Communication

Traceless Templated Amide-Forming Ligations

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Traceless Templated Amide-Forming Ligations

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Supporting Information Placeholder

ABSTRACT: Template assistance allows organic reactions to occur under highly dilute conditions – where intermolecular reactions often fail to proceed – by bringing reactants into close spatial proximity. This strategy has been elegantly applied to numerous systems, but always with the retention of at least one of the templating groups in the product. In this report, we describe a traceless, templated amide-forming ligation that proceeds at low micromolar concentration under aqueous conditions in the presence of biomolecules. We utilized the unique features of an acylboronate–hydroxylamine ligation, in which covalent bonds are broken in each of the reactants as the new amide bond is formed. By using streptavidin as a template and acylboronates and *O*–acylhydroxylamines bearing desthiobiotins that are cleaved upon amide formation, we demonstrate that traceless, templated ligation occurs rapidly even at sub-micromolar concentrations. The requirement for a close spatial orientation of the functional groups – achieved upon binding to streptavidin – is critical for the observed enhancement in the rate and quantity of product formed.

Templated organic reactions are widely used by biological systems to accelerate covalent bond formation by bringing reactants in close proximity and increasing their effective concentration;[1] well-known examples include ribosomal peptide synthesis and DNA ligation. The same principles have been applied in purely synthetic template-promoted reactions[2] and notable successes include DNA-and PNA-templated ligation.[3] Despite the elegance of these approaches and applications to areas such as DNA-templated synthesis, proximity-driven mapping of binding pockets[4] and combinatorial synthesis,[5] their utility for broader synthetic applications is limited by the persistence of at least one of the templating groups (Figure 1a-c).[3d,6,7] For example, a number of template-assisted native chemical ligations (NCL) take place with release of one templating scaffold, but the second must be installed at a distal site of the other reactant (Figure 1b).[8,9] To address this limitation, Diederichsen *et al.* disclosed a photocleavable PNA-templated native chemical ligation (NCL)[10] that allows cleavage of the directing PNA strands from the ligated peptide by irradiation.[11] However, a traceless, templated ligation in which the templating moieties are cleaved concomitantly with bond formation[12] has not been successfully implemented, likely due to the lack of covalent bond-forming reactions suitable for this purpose.

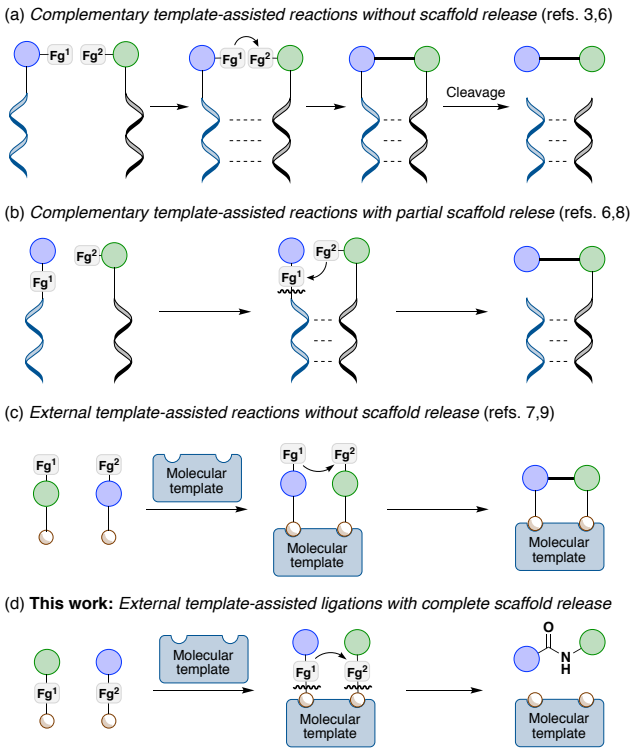

Figure 1. Schematic representation of types of templated reactions. (a) Complementary template-assisted reactions without directing scaffold release. (b) Complementary template-assisted reactions with partial directing scaffold release. (c) External template-assisted reactions without directing scaffold release. (d) *This work*: External template-assisted amide-forming ligations with concomitant release of the directing scaffolds.

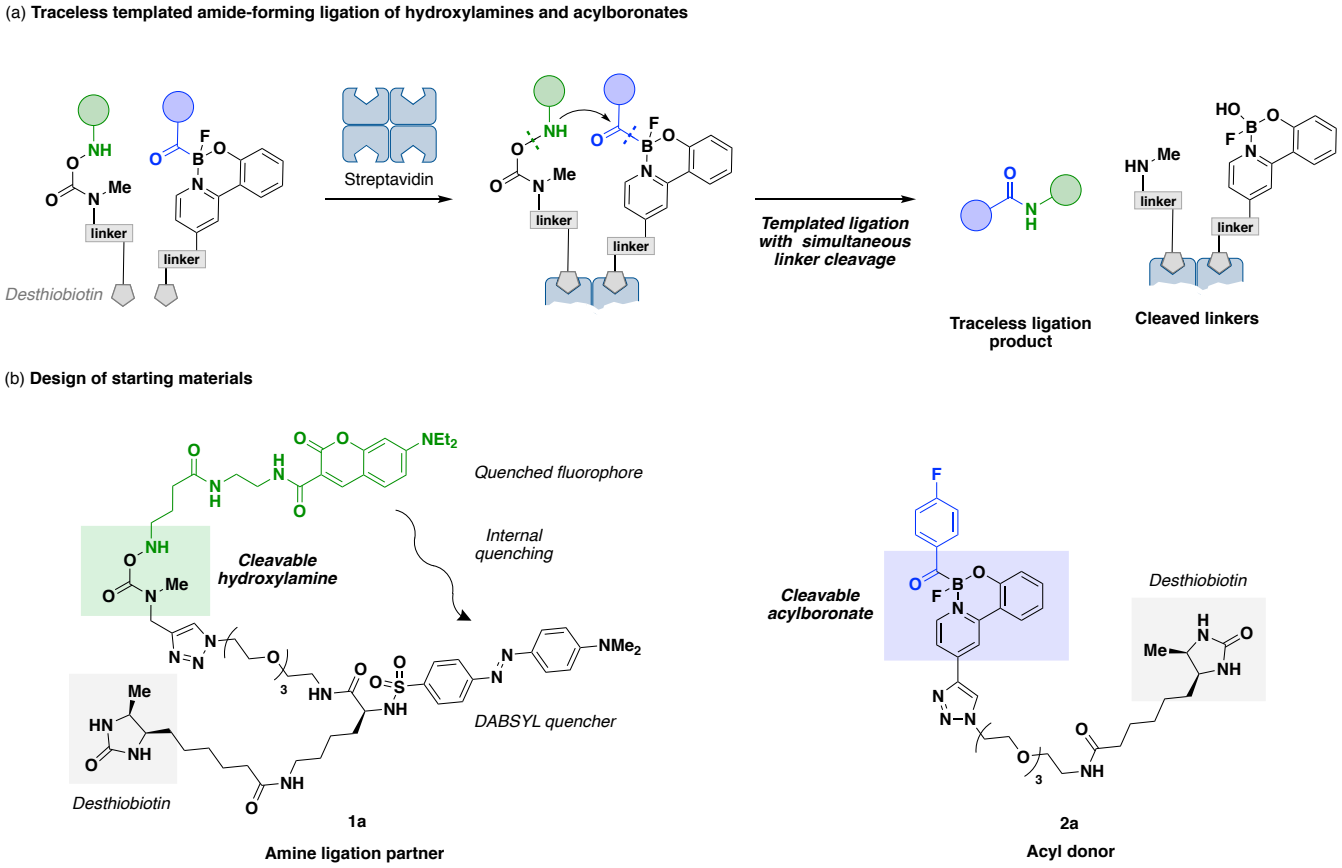

Figure 2. (a) Streptavidin-templated ligation of acylboronates and *O*-acylhydroxylamines, each bearing a desthiobiotin connected to the departing section of their respective functional groups. (b) Specific molecules used for this study (see Supporting Information for detailed synthetic routes).

During the course of our studies on amide-forming ligations from acylboronates,[13,14] we identified variants that fulfilled the rare criteria of simultaneous cleavage of two derivatizable groups during an intermolecular coupling reaction,[15] offering the possibility for true traceless templated reactions. In this manuscript, we demonstrate templated amide-formation in water at low micromolar concentrations using desthiobiotin as the ligand and streptavidin as the template. This work anticipates a generic approach to conjugations that overcome the inherent limitations in the rate of non-enzymatic coupling reactions. Detailed kinetics analysis and modeling show that template-induced proximity of the two reaction partners substantially enhances product formation above the background rate.

The reaction of acylboronates bearing pyridine-derived ligands and *O*-acylhydroxylamines results in the formation of amide bonds with the simultaneous loss of both the ligated boron and the carbamate by N–O bond cleavage (Figure 2a).[13] The template binding groups can be located in regions of the reaction partners that are cleaved during the ligation, which would result in traceless, templated product formation. Importantly, it occurs under aqueous conditions without the need for any other reagents or catalysts – making it suitable for use in the presence of biological molecules.

As our molecular template of choice, we selected the streptavidin-desthiobiotin pair by linking the ligating functional groups to desthiobiotin. The approach builds on a long history of streptavidin as a template for inducing molecular proximity, including elegant work from Ward on the development of streptavidin-based artificial metalloenzymes.[16] Furthermore, Winssinger has previously demonstrated that two distinct reactants can be brought into proximity through a similar system for the template-assisted photocatalyzed azide reduction.[17,18] Streptavidin displays a particular arrangement of its four binding pockets, located so that two of them are in spatial proximity to each other.[19] Its use has the disadvantage of being able to form non-productive complexes, but considerably simplifies the synthesis of the substrates and employs a readily available template with a low dissociation constant (K_D ~ 10^{-14} M for biotin and ~10^{-11} M for desthiobiotin) and fast association kinetics (k^{on} >10^6 M^{-1} s^{-1}).[20]

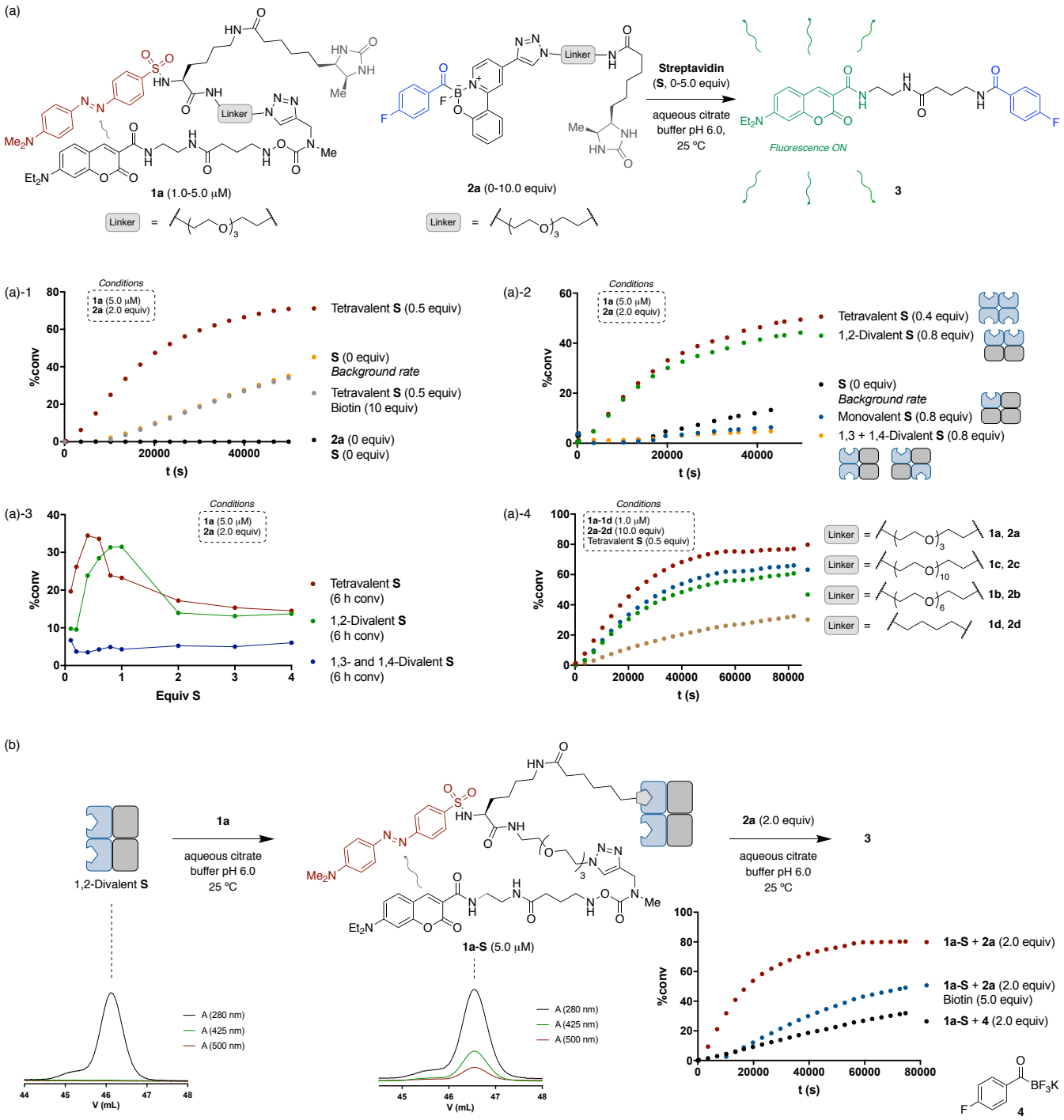

Figure 3. (a) Experiments performed using hydroxylamines **1a–d** (1.0–5.0 μM), acylboronates **2a–d** (0–10.0 equiv) and expressed streptavidins (**S**, 0–4.0 equiv) in aqueous citric acid / sodium citrate buffer pH 6.0 (25 mM) at 25 °C. Product formation monitored by fluorescence (λ^{ex} = 430 nm, λ^{em} = 485 nm). All plots are mean values from three replicates (error bars are omitted for clarity). (a)-1: Conversion plot comparing the reaction in the presence or absence of added biotin (10.0 equiv). Performed with **1a** (5.0 μM), **2a** (2.0 equiv) and tetravalent **S** (0.5 equiv). (a)-2: Performance of different streptavidin mutants in the reaction. Performed with **1a** (5.0 μM) and **2a** (2.0 equiv). (a)-3: Streptavidin loading studies. Performed with **1a** (5.0 μM) and **2a** (2.0 equiv). (a)-4: Effect of linker length. Performed with **1a–1d** (1.0 μM), **2a–2d** (10.0 equiv) and tetravalent **S** (0.5 equiv). (b) Isolation of the association complex **1a–S** between 1,2-divalent streptavidin and **1a**; FPLC traces of the purified protein at different wavelengths. A conversion plot over time for the reaction of **1a–S** (5.0 μM) with **2a** (2.0 equiv) is shown on the bottom-right corner.

The association of two different biotin-equipped starting materials with streptavidin could produce a number of different spatial combinations, most of which lead to non-productive associations. While using a weakly associating ligand may allow it to dissociate from unproductive arrangements, such a system would unnecessarily complicate the analysis of the ligation and kinetics. We therefore elected to use desthiobiotin and adopted two approaches to minimize non-productive binding arrangements during our validation of a true traceless templated ligation. First, we employed a small excess of one ligation partner to ensure that as many productive orientations as possible were formed. Second, we followed the convenient procedure of Howarth and co-workers to express and separate streptavidin mutants having different valencies and spatial arrangements of the binding pockets.[21]

To accurately monitor the rate of ligation we designed starting materials whose conversion could be determined at low concentrations by real-time fluorescence measurements (Figure 2b). Hydroxylamine **1a** was equipped with a coumarin fluorophore attached to the N-atom and a 4-(dimethylaminophenylazo) benzenesulfonyl (DABSYL) quencher was connected to the cleavable part of the molecule – the *O*-acyl side – along with a desthiobiotin and a polyethylene glycol (PEG) linker. This design allows for internal fluorescence quenching of the coumarin moiety (FRET effect); as the reaction proceeds the fluorophore and the quencher on **1a** are separated by the N–O bond cleavage, resulting in an increase in the fluorescence signal output (Figure 3a). Acylboronate **2a** bearing a desthiobiotin attached to the ligand on boron via a short PEG linker could be easily prepared in two steps from commercially available potassium acyl trifluoroborate (KAT) reagent **4**.[22] As the rate of KAT ligations are strongly influenced by pH,[23] we chose aqueous buffers at pH 6.0 – as the expected rate was low enough to benefit from templation with a moderate background rate at low micromolar concentrations. Streptavidin is well known to be stable at this pH.[24]

With the starting materials in hand, we screened a number of streptavidin mutants and reaction conditions. In a typical experiment, **1a** (1.0 equiv) was initially mixed with streptavidin in aqueous citrate buffer (pH 6.0) for 5 min followed by addition of **2a** (2.0 equiv). The reaction was monitored by fluorescence (λ^{ex} = 430 nm, λ^{em} = 485 nm) over time. The coupling of **1a** and **2a** in the presence of expressed tetravalent streptavidin (Figure 3a-1, dark red dots) was accelerated over the background (orange dots), and as compared to a negative control experiment in which excess biotin was added to block streptavidin binding pockets (grey dots). The stability of reagent **1a** and streptavidin during the course of the reaction was confirmed by fluorescence measurements (Figure 3a-1, black dots). After reaching the plateau, experiments were analyzed by LC-MS to confirm the final conversion values (see the Supporting Information). Only desired amide product **3**, unreacted starting materials **1a** and **2a**, and byproducts derived from the cleavage of **1a** and **2a** could be observed by LC-MS analysis of the mixture.

The reaction exhibited templated ligation only in the presence of tetravalent and 1,2-divalent ("*cis*") streptavidins; monovalent and 1,3- or 1,4 ("*trans")* streptavidins did not show any enhancement over the background reactivity (Figure 3a-2). Streptavidin loading studies showed that the optimal loading is 0.5 equiv relative to **1a** for tetravalent streptavidin and 1.0 equiv for the 1,2-divalent mutant. This is in excellent agreement with the number of possible matching combinations of starting materials (Figure 3a-3). We examined a variety of hydroxylamines and acylboronates having different linker lengths between the desthiobiotin unit and the ligating functional groups (Figure 3a-4); optimal performance was found when a short PEG linker was employed (**1a** and **2a**). The use of longer PEG chains (**1b**, **1c** and **2b**, **2c**) resulted in slightly diminished performance and a short alkyl spacer (**1d** and **2d**) showed no acceleration over the background rate.

To further confirm our hypothesis that streptavidin assists the amide formation by binding both **1a** and **2a**, we formed a 1:1 complex (**1a**–**S**) of 1,2-divalent streptavidin and **1a** and isolated it by FPLC. Upon addition of 2.0 equiv of **2a** at 5.0 μM (Figure 3b), we observed rapid formation of the amide product and good overall conversion. Addition of biotin to block the binding pockets again resulted in a considerable decrease of initial rate and conversion. This result shows clearly that productive binding of the acylboronate and hydroxylamine in adjacent streptavidin binding sites is responsible for the increase in the observed rate of amide formation.

To a first approximation, the formation of amide **3** is expected to depend on two processes: 1) template-assisted formation of **3** from the productive association of reagents **1** and **2** into two proximal streptavidin pockets – a step governed by the first-order rate constant k^{T}. 2) The background reaction of **1** and **2**, represented by the second-order rate constant k^{B}.

To provide a mathematical model for the effect of templation on the rate of product formation, we adopted pseudo-first order conditions ($[\mathbf{2}] \geq 10 \cdot [\mathbf{1}]$). The corresponding integrated rate equation adopts a bi-exponential form as shown in Equation 1.

$$\%\ conv = \frac{100 \cdot [\mathbf{3}]}{[\mathbf{1}]_0} = F \cdot \left[1-e^{(-k^{T} \cdot t)}\right] + (100-F) \cdot \left[1-e^{(-k^{B} \cdot [\mathbf{2}]_0 \cdot t)}\right] \quad (1)$$

F = initial fraction of productive complex = $\frac{100 \cdot [\text{Productive complex}]_0}{[\mathbf{1}]_0}$

k^{T} = first order rate constant of templated amide formation (s^{-1})

k^{B} = pseudo-first order background rate constant ($M^{-1}\ s^{-1}$)

$[\mathbf{2}]_0 >> [\mathbf{1}]_0$

Assumed irreversible binding of **1** and **2** with **S**

The first exponential term corresponds to product formation from template assistance – a parameter directly proportional to the fraction of productive associations of starting materials **1** and **2** to streptavidin (F parameter). The second term expresses the conversion of product due to non-templated, background processes. Under first-order conditions, the background exponent contains the concentration of reagent **2** as a constant parameter.

To further evaluate the validity of our model, we conducted kinetics experiments under pseudo-first order conditions (Figure 4). We compared a number of reaction plots conducted at different concentrations but with comparable setup and reagent equivalencies. The conversion over time curve at 5.0 μM was fitted to Equation 1 with 85% templation amplitude (F factor) and a templation rate constant of $k^{T} \approx 4 \cdot 10^{-5}\ s^{-1}$ by constraining the background rate constant (k^{B}) to 0.2 $M^{-1}\ s^{-1}$ (red dots).[25] A slightly lower templation amplitude and constant (k^{T} and F) were obtained when fitting Equation 1 to the plot measured at 1.0 and 0.5 μM concentration (blue and green dots respectively). The background reaction followed a monoexponential growth (black dots). The background rate constant was lower in the presence of streptavidin, a factor that we attributed to the increased steric hindrance of bound reagents compared to when they are in solution.

In conclusion, we have established a streptavidin-templated reaction of desthiobiotin-equipped hydroxylamines **1** and acylboronates **2** with traceless formation of the corresponding amide. The reaction achieves high levels of conversion, even at 500 nM – far above the conversion expected from the background reaction alone. The present approach to templated-ligation establishes that successful traceless templated reactions can provide substantial enhancement in conversion and can be implemented with a straightforward streptavidin–desthiobiotin binding pair. This approach will be useful for applications in both signaling and chemoselective conjugations under dilute aqueous conditions.

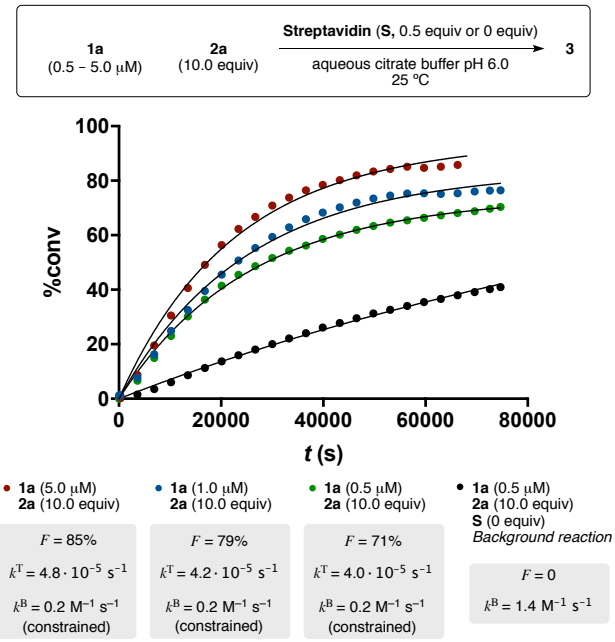

Figure 4. Kinetics experiments under pseudo-first order conditions fitted to Equation 1. Dots represent mean data points from three experiment replicates performed with **1a** (0.5–5.0 μM), **2a** (10.0 equiv) and tetravalent streptavidin (**S**, 0–0.5 equiv) in aqueous citrate buffer pH 6.0. Error bars are omitted for clarity. Lines are non-linear fits from Equation 1 using the fitted parameters inside the grey boxes (bottom part).

ASSOCIATED CONTENT

Supporting Information. Experimental procedures, supplementary results and spectroscopic data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org..

AUTHOR INFORMATION

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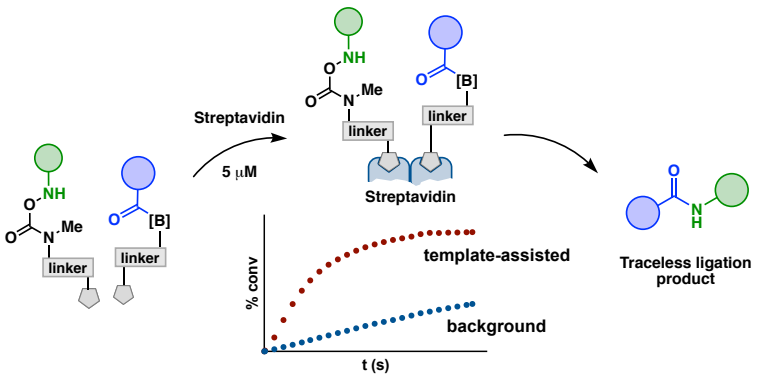
TOC Graphic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