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Short communication

Solid-phase synthesis, isolation and analysis of a mouse protein, the macrophage migration inhibitory factor

Thomas Kaiser[a,b,*], Peter Luppa[a], Wolfgang Voelter[b]

[a]*Institut für Klinische Chemie und Pathobiochemie, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany*
[b]*Abteilung für Physikalische Biochemie, Physiologisch–Chemisch Instituts, Universität Tübingen, Hoppe-Seyler-Strasse 4, 72076 Tübingen, Germany*

Abstract

Murine macrophage migration inhibitory factor (mMIF) is an M_r 12 500 protein composed of all natural amino acid residues. Using the fluorenylmethoxycarbonyl chemistry for solid-phase peptide synthesis (SPPS) under special conditions, a stepwise approach was very successful leading to a crude product in unexpected high purity. After RP-HPLC isolation, a little mass difference of −12 u was determined for the received protein by matrix-assisted laser desorption ionization MS and ion spray MS and the failure sequence [Ser15]-mMIF identified by Edman degradation. The total solid-phase synthesis was repeated in the stepwise manner under the same conditions leading to the expected mMIF protein in high purity, which was confirmed by different analytical methods. Our results shows the reproducibility of our SPPS approaches to proteins and point out the importance of high-resolution mass spectrometry for a rapid and accurate analysis of such biopolymers.

Keywords: Solid-phase peptide synthesis; Protein synthesis; Migration inhibitory factors; Proteins

1. Introduction

Macrophage migration inhibitory factor (MIF), the first discovered lymphokine [1,2], turned out to be a pro-inflammatory cytokine, secreted by the pituitary and the macrophage during lethal endotoxemia and is furthermore a potent protein mediator of inflammation in a number of infection conditions [3]. The protein is not only a critical component in the immune system, mediating both, inflammation and immunity. MIF was further estimated to be especially expressed in differentiating epithelial cells and also in lens cells throughout development and thought to be involved in the cell proliferation process [4]. The murine factor (mMIF) is an M_r 12 502 protein with 115 amino acid residues composed of all natural amino acid components (see Fig. 1) and adopts approximately 47% β-sheet conformation. ^{1}H nuclear magnetic resonance (NMR) studies and the crystal structure of rat MIF, with 98.5% similarity compared to mMIF, confirmed extended regions of secondary structure, whereby the crystal data identified two α-helices (17–31 and 68–88) and five β-sheet domains (1–7, 37–42, 46–50 and 56–63) [4,5]. For further studies on structure–activity relationships, we decided to synthesize this multifunctional and especially from the synthetic point of view challenging polypeptide using the solid-phase peptide synthesis (SPPS) methodology.

*Corresponding author. Corresponding address: Institut für Klinische Chemie und Pathobiochemie, Technische Universität München, Ismaninger Strasse 22, 81675 Munich, Germany.

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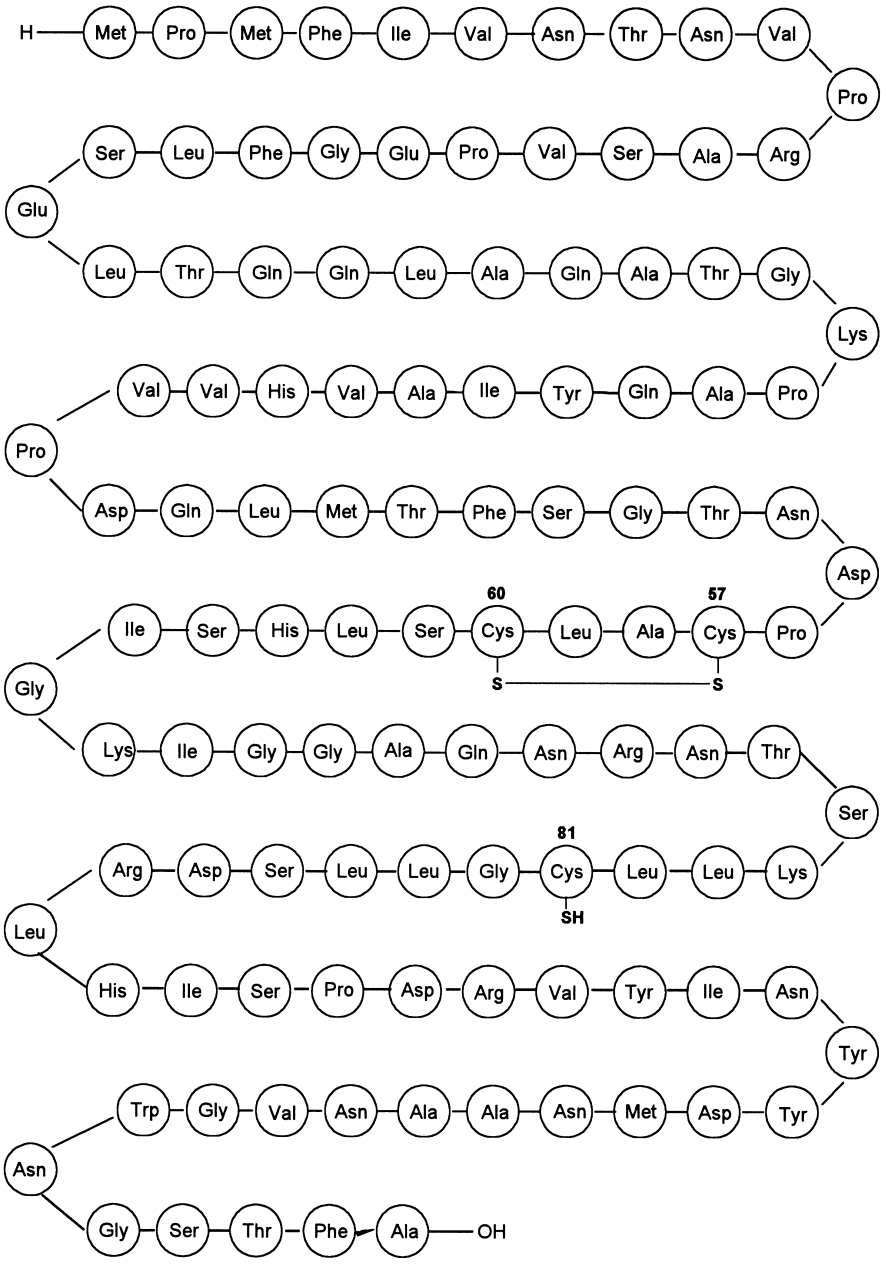

Fig. 1. Primary structure of mMIF including a predicted disulfide bridge mMIF(60–57) [6].

2. Experimental, results and discussion

For the SPPS approach we applied the fluorenylmethoxycarbonyl (Fmoc)/*tert*.-butyl (*t*Bu)-strategy [7] with common protecting groups: *tert*.-butyl ether (*t*Bu) for Ser, Thr and Tyr; *tert*.-butyl ester (O*t*Bu) for Glu and Asp; benzyloxycarbonyl (Boc) for Lys and Trp; trityl (Trt) for Gln, Asn, His and Cys; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for Arg and 2-(1H-benzotriazol-1yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate (TBTU)-activation for amino acid coupling. Preliminary stepwise attempts showed extraordinary experimental problems during the assembly of the amino acid derivatives in the C-terminal region of this protein. Different approaches, ''batchwise'' as well as by the ''continuous flow'' method, using maximum protection strategy, different derivatized polystyrene resins and varying coupling procedures were insufficient and characterized mMIF(93–115) to be a typical ''difficult sequence'' peptide [8]. Therefore, we decided to follow a fragment condensation strategy [9], using Fmoc chemistry for SPPS with maximum protection and different types of polystyrene resins as solid support [10]. To receive the cystin-protein [6] (see Fig. 1) the protected fragment mMIF(53–69)(Cys^{57}–Cys^{60}) with the predicted disulfide bridge was chosen [10]. The progress of these approaches for the total synthesis with our fragment condensation strategy was limited and the protein could not be synthesized in this way.

Surprisingly, another stepwise elongation approach on an automatic ECOSYN P peptide synthesizer (Eppendorf, Hamburg, Germany) using a TentaGel resin (RAPP-Polymere, Tübingen, Germany) as solid support showed that practical problems during the synthesis of mMIF could be better overcome by a step-by-step assembly of the chosen amino acid derivatives. For the total synthesis of mMIF in the stepwise manner, a TentaGel-R-trityl resin, loaded with 0.12 mmol Fmoc-Ala/g, was chosen and the Fmoc deprotection performed with 30% piperidine/dimethylformamide (DMF). For acylation TBTU–diisopropylethylamine (DIEA) double couplings [Fmoc–amino acid–TBTU–hydroxybenzotriazol (HOBt)–DIEA, 6:6:1:10) were employed, except for the preformed symmetrical anhydride (PSA) acylation to incorporate the Fmoc-

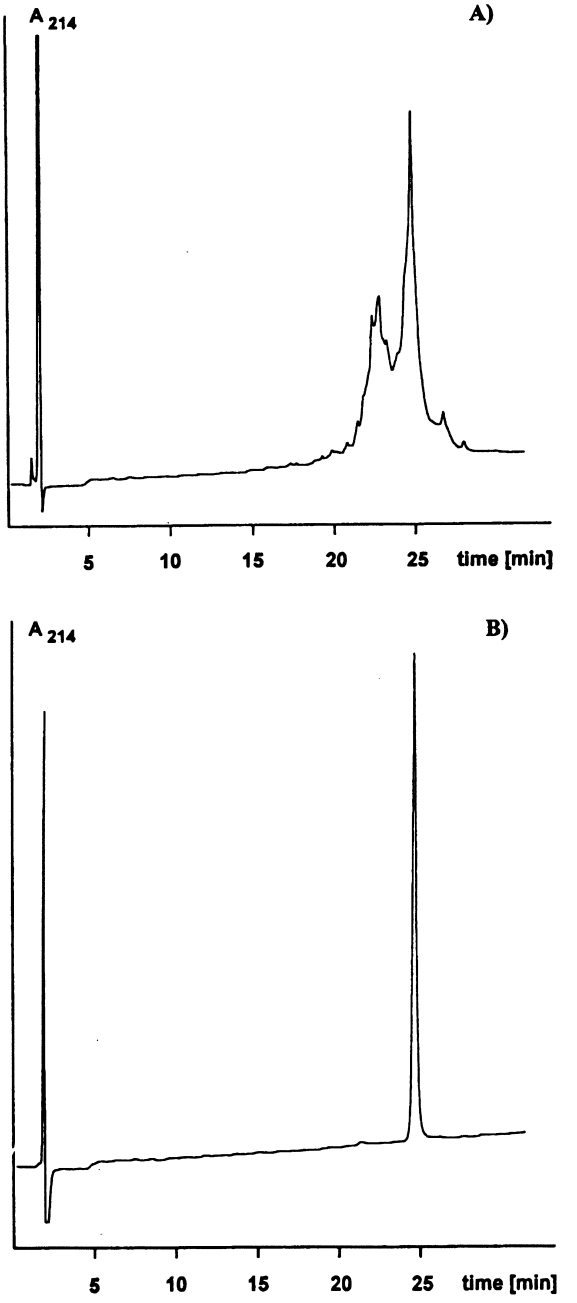

Fig. 2. RP-HPLC profile of the fully deprotected crude product (A) of the total stepwise synthesis and of the isolated (Ser15)-mMIF protein (B). Chromatographic conditions: system: high-precisions pump Model 480 and BT 3030 UV detector (Gynkotek, Munich, Germany), column: Nucleosil 5 μm 100-C_{18} 120×4.6 mm (Macherey–Nagel, Düren, Germany); eluents: 0.05% TFA in water (A), 0.05% TFA in CH_3CN (B); gradient: 0–1 min 95% A, 1–31 min 95–60% A; flow-rate: 1 ml/min; UV detection at 214 nm.

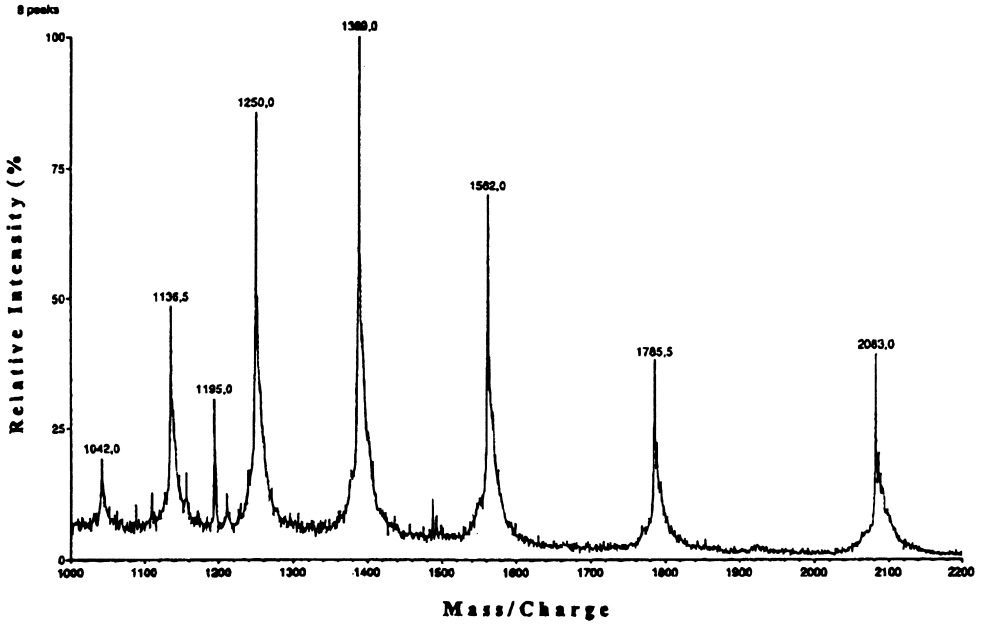

Fig. 3. IS-MS (API III/TAGA 6000 E from Sciex, Toronto, Canada) of the isolated [Ser15]-mMIF product (Fig. 2B), solved in 80% HPLC eluent B (see Fig. 2). *m/z* values of the multicharged ion spray spectrum: 1042,0 $[M+12H]^{12+}$, 1136,5 $[M+11H]^{11+}$, 1250,0 $[M+10H]^{10+}$, 1398,0 $[M+9H]^{9+}$, 1562,0 $[M+8H]^{8+}$, 1785,5 $[M+7H]^{7+}$, 2083,0 $[M+6H]^{6+}$. Calculated average molecular mass: 12.490,6 ($\Delta=-12,4$ to the mMIF mass).

Table 1
Sequence analysis by Edman degradation (Applied Biosystems 473A, Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) of the N-terminal region (amino acids 1–23) of the HPLC-isolated product

Cycle No.	Expected amino acid	Detected amino acid
1	Met	Met
2	Pro	Pro
3	Met	Met
4	Phe	Phe
5	Ile	Ile
6	Val	Val
7	Asn	Asn
8	Thr	Thr
9	Asn	Asn
10	Val	Val
11	Pro	Pro
12	Arg	Arg
13	Ala	Ala
14	Ser	Ser
->15	Val	Ser
16	Pro	Pro
17	Glu	Glu
18	Gly	Gly
19	Phe	Phe
20	Leu	Leu
21	Ser	Ser
22	Glu	Glu
23	Leu	Leu

Cys(Trt)-OH residues, which are sensitive to racemization [11]. During the synthesis, DMF was used as solvent only. The total synthesis was performed without interruptions and controlled by cleaving small amounts of synthetic material from the resin at the positions Asp^{93}, Leu^{83}, Gly^{70}, Thr^{53} and Met^{48}. The completely deprotected mMIF peptide segments already indicated a successful progress of the synthesis by the investigation of reversed-phase high-performance liquid chromatography (RP-HPLC) in combination with matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS) and ion spray (IS) MS. After a final two-step cleavage and deprotection [(1) 20% trifluoroacetic acid (TFA)–dichloromethane and (2) TFA–ethandithiol (EDT)–thioanisol (TA)–triisopropylsilane (TIS)–water–phenol, 80:8:2:1:1:3, 5 h, room temperature (RT)], the crude product of this total stepwise synthesis was obtained in unexpected high purity (~50%, according to analytical RP-HPLC, see Fig. 2A) with 23% yield, calculated from the loading of the used Fmoc-Ala resin. The desired main product could easily be separated by RP-HPLC and after lyophilization, the isolated synthetic product showed a sharp RP-HPLC signal (Fig. 2B).

After RP-HPLC isolation of the main product, a small mass difference was determined for the synthetic protein using MS techniques (IS-MS: m/z: 12.490,6; $\Delta=-12$, Fig. 3; MALDI–MS: m/z: 12491,9; $\Delta=-11$, spectrum not shown). These results point out the importance of high resolution mass spectrometry for a rapid and accurate analysis of such molecules. Further Edmann degradation (Table 1) identified the failure sequence as [Ser15]-mMIF in agreement with the found mass difference. Therefore, the total solid-phase synthesis was performed again under the same conditions leading to the expected mMIF protein in high purity (>45% HPLC, Fig. 4) with an overall yield of 26%. These results show a good reproducibility in yield and purity for the two stepwise protein synthesis approaches. Synthetic mMIF was confirmed by the above mentioned analytical methods (see Figs. 5 and 6) and also by amino acid analysis (LC 3000, Eppendorf, Hamburg, Germany). As it is still not clear whether the native protein has the predicted cystin bridge [6] (Fig. 1), no attempts for selective oxidation were performed after isolation of the protein.

The syntheses demonstrate the powerful strategy of our applied solid-phase method producing proteins with over 100 amino acid residues. However, it is a prerequisite that efficient analytical methods like HPLC, MALDI–MS and IS-MS are available, allowing to control the purity and identity of the synthetic biopolymers.

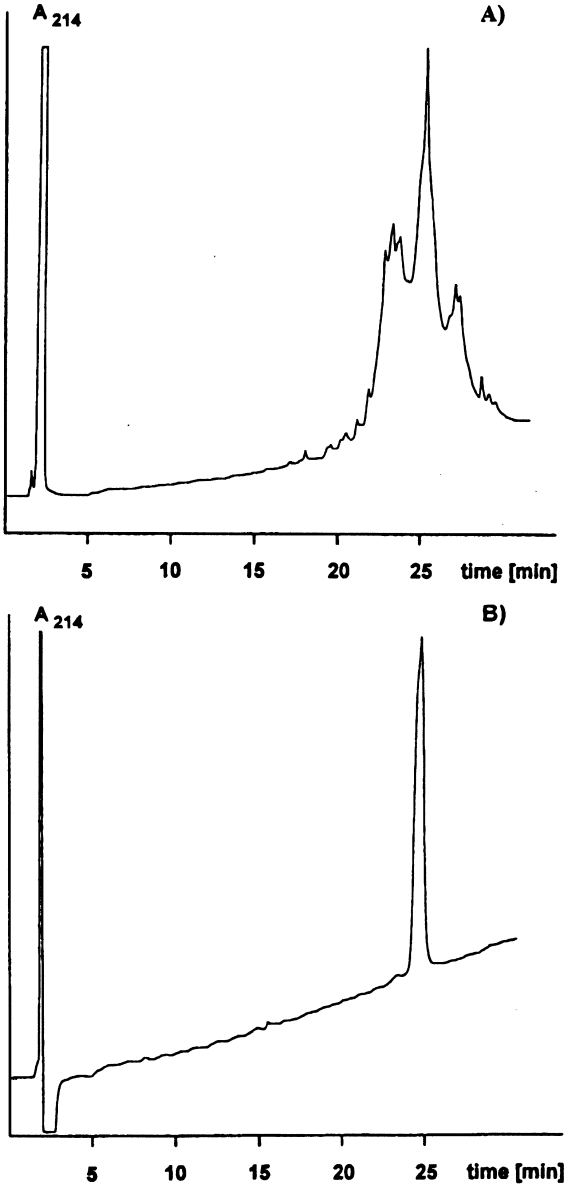

Fig. 4. RP-HPLC profile of the fully deprotected crude product (A) of the total stepwise synthesis and of the isolated mMIF protein (B). Chromatographic conditions as in Fig. 2.

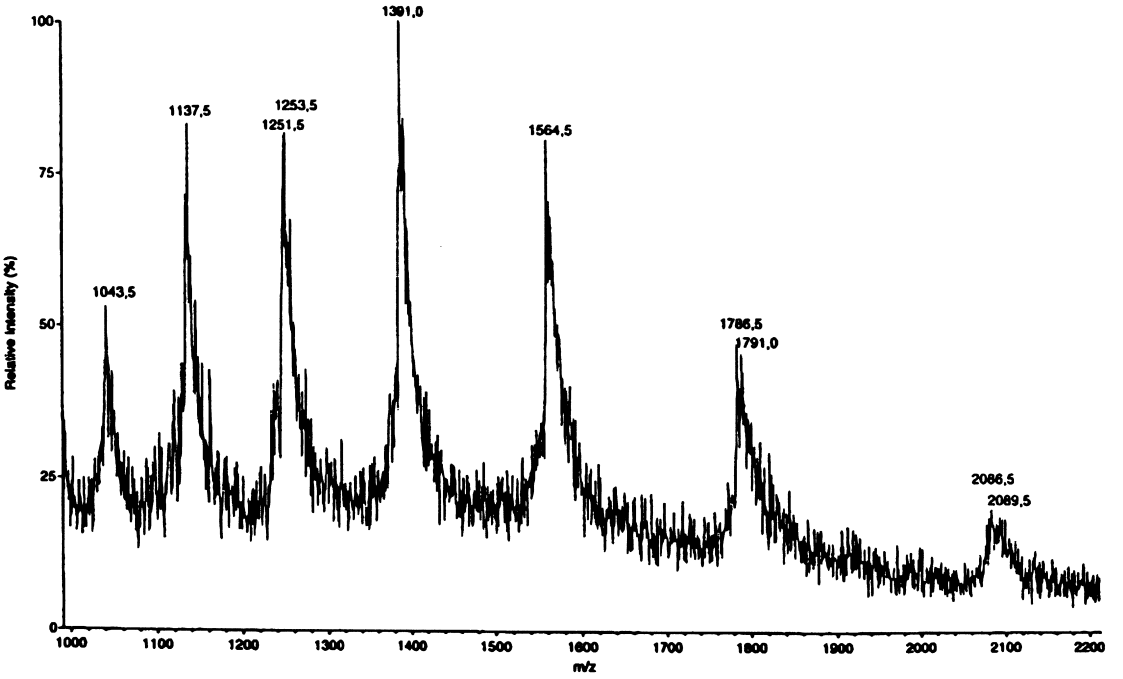

Fig. 5. IS-MS of the isolated product (Fig. 4B). Conditions as in Fig. 3. m/z values of the multicharged ion spray spectrum: 1043,5 $[M+12H]^{12+}$, 1137,5 $[M+11H]^{11+}$, 1251,5 $[M+10H]^{10+}$, 1391,0 $[M+9H]^{9+}$, 1564,5 $[M+8H]^{8+}$, 1786,5 $[M+7H]^{7+}$ and 2086,5 $[M+6H]^{6+}$. Calculated average molecular mass: 12504 ($\Delta=+2$ to the mMIF mass).

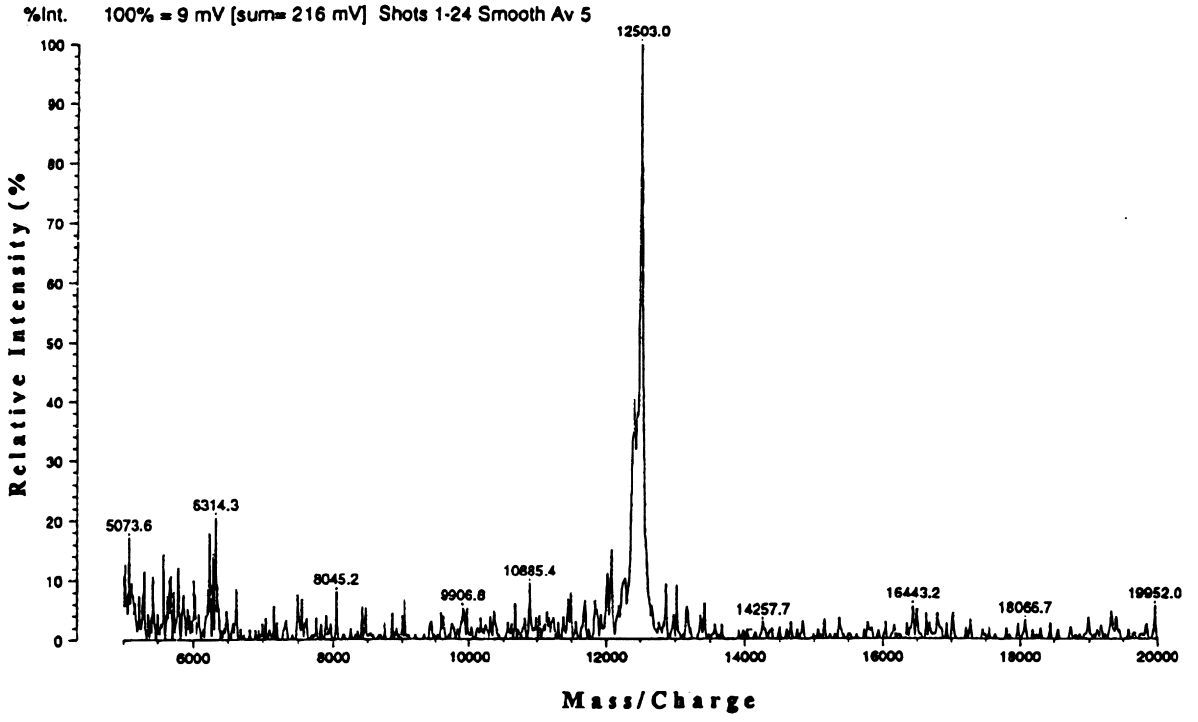

Fig. 6. Matrix-assisted laser desorption mass spectrum of purified synthetic mMIF (Kratos MALDI III, Shimadzu, Duisburg, Germany). Matrix: α-cyano-cinnamic acid (sandwich method), number of shots: 1–24. m/z: 12 503.0 $[M+H]^{+}$.

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