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The Total Chemical Synthesis of Monocyte Chemotactic Protein-1 (MCP-1)

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The affinity-based N^{α}-amino protecting group tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl (Tbfmoc) has been utilized as a hydrophobic probe to allow the simple, quick and highly effective isolation of a 76 residue cysteine-containing protein (MCP-1). The base-labile Tbfmoc group can be removed under very mild conditions, which preserve the thiol-containing protein in the reduced state. Oxidative folding was then used to furnish the biologically active β-chemokine MCP-1.

Keywords: Tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl; Tbfmoc; peptide synthesis; solid-phase synthesis; MCP-1

The stepwise synthesis of large polypeptides by the solid-phase method has always been seen as a major objective in peptide chemistry [1]. However, the main obstacle to the success of such syntheses has always been the difficulty in separating the desired final product from truncated and deletion sequences which can accumulate on the resin during synthesis [2]. The physical and chemical similarities of the desired sequence and such impurities can often require protracted purification in order to isolate the target sequence [3], and this in turn can result in low yield of isolated product.

The incorporation of capping steps within each cycle would not only eliminate the deletion peptides but, additionally, would allow the design and introduction of a labile N^{α}-protection group, which could be used as a vehicle for subsequent affinity-based purification of the product [4–6]. We recently reported the use of the base labile N^{α}-protecting group tetrabenzo[a,c,g,i]fluorenyl-17-methoxycarbonyl (Tbfmoc) to simplify the purification of synthetic peptides and proteins produced using Fmoc/*t*-Bu-based SPPS [6]. The Tbfmoc group (**7**; Scheme 1) is incorporated onto the N-terminus of the resin-bound polypeptide sequence by reaction with chloroformate (**6**). After cleavage of the Tbfmoc-labelled polypeptide from the resin, the desired sequence can then either be purified by exploiting the high affinity of the large, essentially planar, aromatic Tbfmoc group for porous graphitized carbon or by using the hydrophobic nature and specific UV absorbance (364 nm) of the Tbfmoc system to simplify the chromatographic purification by HPLC. In our previous communication [6] we stated the requirement for cysteine residues to remain protected during purification. We now report the use of our Tbfmoc methodology to aid the purification of small proteins containing free thiols and illustrate its utility by producing the biologically significant β-chemokine MCP-1.

Abbreviations: AA, amino acid; Acm, acetamidomethyl; AcOH, acetic acid; Bu^{t}, tert-butyl; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DTT, dithiothreitol; EDT, ethane-1,2-dithiol; HEPES, *N*-(2-hydroxyethyl-l-piperazine *N*-ethane)-sulphonic acid; PMC, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; SPPS, solid-phase peptide synthesis; hTGF, human transforming growth factor α; TIS, triisopropylsilane; Trt, triphenylmethyl.

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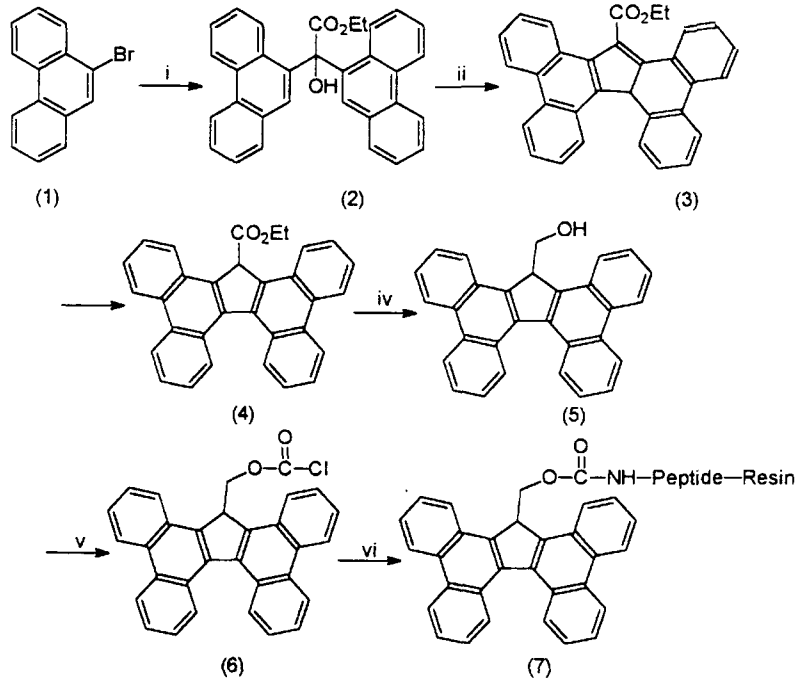
Chemokines are proinflammatory cytokines which attract and activate specific types of leukocytes [7] and can be grouped into two subfamilies, α and β, based on differences in both their primary structure and chromosomal location [8]. In contrast to α-chemokines, such as IL-8, which attract and activate neutrophils, β-chemokines attract and activate monocytes and lymphocytes. In chemokine α-proteins, a single amino acid separates the two cysteine residues nearest the N-terminus and hence they are often referred to as C–X–C proteins. In the case of the β-chemokines the two cysteines nearest the amino terminus are adjacent to each other and are referred to as C–C proteins.

Opinion seems to be divided on the best approach towards the batch SPPS of cysteine-containing proteins. Boc-based strategy has in the past relied mainly on temporary thiol protection which is removed on cleavage of the polypeptide from the solid support. This approach has successfully demonstrated for the synthesis of hTGF-α (50 residues, 3 disulphide bridges) [9], interleukin-3 (140 residues, 2 disulphide bridges) [10], interleukin-8 (72 residues, 2 disulphide bridges) [11] and neutrophil-activating peptide 2 (70 residues, 2 disulphide bridges) [11] as examples. The Fmoc-based SPPS of large, cysteine containing polypeptides has on the other hand relied mainly on the use of the semi-permanent acetamidomethyl (Acm) protecting group, as described for the synthesis of hTGF-α (50 residues, 3 disulphide bridges) [12]. Our approach to the synthesis of such compounds has identified the requirement for unambiguous removal of thiol protection as being a major factor in the outcome of subsequent attempts at oxidative folding and it is for this reason that we chose to incorporate the cysteine residues present in the sequence of MCP-1 (Figure 1) as the triphenylmethyl (Trityl)-protected derivative.

Cloning and sequencing of the MCP-1 gene [13] has shown that the cDNA codes for a 99 residue protein comprising a 23 residue signal sequence and a 76 amino acid sequence which corresponds to that of MCP-1. The signal sequence is post-translationally removed to produce the MCP-1 protein with an N-terminal glutamine residue which can spontaneously afford the blocked N-terminus [14]. For our synthetic protein we chose to incorporate the N-terminal residue as glutamine in order to use our Tbfmoc



Scheme 1 (**1**) Mg, THF, $(CO_2ET)_2$; (**2**) TFA/CH_2Cl_2; (**3**) Et_3N/CH_2Cl_2; (**4**) diisobutylaluminium hydride/CH_2Cl_2; (**5**) triphosgene/*N*,*N*-dimethylaniline/CH_2Cl_2; (**6**) peptide–resin/diisopropylethylamine/CH_2Cl_2; (**7**) Tbfmoc group.

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Gln.Pro.Asp.Ala.Ile.Asn.Ala.Pro.Val.Thr
Cys.Cys.Tyr.Asn.Phe.Thr.Asn.Arg.Lys.Ile
Ser.Val.Gln.Arg.Leu.Ala.Ser.Tyr.Arg.Arg
Ile.Thr.Ser.Ser.Lys.Cys.Pro.Lys.Glu.Ala
Val.Ile.Phe.Lys.Thr.Ile.Val.Ala.Lys.Glu
Ile.Cys.Ala.Asp.Pro.Lys.Gln.Lys.Trp.Val
Gln.Asp.Ser.Met.Asp.His.Leu.Asp.Lys.Gln
Thr.Gln.Thr.Pro.Lys.Thr
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Figure 1. Amino acid sequence of MCP-1.

methodology to purify the protein at the completion of synthesis. Assembly of the MCP-1 sequence (Figure 1) was carried out on a 0.086 mmol scale using Fmoc-Thr(OBu^t) functionalized 4-alkoxybenzylalcohol resin [15]. All amino acids were double coupled and, at the completion of synthesis before removal of the N-terminal Fmoc group, the total resin-bound product was sonicated in the presence of acetic anhydride/HOBt/DIEA in order to cap any remaining free amino groups. The N-terminal Fmoc group was then removed and, after thoroughly washing the resin, the Tbfmoc group was introduced via the chloroformate (**6**).

A portion of the dry Tbfmoc functionalized resin-bound product was then cleaved from the resin by treatment with a mixture of EDT/thioanisole/water/TIS/phenol/TFA for 4 h at room temperature. The crude Tbfmoc-labelled protein was then isolated, after removal of the resin by filtration, by rapid concentration of the filtrate *in vacuo* and diethyl ether preciptiation. The crude solid obtained was then immediately dissolved in aqueous acetic acid (50%), applied to the top of a size exclusion column (Sephadex G-50) and eluted with aqueous acetic acid (30%). The protein-containing fractions were pooled and the Tbfmoc-labelled material isolated by HPLC whilst monitoring at 364 nm to allow detection of the characteristic Tbfmoc chromophore. The purified Tbfmoc-labelled protein was then lyophilized prior to removal of the Nα-protecting group. Previously we have reported the use of piperidine to remove the base labile Tbfmoc group [7]; however, recent work [16] has shown that removal of the Tbfmoc group can be accomplished at pH 8.5 in 6 M guanidine hydrochloride solution in a few hours. In the case of cysteine-containing proteins these conditions require addition of a reducing agent such as dithiothreitol (DTT) so that the highly oxidizable thiol groups can be preserved in the reduced state, hence avoiding problems of solubility and the formation of polymeric aggregates.

Figure 2 illustrates the typical peak profiles obtained during the removal of the Tbfmoc group under these reducing conditions. The broad hydrophobic peak due to the Tbfmoc-labelled protein gradually collapses to give a much sharper earlier-eluting peak, which corresponds to the fully reduced and denatured protein. In the case of MCP-1, monitoring of the Tbfmoc removal by HPLC showed that complete deprotection was accomplished in 4 h and the crude reduced/denatured MCP-1 (Figure 3) was then isolated after desalting (Sephadex G-50, 30% AcOH) followed by lyophilization.

The crude MCP-1 was folded using the general method of Jaenicke and Rudolph [17], during which the progress of the folding reaction was monitored by HPLC. The folded oxidized protein, which eluted earlier than the fully reduced protein on HPLC, was then isolated by HPLC and lyophilized to give the purified MCP-1 (Figure 4).

The purified synthetic MCP-1 was fully characterized by mass spectrometry and amino acid analysis. In addition, mass spectral analysis of the fragments produced on enzymatic digest of the synthetic protein confirmed the presence of fragments due to the expected C11-C36 and C12-C52 disulphide bridges. Further confirmation of the correctly folded structure was also obtained from the similar behaviour of both synthetic and recombinant protein when assayed for their ability to bind to MCP-1 receptors on the

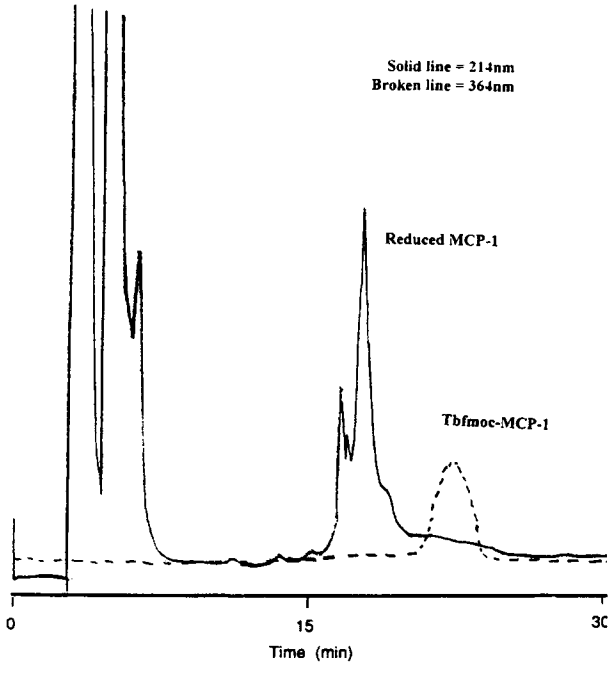

Figure 2. HPLC of Tbfmoc deprotection reaction to give crude reduced MCP-1. Broken line = Tbfmoc-MCP-1 at $t = 0$. Solid line = MCP-1 crude reduced/denatured at $t = 4$ h.

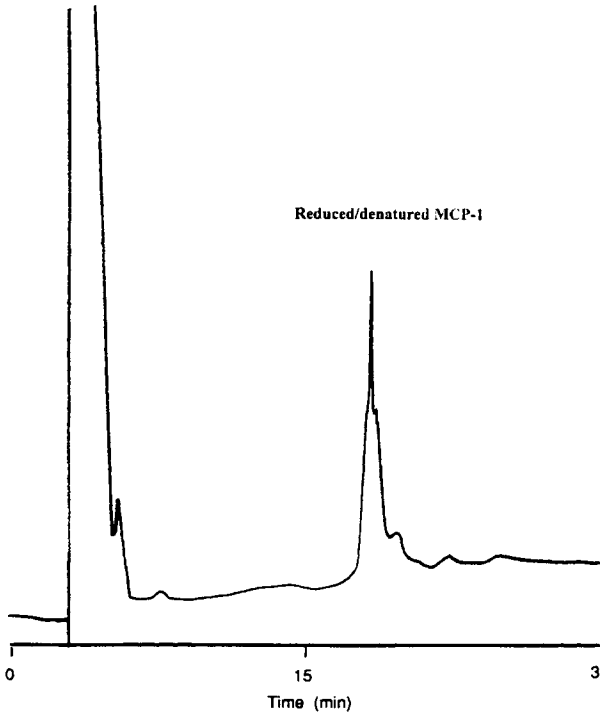

Figure 3. HPLC of crude reduced/denatured MCP-1 after Tbfmoc deprotection and desalting by size exclusion chromatography (Sephadex G-50, 30% AcOH).

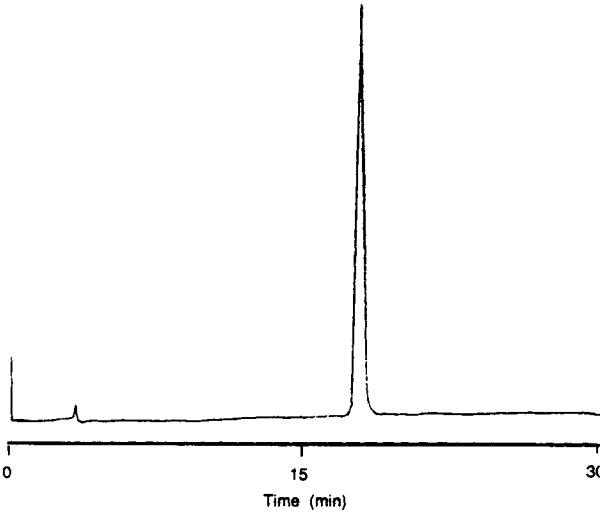

Figure 4. HPLC of pure folded synthetic MCP-1.

surface of human peripheral blood monocytes [18] and also when assayed for calcium mobilization [18]. The synthetic MCP-1 was found to bind to 3552 receptors/cell with an affinity of 1.27 nM, compared with the average affinity of 0.5 n/M for expressed material, and gave an intracellular calcium mobilization value of 484 nM Ca^{2+} for 10 nM of synthetic protein, which is the same as for the expressed material.

In conclusion, we have demonstrated that the Tbfmoc-based purification of synthetic proteins offers a simple, quick and highly effective method for isolating biologically significant target molecules after chemical synthesis. The ease of synthesis of the key reagent (TbfmocCl) [6] and the flexibility of purification method, either by affinity-type binding to PGC [6] or by reverse-phase HPLC, should allow the widespread application of this methodology to the problem of purification of synthetic proteins. (Porous graphitized carbon, PGC, was supplied by Shandon Scientific, Runcorn, UK.)

EXPERIMENTAL PART

General

All amino acids and the 4-alkoxybenzylalcohol functionalized polystyrene resin were purchased from Bachem, Bubendorf, Switzerland. The following side-chain protecting groups were used: Arg(Pmc), Asp(O-Bu^t), Asn(Trt), Gln(Trt), Glu(OBu^t), His(Trt), Lys(Boc), Ser(Bu^t, Thr(Bu^t) and Tyr(Bu^t). Peptide synthesis grade DMF, 1,4-dioxan and piperidine were purchased from Rathburn Chemicals, Walkerburn, Scotland. Peptide synthesis grade TFA was purchased from Applied Biosystems Ltd. All remaining chemicals were obtained from Aldrich. Laser desorption time of flight mass spectra were obtained using a PerSeptive Biosystems LaserTec Benchtop II system. Amino acid analysis was carried out on an LKB 4151 Alpha Plus amino acid analyser equipped with an LKB 2220 Recording Integrator. Gel filtration was carried out using a Microperpex 2132 peristaltic pump and two LKB Uvicord 2138s spectrophotometers at 277 and 365 mm. HPLC was performed using an Applied Biosystems 151A HPLC system.

Solid-phase Peptide Synthesis

All polypeptides were synthesized using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy of N^{α}-protection on an Applied Biosystems 430A peptide synthesizer fitted with a UV monitoring system as described previously. All amino acids were double-coupled as the symmetrical anhydride (1 mmol of amino acid) followed by the HOBt active ester (0.5 mmol of amino acid). The exceptions to this were the amino acids asparagine, glutamine and histidine, which were coupled twice via their HOBt esters and glycine, which was coupled singly as a symmetrical

anhydride (2 mmol of amino acid). Synthetic procedures were pre-programmed into the ABI 430A synthesizer prior to the commencement of synthesis using our own optimized in-house synthetic cycles. Each synthetic cycle involved (a) a capping step; (b) deprotection of the base labile Fmoc group and (c) coupling of the next protected amino acid. The pre-programmed synthetic cycles are summarized below.

Capping. The resin was vortexed with a solution of acetic anhydride (0.5 M), DIEA (0.125 M) and HOBt (0.2% w/v) in DMF (10 ml) for 10 min before the capping solution was drained from the vessel and the resin washed by six portions of DMF.

Deprotection. The resin was vortexed with a solution of 20% piperidine/DMF for 3 min before being drained. An aliquot of the deprotection solution was then sent to a UV detector in order to quantify the amount of fulvene–piperidine adduct present and hence subsequently gave an indication of the percentage incorporation of each residue. Deprotection was then repeated for a second time for 1 min in order to establish if the N^{α}-protecting group had been completely removed. Finally the resin was washed with six portions of DMF/1,4-dioxan (1;1).

Coupling. The resin was vortexed with a solution of 0.5 mmol Fmoc amino acid preformed symmetrical anhydride (formed from 1 mmol Fmoc AA and 0.5 mmol DIC in the activator vessel). The first coupling cycle was allowed to continue for 30 min before the solution was drained from the reaction vessel and the resin washed with two portions of DMF. The resin was then vortexed for a second 30 min period with 0.5 mmol of Fmoc amino acid HOBt active ester (preformed from 0.5 mmol Fmoc AA, 0.5 mmol HOBt and 0.5 mmol DIC) before being drained and washed with four portions of DMF.

Coupling of the C-terminal Amino Acid onto 4-Alkoxybenzylalcohol (Wang) Resin

A solution of Fmoc amino acid (1 mmol) and *N,N'*-diisopropylcarbodiimide (DIC) (0.5 mmol) in DMF (20 ml) was stirred for 15 min at room temperature, then 4-benzyloxybenzylalcohol functionalized polystyrene resin (Wang) (1.0 g, 0.8 mmol) was added, together with a catalytic amount of 4-(*N,N'*-dimethylamino)-pyridine and the mixture was then sonicated for 1–2 h. The functionalized resin was then removed by filtration and sequentially washed with DMF, 1,4-dioxan and dichloromethane, before being dried under vacuum. The loading of the functionalized resin was then determined by treating a known weight of resin with 20% piperidine/DMF in a 10 ml volumetric flask, for 20 min in a sonic bath. The UV absorbance of the supernatant was then measured at 302 nm and the loading calculated using the Beer–Lambert law ($\varepsilon_{302} = 15{,}400$ for fulvene–piperidine adduct). This procedure generally gives a resin with a functionality in the region of 0.1 mmol/g.

MCP-1

The synthesis was carried out on a 0.086 mmol scale using Fmoc-Thr($O^{t}Bu$) functionalized 4-alkoxybenzylalcohol resin (1.0 g, 0.081 mmol/g). All amino acid side chains were protected as described previously. All amino acids were double coupled and coupling of residues 46–39 and 10–1 was extended for a further 30 min. All amino acids were incorporated as described above, with the exception of glutamine.[1] which was incorporated unprotected. At the completion of the assembly the N-terminal Fmoc group was left on and the total resin-bound product was treated with a mixture of acetic anhydride (0.5 M), DIEA (0.125 M) and HOBt (0.2%w/v) in DMF (10 ml) for 30 min in a sonic bath. The resin was then returned to the synthesizer vessel and the N-terminal Fmoc group was removed automatically. The resin-bound product was then sequentially washed with DMF, 1,4,-dioxan, dichloromethane and dried. The dry resin-bound protein was then added to a suspension of TbfmocCl (400 mg, 0.88 mmol) in dichloromethane (20 ml) and DIEA (70 μl, 0.4 mmol) was added before the reaction flask was sealed, covered in aluminium foil and sonicated for 3 h with occasional mixing. The Tbfmoc–peptide–resin (*ca.* 2 g) was then filtered off, washed thoroughly with dichloromethane and dried.

A 500 mg portion of the dry Tbfmoc resin-bound protein was then added to a mixture of EDT (2 ml), thioanisole (0.5 ml), phenol (0.75 g), water (0.5 ml), TIS (0.5 ml) and stirred for 15 min, before TFA (10 ml) was added and the mixture stirred for a further 4 h whilst protected from daylight. The resin was then removed by filtration and the filtrate concentrated *in vacuo* to give an oil, which yielded solid Tbfmoc–protein on trituration with diethyl ether. The solid Tbfmoc–protein was then filtered off, washed with diethyl ether and dissolved in aqueous acetic acid (50%) before being applied to the top of a column of Sephadex G-50 (10 × 800 mm) and eluted with aqueous acetic acid (30%). Pure Tbfmoc–protein was obtained by semi-preparative

HPLC (Vydac C18, 250 × 22 mm, 10 μm, A = H_2O, B = CH_3CN, 0.1% TFA; 9 ml/min, 30–90% B over 25 min, λ = 364 nm) and lyophilized. The Tbfmoc was then deprotected under reducing conditions (6 M Gdm–HCl, pH 8.5 Tris, 0.1 M, containing excess DTT) for 4 h at 37°C before being desalted (Sephadex G–50, 30% AcOH). The protein-containing fractions were then pooled and lyophilized prior to folding.

Folded oxidized MCP-1 was obtained by dissolving the fully reduced protein in 1 M guanidine–HCl (0.1 M, Tris, pH 8.5, 250 ml) containing 0.1 mmol EDTA, 0.3 mmol reduced and 3 mmol oxidized glutatione. After stirring for three days at room temperature the folding solution was acidified (AcOH) and loaded onto a semi-preparative HPLC column (Vydac C18, 250 × 22 mm, 10 μm). Elution with a linear gradient of CH_3CN/0.1%TFA gave pure folded MCP-1 (4 mg) as a white solid after lyophilization; Asx_8 7.84, Thr_7 6.49, Ser_5 4.78, Glx_8 9.90, Pro_5 4.93, Ala_6 6.77, Cys_4 3.44, Val_5 4.73, Met_1 1.02, Ile_6 5.76, Leu_2 2.29, Tyr_2 1.37, Phe_2 2.23, His_1 1.06, Lys_9 8.27, Arg_4 4.13, Trp_1 (N/A); *m/z* (laser desorption) 8681.2 , $C_{379}H_{614}N_{109}O_{114}S_5$ requires 8682; HPLC (Vydac C18, 250 × 4.6 mm, 5 μm, A = H_2O, B = CH_3CN, 0.1% TFA; 1 ml/min, 10–90% B over 30 min λ = 214 nm), *Rt* = 15.8 min, 52.5%B.

Disulphide Mapping by Partial Tryptic Digest

As a general method, the protein of interest (0.5 mg) was dissolved in ammonium acetate buffer (0.1 M, pH 7.5) containing 0.1 mg of trypsin. This sample was then incubated at 37°C for 4–7 h before the digestion was stopped by adding 6 M HCl (5 μl). The sample was then lyophilized and analysed by laser desorption mass spectrometry.

Receptor Binding Protocol

Human peripheral blood monocytes were isolated by a combination of Ficoll-Hypaque gradient centrifugation and centrifugal elutriation as previously described [18]. Cells were suspended at 1 × 10 **7** cells/ml in binding buffer (RPMI 1640 medium with 0.0255 human serum albumen and 10 mM HEPES buffer). Each assay tube combined 50 μl of buffer, 25 μl radiolabelled MCP-1 (^{125}I-MCP-1, DuPont NEN, 2200 Ci/mmole, 50 pmole final assay concentration), 25 μl unlabelled MCP-1 and 100 μl of cells. After 1 h at room temperature, cells were spun through a mixture of silicone:paraffin oil (80:20) to separate bound from free counts. The cell pellet was then counted in a gamma scintillation counter. Cold competition Scratchcard curves were generated for all samples. The concentration of unlabelled MCP-1 used for these studies ranged from 1 pmole to 100 nmole (minimum of 12 concentrations). The Kd and Bmax were calculated using the LIGAND software package.

Calcium Mobilization Protocol

Isolated human monocytes were incubated at 1 × 10^7 cells/ml in RMPI 1640 medium containing 5 μg/ml Indo-1 (Molecular Probes). Cells were incubated at 37°C for 20–30 min. An equal volume of media was added and incubation continued at 37°C for a further 20–30 min. The cells were washed twice with Dulbecco's phosphate-buffered saline containing no calcium and magnesium, before being resuspended at 5 × 10^6 cells/ml in the same buffer containing 1 mmole calcium, 1 mmole magnesium and 5 mmole glucose. The cells were placed into a 2 ml cuvette and monitored in a Perkin Elmer 5050 spectrofluorimeter for differential fluorescence at 340 and 390 nm. The MCP-1 was added in 1% of the final volume to the cells within 2 h of labelling. Fluorescence values were converted to nmoles of intracellular calcium as described previously [18]. Addition of 10 μmole ionomycin followed by 2 mmole manganese chloride were used to establish the F_{max} and F_{min}, respectively. The data obtained (Table 1) for the synthetic MCP-1 are comparable to that obtained with a purified recombinant MCP-1 standard.

Table 1 Calcium Mobilization Assay

	Concentration (nM)	Intracellular Ca (nM)
Synthetic MCP-1	1	121
	10	484
	100	2563

Acknowledgements

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