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Water-Driven Chemoselective Reaction of Squarate Derivatives with Amino Acids and Peptides

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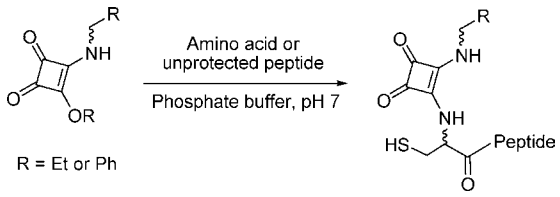
ABSTRACT



Here, we report a new class of highly chemoselective reactions between squarate derivatives and the amino acid cysteine or unprotected peptides with a N-terminus cysteine that proceed most efficiently in entirely aqueous solution at neutral pH. Kinetic and structural studies reveal that the presence of hydrogen bonding in water is primarily responsible for both the high yield and fast rate of the reaction.

Chemoselective transformations in aqueous environments are the most important reactions for sustaining life. While most organic synthesis is conducted in toxic organic solvents, studies have shown that hydrogen bonds can catalyze or facilitate organic reactions albeit in nonaqueous environment.[1] In addition, water has been found to enhance the reactivity of many types of organic reactions.[2] Particularly, the recently reported "on water" reactions[3] appear to be driven by the enhanced hydrogen bonding at the organic−aqueous interface.[4]

In this work, we report a new class of chemoselective reactions between squarate derivatives and amino acids (or unprotected peptides) in an entirely aqueous environment at neutral pH, whereby chemoselectivity and rate enhancement appear to be imparted by hydrogen bonding of the abiotic reactant in an aqueous solution. We also present a kinetic study that reveals how water promotes this reaction relative to other protic and aprotic polar solvents, as well as how hydrogen bonding can catalyze this reaction in organic solvents.

Designed chemoselective reactions that can proceed in water are relatively scarce, but have been found to bear enormous potential for studying and interfacing with biological systems. Past examples include synthesizing a whole protein without protecting groups,[5] orthogonal ligation methods for peptides and proteins,[6] decoration of cell surfaces by modified Staudinger ligation,[7] click chemistry,[8] modifying

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proteins by metal-catalyzed organic reactions,[9] and site-selective peptide modification.[10] Furthermore, development of chemoselective reactions in water with abiotic molecular structures are important because new structural elements can be incorporated into complicated living systems.

Due to the extensive π-conjugation with the carbonyl groups and the ring strain of the cyclobutene group, esters derived from squaric acids (squarate derivatives) are more reactive than generic esters.[11] Interestingly, substituting the squarate derivatives with an amino group (e.g., **1a**) increases long-term stability against hydrolysis in water at pH 7.02 to at least 7 days. Presence of esterases or cell lysates does not promote hydrolysis either (see Supporting Information).

Examining the reactivity of the substitution reaction of amino squarate derivatives (Figure 1) with a mixture of five

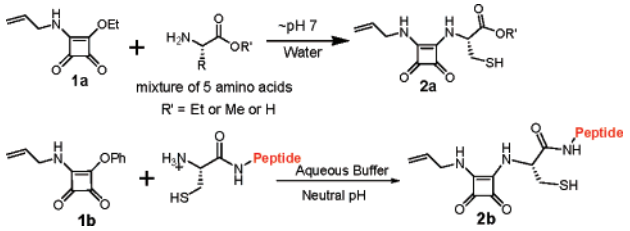

Figure 1. Chemoselective reaction of squarate derivatives with amino acids (a mixture of L-cysteine, L-serine, glycine, L-tyrosine, L-lysine) and with peptides containing N-terminus cysteine (N′-CAGRGDS-C′).

amino acids (L-cysteine, L-serine, glycine, L-tyrosine, and L-lysine), however, indicates that squarate derivatives react exclusively with cysteine in water at neutral pH. When the pH is increased to 8.5, the L-lysine residue also starts to substitute the squarate derivative (see Supporting Information).

Reaction Driven by Hydrogen Bonding. Measuring the rate of the reaction of **1a** and L-cysteine ethyl ester in different solvents (D_2O, CD_3OD, DMSO-d_6 ,and THF-d_8) reveals that the reaction is driven by the presence of hydrogen bonds in the solvent rather than the dielectric properties of the solvent (Figure 2). *First*, this reaction does not proceed in polar aprotic solvents (DMSO-d_6 and THF-d_8), but proceeds in protic solvents. *Second*, this reaction proceeds with the fastest rate and highest yield in water. As DMSO

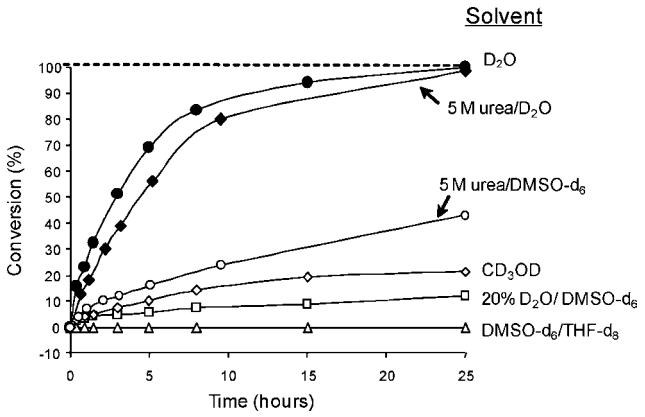

Figure 2. Plot of conversion of **1a** to **2a** in different solvents versus time.

has a higher dielectric constant than methanol, the dielectric constant or polarity of the solvent does not seem to play a critical role in facilitating this reaction. Furthermore, as both the amino acid and squarate derivatives are highly soluble in water, the hydrophobic packing proposed for enhanced Diels−Alder reaction in water does not seem to be a viable rationale for this reaction.[2e,f]

Urea-Catalyzed Coupling via Hydrogen Bonding. To further investigate the effect of hydrogen bonding on the reaction, we measured the rate of the reaction by adding 20% D_2O or 5 M urea into DMSO-d_6. In both cases, the reaction is revitalized for both the yield and the rate of the reaction (Figure 2 and Table 1). Interestingly, when urea is added to

Table 1. Rates of the Reaction of **1a** and L-Cysteine Affording **2a** in Different Solvents

solvent	ϵ^a	k ($\times 10^3$)^b	relative rate	% conversion^c
D_2O	80.0	9.0	30.0	100 (80^d)
5 M urea/D_2O	–	5.5	18.3	100
5 M urea/DMSO-d_6	–	1.0	3.3	43
CD_3OD	32.6	0.6	2.0	22
20% D_2O/DMSO-d_6	–	0.3	1.0	15
DMSO-d_6	48.0	–	–	–^{e}

^{a} Dielectric constant at 25 °C. ^{b} Initial rate constant (see Supporting Information). ^{c} Calculated from the ^{1}H NMR peak integration (see Supporting Information). ^{d} Isolated yield. ^{e} No detectable conversion is observed.

water, the initial rate of the reaction is slightly suppressed, but the complete conversion of the reactant is still obtained in the same time frame (Table 1). This result is consistent with the notion that urea, functioning as a weaker hydrogen-bond donor than water, promotes the reaction in DMSO but disrupts the initial rate of the reaction in water. Urea is known to catalyze carbon−carbon bond formation by activating aldimines[12a,b] and ketoimines[12c] via hydrogen bonding in Mannich[12] and hydrocyanation reactions. In this work we demonstrate that urea can also be used to catalyze the amide

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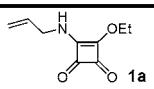
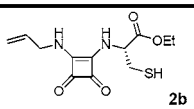
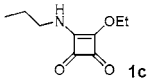
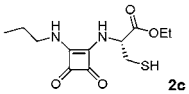
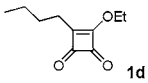
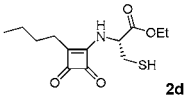
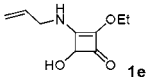
formation between squarate derivatives and cysteine in organic solvents. Although the catalysis by urea is slower in DMSO-d_6 than D_2O, this result appears to support the proposition that the electrophilicity of the squarate substrates is enhanced by hydrogen bonds.

Effects of Squarate Substituents and Leaving Groups. Examining the reactivity of different squarate derivatives indicates that this reaction tolerates a wide range of substitutents. But the presence of both of the carbonyl groups is necessary for facilitating this chemoselective reaction in water at pH 7.02 (Table 2, entry 4). In our attempt to extend this reaction to peptides containing N-terminus cysteine, we observed that the leaving group on squarate is a critical component for ligating peptides to the squarate derivatives. When peptides were used instead of amino acids, the ligated product was obtained in high yield only when the phenoxy derivative was used instead of the ethoxy derivative (Figure 1).

Table 2. Ligation of Different Squarate Derivatives with L-Cysteine Ethyl Ester in Phosphate Buffer (pH 7.02)

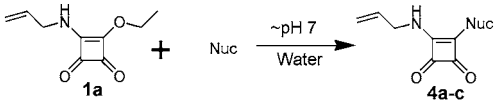
entry	squarate derivative	time	product	yield
1	 **1a**	0.5 h	 **2b**	75%[a]
2	 **1c**	0.5 h	 **2c**	80%[b]
3	 **1d**	1.0 h	 **2d**	80%[b]
4	 **1e**	24 h	----	---[c]

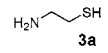
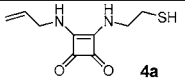
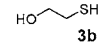
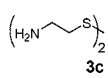
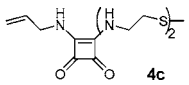
[a] Isolated yield; small amounts of hetero- and homodimer were also isolated. [b] Isolated yield; the product was recovered as homodimer of disulfide. [c] No detectable conversion was observed.

Requirement of the Nucleophile. To determine the requirement for the nucleophiles, we investigated the reaction of **1a** with β-mercaptoethyl amine, β-mercaptoethanol, and cystamine (Table 3). The reaction between **1a** and β-mercaptoethylamine resulted in the formation of ligated product **4a**. When β-mercaptoethanol was used in place of β-mercaptoethylamine, no detectable conversion was observed. When cystamine was used as the nucleophile, only trace amount (~5% yield) of the product was obtained.

For β-mercaptoethylamine, the thiol group has a pK_a of 8.50,[13a] and the ligation result is consistent with the thiolate first reacting with the squarate derivative (**1a**) to form a covalent intermediate, followed by an intramolecular displacment by the amino group (S → N acyl transfer) to afford the final product.[5d]

Table 3. Reaction of **1a** with Different Nucleophiles To Determine Functional Group Requirement of This Chemoselective Ligation



entry	Nuc	time	product	yield[a]
1	 **3a**	5.0 h	 **4a**	80%
2	 **3b**	5.0 h	---	---[b]
3	 **3c**	5.0 h	 **4c**	~5%

[a] Isolated yield after purification by filtration and washing with water. [b] No detectable conversion was observed.

For β-mercaptoethanol, the thiol group is less acidic (pK_a 9.61)[13b] compared to β-mercaptoethylamine and thus does not efficiently react with squarate derivative, **1a**. The low yield (~5%, entry 3, Table 3) of the reaction between cystamine and **1a** is consistent with the lack of the thiol group in the solution containing disulfide amine (cystamine). Furthermore, it is known that the substitution by an amine on monoamine squaric acid derivative is slow.[14]

Together these results indicate that both the amine and thiol groups in close proximity are required for the chemoselective reaction to occur in aqueous buffer at neutral pH. For mechanistic consideration, we believed that this chemoselective reaction is likely enabled when the pK_a of the thiol group is lowered by having an amino group in close proximity in an aqueous solvent, and the reaction proceeds via an intramolecular S → N acyl transfer.[5d]

The initial step of the reaction involves attack of the thiolate ion on the electrophilic carbon of the squarate to form a thioester-type intermediate. This intermediate then undergoes facile intramolecular S → N acyl transfer to form a stable ligated product (Figure 3).

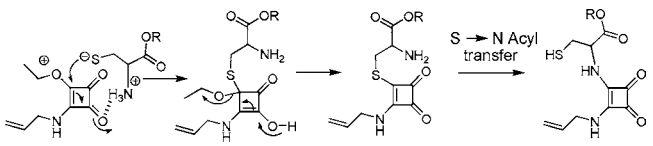


Figure 3. Proposed mechanism for the chemoselective reaction of squarate derivative with cysteine.

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It is interesting that, while the squarate derivatives are activated sufficiently for the nucleophilic attack by thiolate ions in water at neutral pH, they are also stable against hydrolysis in water.

Coupling Peptides Using Squarate. We explored the utility of 3,4-diphenoxy-1,2-cyclobutenedione as a linker to couple two peptides together. First, we coupled a peptide containing a lysine (NH_2-A**K**GRGDS-COOH) residue onto the diphenoxy squarate in DMSO/Et_3N to obtain a monosubstituted product. No disubstitution was observed as the second substitution is much slower than the first.[14] After purifying the lysine−phenoxy squarate conjugate by extraction, we coupled a second peptide containing N-terminus cysteine (NH_2-**C**AGRDGS-COOH) in phosphate buffer at pH 7 (Figure 4).

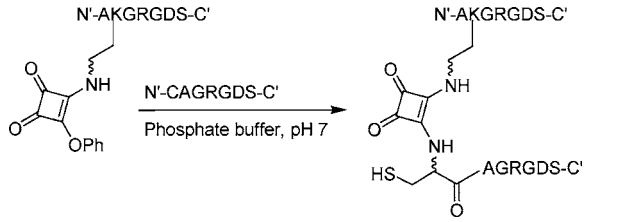

Figure 4. Schematic representation to stitch two peptides together using rigid squarate linker.

We note that the maleimide group also offers selective coupling with the thiol group.[15] Comparing the squarate coupling to the maleimide coupling in aqueous environment, we did not observe a noticeable difference in the kinetics and the concentration requirement by using alkoxy squarate (results not included). However, because squarate coupling proceeds preferentially in water and is pH dependent, the squarate chemistry may offer further selectivity between different thiol groups in more complex biological conditions. This topic and the use of this reaction to synthesize new non-peptidic mimics of integrin antagonists are the subjects of our ongoing research.

To conclude, we have demonstrated a new chemoselective reaction between squarate derivatives and N-terminus cysteine residues in water at neutral pH. The reaction is primarily promoted by the hydrogen bonds rather than by the high dielectric property of water. By using this chemoselective ligation reaction we have developed a rigid linker to ligate two unprotected peptides together. We believe that this class of water-driven reactions has the potential for developing biocompatible reagents for *in vivo* studies, and for developing useful nonnatural structural moieties for modifying biomolecules.

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Supporting Information Available: Kinetics studies, characterization of the stability of squarate derivatives in esterase and cell lysates; experimental procedures and spectral data for all the new compounds; MS and HPLC traces of reactions with peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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