

Squaric Acid Diethylester: A Simple and Convenient Coupling Reagent

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Z. Naturforsch. **46c**, 498–501 (1991); received March 6, 1991

Squaric Acid Diethylester, Squaric Acid Monoamides, Squaric Acid Diamides, Bifunctional Coupling Reagent, Covalent Modification

A new method for the covalent modification of polymers in a two-step process is described. Squaric acid diethylester reacts with primary or secondary aliphatic amines selectively to ester amides. It is shown for the first time that this class of compounds react with polymeric systems containing amine functionalities (*e.g.* ϵ -amino groups of proteins) in aqueous solution, forming stable squaric acid diamide linkages. Three different monoamides were synthesized and the reaction rates of diamide formation versus hydrolysis were determined. Detailed kinetic studies revealed that unwanted hydrolytic side-reactions are more than ten times slower than the formation of squaric acid diamides.

Introduction

The development of suitable procedures for the covalent coupling of biopolymers (*e.g.*, antibodies or enzymes), cell membranes or other cellular macromolecules to synthetic chemicals or naturally occurring compounds is of general interest for basic research as well as for diagnostic or therapeutic applications. Our group is engaged in developing new strategies for site-directed drug delivery *in vivo* based, *e.g.*, on tumor-selective pH-reduction [1–6], in order to achieve drug-prodrug conversion preferentially in malignant tissues. In this context, acid-sensitive chemical linkers between tumor-targeting monoclonal antibodies (Mab) and low and high molecular weight cytotoxic compounds need to be designed for acid-catalyzed release of the toxic molecules *in situ*. Covalent coupling of acid-sensitive structures under slightly alkaline conditions requires particularly gentle methods. Here we report on the use of squaric acid diethylester (**1**) as a versatile coupling reagent and describe suitable

reaction conditions for linking relevant molecules to biopolymers.

Materials and Methods

¹H NMR: Varian XL 200, VXR 200 (internal TMS). – MS: Varian MAT 311 A; high resolution: Varian MAT 731. – IR: BIO-RAD FTS-7. – UV: Hitachi 150-20 spectrophotometer. – Melting points: Büchi 510. – HPLC: Du Pont Instruments 850, Waters 600 E System. – Detection systems: Photodiode array detector 990, Waters 481 wavelength detector. Columns: Lichrosorb RP 18, 10 μ m; solvent system: A: 0.1 M ammonium formate (pH 5.0), B: 100% methanol (flow rate, 1 ml/min); Protein PAK 200 (300) sw (Waters); isocratic solvent system: 0.1 M Na₂SO₄, 0.02 M NaH₂PO₄, pH 6.8 (flow rate, 1 ml/min).

Chemicals: Squaric acid diethylester and *p*-nitrobenzylamine were obtained from Aldrich, Steinheim, F.R.G.; bovine serum albumin (BSA), poly-D-lysine, and biocytin (ϵ -lysine-biotin) were from Sigma, München, F.R.G. All chemicals were of analytical grade.

Preparation of squaric ester monoamides (5, 6 and 7): To a solution or suspension of the amine *p*-nitrobenzylamine (HCl-salt, 1 mmol; 188.6 mg) biocytin (1 mmol; 372.5 mg) or 2',3'-O-isopropyliden-5'-amino-deoxyadenosine synthesized according to [12] (1 mmol; 430.0 mg) in 5 ml dry ethanol, 2.5 equiv. of squaric acid diethylester and 1–2 equiv. of triethylamine were added under stirring at room temperature. The progress of the reactions was monitored by HPLC. Reactions were completed after 2–24 h. After removal of the solvent, white crystalline solids were obtained: Amidester (**5**): m.p. 140 °C (dec.). UV Ig ϵ , nm: MeOH 4.44, 272. IR λ_{\max} , cm⁻¹: KBr 1820, 1710, 1680. ¹H NMR ([D₆]DMSO): δ 1.40 (3H, t, J = 7.6 Hz, OCH₂CH₃), 4.75 (4H, m), 7.50 (1H, d, J = 9.0 Hz), 8.25 (1H, d, J = 9.0 Hz), MS (rel. int.): m/z 276 (40) (calcd. for C₁₃H₁₂N₂O₅ 276.0746; found 276.0746).

Amidester (**6**): m.p. 87 °C (dec.). UV Ig ϵ , nm: MeOH 4.34, 271. IR λ_{\max} , cm⁻¹: KBr 1818. ¹H NMR ([D₆]DMSO): δ 1.35 (3H, m, CH₃), 1.55–1.80 (12H, m), 2.25 (2H, t, J = 7.0 Hz), 2.78 (1H, d, J = 13 Hz), 2.91 (1H, m), 2.99 (1H, dd, J = 5.0, 13.0 Hz), 3.19 (1H, t, J = 7.0 Hz), 3.33 (1H, m), 3.72 (1H, t, J = 7.0 Hz), 4.20 (2H, m), 4.42 (1H, m), 4.55 (1H, m), 4.70.

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Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0500–0498 \$ 01.30/0



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Amidester (7): m.p. 127 °C (dec.). UV Ig ϵ , nm: MeOH 4.53, 257. IR λ_{\max} , cm^{-1} : KBr 1830, 1700, 1630. ^1H NMR (200 MHz, $[\text{D}_6]\text{DMSO}$): δ 1.25 (3H, m, OCH_2CH_3), 1.35 (3H, s, CH_3), 1.55 (3H, s, CH_3), 3.70 (2H, m), 4.31 (1H, m), 4.55 (2H, m), 5.10 (1H, dd, $J=3.5, 6.5$ Hz), 5.43 (1H, dd, $J=2.5, 6.5$ Hz), 6.18 (1H, d, $J=2.5$ Hz), 7.4 (2H, s, br), 8.25 (1H, s), 8.32 (1H, s), 8.90 (1H, m). MS (rel. int.): m/z 430 (20) (calcd. for $\text{C}_{19}\text{H}_{22}\text{N}_6\text{O}_6$ 430.1600; found 430.1600).

Kinetic measurements

(i) Hydrolysis and diamide formation

Initial concentrations of **5**, **6**, and **7** were 1.5×10^{-5} mol/l and of poly-D-lysine (MW: 40,000, Lot. Nr. 57 F-5030) 1.5 mg/ml buffer, respectively. All reactions were carried out in 150 mM salt solutions at 37 °C, and monitored and recorded continuously by spectrophotometry (hydrolysis: 285 nm; conjugation: 300 nm) until the endpoints were reached. The pH of the solution after the reaction was identical to the initial pH. All time-concentration plots over the pH range 8–11 were found to be *pseudo* first-order, and the values of k_{obs} were obtained from linear plots. For convenience, the half-life values of all reactions were calculated according to $t_{1/2} = 0.693/k_{\text{obs}}$.

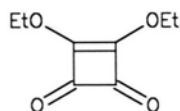
(ii) Coupling to BSA

Initial concentrations of BSA and **6**: 4.5 mg/ml buffer. All coupling experiments (at pH 7.0, 8.0 and 9.0) were carried out in 150 mM salt at 37 °C. Aliquots were taken at different times and subjected to gel filtration (Protein PAK 200 sw, Waters). The photodiode array detection system was used to calculate the biotin/BSA molar ratios as a func-

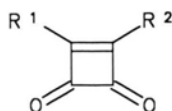
tion of time and pH (wavelength 220–350 nm and internal standards).

Results and Discussion

It is well known [7–8], that 3,4-diethoxycyclobut-3-en-1,2-dione (**1**) (squaric acid diethylester) reacts selectively and under very mild reaction conditions with primary (or secondary) amines to give stable monoamides (**2**), and with an excess of amines to give stable vinylogous squaric acid diamides (**3**) [9]. However, very little is known [10] about the kinetics of diamide formation (**3**) and the hydrolytic reaction to the monoamide (**4**) in *aqueous buffer solutions*. To evaluate the kinetics of diamide formation (conjugation with poly-D-lysine) *versus* hydrolysis, 3 different monoamides were synthesized: a benzylamine derivative (**5**), a substituted biotin compound (**6**), and an adenine derivative (**7**). UV-spectroscopy was the method of choice because of the characteristic difference of the respective UV-spectra along with the high extinction coefficients of the squaric acid moiety. The formation of diamide is accompanied by a bathochromic shift of the UV maxima from 275 to about 300 nm. In contrast to diamide formation, the hydrolytic side-reactions only exhibit a bathochromic shift of about 5 to 10 nm. In Fig. 1 the pH-rate profiles for diamide formation are shown in comparison with the profiles for the hydrolytic side-reaction. All experiments were carried out under *pseudo* first-order reaction conditions. Over the pH range investigated, the observed *pseudo* first-order rates of both hydrolysis and diamide formation are a linear function of the pH, indicating that the coupling reaction (diamide formation) is at least one order of magnitude faster than the unwanted hydrolytic side-reactions. All



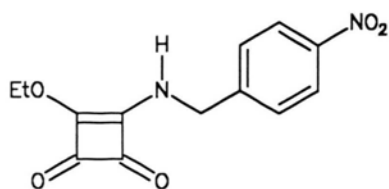
1



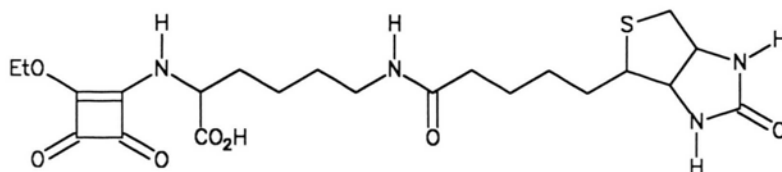
2: $\text{R}^1 = \text{OEt}$, $\text{R}^2 = \text{NH-ALK}$

3: $\text{R}^1 = \text{NH-ALK}$, $\text{R}^2 = \text{NH-ALK}$, POLYMER-NH

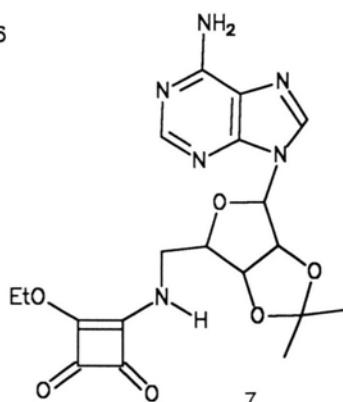
4: $\text{R}^1 = \text{OH}$, $\text{R}^2 = \text{NH-ALK}$



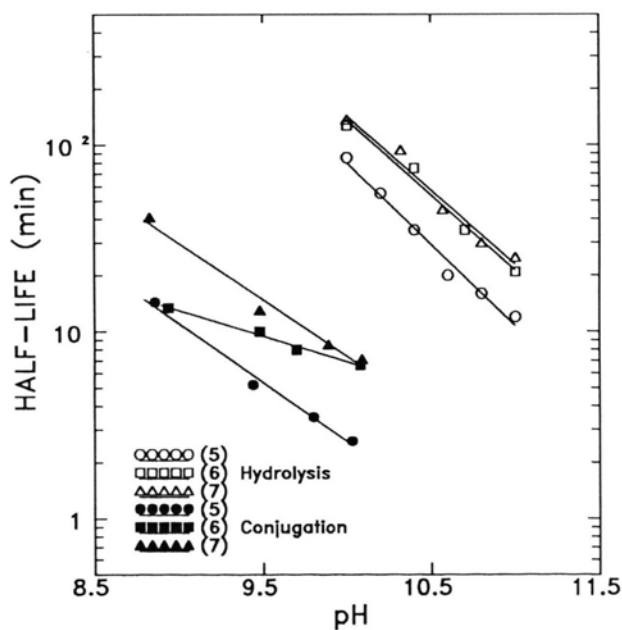
5



6



7



squaric ester monoamides investigated showed reaction profiles very similar to those of **5**, **6** and **7** depicted in Fig. 1. Detailed quantitative kinetic and thermodynamic measurements in aqueous buffer solution [11] revealed that the second-order rate constant for diamide formation (*e.g.*, for *n*-propylamine as the amine component) in the case of, *e.g.*, compound **7** is about 13 times higher ($k_{(\text{diamide})} = 0.37 \text{ l mol}^{-1} \text{ sec}^{-1}$ and $k_{(\text{hydr})} = 0.029 \text{ l mol}^{-1} \text{ sec}^{-1}$ [11]) than the rate constant for hydrolysis of **7**. These observations prompted us to carry out further experiments to corroborate the results. Fig. 2 summarizes the data obtained

Fig. 1. Log half-life vs. pH plots of **5**, **6** and **7**, as calculated from $k_{(\text{obs})}$ for (i) hydrolysis (open symbols) and (ii) conjugation (closed symbols) with poly-D-lysine. Initial concentrations of **5**, **6** and **7**, respectively, were $1.5 \times 10^{-5} \text{ mol/l}$. The concentration of poly-D-lysine was 1.5 mg/ml. All reactions were carried out at a concentration of 150 mM salt and at 37 °C.

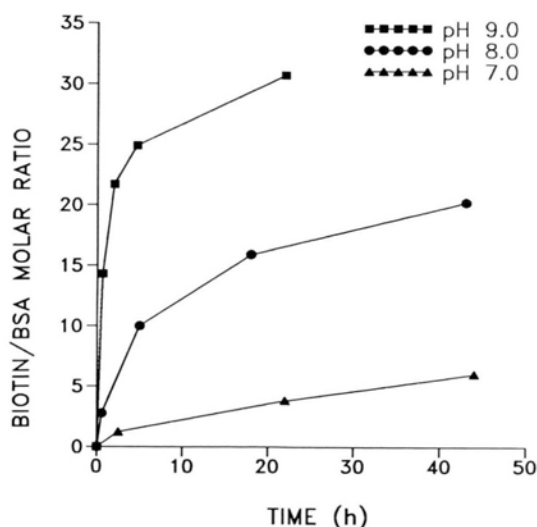


Fig. 2. Biotin/BSA molar ratios as a function of time and pH. Initial concentrations: 4.5 mg BSA/ml buffer and 4.5 mg of the biotin derivative (**6**)/ml buffer. All reactions were carried out at a concentration of 150 mM salt and at 37 °C.

for the coupling of **6** with BSA. The “coupling ratio” (mol **6**/mol BSA) can be manipulated easily by choosing different reaction conditions, *e.g.*, by variation of the pH of the buffered aqueous solution (Fig. 2). Since the UV spectra allow a clear distinction between different squaric acid derivatives, the reaction kinetics can be monitored easily and quantified by UV spectroscopy. These results underline the potential of the new conjugation procedure for the controlled coupling of many different compounds to (bio)polymers for a variety of purposes.

Acknowledgements

This work was supported by grants from the Dr. Mildred Scheel-Stiftung für Krebsforschung and by the Federal Ministry for Research und Technology (0318849 A).

We thank Prof. Dr. L. F. Tietze (Universität Göttingen) and his coworkers for kindly providing laboratory facilities and for fruitful discussions.

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