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# Conjugation of carbohydrates to proteins using di(triethylene glycol monomethyl ether) squaric acid ester revisited

Peng Xu, Michael N. Trinh, Pavol Kováč*

*NIDDK, LBC, National Institutes of Health, Bethesda, MD 20892-0815, USA*

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ABSTRACT

Properties of di(triethylene glycol monomethyl ether) squarate relevant to conjugation of carbohydrates to proteins have been reinvestigated and compared with those of dimethyl squarate. It is concluded that the commercially available, crystalline dimethyl squarate remains the most convenient and efficient reagent for conjugation of amine-containing carbohydrates to proteins by a two-step or one-pot conjugation protocol.

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The method for coupling two amines through the squaric acid residue was introduced in Germany by Tietze's group in 1991 [1]. The conjugation takes place in two steps (Scheme 1). First, at neutral conditions, the first amine reacts with a squaric acid diester to form a monoamide monoester. The latter can react at basic conditions, for example pH 9 [1]. With the same or a different amine to form a diamide. The same laboratory soon recognized [2] that when one of the amines is a functionalized carbohydrate and the other one is a protein important tools in the life sciences, the neoglycoconjugates, could be obtained. The potential of the method was initially not duly recognized, and it took quite a while before this method was rediscovered [3] and then became widely used. Currently, the squaric acid chemistry-based conjugation is considered one of the most powerful methods for making glycoconjugates [4–6]. Many experimental vaccines and tools for the life sciences have been prepared in this way. Although other squaric acid diesters have been used [7,8], dimethyl (e.g. Refs. [9–12]) and diethyl (e.g Refs. [13–22]) squarates have been the most popular reagents in this regard.

We concluded [11] previously that among squarate diesters currently in use, the commercially available dimethyl squarate (**1**, Fig. 1) was the most convenient reagent for making glycoconjugates. Here, the term 'squarate reagent' represents squaric acid diesters and the term 'conjugation reagent' is used to describe monoamide monoesters formed from a diester and an amine.

When treated with squarate reagents, amines readily yield conjugation reagents which are, unfortunately, prone to saponification at the conditions of conjugation (pH $\geq$ 9). Thus, conjugation requires use of variable excess of the monoester reagent. When conjugating small oligosaccharides, the rate of the conjugation is relatively high, and the reaction usually takes only a few hours to complete. Therefore, large excess of the conjugation reagent is not required. However, when larger oligosaccharides or polysaccharides are being converted, because of the slower reaction rate, considerable excess of the conjugation reagent is necessary, which eventually ends up as expensive waste.

Wurm et al. [23] have introduced a novel squaric acid-based water-soluble squarate reagent, squaric acid di(tri(ethylene glycol) monomethyl ether)ester (**2**, Fig. 1). They describe **2** as less prone to hydrolysis than **1** and used the former in a one-pot ligand–protein conjugation in aqueous reaction medium. While one-pot conjugation in water was reported before [10], we were intrigued by the claimed outstanding hydrolytic stability of conjugation reagents made from **2**. Having a more stable conjugation reagent would be beneficial in connection with our squaric acid chemistry-driven development of glycoconjugate vaccines from synthetic [12,24] and particularly bacterial [22,25,26] carbohydrates.

Careful examination of the just cited communication [23] revealed that the studies with **2**, including hydrolysis kinetics,

* Corresponding author.
*E-mail address:* kpn@helix.nih.gov (P. Kováč).

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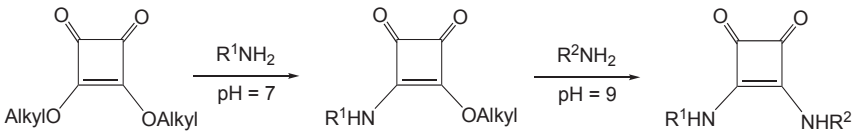


**Scheme 1.** Conjugation of amines by squaric acid diester chemistry.

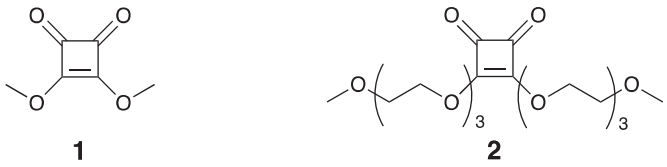


**Fig. 1.** Structures of squaric acid dialkyl esters **1** and **2.**

were performed with crude, unpurified material. This prompted us to firstly, prepare reagent **2** in pure state (c.f. Fig. 2 for comparison of the relevant part of the $^1$H NMR spectra of **2** [23] with the reagent prepared as described here) and characterize it, secondly, to verify the hydrolytic stability of conjugation reagent prepared from **2** at the conditions of conjugation and, thirdly, compare the utility of the conjugation reagents made from **1** and **2** for making neoglycoconjugates.

Accordingly, compound **2** was prepared from squaric acid (**3**) and alcohol **4** as described [23] (Scheme 2) and obtained in the analytically pure state, after chromatography. It is worth mentioning that due to its high boiling point, compound **4** used in excess during the preparation could not be completely removed without chromatography. That also afforded a small amount of **5**, which originated from the impurity in the commercial reagent used. Thus, chromatography, which is in this case not a trivial task and was not included in the previous protocol [23], is necessary in order to obtain compound **2** in the pure state. Alcoholic solvents should be avoided during chromatography, to prevent formation of transesterification products. When MeOH was used for purification, variable amount of by-product **6** was isolated. Eventually, MeCN–$CH_2Cl_2$ was used for the purification of **2**.

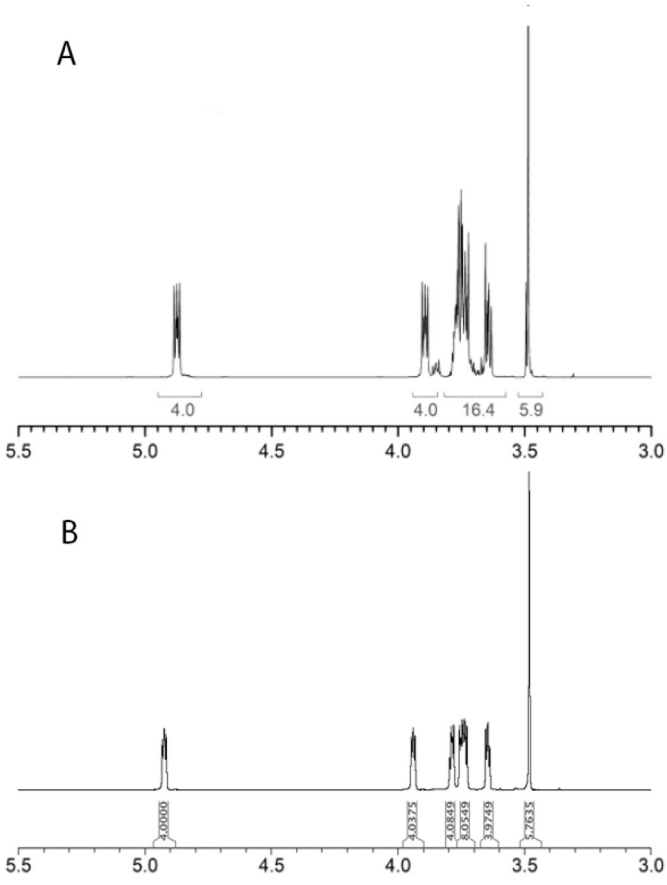


**Fig. 2.** Comparison of the $^1$H NMR spectra of **2. A** Material reported [23]. **B** Material described here.

A comparison of literature data [23,27] shows that the hydrolytic stability of squarates **1** and **2** is comparable. About 90% of **1** was found to hydrolyze to the corresponding monoacid after 16 h in 0.5 M pD 7 buffer [27], while it was reported [23] that over 70% of **2** hydrolyzed after ~ 13 h when kept at pD 7 (NMR). However, because both **1** and **2** are relatively cheap commodities, the stability of conjugation reagents at the conditions of conjugation is much more important than that of dialkyl squarate reagents. Therefore, we next evaluated the hydrolytic stability of conjugation reagents prepared from **1** and **2**. Wurm et al. reported [23] that conjugation reagent **7** (Fig. 3) made from compound **2** has an estimated half-life in 0.01 M pD 9.5 buffer of 11–12 days. They concluded, from the known [11] half-life data for **8** (2–3 days) that the squarate monoester amides based on squaric acid di(tri(ethylene glycol) monomethyl ether)ester **2** are more stable than those based on **1**, and are, therefore, more useful conjugation reagents. However, the comparison presented and the conclusions they made [23] are unsound because the hydrolytic stabilities of the two families of conjugation reagents were determined at different conditions [11,23]. When Hou et al. tested the stability of **8** (Fig. 3) [11] the reaction medium was a 0.5 M pH 9.0 buffer, where the pH remained relatively stable during the course of the hydrolysis. However, Wurm et al. performed the stability experiments in 0.01 M pH 9.5 buffer. Considering the amount of **7** (43 μmol) and the amount of buffer used (0.7 mL), the capacity of the buffer must have gradually become insufficient as the hydrolysis of **7** progressed. As a result, the pD must have fallen well below 9.5 (this was later proved experimentally, see below). Also, it is not clear why they determined the stability of **7** in 0.01 M buffer (pH 9.5) when they performed the conjugation with the same reagent [23] in 0.1 M buffer (pH 9.1).

In order to objectively compare the hydrolytic stability of the two conjugation reagents discussed, we determined stability of conjugation reagents **8** and **10** at pH 9.5 in 0.01 M buffer [23] and also at the conjugation conditions we normally use (0.5 M pH 9.0 buffer). Compound **10** was obtained by derivatization of amine **9** [11] with squarate reagent **2** (Scheme 3). The reaction was uneventful and compound **10** was obtained in 70% yield. In parallel experiments, reagents **8** and **10** were treated under the two different conditions just mentioned (Fig. 4). The progress of the hydrolyses was judged ($^1$H NMR) by monitoring the decrease of the intensity of the squarate ester peaks at δ ~ 4.3 (the signal for the vinylogous methyl squarate group in the squarate derivative **8** appears as two singlets [28]) and at 3.4 ppm (singlet for the methyl group at position 19′ in **10**).