Conformational Studies of Synthetic Polyethylene Glycol Bound Substance P and its Lower Analogues

Piotr Koziej*, Manfred Mutter**

Institute of Organic Chemistry, Johannes-Gutenb-F Universität, D-6500 Mainz
Hans-Ulrich Gremlich, Günter Hölzemann

Medical Chemistry Research Dept., E. Merck, Postfach 4419, D-6100 Darmstadt
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The synthesis of Substance P (SP) bound to the C-terminal methionine to polyethylene glycol monomethyl ether (M-PEG) is described. SP-PEG-M is obtained in good yield by the liquid-phase peptide synthesis. Gel permeation chromatography reveals that aggregated forms of the PEG-bound peptides are absent in CHCl₃. CD and IR spectra point to a β-turn located at the N-terminal part of SP-PEG-M. In binding studies and in vivo assays the described compounds are devoid of biological activity.

1. Introduction

Although Substance P (SP) is known since many decades [1], its physiological role as well as its biological active conformation still remains to be fully elucidated [2]. The synthesis of the undecapeptide and of several analogues has been performed by various procedures, e.g. the classical solution methods [3], the solid-phase synthesis [4] and the liquid-phase method [5] using polyethylene glycol as solubilizing polymeric protecting group [6].

Structure activity studies have shed light on the relative importance of every single amino acid in relation to the biological activity [7]; most notably, it has been found that the C-terminal hexapeptide is still active in a variety of tissues [8].

An “U-turn” having a hydrogen bond between Gln-NH [6] → OC-Met [11] has been proposed as the biological active conformation on the basis of NMR measurements, conformational energy calculations and structure activity studies [9]. CD measurements of SP in water show conformational changes in the presence of sodium dodecyl sulfat (SDS) [10].

Conformationally restricted cyclic analogues have only limited biological activity [11, 12].

In this paper we want to report the synthesis of SP and its C-terminal partial sequences with polyethylene glycol monomethyl ether (M-PEG) directly bound to the C-terminal methionine (Fig. 1) via an amide bond. The PEG used had a molecular weight Mr ~5000. CD and IR measurements in 2,2,2-trifluoroethanol (TFE) were performed in order to elucidate the conformational properties of these analogues. The biological activity was assessed in comparison to SP after intrathecal application in rats and in binding studies.

2. Results and Discussion

The free hydroxy group of M-PEG of molecular weight 5000 was converted via three steps into an amino group [13]. The yield of the conversion was 75% as detected by microtitration with HClO₄. Acylation of the “amino-polymer” with Boc-Met-OH using DCC/HOBt [14] resulted in an amide bond between the terminal methionine and PEG (see Fig. 1). The Boc group was cleaved by HCl/ACOH in the presence of 10% ethanedithiol under a nitrogen atmosphere.

Symmetrical anhydrides of Boc protected amino acids were used for stepwise synthesis according to the liquid-phase procedure [5, 6]. Boc protected

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Fig. 1. Amino acid sequence of PEG-bound Substance P.

* On leave from Mazowieckie Zakłady Chemiczne, Warszawa-Pruszkow Lipowa 42, Poland.
** Reprint requests to Prof. Dr. M. Mutter, Institute of Organic Chemistry, University of Basel, St.-Johanns-Ring 19, CH-4056 Basel, Switzerland.
glutamine had to be coupled as its nitrophenyl ester. A different strategy was applied for the lysine and proline residues. The base labile fluorenylmethyl-oxycarbonyl (Fmoc) group was used for N\textsubscript{r}-protection, whereas the N\textsubscript{r}-group of lysine was Boc protected. The synthesis was completed with Boc-Arg(Mtr)–OH, thus allowing the cleavage of all protecting groups with trifluoroacetic acid in one single step.

Before the evaluation of the conformation, the aggregation behaviour of SP-PEG-M had to be investigated. M. Rueger et al. [15] reported the self-association of SP, which strongly influences the appearance of the CD spectra. It has been demonstrated, that gel permeation chromatography (GPC) is a versatile tool to detect aggregation of PEG-bound peptides [16]. Fig. 2 shows the GPC of “amino-PEG” together with that of SP-PEG-M in CHCl\textsubscript{3}. The appearance of only one single peak indicates, that aggregated forms are absent even in this \(\beta\)-sheet promoting solvent. To record the circular dichroic (CD) spectra, samples of the polymer-bound peptides were side-chain deprotected and dissolved in 2,2,2-trifluoroethanol (TFE). Fig. 3 shows the spectra of polymer-bound octa- to undecapeptides in this solvent.

With increasing chain length of the peptide two pronounced negative Cotton effects at 223 nm and 204 nm are observed. The minimum at 223 nm (\(n\rightarrow\pi^*\) transition) develops from the octa- to undecapeptide and is absent at lower chain length.

A comparison of the CD spectra with data obtained from conformationally restricted cyclic peptides [17] suggests a \(\beta\)-turn geometry for SP-PEG-M. From the chain length dependence for the onset of secondary structure formation, the postulated \(\beta\)-turn is located in the N-terminal region of SP: The conformational transition from an unordered form to an ordered \(\beta\)-turn structure occurs in going from the octapeptide to the undecapeptide. This finding is in harmony with the strongly \(\beta\)-turn inducing potential of the proline residue in position 2 [18a]. Moreover, empirical \(\beta\)-turn predictions taken from X-ray data of a large number of proteins indicate,

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**Fig. 2.** GPC of “amino-PEG” and Substance P-PEG in CHCl\textsubscript{3} (see text).

**Fig. 3.** Circular dichroic spectra of Substance P-PEG and C-terminal derivatives. 1 octa-peptide; 2 nona-peptide; 3 deca-peptide; 4 undeca-peptide; \(c = 1\) mg peptide/ml.
that the dipeptide Pro\(^3\)–Lys\(^3\) has a very high \(\beta\)-turn probability [18]. Taking these considerations into account, the experimental data suggest the existence of a \(\beta\)-turn in SP at the N-terminus of the type Arg\(^1\)–Pro\(^{+1}\)–Lys\(^{+2}\)–Pro\(^{+3}\).

As a further conformational probe infrared spectra of SP-PEG-M, free SP and their C-terminal derivatives in 2,2,2-trifluoroethanol-d\(_3\) (TFE-d\(_3\)) were recorded.

In Fig. 4, the amide I absorption bands of the various compounds, which are sensitive to conformational transitions [19], are depicted. The amide I band of the PEG-bound octapeptide (a) is centered near 1651 cm\(^{-1}\) (Table I) and does not shift significantly from going to the decapeptide (b) resp. to the undecapeptide (c). The appearance of an absorption band near 1650 cm\(^{-1}\) is characteristic for a random-coil conformation or an \(\alpha\)-helical structure. From data about the critical chain length of helix-formation, as well as from conformational energy calculations, the onset of a helix structure in these short oligopeptides is very unlikely. However, the existence of a \(\beta\)-turn as indicated by the CD spectra would be in harmony with IR data [27, 28]. Most notably, the appearance of a shoulder near 1660 cm\(^{-1}\) at the undecapeptide level strongly favours such an interpretation. On the other hand, the PEG-free peptides (d–f) show a rather different spectral behavior compared to the PEG-bound derivatives: Both the drastic decrease in the ellipticities of the negative Cotton effects in the CD spectra (not shown here) as well as the shift of the amide I band (Fig. 4) from 1650 cm\(^{-1}\) for the octapeptide to 1662 cm\(^{-1}\) for SP itself (Table I) can be explained by an increasing tendency of the peptide for aggregation in TFE. This pronounced tendency to aggregate is a well known phenomenon for Substance P [15]. Taking into account a solvent induced shift of 7 cm\(^{-1}\) to lower wavenumbers for the amide I band in TFE-d\(_3\) [20], the effective amide I absorption for SP itself amounts to 1669 cm\(^{-1}\). This frequency is characteristic for an \(\alpha\)-helix [21] as well as a \(\beta\)-turn (of the parallel type) [29]. For the same reasons as outlined above the onset of a helical structure can be excluded.

In summarizing, the spectroscopic data indicate, that the lower homologs of SP adopt predominantly a r.c. conformation in the membran mimicking solvent TFE. On the other hand, a \(\beta\)-turn formation in the N-terminal region of Substance P is indicated by

### Table I. Center of the amide I absorption band [cm\(^{-1}\)] of SP-PEG-M, free SP and their C-terminal derivatives in 2,2,2-trifluoroethanol-d\(_3\) at room temperature.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>M-PEG-Peptide</th>
<th>Free-Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexa-Peptide</td>
<td>1653</td>
<td>–</td>
</tr>
<tr>
<td>Hepta-Peptide</td>
<td>1650</td>
<td>–</td>
</tr>
<tr>
<td>Octa-Peptide</td>
<td>1650</td>
<td>1650</td>
</tr>
<tr>
<td>Nona-Peptide</td>
<td>1650</td>
<td>–</td>
</tr>
<tr>
<td>Deca-Peptide</td>
<td>1650</td>
<td>1651</td>
</tr>
<tr>
<td>Undeca-Peptide</td>
<td>1649</td>
<td>1662</td>
</tr>
</tbody>
</table>

Fig. 4. Amide I absorption bands of SP-PEG-M, free SP and their C-terminal derivatives in 2,2,2-trifluoroethanol-d\(_3\) at room temperature.

a) M-PEG-Octa-Peptide; b) M-PEG-Deca-Peptide; c) M-PEG-Undeca-Peptide; d) Octa-Peptide; e) Deca-Peptide; f) Undeca-Peptide.

In a), b), and c), spectra of pure 2,2,2-trifluoroethanol-d\(_3\) and of pure M-PEG–NH\(_2\); in d), e), and f), spectra of pure 2,2,2-trifluoroethanol-d\(_3\) have been subtracted. Concentrations have been 1 mg peptide in 1 cm\(^3\) solution.
CD and IR data. This intrinsic tendency to form a folded structure at the N-terminus appears more pronounced in the unaggregated, PEG-bound peptide compared to free SP. Obviously, due to the strong solubilizing effect of the flexible PEG chain, aggregation is not possible for this peptide.

The biological activity of the hexa- to undecapeptide derivatives in comparison to Substance P was evaluated after intrathecal application in rats. The described compounds did not induce scratching in concentrations of 6 nmol (Substance P: 2 nmol) and were devoid of analgesic activity in the hot plate test at the same concentrations. In binding studies the M-PEG derivatives were not able to displace bound (1H)-Substance P from its binding site [23].

We may conclude from these experiments that M-PEG bound to the C-terminal methionine of SP changes the solution properties of the native peptide in a way that receptor binding is no longer possible. This result is not unexpected, as the importance of the C-terminal primary amide has been shown in a series of analogues modified at the C-terminus [24]. The situation might be different, if M-PEG is bound to the N-terminal amino acid; so far, this compound has not yet been accessible synthetically.

3. Experimental Part

Polyethylene glycol monomethyl ether was obtained from Hoechst AG and dried before use. Protected amino acids were purchased from Bachem. Boc-glutamine p-nitrophenyl ester was recrystallized before coupling. Dichloromethane, which was used as a solvent for all coupling steps, was stored over molecular sieve.

At each coupling step a Kaiser-test [25] was performed. The following solvents were used for TLC: n-butanol/AcOH/water 3:1:1; chloroform/methanol/AcOH 90:8:2. Amino acid analyses were made after coupling and deprotection of residues 1, 6 and 11. Norleucine was used as internal standard.

Solvents for CD and IR measurements were 2,2,2-trifluoroethanol (TFE) and 2,2,2-trifluoroethanol-d$_3$ (TFE-d$_3$) (E. Merck, Uvasol quality).

The optical rotations were measured with an automatic Jasco spectropolarimeter Model J-500 A. The infrared spectra were recorded on a Bruker IFS 45 FT-spectrometer. Peptide concentrations have been 1 mg peptide in 1 cm$^3$ solution. For the GPC experiments, α-Styragel 10$^{-3}$ Å columns were used (Waters). The UV absorbance at 254 nm was recorded with an HP 3390 integrator. The solvent for this investigation was chloroform (E. Merck, Uvasol quality). Approximately 50 µl of the mixture of 2.5 mg of M-PEG-peptide in 1 cm$^3$ of chloroform was applied to the column.

"Amino-PEG" (M-PEG–NH$_2$)

15 g of dry polyethylene glycol monomethylether with a molecular weight of app. 5000 was converted to "amino-PEG" by the known procedure [13]. 12 g of "amino-PEG" were obtained with a 75% conversion to the amino group as detected by micro titration in dichloromethane/AcOH 9:1 with HClO$_4$.


12 g "amino-PEG" (approx. 3 mmol amino groups) was acylated by Boc–Met–OH using DCC/HOBt [14]. After completion of the coupling (negative ninhydrin test) Boc–Met–PEG–M was purified by recrystallization from ethanol/ether 1:1 at 0°C and from dichloromethane precipitated by ether at 0°C. The Boc group was split off by HCl/AcOH with the addition of 10% ethanedithiol under a nitrogen atmosphere. After neutralization chain elongation was performed according to the standard procedures of liquid-phase peptide synthesis [26] using symmetrical anhydrides of Boc–Leu–OH, Boc–Gly–OH, Boc–Phe–OH. Boc-glutamine was coupled via its p-nitrophenyl active ester. To complete the synthesis, symmetrical anhydrides of Boc–Pro–OH, Fmoc–Lys(Boc)–OH, Fmoc–Pro–OH and Boc–Arg(Mtr)–OH were used. The Fmoc group was split off by 15% piperidine in dimethylformamide. All coupling steps were checked for completion (>99.5%) by the fluorescamine test.

SP-PEG-M was obtained by deprotection of all side chain protecting groups using trifluoroacetic acid in the presence of thioanisol under a nitrogen atmosphere.

Table II. Amino acid analyses of PEG-bound peptides.

<table>
<thead>
<tr>
<th>Amino acids</th>
<th>Coupling steps 1</th>
<th>Coupling steps 6</th>
<th>Coupling steps 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nle</td>
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<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Met</td>
<td>0.98</td>
<td>0.95</td>
<td>0.96</td>
</tr>
<tr>
<td>Leu</td>
<td>1.05</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Gly</td>
<td>0.96</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Phe</td>
<td>1.98</td>
<td>2.03</td>
<td></td>
</tr>
<tr>
<td>Gln</td>
<td>1.01</td>
<td>2.02</td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td></td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>Lys</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arg</td>
<td>0.87</td>
<td></td>
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atmosphere for one hour at room temperature. The peptide PEG ester was purified by column chromatography on Sephadex LH 20 in methanol. The results of the amino acid analyses are summarized in Table II.

We gratefully acknowledge the biological testing by Prof. Dr. A. Wild, E. Merck, Department of Pharmacology, and Dr. H. E. Greiner, E. Merck, Department of Medical Biochemistry, Neurobiochemical Unit.