A Disulfide Intercalator Toolbox for the Site-Directed Modification of Polypeptides

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A Disulfide Intercalator Toolbox for the Site-Directed Modification of Polypeptides

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Abstract: A disulfide intercalator toolbox was accomplished offering the site-specific attachment of a broad variety of functional groups onto proteins or peptides under mild, physiological conditions. The peptide hormone somatostatin (SST) served as model compound allowing intercalation into the available disulfide bridge. A versatile somatostatin library was accomplished *via* two functionalization schemes starting from the intercalator or the reactive SST precursor before or after bioconjugation. A tetrazole-SST derivative was achieved that reacted in a photoinduced cycloaddition in mammalian cells, which was followed by live cell imaging.

Introduction

Peptides and proteins represent versatile biomacromolecules that receive an emerging interest in medicine as well as in materials science.[1] Due to their unique chemical, physical, and biological properties, polypeptides have been used as e.g. drug molecules,[2] tissue engineering scaffolds,[3] drug delivery matrices[4] and detectors and transducers in biosensoring technology.[5] They reveal many distinct advantages such as high target affinities, specificity and biocompatibility but still their applications are often limited by low chemical and metabolic stability or other unfavourable pharmacokinetic parameters.[6] Therefore, there has been an increasing interest in the development of synthetic procedures that open access to chemically modified polypeptides containing additional substituents at predefined positions.[7] In this way, the aforementioned limitations could be addressed and in addition, novel features that e.g. allow controlling bioactivity or self-assembly could be introduced.[8] For most applications the formation of a homogeneous product is crucial. Thus, the chemical modification needs to be introduced at a distinct site in high yields, which also facilitates purification. Most approaches are based on the attachment of a bioorthogonal anchor group that can react in the presence of abundant functionalities such as amines, carboxylic acid or hydroxyl groups. Often, a single accessible cysteine residue is introduced by genetic engineering since its thiol group selectively reacts with maleimide moieties under pH control.[9] Probably one of the most elaborate strategies is based on the incorporation of reactive, non-canonical amino acids at distinct positions in the polypeptide sequence.[10] In this way, halogen,[11] ethynyl[12] or azido[13] groups have been attached that allow further post modifications. Since only very few native proteins contain unpaired and accessible cysteine residues, recombinant techniques are often the only choice to impart an appropriate bioorthogonal anchor to the polypeptide surface.

A less common approach involves the functionalization of disulfide groups. Disulfides are abundant in proteins and cyclic polypeptides and theoretical evaluation of protein databases revealed that most therapeutically relevant proteins offer at least one disulfide bond close to the surface.[14] Previously, the group of Brocchini has demonstrated that disulfide bridges of proteins can be addressed with high site-specificity.[15] Bis-alkylation conjugation reagents, often referred to as intercalators, have been introduced that react with accessible disulfide bonds in a two-step reaction mechanism (Scheme 1):[14] First, the disulfide bond is reduced followed by disulfide rebridging *via* two successive Michael addition reactions by retaining the tertiary

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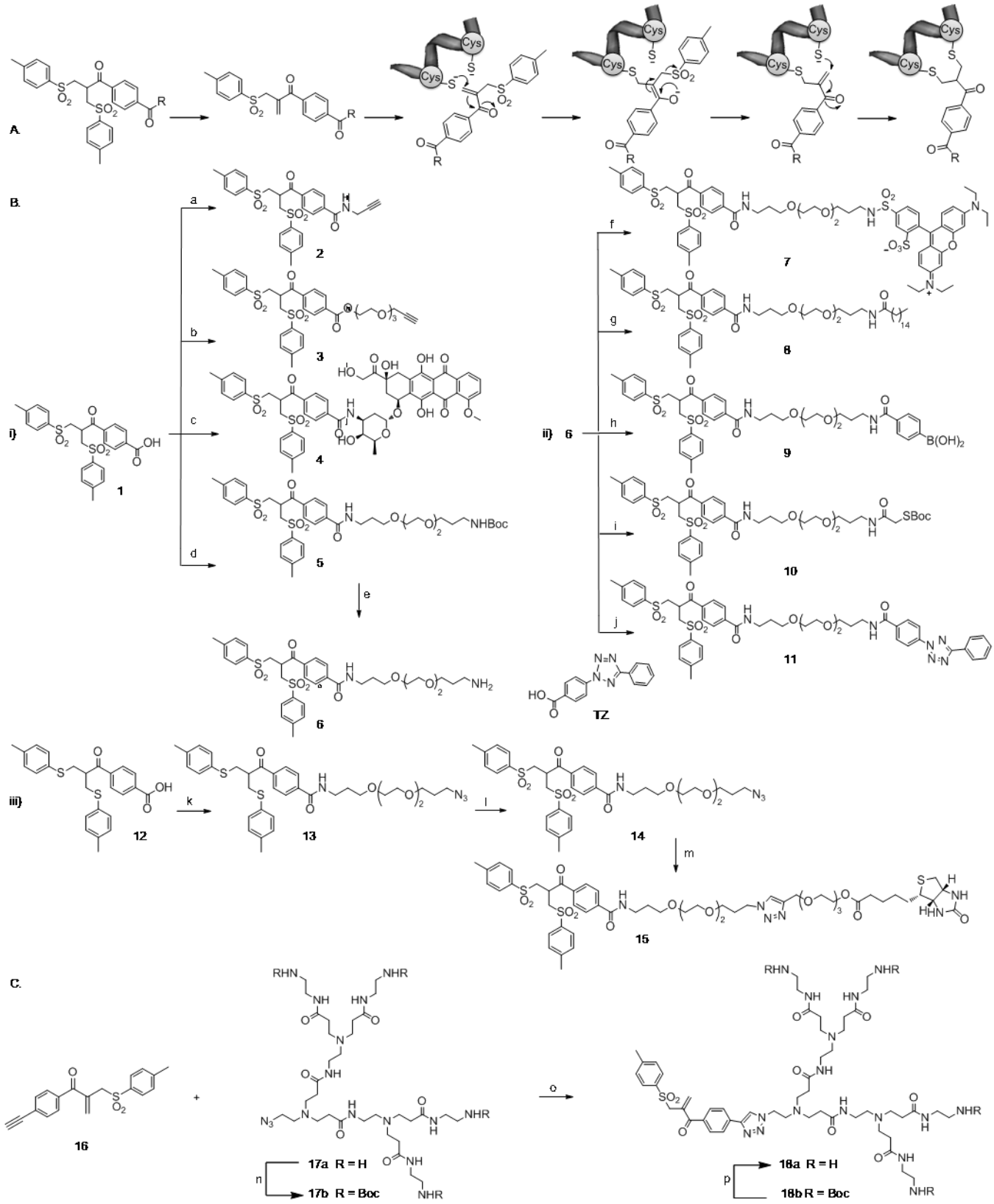

Scheme 1. A. Mechanism of disulfide intercalation. B. Synthesis of various intercalators with different functionalities. a. HBTU, DIEA, propargylamine, 78%; b. **33**, EDC, DMAP, 68%; c. doxorubicin, HBTU, DIEA DMF, 68%; d. **36**, HBTU, DIEA, DMF, 82%. e. TFA, DCM, 95%; f. Sulforhodamine B acid chloride, DIEA, DMF, 45%; g. palmitic acid, HBTU, DIEA, DMF, 78%; h. 4-carboxyphenylboronic acid, HBTU,DIEA, DMF, 60%; i. **34**, HBTU, DIEA, DMF, 63%; j. **TZ**, HBTU, DIEA, DMF, 62%. k, HBTU, DIEA, DMF, 70%; l. oxone, MeOH/H_2O 1:1, 90%; m. **35**, $CuSO_4$, sodium ascorbate, 84%; n. Boc_2O, Et_3N, MeOH, 23 °C, quant.; o. $[Cu(CH_3CN)_4]PF_6$, Et_3N, THF, 70 °C, 1 h, then 23 °C, 10 h, 56%; p. TFA, DCM, quant.

structure of the protein.[14-15] The broad applicability of disulfide intercalators has been demonstrated by the successful modification of peptide hormones, antibodies or therapeutic proteins.[16] We have further investigated the reaction mechanism of disulfide intercalation[16c] by NMR spectroscopy and have reported the introduction of a single iodo and ethynyl group by applying the appropriately functionalized bis-alkylation reagents. In addition, the combination of disulfide intercalation and maleimide chemistry even allowed the cross-conjugation of biomolecules[17] which has been shown particularly attractive for the design of tumor responsive therapeutics.[18] However, up to now, little to no solubility of most intercalators in aqueous solution as well as a lack of more versatile and easily adaptable reaction schemes currently limits broader usage of this promising approach.

Herein, we report the synthesis of a versatile toolbox of bis-alkylation reagents (Scheme 1, compounds **3, 4, 6,7,8,9,10,11,14,15,18a,18b**) offering improved solubility and multiple functionalities that "reanneal" the original disulfide sites and thus introduce the desired function at a predefined position of peptides or proteins (Fig 1A). The burgeoning interest in bioorthogonal chemistry that responds to environmental stimuli motivated us to consider phenyl boronic acids (PBA) and tetrazole units. PBA reacts with salicylhydroxamic acid (SHA) with fast kinetics and high binding affinity in response to pH changes. The photoinduced, bioorthogonal nitrile imine mediated tetrazole-ene cycloaddition reaction (NITEC) offers photoinduced reactions under UV irradiation.[19] The great potential of the NITEC approach has been demonstrated for instance for site-specific (bio)polymer labeling, patterning of various biosubstrates for controlled cell-attachment[20] and light responsive materials,[21] or probing protein dynamics and function as well as to investigate biological processes inside living cells.[22]

Disulfide intercalation has been studied applying the model polypeptide hormone somatostatin (SST) with emphasis on sterical restrictions of the intercalation reaction. SST has been selected as model polypeptide due to its important biological activity, the presence of only one well-accessible disulfide bond as well as its efficient uptake into cells and trafficking into the cytosol, which facilitates investigating chemical reactions even inside living cells. The biological effects of SST are mediated *via* five G protein-coupled membrane bound receptors, SSTR1 to SSTR5 that are aberrantly expressed in high levels on various tumors such as breast, lung, renal, pancreatic, prostate and neuroendocrine cancers in comparison to normal tissues.[23] SST conjugates are of emerging interest for radiotherapy and tumor imaging by labeling SST with radionuclides and for chemotherapy by conjugating SST with antineoplastic agents.[24]

Results and Discussion

Synthesis of the intercalator toolbox.

The intercalation reaction into accessible disulfide bonds of proteins and peptides is based on two consecutive Michael additions. Reagents containing bissulfone groups undergo an elimination reaction under the formation of the reactive monosulfone derivative and *p*-toluene sulfinic acid as byproduct (Scheme 1A). This reaction usually proceeds *in situ* under weak basic conditions. The respective monosulfone subsequently reacts with a free thiol group, which eliminates the second *p*-toluene sulfinic acid group under the formation of a second Michael system. In the presence of a second thiol group in close vicinity, the second Michael addition occurs with the concomitant formation of a C3-rebridged bissulfide. One refers to an intercalation reaction in case both consecutive reactions occur. There are several advantages associated with the introduction of functionalities already at the intercalator. A large variety of substituents can be attached, purification proceeds by chromatography and removal of catalyst traces are usually less challenging. Condensation reactions that provide intercalator libraries proceed smoothly before intercalation but they are not advisable thereafter due to the ubiquitous presence of amines and carboxylic acid groups in proteins and peptides.

Amine bissulfone **6** represents an attractive building block for generating further intercalator derivatives by simple condensation of the desired functionalities. Its synthesis was accomplished in 78% yields by reacting bissulfone **1** with N-Boc-4,7,10-trioxa-1,13-tridecanediamine (**36, SI**) in the presence of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N*,*N*-di*iso*propylethylamine (DIEA), followed by a deprotection of Boc in TFA. Exemplarily, the widely used chromophore sulforhodamine B acid chloride, tetrazole **TZ**, palmitic acid, 4-carboxyphenylboronic acid and a Boc-protected thiol **34 (SI)** were attached yielding the corresponding intercalators **7**, **8**, **9, 10** and **11** after purification by column chromatography in moderate to good yields in a single reaction step (Scheme 1B).

In the case of functionalities that contain many reactive primary amino or carboxylic acid groups should be allowed to react with the intercalator, cycloaddition reactions involving azide or ethynyl groups represent a better alternative since they are absent in almost all naturally existing molecules and therefore react with high chemical selectivity in high yields.[25] The fastest approach toward ethynyl-bissulfones represents the condensation of propargylamine to bissulfone **2** in the presence of HBTU and DIEA providing alkyne-bissulfone **2** in 78% yields. However, **2** is limited by poor labelling efficiency, most likely due to the high lipophilicity of the respective monosulfone (Fig S10B).

To address this limitation, bifunctional linker **38 (SI)** was designed to improve the water solubility of the intercalator. A single amine group of 1,13-diamino-4,7,10-trioxatridecane was Boc-protected whereas the other one was converted into an azide. The most common method for converting amines to azides involves diazo transfer reagents such as trifluoromethanesulfonylazide (TfN_3) serving as "diazo donor".[26] However, due to the explosive nature and relatively poor shelf life of TfN_3, imidazole-1-sulfonylazide **32 (SI)** has been applied instead.[27] **36 (SI)** was treated with imidazole-1-sulfonylazide **32 (SI)** in the presence of copper sulfate and potassium carbonate to give the corresponding azido-derivative **37 (SI)**. After Boc deprotection, the bifunctional linker **38 (SI)** was obtained which was condensed to bissulfide **12** in the presence of HBTU and DIEA in 70% yield. Subsequent oxidation of bissulfide **13** applying oxone yielded the corresponding azido-bissulfone **14** in 90% yields. The analogous intercalator ethynyl-bissulfone **3** with improved water solubility was synthesized accordingly (supporting information, Scheme S1). Azido-bissulfone **14** was then reacted with ethynyl-biotin **35 (SI)**, which was synthesized from ethynyltriethylene glycol **33 (SI)** using 1-ethyl-3-(3-dimethyl

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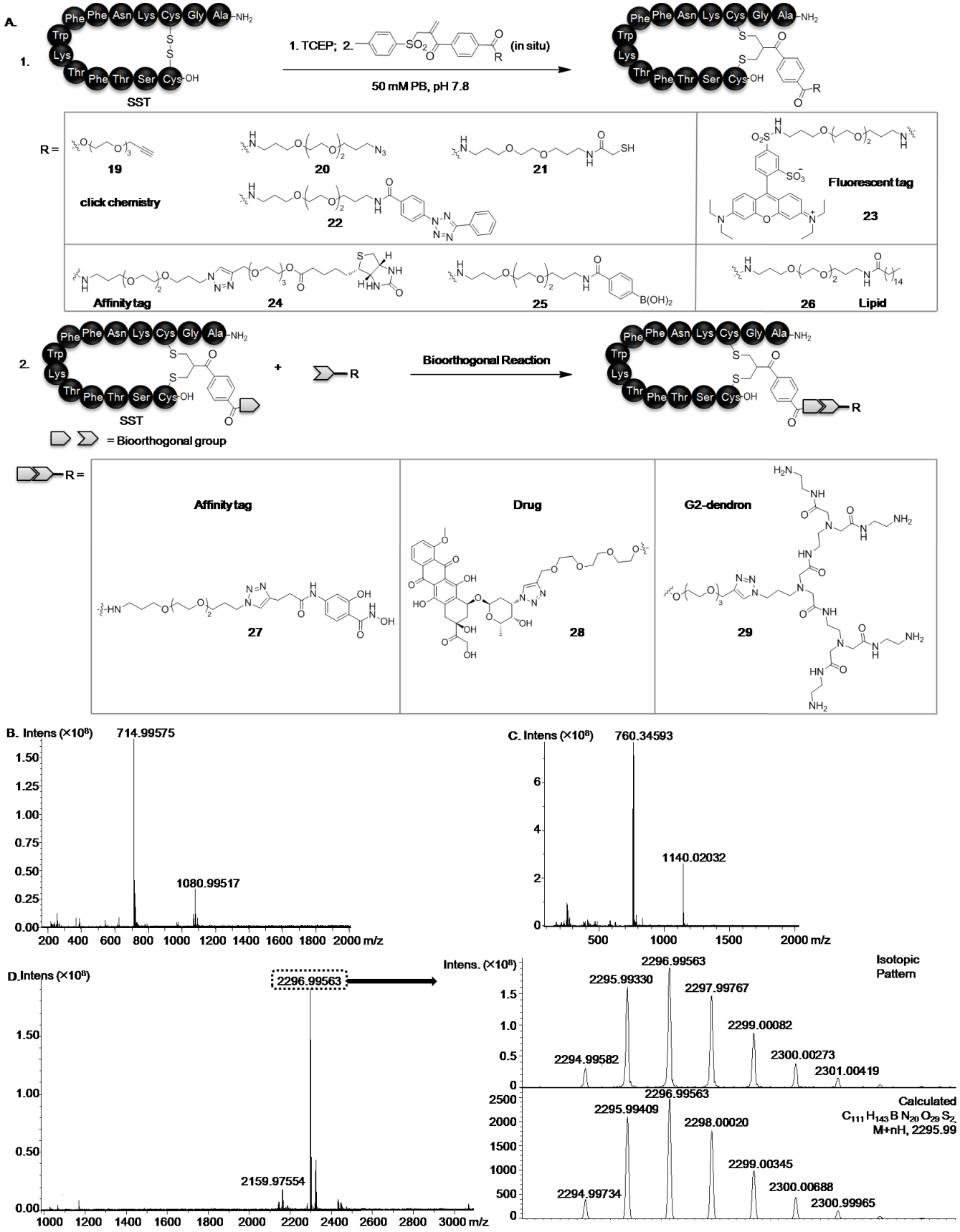

Fig 1. A. Site-specific functionalization of SST; 1. Direct intercalation of SST applying functionalized intercalators. 2. Introduction of bioorthogonal groups onto SST and post-modification *via* bioorthognal reactions; B. HR-ESI-MS of PBA-SST **25** (calc. exact mass: 714.99514 $[M\text{-}2H_2O+3H]^{3+}$, formula: $C_{104}H_{137}BN_{20}O_{25}S_2$; 1080.99436 $[M\text{-}H_2O+2H]^{2+}$, formula: $C_{104}H_{139}BN_{20}O_{26}S_2$); C. HR-ESI-MS of tetrazole-SST **22** (calc. exact mass: 760.34745 $[M+3H]^{3+}$, 1140.01753 $[M+2H]^{2+}$, formula: $C_{111}H_{144}N_{24}O_{25}S_2$; D. HR-MALDI-MS of PBA-SST **25** using DHB as matrix (calc. exact mass: 2296.99563 $[M$+DHB-$2H_2O+H]^+$, formula: $C_{111}H_{143}BN_{20}O_{29}S_2$).

aminopropyl)carbodiimide (EDC) activation and 4-dimethylaminopyridine (DMAP) catalyst as shown in Scheme S1.

Since the alkyne is electron deficient, it readily reacts with azidobissulfone **14** in Cu(I) catalyzed cycloadditions in THF/H_2O to afford biotin bissulfone **15** in 84% yield. In addition, more bulky substituents such as the anti-cancer drug doxorubicin (DOX) as well as a bulky polyamidoamine (PAMAM) dendron were attached to afford **4** and **18a–b**, respectively. **18b** was synthesized *via* Cu(I) catalyzed cycloaddition in DCM/ACN in 56% yield. Deprotection of the Boc group in TFA/CH_2Cl_2 1:1 solution to yielded the dendritic intercalator G2-PAMAM$(NH_2)_4$·TFA salt **18a** in quantitative yield. Doxorubicin (DOX) was attached to bissulfone **1** to afford DOX-bissulfone **4** *via* HBTU coupling in 68% yield. In total, 13 novel bissulfone intercalators have been prepared allowing the introduction of e.g. fluorescence labels, bioorthogonal reactive groups, affinity tags, polymer or drug substituents.

Synthesis of a SST library

SST was derivatized by applying intercalators that contained affinity tags, reactive bioorthogonal groups, a fluorescent probe or a fatty acid (Fig. 1A) in moderate yields. First, the disulfide of SST was reduced by TCEP and then allowed to react with the respective intercalator under slightly basic conditions (pH 7.8) *via* two successive Michael addition reactions. Azido or ethynyl bissulfones (**14**, **3**) were incubated in 50 mM phosphate buffer (pH 7.8) containing 40% ACN to allow elimination of *p*-toluene sulfinic acid thus yielding the corresponding monosulfones *in situ*. Reduction by TCEP and intercalation by the respective monsulfones gave the respective azido or ethynyl SST derivatives (Fig 1A).

SH-SST **21** was produced in a similar fashion after intercalation of the Boc protected SBoc-bissulfone **10** followed by Boc-deprotection in 80% TFA solution for 5 h. Thiol-maleimide chemistry is widely used in bioconjugation reactions due to fast reaction kinetics in aqueous media, the lack of byproducts and the improved stability of the thioether addition product. Therefore, **21** represents an attractive SST building block that gives access to more sophisticated SST derivatives and which was prepared by purely chemical approaches without the necessity to express a SST cysteine mutant.

PBA-SST **25** containing a single boronic acid group was prepared as depicted in Fig 1A and characterized by HR-MS. HR-ESI-MS reveals the dehydration products with mass signals at 714.99575 $[M\text{-}2H_2O+3H]^{3+}$, 1080.99517 $[M\text{-}H_2O+2H]^{2+}$ (Fig 1B) due to thermally induced dehydrations of boronic acid or cyclization to boroxines as reported before.[28] The isotopic pattern of the dehydration products matched the calculated pattern (Fig S16). In addition, HR-MALDI-MS using α-cyano-4-hydroxycinnamic acid (CHCA) as matrix gave the dehydration product with the mass signal of 2142.96879 $[M\text{-}2H_2O+H]^+$ (Fig S15), whose isotopic pattern fits the theoretical pattern. By using 2,5-dihydroxybenzoic acid (DHB) both as matrix and derivatizing reagent due to its complexation to boronic acids, only the final mass signal 2296.99563 $[M+DHB\text{-}2H_2O+H]^+$ was detected owing to the formation of the borate ester with DHB (Fig 1D) supporting that a single boronic acid group was introduced successfully.[28] The attachment of boronic acids into polypeptides is of great interest for biosensing,[29] therapy[30] and pH-responsive materials[31] since boronic acids efficiently reversibly react with bifunctional alcohols,[32] amines[33] or other nucleophiles[34] under physiological conditions. They offer great opportunities to construct stimulus responsive materials based on their bioorthogonal click chemistry.

Interestingly, direct intercalation of **18a**, **18b**, and **4** bearing the sterically demanding G2 PAMAM dendron or the drug doxorubicin was not successful. In both cases, complex product mixtures still containing unreacted starting materials were obtained repetitively (Scheme S2, Fig S7, S8). We presume that the intercalation reaction is limited by the sterical demand of the substituent attached to the intercalator. In case the substituent is too bulky, it might not be able to reach the reduced disulfide residues anymore, since no addition products were observed by LC-MS. Therefore, bulky substituents need to be introduced after the intercalation reaction into the polypeptide. Both azido- and ethynyl-SST (**20**, **19**) offer improved water-solubility and enable further bioorthogonal modifications. Therefore, azido-SST (**20**) was conjugated to G2 PAMAM dendron (**44, SI**) and azido-DOX[18] *via* Cu(I) catalyzed cycloaddition in water. G2 PAMAM-SST (**29**) and DOX-SST (**28**)[18] were isolated in 30% and 47% yield, respectively and characterized by MALDI-MS (Fig S18B) with no Cu(I)-catalyst remaining in the sample.

Cu(I) catalyzed cycloaddition reactions have the inherent disadvantage that the Cu(I)-catalyst needs to be removed entirely due to its cytotoxicity. Even though removal of the catalyst could be accomplished easily at the level of the intercalator as well as for peptides like SST by applying column chromatography or HPLC, purification of proteins can be tedious and product loss or reduced bioactivity can occur. Therefore, there is an increasing interest in the synthesis of alternative, catalyst-free reaction schemes that can be accomplished at the level of the biomacromolecule. In principle, thiol, SHA, PBA, and tetrazole substituents that were introduced by intercalation offer site-directed reactions at the level of the protein. Proteins or peptides containing these functional groups such as SST derivatives (**21, 22, 25, 27**) would offer bioconjugation without the application of cytotoxic metal catalysts.

Phototriggered ligation reaction of tetrazole-SST (22) inside live cells.

The photoinduced 1,3-dipolar cycloaddition of tetrazole derivatives with maleimides usually proceeds within 5 minutes at ambient temperature and represents one of the contemporary tools for rapid modular ligation conjugation.[35] It occurs under the formation of a photoluminescent product that also allows following the reaction kinetics.[22b, 36] Tetrazole-SST (**22**) was accomplished by sequential reduction and intercalation of SST using TCEP and **11**, respectively and followed by HPLC purification. Tetrazole-SST (**22**) was obtained in 41 % yield and it was characterized by HR-ESI-MS (Fig 1C). Bioconjugation of **22** and 6-maleimideocaproic acid in 10-fold excess proceeded *via* tetrazole-ene cycloadditon in aqueous media under UV irradiation for 2 h (Fig 2A). No formation of side products was observed (Fig S20A). **30** was isolated by HPLC (Fig S20B) in moderate (50%) yield and characterized by HR-ESI-MS (Fig 2B).For proof of concept, we have investigated whether this reaction could also be accomplished in a biological environment. Since SST readily penetrates cellular membranes expressing SST2 receptors *via* receptor-mediated endocytosis, a model NITEC reaction was performed inside living cells.

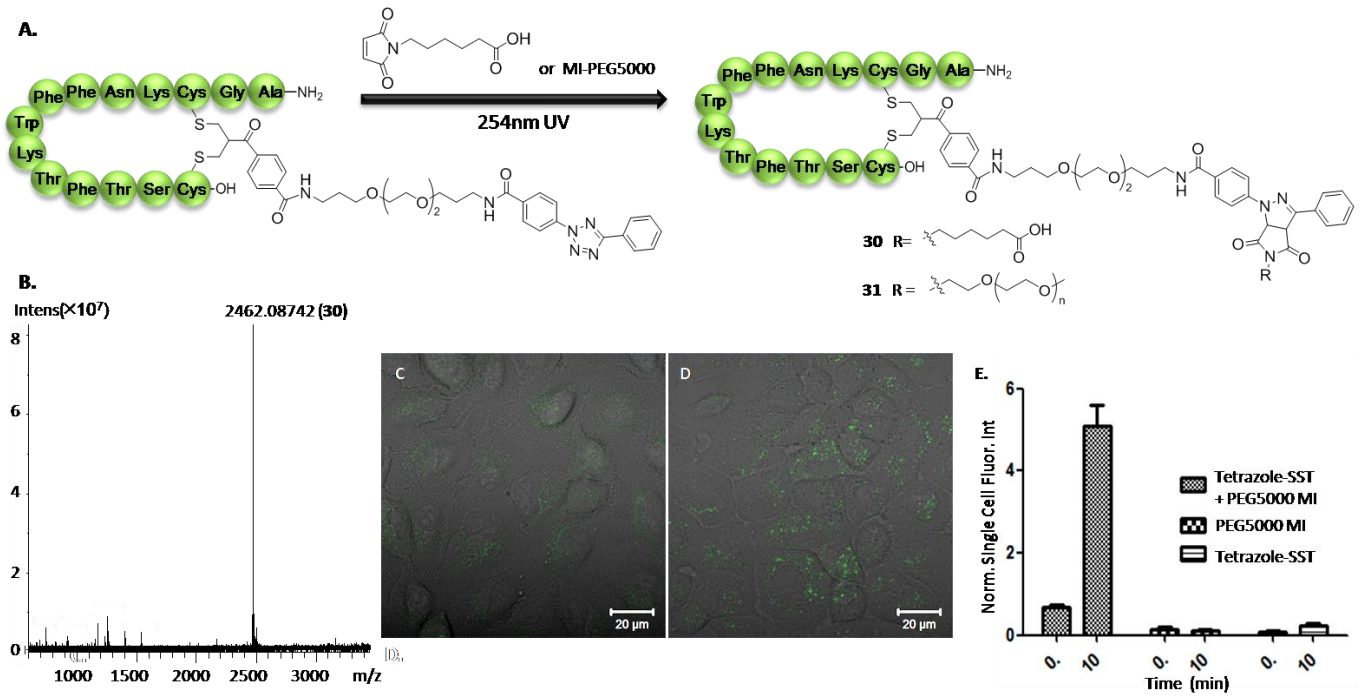

Fig 2. A. Synthetic scheme of the tetrazole-ene cycloadditon of tetrazole-SST **22** with 6-maleimideocaproic acid or MI-PEG5000 under UV irradiation at 254 nm; B. The HR-MALDI-MS of the click product **30** using CHCA as matrix (calc. mass: 2461.09888, formula: $C_{121}H_{157}N_{23}O_{29}S_2$); C. Confocal image of A549 cells incubated with 2 mM MI-PEG5000 and 0.1 mM tetrazole-SST before UV irradiation; D. Incubated with 2 mM MI-PEG5000 and 0.1 mM tetrazole-SST under UV irradiation for 10min; E. The normalized single cell fluorescence intensity (Single cell fluorescence intensity/area of cell) was quantified by ImageJ software based on the confocal images (Fig S22), data are plotted as means ± standard errors of the means (SEM) using GraphPad Prism5 Software (n=10).

Tetrazole-SST **22** (0.1 mM) and the membrane permeable derivative methoxypolyethylene glycol maleimide (2 mM, M_n=5000, MI-PEG5000) were incubated with A549 cells for 4 h. Thereafter, cells were washed thoroughly to remove any remaining tetrazole-SST and MI-PEG5000 that were not uptaken. The tetrazole-ene cycloadditon inside cells was initiated by 254 nm UV irradiation and investigated by confocal microscopy after 10 min. The emission inside A549 cells incubated with both tetrazole-SST (**22**) and MI-PEG5000 under UV irradiation for 10 min was significantly enhanced as compared to A549 cells incubated with both reagents without UV irradiation (Fig 2C, D). The emission intensity inside the cells under UV irradiation for 10 min was quantified by the normalized single cell fluorescence intensity (single cell fluorescence intensity/area of cell) using the ImageJ software (Fig S22, Table S1).[37] It was at least seven times higher compared to the control experiments (Fig 2E). The weak emission of A549 after incubation with tetrazole-SST (Fig 2E) was most likely due to the weak emission of the nitrile imine intermediate. These results clearly support that the photoinduced click reaction was accomplished inside living cells. With the tetrazole-intercalator, a chemically addressable, bioorthogonal group can be readily introduced into polypeptides or proteins of interest in a site-directed fashion. The tetrazole group can undergo photoinduced 1,3-dipolar cycloaddition reactions as demonstrated whereby a new chromophore is formed. This reaction is of potential interest to probe protein function and dynamics inside living cells or to control chemical reactions both spatially and temporally in real time inside a living system by light irradiation.

Conclusion

We have designed a versatile intercalator library that rebridges disulfide bonds and introduces various functionalities onto polypeptides in a site-specific fashion. In total 13 novel intercalators were prepared containing bioorthogonal groups such as ethynyl, azido, phenyl boronic acid (PBA), tetrazole groups or bioaffinity tags like biotin and or imaging probes such as rhodamine. They were connected by triethyleneoxide linkers that impart attractive water-solubility, which is particularly suitable for peptide and protein chemistry. The successful functionalization of the peptide hormone somoatostatin (SST) containing a single disulfide bridge was demonstrated by applying the intercalator toolbox and various new SST conjugates were developed, which could be of great significance for biomedical research. Tetrazole-SST allowed photoinduced reaction under UV irradiation for bioconjugation *via* the photo-induced, bioorthogonal tetrazole-ene cycloaddition reaction (NITEC). Since SST penetrates into cells by receptor-mediated uptake, photoinduced NITEC reaction was accomplished in living cells, which was followed by life cell imaging.

In summary, we have presented a versatile and robust synthetic strategy for introducing desired functional groups into polypeptides that contain accessible disulfide bonds. Due to the improved water-solubility of these intercalators, site-directed functionalization of proteins could be envisaged since most proteins possess at least one accessible disulfide bridge. Therefore, our approach complements the introduction of reactive functionalities such as free thiols, ethynyl or azido groups *via* recombinant techniques offering the advantage that the reaction can be accomplished at the native protein and no protein mutant needs to be expressed.

Experimental Section

General Experimental

Unless otherwise noted, all operations were performed without taking precautions to exclude air and moisture. All solvents and reagents were purchased from commercial sources and were used without further purification. Reaction progress was monitored by thin layer chromatography (TLC) using Merck 60 F254 pre-coated silica gel plates illuminating under UV 254 nm or using appropriate stains. Flash column chromatography was carried out using Merck silica gel 70-230 mesh. NMR spectra were measured on Bruker 400 MHz or 500 MHz NMR spectrometer and the chemical shifts were referenced to residual solvent shifts in the respective deutero solvents. Chemical shifts are reported as parts per million referenced with respect the residual solvent peak. MALDI-TOF-MS spectra were acquired on a Bruker Reflex III. HR-MALDI-MS and HR-ESI-MS were recorded on a Solarix (Bruker) FTICR-MS. LC-MS analysis was performed on a Shimadzu LC-MS 2020 equipped with an electrospray ionisation source and a SPD-20A UV-Vis detector (Shimadzu, Duisburg, Germany). FD-MS (8V) spectra were obtained on a VG Instruments. ZAB 2 SE-FPD. The absorbance and emission were measured on Microplate Readers (Tecan Infinite M1000 PRO). The azido-G2 dendron (**45**) and 2-hydroxy-4-pent-4-ynamido-N-(trityloxy)benzamide were synthesized according to previous literature.[38]

Synthesis of intercalator with different functionalities

Synthesis of *N*-(prop-2-yn-1-yl)-4-(3-tosyl-2-(tosylmethyl)propanoyl)benzamide (2): Bis-sulfone (20 mg, 0.04581 mmol, 1 eq.) dissolved in 1ml of DMF was added HBTU (24 mg, 0.06392 mmol, 1.6 eq.) and DIEA (13 µl, 0.0799 mmol, 2 eq.) at 0°C under argon. The reaction mixture was stirred for 10 min before propargyl amine (3 µl, 0.04794 mmol, 1.2 eq.) was added. The resulting mixture was stirred overnight at RT. The solvent was reduced under high vacuum, and the product was dissolved in DCM. The organic phase was washed with $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and the product was purified using column chromatography to afford 19 mg of **2** in 78% yield. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 2.41 (s, 6H), 3.49 (q, *J* = 6.9 Hz, 2H), 3.62 (q, *J* = 6.9 Hz, 2H), 4.27 (q, *J* = 2.5 Hz, 2H), 4.37 (m, 1H),7.36 (d, *J* = 8.2 Hz, 4H), 7.69 (d, *J* = 8.2 Hz, 6H), 7.77 (d, *J* = 8.2 Hz, 2H) ppm; LC-MS m/z = 538 [*M*+H]$^+$, 382 [*monosulfone*+H]$^+$ (Fig S1).

Synthesis of 2-(2-(2-(prop-2-yn-1-yloxy)ethoxy)ethoxy)ethyl 4-(3-tosyl-2-(tosylmethyl) propanoyl)benzoate (3): Bissulfone (**1**, 200 mg, 0.4 mmol, 1 eq.), DMAP (6 mg, 48 µmol, 0.12 eq.), 1-ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC, 128 mg, 0.48 mmol, 1.2 eq.) and ethynyltriethylene glycol (**33**, 90 mg, 0.48 mmol, 1.2 eq.) were dissolve in 4 ml of anhydrous DCM under argon at 0°C. The reaction mixture was stirred at RT overnight. The mixture was purified by column chromatography using hexane/EA 1:2 to afford 182 mg of product **3** as pale yellow oil in 68% yield. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 2.39 (s, 6H), 3.44 (m, 1H), 3.65 (m, 8H), 3.83 (m, 4H), 4.07 (m, 2H), 4.32 (m, 2H), 4.49 (m, 2H), 7.34 (d, *J* = 8.1 Hz, 4H), 7.66 (m, 6H), 8.09 (d, *J* = 8.4 Hz, 2H) ppm; ^{13}C-NMR (400MHz, $CDCl_3$): δ = 21.4, 30.5, 60.1, 63.6, 64.4, 69.3, 69.4, 69.5, 77.0, 78.5, 128.5, 128.9, 129.9, 130.0, 134.6, 139.6, 141.1, 145.2, 165.7, 201.9 ppm; MALDI-TOF-MS m/z = 671.39 [*M*+H]$^+$.

Synthesis of DOX-intercalator (4)

Bissulfone (**1**, 50 mg, 0.1019 mmol, 1 eq.) dissolved in 1ml of anhydrous DMF was added HBTU (63 mg, 0.1657 mmol, 1.9 eq.) and DIEA (46 µl, 0.2786 mmol, 3.2 eq.) at 0°C under argon. The reaction mixture was stirred for 10 min before doxorubicin hydrochloride (384 mg, 1.2 mmol, 1.2 eq.) was added. The resulting mixture was stirred overnight at RT. The solvent was removed under high vacuum, and the product was dissolved in DCM. The organic phase was washed with $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and the crude product was purified by column chromatography using 5% methanol in chloroform to afford 60 mg of **4** in 68% yield. The product was characterized by LC-MS, revealing monosulfone has already partially formed during the column chromatography due to elimination of *p*-toluenesulfinic acid. LC-MS m/z = 868 [*monosulfone*-H]$^-$, 1024 [*M*-H]$^-$. (Fig S2).

Synthesis of tert-butyl (1-oxo-1-(4-(3-tosyl-2-(tosylmethyl)propanoyl)phenyl)-6,9,12-trioxa-2-azapentadecan-15-yl)carbamate (5): Bissulfone (**1**, 500 mg, 1 mmol, 1 eq.) dissolved in 5 ml of anhydrous DMF was added HBTU (606 mg, 1.6 mmol, 1.6 eq.) and DIEA (330 µl, 2 mmol, 2 eq.) at 0°C under argon. The reaction mixture was stirred for 10 min before Boc-ethylene glycol (**36**, 384 mg, 1.2 mmol, 1.2 eq.) was added. The resulting mixture was stirred overnight at RT. The solvent was removed under high vacuum, and the product was dissolved in DCM. The organic phase was washed with $NaHCO_3$, brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and the crude product was purified by column chromatography using 10% methanol in chloroform to afford 658 mg of **5** in 82% yield. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.36 (s, 9H), 1.61-1.67 (m, 2H), 1.82-1.88 (m, 2H), 2.74 (s, 6H), 3.11-3.13 (m, 2H), 3.40 (t, *J* = 5.95 Hz, 2H), 3.44-3.45 (m, 2H), 3.54-3.63 (m, 11H), 4.29 (s, 4H), 7.28-7.31 (m, 2H), 7.57-7.66 (m, 4H), 7.71-7.78 (m, 4H), 7.82-7.84 (m, 2H) ppm; ^{13}C NMR (100MHz, $CDCl_3$): δ 21.6/21.7, 28.4, 28.7, 29.6, 38.3, 39.0, 57.7, 69.4, 70.0, 70.2, 70.4, 70.6, 78.8, 127.1, 128.3, 129.6, 129.9/130.2, 135.2/135.9, 135.7/135.8, 138.3, 138.4, 145.1/145.5, 156.0, 166.2, 194.2 ppm; LC-MS m/z = 669 [*monosulfone* + H]$^+$, 825 [*M* + Na]$^+$.

Synthesis of N-(3-(2-(2-(3-aminopropoxy)ethoxy)ethoxy)propyl)-4-(3-tosyl-2-(tosylmethyl)propanoyl) benzamide (6): Compound **5** (500 mg, 0.623 mmol, 1 eq.) was dissolved in 3 ml of DCM and TFA (476 µl, 10 eq.) was added. The DCM and TFA were removed under vacuum at 30°C to afford 416 mg of amine bissulfone **6** as yellow oil in 95% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.84-1.90 (m, 2H), 1.94-1.99 (m, 2H), 2.41 (s, 6H), 3.20-3.25 (m,2H), 3.47-3.55 (m, 4H), 3.57-3.62 (m, 8H), 3.72 (t, *J* = 5.22 Hz, 2H), 4.33 (s, 4H), 7.33-7.35 (m, 2H), 7.59-7.67 (m, 4H), 7.69-7.77 (m, 4H), 7.83-7.85 (m, 2H) ppm**;** ^{13}C-NMR (100MHz, $CDCl_3$): δ = 21.74, 26.15, 29.30, 38.01, 40.97, 57.91, 69.04, 69.42, 69.74, 69.80, 70.28, 71.01, 127.33, 128.43, 129.78, 130.12, 135.01, 135.66/135.82, 137.53, 138.98, 145.48, 167.68, 194.50 ppm. LC-MS m/z = 547 [*monosulfone* + H]$^+$, 703 [*M*+H]$^+$

Synthesis of Rhodamine-bissulfone (7): Sulforhodamine B acid chloride (60 mg, 0.102 mmol, 1 eq.) was dissolved in 2 ml of anhydrous DMF at 0℃ under argon. Then, amine bissulfone (**6**, 100 mg, 0.142 mmol, 1.4 eq.) and DIEA (50 µl, 0.306 mmol, 3 eq.) was added sequentially. The reaction mixture was stirred at RT overnight. The solvent was removed under vacuum and the mixture was purified by column chromatography by using 10% methanol in chloroform to afford 57 mg of the product **7** as pink solid in 45% yield. The product was characterized by LC-MS, revealing monosulfone has already partially formed during the column chromatography due to elimination of *p*-toluenesulfinic acid. LC-MS m/z = 1087 [monosulfone + H]$^+$, 1243 [bissulfone + H]$^+$ (Fig S3).

Synthesis of Palmityl-bissulfone (8): Palmitic acid (134 mg, 0.5122 mmol) dissolved in 3 ml of anhydrous DMF was added HBTU (312 mg, 0.82 mmol) and DIEA (169 µl, 1.024 mmol) at 0°C under argon. The reaction mixture was stirred for 10 min before **6** (300 mg, 0.4268 mmol) was added. The resulting mixture was stirred overnight at RT. The solvent was reduced under high vacuum and the product was dissolved in DCM. The organic phase was washed with saturated $NaHCO_3$ twice, brine and dried over anhydrous Na_2SO_4. The crude product was purified by chromatography column to afford 313 mg of the product in 78% yield. The product was characterized by LC-MS, revealing monosulfone has already partially formed during the column chromatography due to elimination of *p*-toluenesulfinic acid. LC-MS m/z = 941 [*M*+H]$^+$, 963 [*M*+Na]$^+$, 785 [*monosulfone* + H]$^+$, 807 [*monosulfone* + Na]$^+$ (Fig S4).

Synthesis of PBA-bissulfone (9): 4-Carboxyphenylboronic acid (58 mg, 0.3415 mmol) dissolved in 2 ml of anhydrous DMF was added HBTU (208 mg, 0.5464 mmol) and DIEA (113 µl, 0.683 mmol) at 0°C under argon. The reaction mixture was stirred for 10 min before **6** (200 mg, 0.2845 mmol, 1.2 eq.) was added. The resulting mixture was stirred overnight at RT. The solvent was reduced under high vacuum and the product was dissolved in $CHCl_3$. The organic phase was washed with saturated $NaHCO_3$ twice, brine and dried over anhydrous Na_2SO_4. The crude product was purified by chromatography column using 4% MeOH in $CHCl_3$ as eluting solvent combination. The solvent was removed under vacuum to afford 145 mg of **9** in 60% yield. LC-MS m/z = 695 [*monosulfone* + H]$^+$, 717 [*monosulfone* + Na]$^+$, 851[*M* + H]$^+$, 875 [*M* + Na]$^+$ (Fig S5).

Synthesis of SBoc-bissulfone (10): Compound **34** (98 mg, 0.4268 mmol) dissolved in 2 ml of anhydrous DMF was added HBTU (312 mg, 0.82 mmol) and DIEA (169 µl, 1.024 mmol) at 0°C under argon. The reaction mixture was stirred for 10 min before amine-bissulfone (**6**, 300 mg, 0.4268 mmol) in 1 ml of anhydrous DMF was added. The resulting mixture was stirred overnight at RT and the solvent was removed under high vacuum. The product was dissolved in $CHCl_3$ and washed with $NaHCO_3$ (3×), brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and the crude product was purified by column chromatography using 5% methanol in $CHCl_3$ to afford 236 mg of **10** in 63% yield. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.47 (s, 9H), 1.70-1.74 (m, 2H), 1.90-1.93 (m, 2H), 2.40/2.47 (s, 6H), 3.29-3.34 (m, 2H), 3.41-3.44 (m, 2H), 3.45-3.50 (m, 4H), 3.57-3.67 (m, 10H), 4.32 (s, 2H), 7.30-7.42 (m, 4H), 7.62-7.72 (m, 4H), 7.76-7.82 (m, 2H), 7.87-7.89 (m, 2H) ppm; ^{13}C-NMR (100MHz, $CDCl_3$): δ = 21.8/21.9, 28.3, 28.9, 29.1, 34.8, 38.0, 39.3, 57.8, 69.6, 70.2, 70.52, 70.5, 70.4, 70.8, 86.1, 127.2, 128.4, 129.8, 130.0/130.3, 135.4/136.0, 135.9, 138.5, 139.8, 145.2/145.7, 166.3, 168.6, 194.4 ppm. MALDI-TOF-MS m/z = 899.42 [*M*+Na]$^+$

Synthesis of tetrazole-bissulfone (11): 4-(2-phenyl-2H-tetrazol-5-yl)benzoic acid (**TZ**, 20 mg, 0.07512 mmol, 1 eq.) dissolved in 1 ml of anhydrous DMF was added HBTU (46 mg, 0.1202 mmol, 1.6 eq.) and DIEA (25 µl, 0.1502 mmol, 2 eq.) at 0°C. The reaction mixture was stirred for 10 min before **6** (63 mg, 0.09014 mmol, 1.2 eq.) was added. The resulting mixture was stirred overnight at RT. The solvent was reduced under high vacuum and the product was dissolved in ethyl acetate. The organic phase was washed with saturated $NaHCO_3$ twice, brine and dried over anhydrous Na_2SO_4. The crude product was purified by chromatography column using 4% MeOH in $CHCl_3$ as eluting solvent combination. The solvent was removed under vacuum to afford 44 mg of the product in 62% yield. The product was characterized by LC-MS, revealing monosulfone has already partially formed during the column chromatography due to elimination of *p*-toluenesulfinic acid. LC-MS m/z = 795 [*monosulfone*+H]$^+$, 951 [*M*+H]$^+$ (Fig S6).

Synthesis of *N*-(3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)-4-(3-(p-tolylthio)- 2-((p-tolylthio) methyl) propanoyl) benzamide (13): Bis-sulfide (**12**, 380 mg, 0.866 mmol, 1 eq.) dissolved in 4 ml of anhydrous DMF was added HBTU (530 mg, 1.386 mmol, 1.6 eq.) and DIEA (290 µl, 1.732 mmol, 2 eq.) at 0°C under argon. The reaction mixture was stirred for 10 min before **38** (210 mg, 0.866 mmol, 1.2 eq.) was added and the resulting mixture was stirred overnight at RT. The solvent was reduced under high vacuum and the crude product was dissolved in DCM. The organic phase was washed with saturated $NaHCO_3$ twice, brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and crude product was purified by flash chromatography to afford 403 mg of **13** in 70% yield. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.72-1.97 (m, 2H), 1.85-1.90 (m 2H), 2.32 (s, 6H), 3.09-3.24 (m, 2H), 3.30 (t, *J* = 6.7 Hz, 2H), 3.41-3.47 (m, 4H), 3.54-3.66 (m, 10H), 3.73-3.80 (m, 1H), 7.03 (d, *J* = 8.0 Hz, 4H), 7.10 (d, *J* = 8.1 Hz, 4H), 7.56 (d, *J* = 8.4 Hz, 2H), 7.73 (d, *J* = 8.4 Hz, 2H) ppm; ^{13}C-NMR (125MHz, $CDCl_3$): δ = 21.1, 28.6, 29.0, 36.4, 39.4, 45.6, 48.4, 67.9, 70.2, 70.3, 70.4, 70.4, 127.2, 128.5, 129.8, 131.2, 131.5, 137.2, 138.5, 138.9, 166.03, 200.33 ppm; ESI-MS m/z = 665 [*M*+H]$^+$, 687 [*M*+Na]$^+$.

N-(3-(2-(2-(3-azidopropoxy)ethoxy)ethoxy)propyl)-4-(3-tosyl-2-(tosylmethyl)propanoyl) benzamide (14): 13 (250 mg, 0.376 mmol, 1 eq.) and oxone (1.39 g, 2.256 mmol, 6 eq.) were added to a 1 ml solution of methanol/deionized water 1:1. The reaction mixture was allowed to stir at RT for 48 h. The mixture was diluted by deionized water (60 ml) and extracted with chloroform (4x50 ml). More water was added to dissolve all the inorganic solids and the aqueous layer was extracted once with chloroform (50 ml). The chloroform extracts were combined and wash once with brine. Then the organic chloroform layer was dried over anhydrous sodium sulfate and filtered. The solvent was removed under vacuum to afford 247 mg of **14** in 90% yield. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.72-1.79 (m, 2H), 1.87-1.92 (m, 2H), 2.47 (s, 6H), 3.28-3.32 (t, *J* = 6.7 Hz, 2H), 3.42-3.49 (m, 6H), 3.57-3.69 (m, 12H), 4.30-4.35 (m, 1H), 7.34 (d, *J* = 8.0 Hz, 4H), 7.62-7.67 (m, 6H), 7.78 (d, *J* = 6.6 Hz, 2H) ppm; ^{13}C-NMR (125 MHz, $CDCl_3$): δ = 21.8, 28.5, 29.0, 35.5, 48.4, 55.5, 67.9, 70.2, 70.3, 70.5, 71.0, 127.6, 128.3, 128.7, 130.2, 132.9, 135.3, 136.0, 145.6, 165.8, 202.5 ppm; MALDI-TOF-MS m/z = 729.65 [*M*+H]$^+$.

Synthesis of 2-(2-(2-((1-(1-oxo-1-(4-(3-tosyl-2-(tosylmethyl)propanoyl)phenyl)-6,9,12-trioxa-2-azapentadecan-15-yl)-1H-1,2,3-triazol-4-yl)methoxy)ethoxy)ethoxy)ethyl 5-((3aS,4S,6aR)-2 - oxohexahydro-1H-thieno [3,4-d]imidazol-4-yl)pentanoate (15): Compound **14** (200 mg, 0.276 mmol, 1 eq.) and ethynylbiotin (**35**, 136 mg, 0.328 mmol, 1.2 eq.) were mixed in 2 ml of THF/H_2O 1:1. sodiumascorbate (56 mg, 0.276 mmol, 1 eq.) and copper sulfate (4.4 mg, 27.6 µmol, 0.1 eq.) were added to the mixture sequentially. The reaction mixture was stirred at RT for 24 h. The solvent was removed under reduced pressure by rotary evaporator. The residue was dissolved in $CHCl_3$ and the precipitate was removed by filtration. The crude product was purified by column chromatography using 10% methanol in chloroform to afford 265 mg of the product **15** as slightly yellow oil in 84% yield. ^{1}H-NMR (400MHz, $CDCl_3$): δ = 1.86-1.93 (m, 4H), 2.06-2.12 (m, 2H), 2.33 (t, 2H), 2.71 (d, 1H), 2.47 (s, 6H), 2.86-2.91 (m, 1H), 3.11-3.15 (m, 1H), 3.39 (t, 2H), 3.46-3.51 (m, 4H), 3.56-3.68 (m, 24H), 4.19-4.21 (m, 2H), 4.26-4.30 (m, 1H), 4.35-4.42 (m, 3H), 4.46-4.49 (m, 1H), 4.64 (s, 2H), 5.03 (s, 1H), 5.50 (s, 1H), 7.35 (d, 4H), 7.49 (t, 1H), 7.61 (s, 1H), 7.67 (d, 6H), 7.82 (d, 2H) ppm; ^{13}C NMR (400MHz, $CDCl_3$): δ = 195.0, 173.5, 165.7, 163.5, 145.4, 144.5, 139.4, 135.8, 135.0, 130.0, 128.4, 128.0, 127.5, 123.1, 70.3, 70.3, 70.2, 70.2, 70.0, 69.9, 69.4, 68.9, 67.0, 59.9, 55.4, 55.3, 46.9, 40.3, 38.8, 35.3, 33.6, 30.0, 28.6, 28.1, 28.0, 24.5, 21.6 ppm. MALDI-TOF-MS m/z = 1143.61 [*M*+H]$^+$.

Synthesis of Dendritic intercalator G2-PAMAM(–NHBoc)$_4$ (18b)

A degassed solution of **17b** (142 mg, 121 µmol) and intercalator **16** (44 mg, 136 µmol) in 4.5 ml MeCN/CH_2Cl_2 2:1 was treated with 2,6-lutidine (5 µl, 4.3 mg, 36 µmol) and $[Cu(MeCN)_4]PF_6$ (4.2 mg, 11 µmol) at 23°C under argon atmosphere. After 10 min the reaction mixture turned green and the solution was heated to 70°C for 1 h. After stirring for further 10 h at 23°C, the reaction mixture was diluted with 30 ml of CH_2Cl_2, washed with $NaHCO_3$ (0.7 M, 3 x 10 ml), water (1 x 20 mL), and brine (1 x 20 mL), dried over $MgSO_4$, filtered, and concentrated. Flash chromatography (SiO_2, gradient 13%–23% MeOH in CH_2Cl_2) yielded 102 mg of the dendritic intercalator **18b** as a colorless solid in 56% yield. R_f = 0.21(18% MeOH in CH_2Cl_2); ^{1}H-NMR (300 MHz, CD_2Cl_2): δ = 8.38 (s, 1H, $H_{triazole}$), 7.98 (d, *J* = 8.0 Hz, 2H), 7.79 (m, 4H), 7.47 (broad, 6H, NH_{amide}), 7.39 (d, *J* = 8.0 Hz, 2H), 6.08 (s, 1H, H_{olefin}), 5.99 (s, 1H, H_{olefin}), 5.71 (s, 4H, NH_{Boc}), 4.52 (s, 2H, triazole-CH_2), 4.34 (s, 2H, CH_2SO$_2$), 3.26 (m, 12H, CH_2NH$_{amide}$), 3.18 (d, *J* = 4.7 Hz, 10H, CH_2NH$_{carbamate}$, triazole–CH$_2$CH_2N), 2.96 (s, 4H, NHCH$_2$CH_2N), 2.75 (s, 12H, CH_2CO), 2.44 (s, 3H, Ph-CH_3), 2.36 (s, 8H, NCH_2CH$_2$CO$_{outer}$), 2.27 (s, 4H, NCH_2CH$_2$CO$_{inner}$), 1.40 (s, 36H, C$H_{3,Boc}$) ppm; FT-IR $\tilde{\nu}$ = 544, 667, 710 (s), 796, 860, 955 (s), 1039, 1167 (vs), 1250, 1365, 1454, 1525 (s), 1645 (vs), 1685, 2330, 2362, 2827, 2978, 3084, 3300 cm^{-1}(vb); FD-MS m/z = 1518.6 [*M*+Na]$^+$; MALDI-TOF-MS m/z = 1497 [*M*+H]$^+$, 1535 [*M*+K]$^+$; HR-ESI-MS m/z: 1517.8171 (100%), 1518.8258 (90%), 1519.8469 (31%), 1520.8556 (11%), calc. for [*M*+Na]$^+$: 1517.8166 (100%), 1518,8200 (76%), 1519,8233 (29%), 1520,8267 (7%).

Synthesis of Dendritic intercalator G2-PAMAM(–NH$_2$)$_4$TFA salt (18a)

18b (27 mg, 18 µmol) was dissolved in 1 ml of TFA/CH_2Cl_2 1:1, stirred for 2 h at 23°C, and concentrated to obtain 44 mg of **18b** in quantitative yield as a colorless solid, which is soluble in MeOH or water, but not in CH_2Cl_2. ^{1}H-NMR (300 MHz, d_3-MeOD): δ = 8.67 (s, 1H, $H_{triazole}$), 8.04 (d, *J* = 8.3 Hz, 2H), 7.86 (m, 4H), 7.48 (d, *J* = 8.2 Hz, 2H), 6.09 (s, 1H, H_{olefin}), 6.00 (s, 1H, H_{olefin}), 5.05 (N*H*CO), 4.50 (s, 2H, SO$_2$CH_2), 3.96 (N*H*CO), 3.59 (s, 8H), 3.49 (t, *J* = 5.9 Hz, 16H), 3.36 (4H), 3.09 (t, *J* = 5.8 Hz, 8H), 2.91–2.67 (m, 12H), 2.47 (s, 3H, Ph-CH_3) ppm; MALDI-TOF-MS m/z = 1095 [*M*+H]$^+$, 1117 [*M*+Na]$^+$. (calc. mass: 1094.62).

Functionalization of SST

Generally, bissulfones with different functitonalities are preincubated in 50 mM PB (pH 7.8) overnight to allow the formation of corresponding monosulfone. SST is reduced by 2 eq. TCEP for 30 min and subsequently reacted with formed monosulfone to afford SST conjugates with corresponding functionalities (ethynyl, azido, biotin, SBoc, Rhodamine, tetrazole, palmityl and PBA) (Fig. 2). The SST derivertives are purified by HPLC and characterized by MALDI-MS, LC-MS or HR-MS.

Ethynyl-SST (19): MALDI-TOF-MS m/z = 1998.22 [M+H]$^+$ (calc. mass: 1996.87, CHCA as matrix, Fig S9).

Azido-SST (20): MALDI-TOF-MS m/z = 2056.40 [M+H]$^+$ (calc. mass: 2054.94, CHCA as matrix, Fig S10).

SH-SST (21): MALDI-TOF-MS m/z = 2104.69 [M+H]$^+$ (calc. mass: 2102.93, CHCA as matrix, Fig S11).

Tetrazole-SST (22): HR-ESI-MS m/z = 760.34593 [M+3H]$^{3+}$, 1140.02032 [M+2H]$^{2+}$; LC-MS m/z = 760 [M+3H]$^{3+}$, 1140 [M+2H]$^{2+}$ (calc. mass: 2278.02052), Fig 1C and Fig S12.

Rhodamine-SST (23): MALDI-TOF-MS: m/z= 2570.80 [M+H]$^+$ (calc. mass: 2569.09, CHCA as matrix, Fig S13).

Biotin-SST (24): MALDI-TOF-MS m/z = 2470.73 [M+H]$^+$ (calc. mass: 2469.12, CHCA as matrix, Fig S14).

PBA-SST (25): HR-ESI-MS: m/z = 714.99575 [M-2H$_2$O+3H]$^{3+}$; 1080.99517 [M-H$_2$O+2H]$^{2+}$. HR-MALDI-MS: m/z = 2296.99563 [M+DHB-2H$_2$O+H]$^+$ (DHB as matrix); m/z = 2142.96879 [M-2H$_2$O+H]$^+$ (CHCA as matrix), calc. mass = 2177.98477, Fig 1B, 1D and Fig S15, S16.

Palmityl-SST (26): MALDI-TOF-MS: m/z = 2268.75 [M+H]$^+$, 2290.82 [M+Na]$^+$ (calc. mass: 2267.18, CHCA as matrix, Fig S17).

SST conjugates *via* click chemistry

Synthesis of G2-SST (29): N_3-G2 (**45**, 4 mg, 5.71 µmol) and ethynyl-SST (**19**, 5 mg, 2.50 µmol) are dissolved in 2 ml of H_2O. To this solution, sodium ascorbate (4 mg, 20.2 µM) and $CuSO_4$ (2 mg, 12.5 µM) were added sequentially. The resulting reaction mixture was stirred for 24h at RT. The mixture was concentrated and purified by Prep HPLC using an Atlantis Prep OBD T3 Column (19 x 100 mm, 5 µm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-5 min), raising to 23% B in 2 min, 23% B for 10 min, raising to 26% B in 0.5 min, 26% B for 10 min and finally reaching 100% B in 5 min with a flow rate of 10 ml/min. The absorbance was monitored at 280 nm and 254 nm. The retention time for **29** was 37.3 min. 2 mg of the product **29** was obtained from lyophilisation in 30% yield. The product was characterized by MALDI-TOF-MS using CHCA as matrix. MALDI-TOF-MS m/z=2697.41 [*M*+H]$^+$, 2719.33 [*M*+Na]$^+$ (calc. mass: 2697.33, CHCA as matrix, Fig S18).

Synthesis of SHA-SST (27): 2-hydroxy-4-pent-4-ynamido-N-(trityloxy)benzamide (2.4 mg, 4.89 µmol, 2 eq.) was dissolved in 40 µl of DMSO and further diluted in 1 ml of DCM. To this solution, azido-SST (**20**, 5 mg, 2.43 µmol, 1 eq.) in 1 ml of H_2O was added and followed by adding sodium

ascorbate (1 mg, 5.05 μmol, 2 eq.) and $CuSO_4$ (0.4 mg, 2.51 μmol, 1 eq.) sequentially. The resulting mixture was shaked for 24 h at RT. The mixture was concentrated and purified by Prep HPLC using an Atlantis Prep OBD T3 Column (19 x 100 mm, 5 μm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-10 min), raising to 40% B in 5 min, 40% B for 9 min, raising to 45% B in 1 min, 45% B for 10 min and finally reaching 100% B in 5 min with a flow rate of 10 ml/min. The absorbance was monitored at 280 nm and 254 nm. 4 mg of Trt protected SHA-SST was obtained from lyophilisation and its retention time was 29,789 min. The Trt protected SHA-SST was dissolved in 1 ml of ACN/H_2O 1:1 and followed by adding 200 μl of TFA and 120 μl of tri*iso*propylsilane. After 12 h reaction at RT, the mixture was concentrated and purified by Prep HPLC using an Atlantis Prep OBD T3 Column (19 x 100 mm, 5 μm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-5 min), raising to 36% B in 5 min, 36% B for 8 min and finally reaching 100% B in 5 min with a flow rate of 10 ml/min. The absorbance was monitored at 280 nm and 220 nm. 2 mg of product **27** was obtained from lyophilisation in 35% overall yield and its retention time is 13.12 min. The product was characterized by HR-ESI-MS and HR-MALDI-MS using CHCA as matrix. HR-ESI-MS m/z = 577.01294 $[M+4H]^{4+}$, 769.01462 $[M+3H]^{3+}$, 1153.01891 $[M+2H]^{2+}$, HR-MALDI-MS m/z = 2305.01095 $[M+H]^{+}$ (calc. mass: 2304.02091), Fig S19.

Synthesis of compound 30: Tetrazole-SST (**22**, 2 mg, 0.878 μmol, 1 eq.) was dissolved in 1 ml of H_2O and 6-maleimideocaproic acid (1.9 mg, 9 mmol, 10 eq.) in 200 μl of DMSO was added. The resulting mixture was irradiated by UV lamp (254 nm) for 2 h. The mixture was purified by analytical HPLC using an MerckChroCART 125-4 Column with the mobile phase starting from 95% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0-2 min), raising to 35% solvent B in 3 min and then to 38% solvent B in 13 min, keeping at 38% solvent B for 4 min, decreasing to 5% solvent B in 2 min and finally balancing the column with 5% solvent B for 1 min with a flow rate of 1 ml/min. The absorbance was monitored at 220 nm. The retention time of conjugate **30** was 19.08 min (Fig S20). The product was characterized by HR-ESI-MS and HR-MALDI-MS. HR-ESI-MS m/z = 821.37410 $[M+3H]^{3+}$, 1231.55805 $[M+2H]^{2+}$ (Fig S21); HR-MALDI-MS m/z = 2462.08742 $[M+H]^{+}$ (calc. mass: 2461.09888,CHCA as matrix, Fig 2B).

Confocal study of phototriggered reaction in live cell

Thirty thousands of A549 cells were plated in a μ-Slide 8-well chambered coverslip (ibidi, Martinsried, Germany) in 300 μl medium. The cells were cultured overnight to allow adhesion. The next day, 0.1 mM of tetrazole-SST, 2 mM of MI-PEG5000 or both compounds were added respectively with fresh medium. The cells were then further incubated for 4 h in the incubator. Before imaging, cells were washed with PBS buffer for 3 times. Then cells were irradiated under 254 nm wavelength UV lamp for 0 min, 10 min or 20 min. Imaging was performed using a LSM 710 laser scanning confocal microscope system (Zeiss, Germany) coupled to an XL-LSM 710 S incubator and equipped with a 63× oil immersion objective. The fluorescence of formed **31** was recorded using a 440-510 nm filter and a Diode405-30 laser for excitation. The acquired images were processed with Image-J software. The phototriggered reaction was monitored by the normalized single cell fluorescent intensity (Single cell fluorescence intensity/area of cell) using the ImageJ software to quantify the fluorescent conjugate **31** generated in situ. Data are plotted as means ± standard errors of the means (SEM) using GraphPadPrism5 Software.

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Keywords: site-specific protein modification • intercalation • disulfide functionalization • somatostatin analogues • NITEC

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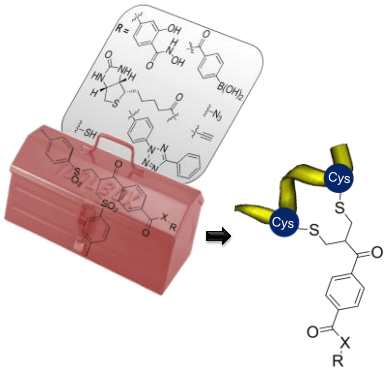
A versatile disulfide intercalator toolbox was accomplished offering the site-specific modification of proteins or peptides with various functional groups under mild, physiological conditions. Functionalization of the peptide hormone somatostatin with a tetrazole substituent even facilitated the photo-induced, bioorthogonal tetrazole-ene cycloaddition reaction (NITEC) in living cells, which was followed by life cell imaging.



Site-specific protein chemistry

T. Wang, Y. Wu, S. L. Kuan, O. Dumele, M. Lamla, D. Y. W. Ng, M. Arzt, J. Thomas, J. O. Mueller, C. Barner-Kowollik, T. Weil**

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A Disulfide Intercalator Toolbox for Site-Directed Modification of Polypeptides