Water-driven ligations using cyclic amino squarates: a class of useful S_N1-like reactions†

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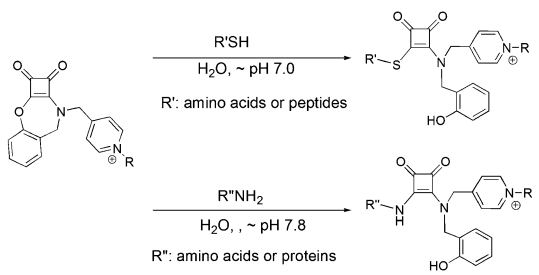
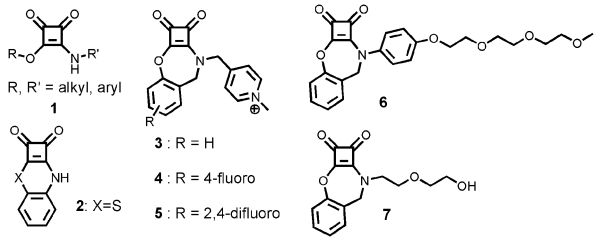
We report a class of water-soluble and -stable cyclic amino squarates that ligate with cysteine or lysine residues without side-products in an entirely aqueous environment. The ligations include addition–elimination reactions that are promoted by water in a way similar to S_N1 reactions. The structural versatility of the reactants allows the potential recognition of selected amino acid residues on proteins.

Organic reactions in entirely aqueous environment[1] are important for green chemistry and for applications in life sciences, including bioconjugation, protein modification or even reactions in a living system.[1c] The S_N1 substitution reactions typically proceed best in water; however, these reactions are generally not very useful. Here, we describe a new class of substitution reactions that are driven by water in a way similar to S_N1 reactions. This advance relies on the use of cyclic amino squarates, which are both soluble and stable in water, to ligate with thiol or amino groups under different reaction conditions without the formation of side products (Scheme 1).

Squaric acid derivatives have enabled many studies in bioorganic chemistry, including developments in bioconjugation methods,[2] phosphate ester mimics,[3] and sensors.[4] Recently, we discovered that amino phenoxy squarates react selectively with cysteine residues in water, but not in organic solvents.[1p] This class of reactions suggests that the preference of water is not limited to S_N1 reactions, but that using water to promote other types of reactions is feasible, probably either by activation of the reactants or stabilization of the transition states. This class of chemoselective reactions is relatively slow (finishes in ~1 h), but highly chemoselective, which allows surface reactions to distinguish cysteine groups at the N-terminus positions over other locations in a peptide.[2d] Here, we further report that by introducing strain into the reactant structure, water can facilitate the reactions with a faster rate and also enable coupling with weaker nucleophiles, such as amino groups at near neutral pH, which has many biological applications.



Scheme 1

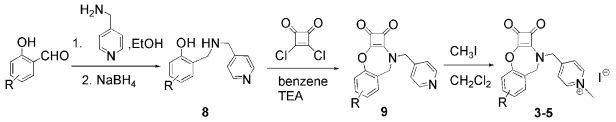


Scheme 2

We first targeted cyclic amino squarate with a six-member ring (Scheme 2, **2**). Cyclic amino squarate **2** reacted with amino groups in organic solvents (see ESI†), but was not soluble in water. In addition, thiol groups are not desired in the ligation product because of disulfide formation. We have been unable to synthesize the corresponding cyclic amino squarate with an oxygen bridge (X = O), probably due to the high strain in the heteroatom ring. These observations prompted us to hypothesize that including a seven-member ring, as shown in molecules **3** to **7**, may facilitate both solubility and ligation in water.

Cyclic amino squarates **3–5** are readily accessible synthetically (Scheme 3). Briefly, amine **8**, which was obtained by a one-pot reductive amination of 2-formylphenol and 4-aminomethyl-pyridine, coupled with 3,4-dichloro-3-butene-1,2-dione[5] to afford the 7-membered ring cyclic squarate pyridine derivatives. Treatment of **9** with organoiodides readily produced the desired products. The pyridinium facilitates water solubility and can be used to tether small drug molecules or ligands to the cyclic amino squarate molecule. As oligo(ethylene glycol) is often used as water-soluble linkers, we also synthesized compounds **6** and **7** (see ESI†). However, these two compounds exhibited sluggish water solubility. We note that an often neglected issue with oligo(ethylene glycol) is its propensity to form complexes with divalent (or higher valent) metal ions.[6] For these reasons, using other moieties, such as positive charges from pyridinium groups, to facilitate water solubility is worth exploration.

Coupling of cysteine amino acids and peptides containing cysteine residues to the cyclic amino squarate (Scheme 2, **3**) was about 12 times faster than with the open squarate



Scheme 3

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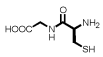
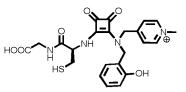
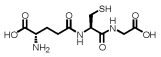
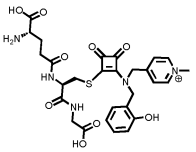
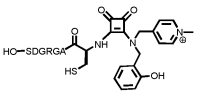
† Electronic supplementary information (ESI) available: Experimental procedures and spectral data for all the new compounds. See DOI: 10.1039/c0cc03989f

Table 1 Ligation of cyclic amino squarate derivative **3** with unprotected thiol-containing peptides in water (Scheme 1)[a]

Entry	Reactant	Product	Time/min
1			5[b]
2			60[c]
3	N′-CAGRGDS-C′		180[c]

[a] Reaction conditions: H_2O, r.t. [b] Quantitative. [c] >95% conversion.

molecule, **1**.[2d] In the presence of a β-amino group, the thiolate attack on the squarate was followed by an intramolecular S to N squarate transfer. Table 1 shows selected results of cysteine coupling with **3**. This class of reactions is mild, selective, quantitative, and without side products (Table 1).

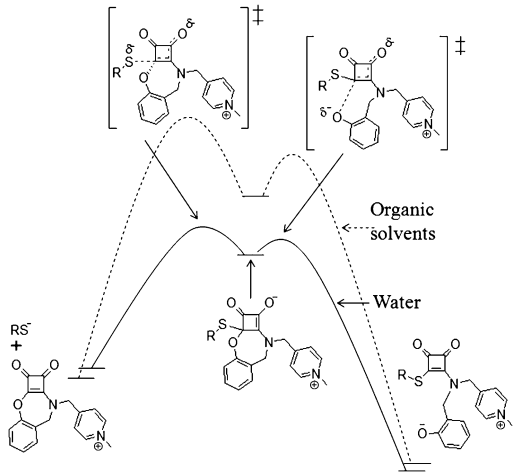
To understand in some detail the nature of this reaction, we measured the rate of the reaction in different deuterated solvents, including D_2O, D_2O/DMSO-d_6 (v/v, 1 : 4; molar ratio, 1.05 : 1), DMSO-d_6, CD_3OD, D_2O/CD_3CN (v/v, 1 : 3; molar ratio, 1.05 : 1) and CD_3CN (Table 2). The results showed that the ligation proceeds with the highest rate in water, or when water is present.

As these solvent effects are almost identical to those observed in S_N1 reactions,[7] this water-driven reaction appears to be promoted by water stabilizing a charged intermediate and polar transition states. We believe that the presence of multiple resonance structures (as opposed to that of generic carbonyl substitution reactions) stabilizes the intermediates and transition states for this reaction, which is similar to the way tertiary carbocations stabilize the intermediates of a typical S_N1 reaction. When the energy of the intermediate is low enough, water solvation of charges becomes effective to facilitate the chemical transformation (Scheme 4). In contrast, the most commonly used *N*-hydroxysuccinimide (NHS)-activated ester for modifying lysine residues[8] primarily activates the reactants rather than stabilizing the transition states, and also reacts with the solvent water. In the presence of multiple nucleophiles such as sugar moieties that can promote hydrolysis,[9] the NHS ester method becomes unreliable. Parallel to chemical

Table 2 Reactions of compound **3** with L-cysteine ethyl ester dihydrochloride in different deuterated solvents

Entry	Solvent	Time[a]
1	D_2O	5 min
2	D_2O/DMSO-d_6 (1/4)	5 min
3	DMSO-d_6	1 h
4	CD_3OD	16 h
5	D_2O/CD_3CN (1/3)	3 h
6	CD_3CN	—[b]

[a] >95% conversion. [b] No observable conversion.



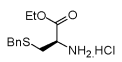
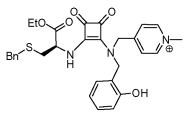
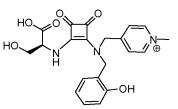
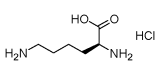
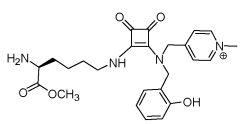
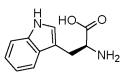
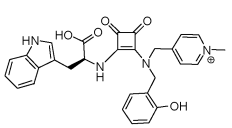
Scheme 4

reactivity, squarate **3** exhibited a positive solvatochromism (red shift from 250 to 264 nm in UV absorption) when the solvent polarity is increased from CH_3CN to water (see ESI†). This result suggests that the excited state of the molecule, which is more polar, is better stabilized by solvation than the ground state of the molecule in water.[10]

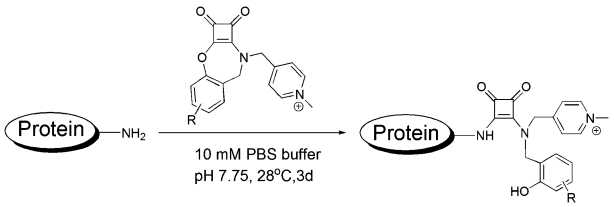
At a slightly higher pH (~7.8), the cyclic amino squarate also ligated effectively with amino groups and with proteins in water, albeit at a slow reaction rate (Table 3). Because of this reactivity in water, this class of molecules provides a bioorganic reaction tool for modifying proteins through lysine residues.

To demonstrate the ability of modifying proteins, we explored modifying lysozyme and ribonuclease A with **3–5** (Scheme 5). The proteins (1 equiv. of lysozyme or ribonuclease A) were incubated with **3**, **4** or **5** (50 equiv.) in 10 mM PBS buffer (pH 7.75) at 28 °C for 3 days. The products were dialyzed,

Table 3 Ligation of cyclic amino squarate derivative **3** with amino groups in water (Scheme 1)

Entry	Reactant	Product	Time/d
1			6[a,b]
2			6[a,b]
3			3[c,d]
4			6[a,b]

[a] Reaction conditions: H_2O, rt. [b] >95% conversion. [c] 10 mM PBS buffer, pH 7.75. [d] ~30% conversion.



Scheme 5

Table 4 Number of lysine modifications per protein using **3**, **4** or **5** determined by MALDI-TOF[a]

	3	**4**	**5**
Lysozyme	1[b]	1[b]	1[b]
RNase A	5	3	2

[a] Reaction conditions: 10 mM PBS buffer (pH 7.75), rt, 3 d. [b] The yield of modification increased as fluoro-substitution increases in squarates **3** to **5**.

lyophilized and examined by MALDI-TOF. Interestingly, while both lysozyme and RNase A were modified with cyclic amino squarate, the efficiency for the modification was different between the two proteins. Higher yield was seen for lysozyme with one lysine modification as the number of fluoro-substitution was increased on the cyclic amino squarate. In contrast, the extent of lysine modifications for RNase A decreased from 5 to 2 when the number of fluoro-substitution was increased (Table 4).

Due to the difference in the microenvironment on the surface of a protein, lysine residues differ in properties such as pK_a and nucleophilicity. The structural versatility of the cyclic amino squarates provides an opportunity for the molecules to recognize selected regions and amino acid residues prior to ligation—a subtle selectivity often difficult to achieve.

To conclude, we have developed a new class of ligation reactions in entirely aqueous solutions, for which the reaction is likely promoted by stabilization of the transition states. The thiol-containing molecules react with cyclic amino squarate molecules significantly faster than with acyclic amino squarate.[1p] We believe that the enhanced reactivity is due to the incorporated ring strain in the 7-membered ring. Our discovery suggests that using water to enable useful reactions in a way similar to S_N1 reactions is possible.[11] This work also suggests the promising likelihood of using water to promote other types of reactions by building reactants that fulfill certain structural requirements such as molecular strain in the reactant structures, and resonance stabilization in the transition states and intermediates. As the cyclic amino squarate is stable and can be readily derivatized with small molecules through pyridinium moiety, this reaction is useful for applications involving difficult bioconjugation.

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Notes and references

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