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Chemical synthesis of Maxadilan, a non-mammalian potent vasodilatory peptide consisting of 61 amino acids with two disulfide bridges, and its related peptides

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This paper is dedicated to the memory of Professor Bruce Merrifield, a pioneer and one of the most respected experimental scientists, who made extraordinary contributions to high throughput chemical synthesis.

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Abstract

A potent and persistent non-mammalian derived vasodilator, maxadilan (Maxa) consists of 61 amino acids with two disulfide linkages and acts as an agonist of the type I receptor of pituitary adenylate cyclase activating polypeptide (PACAP), although there is very little sequence similarity. The total chemical syntheses of Maxa, its disulfide isomers and various fragments have been performed successfully by highly efficient solid-phase peptide synthesis (SPPS). A "difficult sequence", envisaged in the middle region of Maxa, could be overcome by improved synthesis protocols. After assembly peptides were liberated from the resin by cleavage. Peptides having disulfide(s) were purified by two steps of preparative HPLC using cation exchange followed by reverse phase columns. Purified peptides were characterized by HPLC, Edman-sequencing, amino acid analysis and mass spectrometry in addition to disulfide form determination. The peptides obtained were used for recognition studies by the melanophore assay to confirm the native disulfide form. Peptide libraries related to Maxa, produced in the present study, will be useful for the elucidation of the structural requirements of Maxa for interaction with the PACAP type 1 receptor (PAC1).

Keywords

vasodilator, maxadilan, PAC1 receptor, disulfide isomer, highly efficient solid-phase synthesis, difficult sequence, melanophore assay

Abbreviations

HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridin-1-ylmethylene]-*N*-methylmethan-aminium hexafluorophosphate *N*-oxide, HOBt: 1-hydroxybenzotriazole, LC-IT-MS: liquid chromatograph with on-line ion-trap mass spectrometry, MALDI-TOF MS: matrix assisted laser desorption ionization time of flight mass spectrometry, Maxa: maxadilan, PACAP: pituitary adenylate cyclase activating polypeptide, PAC1: PACAP type 1 receptor, SPPS: solid-phase peptide synthesis, RP-HPLC: reverse-phase high performance liquid chromatography, PyBOP: benzotriazole-1-yl-oxy-tris (pyrrolidino)phosphonium hexafluorophosphate, TFA: trifluoroacetic acid, TMP: ,4,6-trimethylpyridine.

INTRODUCTION

Blood feeding or hematophagus arthropods have developed a variety of anti-hemostatic compounds to ensure success in obtaining blood from their hosts. Identifying such compounds associated with these mechanisms will provide new tools for studying receptor biology and lead to new therapeutic agents for cutaneous and vascular biology. Maxadilan (Maxa) is a potent vasodilator peptide, which was isolated from salivary gland lysates of the bood feeding sand fly *Lutzomyia longipalpis*, a vector of leishmaniasis (Lerner et al. 1991) and later cloned (Lerner et al. 1992). Although Maxa has four cysteinyl residues, the exact disulfide form of the natural material (wild type) are not precisely known. Hence chemical synthesis is indispensable. Maxa aids the fly in obtaining blood from the skin of its vertebrate hosts and

has been demonstrated as a ligand of the type 1 receptor of pituitary adenylate cyclase activating polypeptide (PAC1) (Moro and Lerner 1997), although Maxa and pituitary adenylate cyclase activating polypeptide (PACAP) do not show significant sequence similarity (Fig. 1). PACAP, a neuropeptide with vascular and endocrine activities, was isolated from ovine hypothalamus through its potent activity in stimulating cAMP production in rat anterior pituitary cells (Miyata et al. 1989 and 1990). Two functional forms of PACAP have been identified, namely PACAP-38, a C-terminally amidated 38 amino acid residue peptide and PACAP-27, the 27 amino acids N-terminal peptide amide of PACAP-38 which shares 68% sequence homology with vasoactive intestinal polypeptide (VIP). PACAP and its receptors are members of the secretin/glucagon/VIP family of ligands and receptors. The receptors are members of the G-protein coupled/seven transmembrane spanning family and three classes of these receptors have been identified, (reviewed in Rawlings and Hezareh 1996). PACAP has been implicated in a number of physiological processes including neurotransmission, vasodilatation, immuno-modulation and insulin secretion. However, the absence of type-specific receptor agonists and antagonists has hindered until now the progress of further biochemical studies. Clearly Maxa may provide such a specific agonist of PAC1. The total synthesis of Maxa and its related peptides should provide useful tools for studying the physiological actions of the PACAP receptor system and may provide new insights into the pathophysiology and treatment of skin diseases that have a vascular and possible neurocomponent. Structural requirements for ligand-receptor interactions will allow the development of drugs that either stimulate or block these receptors. The action of Maxa is an endothelium-independent vasodilator with immuno-modulatory properties (Qureshi et al. 1996) that binds to PAC1 inducing the accumulation of intracellular cAMP and subsequent vasodilatation (Moro and Lerner 1997).

Fig. 1 Primary structure of Maxadilan and PACAP with related peptides

Previously we have synthesized numerous PACAP/VIP peptides and their related derivatives, elucidated their solution structures and compared many aspects of their biological actions, (summarized in Nokihara et al. 1998; Wray et al, 1998). The discrimination between two distinct PACAP receptor types was found to be in positions 4 and 5 of the N-terminal region (Ando et al. 1996). It is interesting that Maxa is recognized only by PAC1 and hence the development of potent agonists and antagonists to PAC1 from this source may have important clinical applications. The present paper describes construction of a mini-library of Maxa derivatives (Fig 2.); the total chemical syntheses of Maxa (61 amino acids with two disulfides in positions 1-5 and 14-51: predicted for wild type Maxa), its disulfide analogs (mono disulfide, two disulfide isomers and linear derivatives without disulfides) and various partial fragments, N- and C- terminal as well as central region fragments (C-terminal fragments with or without disulfide, C-terminal disulfide dimer, middle fragments with a disulfide, N-terminal fragments with and without a disulfide, N-terminal disulfide dimer, without middle fragment with a disulfide, N-linked to C-terminal fragment, and two chains connected with disulfide bond between 14-51). These peptides have been prepared by an efficient solid-phase synthesis using the Fmoc-strategy.

MATERIALS AND METHODS

General

Chemicals for peptide assembly including resin were SynProPep® products from Shimadzu Corp. (Kyoto, Japan). Additionally, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b] -pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide were obtained from PerSeptive Biosystems (Framingham, MA), 2,4,6-trimethylpyridine (TMP) was purchased from

Aldrich (Milwaukee, WI). All other solvents and chemicals were of analytical grade from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemicals (Osaka, Japan) and used as received. Endoproteinase Lys-C (EC 3.4.21.50) from *Achromobacter lyticus* was purchase from Wako Pure Chemicals.

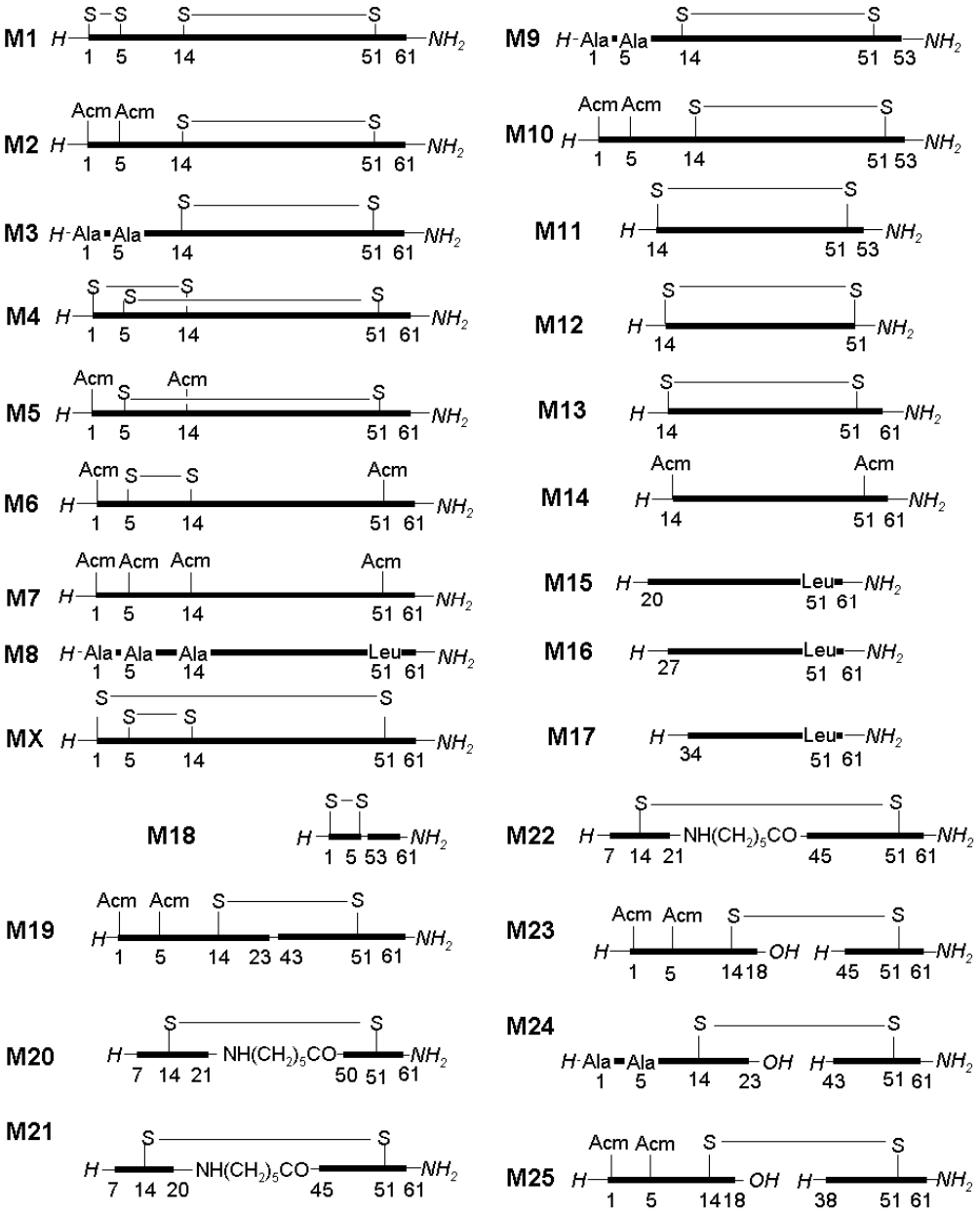

Fig. 2 Schematic drawing of the Maxa mini- library prepared in the present study. MX could not be obtained.

Peptide chain sssembly

The side-chain protecting groups used were 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg, t-Bu for Asp, Glu, Ser, Thr and Tyr, and trityl (Trt) for Asn, Gln and His, and Boc for Lys. Cys was protected by Trt and/or Acm group, and a pair of the same protecting groups was selectively removed to generate disulfide. Parallel simultaneous assembly of peptide chains was performed on the TentaGel® S-RAM resin (100 mg, 0.2 meq/g for syntheses of the C-terminus of Maxa). For the N-terminal elongation of 61 amino acid peptides, a half of peptidyl resin was used from position 34. For syntheses of the middle and N-terminal fragments of Maxa the same resin (50 mg, 0.2 meq/g) was used. The solid-phase synthesis was carried out using a synthesizer, Shimadzu PSSM-8 System (Kyoto,

Japan), by the Fmoc-strategy with the modified protocols: Instead of DMF as a solvent for synthesis, modified DMF (designated DMF*) consisting 40% DMF, 9% DMSO, 30% N-methylpyrrolidone, 20% dichloromethane, 1% triton X-100, 2M ethylene carbonate (Tokyo Kasei Co. Ltd, Tokyo), was used throughout the syntheses. Deprotection of Fmoc-groups was carried out using 2% 1,8-diazobicyclo [5, 4, 0]-undec-7-ene (DBU), 2% piperidine in DMF*. N-methylmorpholine in the standard protocol was replaced by TMP (1 mol/mL in DMF*), benzotriazole-1-yl-oxy-tris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP) was replaced by HATU. 1-Hydroxybenzotriazole (HOBt) as an additive was absent. All other conditions were the same as in the standard protocol. During the assembly of the 61 amino acid residue peptide, a half of the peptidyl resin was removed from the reaction vessel and cleaved to monitor the assembly. The C-terminal fragments (34-61, 27-61 and 20-61) were then further assembled from the remaining resin (10 □mol).

Cleavage

After assembly the peptidyl resin was suspended in a cold cocktail (twice volume of peptide resin) consisting of 82.5 % TFA, 5 % water, 5 % thioanisole, 3 % thiophenol, 2.5 % ethane dithiol and 2 % methylethylsulfide and allowed to stand at room temperature for 8 h with occasional shaking. After filtration peptides were precipitated with cold diethyl ether and separated by centrifugation (2500 rpm, 10 min), washed five times with diethyl ether and finally dissolved in 30 % acetonitrile in 0.005 N HCl and freeze dried to give crude peptide (yield 75-80 % of calculated amounts).

Disulfide linkage formation

The cleaved peptides, with *S*-Trt protection of Cys residues, were dissolved in 1 M NH_4OAc, 8M Urea (pH 7.6) at a concentration of 1 mg/mL. With vigorous stirring 0.1M K_3FeCN_6 in 1 M NH_4OAc was added dropwise (ca 400 □L for ca 10 □mol peptide) and the resulting mixture was stirred for a further 30 min at room temperature, then quenched by addition of AcOH (until pH 4.5). The mixture was desalted by column chromatography using Sephadx®G25 (fine) with 5% AcOH. Each fraction (10 mL) was collected and an aliquot was monitored by HPLC and analysed by mass spectrometry. The fractions containing the desired material were pooled and freeze dried to give crude oxidized peptides (yield ca 60% of theoretical value). These were purified as described below.

The second disulfide linkage was formed from the peptide with a pair of *S*-Acm groups, and was obtained after cleavage followed by the above disulfide formation with subsequent purification. Typically, Cys(Acm) Maxadilan (3.0 mg) was dissolved in 0.01N HCl (90 □L) and AcOH (360 □L). With vigorous stirring at room temperature 0.5 M iodine in MeOH 44 □L (50 eq) was added dropwise and stirred for 30 min. The mixture was quenched with ascorbic acid (7 mg) in 1M sodium citrate pH 5.0 (1 mL). The resulting mixture was diluted to 10 mL with water and purified as described below.

Purification of Synthetic Peptides

Purification was performed using a HPLC-system, Model LC10A (Shimadzu). All synthetic peptides were analyzed by RP-HPLC employing a SynProPep® RPC18 column (4.6 X 150 mm) (Shimadzu) using a linear gradient (gradient rate: 1 %/min) with 0.01N HCl and acetonitrile at a flow rate of 1.0 mL/min and monitored at 210 nm. The crude peptides with disulfide linkages were initially separated on an ion-exchange column, using a Poros SP/M column (4.6 X 100 mm) with two buffer systems consisting of a starting buffer (A = 20 mM $NaHPO_4$ pH 6.0) and final buffer (B = 1M NaCl, 20 mM $NaHPO_4$ pH 6.7) with a linear gradient of A/B = 100/0 (0-5 min), 100/0 -0/100(5-30 min) at a flow rate of 5 mL/min and monitored at UV 230 nm. Major fractions were pooled and further separated by multiple injections on SynProPep® RPC18 (20 X 150 mm) using a linear gradient of 0.01N HCl and acetonitrile (acetonitrile: 20-25 % in 15 min) at a flow rate of 9.9 mL/min and monitored at 230 nm. In each case the major peak was collected and freeze dried to give Maxa-related peptides. The yield of Maxa(1-61) with *S*-Acm(1, 5), disulfide (14-51) was ca 10 %, Maxa(1-61) with *S*-Acm(1, 51), disulfide (5-14) was ca 5 % and Maxa(1-61) with *S*-Acm(1, 14), disulfide (5-51) was ca 5 % calculated from the lyophilized major fraction separated on Sephadex® G25. After formation of the second disulfide bond and subsequent purification, yields were as follows: 0.8 mg Maxa (1-61) with disulfides (1-5, 14-51), in which the first

linkage was 14-51, 0.4 mg Maxa (1-61) with disulfides (1-14, 5-51), in which the first linkage was 5-51, Maxa (1-61) with disulfides (1-51, 5-14) could not be obtained. Other fragments containing disulfide linkage(s) were synthesized and purified in a similar manner.

Characterization of synthetic peptides including disulfide forms

The purified peptides were characterized by RP-HPLC and LC-ITMS on LCQ-DECA (Finnigan Corp, CA) or MALDI-TOF MS on a Voyger Eliute DE (PerSeptive Biosystems, MA) in the linear mode with sinapinic acid or □-cyano-4-hydroxycinnamic acid as matrix. Sequence analyses of the purified materials were performed on a gas-phase protein sequencer, Model PPSQ-10 (Shimadzu) according to the manufacture's protocol to confirm the desired N-terminal sequence as well as disulfide forms described previously (Nokihara et al. 1992). Enzymatic cleavage was performed using lysylendopeptidase for 14 h at 37□C in 0.1 M N-ethylmorpholine/ AcOH buffer (pH 8.5) at a molar ratio of ca 1:50 (enzyme : substrate). The digests were quenched with 1N HCl and sequenced to confirm the disulfide forms as described previously (Nokihara et al.1997a). Peptide content was determined by amino acid analysis, which was performed after gas-phase acid hydrolysis (Bidlingmeyer et al.1984) followed by dabsylation (Chang et al.1981) and HPLC-separation using a TSK Octadodecyl-2PW column (4.6 X 150 mm).

Melanophore assay:

Frog fibroblasts were cultured at room temperature for 3-4 days in 0.7x Lebowitz-15 medium, 20% fetal bovine serum, 1x glutamine, and 1x penicillin-streptomycin. This conditioned medium was used to maintain a stable line of melanophores expressing the rat PAC1. Maxa-related peptides were assayed for functional activity by measuring pigment dispersion in melanophores stably expressing the rat PAC1 receptor. Briefly, PAC1 expressing melanophores were plated in 96 well plates at a density of approximately 40,000 cells per well. Two hours prior to the addition of Maxa or related peptides, the medium was changed and melanosomes were aggregated with 2 nM melatonin, which targets the endogenous Gi linked melatonin receptor. Baseline readings were taken with a plate reader (Tecan, Maennedorf, Switzerland) at 620 nm to obtain the initial transmittance (T_i) values at time zero before the addition of ligands. For the agonist assay, Maxa and mutants were diluted from stock solutions and added to the wells in 10-fold dilutions in triplicate. The antagonist assay was performed by adding 10-fold dilutions of the mutants in the presence of 0.08 nM Maxa. Final transmittance (T_f) readings were taken after one hour. EC-50 and IC-50 values were generated as described previously (Pereira et al. 2002).

RESULTS AND DISCUSSION

Maxa and related large peptides were synthesized based on our previous experience of the preparation of high quality large peptides by solid-phase synthesis (Nokihara and Semba 1988; Nokihara 1990; Nokihara et al. 1997b). In particular cross contamination-free manipulation and efficient coupling protocols with efficient mixing and agitation by fine bubbling of nitrogen from the bottom of the reaction vessels through an extra mounted membrane filter, implemented on an automated simultaneous multiple synthesizer, Model PSSM-8 (Shimadzu) was used. The first run of the total synthesis used optimized protocols that had been employed previously. The HPLC profiles of the crude material obtained by cleavage followed by the first disulfide bond formation are illustrated in Fig 3 (left). Further changes in coupling reagents described below and the modified solvent used for deprotection and coupling, which may suppress aggregation of elongated peptide chains, were then made to the protocols to afforded higher efficiency. The major byproducts generated in the assembly on a polymer support are undoubtedly the deletion peptides, produced by unused reagents or incomplete acylation and/or Fmoc-deprotection. Additionally, diastereomers produced through racemization, can be minimized by faster coupling with HATU (Carpino et al.1994) with TMP (Carpino and El-Faham 1995). Throughout assembly the single coupling but no-capping protocol was employed in the special solvent mixture. Several side reactions have been reported during cleavage, although these can be successfully suppressed by the use of appropriate scavenger cocktails and careful

operation, *ie*. the cleavage cocktail was added at low temperature with immediate mixing. Fig. 3 shows HPLC profiles of crude S-protected Maxa after cleavage followed by the first disulfide formation (position 14-51). Some improvement of the syntheses can bee seen (Fig 3 right) (Nokihara et al. 2001a). The different retention times of the desired material is a consequence of the different dead volumes associated with the different HPLC columns and HPLC systems used for these analyses. The major peak from both syntheses have the expected mass.

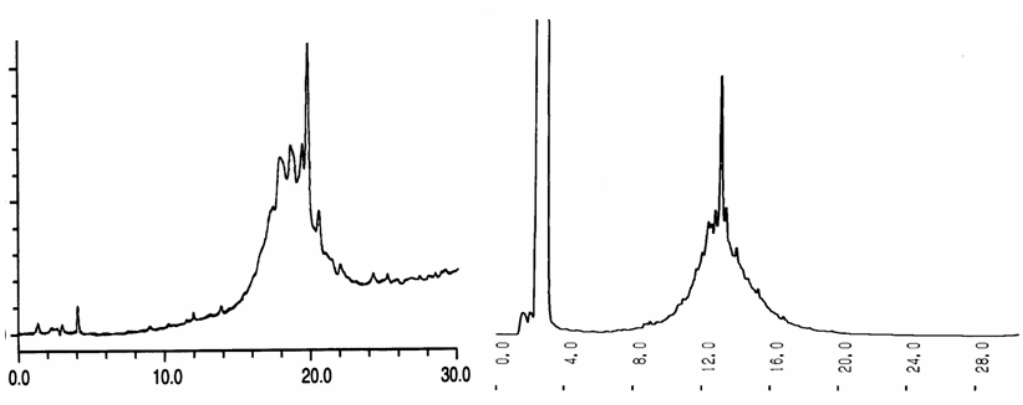

Fig. 3 Crude 61 amino acid peptides M2, Maxa(1-61)-NH_2 with S-Acm(1, 5) and disulfide (14-51), after cleavage followed by first disulfide formation. (Column: left: TSK ODS120T, 4.6 x 150 mm, flow rate 1.0 mL/min, monitored at 210 nm, linear gradient: A= 0.01N HCl, B= acetonitrile, A: 85-55 in 30 min; right SynProPep® RPC18 4.6 id x 150 mm, flow rate 1.0 mL/min, monitored at 210 nm, linear gradient: A= 0.01N HCl, B= acetonitrile, A: 85-55 in 30 min). The crude Maxa shown at the left was prepared by conventional synthetic protocols employed in the syntheses of PACAP related peptides (Nokihara 1998). The crude Maxa shown at the right was prepared by the present improved method.

Maxa is predicted to have two disulfide linkages as four cysteinyl residues were identified from cDNA analysis (Lerner et al. 1992). Different side chain protecting groups were employed in order to ensure the homogeneity of the synthesized disulfide linkages and to allow confirmation of the natural disulfide forms and/or conformational identification as performed in our previous synthesis of guanylin and its disulfide isomers (Nokihara et al. 1997a). Simultaneously, a considerable number of full length linear forms of the 61 amino acid peptide were also synthesized to elucidate the importance of the cysteine residues and the disulfide linkages. In order to monitor the assembly efficiency during synthesis, samples of the intermediate peptidyl resin were removed, cleaved and characterized.

Despite the low similarity between the primary sequences of PACAP and Maxa, one possible sequence alignment suggested positions 34-35 of Maxa aligned with positions 6-7 of PACAP (Fig. 1), and this appeared to be important as PACAP(6-27) is known to have antagonistic properties. We also speculated that in an alternative alignment His at position 20 in Maxa may align with the N-terminal His of PACAP. Thus, to explore these possible correlations with Maxa's biological actions, selective N-terminal deleted fragments Maxa(34-61), Maxa (27-61) and Maxa (20-61) were synthesized in which Cys-51 was replaced by Leu. As one of the disulfide linkage is predicted between positions 14 and 51, various N- and C- terminal truncated fragments, in addition to a number of open and internally modified fragments, were selected to study the importance of the central unit of Maxa. All synthetic peptides prepared in the present study are summarized in Fig. 2. Somewhat surprisingly disulfide formation from the tetra-sulfhydryl Maxa obtained from tetra Cys(Acm)^{1,5,14,51} , M7 in Fig. 2, did not efficiently generate the desired Maxa nor its disulfide isomers, although random disulfide formation of several natural peptides and proteins, which contain fully reduced thiol groups normally form the folded product with the native cystine connectivity (Kimura 2004). Normally nature generates the most thermodynamically stable three dimensional structures in aqueous neutral media.

Although a highly efficient SPPS (Nokihara et al. 1997b) has been carried out, the total overall yield was poor and chromatograms of cleaved crude products gave unsatisfactory peptide patterns,

apart from the C-terminal half-size Maxa such as M17 in Fig. 2. In the syntheses from position 34 in the N-terminal direction a portion of the peptidyl resins were cleaved and characterized. A "difficult sequence" was envisaged in the middle region of Maxa, specifically from position 34, while monitoring using peptide-cleavage indicated gradually poorer quality products. Various secondary structure prediction programs consistently suggest the full length molecule consists of a β-sheet sandwiched between two α-helices (Fig. 4). In fact, the "difficult sequence" observed during the total synthesis of Maxa involved β-structures and thus aggregation caused by hydrogen bond formation. Hence we have taken various steps to improve the yield and quality of the cleaved peptides, and to improve the synthesis efficiency (Nokihara et al. 2001b).

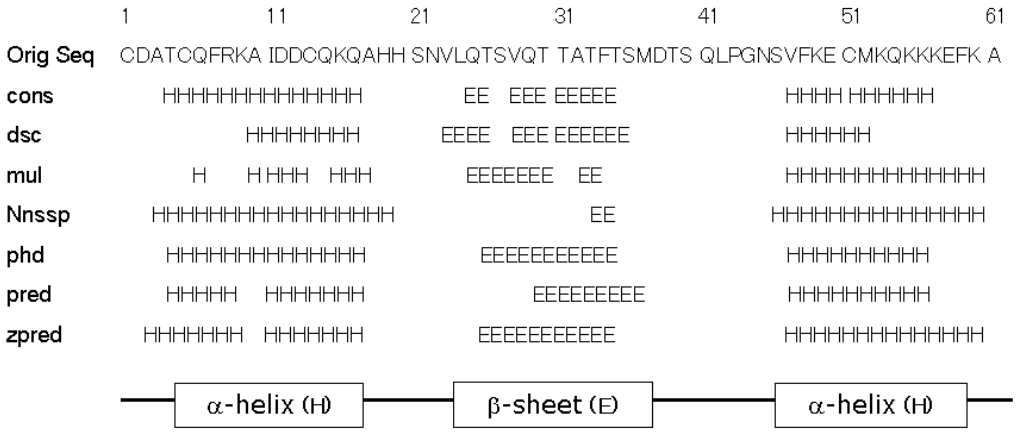

Fig. 4. Prediction of the secondary structure of Maxa peptides used programs in the public domain; http://www.hgmp.mrc.ac.uk. This site is maintained by the UK Human Genome Mapping Program. (cons: Conservation number; phd: PHD prediction; dsc: DSC prediction; pred: PREDATOR prediction; nnssp: NNSSP prediction; zpred: Zpred prediction)

After cleavage and disulfide formation by oxidation followed by desalting, the desired materials were purified by two steps of preparative column chromatography, *ie*, ion-exchange prior to RP that is a very powerful purification step for longer peptides as we have shown previously for a 126 amino acid small protein (Nokihara, K.1990). Characterization of the disulfide forms, following a procedure used by us previously (Nokihara, K., et al., 1992) indicated only two of the three possible disulfide isomers, namely S-S 1-5/14-51 and 1-14/5-51, could be synthesized. The disulfide with 1-51/5-14 could not be produced, although single disulfides containing Maxa-derivatives (disulfide 14-51, 5-14 and 5-51) were prepared. Clearly the distance difference between positions 1 and 51 is too great to allow significant formation of this disulfide linkage in the linear molecule, and probably indicates the smaller ring disulfide linkages are formed initially.

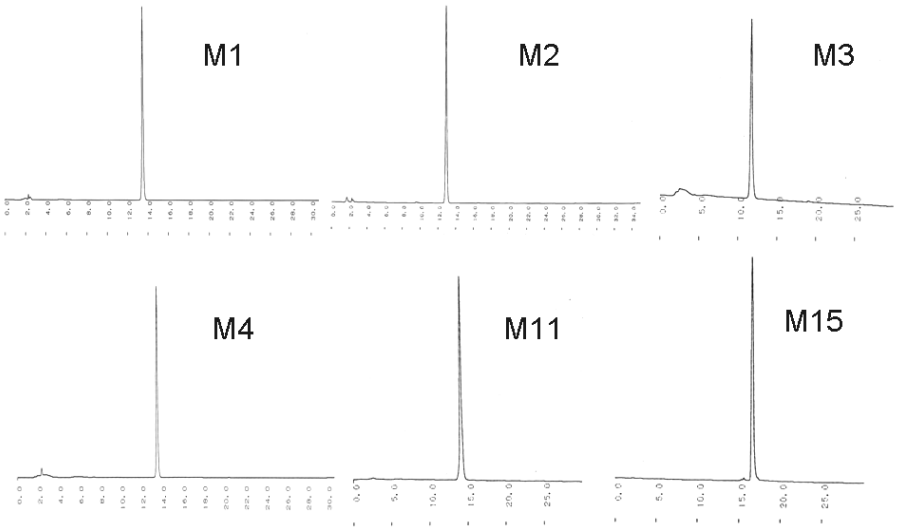

Fig. 5. Representative HPLC-profiles of synthetic Maxa-related peptides after purification: M1: [S-S$^{1-5,\ 14-51}$] Maxa(1-61) -NH$_2$, wild type; M2: [Cys(Acm)$^{1,\ 5}$, S-S$^{14-51}$] Maxa(1-61)-NH$_2$; M3: [Ala$^{1,\ 5}$,S-S$^{14-51}$] Maxa(1-61)-NH$_2$; M4: [S-S$^{1-14,\ 5-51}$] Maxa(1-61) -NH$_2$, M11: [S-S$^{14-51}$] Maxa(14-53)-NH$_2$; M15: [Leu^{51}] Maxa(20-61)-NH$_2$. HPLC conditions were as described in the experimental section.

After purification, HPLC analyses of the listed peptides (Maxa mini-libraries) showed >96 % purity and Edman-sequencing indicated the expected sequences. HPLC-profiles of several representatives of the Maxa-mini library are shown in Fig. 5. A number of N- and C-terminal fragments were connected by disulfide bonds between positions 14 and 51. In these syntheses a number of different products were produced corresponding to all possible combinations of the interacting fragments (the example in Fig. 6 shows the mixture produced from the N- and C-terminal fragments connected through a disulfide bridge at positions 14 and 51). In each case the reaction mixture could be efficiently monitored by LC-ITMS and the desired product isolated. Mass-spectrometric data for all the synthesized peptides are summarized in Table 1.

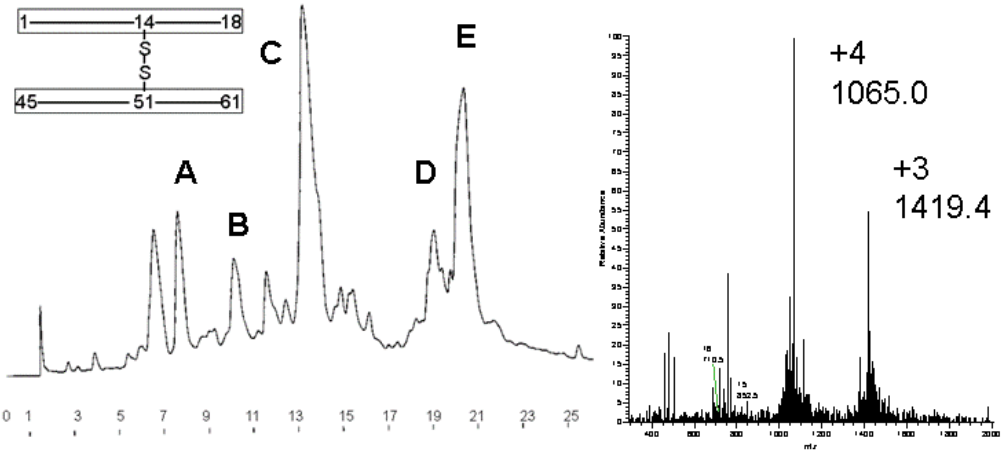

Fig. 6 Left: separation profile of preparative purification after disulfide connection of N-terminal fragment, [Acm$^{1,\ 5}$ Cys(SH)^{14}] Maxa(1-18), and [Cys(SH)^{51}] Maxa(45-61)-NH$_2$. A = dimer of C-terminal fragment ; B = C-terminal fragment; C = desired peptide; D = dimer of N-terminal fragment; E = N-terminal fragment. Right: ITMS of C, deconvoluted mass: 4256.0 (M). Column: SynProPep® RPC18 (20x150 mm), 0.1NHCl/ acetonitrile 85/15 - 70/30 (30 min), Flow 9.9mL/min, 210 nm

Recently clones for numerous G-protein coupled receptors have been available for drug discovery, thus demands for rapid functional assays are increasing. A new approach has been developed for visualizing functional ligand-receptors interactions which provides rapid and quantitative data for functional interactions between ligands and receptors. This method has been designated the melanophore assay (reviewed by Lerner, 1994). This melanophore based pigment-translocation assay provides a straightforward means of visually evaluating functional interactions between ligands and their receptors. The peptides prepared in the present study were tested using the melanophore assay. Fig. 7 shows representative melanophore assay data for M1, M2, M4 and M8. All the biological data are summarized in Table 1. The synthetic peptide M1 corresponding to the wild type peptide has the highest affinity of all synthetic peptides, while loss of the N-terminal disulfide is not critical. Loss of activity is apparent for N- and C-terminal truncated molecules with a disulfide (14-51), as well as central fragments with a disulfide (14-51) and Maxa without the central region. Apparently the flexibility of both terminal regions of the molecule plays an important roll for peptide recognition, with the N-terminal region between 7 and 14 and the C-terminal region from 45 being important. Antagonistic effects were observed in a two-chain peptide 1-23 and 43-61 linked with a disulfide bridge between 14 and 51, while a similar molecule with 1-18 instead of 1-23 was weaker. Dimers of the N- and C-terminal fragment showed weak agonistic effects and an N-terminal region connected to C-terminal region via amino hexanoic acid showed weak agonistic effects.

Table 1. List of the synthetic Maxa mini-libraries. Mass-spectrometric analyses were carried out after purification. EC50 is medium effective concentration in melanophore assays.

No	Peptide	Mwt Calcd.(av)	Found (M+H)	EC50 (nM)
M1	[S-S$^{1\text{-}5,14\text{-}51}$] Maxa(1-61)-NH2 (Wild Type)	6865.8	6866.25	0.01
M2	[Cys(Acm)^{1,5}, *S-S*$^{14\text{-}51}$] Maxa(1-61)-NH_2	7010.0	7010.20	0.2
M3	[Ala^{1,5}, S-S$^{14\text{-}51}$] Maxa(1-61)-NH_2	6803.7	6804.50	0.15
M4	[S-S$^{1\text{-}14,\ 5\text{-}51}$] Maxa(1-61)-$NH_2$	6865.8	6866.11	0.02
M5	[Cys(Acm)^{1,14}, S-S$^{5\text{-}51}$] Maxa(1-61)-NH_2	7010.0	7011.20	0.04
M6	[Cys(Acm)^{1,51}, S-S$^{5\text{-}14}$] Maxa(1-61)-NH_2	7010.0	7010.85	1.1
M7	[Cys(Acm)^{1,5,14,51}] Maxa(1-61)-NH_2	7154.1	7154.70	Not tested (NT)
M8	[Ala^{1,5,14}, Leu^{51}] Maxa(1-61)-NH_2	6783.7	6784.22	100
M9	[Ala^{1,5},S-S$^{14\text{-}51}$] Maxa(1-53)-NH_2	5815.5	5816.01	20
M10	[Cys(Acm)^{1,5}, S-S$^{14\text{-}51}$] Maxa(1-53)-NH_2	6021.8	6021.36	NT
M11	[S-S$^{14\text{-}51}$] Maxa(14-53)-NH_2	4412.0	4412.72	NT
M12	[S-S$^{14\text{-}51}$] Maxa(14-51)-NH_2	4152.6	4153.78	NT
M13	[S-S$^{14\text{-}51}$] Maxa(14-61)-NH_2	5400.2	5401.50	10
M14	[Cys(Acm)$^{14,\ 51}$] Maxa(14-61)-NH_2	5544.4	5545.38	inactive
M15	[Leu^{51}] Maxa(20-61)-NH_2	4716.4	4717.30	inactive
M16	[Leu^{51}] Maxa(27-61)-NH_2	3936.6	3937.50	inactive
M17	[Leu^{51}] Maxa(34-61)-NH_2	3247.8	3247.88	inactive
M18	[S-S$^{1\text{-}5]}$ Maxa(1-5+53-61) -NH_2	1625.0	1625.60	inactive
M19	[Cys(Acm)^{1,5}, S-S$^{14\text{-}51}$] Maxa(1-23+43-61)-NH_2	4967.8	4967.63	inactive
M20	[S-S$^{14\text{-}51}$] Maxa[7-21-NH$(CH_2)_5$CO-50-61]-NH_2	3374.0	3373.38	inactive
M21	[S-S$^{14\text{-}51}$] Maxa[7-20-NH$(CH_2)_5$CO-45-61]-NH_2	3862.6	3864.4 (M)	500 nM - 5μM
M22	[S-S$^{14\text{-}51}$] Maxa[7-21-NH$(CH_2)_5$CO-45-61]-NH_2	3949.7	3949.57	inactive
M23	[Cys(Acm)^{1,5}] Maxa(1-18) / S-S$^{14\text{-}51}$/ Maxa(45-61)-NH_2	4257.0	4256.42	inactive
M24	[Ala^{1,5}] Maxa(1-23) / S-S$^{14\text{-}51}$/ Maxa(43-61)-NH_2	4779.5	4778.54	positive antagonist
M25	[Cys(Acm)^{1,5}] Maxa(1-18) / S-S$^{14\text{-}51}$/ Maxa(38-61)-NH_2	4955.7	4956.00	inactive

CONCLUSION

Before the discovery of Maxa and its relationship to PACAP, binding and functional studies in the PACAP/VIP field were difficult to ascribe to a particular ligand or receptor. Three types of PACAP/VIP receptors are known: Namely PAC1 which binds PACAP-38 and 27, PAC2 which is the classical VIP-receptor and shows similar binding of PACAPs and VIP, and PAC3 which binds helodermin, a non-mammalian vasodilation peptide (Rawlings and Hezareh 1996). PAC1 is believed to be the most important since it is widespread in brain. To date Maxa is the only known agonist beside PACAPs. We have demonstrated that chemically synthesized Maxa is recognized by PAC-1 receptors, although Maxa and PACAP have no significant sequence similarity. Maxa is predicted to have two disulfide linkages, while four cysteinyl residues were identified through cDNA (Lerner and Shoemaker 1992). Therefore we have designed and synthesized different disulfide linkages. Disulfides at positions 1-5 and 14-51 (M1) and 1-14 and 5-51 (M4) showed high affinities to PAC-1 receptors in the melanophore assay, although M1 is slightly higher. The present mini-library of derivatives have been shown to contain a number of active molecules that will be useful as substrates to explore the receptor PAC1 pharmacology with respect to its biological function and structure. More detailed pharmacological studies will be published elsewhere.

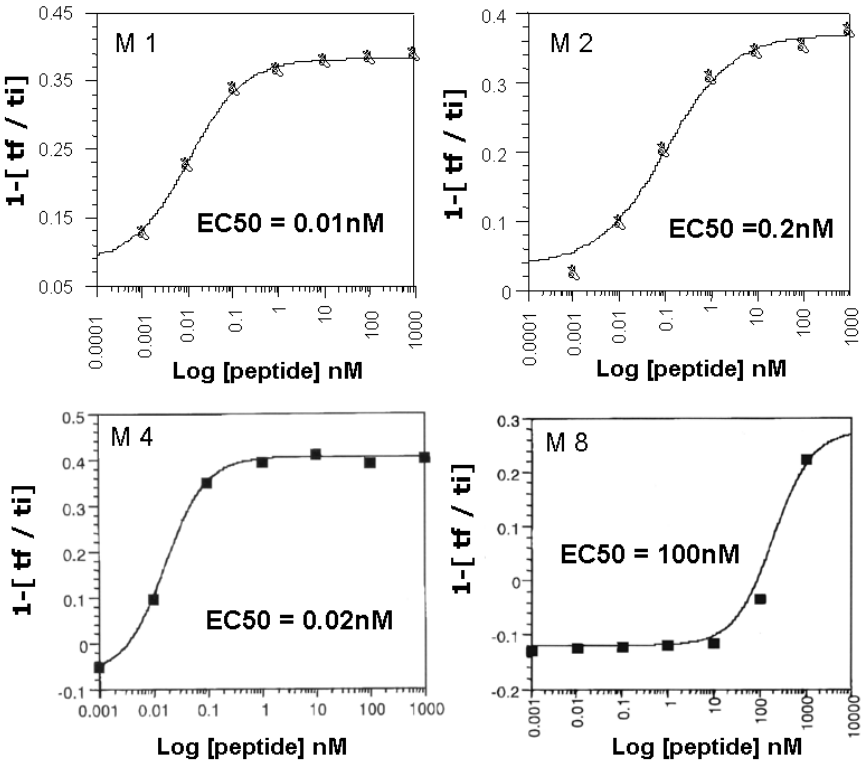

Fig. 7 Representative Melanophore assay results for putative wild type Maxa having 61 amino acids with two disulfides, M1 and its related peptides, M2, M3 and M8.

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