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A Cleavable Amino−Thiol Linker for Reversible Linking of Amines to DNA

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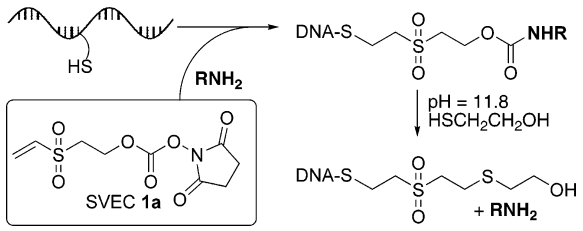
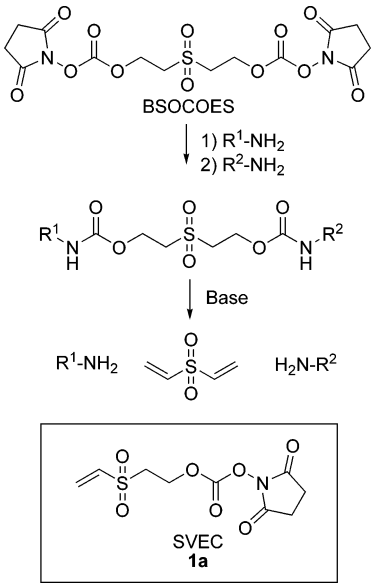


A cleavable heterobifunctional cross-linker for the reversible conjugation of amines to thiol-modified DNA has been developed and tested. The succinimidyl 2-(vinylsulfonyl)-ethyl carbonate (SVEC) was prepared in three steps and tested for its ability to react with amines and thiols. The linker was efficient for binding leucine to a thiol-modified DNA sequence and for releasing the amino acid at pH 11.8.

Introduction

Bifunctional chemical linkers are important for covalently attaching molecules to organic supports such as resins or biomolecules.[1,2] In a part of our ongoing interest in DNA-programmed synthesis,[3] a cleavable amino-thiol cross-linker with the following properties was required: (i) it should be capable of straightforward conjugating an amino functionality to a thiol modified DNA sequence, (ii) it should be cleavable under DNA compatible conditions to release the free amino group and, (iii) after cleavage it should leave a nonreactive functional group at the DNA strand. Several commercially available heterobifunctional linkers fulfill the first demand. They typically contain a *N*-hydroxysuccinimide ester for reaction with the amine and a maleimide, vinylsulfone, pyrimidyl disulfide, α-haloester, or α-haloamide for reaction with the thiol. However, to the best of our knowledge, none of the existing cross-linkers also fulfill the second requirement. The bifunctional linker BSOCOES which is commercially available can almost do the job (Scheme 1).[2] In spite of being an amine−amine linker it is capable of subsequently liberating a free amine by base-catalyzed elimination at a pH of 11−12. However, cleavage of this linker liberates two free amino groups which have to be separated before further chemical functionalization. In work by Liu et al. the BSOCOES linker was applied for attachment of amines to amino-modified oligonucleotide-sequences.[4] To circumvent the presence of two amino groups after cleavage, the oligonucleotide contained a biotin label for removal of the sequence from solution by avidine beads.

SCHEME 1. Chemistry of the Bifunctional Linker BSOCOES[2] and Structure of the New Heterobifunctional SVEC Linker 1a



Described herein is the preparation and application of a new amino−thiol cross-linker, the succinimidyl 2-(vinylsulfonyl)-ethyl carbonate **1a** (SVEC), which fulfills the three requirements listed above (Scheme 1). The linker contains an *N*-hydroxysuccinimide carbonate for coupling to the amine and a vinylsulfone for subsequent coupling to the thiol. We demonstrate that it can be used for the reversible linking of amines and amino acids to thiol-modified DNA sequences.

The linker was synthesized in a straightforward manner starting from 2-mercaptoethanol and vinyl bromide (Scheme 2). The first step of the synthesis was performed according to a method described by Schöberl and Biedermann.[5] The reaction between 2-mercaptoethanol and vinyl bromide involves a

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(2) See www.piercenet.com
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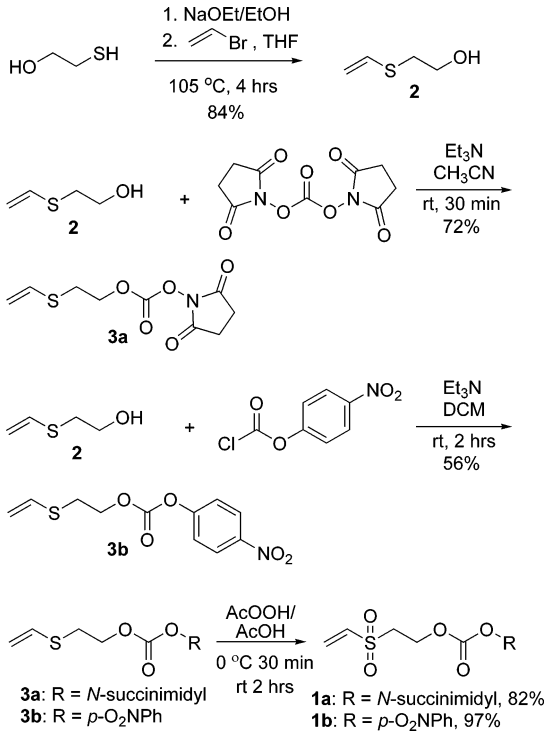
SCHEME 2. Synthesis of the Linkers 1a and 1b



substitution at an unsaturated carbon to give **2**. The 2-mercaptoethanol is first added to an excess of sodium ethanoate. Vinyl bromide, which is a gas at room temperature, was added dissolved in THF, and the reaction was heated in an autoclave at 105 °C. The product was obtained in 84% yield.

In the second step the carbonate is prepared by reaction with *N*,*N*′-disuccinimidyl carbonate.[6−8] The carbonate reacts with **2** in acetonitrile to give **3a** in 76% yield. This is comparable to yields seen in the literature for analogous reactions.[6−8] For comparison we also prepared the corresponding *p*-nitrophenyl carbonate **3b** by a slightly modified procedure.[9,10]

Finally, the sulfides **3a** and **3b** are oxidized to the respective sulfones **1a** and **1b**. The oxidation is performed by treatment of the sulfides with peracetic acid at 0 °C for 30 min and subsequently 2 h at room temperature.[10] The extracted crude products are pure, and no further purification is required. Oxidation of the olefin to the epoxide is not observed. In our first attempt to prepare **1a**, compound **2** was oxidized to the corresponding (2-hydroxyethyl)vinylsulfone; however, the resulting sulfone was unstable and tended to polymerize. By the present procedure, the yield of *p*-nitrophenyl 2-(vinylsulfonyl)-ethyl carbonate (PNVEC) **1b** is nearly quantitative (97%), while SVEC **1a** is obtained in a slightly lower yield of 82%. This is likely due to loss of compound during the extraction, since the succinimidyl group is more polar than the *p*-nitrophenol and has a greater solubility in the aqueous phase. The two sulfone products, **1a** and **1b**, are crystalline, and stable at 4 °C for several months. The synthesis can easily be performed on a 2 g scale.

To investigate the performance of the prepared linkers, reactions with amines and thiols have been carried out. Initial experiments showed that if the linkers were first mixed with a thiol, the thiols preferently react with the carbonate. Thus, the linkers must be reacted with the amine first and subsequently with the thiol. Furthermore, it was observed that the succinimidyl carbonate **1a** was much more reactive toward amines such as, for example, *t*-butyl phenylalanine **4a**, than p-nitrophenyl carbonate **1b**, and **1a** allows reactions to be completed in just 15 min at room temperature. For comparison the reaction of **1b** with **4a** under similar conditions only led to 50% conversion after 2 h as monitored by ^{1}H NMR. Therefore, the succeeding experiments have been performed exclusively with linker **1a**. Experiments also showed that the amines do not react with the vinylsulfone moiety of **1a**. Base is required for the reactions to proceed and it was observed that the linkers and their carbamate products are stable in the presence of weak bases such as diisopropylamine and sodium bicarbonate.

To demonstrate the ability of the linker to attach an amine functionality to a thiol and to provide well-defined products, the linking of three amino acids to benzylthiol was demonstrated (Scheme 3). The goal is to link free amino acids to DNA via the linker as shown in the last part of this Note; however, for the initial experiments the amino acid was *t*-butyl protected at the acid moiety to improve solubility in organic solvents.

Reactions of **1a** with a slight excess of *t*-butyl protected phenylalanine **4a**, leucine **4b,** and tyrosine **4c** on a ∼100 mg scale for 15 min at room temperature were performed (Scheme 3). Analysis of the products by ^{1}H NMR revealed quantitive conversion. Excess of the amines was removed by acidic extraction to provide the products **5a**−**c** in 76−94% yield after purification by column chromatography.

The three amino acid linker conjugates **5a**−**c** were next subjected to reaction with benzylthiol to test the performance of the vinylsulfone. ^{1}H NMR spectra of the three reaction mixtures of the thiol additions showed quantitative reactions with the vinylsulfone after 1 h at room temperature in acetonitrile. Reactions were performed on a ∼50 mg scale, and after purification the products **6a**−**c** were obtained in 67−95% yields.

As shown in the following, the linker is also very useful for attachment of an amino acid to a thiol-modified DNA sequence and for subsequent cleavage to release the amino acid. The 21 bp DNA sequence **8** is obtained from a commercially purchased sequence containing an internal amine spacer at a thymine base (Scheme 4). The amine was extended with a disulfide modifier, and after cleavage of the disulfide the thiol modified sequence **8** was obtained.

The conjugation procedure is straightforward and the first two steps performed in one pot (Scheme 4). The linker **1a** is mixed with 1.5 equiv of leucine to form the linker−amino acid intermediate **7**. After 15 min the DNA sequence **8** is added in a ratio of **7** and **8** of 200:1 and incubated at room temperature for 16 h. The resulting conjugate **9** was purified by HPLC. The shift in mass from **8** to conjugate **9** is verified by MALDI-TOF mass spectrometry. The conjugation of leucine with a 5′-thiol modified oligonucleotide sequence using linker **1a** was also performed (data not shown).

Finally, it was demonstrated that the amino acid can be cleaved from the DNA sequence. Incubating conjugate **9** at pH

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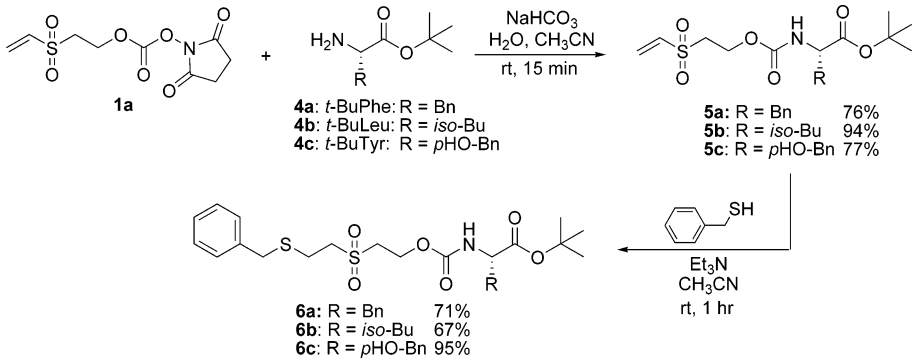
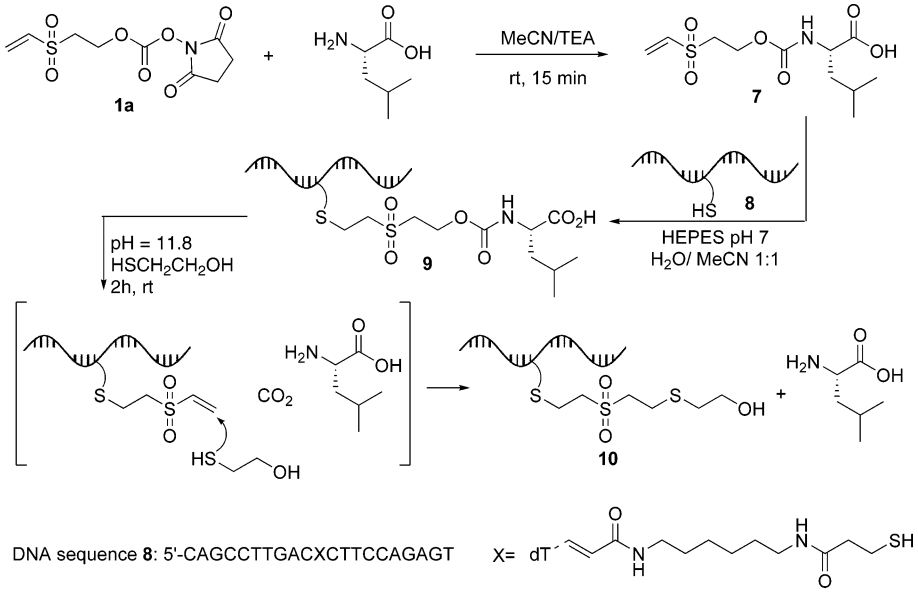
SCHEME 3. Testing of Linker 1a's Ability to Conjugate Amino Acids to Benzylthiol



SCHEME 4. Demonstration of the Attachment of Leucine to a Thiol-Modified DNA Sequence and Subsequent Cleavage of the Linker



11.8 for 2 h in the presence of 2-mercaptoethanol led to cleavage of the carbamate. The intermediately formed reactive Michael acceptor, the vinyl sulfone, is converted by reaction with 2-mercaptoethanol into the corresponding sulfide. Simultaneously, liberation of carbon dioxide from the carbamate leads to liberation of the free amino acid. Cleavage of the carbamate and reaction of the remainder with 2-mercaptoethanol was verified by mass spectrometry after precipitation of **10**.

It is our belief that the SVEC **1a** linker reported here can be useful for conjugation of amines to thiols and biomolecules containing thiols. It may indeed be useful for the transfer of amino acids from one biomolecule to another by reaction of, for example, the carboxylic acid group in **9** (Scheme 4) with an amino group on another sequence followed by cleavage of the linker. In another aspect the vinylsulfone, which is liberated after basic cleavage of the carbamate may, in the absence of 2-mercaptoethanol, be used for conjugation with another thiol.

In conclusion, we have prepared a new type of cleavable amino-thiol linkers SVEC (**1a**) and PNVEC (**1b**). Both linkers are synthesized in three steps from commercially available starting materials. In particular **1a** was efficient for linking amino acids to a thiol as demonstrated in three examples. Most importantly it is straightforward and efficient to conjugate amino acids to thiol-modified DNA by the use of **1a** as demonstrated for leucine. It was furthermore demonstrated that the linker is efficently cleaved at pH 11.8 and that the reactive vinylsulfone residue on the DNA sequence is passivated by reaction with 2-mercaptoethanol.

Experimental Section

Succinimidyl 2-Vinylsulfanylethyl Carbonate (3a). To a stirred solution of **2**[5] (1.100 g, 10.56 mmol) in dry acetonitrile (40 mL), *N*,*N*′-disuccinimidylcarbonate (3.953 g, 15.43 mmol) is added at room temperature. To the reaction mixture, which is a cloudy suspension, triethylamine (3.206 g, 31.7 mmol) is added, and after a couple of minutes the reaction mixture turns clear. Analysis by TLC (eluent: diethyl ether) shows that the reaction is complete after 30 min. The solvent is removed in vacuo. The residue is dissolved in 50 mL CH_2Cl_2 and washed with 3 × 25 mL saturated $NaHCO_3$. The organic phase is washed with 30 mL brine and dried with anhydrous $MgSO_4$, and the solvent is removed in vacuo. The crude product is purified with flash column chromatography to yield **3a** (1.868 g, 7.62 mmol, 72%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.29 (dd, J_c = 10.0 Hz, J_t = 16.8 Hz, 1H), 5.29 (d, J_t = 10.0 Hz, 1H), 5.25 (d, J_t = 16.8 Hz, 1H), 4.45 (t, J = 7.2 Hz, 2H), 3.03 (t, J = 7.4 Hz, 2H), 2.84 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 25.7, 29.3, 69.2, 113.3, 130.7, 151.6, 168.9.[11]

Succinimidyl 2-Vinylsulfonylethyl Carbonate (SVEC) (1a). To a stirred solution **3a** (1.868 g, 7.62 mmol) in glacial acetic acid

(11) We were not able to obtain HRMS or MS of **3a** or **3b**

(4 mL), peracetic acid (3.20 mL of 32% in dilute acetic acid, 15.24 mmol) is added dropwise at 0 °C. After 30 min the cooling bath is removed, and the reaction is continued at ambient temperature. The reaction is followed by TLC (eluent: EtOAc). The sulfone product has a higher retention factor than the sulfoxide intermediate. After 2 h, the only spot seen on the TLC plate is from the sulfone product, and the solvent is removed in vacuo. The residue is dissolved in 45 mL water and extracted with 3 × 45 mL dichloromethane. The organic phase is washed with brine and dried over sodium sulfate, and the solvent is evaporated off. To remove remaining acetic acid, toluene is added to the crude product and evaporated off as an azeotropic mixture to yield the crystalline product **1a** (1.737 g, 6.26 mmol, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.64 (dd, J_c = 9.6 Hz, J_t = 16.4 Hz, 1H), 6.45 (d, J_t = 16.4 Hz, 1H), 6.22 (d, J_c = 9.6 Hz, 1H), 4.65 (t, J = 5.8 Hz, 2H), 3.37 (t, J = 5.8 Hz, 2H), 2.78 (s, 4H). ^{13}C NMR (100 MHz, CD_3CN): δ 26.1, 53.1, 65.1, 131.7, 137.5, 152.0, 170.5. HRMS: *m*/*z* calcd for $C_9H_{11}NNaO_7S$ $(M + Na)^+$, 300.0154; found, 300.0127.

Reaction of *O*-*t*Bu-Leu with 1a (5a). To a solution of SVEC **1a** (102.3 mg, 0.37 mmol) in acetonitrile (5 mL), is added **4a**·HCl (101.0 mg, 0.392 mmol) and water (5 mL). Sodium bicarbonate (66 mg, 0.784 mmol) (1 equiv if the free amino acid is used and 2 equiv if the hydrochloride salt of the amino acid is used) is added, and the reaction is complete within minutes according to TLC analysis. The CH_3CN is removed in vacuo, and the remaining aqueous solution is diluted with brine (5 mL). The aqueous phase is extracted two times with DCM (10 mL). The organic phases are combined, washed with brine, dried with Na_2SO_4, and concentrated in vacuo. The crude product is purified by flash column chromatography (eluent: ethylacetate/pentane 3:2) to give **5a** as a colorless oil in 76% yield (107 mg, 0.28 mmol). ^{1}H NMR (400 MHz, CD_3Cl): δ 7.32−7.15 (m, 5H), 6.61 (dd, J_c = 10.00 Hz, J_t = 16.40 Hz, 1H), 6.42 (d, J_t = 16.80 Hz, 1H), 6.01 (d, J_c = 9.80. Hz, 1H), 5.21 (d, J = 8.00 Hz, 1H), 4.46 (m, 3H), 3.31 (t, 5.60 Hz, 1H), 3.07 (dt, J = 6.00 Hz, J = 10.00 Hz, 2H), 1.42 (s, 9H). ^{13}C NMR (100 MHz, CD_3Cl): δ 28.2, 38.5, 54.1, 55.3, 58.5, 82.8, 127.3, 128.7, 129.7, 130.8, 136.1, 136.9, 154.9, 170.6. HRMS: *m*/*z* calcd for $C_{18}H_{25}NNaO_6S$ $(M + Na)^+$, 406.1300; found, 406.1301.

Addition of Benzylthiol to 5a (6a). To a stirred solution of **5a** (47.2 mg, 0.123 mmol) in acetonitrile (6 mL) and water (1 mL), benzylthiol (18 mg, 0.145 mmol) and triethylamine (14.67 mg, 0.145 mmol) are added. After 1 h acetonitrile is removed in vacuo. Additional water (5 mL) is added and extracted two times with the double volume of diethyl ether (10 mL). The organic phase is dried with Na_2SO_4 and concentrated in vacuo. The crude product is purified by flash column chromatography (eluent: EtOAc/pentane 1:1) to give **6a** as a colorless oil in 71% yield (44.1 mg, 0.087 mmol). ^{1}H NMR (400 MHz, CD_3Cl): δ 7.29 (m, 8H), 7.16 (d, J = 6.8 Hz, 2H), 5.28 (d, J = 8.00 Hz, 1H), 4.50 (q, J = 6.67 Hz, 1H), 4.44 (dt, J = 2.22 Hz, J = 5.78 Hz, 2H), 3.74 (s, 2H), 3.24 (dt, J = 2.26 Hz, J = 5.78 Hz, 2H), 3.08 (m, 3H), 2.83 (m, 2H), 1.40 (s, 9H). ^{13}C NMR (100 MHz, CD_3Cl): δ 23.5, 28.2, 36.9, 38.6, 53.2, 54.3, 55.4, 58.5, 82.8, 127.3, 127.8, 128.8, 129.0, 129.1, 129.7, 136.1, 137.6, 154.8, 170.5. HRMS *m*/*z* calcd for $C_{25}H_{33}NNaO_6S_2$ $(M + Na)^+$, 530.1647; found, 530.1626.

Conjugation of Sequence 8 with SVEC-Leu to Form 8-(SVEC)-Leu (9). A 20 mM solution of leucine attached to the linker **1a** (SVEC) was prepared by mixing the **1a** (80 μL of a 50 mM solution in MeCN) with leucine (100 μL of a 60 mM aqueous solution) in the presence of *N*,*N*-diisopropyl ethylamine (20 μL of a 400 mM solution in MeCN) for 15 min at room temperature. This solution can be stored for several days at 4 °C. Conjugation to an oligonucleotide was achieved by mixing the SVEC-Leu solution (50 μL) with the thiol functionalized oligonucleotide **8** dissolved in 333 mM HEPES buffer pH 7.0. The solution was incubated at room temperature for 16 h. The conjugate **9** was purified by EtOH precipitation, purified by HPLC, and identified by MALDI -TOF mass spectrometry: *m*/*z* calcd for **9** $(M)^+$, 6891.3; observed, 6890.8.

Cleavage of Conjugate 9 under Formation of 10. The linker was cleaved by treating the conjugate **9** (80 pmol) with CAPS buffer pH 11.8 (100 mM) and 2-mercaptoethanol (40 mM) in a total reaction volume of 10 uL. After incubation for 2 h at room temperature, the mixture was diluted to 40 uL with water. EtOH precipitated from ammonium acetate buffer pH 8.6 (2.5 M), and the cleaved conjugate was identified by MALDI-TOF mass spectrometry: *m*/*z* calcd for **10** $(M)^+$, 6794.6; found, 6795.1.

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Supporting Information Available: General experimental procedures, full characterization of all new compounds, procedures for DNA-modifications and purifications, MALDI-TOF spectra, HPLC chromatogram, and copies of ^{1}H NMR and ^{13}C NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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