Unambiguous synthesis of stromal cell-derived factor-1 by regioselective disulfide bond formation using a DMSO–aqueous HCl system

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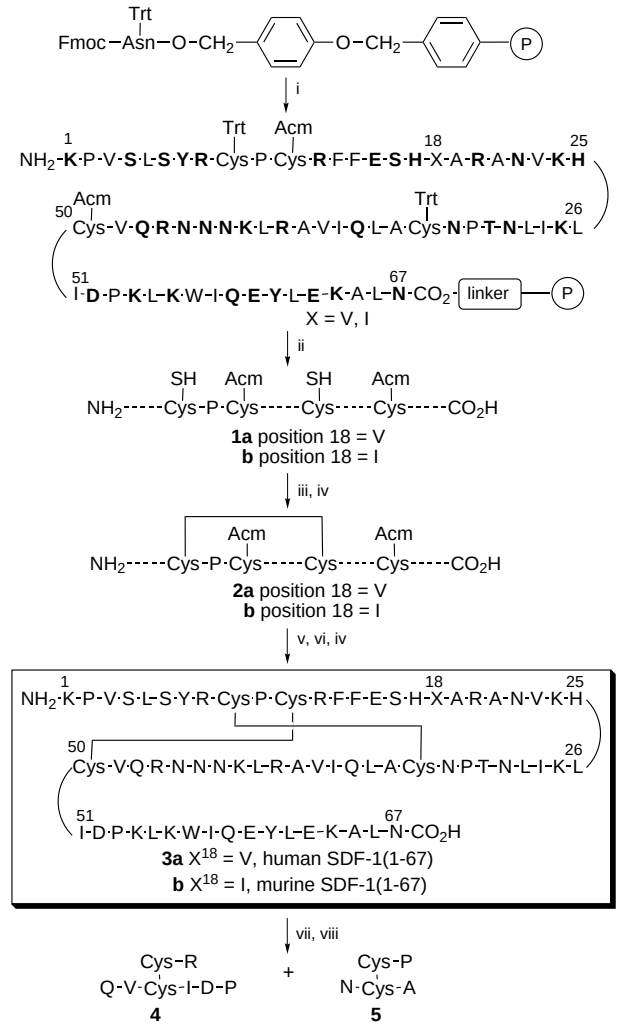
Total synthesis of human and murine stromal cell-derived factor-1s (SDF-1s) (residues 1–67) containing two disulfide bonds and an oxidation-sensitive Trp residue was achieved unequivocally by stepwise disulfide-forming reactions: A silver trifluoromethanesulfonate (AgOTf)–(DMSO)–aqueous HCl system was used in combination with air oxidation to form regioselectively each disulfide bond.

SDF-1,[1] a lymphocyte chemoattractant, belongs to the CXC-chemokines between two subfamilies, the CXC- and CC-chemokines.[2] Recently, it was reported that the CXC-chemokine receptor, CXCR4/fusin, and the CC-chemokine receptor, CCR5, serve as coreceptors in association with CD4 for the entry of T-cell line-tropic (T-tropic) and macrophage-tropic (M-tropic) strains of HIV-1, respectively,[3] and that SDF-1 blocks infection of T-cells by T-tropic HIV-1.[4] Thus, SDF-1 and its derivatives have the potential to become attractive candidates for the chemotherapy and prophylaxis of HIV-1 infection. The human SDF-1 (residues 1–67), having two intramolecular disulfide bonds and Trp (the sequence shown in Scheme 1), was synthesized by Springer and co-workers, and was identical in activity to the purified natural murine SDF-1.[4a] For the synthesis of human SDF-1, the researchers employed random disulfide bond formation of the fully reduced SDF-1 ([Cys(SH)^{9,11,34,50}]-SDF-1) by air oxidation. However, it is desirable that each disulfide bond be formed regioselectively in order to suppress the formation of disulfide isomers, particularly since Cys^{9} and Cys^{11} are present in close proximity in human SDF-1. The natural SDF-1 contains two disulfide bonds between Cys^{9} and Cys^{34} and between Cys^{11} and Cys^{50}.[5] It would be valuable to define the activity of this peptide whose disulfide bridges are correctly formed. Thus, we attempted to synthesize SDF-1(1–67) by regioselective disulfide bond formation. The differential *S*-protection of four Cys residues and the following stepwise disulfide bond formation must be carried out regioselectively. Several disulfide bond-forming reactions, *i.e.* iodine,[6] thallium(III) trifluoroacetate,[7] DMSO[8] and sulfoxide-silylating reagents,[9] have been found to convert some *S*-protected Cys into cystine by us and others. The regioselective syntheses using these reactions in combination with a conventional air oxidation method have been reported; however, one potential limitation to the use of these reactions is the oxidative decomposition of Trp.

We have recently developed a new procedure for disulfide bond formation in *S*-Acm-containing‡ peptides[10] using an AgOTf– DMSO–aq. HCl system.[11] In this protocol, *S*-Acm groups are removed from Cys(Acm) residues by AgOTf[12] and subsequent DMSO–aq. HCl treatment effects two reactions: the conversion of Cys(Ag) residues into Cys(SH) residues besides precipitation of AgCl by the action of HCl, and the disulfide bond formation of Cys(SH) residues by the action of DMSO in aqueous media.[13] Under these reaction conditions, no significant side reactions are observed with oxidation-sensitive amino acids such as Met, Tyr and Trp. Thus, in the present study, we synthesized human and murine SDF-1s(1–67) by the regioselective disulfide bond formation using a combination of the AgOTf–DMSO–aq. HCl system with air oxidation in order to investigate the feasibility of practical applications of this strategy to relatively large and complicated peptides.

SDF-1(1-67) has a single substitution of Ile to Val at a position 18 between mouse and human. The peptidyl resins of human and murine SDF-1s were constructed by Fmoc-based† solid-phase synthesis on *p*-alkoxybenzyl alcohol resins[14] (Scheme 1). To form regioselectively two disulfide bridges, two different protecting groups were employed with suitable pairs for the protection of four SH groups of the Cys residues: Trt‡ groups for Cys^{9} and Cys^{34}, and Acm groups for Cys^{11} and Cys^{50}. *S*-Trt groups are removable *via* Me_3SiBr treatment,[15]



Scheme 1 *Reagents and conditions*: i, Fmoc-based solid phase synthesis; ii, Me_3SiBr–thioanisole–TFA; iii, air oxidation; iv, HPLC purification; v, AgOTf–TFA; vi, DMSO–aq. HCl; vii, trypsin; viii, prolyl endopeptidase. Bold letters indicate protected amino acids.

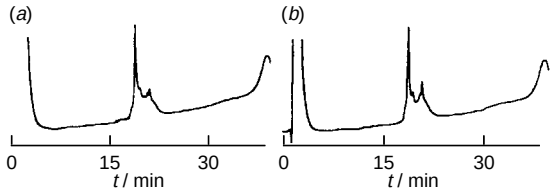


Fig. 1 Analytical HPLC of the crude (*a*) human and (*b*) murine SDF-1s. HPLC conditions: a μBondasphere 5 μC18-100 Å (3.9 × 150 mm) column, a linear gradient of MeCN (25–55%, for 30 min) in 0.1% aq. TFA at a flow rate of 1 cm^3 min^{-1}, a Waters LC module 1 equipped with a Waters 741 Data Module, UV absorption measurement at 220 nm.

whereas *S*-Acm groups are stable during to Me_3SiBr treatment, but can be removed *via* AgOTf treatment. For the side-chain protection of the other amino acid residues, the following groups were used: Trt groups for Asn and Gln, Boc groups for Lys and His, Bu^t groups for Glu, Tyr, Asp, Thr and Ser, and Pmc‡ groups for Arg. The protected resins were treated with 1 mol dm^{-3} Me_3SiBr–thioanisole–TFA to cleave peptides from the resins and remove all the protecting groups except for the *S*-Acm groups.[15] The resulting crude dihydropeptides, [Cys(SH)^{9,34}, Cys(Acm)^{11,50}]-SDF-1s (Scheme 1, **1a**,**b**), were air-oxidized to establish the first disulfide bond. After HPLC purification, monocyclic products, [Cys(Acm)^{11,50}]-SDF-1s (**2a**,**b**), were subjected to sequential treatment with AgOTf–TFA and 50% DMSO–1 mol dm^{-3} aq. HCl (v/v) to establish the second disulfide bond. Each crude bicyclic product exhibited a sharp main peak on analytical HPLC (Fig. 1) without significant side products. HPLC purification gave human and murine SDF-1s (**3a**,**b**) in satisfactory yields.§ The effective concentrations (EC_{50}) of the synthetic human and murine SDF-1s for 50% protection in an assay of HIV-induced cytopathogenicity were 463 and 201 μmol m^{-3}, respectively, and for 50% inhibition of HIV-1 p24 antigen production were 630 and 750 μmol m^{-3}, respectively. Furthermore, these peptides have the ability to induce an increase in intracellular Ca^{2+} through CXCR4/fusin (data not shown). These activities are comparable with those reported by Springer and co-workers.

In conclusion, total synthesis of human and murine SDF-1s (1-67) has been accomplished by stepwise and regioselective disulfide bond formation using air oxidation and the AgOTf–DMSO–aq. HCl system. This combination protocol has proven to be useful for preparation of relatively large and complicated bis(disulfide) peptides, particularly when Trp residues are present.

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Footnotes and References

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‡ *Abbreviations*: Acm = acetamidomethyl, Fmoc = fluoren-9-ylmethoxycarbonyl, Trt = triphenylmethyl, Pmc = 2,2,5,7,8-pentamethylchroman-6-ylsulfonyl.

‡ Human SDF-1(1-67) **3a** was prepared by the following procedure: The protected human SDF-1(1–67) resin (203 mg, 13.5 μmol) was treated with 1 mol dm^{-3} Me_3SiBr–thioanisole–TFA (20 cm^3) in the presence of *m*-cresol (1 cm^3, 860 equiv.) and ethane-1,2-dithiol (0.4 cm^3, 520 equiv.) at 4 °C for 2 h. After removal of the resin by filtration, the filtrate was concentrated *in vacuo*. Ice-cold dry Et_2O (30 cm^3) was then added, and the resulting powder was collected by centrifugation. After washing three times with ice- cold dry Et_2O (3 × 20 cm^3), the product **1a** was dissolved in 50% AcOH (2 cm^3). Subsequently, the total solution volume was adjusted to 400 cm^3 with H_2O and the pH was then adjusted to 8 with conc. NH_4OH. After 1 day of air oxidation, the pH of the solution was adjusted to 5 with AcOH. The crude product was purified by preparative HPLC [Waters Delta Prep 4000 on a Cosmosil packed column (5 μC18-100 Å, 20 × 250 mm) using a linear gradient of MeCN (31–33%, for 30 min) in 0.1% aq. TFA at a flow rate of 7 cm^3 min^{-1}]. The solvent was removed by lyophilisation to give [Cys(Acm)^{11,50}]-human SDF-1 **2a** as a fluffy white powder: (17.4 mg, 1.80 μmol). [Cys(Acm)^{11,50}]-human SDF-1 (17.2 mg, 1.78 μmol) was treated with AgOTf (45.6 mg, 100 equiv.) in TFA (2 cm^3) in the presence of anisole (0.02 cm^3) at 4 °C. After 2 h, ice-cold dry Et_2O (30 cm^3) was then added. The resulting product was washed three times with ice-cold dry Et_2O (3 × 20 cm^3), and then treated with 50% DMSO–1 mol dm^{-3} aq. HCl (v/v, 17 cm^3) at room temperature for 7 h. After removal of the AgCl precipitate by filtration, the filtrate was diluted with H_2O (150 cm^3). The crude peptide was purified by preparative HPLC [a linear gradient of MeCN (30–32%, for 30 min)] to give human SDF-1 **3a** as a fluffy white powder [2.1 mg, 0.245 μmol, 13.8% (calculated from **2a**)]: [IS-MS (reconstructed): Found, 7827.5. Calc. for $C_{350}H_{566}N_{104}O_{92}S_4$, 7826.5]. Its disulfide pairings proved to be identical with those of the natural peptide based on ion spray mass analysis of peptide fragments (**4** and **5** in Scheme 1) derived from the successive digestion treatment of the synthetic peptide by trypsin (TRCK-treated type XIII from bovine pancreas) and prolyl endopeptidase (from Flavobacterium). $[\alpha]_D^{28}$ −92.3 (*c* 0.1, H_2O).

Murine SDF-1(1-67) **3b** was prepared by the same procedure as described above, [16.4% (calculated from (**2b**)]: [IS-MS (reconstructed): Found, 7841.0. Calc. for $C_{351}H_{568}N_{104}O_{92}S_4$, 7840.6]. Its disulfide pairings proved to be identical with those of the natural peptide. $[\alpha]_D^{28}$ −84.8 (*c* 0.1, H_2O).

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