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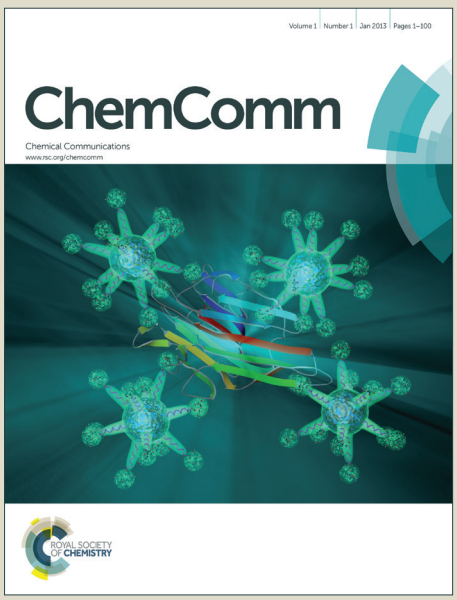

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Propargylamine-Isothiocyanate Reaction: Efficient Conjugation Chemistry in Aqueous Media

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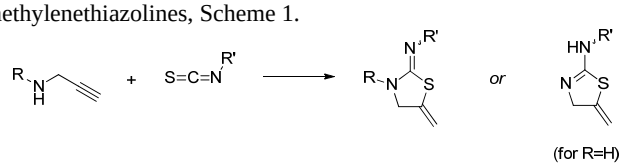
A coupling reaction between secondary propargyl amines and isothiocyanates in aqueous media is described. The reaction is high-yielding and affords cyclized products within 2-24 hours. A functionalized ether lipid was synthesized in 8 steps, formulated as liposomes with POPC and conjugated to FITC under mild conditions using this method.

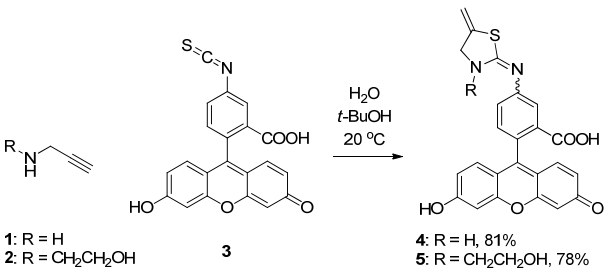
For bioconjugation purposes, and in the development of green chemistry, it is important to have a toolbox of efficient high-yielding reactions that can occur in aqueous media.[1] It is an advantage, if these reactions are stoichiometric in nature, as many bioactive molecules used in conjugation reactions represent a significant investment, financially and/or in terms of effort. Examples of such reactions are the Cu-catalyzed[2] (or strain promoted)[3] Huisgen cycloaddition, the Staudinger ligation,[4] thiol addition to maleimide[5] and amide formation from NHS† esters and amines.[6] Another popular method, which is used frequently for labeling biomolecules with fluorescent dyes, is the reaction between isothiocyanates and amines to afford a thiourea functionality while forging a covalent linkage between the dye and the molecule containing the amino group.[7] We are interested in expanding the conjugation chemistries available and came across an old reaction, which hitherto has only been described in organic solvents, often under forcing condition: the reaction between isothiocyanates (ITCs) and propargyl amines to afford 2-imino-4-methylenethiazolidines or 2-amino-4-methylenethiazolines, Scheme 1.



Scheme 1. Propargyl amine-isothiocyanate reaction to form thiazolidines.

This transformation was first disclosed by Dillard and co-workers in 1964,[8] and later studied by Eloy and Deryckere.[9] In 1976 the group of Honkan[10] published a study on the synthesis of different thiazolidine derivatives related to clonidine, a compound with antihypertensive properties and CNS depressants activity. They tested a variety of different propargyl amine derivatives which in general yielded the 4-methylenethiazolidine product, but in some cases a small quantity of endocyclic double bond isomers was isolated and for two reactions this was the sole product observed. We were interested in studying whether this transformation could be used as a conjugation method in aqueous media, since isothiocyanates are often used for functionalizing amines and a number of ITC-functionalized fluorophores are commercially available. Here we report the successful application of this reaction in water and demonstrate, as an example of its use, that it is possible to functionalize liposomes with a fluorophore in a very convenient manner using this conjugation method. We also evaluate the scope of the reaction, with regards to different buffer systems and a polyfunctional decapeptide carrying a propargyl amine.

Mixing propargyl amine (**1**) and fluorescein isothiocyanate (FITC, **3**) in water/tert-butanol 3:2 and stirring for 4 hours afforded the desired thiazolidine product **4** in 81% isolated yield after column chromatography (Scheme 2). Due to the modest solubility of FITC in water, initial investigation of the reaction was done in mixed organic/aqueous solvent systems The thiourea product was not formed and no evidence of alkene isomerization to the iminothiazole was observed.



1: R = H
2: R = CH_2CH_2OH

3

4: R = H, 81%
5: R = CH_2CH_2OH, 78%

Scheme 2. Initial reactions with FITC.

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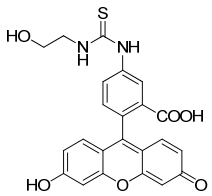
Having established the feasibility of this transformation in the presence of water, we next tested the performance of a secondary amine, *N*-propargyl ethanolamine (**2**).[11] Under identical reaction conditions, the reaction was done in 2 hours and **5** was subsequently isolated in 78% yield.

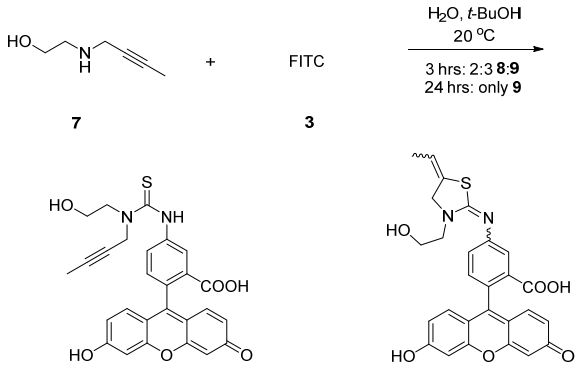
Based on these initial promising results we decided to investigate whether the thiazolidene formation was competitive with the formation of thioureas in the presence of a primary amine as a competing nucleophile. Incubation of FITC with a 1:1 mixture of ethanolamine and propargyl amine **2** afforded a 1:10 mixture of thiourea **6** (Figure 1) and thiazolidine **5**, demonstrating that the thiazolidene formation was the dominant reaction pathway.



6

Figure 1. Minor thiourea product formed from ethanolamine.

We also subjected 2-(but-2-ynylamino)ethanol **7** (ref. 12) to our standard reaction conditions and after 3 h isolated a 2:3 mixture of the thiourea **8** and the thiazolidine **9** (Scheme 3). Increasing the reaction time to 24 h led to complete formation of the thiazolidine with no thiourea product detected, which strongly indicates that the reaction takes place in a step-wise fashion where the thiazolidene is formed from an initial thiourea intermediate.



Scheme 3. Experiment with an internal triple bond.

The performance of the reaction in a range of aqueous buffers was also evaluated. The results are summarized in Table 1 and reveal some interesting differences.

Table 1. Competition experiments in aqueous solvents.[a]

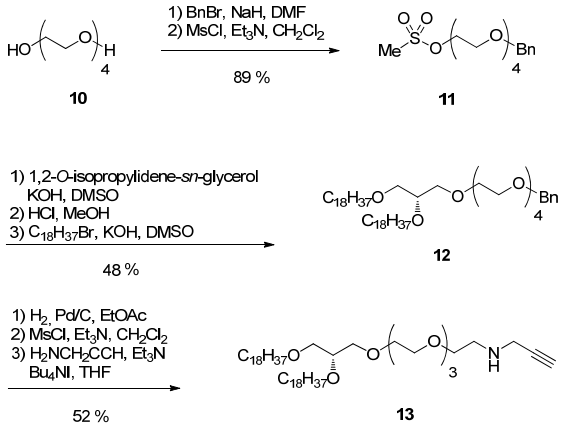
Entry	Solvent	Conversion FITC 24h	Ratio 5:6
1	H_2O	72 %[b]	2:1
2	HEPES, pH 7.0	96 %	2:1
3	Borate, pH 8.3	>98 %	3:1
2	PBS, pH 7.4	>98 %	9:1

[a] Mixtures of ethanolamine (1 equiv.), propargyl amine **2** (1 equiv.) and FITC (0.5 equiv.) in four different aqueous solutions (0.5 mM FITC) were reacted at 20 °C and monitored by HPLC and LC-MS (see supporting information). [b] Conversion >98% after 48h.

The reaction is generally complete after 24 h, but in non-buffered water, it is slower (entry 1). The selectivity between the thiazolidine product **5** and the thiourea **6** ranged between 2:1 (entry 1 and 2) and 9:1 (entry 4), performing best in PBS buffer.

We next wanted to test whether this reaction could be used for bioconjugation under mild, aqueous conditions at ambient temperature. Liposome surface functionalization was chosen as model system since it assesses both the performance in buffer and the feasibility of conjugation to nanoparticles. We decided to synthesize the propargyl amine functionalized lipid **13** that could be incorporated in a lipid bilayer and subsequently conjugated to FITC. The synthesis can be seen in Scheme 4 and started from tetraethylene glycol **10**, which was monobenzylated and mesylated to afford **11** in 89% yield, an improvement over a published method.[13] Reaction of isopropylidene glycerol and **11** followed by deprotection and alkylation with 1-bromooctadecane yielded the *sn*-3 PEGylated dietherlipid **12**. Hydrogenolysis, mesylation and nucleophilic displacement with propargyl amine afforded the desired lipid **13**.

The propargyl amine functionalized lipid **13** (1 %) was mixed with POPC (99 %) and formulated as liposomes by extrusion in PBS buffer using the dry lipid film technique,[14] yielding clear dispersions. The particle size of the formulated lipids was measured by DLS. The formed liposomes had a mean diameter of 145 nm (PDI: 0.245). The functionalized liposomes (c = 12.5 mM) were incubated with FITC (31.25 µM; 0.5 equiv.) and samples were taken for HPLC analysis after 5, 30, 60, 120, 240 and 1440 minutes. The analysis showed that after 5 min, more than 50 % of the FITC (**3**, RT = 6.0 min) had been converted to a major (RT = 18.6 min) and a minor (RT = 17.0 min) product. After 4 h, all the FITC had been converted to the two products and after 24 h, only the fast eluting (RT = 17.0 min) product was detected, see Figure 2. We performed the reaction between the propargyl amine functionalized lipid **13** and FITC in solution (see supporting information), which enabled us to identify the faster eluting compound (RT = 17.0 min) as the desired product. HPLC-ESI-MS showed that both peaks had the same mass, 1198.8, which allowed us to assign the peak at RT = 18.6 min as the intermediate thiourea. The integrity of the liposomes after 24 h incubation with FITC were verified by DLS and showed no significant change in the average size or polydispersity of the particles (mean diameter: 148 nm, PDI: 0.231). Fluorescent microscopy of FITS-functionalized liposomes can be seen in Figure S1 in supporting information.



Scheme 4. Synthesis of the propargyl amine functionalized lipid **13**.

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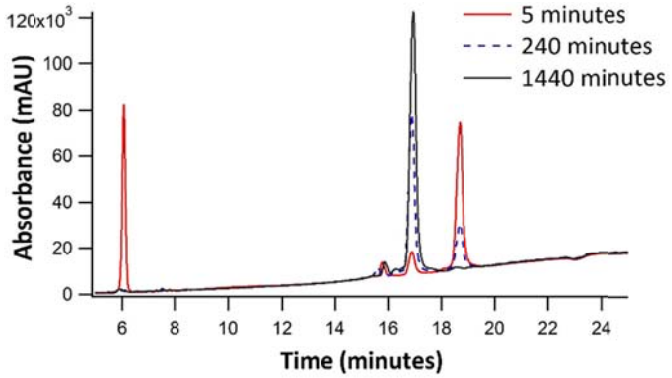
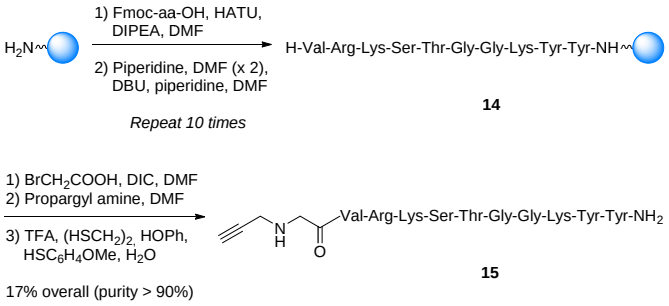


Figure 2. Example of the monitoring of a conjugation reaction over time by HPLC at 254 nm. The decreasing peak with RT=6.0 min belongs to the free fluorescein isothiocyanate (FITC, **3**), while the peaks with RT=17.0 min and RT=18.6 min are the 2-imino-4-methylenethiazolidine and the intermediate thiourea, respectively.

For conjugation purposes it is common that several functional groups are present and we wanted to evaluate the scope and limitation of the approach reported here. Therefore, we prepared the protected resin-bound decapeptide **14**, see Scheme 5 (side-chain protection groups omitted for clarity). This was functionalized through reaction with bromoacetic acid and propargyl amine[15] and cleaved from the resin with Reagent K[16] to afford decapeptide **15**. The eight amino acid residues from the *N*-terminal end are identical to residues 7-14 in histone class H3 tails, known for a high number of lysines.[17] The C-terminal tyrosines were added to ease detection by HPLC-DAD.



Scheme 5. Synthesis of the propargyl amine functionalized peptide **15**.

When peptide **15** was reacted with an excess of FITC in PBS buffer, full conversion of the peptide was observed and products of both mono- and difunctionalization was observed. In contrast, when FITC was the limiting reagent, only monofunctionalization was observed, which could be confirmed by MALDI-TOF-MS (see supporting information). However, it was also clear from these experiments that the reaction did not have a high selectivity for the propargyl amine. Three main products could be detected by LC-MS in an approximate ratio of 1:1:2. Trypsin digestion of the product mixture confirmed that the *N*-terminal thiazolidine was formed as a major product (see supporting information).

In summary, the results described here offer a new way of conjugating isothiocyanates, exemplified by FITC, to propargyl amine functionalities. The reaction is atom economic, takes place under mild reaction conditions in aqueous solutions without the need for additives or catalysts and is compatible with complex structures as seen for the functionalized lipid **13** incorporated in the bilayer of liposomes. Our investigations also show that in the presence of several competing nucleophiles, such as the primary amine of lysine side-chains, the selectivity of the reaction is limited. Nonetheless, we believe this convenient reaction can find use in conjugation chemistry where mild reaction conditions are required.

Acknowledgements

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Notes and references

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† Abbreviations: CNS, central nervous system; DAD, diode-array dectector; DIPEA, diisopropylethylamine; DLS, dynamic light scattering; ESI-MS, electrospray-ionization mass spectrometry; FITC, fluorescein isothiocyanate; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HPLC, high-performance liquid chromatography; ITC,,isothiocyanate; MALDI-TOF-MS, matrix-assisted laser-desorption mass spectrometry; NHS, *N*-hydroxysuccinimide; PBS, phosphate buffered saline; PDI, polydispersity index; PEG, polyethylene glycol; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RT, retention time, TFA, trifluoroacetic acid,

Electronic Supplementary Information (ESI) available: Detailed description of the synthetic approach to compounds **4**–**9** and **11**–**13** and their characterization. See DOI: 10.1039/c000000x/

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