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MULTIPLE PEPTIDE SYNTHESIS USING CELLULOSE-BASED CARRIERS: SYNTHESIS OF SUBSTANCE P - DIASTEREOISOMERS AND THEIR HISTAMINE-RELEASING ACTIVITY

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ABSTRACT:
Manual multiple peptide synthesis on cotton disks was used to synthesize the complete D-amino acid replacement set of substance P. The influence of D-amino acid substitutions on HPLC-retention times and on the ability to release histamine from rat peritoneal mast cells was investigated.

Large numbers of peptides are required for screening purposes in many research areas, particulary in immunology and pharmacology. The characterization of an antibody epitope may require the synthesis of several hundred oligopeptides, which can be realized by synthesis and testing on polyethylene rods or cellulose disks without the isolation of individual peptides[2,3]. However, the elucidation of structure-activity relationships in pharmacology often requires the isolation of mg-amounts and careful characterization of each individual peptide.

There are now a number of techniques for simultaneous multiple peptide synthesis based on segmentation of polystyrene-based resins via polypropylene bags[4], syringes[5] or microtiter plates[6]. Planar carriers such as cellulose paper[7,8], cotton[9] or membranes[10] should be especially suited for segmentation because their size can be chosen individually according to the desired amount of peptide. Cellulose and cotton carriers are inexpensive and their hydroxyl groups can be easily modified for peptide synthesis[7,8]. Moreover, cellulose-linked peptides are useful in the characterization of antibody-peptide interactions[3,11].

In a previous study[9] we compared the utility of cotton strips, Whatman 540 paper and polypropylene membranes coated with hydroxypropyl acrylate[10]. When the Fmoc/^{t}Bu-technique[12] is applied, cotton shows suitable stability towards solvents and reagents, and can be modified by appropriate linkers, resulting in a convenient substitution level (about 2-3 μmol/cm^2 = 0.1-0.15 mmol/g). Moreover, cotton exhibits a pronounced ability to absorb solvents thus allowing the coupling to be performed simply by wetting the carrier and eliminating conventional reaction vessels. Bromphenol blue[13] is a suitable indicator for monitoring the coupling reaction[9].

Mast cell-triggering by peptides has been characterized to be a receptor-independent process[14,15], and it has been suggested that cationic peptides activate G proteins directly via an interaction with the C-terminus of α-subunits of G proteins by mimicking the agonist-bound receptors[15]. This direct interaction should depend on distinct stereochemical features of the peptide[16]. To test the stereochemical requirements of mast cell activation by peptides, we synthesized the complete D-amino acid replacement set of substance P[17] on cotton disks and investigated the histamine-releasing activity of the peptides. The initial results presented here point to rather low stereochemical requirements of mast cell degranulation, not supporting a mechanism of direct G protein-peptide interaction.

(1) H - Arg - Pro - Lys - Pro - Gln - Gln - Phe - Phe - Gly - Leu - Met - NH_2

(Substance P)

(2) H - Arg - Pro - Lys - Pro - Gln - Gln - Phe - Phe - Gly - Leu -DMet - NH_2

(3) H - Arg - Pro - Lys - Pro - Gln - Gln - Phe - Phe - Gly -DLeu - Met - NH_2

(4) H - Arg - Pro - Lys - Pro - Gln - Gln - Phe -DPhe - Gly - Leu - Met - NH_2

(5) H - Arg - Pro - Lys - Pro - Gln - Gln -DPhe - Phe - Gly - Leu - Met - NH_2

(6) H - Arg - Pro - Lys - Pro - Gln -DGln - Phe - Phe - Gly - Leu - Met - NH_2

(7) H - Arg - Pro - Lys - Pro -DGln - Gln - Phe - Phe - Gly - Leu - Met - NH_2

(8) H - Arg - Pro - Lys -DPro - Gln - Gln - Phe - Phe - Gly - Leu - Met - NH_2

(9) H - Arg - Pro -DLys - Pro - Gln - Gln - Phe - Phe - Gly - Leu - Met - NH_2

(10) H - Arg -DPro - Lys - Pro - Gln - Gln - Phe - Phe - Gly - Leu - Met - NH_2

(11) H -DArg - Pro - Lys - Pro - Gln - Gln - Phe - Phe - Gly - Leu - Met - NH_2

Figure 1. D-Amino acid replacement set of substance P

The synthesis was performed on eleven cotton disks (each 15 cm^2), loaded with glycine by reaction with Fmoc-Gly/DIC/HOBt/NMI, acetylation of residual hydroxyl groups with Ac_2O/NMI, Fmoc-deprotection with 20% piperidine/DMF and subsequent introduction of a trialkoxybenzhydryl-type linker[18] as described earlier[9,18]. The cotton disks had a substitution of about 25 μmol/disk as determined by UV-measurement after Fmoc-deprotection with 20% piperidine/DMF. For chain elongation, in each cycle ten cotton disks were allowed to react simultaneously with the corresponding Fmoc-amino acid-HOBt ester and the eleventh disk was treated separately with the corresponding D-amino acid derivative. Deprotection and washes were carried out together for all carriers of the set[9]. Final deprotection of side-chain protecting groups and cleavage from the cotton carrier were performed for each cotton disk separately by treatment with 95% TFA/ 2.5% thioanisole/2.5% dimethylsulfide. Following work-up[9] the crude peptides were obtained with a purity of

50-80% (analytical HPLC[20]). Because in a few cases only moderate product qualities were obtained (about 50% according to HPLC), the synthesis was repeated to study the influence of double-couplings and acetylations of remaining uncoupled amino groups. Figure 2 demonstrates the improvement in the purity of the crude products. However, in most cases double couplings and acetylations were not necessary and single step purification by preparative HPLC[20] yielded mg-quantities of the desired peptides.

Although the present results demonstrate the efficiency of multiple peptide synthesis on cotton carriers, the method needs further improvement. Thus, bromphenol blue monitoring does not generally permit a definite decision as to whether the coupling reaction is complete. Moreover, we occasionally observed a decrease of the substitution level during synthesis, possibly due to the sensitivity of the linker toward HOBt[21].

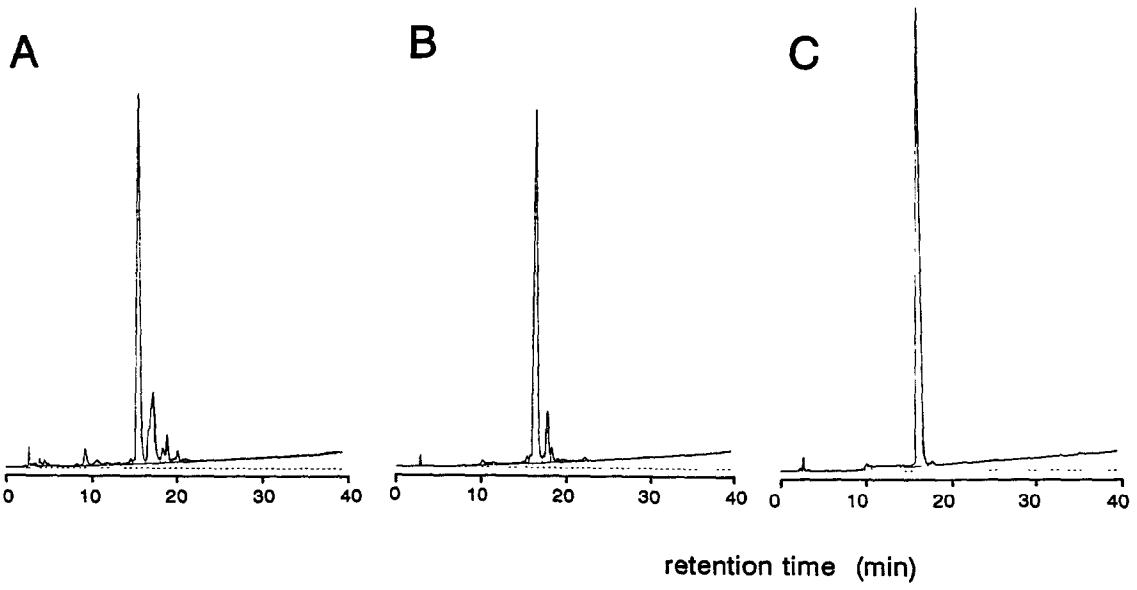

Figure 2. Synthesis of [D-Leu][10]-substance P on cotton-disks. Analytical HPLC of products obtained after synthesis without (A) and with acetylations (B). Purified sample (C).

The availability of all diastereoisomers of substance P allowed a study of the influence of D-configuration on retention behavior in HPLC-analysis. All ten diastereoisomers were separated from substance P, with D-Phe causing the most dramatic increase in retention time. Generally the introduction of D-amino acid residues into the lipophilic C-terminal domain of substance P improves the interaction with hydrophobic phases. In the polar N-terminal part of substance P, which is not thought to be involved in the interaction of the peptide with the stationary phase, D-configuration results predominantly in shorter retention times.

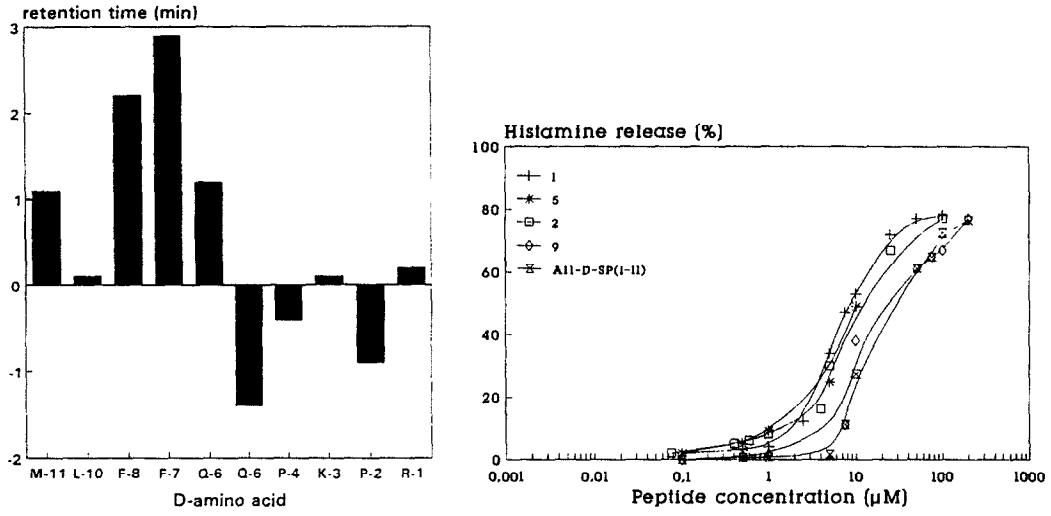

Figure 3. Influence of D-amino acid substitution on the HPLC-retention time of substance P[20]

Figure 4. Histamine-releasing activity of D-amino acid-containing substance P analogs on rat peritoneal mast cells

In order to study the stereochemical requirements of mast cell degranulation, we investigated the histamine-releasing activity of substance P-diastereoisomers on rat peritoneal mast cells. In recent studies[24] we supported earlier suggestions[22] on the significance of lipophilic domains and positive charges for mast cell triggering by peptides, whereas the formation of distinct secondary structures like α-helicity is obviously not a prerequisite for the peptide-induced activation of mast cells[23,24]. Because specific peptide receptors are seemingly not involved in mast cell activation[14,15,25], direct interaction of the G-protein α-subunits with cationic peptides was postulated to be involved in the process of histamine release[15,16]. Figure 4 demonstrates that D-Phe[7]-, D-Lys[3]-, D-Met[11]- and all-D-substance P[24] exhibit nearly equipotent activity when compared with the native peptide, indicating that the stereochemical requirements in the process of peptide-induced mast cell activation are negligible.

Thus, our results do not support a mechanism of G protein activation by direct peptide - G protein interaction[15,16], because G protein activation, thought to be caused by interaction of peptides with the C-terminal domain of the α-subunit[16], should depend on the structural specificity of the cationic peptide.

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12 Abbreviations: Fmoc, 9-fluorenylmethoxycarbonyl; ^{t}Bu, *tert.*-butyl; DIC, N,N'-diisopropylcarbodiimide; HOBt, 1-hydroxybenzotriazole; NMI,1-methylimidazole; Ac_2O, acetic acid anhydride; DMF, N,N-dimethylformamide; TFA, trifluoroacetic acid.

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20 Analytical HPLC: Polyencap A300, 5 μM, 250x4 mm I.D. (Bischoff Analysentechnik GmbH, Germany), mobile phase A: 0.1% TFA in water, B: 0.1% TFA in 50% acetonitrile/50% water (v/v), linear gradient 20-80% B in 40 min., 1ml/min, detection at 220 nm. Preparative purification: PLRP-S 300A, 10 μM, 250x25 mm I.D. (Polymer Laboratories, UK), mobile phase A: 0.1% TFA in water, B: 0.1% TFA in 50% acetonitrile/50% water (v/v), linear gradient 20-80% B in 70 min., 10 ml/min, detection at 220 nm, Peptides were characterized by FAB-MS spectra using the $[M+H]^+$ peaks which corresponded to the calculated values.

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