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# β-Eliminative Releasable Linkers Adapted for Bioconjugation of Macromolecules to Phenols

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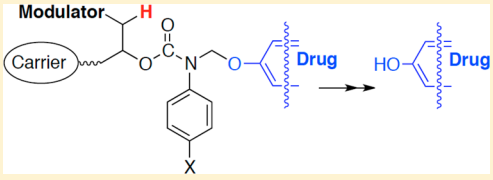
Supporting Information

**ABSTRACT:** We recently reported a chemical approach for half-life extension that utilizes sets of releasable linkers to attach drugs to macromolecules via a cleavable carbamate group (Santi et al., *Proc. Nat. Acad. Sci. U.S.A.* **2012**, *109*, 6211−6216). The linkers undergo a β-elimination cleavage to release the free, native amine-containing drug. A limitation of the technology is the requirement for an amino group on the drug in order to form the carbamate bond, since most small molecules do not have an amine functional group. Here, we describe an approach to adapt these same β-elimination carbamate linkers so they can be used to connect other acidic heteroatoms, in particular, phenolic hydroxyl groups. The approach utilizes a methylene adaptor to connect the drug to the carbamate nitrogen, and an electron-withdrawing group attached to carbamate nitrogen to stabilize the system against a pH-independent spontaneous cleavage. Carbamate cleavage is driven by β-elimination to give a carboxylated aryl amino Mannich base which rapidly collapses to give the free drug, an aryl amine, and formaldehyde.




## INTRODUCTION

Conjugation of drugs to macromolecular carriers is a proven strategy for improving pharmacokinetics. In one approach, a drug is covalently attached to a long-lived circulating macromolecule − such as polyethylene glycol (PEG) − through a linker that is slowly cleaved to release the native drug.[1,2] We have recently reported such conjugation linkers that self-cleave by a nonenzymatic β-elimination reaction in a highly predictable manner, and with half-lives of cleavage spanning hours to over a year.[3] In this approach, a macromolecular carrier is attached to a linker that is attached to a drug via a carbamate group (**1**; Scheme 1); the β-carbon has an acidic carbon−hydrogen bond (C−H) and also contains an electron-withdrawing p$K_a$ "modulator" (Mod) that controls the acidity of that C−H. Upon hydroxide-ion catalyzed proton removal (**2**), a rapid β-elimination occurs to cleave the linker−carbamate bond and release the free drug or pro-drug and a substituted alkene **3**. The rate of drug release is proportional to the acidity of the proton, and that is controlled by the chemical nature of the modulator; thus, the rate of drug release is controlled by the p$K_a$ modulator. Both *in vitro* and *in vivo* cleavages were linearly correlated with electron withdrawing effects of the modulators and, unlike ester bonds commonly used in releasable linkers,[2] were not catalyzed by general bases or serum enzymes. It was also shown that with releasable conjugates of this type, the half-life of a rapidly cleared drug is transformed into that of the conjugate itself; there is a longer half-life and diminished $C_{max}$ compared to repeated bolus doses of a short-lived drug needed to maintain a therapeutic level.

While the β-eliminative linker technology well serves primary and secondary amine-containing drugs that can form carbamates, it is not suitable for most small molecules or functional groups of peptides other than amine groups. We were in need of a method to attach other functional groups − particularly phenolic hydroxyls − of drugs to macromolecules via β-eliminative linkers, and an understanding of the chemistry of such conjugates. In the present work, we describe a synthetic approach for adapting our β-eliminative carbamate linkers to attach phenols to macromolecular carriers. Using *p*-nitrophenol (pNPhOH) as a model leaving group, we describe methods of synthesis, mechanistic studies that define pathways and kinetics of cleavage, and structure activity relationships that allow rational design of conjugates of phenol-containing drugs with predictable rates of release.

## EXPERIMENTAL PROCEDURES

**General.** Materials purchased were the purest grade commercially available. HPLC analyses were by reverse phase using UV detection. Details are provided in SI.

**Synthesis.** *O-[7-Azido-1-(phenylsulfonyl)heptan-2-yl]-N-phenyl-carbamate (**12A**).* To a stirred solution of the 1-(phenylsulfonyl)-7-azido-2-heptanol (0.150 g; 0.504 mmol) and triphosgene (0.210 g; 0.708 mmol) in anhydrous tetrahydrofuran (6 mL) was added pyridine (90 uL; 1.11 mmol) slowly over 5 min. The resulting suspension was stirred at ambient temperature for 35 min at which time analysis of the reaction mixture by TLC (50% ethyl acetate/50% hexanes; UV) showed complete disappearance of the alcohol, $R_f$ = 0.53, and the emergence of a new spot at $R_f$ = 0.76. The precipitated pyridine hydrochloride was removed by filtration and washed with tetrahydrofuran (2 mL). The filtrate was concentrated on the rotovap.

The crude chloroformate was dissolved in anhydrous tetrahydrofuran (6 mL) and aniline (55 μL; 0.603 mmol) added. To this solution was added pyridine (98 μL; 1.21 mmol). The suspension was stirred at room temperature for 2 h at which time analysis of the reaction mixture by TLC (50% ethyl

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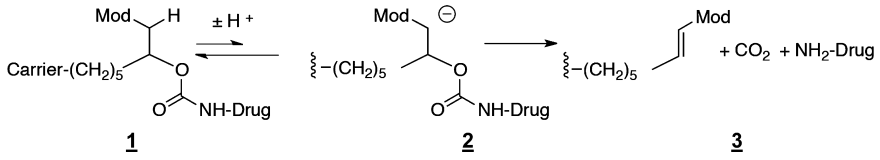
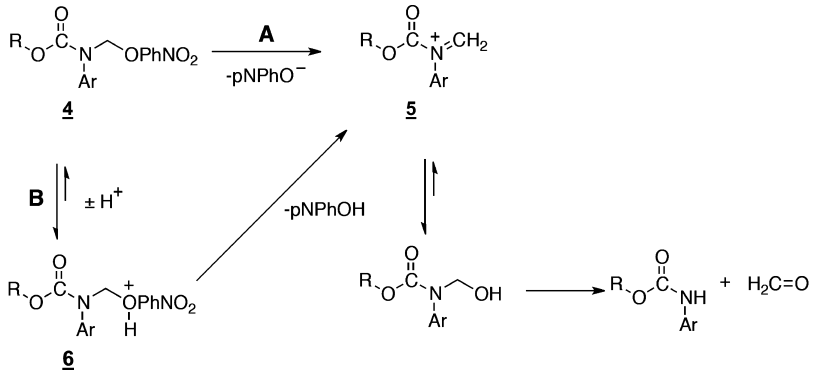
**Scheme 1**




**Scheme 2**




acetate/50% hexanes; UV) showed loss of the starting chloroformate at $R_f$ = 0.76 and the appearance of a new product spot at $R_f$ = 0.50. The precipitated pyridine hydrochloride was removed by filtration and washed with tetrahydrofuran (2 mL). The filtrate was concentrated and the residue partitioned between ethyl acetate and water (20 mL each). The phases were separated and the aqueous phase was extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with water, 1 M hydrochloric acid, and saturated sodium chloride solution (10 mL each) and dried over magnesium sulfate. The solvent was evaporated to give a pale yellow oil (0.288 g) that was purified using a Thomson Instruments single step silica gel column (12 g) eluting with 30% ethyl acetate/70% hexanes to give the carbamate as a colorless oil (0.190 g; 0.454 mmol; 90%). $^1$H NMR ($CDCl_3$) 1.24 (4H, m), 1.56 (2H, m), 1.73 (2H, m), 3.29 (2H, t, J = 6.8 Hz), 3.31 (1H, dd, J = 3.5 Hz, J = 14.7 Hz), 3.50 (1H, dd, J = 7.9 Hz, J = 14.7 Hz), 5.20 (1H, m), 6.19 (1H, br. s), 7.06 (1H, m), 7.25 (4H, m), 7.51 (2H, m), 7.58 (1H, m), 7.93 (2H, m). HPLC purity = 99% at 254 nm.

Compounds **12B,C** and 6-azidohexyl-*N*-aryl carbamates **16A-C** were prepared in a similar fashion, and are described in SI.

*O-[7-Azido-1-(phenylsulfonyl)heptan-2-yl]-N-(4-nitrophenoxymethyl)-N-phenylcarbamate (**14A**).* *O*-[7-Azido-1-(phenylsulfonyl)heptan-2-yl]-*N*-phenylcarbamate (0.040 g; 0.096 mmol) was dissolved in anhydrous tetrahydrofuran (0.5 mL) and *freshly distilled* chlorotrimethylsilane (0.5 mL) and paraformaldehyde (0.007 g; 0.233 mmol) were added. The reaction mixture was heated at 65 °C for 41 h at which time HPLC analysis (after a MeOH quench) showed consumption of the starting material and the appearance of a new product peak. The reaction mixture was concentrated under reduced pressure. Ethyl acetate (5 mL) was added to the residue and the solution reconcentrated. This was repeated once more. 50% Ethyl acetate/50% hexanes (3 mL) were then added and the mixture filtered. The filtrate was concentrated under reduced pressure and the crude chloride dissolved in anhydrous *N,N*-dimethylformamide (0.25 mL).

The above solution was added to a solution of sodium *p*-nitrophenolate (0.025 g; 0.155 mmol) in anhydrous *N,N*-dimethylformamide (0.25 mL). The resulting orange solution was stirred at ambient temperature for 1 h. It was then diluted with water (10 mL) and extracted with ethyl acetate (3 × 10 mL). The combined organics were washed with brine, dried over magnesium sulfate, and concentrated. The crude product was purified using a Thomson Instruments single step silica gel column (4 g) eluting with 40% ethyl acetate/60% hexanes to furnish the phenyl ether as a pale yellow oil (0.013 g; 0.024 mmol; 25%). $^1$H NMR ($CDCl_3$) 1.21 (4H, m), 1.40 (4H, m), 3.17 (3H, m), 3.35 (1H, m), 5.24 (1h, m), 5.61 (2H, br. s), 6.89 (2H, d, J = 9.4 Hz), 7.04 (1H, m), 7.06 (1H, m), 7.33 (2H, m), 7.57 (2H, t, J = 7.7 Hz), 7.67 (1H, m), 7.91 (2H, m), 8.15 (3H, m).

Compounds **14B,C** and 6-azido-*N*-aryl carbamates **16A-C** and **24** were prepared in a similar fashion, and are described in SI. An alternate, improved procedure for *N*-chloromethylation of carbamates is also provided in SI.

*O-[7-PEG$_{40\ kDa}$-1-(phenylsulfonyl)heptan-2-yl]-N-(4-nitrophenoxymethyl)-N-arylcarbamates (**15**) and O-[6-PEG$_{40\ kDa}$-1-hexyl-N-(4-nitrophenoxymethyl)-N-arylcarbamate (**17**).* To a stirred solution of PEG$_{40\ kDa}$-DBCO (800 μL of a 3.13 mM solution in acetonitrile; 2.5 μmol) was added of the azide **14** or **16** (33 μL of a 150 mM solution in acetonitrile; 4.95 μmol). The solution was stirred at ambient temperature for 24 h. HPLC analysis of the reaction solution showed that the DBCO maxima at 309 and 291 were undetectable at this time. Me-tBu ether (15 mL) was added to the reaction solution and it was stirred for 30 min. The solid PEG-conjugate was collected, washed with Me-tBu ether (2 × 5 mL), and dried under high vacuum.

Properties of **15A-C** and **17A-C** are described in SI.

**Kinetic Studies.** Kinetic studies were performed by spectrophotometric determination of $pNPhO^-$ at pH > 7.4 and analysis by HPLC, as detailed in SI.

**Rate Equations.** The derivation of rate equations for base-catalyzed reactions of β-eliminative 4-nitrophenoxymethyl carbamate linkers are given in SI.

## RESULTS

**Chemistry.** The approach described here extends from the work of Majumdar and Sloan[4] who reported the synthesis and properties of N-substituted-N-aryloxymethyl alkyl carbamates of **4** (Scheme 2, path A), containing a methylene bridge connecting the carbamate nitrogen with an oxygen of a phenol, and a N-aryl group that acts as a "stabilizer" against spontaneous hydrolysis.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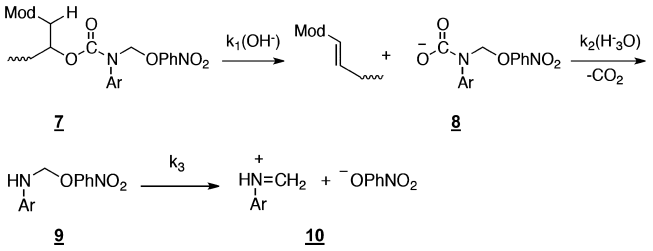
These were prepared by N-chloromethylation of N-alkyl/aryl-O-methyl carbamates, followed by reaction with a phenoxide anion. Solvolysis to give the phenol and the iminium ion (**5**) occurred by a slow pH-independent cleavage of the N-methylol bond over pH 4.6 to 8.8 that proceeds by a spontaneous $S_N1$ mechanism. Subsequently, as with related carbinolamides,[5,6] after addition of water to the iminium ion the N-hydroxymethyl carbamate should undergo acid- or base-catalyzed conversion to the carbamate and $CH_2O$. The $S_N1$ reaction is facilitated by the leaving group ability of the phenol, and shows a negative linear correlation with the $pK_a$ of the phenol (slope = −0.93). The reaction rate is decreased by electron withdrawing groups on the carbamate nitrogen, but the effects were not thoroughly characterized. Although not previously reported, we show below that an analogous acid-catalyzed reaction occurs that proceeds by a similar pathway (Scheme 2, path B) involving a protonated intermediate such as **6**, or alternative described below. Analogous N-substituted amidomethyl esters of carboxylic acids also undergo solvolysis by similar $S_N1$ and acid catalyzed mechanisms.[7]

In the present work, we prepared and studied carbamates analogous to **4** that contain a $\beta$-eliminative group and can undergo base-catalyzed cleavage of the O-alkyl bond (**7**, Scheme 3).

**Scheme 3**




We reasoned that if the $S_N1$ reaction of such carbamates could be sufficiently suppressed by an N-aryl stabilizer, cleavage of the C−O bond of the carbamate would be driven by $\beta$-elimination to provide the carboxylated Mannich base **8**. We assumed that the carboxylated secondary aryl amines **8** would undergo an acid-catalyzed decarboxylation as described for their well-studied primary aryl-amine counterparts,[8,9] and that the resultant N-aryl Mannich base **9** would convert to the free drug and iminium ion, which would dissociate to the aryl amine and $CH_2O$.[10]

Based on the above considerations, we designed and executed the synthetic sequence for the N-aryl-N-p-nitrophenoxymethyl-(pNPhOCH$_2$)-carbamate conjugates depicted in Scheme 4. pNPhOH was selected as the leaving group because it can be easily detected by UV, and is as acidic a phenol as we would anticipate being present in most drugs. As aryl amine "stabilizer" components of carbamates, we used aniline ($pK_a$ 4.6), p-$NH_2PhC(O)NEt_2$, ($pK_a$ 3.5), and p-$NH_2PhSO_2N(CH_2)_2O$ ($pK_a$ 1.6) because of the anticipated suppression of $S_N1$ hydrolysis provided by the electron withdrawing aryl groups.[4] For the $pK_a$ modulator that drives $\beta$-elimination we used the phenylsulfone ($PhSO_2$-) moiety that has been extensively studied as a $pK_a$ modulator in cleavage of carbamates of primary amines.[3] As controls that cannot undergo $\beta$-elimination but could undergo $S_N1$ and acid-catalyzed hydrolysis, we used analogous linkers that did not contain a modulator.

Azidoalcohol **11** containing Mod=$PhSO_2$− was converted to its chloroformate by treatment with triphosgene, and then reacted with aryl amines to give N-aryl carbamates **12A-C**. The carbamates were treated with TMSCl/$CH_2O$ to provide the reactive N-chloromethyl carbamates **13A-C**. Reaction of **13A-C** with pNPhONa gave the corresponding pNPhOH ethers **14A-C**. Using Cu-free [3 + 2] dipolar cycloaddition, connection of **14A-C** to mPEG$_{40\ kDa}$-dibenzocyclooctyne(DBCO)[3,11] to form the PEG conjugates **15A-C** was achieved in near-quantitative yield. Starting with 6-azido-1-hexanol, a similar sequence of conversions gave the stable azidohexylcarbamates **16A-C** without a modulator that were converted to PEG-conjugates **17A-C**. The generality of this synthesis is demonstrated by its application to the tyrosine analog, N-(6-DNP-aminocaproyl)-Tyr, of **14B** with phenol $pK_a$ ~ 10 (**24**; see SI), and the anticancer agent SN38 ($pK_a$ ~ 8.6).[12]

**Solvolysis Products of N-aryl-N-pNPhOCH$_2$-carbamates.** The solvolysis products of the N-(pNPhOCH$_2$)carbamates studied here identifies the pathways of pNPhOH release. That is, the spontaneous (Scheme 2, path A) or acid catalyzed (Scheme 2, path B) reactions should both provide pNPhOH and an N-aryl carbamate, whereas $\beta$-eliminative cleavage (Scheme 3) should result in formation of pNPhOH, the aryl amine and the alkenyl sulfone ($PhSO_2CH$=CHR). In accordance with these expectations, in 1 N HCl, the azido-linker-carbamate **14C** gave pNPhOH and the N-arylcarbamate **12C**; the carbamate product remained stable in 1 N HCl for at least 140 h thereafter. Under similar conditions, the corresponding PEG conjugate **15C** released only pNPhOH and the other product was assumed to remain coupled to PEG as the N-aryl carbamate. At pH 4−9, O-alkyl-N-aryl carbamates analogous to **16C** have previously been shown to undergo spontaneous $S_N1$ hydrolysis to yield the N-aryl carbamate and pNPhOH.[4] At pH 8.4, the azido-linker-carbamate with a $PhSO_2$− modulator **14C** yields the pNPhOH, aryl amine, and alkenylsulfone as expected from a $\beta$-elimination reaction; at pH > about 4 the corresponding PEG conjugates **15A-C** released pNPhOH and aryl amine leaving a PEG-alkenyl sulfone conjugate.

**Solvolysis Kinetics and Mechanisms of N-aryl-N-pNPhOCH$_2$-carbamates with Stable Linkers (17A-C).** The pH-log $k_{obsd}$ profiles for solvolysis of the PEG$_{40\ kDa}$-pNPhOCH$_2$-carbamates containing stable linkers (Figure 1) fit eq 1.

$$\log k_{obsd} = \log(k_{H+}[H_3O^+] + k_{H2O}) \quad (1)$$

There is an uncatalyzed release of pNPhOH at ~ pH 4.5 to 9.3 at 37° (Table 1; Figure 1) as observed in studies of related O-Me carbamates at pH 4.6 to 8.8,[4] and a hydronium ion catalyzed rate at lower pH values. The uncatalyzed, spontaneous rates are suppressed by electron withdrawing *p*-substituents of the N-aryl moiety of the carbamate (−H > −CON(Et)$_2$ > −$SO_2N(CH_2)_2O$) and related to the $pK_a$ of the arylamine component by eq 2.

$$\log k(h^{-1}) = 0.29pK_a - 3.95 \qquad (R^2 = 0.990) \quad (2)$$

With **17A**, the solvolysis rate shows a significant temperature effect, being about 36-fold slower at 37 °C than that reported for the analogous O-Me carbamate at 46 °C.[4] The data are in accordance with a mechanism that proceeds by pH-independent, spontaneous loss of pNPhOH with involvement of electrons of the carbamate nitrogen to generate an iminium ion intermediate (Scheme 2, path A). It has previously been shown that log *k* of the $S_N1$ reaction shows an excellent correlation with the $pK_a$ (slope = −0.93) of the departing phenol.[4] If we assume the effects of the stabilizer and leaving group abilities are independent of each other, bilinear eq 3 that uses the two linear free energy relationships for these reactions (above) allows estimation of the spontaneous solvolysis rate of any phenolic leaving group with the stabilizers described here.

$$\log k(h^{-1}) = -0.935 \times pK_a(\text{leaving group}) + 0.29 \times pK_a(\text{stabilizer}) + 2.71 \quad (3)$$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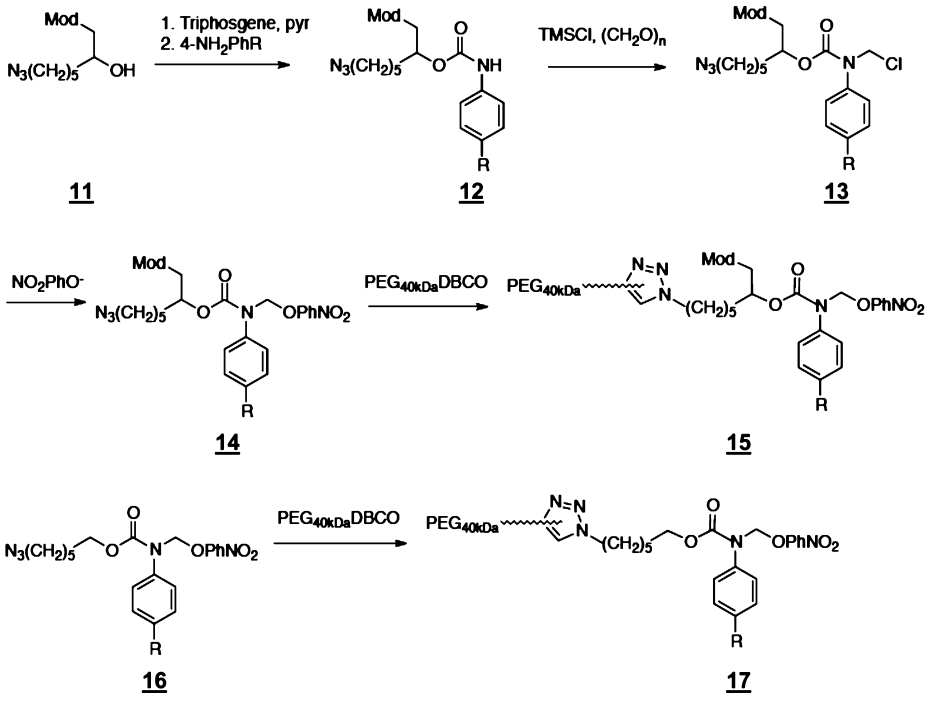
Scheme 4




**Mod = $PhSO_2$-**

**A**, R = H; **B**, R=-C(=O)N(Et)$_2$; **C**, R= -$SO_2N(CH_2CH_2)_2O$

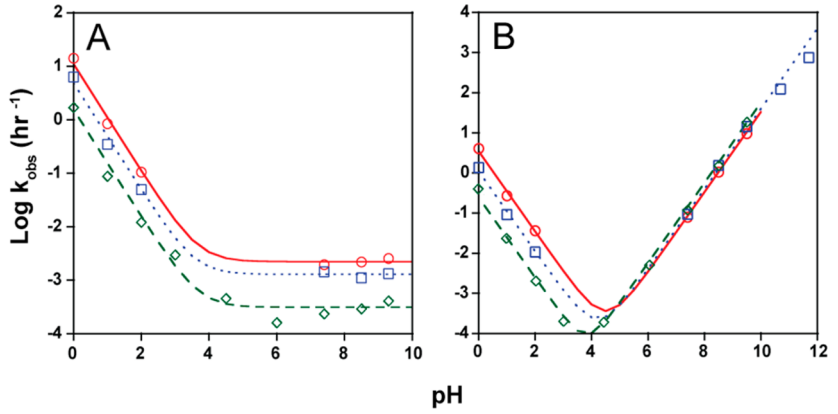



**Figure 1.** pH-log $k_{obsd}$ profiles for solvolysis of N-aryl-N-(pNPhO$CH_2$)carbamates: (A) stable linkers **17**; (B) $\beta$-eliminative linkers with $PhSO_2$-modulator **15A** (red), **B** (blue) and **C** (green).

Rate constants for the hydronium ion catalyzed cleavage of stable $PEG_{40\ kDa}$-$NO_2PhOCH_2$-carbamates (**17A-C**) are provided in Table 1. As with the spontaneous solvolysis at neutral pH, electron withdrawing substituents on the carbamate nitrogen retarded the solvolysis and were related to the p$K_a$ of the arylamines by eq 4

$$\log k_{H+}(M^{-1}\,h^{-1}) = 0.315pK_a - 0.42 \qquad (R^2 = 0.997) \tag{4}$$

which is essentially identical to that of the uncatalyzed reaction.

The acid-catalyzed mechanism of solvolysis must involve pre-equilibrium protonation, but it is difficult to precisely place the position of protonation because of several possible kinetically equivalent mechanisms. The simplest mechanism is rationalized as protonation of the leaving pNPhOH ether oxygen followed by rapid collapse to the iminium ion (Scheme 5, path A). However, the carbonyl oxygen of a carbamate is the most basic site for protonation,[13] with an estimated p$K_a$ in analogous amides of $\sim -1$. Scheme 5, path B, shows a possible mechanism involving initial protonation of the carbonyl group of the carbamate (**1**) and formation of a transient $sp^3$ hybridized tetrahedral intermediate (**20**), analogous to that which occurs in acid-catalyzed amide hydrolysis.[14] In amide hydrolysis, it is well established that protonation of the amine in the tetrahedral intermediate is required for its departure and that amine basicity plays a profound role in collapse of the intermediate to the carboxylic acid. The p$K_a$ of the amine in a tetrahedral intermediate is reduced some 3- to 5-units compared to its free state,[15] and in the case of **20**, would be lowered by another ~2 units by the N-aryloxy substituent.[16] This could effectively


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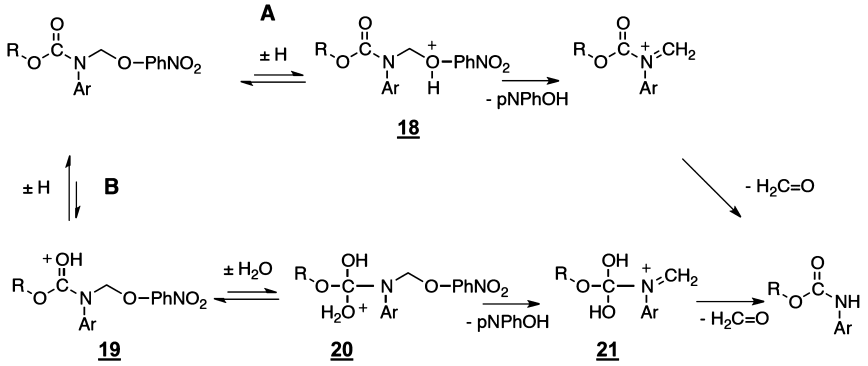
**Table 1. Rates of pNPhOH Release from PEG-Conjugates**

| | N-aryl group | Mod | $k_{H3+0}$ $M^{-1}$ $h^{-1}$ | $k_{H20}$, $h^{-1} \times 10^3$ | $k_{OH^-}$, $M^{-1}$ $h^{-1} \times 10^{-3}$ | $t_{1/2}$ h, pH 7.4 |
|---|---|---|---|---|---|---|
| **15A** | Ph- | $PhSO_2$- | 3.5 ± 0.70 | ND | 334 ± 44 | 8.3 ± 1.1 |
| **15B** | $Et_2NCOPh$- | $PhSO_2$- | 1.1 ± 0.23 | ND | 404 ± 82 | 6.8 ± 1.4 |
| **15C** | $O(CH_2)_2NSO_2Ph$- | $PhSO_2$- | 0.25 ± 0.08 | ND | 572 ± 63 | 4.8 ± 0.5 |
| **17A** | Ph- | none | 11.0 ± 2.9 | 2.23 ± 0.31 | | 311 ± 42 |
| **17B** | $Et_2NCOPh$- | none | 4.9 ± 1.4 | 1.29 ± 0.17 | | 536 ± 69 |
| **17C** | $O(CH_2)_2NSO_2Ph$- | none | 1.5 ± 0.07 | 0.31 ± 0.09 | | 2214 ± 636 |

**Scheme 5**




prevent amine protonation necessary for its departure, but electrons of the tetrahedral nitrogen might still facilitate iminium ion formation with concomitant elimination of pNPOH. Finally, the protonated carbamate **19** might undergo $S_N2$ displacement to form O-hydroxymethyl-pNPOH, in a manner analogous to that proposed for hydrolysis of N-acylimines.[17] However, the detection of a N-hydroxymethyl-carbamate intermediate in a related work argues against this mechanism.[12]

**Solvolysis Kinetics and Mechanism of N-aryl-N-pNPhOCH$_2$-carbamates with $\beta$-Eliminative Linkers (15A-C).** The pH-log $k_{obsd}$ profiles for solvolysis of $PEG_{40\ kDa}$-$NO_2PhOCH_2$-carbamates containing $\beta$-eliminative linkers **15A-C** (Figure 1) are best described by eq 5.

$$\log k_{obsd} = \log(k_{H+}[H_3O^+] + k_o + k_{OH-}[OH^-]) \qquad (5)$$

The rate of release of pNPhOH was first order in hydronium ion from pH 0 to ~4 at 37°. The rate constants (Table 1) are 3- to 6-fold slower than those of the stable carbamates, so there is a modest inhibition of the rate by the modulator. Electron withdrawing substituents on the carbamate nitrogen retarded the solvolysis and were related to the p$K_a$ of the N-arylamines by the eq 6 which is very similar to that described for the stable linker. Thus, the acid-catalyzed mechanisms of **15A-C** solvolysis are concluded to also occur by the mechanism depicted in Scheme 2, path B

$$\log k_{H+}(M^{-1}\,h^{-1}) = 0.358pK_a - 1.16 \qquad (R^2 = 0.997) \qquad (6)$$

As with analogous primary carbamates,[3] $PEG_{40\ kDa}$-$NO_2PhOCH_2$ N-aryl carbamates with Mod=$PhSO_2$−, **15A-C**, showed release rates of pNPhOH that were first order in hydroxide ion between pH ~4 to 11.7 (Table 1). Thus, the base-catalyzed reaction involves $\beta$-eliminative cleavage of the O-alkyl bond of the carbamate to form a carbamic acid that collapses to $CO_2$, $CH_2O$, the aromatic amine, and pNPhOH (Scheme 3). The rates of $\beta$-elimination were only slightly accelerated by electron withdrawing N-substituents, which can be attributed to the more acidic carbamic acid leaving groups; the rates follow the equation $\log k_{OH-} = -0.075\ pK_a + 0.53$ ($R^2 = 0.972$). Notably, although the rate dependence on p$K_a$ of the aryl amine is small, it opposes the retarding effect observed in spontaneous hydrolysis. The base-catalyzed rate of $\beta$-elimination is suppressed only 2.5-fold by a phenol having poorer leaving group ability since an O-alkylated tyrosine analog of **14B** (**24**) showed $t_{1/2}$ = 17 h at pH 7.4 (SI).

At pH 4.5, near the nadir of the pH-log $k$ profile (~pH 3.8), the observed rate of hydrolysis of **15C** ($0.23 \times 10^{-4}$ $h^{-1}$) is ~15-fold slower than the spontaneous hydrolysis rate of **17C** containing a stable linker ($3.4 \times 10^{-4}$ $h^{-1}$) and a fit of the data in Figure 1B using the latter rate constant for $k_O$ was poor. Thus, the stable linker is not a perfect surrogate for the spontaneous hydrolysis rate of **15C**, containing Mod=$PhSO_2$−.

The rates of base catalyzed $\beta$-eliminative cleavage of the N-aryloxy-N-aryl carbamates **15A-C** are some ~3- to 6-fold faster than analogous carbamates of primary aliphatic amines.[3] This is attributed the lower p$K_a$ of N-carbamic acids of N-aryl vs primary amines and hence greater leaving group ability of the N-aryl carbamates. The good leaving group ability of the N-aryl carbamates are caused by two effects: (a) electron withdrawing substituents on the N-aryl group decrease the p$K_a$ of carbamic acids,[8] manifested here by rate differences of up to 1.7-fold for $\beta$-elimination of reactants with differing stabilizers, and (b) even lower p$K_a$ values expected for carbamic acids of Mannich bases[10] of and N-hydroxymethylated[16] aryl amines, which are ~2 units lower than those of the parent aryl amine; N-aryloxymethylation should cause a similar p$K_a$ depression and consequent increase in carbamate leaving group ability. Indeed, $\beta$-elimination of the N-aryloxymethyl carbamate **14B**, containing a N-aryloxy group, solvolyzes some 4-fold faster than the azido-linker-N-arylcarbamate **12B** ($t_{1/2}$ = 51 h, pH 7.4), not containing a N-aryloxy group.

**Detection of Intermediates.** We desired to determine whether decarboxylation of the carbamic acid **8** ($k_2[H_3^+O]$, Scheme 3) or collapse of the Mannich base intermediate **9** ($k_3$, Scheme 3) might be slow enough to allow their accumulation at physiologic pH. Several indirect lines of evidence indicated this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was likely not the case. First, the rates of decarboxylation of primary aryl amine carbamates are rapid at physiological pH, with $t_{1/2}$ values of several minutes for the slowest.[8] Second, we did not detect intermediates in any of the aforementioned studies using HPLC − although we recognize the chromatography support could have induced instabilities. Third, as reported for primary aryl amine carbamates,[8] we attempted to trap a stable carbamate by allowing its formation from azido-linker carbamates **14A**, **B** in strong base, and then monitoring for spectral changes accompanying decarboxylation upon neutralization. However, upon dissolution of **14B** at pH 12, pNPhOH was released as rapidly as we could perform a measurement, and no further spectral changes were detected upon neutralization. These results, albeit indirect, suggest that both decarboxylation and Mannich base decomposition occurred very rapidly at basic pH.

We next attempted to detect an intermediate that follows $\beta$-elimination by a kinetic approach, following the release of $pNPhO^-$ at 405 nm that accompanies cleavage of the ether bond at basic pH and is immune to potential isolation artifacts. The rate of pNPhOH formation in the three sequential irreversible reactions depicted in Scheme 3 is expressed by eq 7. The differential equation was derived by reported approaches[18] (see SI) and simulated at different pH values using MS Excel. Here, the $F$ terms represent amplitude coefficients for each of three exponentials representing each rate constant (see SI). Over the pH range where the hydroxide-catalyzed $\beta$-elimination is slow compared to the others $F_1$ approaches 1 and the other coefficients approach 0; the expression becomes eq 8, and the pH-log rate profile has a slope of +1. If, at higher pH, the hydroxide-catalyzed rate becomes faster than a subsequent reaction, the rate will be governed by the decarboxylation reaction ($F_2 \times e^{-k2[H+]\ t}$) or pH independent Mannich base decomposition ($F_3 \times e^{-k3t}$). In such cases, as the $F$ associated with an exponential approaches 1 and the others approach 0, the pH-log rate profile will have a slope of −1 (eq 9) if the decarboxylation becomes rate determining, or 0 if the Mannich base decomposition becomes rate determining (eq 10). Thus, the rate obtained at the highest pH where pNPhOH release remains first order in hydroxide allows assignment of a lower limit on the rate of reaction of either intermediate at that pH. Since the rate of reaction either remains the same (rate-determining Mannich base collapse[10]) or decreases (rate-determining decarboxylation[8]) at lower pH, this rate also corresponds to a lower limit of the overall rate at pH 7.4.

$$dP/dt = [1 - F_1 \times e^{-k1[OH-]t} - F_2 \times e^{-k2[H+]t} - F_3 \times e^{-k3t}] \quad (7)$$

$$dP/dt = 1 - e^{-k1[OH-]t} \quad (8)$$

$$dP/dt = 1 - e^{-k2[H+]t} \quad (9)$$

$$dP/dt = 1 - e^{-k3t} \quad (10)$$

The log $k_{obsd}$ vs pH profile for pNPhOH formation from **15B**, Mod=$PhSO_2-$, shows a slope of +1 from pH 7.4 to at least pH 11.7, the highest pH we could conveniently monitor the reaction. Here, $t_{1/2}$ ~5 s so neither intermediate has a $t_{1/2}$ > 5 s at that pH. At lower pHs, the acid-catalyzed decarboxylation would be even more rapid,[8] and the rate of Mannich base breakdown would be pH-independent until the aryl amine is protonated at pH < 5.[10] Thus, we conclude that both intermediates decompose rapidly and do not accumulate to any significant extent at pH 7.4.

## DISCUSSION

In the present work, we adapted $\beta$-eliminative linkers originally designed to deliver amine-containing drugs from carbamate-containing linkers so they can be used with phenol-containing drugs. The functional components of the adapted linkers include (a) an electron-withdrawing modulator that, as before,[3] governs the rate of $\beta$-elimination and drug release, (b) a methylene adaptor that connects the carbamate nitrogen to the phenol oxygen of the drug, and (c) an electron-withdrawing stabilizer attached to the carbamate nitrogen that inhibits undesirable spontaneous cleavage of the adaptor−oxygen bond. Upon $\beta$-elimination, a carboxylated Mannich base of the drug is released that collapses to provide the free phenol-containing drug.

The synthesis follows that reported for primary carbamates[4] to give aryl amino carbamates **12**; intermediate **12** contains an azido group for coupling to a macromolecule, a modulator, and a carbamate of an electron withdrawing aryl amine. N-Chloromethylation of **12** provided **13**, which readily alkylates the oxygen of a phenol in the presence of base. After purification, the azido-linker-phenol conjugate **14** is coupled by Cu-free click chemistry to PEG modified with a cyclooctyne to give the bioconjugate **15**. For stable analogs not containing a modulator (**17**), analogous synthetic sequences were performed starting from an appropriate O-(6-azidohexyl)-N-aryl carbamate. As indicated by previous studies,[4] the synthetic approach should also yield conjugates of drugs containing other acidic heteroatoms and, indeed, we have prepared conjugates of alcohols and thiols by this approach.

Solvolysis of conjugates with stable linkers **17** at all pH values and those containing a $PhSO_2-$ modulator **15** in acidic media gave pNPhOH and the N-aryl carbamate as final products, indicating initial cleavage of the methylene adaptor oxygen bond and formation of an iminium ion intermediate. In contrast, solvolysis of the conjugate containing a $PhSO_2-$ modulator at pH > about 4 yielded pNPhOH, the alkenylsulfone and aryl amine as products, indicating initial $\beta$-eliminative O-alkyl cleavage of the carbamate bond.

Previous and current kinetic studies of the solvolysis covering a wide pH range allow a more detailed analysis of the reaction mechanisms. The kinetics for solvolysis of the $\beta$-eliminative conjugates is described by eq 11.

$$k_{obs} = k_{H+}[H_3O^+] + k_{H2O} + k_{OH-}[OH^-] \quad (11)$$

The first term of eq 11, $k_{H+}[H_3O^+]$, is the specific acid catalyzed reaction involving protonation of the reactant followed by release of pNPhOH and the N-aryl carbamate. The rates are lowered by electron withdrawing groups on the aryl carbamate nitrogen that destabilize the iminium ion intermediate, and show a linear relationship with the $pK_a$ of the parent aryl amine. The results are in accordance with equilibrium protonation of the leaving group ether-oxygen, followed by rate determining cleavage to pNPOH and the unstable carbamate iminium ion (Scheme 5); the iminium ion intermediate would rapidly hydrate to the N-hydroxymethyl carbamate which would rapidly lose formaldehyde in acidic media.[6]

The second term of eq 11, $k_{H2O}$, is the spontaneous uncatalyzed $S_N1$ counterpart of the acid catalyzed reaction that also gives pNPhOH and the N-aryl carbamate as products (Scheme 2). The exact contribution of this pathway cannot be ascertained with linkers containing a $pK_a$ modulator that causes a competing, more rapid $\beta$-elimination reaction. Thus, it was studied with surrogate stable linkers having varying N-aryl


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stabilizers (**17A-C**). As with the acid catalyzed reaction, the rates are retarded by electron withdrawing groups that destabilize the iminium ion intermediate, and show a linear inverse relationship with the p$K_a$ of the aryl amine component that is nearly identical to that in the acid-catalyzed reaction. Majumdar and Sloan have also reported that the $S_N1$ reaction shows an excellent correlation with the p$K_a$ (slope = −0.93) of the departing phenol.[4]

The third term of eq 11, $k_{OH-}[OH^-]$, is the specific base catalyzed $\beta$-eliminative cleavage driven by the electron withdrawing effect of the modulator. The initial step is the cleavage of the O-alkyl bond of the carbamate to give a carboxylated Mannich base which undergoes decarboxylation and subsequent collapse[8] of the Mannich base to the aryl amine, formaldehyde, and free drug (Scheme 3). Using the $PhSO_2-$ modulator and N-aryl stabilizers, $\beta$-elimination reactions occur at physiological pH about 3- to 6-fold faster than analogous primary carbamates;[3] this can be ascribed to the lower p$K_a$ of the carbamic acid leaving groups by the electron withdrawing aryl amine enhanced further by the N-aryloxy group.[10,16] In contrast to the acid-catalyzed and $S_N1$ solvolysis, the $\beta$-eliminative rates show only a small dependence on the p$K_a$ of the aryl amine stabilizer.

Once formed *in vivo*, the two intermediates of the $\beta$-elimination reaction − the carboxylated Mannich base and the Mannich base − should ideally convert to products at a rapid rate; otherwise, they might accumulate and distribute or clear with different pharmacokinetics than the native drug. Studies[8] show that the rate of decarboxylation of N-aryl carbamic acids is sufficiently rapid that such intermediates should not accumulate to any significant extent at neutral pH. Indeed, in the present case, we could estimate that the carbamic acid **8** decarboxylated with a $t_{1/2}$ <5 s at physiologic pH. In the only direct mechanistic study of cleavage of N-aryl Mannich bases we are aware of, Bundgaard and Johansen[10] showed that the rates of collapse are pH independent above their p$K_a$, and inhibited when protonated. Rates of breakdown of N-aryl Mannich bases of imides are depressed by reducing the p$K_a$ of the aryl amine, with a 9.3-fold change per p$K_a$ unit. The rates are likewise decreased by increasing the p$K_a$ of the leaving imide group, with a change of about 13-fold per p$K_a$ unit. With conjugate **15B**, pNPhOH is a sufficiently good leaving group (p$K_a$ 7.14) that, based on these studies, we would not expect Mannich base accumulation with any of the stabilizers used; indeed, using a kinetic approach we could estimate that the Mannich base formed from **15B** also has a $t_{1/2}$ of <5 s at pH 7.4.

In designing linkers for *in vivo* use, it is important that the stabilizer is sufficiently electron withdrawing to depress the rate of the $S_N1$ reaction such that it does not compete with $\beta$-elimination − i.e., $k_{OH}(OH^-) \gg k_{H2O}$. For example, O-Me-N-methyl-N-pNPhOCH$_2$-carbamate hydrolyzes some 24-fold faster than the corresponding N-phenyl carbamate,[4] which from our data (Table 1) gives the $S_N1$ reaction of the N-alkyl carbamate a $t_{1/2}$ of about 13 h at neutral pH; thus, with a N-Me stabilizer and the $PhSO_2-$ modulator used here ($t_{1/2}$ ∼8.3 h at pH 7.4) only ∼40% of the product would emanate from $\beta$-elimination. Likewise, with the N-Ph modulator **15A**, the $S_N1$ rate is only about 37-fold slower than the $\beta$-elimination reaction at pH 7.4 (Table 1), which would compete with $\beta$-elimination in linkers with longer half-lives. On the other hand, the modulator should not be so electron withdrawing that it unduly stabilizes the Mannich base intermediate.[10] This is not an issue in the present work, but it could become an important consideration with drug leaving groups that are so poor they stabilize the Mannich base intermediate. However, poorer leaving groups also slow the $S_N1$ reaction,[4] so a less electron-withdrawing stabilizer could be used for $S_N1$ stabilization and concomitantly decrease the lifetime of the Mannich base. Thus, a key requirement for effective use of these linkers is an appropriate balance between the leaving group ability of the drug in the context of the modulator used (i.e., $t_{1/2}$ for $\beta$-elimination at pH 7.4). Since this balance may vary with each type of leaving group, it may be necessary to determine the structure activity relationships of the leaving group abilities and $S_N1$ rates on the stabilizer for each drug functional group used in such linkers.

Linker design should also incorporate considerations of the need for long-term storage of drug-containing bioconjugates. Since the hydroxide-catalyzed $\beta$-elimination rate is depressed 10-fold for each pH unit decrease, and another 30-fold for each 10 °C reduction of temperature,[3] linkers can be stabilized for long periods by simply lowering the pH and temperature. However, the stability afforded by lowering pH is limited to the extent where such stabilization exceeds the spontaneous $S_N1$ or acid catalyzed reaction rates.

## ■ CONCLUSION

We have described an approach to adapt $\beta$-eliminative linkers for carbamate-linked amino groups of drugs so they can also be used to attach phenolic hydroxyl groups of drugs. Kinetic studies of solvolysis defined the mechanistic pathways throughout the pH range, and established three key elements for controlled release. These elements are: (a) the user-defined modulator that controls the rate of base-catalyzed $\beta$-eliminative drug release, (b) the leaving group ability of the drug component, and (c) the carbamate stabilizer that controls the rate of spontaneous $S_N1$ solvolysis. In designing bioconjugates using the approach described here, it is essential to understand and appropriately balance these effects to achieve optimized control of drug release.

## ■ ASSOCIATED CONTENT

### Supporting Information

Synthesis of intermediates and PEG conjugates, kinetic procedures and derivation of equations. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

## ■ REFERENCES

(1) Greenwald, R. B., Yang, K., Zhao, H., Conover, C. D., Lee, S., and Filpula, D. (2003) Controlled release of proteins from their poly(ethylene glycol) conjugates: drug delivery systems employing 1,6-elimination. *Bioconjugate Chem. 14*, 395−403.

(2) Filpula, D., and Zhao, H. (2008) Releasable PEGylation of proteins with customized linkers. *Adv. Drug. Delivery Rev. 60*, 29−49.

(3) Santi, D. V., Schneider, E. L., Reid, R., Robinson, L., and Ashley, G. W. (2012) Predictable and tunable half-life extension of therapeutic agents by controlled chemical release from macromolecular conjugates. *Proc. Natl. Acad. Sci. U.S.A. 109*, 6211−6.

(4) Majumdar, S., and Sloan, K. B. (2006) Synthesis, hydrolyses and dermal delivery of N-alkyl-N-alkyloxycarbonylaminomethyl (NANAOCAM) derivatives of phenol, imide and thiol containing drugs. *Bioorg. Med. Chem. Lett. 16*, 3590−4.

(5) Bundgaard, H., and Johansen, M. (1984) Hydrolysis of N-(α-hydroxybenzyl) benzamide and other N-(α-hydroxyalkyl) amide derivatives: implications for the design of N-acyloxyalkyl-type prodrugs. *Int. J. Pharm. 22*, 45−56.

(6) Tenn, W. J., III, French, N. L., and Nagorski, R. W. (2001) Kinetic dependence of the aqueous reaction of N-(hydroxymethyl)benzamide derivatives upon addition of electron-withdrawing groups. *Org. Lett. 3*, 75−78.
(7) Iley, J., Moreira, R., Calheiros, T., and Mendes, E. (1997) Acyloxymethyl as a drug protecting group: Part 4. The hydrolysis of tertiary amidomethyl ester prodrugs of carboxylic acid agents. *Pharm. Res. 14*, 1634−9.
(8) Johnson, S. L., and Morrison, D. L. (1972) Kinetics and mechanism of decarboxylation of N-arylcarbamates. Evidence for kinetically important zwitterionic carbamic acid species of short lifetime. *J. Am. Chem. Soc. 94*, 1323−34.
(9) Ewing, S. P., Lockshon, D., and Jencks, W. P. (1980) Mechanism of cleavage of carbamate anions. *J. Am. Chem. Soc. 102*, 3072−3084.
(10) Bundgaard, H., and Johansen, M. (1981) Prodrugs as drug delivery systems. XIX. Bioreversible derivatization of aromatic amines by formation of N-Mannich bases with succinimide. *Int. J. Pharm. 8*, 183−192.
(11) Debets, M. F., van Berkel, S. S., Schoffelen, S., Rutjes, F. P., van Hest, J. C., and van Delft, F. L. (2010) Aza-dibenzocyclooctynes for fast and efficient enzyme PEGylation via copper-free (3 + 2) cycloaddition. *Chem. Commun. 46*, 97−9.
(12) Santi, D. V., Schneider, E., and Ashley, G. A. (Unpublished Results).
(13) Olah, G. A., and Calin, M. (1968) Stable carbonium ions. LIX. Protonated alkyl carbamates and their cleavage to protonated carbamic acids and alkylcarbonium ions. *J. Am. Chem. Soc. 90*, 401−4.
(14) Brown, R. S., Benneta, A. J., and Slebocka-tilk, N. (1992) Recent perspectives concerning the mechanism of $H_3O^+$- and $OH^-$-promoted amide hydrolysis. *Acc. Chem. Res. 25*, 481−488.
(15) Guthrie, J. P. (1974) Hydration of carboxamides. evaluation of the free energy change for addition of water to acetamide and formamide derivatives. *J. Am. Chem. Soc. 96*, 3608−3615.
(16) Abrams, W. R., and Kallen, R. G. (1976) Equilibria and kinetics of N-hydroxymethylamine formation from aromatic exocyclic amines and formaldehyde. effects of nucleophilicity and catalyst strength upon mechanisms of catalysis of carbinolamine formation. *J. Am. Chem. Soc. 98*, 7777−7789.
(17) Sayer, J. M., and Conlon, P. (1980) The timing of the proton-transfer process in carbonyl additions and related reactions. general-acid-catalyzed hydrolysis of imines and N-acylimines of benzophenone. *J. Am. Chem. Soc. 102*, 3592−3600.
(18) Korobov, V. I., and Ochkov, V. F. (2011) Multi-Step Reactions: The Methods for Analytical Solving the Direct Problem, in *Chemical Kinetics with Mathcad and Maple*,pp 35−72, Springer-Verlag, Vienna.