. DPSO and 100 equiv. anisol in 200 mL of neat TFA (and later TFA : DCM = 1 : 1) to 20 bags and shaking for 18 h (with TFA : DCM = 1 : 1 for 6 h) at room temperature. HF Cleavage was performed with 2.5% thioanisol as a scavenger. The reaction took place at −5 °C for 2 h. The peptides were purified by semi-preparative RP-HPLC and characterized by TOF-MS (Table 2). Amino acid analysis was impractical, because thiol containing amino acids were destroyed during hydrolysis.

*Biological tests.* GPI assay with and without atropine for NK-1 and NK-3 was performed as described (20).

TABLE 2

*Structure and data of the nine peptides of mixture 4*

| Peptide | X[a] | Y[b] | *l* | *m* | *n* | *p* | MW | TOF-MS | K' |
|---|---|---|---|---|---|---|---|---|---|
| 9 | Gly(S2) | Gly(S3) | 4 | 3 | 1 | 3 | 1037.3 | 1035.0 | 4.8 |
| 12 | Cys | HCys | 3 | 4 | 1 | 2 | 1023.3 | 1023.9 | 4.6 |
| 13 | Cys | Gly(S2) | 3 | 4 | 1 | 2 | 1023.3 | 1026.2 | 4.8 |
| 27 | HCys | HCys | 4 | 3 | 2 | 2 | 1037.3 | 1038.4 | 4.6 |
| 28 | HCys | Gly(S2) | 4 | 3 | 2 | 2 | 1037.3 | 1039.6 | 4.7 |
| 31 | HCys | Cys | 3 | 4 | 2 | 1 | 1023.3 | 1022.7 | 4.5 |
| 49 | Gly(S2) | Gly(S3) | 4 | 3 | 2 | 3 | 1051.3 | 1052.0 | 4.9 |
| 52 | Gly(S2) | HCys | 3 | 4 | 2 | 2 | 1037.3 | 1034.1 | 4.9 |
| 53 | Gly(S2) | Gly(S2) | 3 | 4 | 2 | 2 | 1037.3 | 1037.3 | 4.4 |

[a] Amino acid in position 11
[b] Amino acid in bridgehead.

## REFERENCES

1. Gilon. C., Halle. D., Chorev, M., Selinger, Z. & Byk. G. (1991) *Biopolymers* 31. 745–750
2. Gilon. C., Halle. D., Chorev. M., Selinger. Z & Byk. G (1993) in Smith. J A & Rivier. J eds *Peptides Chemistry and Biology 1992*. pp. 476–477, ESCOM, Leiden
3. Gilon, C., Zeltser, I., Rashti-Behar, V., Muller. D., Bitan, G., Halle, D., Bar-Akiva, G., Selinger, Z & Byk, G. (1993) in Yanaihara, N eds., *Peptide Chemistry 1992* pp. 482–485, ESCOM, Leiden
4. Byk, G., Halle, D., Zeltser, I., Bitan, G., Selinger, Z & Gilon, C. (1996) *J. Med. Chem.* 39. 3174–3178
5. Saulitis, J., Mierke, D.F., Byk, G., Gilon, C & Kessler, H (1992) *J. Am. Chem. Soc* 114, 4818–4827
6. Golic-Grdadolnik, S.G., Mierke. D.F., Byk, G., Zeltser, I., Gilon, C & Kessler, H (1994) *J. Med. Chem* 37, 2145–2152
7. Bitan, G., Behrens, S., Mathä, B., Mashriki, Y., Hanani, M., Kessler, H & Gilon, C. (1995) *Lett. Peptide Sci.* 2, 121–124
8. Byk, G. & Gilon, C. (1991) *J. Org. Chem* 57, 5687–5691
9. Muller, D., Zeltser, I., Bitan, G. & Gilon, C. *J. Org. Chem.* (1997), 62, 411–416
10. Bitan, G. & Gilon, C. (1995) *Tetrahedron* 51, 10513–10522
11. Gilon, C., Bitan, G & Muller, D., in Proceedings of the 4th Solid Phase Synthesis Symposium, Edinburgh, 1995, in press
12. Bitan, G., Zeltser, I., Byk, G., Gluhov, E.V., Halle, D., Mashriki, Y., Sukhotinsky, I., Hanani, M., Selinger, Z. & Gilon, C. (1996) *J. Peptide Sci.* 2, 261–269
13. Schwyzer, R. (1992) *Brazil. J. Med. Biol. Res.* 25, 1077–1089
14. Houghten, R.A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 5131–5135
15. Thompson, P.E., Keah, H.H., Gomme, P.T., Stanton, P.G. & Hearn, M.T.W. (1995) *Int. J. Peptide Protein Res* 46, 174–180
16. Akaji. K., Fujino, K., Tatsumi, T. & Kiso, Y. (1993) *J. Am. Chem. Soc.* 115, 11384–11392
17. Camarero, J.A., Giralt, E & Andreu, D. (1995) *Tetrahedron Lett.* 36, 1137–1140
18. Chassaing, G., Convert, O & Laville, S. (1986) *Eur. J. Biochem.* 154, 77–85
19. Young, J.K., Anklin, C & Hicks, R.P (1994) *Biopolymers* 34, 1449–1462
20. Laufer, R., Wormser, U., Friedman, Z.Y., Gilon, C & Selinger, Z. (1985) *Proc. Natl. Acad. Sci. USA* 83, 7444–7448

Address:
Professor *Chaim Gilon*
Department of Organic Chemistry
The Hebrew University of Jerusalem
Givat-Ram
Jerusalem 91904
Israel
Tel: 972-2-6585276
Fax: 972-2-6585345
e-mail: gilon@vms.huji.ac.il