Structural Insights into BET Client Recognition of Endometrial and Prostate Cancer-Associated SPOP Mutants

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Abstract

BET proteins such as BRD3 are oncogenic transcriptional coactivators. SPOP binding triggers their proteasomal degradation. In both endometrial and prostate cancer, SPOP mutations occur in the MATH domain, but with opposed influence on drug susceptibility. In prostate cancer, SPOP mutations presumably cause increased BET levels, decreasing BET inhibitor drug susceptibility. As opposed, in endometrial cancer, decreased BET levels concomitant with higher BET inhibitor drug susceptibility were observed. Here, we present the to our knowledge first co-crystal structure of SPOP and a bromodomain containing protein (BRD3). Our structural and biophysical data confirm the suggested loss-of-function in prostate cancer-associated SPOP mutants and provide mechanistic explanation. As opposed to previous literature, our data on endometrial cancer-associated SPOP mutants do not show altered binding behavior compared to wild-type SPOP, indicating a more complex regulatory mechanism. SPOP mutation screening may thus be considered a valuable personalized medicine tool for effective antitumor therapy.

The bromodomain and extraterminal (BET) family of proteins (BRD2, BRD3, BRD4 and BRDT) are involved in various cellular processes, including the modulation of gene expression. Bromodomain containing proteins read acetylation patterns on histones and recruit other protein machinery to regulate gene transcription. As changes in normal histone modification patterns is frequent in cancer, BET proteins seem to be involved in the epigenetic regulation of cancer.[1] As they are able to sustain malignant transcriptional programs in tumors, they were found to be attractive therapeutic targets[2]. Cellular BET levels are regulated through their interaction with the speckle-type POZ protein (SPOP)[2-4], which is part of a cullin-RING-basedE3 ubiquitin-protein ligase complex[5]. In this complex, SPOP serves as the substrate recognition part that directly binds the substrate via its MATH domain. SPOP also recognizes CUL3 which binds to an E2 ubiquitin-conjugating enzyme[6]. The E2 enzyme carries activated ubiquitin which is transferred to the substrate as the signal for proteasomal degradation[7].

SPOP contains a MATH (mephrin and TRAF homology) domain (Fig.1a) which mediates binding of a number of clients[5]. Cancer-genome sequencing studies have shown that in prostate and endometrial cancer the gene encoding the E3-ligase adaptor protein SPOP is frequently mutated[8,9]. SPOP mutations associated with prostate cancer are located in the substrate binding site of the SPOP MATH domain, whereas endometrial cancer-associated mutation sites are found in distant regions of the MATH domain structure[3].

Recently, Janouskova *et al*. reported paradoxical findings, proposing that mutations located in the same SPOP domain lead to opposing effects in drug susceptibility. They showed that BET protein levels are increased in prostate cancer cells carrying typical SPOP mutations. The mutations presumably cause a loss-of-function in SPOP, impairing BET binding and ubiquitination. The subsequent accumulation of BET proteins results in a malignant transcription program and diminished susceptibility of the cancer cells towards small-molecule BET inhibitors (Fig.1b). As opposed, in endometrial cancer cells, reduced BET protein levels were observed *in vivo*, concomitant with increased drug susceptibility of the cancer cells towards BET inhibitors. The authors suggest a gain-of-function mechanism in endometrial cancer-associated SPOP mutants, resulting in increased BET protein affinity and ubiquitination[3] (Fig.1b). However, the molecular mechanisms that might explain this are unclear.

In this study, we characterized the SPOP-BET protein interaction using biophysical *in vitro* techniques such as nuclear magnetic resonance (NMR) spectroscopy and isothermal titration calorimetry (ITC). To investigate structural effects of cancer-associated mutations in the SPOP-BET protein interface, we co-crystallized SPOP MATH (residues 28-166) with a peptide comprising the SPOP degron of BRD3 (**1**, residues 245-253, KADTTTPTT). To our knowledge, this represents the first bromodomain-SPOP complex structure. BRD3 was chosen because it has been a focus of study in previous SPOP-related literature[3]. It is representative for the BET protein family as its SPOP binding consensus (SBC) sequence is identically found in BRD4 and differs from the BRD2 sequence in only one position. The co-crystal structure of SPOP-BRD3 (Fig.1c) shows that prostate cancer-associated SPOP mutation sites are located directly in the BRD3 binding groove, while endometrial cancer-associated mutation sites are distant from the binding site. A detailed view of the interactions formed between SPOP and BRD3 (Fig.1d) shows that prostate cancer-associated mutation sites are found in SPOP residues involved in critical interactions with BRD3. SPOP Asp130 for example forms hydrogen bonds to BRD3, and was previously shown to be critically involved also in SPOP binding to other substrates[10]. Additionally, aromatic SPOP residues such as Phe133 and Phe102 form a hydrophobic pocket on the SPOP

surface, which is occupied by BRD3 Ala246 and is considered critical for binding. Based on our structure, it is reasonable that the introduction of any prostate cancer-associated SPOP mutation causes significantly weaker BRD3 binding. In contrast, all endometrial cancer-associated SPOP mutation sites are located at positions distant from the BRD3 binding site. Thus, a possible influence on SPOP-BRD3 binding of these mutations needs to be further elucidated.

For a qualitative comparison of BRD3 binding behavior of SPOP mutants, NMR experiments were performed (Fig.2a-f). For wild-type and mutant SPOP proteins, ^{1}H,^{15}N HSQC NMR spectra were recorded both in unbound form and with five-fold molar excess of BRD3 (**1**, residues 245-253). The NMR spectra of all mutant proteins demonstrate that the mutations do not disrupt the tertiary fold of the SPOP MATH domain (Fig.S1). Residues Tyr87, Leu88 and Gln127 are key residues directly in the ligand binding site of SPOP. A comparison of the chemical shift perturbation (CSP) of these signals indicates that prostate cancer-associated SPOP mutants show no or very little CSPs upon BRD3 addition (Fig.2b-c). This demonstrates that these mutants have no or strongly reduced binding affinity to BRD3 compared to the wild-type SPOP protein (Fig.2a). In contrast, all endometrial cancer-associated SPOP mutants show significant CSPs and line-broadening upon BRD3 addition. The shift patterns of these mutants upon BRD3 addition (Fig.2d-f) are highly similar to the one of wild-type SPOP, indicating comparable binding modes and strength.

The BRD3 binding affinity of SPOP mutants was also studied by fluorescence polarization (FP) measurements. Different concentrations of the respective SPOP proteins were mixed with fluorescently labeled BRD3 peptide (**4**). The binding isotherms of the assay (Fig.2g) clearly show the different BRD3 binding behavior of endometrial and prostate cancer-associated SPOP mutants. Almost all endometrial cancer-associated mutants show a binding strength to BRD3 comparable to the wild-type protein. Only SPOP R121Q shows slightly reduced binding strength. However, this mutant still binds stronger than all tested prostate cancer-associated mutants. Their binding behavior is significantly reduced compared to the wild-type protein. These observations are consistent with NMR experiments.

To quantitatively determine the binding affinity and thermodynamic features of BRD3 binding, we performed ITC experiments with SPOP proteins (Table S2, Fig.S2). The dissociation constant (K_D) of the wild-type SPOP MATH domain to the BRD3 peptide (**1**, residues 245-253) was determined as 168 ± 19 µM. When the most common prostate cancer-associated SPOP mutant F133V[9] was titrated, no binding could be detected. In contrast, all tested endometrial cancer-associated SPOP mutants bind to the BRD3 peptide, with K_D values comparable to SPOP wild-type.

In summary, data from NMR, ITC and FP experiments confirmed that prostate cancer-associated SPOP mutants show no or significantly reduced BRD3 binding, while endometrial cancer-associated SPOP mutants showed binding behavior comparable to wild-type SPOP. None of the mutations caused an increase in binding affinity between SPOP and BRD3.

In order to obtain structural insights into the effects of SPOP MATH mutations, we co-crystallized SPOP MATH E47K, M117V and D140N with BRD3 (**1**, residues 245-253). The superposition of the co-crystal structures with the wild-type SPOP-BRD3 complex (Fig.3a) shows almost identical orientation of the SPOP backbone, and highly similar positions of the respective BRD3 ligands. The mutations do not seem to result in an altered SPOP-BRD3 interface, and consistent with our binding studies there is no structural explanation for the previously postulated increase in affinity for the mutations.

As these data were obtained on a relatively short fragment of BRD3, additional ITC experiments with a longer BRD3 construct (residues 24-416) were performed (Fig.S3) to rule out that additional residues could be involved in the interaction. This BRD3 construct is extended around the SPOP binding site, and comprises both bromodomains of BRD3 and the connecting linker where the degron is localized (Fig.1a). The titrations showed very similar K_D values of SPOP mutants E47K, E50K, E78K, D140N and R121Q to BRD3, comparable to wild-type SPOP (K_D= 61 ± 11 µM). The affinities to the extended BRD3 protein (24-416) (Table 1, Fig.3b and S3) are slightly higher than those determined for the BRD3 peptide (**1**, residues 245-253) (Table S2, Fig.S2), indicating that BRD3 residues adjacent to the shorter peptide may provide some contribution to SPOP binding.

Out of all endometrial cancer-associated SPOP mutants, only M117V showed a somewhat increased affinity to the BRD3 protein (K_D= 13 ± 3 µM) compared to wild-type SPOP. As this mutation site is relatively close to the N-terminus of the BRD3 peptide in our co-crystal structure (Fig.1d), it is conceivable that the residue might be involved in binding to larger BRD3 constructs, implying that the mutation affects the contacts formed distant from the core binding interface. Still, all other tested endometrial cancer-associated mutants showed identical BRD3 binding strength to wild-type SPOP, regardless of the used BRD3 construct.

In conclusion, our data confirm the reduced BRD3 binding capabilities of prostate cancer-associated SPOP mutants, and rationalize the effect of these mutations on a structural level. The mutation sites are located in the ligand binding groove of SPOP and are involved in critical contacts. Mutation of any of these residues is thus expected to compromise BRD3 binding. Based on the similarity of different SPOP clients, it is likely that these mutations also impair binding of other SPOP clients[10]. Our work covers most of the known prostate and endometrial cancer-associated SPOP mutation sites. Additionally reported prostate cancer-associated mutation sites include S119 and K134[9] as well as F101 and K135[11]. Adjacent SPOP mutation sites were studied in our work (F133, F102). As all of those mutation sites are located in the ligand binding groove, we expect them to have highly similar effects, impairing BET protein binding. As for endometrial cancer-associated SPOP mutants, additional mutation sites P94[8], E46[12] and G75[13] were reported. Again, adjacent mutation sites were studied in our work (E47 and G75), and a similar behavior of the mutants is assumed.

Our studies with endometrial cancer-associated SPOP mutations did neither show an increased binding affinity nor a detrimental effect on the BRD3 binding interface compared to wild-type SPOP. Only when a large BRD3 construct was used as ligand, a single SPOP mutant (M117V) showed slightly increased affinity in ITC experiments. Based on our structural and biophysical data, it is unlikely that this point mutation could cause the described *in vivo* effect of reduced cellular BRD3 levels in endometrial cancer cells[3]. Moreover, all other mutations do not affect the binding affinity *in vitro*. Therefore, the observed disease phenotypes *in vivo* could be caused by higher-level regulatory mechanisms, where mutations may alter protein localization, bring interactions with additional protein binding partners, or potentially affect liquid-liquid phase-separation of complexes, which were recently shown to be caused by SPOP mutation in cancer cells[14]. Still, in specific tumors, screening for SPOP mutations could offer a vital tool to personalize the therapeutic strategy.

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Author Contributions

M.S.O. and G.M.P. conceived and designed the experiments. M.S.O. and W.H. performed the experiments. W.H. and O.P. conceived and performed chemical synthesis. M.S.O. and G.M.P. recorded and analyzed crystallographic data. M.S.O. and W.H. recorded NMR data, M.S.O. and M.S. analyzed NMR data. M.S.O., W.H., O.P., M.S. and G.M.P. wrote the manuscript.

Competing Interests Statement

The authors declare no competing interests.

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Materials and Methods

Protein Expression and Purification

Human SPOP MATH (residues 28-166) and human BRD3 (24-416) were cloned into pETM-11 vector. SPOP MATH mutants were created using the QuikChange Site-Directed Mutagenesis Kit and Primer Design tool (Agilent Technologies) according to the manufacturer's protocols. Proteins were expressed in *E.coli* BL21 (DE3) cells using ZYM-5052 auto-induction medium. Proteins used for NMR experiments were uniformly labeled with ^{15}N or $^{15}N/^{13}C$ by growing expression cultures in M9-based minimal medium. All proteins were purified from cell lysate via IMAC using a Ni-NTA column. After elution, proteins were dialyzed to TEV protease cleavage buffer (100 mM Tris-HCl pH 8.0, 100mM NaCl, 5 mM β-mercaptoethanol (β-ME)) over night at 4°C after addition of TEV protease (1:50). After cleavage, proteins were subjected to a second Ni-NTA affinity chromatography step. Proteins were further purified via size-exclusion chromatography (SEC) using a HiLoad 16/60 Superdex 75 preparative grade (pg) column (SPOP MATH) or a HiLoad 16/60 Superdex 200 pg columm (BRD3) (Äkta system, GE Healthcare). Crystallization buffer (5 mM Tris, 50 mM NaCl, 5 mM β-ME, pH 8.0) was used as SEC buffer for wild-type SPOP if intended for crystallization. PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 5mM β-ME, pH 7.4) was used as SEC buffer for all other samples.

Crystallization and X-ray Structure Solution

SPOP MATH (wild-type or mutant protein) was mixed with five-fold molar excess of human BRD3 peptide (**1**, residues 245-253, KADTTTPTT). The mix was concentrated to 16 mg/ml. The crystallization conditions were: 0.2 M NaCl, 0.1 M Tris pH 8.5, 25% (w/v) PEG 3350 (wild type SPOP MATH-BRD3), 0.2 M ammonium acetate, 0.1 M Tris pH 8.5, 25% (w/v) PEG 3350 (SPOP MATH E47K-BRD3), 0.2 M sodium formate, 0,1 M Bis Tris propane pH 7.5, 20% (w/v) PEG 3350 (SPOP MATH M117V-BRD3) and 0.2 M NaCl, 0.1 M HEPES pH 7.0, 20% (w/v) PEG 6000 (SPOP MATH D140N-BRD3). Crystals grew within one to two weeks at room-temperature in vapor diffusion sitting drops and were frozen using 20% glycerol as cryoprotectant. Crystals were measured at the ID23-2 and ID30A-1 beamlines of the ESRF, Grenoble, France. The obtained datasets were processed using the XDS and XSCALE software[15]. Molecular replacement was performed using Molrep v.11.0[16]. For the co-crystal structures of SPOP wild type, E47K and M117V with BRD3, PDB entry 3IVV was used as search model. The search model used for the SPOP D140N-BRD3 co-crystal structure was PDB 6I41. Models were rebuilt using Coot v.0.8.8[17] and iteratively refined using Refmac5[18] from the CCP4 suite v.7.0[19]. Final refinement was done with phenix.refine of the PHENIX suite (v.1.14-3260)[20]. Data collection and refinement statistics are given in Table S1. Structure displays were generated using the PyMol Molecular Graphics System v.1.8.6.0, Schrödinger, LLC. Interactions in crystal structures were analyzed using LigPlot+ v.1.4[21].

NMR Spectroscopy

NMR titrations were recorded via $^{1}H,^{15}N$ HSQC experiments on a Bruker Avance 600 spectrometer equipped with a QCI cryogenic probe at 298K using Topspin v.3.2 (Bruker BioSpin). Spectra were processed using NMRPipe v.8.9[22] and analyzed using CCPN Analysis v.2.4.1[23]. Samples consisted of 100 µM ^{15}N-labeled SPOP MATH (wild-type or mutant) in PBS pH 7.4, 10% D_2O, 5 mM β-ME and contained either no ligand (free form), or five-fold molar excess of BRD3 peptide (**1**). Spectra were recorded using acquisition times of 106.5 ms (^{1}H), 60.1 ms (^{15}N) with a total duration of 1h 20min. For the backbone resonance assignment of SPOP MATH, HNCACB and CBCACONH spectra were used. They were recorded on a Bruker Avance 900 spectrometer equipped with a TCI cryogenic probe at 298K using Topspin v.3.2 (Bruker BioSpin) and processed and analyzed as mentioned above. The sample consisted of 300 µM $^{15}N/^{13}C$-labeled wild-type SPOP MATH in PBS pH 7.4, 10% D_2O, 5 mM β-ME. The HNCACB spectrum was recorded with acquisition times of 79.9 ms (^{1}H), 24.0 ms (^{15}N), 4.2 ms (^{13}C) with a total duration of 3d 20h 38min. The CBCACONH spectrum was recorded with acquisition times of 79.9 ms (^{1}H), 24.0 ms (^{15}N), 6.5 ms (^{13}C) with a total duration of 3d 1h 13min.

Isothermal Titration Calorimetry

Titrations were performed using a MicroCal PEAQ-ITC (Malvern) at 25 °C. All samples were dialyzed to identical PBS pH 7.4 buffer for at least 18h. Measurements were done with titrant concentrations between 1 mM and 5.8 mM. Used titrants were SPOP MATH wild-type, E47K, E50K, E78K, M117V, R121Q, F133V and D140N. SPOP S80R could not be measured due to sample instability. Cell sample concentrations varied between 50 µM and 200 µM of BRD3 (**1**, residues 245-253) or BRD3 (residues 24-416). The runs consisted of 19 injections with a volume of 2 µL and a duration of 4 seconds each. The first injection with a volume of 0.4 µL and a duration of 0.8 seconds was discarded in the evaluation. The syringe stirring speed was 750 rpm for all runs. The spacing for different runs varied between 120 or 150 seconds. Raw data were processed and integrated using the MicroCal PEAQ-ITC Analysis Software v.1.0.0.1259 (Malvern). The fitted offset method was used as control during analysis. The resulting values in ΔH/mol were plotted over the molar ratio. The data points were fitted using a one-site binding model based on curve shape and the stoichiometry observed in crystallographic data. All measurements were performed at least in triplicate, as indicated in Tables 1 and S2, respectively.

Fluorescence Polarization Assay

A 12-point serial dilution (50% dilution per step) of SPOP MATH proteins was created from a starting concentration of 1.3 mM in PBS pH 7.4, 5 mM β-ME, keeping a constant concentration of 40 nM fluorescently labeled BRD3 peptide (**4**). Fluorescence polarization of each sample was measured with an EnVision 2104 Multilabel Plate Reader (PerkinElmer) and the corresponding EnVision Software v.1.12. The data were plotted using OriginPro 9.0G (OriginLab Corp.). Curves were normalized to zero by deduction of blank samples.

Peptide Synthesis

Solid-phase peptide syntheses were performed on a CEM Liberty microwave peptide synthesizer. For 0.1 mmol reaction scale, Fmoc-deprotection was performed by treatment with 10% piperazine (w/v) in ethanol: N-methyl pyrrolidinone (10:90) (v/v, 3 ml) under microwave radiation for the first 15 s (155 W, 75 °C) then 50 s (30 W, 90 °C). The peptide-resins were then rinsed with dimethyl formamide (3 x 2 ml). Peptide coupling was achieved by addition of a solution containing amino acid (5.0 eq, 0.2 M in dimethyl formamide, 2.5 ml), DIC (5.0 eq, 0.5 M in dimethyl formamide, 1 ml) and Oxyma (5.0 eq, 1 M in dimethyl formamide, 0.5 ml) to the N^{α}-deprotected peptide resin and agitated under microwave radiation for first 15 s (170 W, 75 °C) then 110 s (30 W, 90 °C, vessel under external nitrogen flow). Prior to the cleavage step, the peptide-resins were rinsed with dimethyl formamide (2 x 15 ml) and DCM (2 x 15 ml). The cleavage was performed manually by the addition of a mixture of trifluoroacetic acid / triisopropyl silane / water (95:2.5:2.5, % v/v/v; 10 ml) and proceeded within 2-3 h. For the precipitation of the crude product, the cleavage filtrate was treated with diethyl ether (3 x 30 ml).

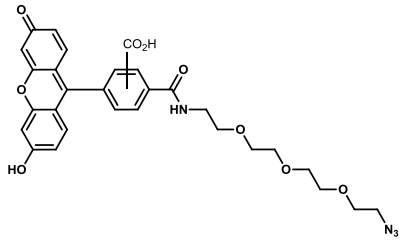
Synthesis of Lys-Ala-Asp-Thr-Thr-Thr-Pro-Thr-Thr-NH_2 (1)

Lys-Ala-Asp-Thr-Thr-Thr-Pro-Thr-Thr-NH_2 (0.1 mmol) was prepared according to the general procedure for peptide synthesis (86 %). HRMS (ESI) *m/e* calcd. for $C_{38}H_{68}N_{11}O_{16}$ (MH^+) 934.4846, found 934.4854.

Synthesis of Lys-Ala-Asp-Thr-Thr-Thr-Pro-Thr-Thr-Pra-NH_2 (2)

Lys-Ala-Asp-Thr-Thr-Thr-Pro-Thr-Thr-Gly(propargyl)-NH_2 (0.1 mmol) was prepared according to the general procedure for peptide synthesis (89 %). For introduction of Pra, Fmoc protected propargylglycine was used (Merck) HRMS (ESI) *m/e* calcd. for $C_{43}H_{73}N_{12}O_{17}$ (MH^+) 1029.5217, found 1029.5211.

Synthesis of Fluorescein-PEG_3-azide (3)



To a solution of 5/6-carboxyfluorescein succinimidyl ester (500 mg, 1.056 mmol, mixture of isomers) in dimethyl formamide (40 ml), diisopropylethyl amine (270 µl, 1.584 mmol, 1.5 eq) and 11-Azido-3,6,9-trioxaundecan-1-amine (254 mg, 1.162 mmol, 1.1 eq) were added. The solution was stirred for 14 h at room temperature. The product was purified by HPLC (water + 0.05% trifluoroacetic acid - acetonitrile + 0.05% trifluoroacetic acid; 5-95% acetonitrile) and obtained as yellow solid (90%). ^{1}H-NMR (600 MHz, CD_3OD, mixture of isomers) δ 2.68 (s, 4H), 3.28-3.33 (m, 4H), 3.49-3.71 (m, 30H), 6.53-6.56 (m, 4H), 6.59-6.62 (m, 4H), 6.69 (t, 4H, J = 2.3), 7.33 (dd, 1H, J = 8.0, 0.6, isomer a), 7.65 (dd, 1H, J = 1.4, 0.7, isomer b), 8.08 (dd, 1H, J = 8.0, 0.6, isomer b), 8.15 (dd, 1H, J = 8.1, 1.4, isomer b), 8.21 (dd, 1H, J = 8.0, 1.7, isomer a), 8.44 (dd, 1H, J = 1.5, 0.6, isomer a); ^{13}C-NMR (600 MHz, CD_3OD, mixture of isomers) δ 26.28, 41.3, 41.24, 51.72,

51.74, 70.27, 70.43, 71.03, 71.11, 71.13, 71.35, 71.43, 71.50, 71.61, 71.64, 71.67, 103.60, 103.61, 110.86, 110.92, 113.67, 123.99, 124.86, 125.66, 126.12, 128.62, 130.13, 130.32, 130.51, 135.57, 137.88, 142.31, 154.03, 168.26, 168.48, 170.59, 174.88; HRMS (ESI) *m/e* calcd. for $C_{29}H_{29}N_4O_9$ (MH^+) 577.1935, found 577.1937.

^1H and ^{13}C NMR spectra were obtained on Bruker Ultrashield spectrometer in the indicated solvent. Column chromatography was carried out on a Büchi Reveleris® PREP. HR-MS were measured on a Q-Tof Premier (Waters) with lockspray ion source (ESI, positive ions) coupled to an Acquity UPLC system (Waters). Commercially available chemicals and solvents were used as received.

Synthesis of Lys-Ala-Asp-Thr-Thr-Thr-Pro-Thr-Thr-Gly(CH_2-triazol-PEG_3-fluorescein)-NH_2 (4)

To a solution of **2** (30 mg, 0.019 mmol) in water (8 ml), **3** (20 mM in *tert*. butanol, 1.0 ml, 0.021 mmol, 1.1 eq) was added. The reaction mixture was degassed by freeze-thaw technique. THTPA (50 mM in water, 0.18 ml, 0.009 mmol, 0.5 eq), copper(II) sulfate (20 mM in water, 0.45 ml, 0.009 mmol, 0.5 eq) and sodium ascorbate (4 mg, 0.019 mmol, 1.0 eq) were added. The solution was stirred for 16 h at room temperature. Organic solvents were removed under reduced pressure and the product was purified by HPLC (water + 0.05% trifluoroacetic acid - acetonitrile + 0.05% trifluoroacetic acid; 1-99% acetonitrile). **4** was obtained as yellow solid (33%). HRMS (ESI) *m/e* calcd. for $C_{72}H_{101}N_{16}O_{26}$ (MH^+) 1605.7073, found 1605.7068.

Accession Numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession numbers 6I41, 6I5P, 6I68 and 6I7A.