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ORIGINAL ARTICLE

An amino acid-based heterofunctional cross-linking reagent

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Abstract We describe the synthesis and characterization of a new lysine-based heterofunctional cross-linking reagent. It carries two readily available aminooxy functionalities and an activated and protected thiol group that is capable of generating reducible disulfides, the former enable bioorthogonal modification of ketones and aldehydes by the formation of an oxime bond. The efficacy of the linker was proven by coupling two doxorubicin molecules to the functionalized amino acid core and the subsequent bioconjugation of this drug conjugate with a thiolated antibody.

Keywords Doxorubicin · Cross-linker · Antibody drug conjugate · Bioconjugation · Anthracycline · Chemoselective

Introduction

Bioconjugation has evolved as a key technique in life sciences for the linkage of two or more molecules, from either natural or synthetic origin, to create novel hybrid materials having the combined properties of its individual components (Hermanson 2008; Hackenberger and Schwarzer 2008). These substances encompass diverse complex structures such as peptide-oligonucleotide (Astriab-Fisher et al. 2002), protein-drug (Kratz 2002), polymer-enzyme (Tong et al. 2013) or antibody-nanoparticle (Ackerson et al. 2006) conjugates. Their widespread application in scientific studies not only opened the opportunity to explore fluorescently labeled enzymes as diagnostic tools in clinical testing, but also led to numerous advantages in targeted drug delivery systems (Gudmand et al. 2010; Zhao et al. 2012).

The conjugation between two or more (bio)molecules is typically achieved by the incorporation of mutually reactive groups into the individual components and the subsequent coupling in solution thereby leading to the formation of amide, oxime, hydrazone, thioether or disulfide bonds (Clave et al. 2008; Singh et al. 2006). Two important requirements must be fulfilled for bioconjugation: (1) the coupling reactions have to be performed in aqueous media at ambient temperature, due to the intrinsic sensitivity of many biological systems against organic solvents and elevated temperatures; (2) the conjugation needs to be highly selective for equimolar proportions of the reactants (Aslam and Dent 1998; Mark 2011).

In this regard, cross-linkers have played an undisputed role for the linkage of various biomolecules and have attracted continuous interest in biochemistry, chemical biology and organic chemistry. Heterofunctional cross-linking reagents that contain a carbonyl-reactive and a sulfhydryl-reactive functionality are relatively new and hold potential to conjugate carbohydrate-containing molecules to thiol-containing proteins or macromolecules. Depending on the reactive group used either stable thioether or cleavable disulfide bonds can be created (Smith et al. 2010; Chalker et al. 2009). Cleavable disulfide bonds can be easily achieved by employing cross-linkers that carry thiol-disulfide exchange functional groups like 2-pyridyl disulfides. The reaction of thiols with 2-pyridyl disulfides is well suited for bioconjugation as it undergoes an irreversible disulfide

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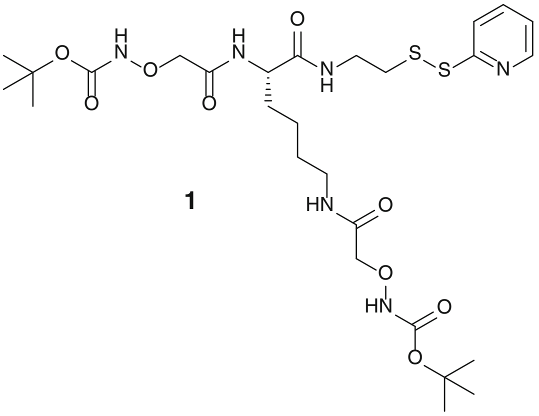

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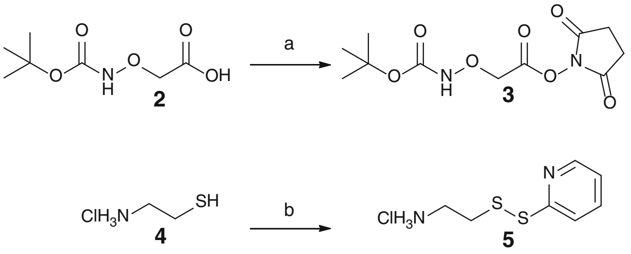
Fig. 1 Heterofunctional cross-linker **1**



Scheme 1 Preparation of the reactive precursors for the modification of lysine. **a** NHS (1.05 eq), DIC (1.15 eq), dichloromethane (DCM), argon, 4 h, r.t., 92 % **b** 2,2′-dipyridyl disulfide (6 eq), methanol, argon, overnight, r.t., quantitative yield

exchange with a free sulfhydryl group and also enables monitoring of the coupling by measuring the characteristic absorbance of the released pyridine-2-thione at 343 nm (Carlsson et al. 1978). Moreover, cleavable disulfides can serve as intracellular delivery systems due to the reductive environment present in the cytosol (Ducry and Stump 2010; Meister and Anderson 1983).

Chemoselective oxime ligation between aminooxy components and aldehydes or ketones is a valuable conjugation reaction that also belongs to the repertoire of bioorthogonal chemistry. The α-effect in aminooxy compounds enables condensation with carbonyls in a highly efficient manner, even in aqueous media (Sander and Jencks 1968). In addition, nucleophilic catalysis of the oxime formation by aniline can further accelerate the condensation, so that complex biological entities can be coupled to each other within a few minutes (Dirksen et al. 2006).

Heterofunctional cross-linkers, which contain three functional groups, are a relatively small but important category of bioconjugation reagents that are often based on the chemical functionalization of an amino acid with at least two bioorthogonal functionalities (Smeenk et al. 2012; Clave et al. 2010). Natural amino acids such as glutamic acid, cysteine or lysine exhibit already different functional groups, however, the preparation of heterofunctional cross-linking reagents often requires enormous synthetic efforts and frequently involves several synthetic steps.

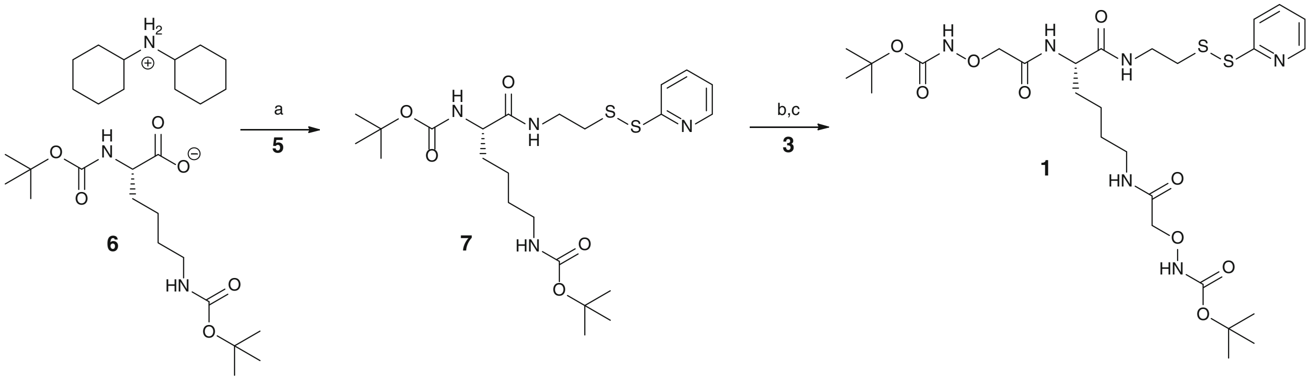
Herein we report the synthesis and characterization of a new heterofunctional cross-linker **1** which employs the scaffold of the amino acid lysine. The reagent carries two aminooxy groups for bioorthogonal modification of carbonyls and a 2-pyridyl disulfide for the chemoselective ligation to thiol-containing biomolecules (Fig. 1). The potency of this cross-linking reagent is demonstrated by the efficient coupling to the anthracycline doxorubicin and immunoglobulin G (IgG).

Results and discussion

Synthesis of lysine-based heterofunctional cross-linking reagent 1

To make the amino acid lysine accessible for bioorthogonal reactions the corresponding carbonyl-reactive functionalities had to be introduced. The preparation of the *N*-hydroxysuccinimide (NHS) active ester **3** from the commercially available (*Boc*-aminooxy)acetic acid was accomplished as described before (Deroo et al. 2003). The activated thiol of cysteamine hydrochloride **4** was obtained by an irreversible disulfide exchange (Scheme 1) (van der Vlies et al. 2010). The reaction was carried out overnight in methanol with an excess of 2,2′-dipyridyl disulfide to avoid side reactions by a stepwise addition of cysteamine hydrochloride. After twofold precipitation in cold diethyl ether, 2-(2-pyridyldithio)ethylamine hydrochloride **5** was obtained in quantitative yield and used without further purification. Compounds **3** and **5** were subsequently coupled to lysine via amide bond formation to create the reactive side chains on the amino acid.

Lysine bears three functional groups, which can be easily and selectively modified if appropriate protective groups are utilized. To prevent side reactions during the amide bond formation either the two amino groups or the carboxylic acid has to be protected. Hence the *N*,*N*′-di-*Boc*-L-lysine dicyclohexylammonium salt was reacted initially with **5** to introduce the activated thiol on the cross-linker precursor **7**. The reaction was carried out in *N*,*N*-dimethylformamide (DMF) with an excess of *N*,*N*-diisopropylethylamine (DIPEA) as base to maintain the free amine of 2-(2-pyridyldithio)ethylamine hydrochloride. The amide bond formation between the protected amino acid derivative and **5** was achieved with a slight excess of *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(*N*-succinimidyl)uronium tetrafluoroborate (TSTU) as coupling reagent. In this initial step the in situ formation of the NHS active ester was mediated by TSTU



Scheme 2 Synthesis of heterofunctional cross-linker **1**. **a** *N*,*N*′-di-*Boc*-L-lysine dicyclohexylammonium salt (1 eq), **5** (1.1 eq), TSTU (1.1 eq), DIPEA (4 eq), DMF, argon, 4 h, r.t., 78 % **b** DCM/TFA (1:1), 1 h, r.t., quantitative yield (c) DIPEA (10 eq), **3** (2 eq), DMF, argon, 3 h, r.t., 81 %

and the desired product was formed within 4 h. To obtain the final cross-linking reagent from **7,** two straightforward consecutive steps were applied as depicted on Scheme 2. At first the α and ε amino groups of the lysine derivative were deprotected. Among the numerous deprotection conditions for the *Boc* group, a solution of equal amounts of dichloromethane (DCM) and trifluoroacetic acid (TFA) was utilized and the deprotection occurred quantitatively within 1 h (Wuts and Greene 2006). After removal of the solvent, the residue was dried for 24 h in high vacuum to remove the remaining TFA. The latter can easily adhere to the oily material causing side reactions, such as deprotection and polymerization of **3** during the subsequent reaction step. The residual oil was dissolved in dry DMF and the pH of the solution was adjusted to 8 with DIPEA. To this solution, two equivalents of **3** were added and the reaction mixture was stirred for 3 h under argon atmosphere. The heterofunctional cross-linker **1** was obtained in high yield as colorless solid, which was characterized, by NMR spectroscopy and MALDI-TOF mass spectrometry. The new linker can thus be synthesized on a gram scale with 58 % yield within four steps from readily available commercial materials. Due to the high reactivity of free aminooxy groups towards omnipresent electrophiles (e.g., laboratory atmosphere) the *Boc* protective group of **1** was cleaved immediately before the corresponding bioconjugation (Viault et al. 2013). Otherwise, the obtained cross-linker can be stored for several months without any decomposition.

Conjugation of **1** to two doxorubicin molecules

To test the efficacy of **1** for bioconjugation, a model drug, namely doxorubicin was attached to the cross-linker. The anthracycline doxorubicin is a widely applied anticancer drug and is an essential component for the treatment of breast cancer, childhood solid tumors, soft tissue sarcomas and aggressive lymphomas (Minotti et al. 2004). However, the therapeutic efficacy of many anthracyclines is restricted by the onset of drug resistance and poor tumor targeting. Different modifications of doxorubicin, like attachment to polymers or targeting molecules, have been carried out to overcome the poor selectivity that results in low therapeutic indexes and substantial toxicities to healthy tissues (Casi and Neri 2012). Doxorubicin bears three different functional groups, which are accessible to chemical modifications: a primary alcohol, an amine and an aliphatic keto group (Fig. 2). The functionalization of the alcohol as well as the amine is often not suitable for biological and therapeutic applications due to the rapid hydrolysis of esters in the blood stream and the reduced DNA intercalation profile of the amine-modified doxorubicin (Mezo et al. 2008). The herein described heterofunctional cross-linker is well suited for the functionalization of doxorubicin utilizing the aliphatic ketone via bioorthogonal oxime bond formation and thus leaving the amine or other functional groups intact.

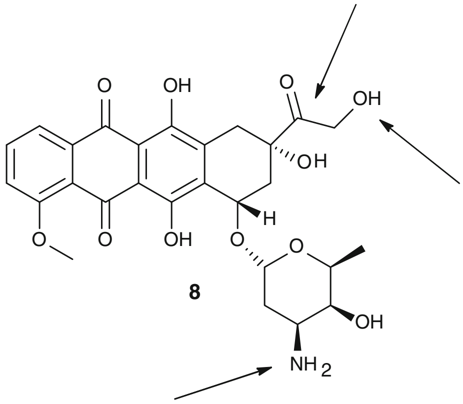
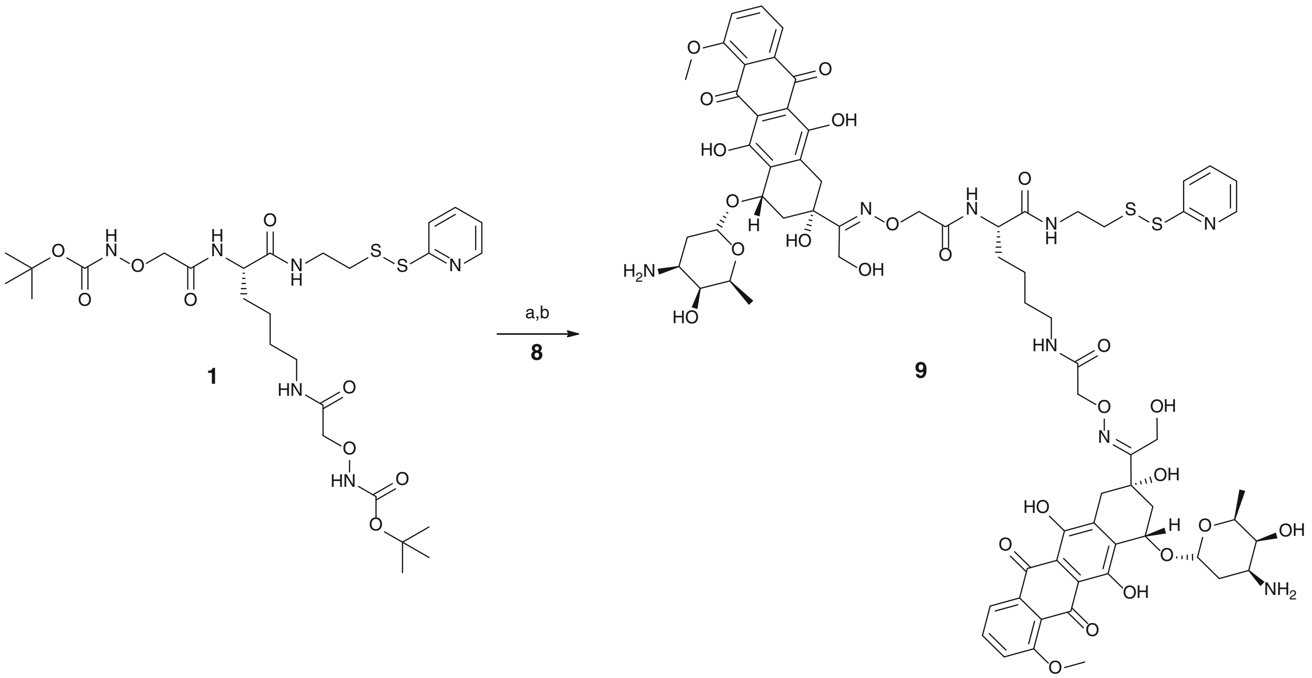


Fig. 2 Reactive sites for chemical modification of doxorubicin

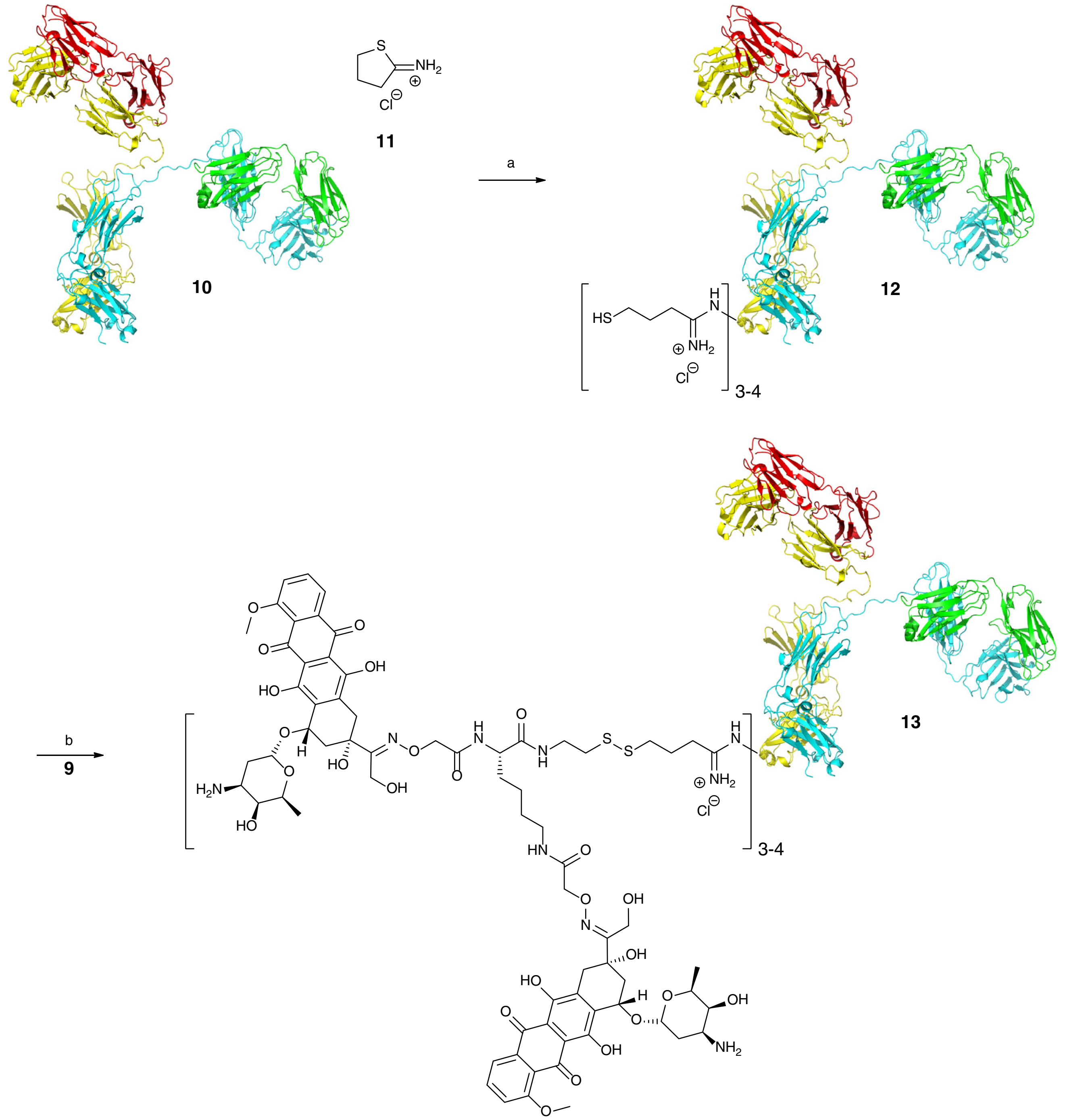


Scheme 3 Bioorthogonal modification of doxorubicin **8** with cross-linking reagent **1**. **a** DCM/TFA (1:1), 1 h, r.t., quantitative yield **b** doxorubicin hydrochloride (2.2 eq), DMF/0.4 M sodium acetate buffer (1:1)—pH 4.8, 24 h, r.t., 71 %

Doxorubicin hydrochloride was dissolved in a mixture of DMF and 0.4 M sodium acetate buffer (pH 4.8) and was added to the deprotected cross-linker. The slightly acidic reaction conditions and the excess of doxorubicin enabled full conversion of the reactants, as seen by reversed phase high performance liquid chromatography (RP-HPLC). The reaction mixture was first purified by gel permeation chromatography (GPC) with Bio-Gel P-2 as stationary phase and triethylammonium acetate (TEAA) buffer (pH 7) as eluent, to remove the excess of doxorubicin. The isolated material was further purified by preparative RP-HPLC to obtain **9** as a red solid in 71 % yield. The product of the bioorthogonal modification **9** was characterized by ^{1}H NMR spectroscopy and mass spectrometry. Additional 2D-NMR experiments (COSY, NOESY and TOCSY) allowed the assignment of all proton signals from the obtained doxorubicin bioconjugate. Moreover, 1 mM solution of **9** in phosphate buffered saline (pH 7.4) was analyzed by RP-HPLC at the characteristic absorption of the drug at 480 nm (supplementary data). The isolated doxorubicin conjugate exhibits a retention time of 23.2 min and a purity of 95.1 % according to the peak integral (Scheme 3).

Site-selective modification of immunoglobulin G

To prove the suitability of the activated thiol-containing doxorubicin bioconjugate for site-selective attachment to proteins, **9** was reacted with thiolated IgG. Antibody drug conjugates play a crucial role in the targeted delivery of drugs to the tumor environment. This emerging class of bioconjugates combines the targeting properties from the antibody with the cytotoxic properties of the utilized drug, mediated by a cross-linking reagent. Usually the cross-linker enables hydrolytic or reductive cleavage of the drug within the tumor site (Ducry and Stump 2010; Alley et al. 2010). The synthesized activated thiol-containing doxorubicin bioconjugate **9** gives the opportunity to access such antibody drug conjugates. Nevertheless, most of the antibodies do not exhibit free sulfhydryl groups for bioconjugation. Typically the cysteine residues of proteins are buried as disulfides, which are very important for the protein structure and function (Brocchini et al. 2008). To make the disulfides accessible for bioconjugation chemistry, reducing agents such as dithiothreitol or *tris*(2-carboxyethyl)phosphine are applied, that can alter the native protein or antibody, respectively. In contrast to disulfides, numerous amino groups from lysine are exposed on the antibody′s surface and they can be selectively modified with 2-iminothiolan **11**, also known as Traut′s reagent, to introduce a precise number of sulfhydryl groups on the proteins surface (Hermanson 2008). These sulfhydryls can be coupled to thiol reactive groups like the 2-pyridyl disulfide of **9**. Therefore, IgG **10** was reacted with 10 equivalents of **11** at 37 °C for one and a half hours in an aqueous buffer solution. This was done according to a literature procedure and led to 3–4 free thiol groups on the antibody's surface (Ji et al. 2010). **12** was obtained in quantitative yield and was always



Scheme 4 Bioconjugation of IgG with **9**. **a 11** (10 eq), 0.1 M PBS buffer (pH 7.2—0.15 M NaCl, 10 mM EDTA), 90 min, 37 °C, quantitative yield **b 9** (20 eq), 0.1 M PBS buffer (pH 7.4), 3 h, 37 °C, quantitative yield

kept in an aqueous buffer solution to prevent degradation. Nevertheless, this solution was not stored, but directly used for bioconjugation with **9** to avoid side reactions involving the free sulfhydryl groups on the antibody's surface. Hence, a solution of **9** in 500 µl of phosphate buffered saline (PBS) was prepared immediately and added to the thiolated antibody, the reaction mixture was incubated for 3 h at 37 °C. To maintain full conversion of the free thiols from **12**, 20 equivalents of **9** were used for the ligation step. Subsequently the excess of **9** was easily removed by GPC on Bio-Gel P-2, due to the intensive red color of the anthracycline doxorubicin. The obtained product fraction containing the doxorubicin carrying IgG **13** was concentrated with ultrafiltration devices (MWCO 10 kDa) and analyzed by sodium

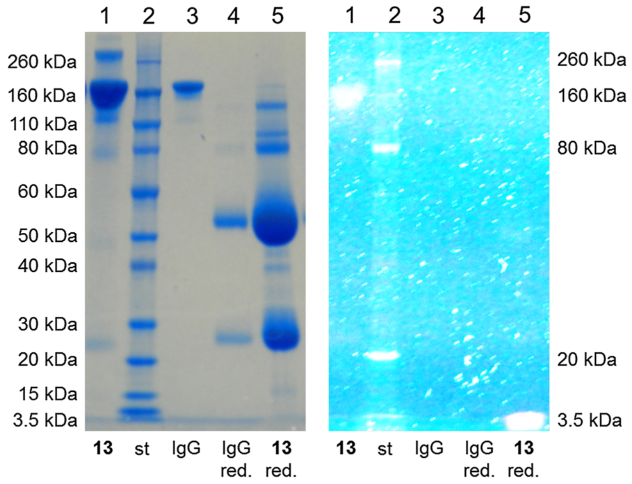


Fig. 3 Comparative study of the antibody drug conjugate **13** and native IgG by SDS-PAGE. *Protein bands* were visualized by Coomassie staining (*left panel*) and fluorescence upon excitation by UV-light (*right panel*)

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Scheme 4).

SDS-PAGE of the antibody drug conjugate **13** is depicted on Fig. 3. The left panel displays the obtained protein bands after staining with Coomassie Brilliant Blue. The intrinsic fluorescence of doxorubicin was employed to visualize drug-related bands after gel electrophoresis (right panel). An appropriate protein ladder for the accomplished SDS-PAGE was applied in lane 2 and the corresponding molecular weights are shown next to the gel. In lane 1 the doxorubicin carrying IgG **13** was analyzed and the achieved intensive band is in good accordance with the obtained band from the utilized native antibody **10** depicted in lane 3 (left panel). Unlike the unmodified antibody the obtained band from **13** was also visible upon UV radiation (bright spot), which distinctly indicates the selective labeling of IgG with the doxorubicin derivative **9**. Furthermore, no low molecular weight doxorubicin-associated substances were detected in the first lane of the right panel, which serves as an efficient proof for the materials purity. The second band from the standard was in good agreement with the molecular weight of IgG (approximately 150 kDa) and **13**. The modification of the thiolated antibody with 3–4 units of **9** led to minor changes in the molecular weight. Nevertheless, **13** exhibited a small band above the product presumably attributed to disulfides of the antibody, which were observed due to the high gel loading to visualize the fluorescence of doxorubicin. Compared to the native antibody, the reduced IgG (lane 4) possessed two explicit bands upon treatment with dithiothreitol. This result was as expected and the obtained bands are corresponding to the molecular weight of the two light (25 kDa) and heavy (50 kDa) chains of the antibody. A similar pattern was observed for the reduction of **13** (lane 5). Moreover, the right panel has shown no fluorescence related to the two different chains, but was clearly visible at the low molecular weight part (bottom) of the gel. Furthermore, no unspecific binding between **9**, as well as doxorubicin, and IgG was observed in a control experiment where the conjugation was carried out in the absence of Traut's reagent (supplementary data). These results from SDS-PAGE display the highly efficient cleavage of the disulfide bond between **9** and **12**, which has been generated by the activated thiol from the cross-linker.

Conclusion

The synthesis and application of a novel amino acid-based heterofunctional cross-linker readily available from commercial sources in excellent yield were described. Bioorthogonal dimerization of appropriate biomolecules with the potential for further modification has been demonstrated. Thereby, stable dimeric drug molecules carrying a protected and activated thiol could be easily prepared and employed in the synthesis of important antibody drug conjugates. The capability of the antibody doxorubicin conjugate as a potential targeted drug delivery system was highlighted by the efficient reductive cleavage of the disulfide bond between the antibody and the drug conjugate, respectively. We believe that this new cross-linker can serve as a powerful reagent in bioconjugation chemistry and will contribute to the rapidly expanding field of chemical biology. This cross-linking reagent can be further employed in additional ligation reactions, involving monoclonal antibodies, oligonucleotides, peptides, carbohydrates, polymers or fluorescent probes.

Experimental section

General

All chemicals, solvents and reagents were purchased from commercial sources (Acros Organics, Alfa Aesar, AppliChem, Deutero, Fisher Scientific, Fluka, Merck, Sigma-Aldrich) and used without further purification. Doxorubicin hydrochloride was purchased from Ontario Chemicals, Inc. (Guelph, Ontario, Canada). Bio-Gel® P-2 was obtained from Bio-Rad Laboratories and immunoglobulin G (100 mg/ml; purity ≥98 %; contains proline and IgA) was a gift from the University Hospital of Mainz. The ultrafiltration devices Amicon Ultra 500 µl (MWCO 10 kDa) were bought from Millipore and the samples were concentrated in an Eppendorf MiniSpin® Plus centrifuge. Column chromatography purifications were carried out on silica gel from

Macherey–Nagel (0.063–0.200 mm) and thin layer chromatography was performed on ALUGRAM® SIL G/UV_{254} sheets with appropriate solvents. ^1H and ^{13}C NMR spectra were recorded on Bruker AMX 300 and Bruker WS 700 (Bruker Avance III) devices. Chemical shifts are expressed in parts per million (ppm) relative to the residual solvent signal: DMSO-d^6 ($\delta_H = 2.50$, $\delta_C = 39.52$) (Gottlieb et al. 1997). Coupling constants (*J*) are given in Hz. MALDI-TOF mass spectrometry was measured on a Bruker Reflex II-TOF spectrometer equipped with a 337 nm nitrogen laser, whereas 2,5-dihydroxybenzoic acid served as a matrix. RP-HPLC was carried out on a Jasco LC-2000Plus System, with appropriate diode array detector (MD-2015) and solvent delivery pumps (PU-2086). Analytical RP-HPLC was conducted with a ReproSil 100 C18 column (250 × 4.6 mm) with 5 μm particle size as a stationary phase and a flow rate of 1 ml/min. Purification of the products was performed on a ReproSil 100 C18 column (250 × 20 mm) with a flow rate of 15 ml/min and 5 μm silica as stationary phase. The applied eluents were 25 mM triethylammonium acetate buffer (pH 7) (A) and acetonitrile (B). RP-HPLC was accomplished with a linear gradient from 0 to 65 % B (40 min). All the substances were detected at 480 nm based on the characteristic absorption of doxorubicin. Incubation of antibody samples was done in an Eppendorf Thermomixer® compact. SDS-PAGE was carried out with NuPAGE® Novex® 4-12 % Bis–Tris Gels (1.0 mm, 12 well) and NuPAGE® MOPS SDS Buffer Kit from Invitrogen. The reduced samples were obtained with NuPAGE® Sample Reducing Agent (10×). Appropriate staining of the antibody bands upon SDS-PAGE was accomplished with Coomassie Brilliant Blue. The obtained antibody bands were compared to the protein ladder "Novex® Sharp Pre-stained Protein Standard" from Invitrogen.

Synthesized compounds

(*Boc*-Aminooxy)acetic acid *N*-hydroxysuccinimide ester (**3**). A slurry of *N*-hydroxysuccinimide (2.79 g, 24.28 mmol) and (*Boc*-aminooxy)acetic acid (4.42 g, 23.12 mmol) in 55 ml of dry DCM was prepared initially. *N,N′*-diisopropylcarbodiimide (3.06 g, 3.76 ml, 24.88 mmol) was added under argon and the clear solution was stirred for 2 h. Additional *N,N′*-diisopropylcarbodiimide (244 mg, 300 μl, 1.99 mmol) was added and the reaction mixture was stirred for further 2 h. Afterwards the precipitated urea was filtered off and washed with a small amount of DCM. The obtained solution was diluted with 200 ml of DCM and washed four times with water. The organic layer was dried with magnesium sulfate and the solvent was removed *in vacuo*, to obtain the product as a colorless solid (6.13 g, 21.27 mmol, 92 %). δ_H(300 MHz; DMSO-d^6) 10.36 (1 H, s, N*H*), 4.82 (2 H, s, O-CH_2), 2.84 (4 H, s, CH_2-CH_2), 1.42 (9 H, s, CH_3); δ_C(75 MHz; DMSO-d^6) 170.0, 165.1, 156.6, 80.6, 69.9, 27.9, 25.5; *m/z* (MALDI-TOF) 357.04 $[M + 3Na]^+$.

2-(2-Pyridyldithio)ethylamine hydrochloride (**5**). 2,2′-Dipyridyl disulfide (25 g, 113.47 mmol) was gradually dissolved in 150 ml methanol and degassed in an ultrasonic bath for half an hour. To this solution cysteamine hydrochloride (2.15 g, 18.91 mmol) was slowly added within 30 min. Afterwards the flask was sealed with a septum and the reaction mixture was stirred overnight under argon. The yellow solution was precipitated twice in cold diethyl ether and the product was obtained as a white crystalline solid (4.21 g, 18.91 mmol, quantitative yield). δ_H(300 MHz; DMSO-d^6) 8.55–8.47 (1 H, m, NC*H*), 8.32 (3 H, br s, NH_3), 7.89–7.72 (2 H, m, SCC*H*, SCCHC*H*), 7.42–7.35 (1 H, m, NCHC*H*), 3.17–3.02 (4 H, m, CH_2-CH_2); δ_C(75 MHz; DMSO-d^6) 158.1, 149.8, 137.9, 121.6, 120.0, 37.7, 34.8; *m/z* (MALDI-TOF) 187.00 $[M + H]^+$.

(*S*)-Di-*tert*-butyl(6-oxo-6-((2-(2-pyridyldithio)ethyl)amino)hexane-1,5-diyl)dicarbamate (**7**). *N,N′*-di-*Boc*-L-lysine dicyclohexylammonium salt (1.52 g, 2.89 mmol), **5** (707 mg, 3.18 mmol) and TSTU (955.9 mg, 3.18 mmol) were dissolved in 35 ml anhydrous DMF. Afterwards DIPEA (1.49 g, 1.96 ml, 11.55 mmol) was added and the reaction mixture was stirred for 4 h at room temperature under argon atmosphere. The suspension was diluted with 250 ml of ethyl acetate and washed three times with 0.2 M hydrochloric acid. Subsequently the clear solution was dried with magnesium sulfate and the solvent was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography using ethyl acetate/hexane (3:1) as eluent, whereby the product was obtained as a colorless oil (1.16 g, 2.25 mmol, 78 %). δ_H(300 MHz; DMSO-d^6) 8.52–8.43 (1 H, m, NC*H*), 8.05 (1 H, t, J = 5.4 Hz, CHCON*H*), 7.88–7.70 (2 H, m, SCC*H*, SCCHC*H*), 7.31–7.18 (1 H, m, NCHC*H*), 6.82 (1 H, d, J = 7.8 Hz, CHN*H*), 6.75 (1 H, t, J = 5.4 Hz, OCON*H*), 3.89–3.73 (1 H, m, C*H*NH), 3.47–3.19 (2 H, m, CH_2CH_2S), 2.98–2.77 (4 H, m, CH_2CH_2S, $OCONHCH_2$), 1.58–1.17 (6 H, m, $CHCH_2CH_2CH_2$), 1.36 (18 H, s, CH_3); δ_C(75 MHz; DMSO-d^6) 172.3, 159.0, 155.5, 155.3, 149.6, 137.8, 121.2, 119.3, 77.9, 77.3, 54.4, 39.6, 37.7, 37.5, 31.6, 29.2, 28.3, 28.2, 22.8; *m/z* (MALDI-TOF) 536.93 $[M + Na]^+$.

Lysine-based heterofunctional cross-linker (**1**). **7** (1.10 g, 2.14 mmol) was dissolved in 15 ml of dry DCM and the equal amount of TFA was added. Afterwards the solution was vigorously agitated for 60 min at room temperature and the reagent was removed in vacuo. The obtained residue was dried for additional 24 h in high vacuum and subsequently dissolved in 25 ml dry DMF.

Consecutively DIPEA (2.76 g, 3.63 ml, 21.37 mmol) and **3** (1.23 g, 4.27 mmol) were added and the solution was stirred for 3 h under argon at room temperature. The reaction mixture was diluted with 300 ml ethyl acetate and washed twice with 0.2 M hydrochloric acid as well as brine. The organic layer was dried with magnesium sulfate and the solvent was removed under reduced pressure. Afterwards the obtained residue was purified by silica gel column chromatography using ethyl acetate/methanol (15:1) and the product was isolated as a colorless solid (1.14 g, 1.73 mmol, 81 %). δ_H(300 MHz; DMSO-d^6) 10.32 (1 H, s, CON*H*O), 10.29 (1 H, s, CON*H*O), 8.50–8.41 (1 H, m, NC*H*), 8.24 (1 H, t, J = 5.4 Hz, CHCON*H*), 8.10 (1 H, d, J = 8.1 Hz, CHN*H*), 7.98 (1 H, t, J = 5.4 Hz, $\mathrm{OCH_2CON}H$), 7.89–7.70 (2 H, m, SCC*H*, SCCHC*H*), 7.29–7.18 (1 H, m, NCHC*H*), 4.38–4.08 (3 H, m, C*H*NH, $\mathrm{NHOC}H_2$), 4.12 (2 H, s, $\mathrm{NHOC}H_2$), 3.49–3.19 (2 H, m, $\mathrm{C}H_2\mathrm{CH_2S}$), 3.08 (2 H, q, J = 6.6 Hz, $\mathrm{NHC}H_2\mathrm{CH_2CH_2}$), 2.89 (2 H, t, J = 6.6 Hz, $\mathrm{CH_2C}H_2\mathrm{S}$), 1.71–1.48 (2 H, m, $\mathrm{CHC}H_2$), 1.47–1.34 (2 H, m, $\mathrm{NHCH_2C}H_2\mathrm{CH_2}$), 1.33–1.21 (2 H, m, $\mathrm{NHCH_2CH_2C}H_2$), 1.40 (18 H, s, $\mathrm{C}H_3$); δ_C(75 MHz; DMSO-d^6) 171.3, 167.9, 167.7, 159.1, 157.0, 156.9, 149.6, 137.8, 121.2, 119.3, 80.6, 80.6, 74.8, 74.6, 52.0, 38.0, 37.8, 37.3, 31.8, 28.7, 27.9, 22.7; *m/z* (MALDI-TOF) 661.11 $[M + H]^+$.

Dimeric doxorubicin bioconjugate (**9**). The cross-linker **1** (217.5 mg, 330 µmol) was dissolved in 10 ml of dry DCM and gradually 10 ml of TFA was added. The solution was stirred for 1 h at room temperature and subsequently the solvent mixture was removed under reduced pressure. Doxorubicin hydrochloride (421.1 mg, 726 µmol) was dissolved in 80 ml DMF/sodium acetate buffer (1:1)—pH 4.8 and was added to the oily residue. Afterwards the reaction mixture was stirred for 24 h at room temperature and the crude product was purified by GPC on Bio-Gel P-2 using 25 mM TEAA buffer (pH 7) as eluent. The isolated fractions were additionally purified by RP-HPLC and the product was obtained as a red solid after precipitation in diethyl ether (354.2 mg, 234.3 µmol, 71 %). δ_H(700 MHz; DMSO-d^6) 8.42–8.38 (1 H, m, NC*H*), 8.17 (1 H, t, J = 4.9 Hz, CHCON*H*), 7.94 (1 H, d, J = 7.7 Hz, CHN*H*), 7.82–7.73 (5 H, m, SCCHC*H*, MeOCCHC*H*C*H*), 7.72–7.66 (2 H, m, $\mathrm{OCH_2CON}H$, SCC*H*), 7.51 (1 H, d, J = 7.0 Hz, MeOCC*H*), 7.47 (1 H, d, J = 7.0 Hz, MeOCC*H*), 7.22–7.17 (1 H, m, NCHC*H*), 5.24–5.17 (2 H, m, CHOC*H*O), 5.16–5.04 (2 H, br s, O*H*), 4.78–4.73 (1 H, m, C*H*OCHO), 4.72–4.66 (1 H, m, C*H*OCHO), 4.46–4.28 (8 H, m, $\mathrm{NOC}H_2$, $\mathrm{N = CC}H_2$), 4.19–4.05 (3 H, m, C*H*NH, MeC*H*), 3.91 (3 H, s, *Me*O), 3.87 (3 H, s, *Me*O), 3.62–3.56 (2 H, m, MeCHC*H*), 3.39–3.20 (6 H, m, $\mathrm{OHCHC}H\mathrm{NH_2}$, $\mathrm{C}H_2\mathrm{CH_2S}$, $\mathrm{OHCCC}H_2$), 3.05–2.89 (4 H, m, $\mathrm{OHCCC}H_2$, $\mathrm{NHC}H_2\mathrm{CH_2CH_2}$), 2.84 (2 H, t, J = 7.0 Hz, $\mathrm{CH_2C}H_2\mathrm{S}$), 2.43–2.32 (2 H, m, $\mathrm{C}H_2\mathrm{CHOCHO}$), 2.24–2.15 (2 H, m, $\mathrm{C}H_2\mathrm{CHOCHO}$), 1.89–1.82 (2 H, m, $\mathrm{NH_2CHC}H_2$), 1.68–1.58 (3 H, m, $\mathrm{NH_2CHC}H_2$, $\mathrm{CHC}H_2$), 1.48–1.41 (1 H, m, $\mathrm{CHC}H_2$), 1.36–1.28 (2 H, m, $\mathrm{NHCH_2C}H_2\mathrm{CH_2}$), 1.20–1.12 (8 H, m, *Me*CH, $\mathrm{NHCH_2CH_2C}H_2$); *m/z* (MALDI-TOF) 1,533.07 $[M + Na]^+$.

Thiolated IgG (**12**). A solution of IgG (5 mg, 0.035 µmol) in 500 µl 0.1 M PBS buffer (pH 7.2) containing 0.15 M NaCl and 10 mM EDTA was prepared. The thiolation reagent **11** (0.96 mg) was dissolved in 1 ml of the same buffer mixture and 50 µl (0.348 µmol) was added to the IgG solution. Afterwards the reaction mixture was incubated in an Eppendorf Thermomixer (300 rpm) at 37 °C for 90 min. This solution was transferred into 500 µl ultrafiltration devices Amicon Ultra (MWCO 10 kDa) and concentrated in an Eppendorf MiniSpin Plus centrifuge (5 min, 10.000 rpm) to 100 µl. Subsequently 400 µl of 0.1 M PBS buffer (pH 7.4) was added and spun again at 10.000 rpm for 5 min. This procedure was repeated one more time and the thiolated antibody was obtained in 100 µl of a viscous buffer solution (5 mg, 0.035 µmol, quantitative yield).

IgG doxorubicin conjugate (**13**). **9** (1.05 mg, 0.695 µmol) was gradually dissolved in 500 µl of 0.1 M PBS buffer (pH 7.4) and added to 100 µl of the thiolated IgG (5 mg, 0.035 µmol) solution. The reaction mixture was incubated in an Eppendorf Thermomixer (300 rpm) for 3 h at 37 °C and subsequently purified by GPC on Bio-Gel P-2 with 0.1 M PBS buffer (pH 7.4). The obtained red product fraction was concentrated in an Eppendorf Mini-Spin Plus centrifuge (10.000 rpm) to 100 µl (5 mg, 0.035 µmol, quantitative yield).

Conflict of interest The authors declare that they have no conflict of interest.

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