

# Photoactivatable Chemokines – Controlling Protein Activity by Light\*\*

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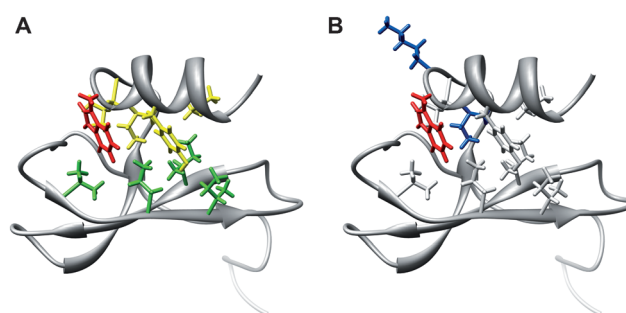
Dedicated to the Bayer company on the occasion of its 150th anniversary

To investigate cellular processes on a molecular level, in situ activatable signaling molecules are important tools to locally address function in a time-dependent manner. Light has turned out to be an ideal, noninvasive trigger that can be applied well-defined in time and space by one- and two-photon excitation techniques. Photomanipulation can be achieved by incorporation of reversible or irreversible photoresponsive elements into molecules as shown for a wide range of applications.<sup>[1]</sup> Over the last three decades the synthesis and application of various caged biomolecules such as second messengers,<sup>[2]</sup> peptides,<sup>[3]</sup> nucleotides,<sup>[4]</sup> and lipids<sup>[5]</sup> were described that contain photocleavable protecting groups. Those may include derivatives of *ortho*-nitrobenzyl, like the 6-nitroveratryloxycarbonyl (Nvoc) group, which allows fast and complete deprotection in aqueous solution with UV light of 365 nm. However, proteins are mostly excluded from site-specific “caging” owing to their size, and only a few strategies have been developed to photomanipulate their structure and/or function.<sup>[1a]</sup>

Here, we provide a novel and straightforward strategy with rationally designed photoresponsive elements that have been incorporated into the backbone of a model protein by expressed protein ligation. The photoprotected *O*-acyl isopeptide backbone modification rearranges spontaneously after UV exposure and leads to the native protein backbone. We used stromal cell-derived factor-1 $\alpha$  (SDF-1 $\alpha$ ), a 68 amino acid disulfide-bridged protein of the chemokine superfamily with particular functions in embryonic development, immune system homeostasis, and revascularization after ischemic conditions. Two G-protein-coupled receptors, CXCR4 and CXCR7, are known to get activated by SDF-1 $\alpha$ , but only CXCR4 was doubtlessly proven to trigger classical G-protein signaling.<sup>[6]</sup> To further investigate and understand the complexity of SDF-1 $\alpha$  signaling in the cellular context, photoactivatable SDF-1 $\alpha$  analogues would be highly

useful, because they would enable the formation of light-induced concentration gradients for high-resolution studies.

The novel strategy provides access to site-specifically caged SDF-1 $\alpha$  derivatives that were rationally designed on the basis of structural data. The C-terminal helix of chemokines is crucial for their biological activity,<sup>[7]</sup> however, we could show that the orientation is more important than its chemical constitution.<sup>[8]</sup> It is assumed that the amphiphilic helix stabilizes the overall structure by interacting with amino acids of the first and second  $\beta$  strand (Figure 1 A) forming the



**Figure 1.** A) Hydrophobic core of SDF-1 $\alpha$  with contributing amino acids from the  $\alpha$  helix (yellow) and the  $\beta$  strands (green). The stabilizing Trp<sup>57</sup> is marked in red. B) The adjacent amino acids of Trp<sup>57</sup>, Lys<sup>56</sup> or Ile<sup>58</sup> (both in blue), were replaced by serine (or homoserine) to introduce the photoswitch. (Protein data base file: 1SDF).

hydrophobic core of SDF-1 $\alpha$ . Therefore, the correct positioning of the conserved Trp<sup>57</sup> is necessary for the formation of the bioactive structure.<sup>[9]</sup> Thus, it was decided to destabilize the surrounding of Trp<sup>57</sup> by incorporation of a flexible backbone modification at the adjacent positions to obtain a reversibly inactive SDF-1 $\alpha$  derivative (Figure 1 B, Scheme 1).

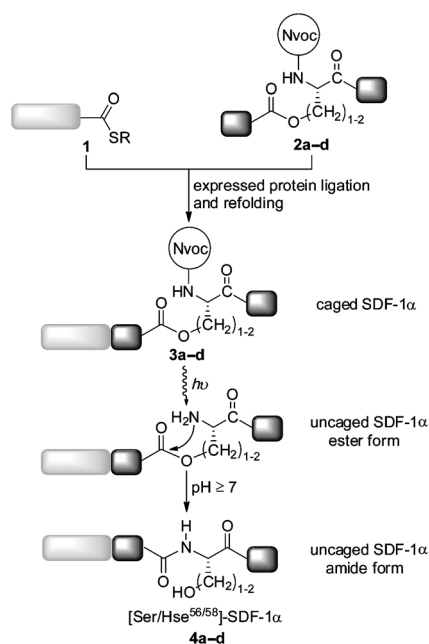
Either Lys<sup>56</sup> or Ile<sup>58</sup> was replaced by L-serine to incorporate photoswitches based on Nvoc-protected *O*-acyl isopeptides<sup>[10]</sup> into the protein backbone. The exchange of the amide bond by an ester bond with the additional methylene group is assumed to provide a higher flexibility<sup>[11]</sup> at the adjacent Trp<sup>57</sup>; the higher flexibility will in consequence lead to an insufficient secondary/tertiary structure formation and thus an inactive protein. Later on, L-homoserine (Hse) was tested as L-serine surrogate to probe a longer homologue that bears an additional methylene group, hence assumed to provide even higher flexibility.

For synthetic reasons SDF-1 $\alpha$  was split into two segments (Scheme 1): Met-[Ala<sup>49</sup>]-SDF-1(1–49), which was prepared as reactive C $\alpha$ -thioester (**1**), and the photoresponsive decapeptide

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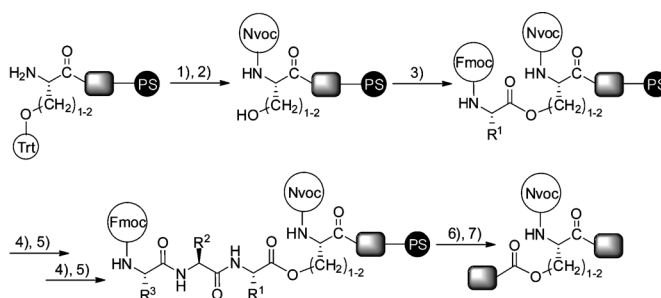


**Scheme 1.** Preparation and photoactivation of caged SDF-1 $\alpha$  analogues. Met-[Ala<sup>49</sup>]-SDF-1(1–49) was prepared as reactive C <sup>$\alpha$</sup> -thioester (1). A series with the following depsipeptide-photoswitch elements was prepared: [Nvoc-Ser<sup>56</sup>] (a), [Nvoc-Ser<sup>58</sup>] (b), [Nvoc-Hse<sup>56</sup>] (c), [Nvoc-Hse<sup>58</sup>] (d).

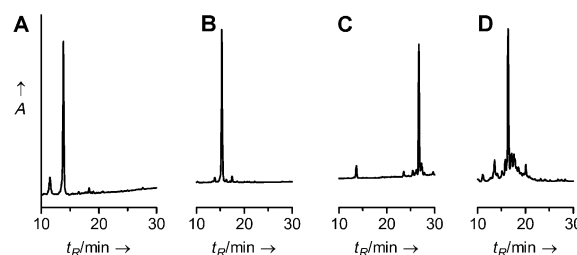
tides [Nvoc-Ser/Hse<sup>56/58</sup>]-SDF-1(50–68) (**2a–d**). Both segments were ligated, refolded, and purified to obtain full-length SDF-1 $\alpha$  analogues (**3a–d**). After Nvoc removal ( $\lambda = 365$  nm) the liberated  $\alpha$ -amino group attacks the ester carbonyl carbon atom and rearranges by an intramolecular O $\rightarrow$ N acyl shift to the native amide bond. This reaction occurs spontaneously but needs the deprotonated  $\alpha$ -amino group.

The N-terminal protein thioester **1** was obtained by using the IMPACT system as described previously.<sup>[12]</sup> The C-terminal *O*-acyl isopeptides [Nvoc-Ser/Hse<sup>56/58</sup>]-SDF-1(50–68) (**2a–d**) were synthesized by Fmoc-based solid-phase peptide synthesis (SPPS; Scheme 2), although the depsipeptide synthesis has been described to be challenging because of critical *O*-acylation and diketopiperazine formation<sup>[13]</sup> (Supporting Information, Figure S5).

In brief, to form the *O*-acyl bond between the side chain of N <sup>$\alpha$</sup> -Nvoc-protected Ser/Hse<sup>56/58</sup> and the following amino acids 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT) was used in combination with *N*-methylimidazole (*N*-MeIm). This activation system has been described for racemization-free loading of hydroxy-functionalized resins with chiral carboxylic acids and amino acids.<sup>[14]</sup> Coupling of Fmoc-Trp(Boc)-OH proceeded fast and efficiently (Figure 2C), however Fmoc-Leu-OH coupling was incomplete (33–37%) and led to low yields. Therefore unreacted hydroxy groups were acetylated to ultimately terminate further elongation. To suppress diketopiperazine formation during critical Fmoc removal of the following amino acid, 1,8-diazabicycloundec-7-ene (DBU)/piperidine in DMF (1:1:8) was applied for 2  $\times$  10 s, and the next Fmoc-aa was coupled in 10-fold excess by using the HOBt/DIC protocol.



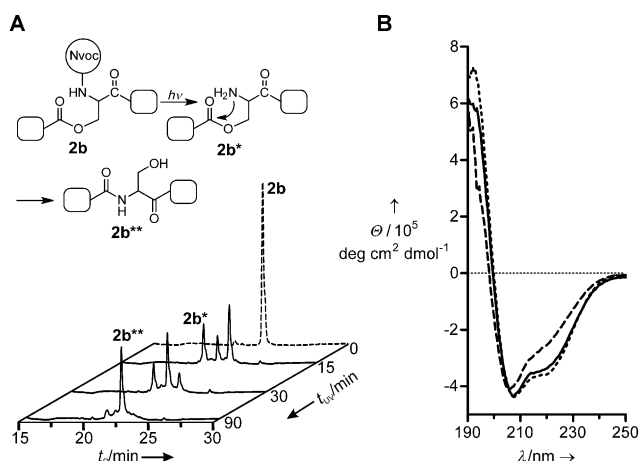
**Scheme 2.** Preparation of caged *O*-acyl isopeptides **2a–d**. Peptides were synthesized by automated SPPS from position 68 to 58 or 56. 1) Nvoc coupling: 3–5 equivalents Nvoc-Cl/HOBt, 5–10 equivalents DIPEA in DMF; 2  $\times$  1 h at room temperature. 2) Trt removal: 1% TFA, 5% TIS in CH<sub>2</sub>Cl<sub>2</sub> ( $\nu/\nu$ ), 10  $\times$  5 min at room temperature. 3) *O*-acylation: 6–10 equivalents MSNT/Fmoc-aa, 0.75 mol % *N*-MeIm in dry CH<sub>2</sub>Cl<sub>2</sub>, 2–3  $\times$  1 h at room temperature. 4) Fmoc removal: DBU/piperidine in DMF (1:1:8), 2  $\times$  10 s. 5) Standard amino acid coupling HOBt/DIC protocol. 6) Standard automated peptide synthesis. 7) Full cleavage: 90% TFA, 10% scavenger mixture (thioanisole/*p*-thiocresole; 7:3), 2 h at room temperature. HOBt = 1-hydroxybenzotriazole, DIPEA = diisopropylethylamine, TFA = trifluoroacetic acid, TIS = triisopropylsilane, DIC = diisopropylcarbodiimide; PS = Wang-polystyrene resin.



**Figure 2.** HPLC chromatograms measured after critical steps of on-resin total synthesis of depsipeptide **2b**. A) After robot synthesis before Nvoc coupling, B) after Nvoc coupling, C) after *O*-acylation, D) crude product. HPLC gradient: 20% CH<sub>3</sub>CN  $\rightarrow$  70% CH<sub>3</sub>CN in 40 min.

After cleavage from the resin and purification of the C-terminal segments **2a–d**, photolysis experiments were performed at different pH values (Supporting Information). By using analytical reverse-phase HPLC it was shown that after 30 min more than 80% of the Nvoc groups were removed and more than 95% after 60 min. UV irradiation of **2a–d** in 10 mM acidic (pH 2.9) or basic (pH 7.4) phosphate buffer led to distinct products differing in their retention time. Both peaks represent uncaged **2a–d** in their ester (pH 2.9) or amide form (pH 7.4). Whereas the first product is formed after illumination, the latter is formed after spontaneous O $\rightarrow$ N acyl rearrangement. This process is significantly slower at pH 7 on ice and allowed direct monitoring of both species (Figure 3A).

Circular dichroism measurements of [Nvoc-Ser<sup>58</sup>]-SDF-1(50–68) (**2b**) in comparison to Ser<sup>58</sup>-SDF-1(50–68) (**6b**) and native SDF-1(50–68) (**7**) revealed reduced helicity of the depsipeptide, which is regained after Nvoc removal by UV (Figure 3B). Scanning within the pH range 6–8 with 25% TFE demonstrated that **2b** and the corresponding **6b** show

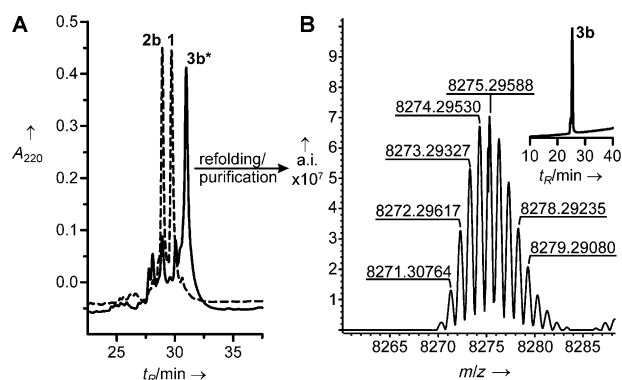


**Figure 3.** A) Photolysis of [Nvoc-Ser<sup>58</sup>]-SDF-1(50–68) (**2b**) and rearrangement of the uncaged peptide from the ester (**2b\***) into the all-amide form (**2b\*\***). Irradiation on ice at pH 7. B) CD spectrum of **2b** (---) compared to SDF-1(50–68) (**7**; —) and the all-amide reference peptide bearing Ser<sup>58</sup> (**6b**; ·····). Spectra were recorded in sodium phosphate (10 mM, pH 7.4) in the presence of 50% 2,2,2-trifluoroethanol (TFE).

the lowest helical content among the series, suggesting the most unstable C terminus (Supporting Information). Next, full-length photoactivatable SDF-1α analogues (**3a–d**) were prepared by expressed protein ligation (Figure 4), and after refolding and purification biological activity was tested.

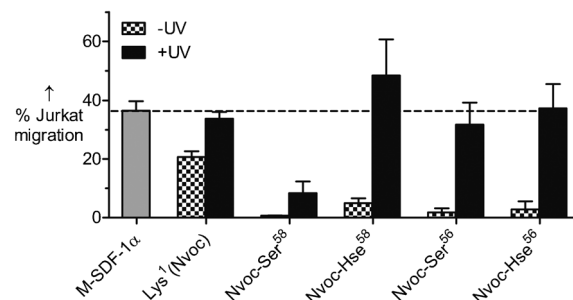
In addition, Lys<sup>1</sup>(Nvoc)-SDF-1α (**5**), in which the ε-amino group of Lys<sup>1</sup> is masked by the bulky Nvoc group, was prepared for control (Supporting Information).<sup>[15]</sup> From previous studies it is known that the N-terminal dipeptide motif Lys<sup>1</sup>-Pro<sup>2</sup> is essential for receptor activation.<sup>[9]</sup>

For biological testing two cell-based assay systems were used, and all analogues were fully characterized prior and after UV illumination with respect to their chemotactic



**Figure 4.** Ligation (100 mM phosphate buffer, 6 M GdmCl, pH 7.3), refolding (rapid dilution in 20 mM Tris buffer, 0.3 M sucrose, 0.2 mM GSH/GSSG each), and purification of [Nvoc-Ser<sup>58</sup>]-SDF-1α (**3b**). A) HPLC chromatogram of the ligation mixture at 0 h (---) and after 24 h (—). **3b\*** indicates unfolded protein. B) Fourier-transform ion-cyclotron resonance (FT-ICR) mass spectrometry isotope pattern of **3b** obtained after refolding confirms disulfide-bridged protein. a.i. = arbitrary intensity.

potential (Figure 5 and Supporting Information, Figure S9) and G-protein signaling (Supporting Information, Figure S10 and Table S2). The chemotaxis assay was performed in a vertical two-chamber system (separated by a membrane)



**Figure 5.** In vitro chemotactic potential tested on CXCR4-positive Jurkat cells. Migrated cells were counted after stimulation for 2 h with SDF-1α analogues (100 nM) with (+) or without (–) UV treatment.

in which the upper chamber was loaded with Jurkat leukemia T cells and the lower chamber with agonist solution (SDF-1α analogues with or without UV). Cells follow the gradient and actively cross the membrane. This experimental setup basically mimics the situation in the blood stream, where cells have to cross the endothelial barrier to reach their destination (e.g. inflamed tissue). Unexpectedly, these experiments revealed that the masked Lys<sup>1</sup>(Nvoc)-SDF-1α (**5**) is already 55% as active as control M-SDF-1α even before Nvoc removal, indicating a more complex activation mode at Lys<sup>1</sup>. In contrast caged SDF-1α analogues **3a–d** showed almost no chemotactic potential, whereas after uncaging the activity of all compounds ranged from 87–132% wild-type activity—with exception of [Nvoc-Ser<sup>58</sup>]-SDF-1α analogue **4b**, which regained only 23% activity. This observation can be explained, because **4b** bears a serine at position 58 that is sterically less bulky than the native isoleucine. Since the side chain of the particular amino acid at position 58 faces towards the hydrophobic core, serine might leave space for water molecules, which can destabilize the core. In contrast, **4d**, which contains homoserine at this position and is nearly isosteric to isoleucine, fills that space and is most likely able to exclude water from this sensitive site. In total homoserine increases stability and leads indeed to the most-potent compound in terms of chemotactic potential. Further evidence for this hypothesis is given by [Nvoc-Ser/Hse<sup>56</sup>]-SDF-1 analogues **4a** and **4c**, which behave nearly identical to the wild type after uncaging. Ser/Hse residues at position 56 become orientated towards the aqueous environment after rearrangement and do neither contribute to the core nor to the helix.

To investigate the G-protein activation on the level of a single signaling pathway, inositol phosphate (IP<sub>x</sub>) accumulation assays were performed and partial agonism and significantly improved potencies were observed for SDF-1α analogues (Supporting Information).

In conclusion, we rationally designed and prepared a set of photoactivatable SDF-1α analogues by introducing for the first time *O*-acyl isopeptides into proteins by expressed

protein ligation. We could show that our compounds switch into their active conformation after UV treatment and regain biological activity in several cell-based assays. Our straightforward approach including defined destabilization of protein helices by depsipeptide segments and self-reorganization of the structure after removal of the photoprotection may be applied to a wide range of middle-sized proteins to study their function under the control of light.

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