A Kinetic Study of Product Cleavage Reactions from the Solid Phase by a Biocompatible and Removable Cleaving Reagent, HCl

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TFA has been widely used as a cleaving reagent in solid-phase organic synthesis. However, it is difficult to remove from the final product, and it is toxic to various cells. To search for an alternative, we studied the kinetics of HCl cleavage reactions of 18 resin-bound compounds on various linkers. HCl is very easy to remove completely from samples, and the residual HCl does not have a toxic effect in cell assays. Most compounds studied in this work can be easily cleaved using a low concentration of HCl (0.9−2.3%) and the minimal amount of time (60−90 min). Even in the most difficult case, a moderate 8% HCl and an extended time (10−15 h) are enough to cleave the product. Therefore, our kinetic studies establish HCl as a biocompatible, removable, and effective substitute for TFA when final compounds are used for biological screening and drug discovery.

Introduction

Combinatorial chemistry,[1] as an enabling discovery technology, has been widely implemented in drug discovery and other research disciplines in the past decade. The synthesis of diverse organic compound libraries on solid supports[2] has been a key methodology in combinatorial chemistry as applied for drug discovery. One crucial component of solid-phase organic synthesis (SPOS) is the linker strategy.[3] Various linkers, such as acid-,[4] base-,[5] or photocleavable[6] linkers, have been developed to accommodate synthesis and cleavage reactions. Among various linkers and cleavage strategies, the application of acid-labile linkers that are cleaved by trifluoroacetic acid (TFA)[4] is probably the most popular approach. However, TFA is difficult to remove completely in the final products by available evaporation methods. The residual amount of TFA in the product can become highly concentrated when the compound is stored as stock solution or dried. In the presence of TFA, compounds with acid-sensitive scaffolds are degraded over time. The analysis of remaining compound showed that compounds are unstable as TFA adducts.[7] The elevated solution-phase pH from the excess TFA or the direct reaction between TFA and the library compounds are responsible for compound decomposition. However, these compounds are essential parts of the diverse chemical space. It is important to keep their integrity to explore their potential in drug discovery. Furthermore, TFA, at a concentration of 10−100 nM, reduced cell numbers and thymidine incorporation into fetal rat osteoblast cultures after 24 h. Similar effects were found in cultures of articular chondrocytes and neonatal mouse calvariae, indicating that the toxic effect of TFA is not specific to one cell type or to one species. When the activities of TFA and HCl were compared in osteoblasts, cell proliferation was consistently less with TFA, resulting in failure to detect a proliferative effect or wrongly attributing an antiproliferative effect.[8] This finding indicated that TFA may cause serious false positive or false negative problems in cell-based high-throughput screening, while HCl is a biologically compatible reagent.

TFA can be introduced by using TFA as a cleaving reagent in solid-phase synthesis or by using TFA as an additive in HPLC purification. Like the superb activity of TFA in cleavage reactions, TFA is also the best additive, so far, to improve chromatography separation. To alleviate the adverse effect of TFA introduced in purification, the use of formic acid instead of TFA has been implemented. Therefore, a substitute acid for TFA in the cleavage of solid-phase-bound library compounds is urgently needed. The proper use of a cleavage agent is critical for the optimal synthesis yield and the compound quality.[9,10] Overuse of the cleaving reagent in terms of concentration and reaction time can degrade the compound and increase impurities in the final product. On the other hand, underuse of the cleaving reagent can leave uncleaved product on the resin and give a low synthetic yield. In this work, we explore the feasibility of using HCl as an alternative in acid cleavage reactions. HCl can be removed easily and completely from samples, and it is also biologically compatible. A better understanding of HCl cleavage kinetics will further validate the use of HCl instead of TFA in solid-phase cleavage reactions. Therefore, we studied the cleavage reaction kinetics of an array of diverse compounds using single-bead FT-IR microspectroscopy.[11] These compounds were linked to 2% PS-DVB (poly(styrene-divinyl-

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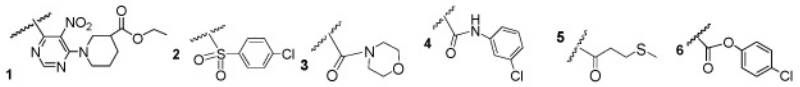
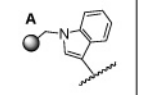
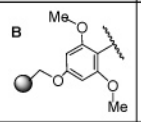
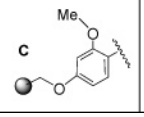
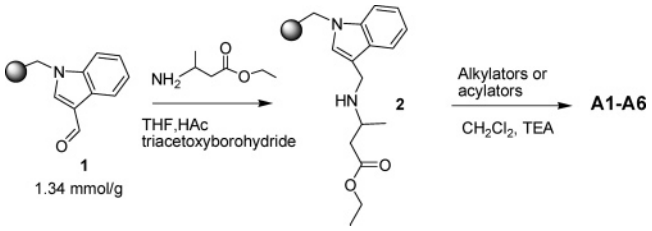
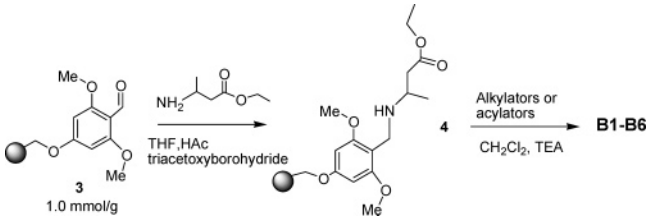
Table 1. A1−C6 Represents the Organic Molecules **1−6** Bound to a Polymer through Linkers **A**, **B**, and **C**

		R					
							
		A1	A2	A3	A4	A5	A6
		B1	B2	B3	B4	B5	B6
		C1	C2	C3	C4	C5	C6

Scheme 1. Synthesis of Resins **A1−A6**



Scheme 2. Synthesis of Resins **B1−B6**



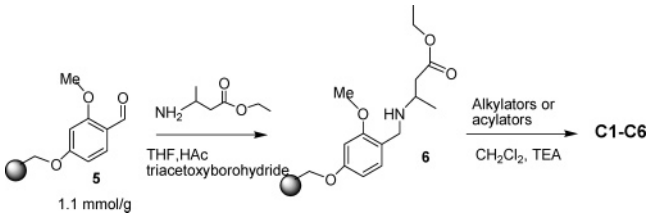
benzene)) resins through three commonly used linkers at different HCl concentrations. We found that HCl is effective at cleaving a wide range of organic compounds on different linkers. Details of our findings are reported in the following sections.

Results and Discussion

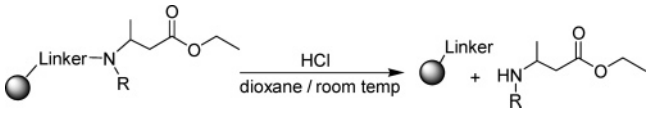
In our study, we investigated the cleavage kinetics of 18 compounds linked to three acid-labile linkers (**A**, **B**, and **C**) (Table 1). Schemes 1−3 show the synthetic routes for these compounds. Scheme 4 shows the general cleavage reaction of these compounds.

1. Synthesis of Resin-Bound Compounds A1−A6, B1−B6, and C1−C6. Linkers **A**, **B**, and **C** have been widely used in SPOS with a nitrogen atom as an anchoring point. Using these linkers, compounds were synthesized by reductive amination of the corresponding resin-bound aldehydes with amines, followed by alkylation or acylation of the secondary amines. All products were characterized by elemental analysis and single-bead FT-IR microspectroscopy. The FT-IR and elemental analysis results are shown in Table 2. The theoretical elemental composition values were calculated on the basis of the resin loading and the molecular structures of the resin and resin-bound compounds. The general agreement of the experimental and theoretical values for synthetic intermediate and compounds are found. For compounds **A6** and **B6**, their IR spectra indicated the formation of the intended compounds. The higher Cl % values are likely caused by the residual amount of solvent DCM used in the synthesis. To further prove this, selected compounds, including **A6**, were cleaved and analyzed by LC/MS to confirm their identity (see Supporting Information Figures S2−S5). The LC/MS data demonstrated the successful synthesis of all compounds.

Scheme 3. Synthesis of Resins **C1−C6**



Scheme 4. General Cleavage Reaction of Resin-Bound Compounds



2. Monitoring the Cleavage Kinetics on Resin and in Solution Simultaneously. The general cleavage reaction is shown in Scheme 4, and the detailed cleavage procedure is described in the following section. After the compound is cleaved, the free compound in solution can be detected by LC/MS. At the same time, single-bead FT-IR can detect the amount of compound that remained on resin by monitoring the intensity of the carbonyl band at ~1730 cm^{-1}. To validate our approach, we studied the cleavage kinetics of resin-bound compound **A2** both on resin and in solution.

The HPLC/UV chromatograms of the cleaved product in solution at various times are shown in Figure 1A. The product appeared in solution at 5 min, and its concentration

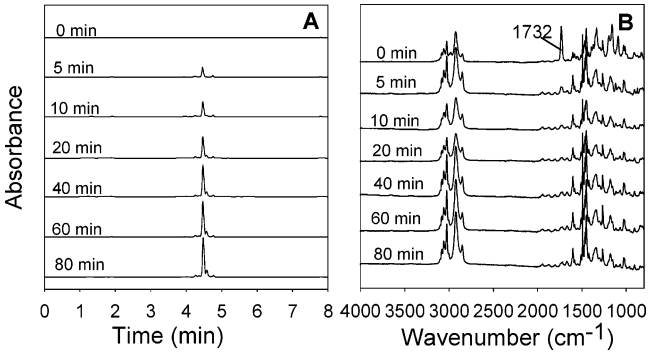


Figure 1. HPLC/UV_{214} chromatograms of the cleaved product and single-bead FT-IR spectra of resin **A2** at various reaction times during the HCl cleavage reaction.

Table 2. Elemental Analysis Results of Resin-Bound Compounds[a]

		theoretical (%)					experimental (%)				
	IR (cm^{-1})	C	H	N	S	Cl	C	H	N	S	Cl
1	1700			1.5					1.3		
2	1731			2.8					3.02		
3	1700			0					<0.02		
4	1731			1.24					1.08		
5	1700			0					<0.02		
6	1731			1.36					1.18		
A1	1731	76.94	7.04	7.48			78.38	6.95	6.51		
A2	1731				2.71					7.71	
A3	1735	83.01	7.55	3.74			81.91	7.57	4.09		
A4	1732	82.12	7.03	3.62			80.57	6.54	4.02		
A5	1731				2.91					3.06	
A6	1726					3.05					7.43
B1	1731	78.04	7.2	4.83			77.5	7.43	5.23		
B2	1731				2.41					3.05	
B3	1735	81.38	7.5	2.22			81.23	7.71	2.4		
B4	1731	80.64	7.11	2.15			80.49	6.71	2.28		
B5	1731				2.58					2.85	
B6	1726					2.72					6.16
C1	1731	79.12	7.22	4.83			78.13	7.29	4.54		
C2	1731				2.65					2.18	
C3	1735	81.57	7.59	2.45			81.24	7.77	2.02		
C4	1731	80.8	7.08	2.37			80.29	6.79	2.06		
C5	1731				2.84					2.36	
C6	1726					3					4.08

[a] All results are an average of duplicate measurements and the relative deviation between the duplicate values is less than 5%.

reached saturation between 20 and 40 min. Single-bead FT-IR spectra of resin **A2** at various times during the cleavage reaction are shown in Figure 1B. Resins were washed thoroughly and dried before FT-IR microspectroscopic measurements. Therefore, noncovalently trapped compounds were undetectable in the IR experiments. From the IR data, the compound was largely cleaved in 5 min, and the cleavage reached completion between 10 and 20 min. After cleavage, the free compound was probably briefly trapped inside the resin before it was released into solution. The rate of diffusion out of resin may depend on the hydrophobic nature of the product. This may account for the difference in the time to reach saturation for the in-solution and on-resin measurements. To further prove that the correct products were released into solution, four other randomly selected resin-bound compounds, **A4**, **A6**, **B4**, and **C2**, were analyzed by LC/MS after cleavage. Their identities were confirmed by LC/MS analysis (Supporting Information, Figures S2−S5).

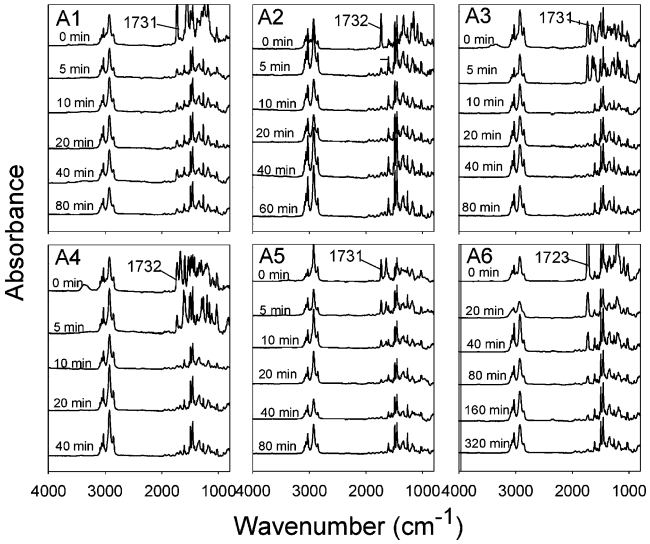


Figure 2. Single-bead FT-IR spectra of resin-bound compounds **A1**−**A6** at various times during the HCl cleavage reaction.

The correct product formation for **A6** also confirms that the compound synthesis was successful and that the high percentage of Cl in the elemental analysis (Table 2) was probably caused by contamination with solvent CH_2Cl_2.

3. Cleavage of Compounds from Various Linkers. The cleavage reactions using HCl in dioxane were carried out for all resin-bound products. HCl induced color changes in the suspension immediately. The color change depended on the compounds and ranged from light pink to dark brown. Single-bead FT-IR spectra of resins at various times during the cleavage reaction were acquired and analyzed. Spectra for **A1**−**A6** are shown in Figure 2**.** Similar data for all other resins are included in Figure S1 in the Supporting Information.

Resins **A1**−**C1** (∼30 mg each and the same for all other resins) were first reacted with 0.9% HCl. A droplet of the suspension was taken at various time intervals, washed with DCM for five times, and used for the single-bead FT-IR analysis. The cleavage of **C1** was finished in 320 min, but the cleavage of **A1** and **B1** had not gone to completion after 24 h, as indicated by the intensity of carbonyl bands in their IR spectra. Higher HCl concentrations (2.3 and then 8%) were then used for **A1** and **B1**. The areas of the carbonyl bands for **A1**−**C1** at various times were integrated. The overlapping peaks were resolved using the peak deconvolution software PeakFit (by AISN Software). The ratio of the peak area integration value with the peak area of an internal standard peak from polystyrene resin at 1947 cm^{-1} was used and plotted against time (Figure 3, **A1**, **B1**, and **C1**). The data sets were fitted to a pseudo-first-order reaction rate equation. The rate constants for **A1**, **B1**, and **C1** are 1.64×10^{-3}, 6.75×10^{-5}, and 1.81×10^{-3} s^{-1}, respectively.

After some preliminary tests, resins **A2** and **C2** were treated with 2.3% HCl, and resin **B2** was treated with 8% HCl (Figure 3, **A2**, **B2**, and **C2**). The data were processed as described above, and the rate constants for **A2**, **B2**, and **C2** were determined to be 2.72×10^{-3}, 3.43×10^{-5}, and 9.70×10^{-4} s^{-1}, respectively.

Resins **A3**, **A4**, **C3**, and **C4** were treated with 0.1% HCl, and **B3** and **B4** were treated with 0.6% HCl. The rate

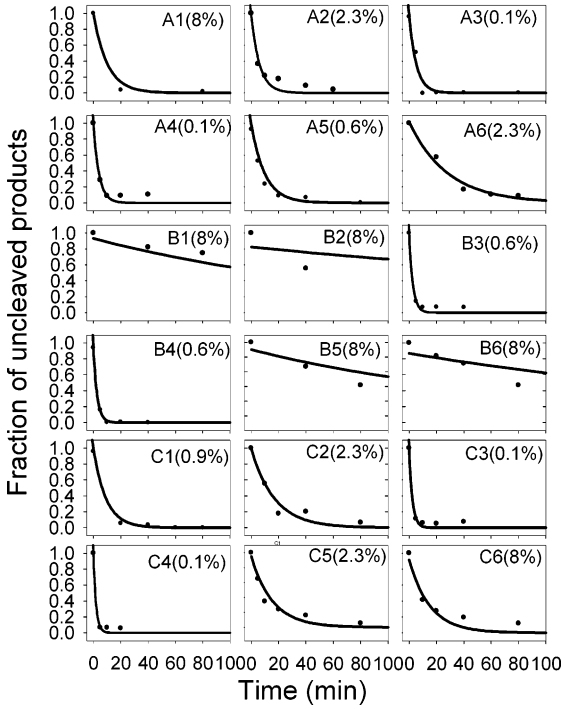


Figure 3. Time courses of the cleavage reaction for all 18 resins. Lines represent the best fit following a pseudo-first-order reaction. The concentration of HCl used in cleavage is shown in each panel.

constants for **A3**, **A4**, **B3**, **B4**, **C3**, and **C4** were determined to be 2.87×10^{-3}, 5.77×10^{-3}, 6.15×10^{-3}, 4.95×10^{-3}, 6.94×10^{-3}, and 8.63×10^{-3} s^{-1} (Figure 3, **A3**, **A4**, **B3**, **B4**, **C3**, and **C4**), respectively.

Resins **A5**, **B5**, and **C5** were treated with 0.6, 8, and 2.3% HCl (Figure 3, **A5**, **B5**, and **C5**). The rate constants were determined to be 1.67×10^{-3}, 8.79×10^{-5}, and 1.06×10^{-3} s^{-1}, respectively. Similarly, resins **A6**, **B6**, and **C6** were treated with 2.3, 8, and 8% HCl (Figure 3, **A6**, **B6**, and **C6**), respectively. Their rate constants were 5.94×10^{-4}, 5.56×10^{-5}, and 8.93×10^{-4} s^{-1}, respectively. For a side-by-side comparison, Figure 3 uses the same time scale (0−100 min) for all data. The complete sets of data points presented on a full time scale, as in the experiments, for each resin are shown in Figure S6 of the Supporting Information.

4. Comparison of Cleavage Kinetics for All Resins. Figure S7 in the Supporting Information shows the cleavage kinetics of the resin-bound compounds associated with linkers **A**, **B**, and **C**. The trend is that the cleavage requires milder conditions (lower concentration of HCl and less time) in the order **A** > **C** > **B** with linker **A** being the most acid-labile linker under our experimental conditions. In addition to the linker, the compounds bound to the linkers can also affect the cleavage reaction rate. For example, on the relatively inert linker **B**, urea compounds **B3** and **B4** tend to be cleaved readily. This effect was previously found using TFA as a cleaving reagent.[9]

A comparison of the cleavage kinetics of various classes of compounds on the same linker also reveals some interesting findings. As shown in Figure S8 in the Supporting Information, urea compounds tend to be cleaved easily (lower HCl concentration and less time). Other compounds show various rates in cleavage reactions. The proper HCl concentration and the cleavage time are identified for all resin-bound compounds.

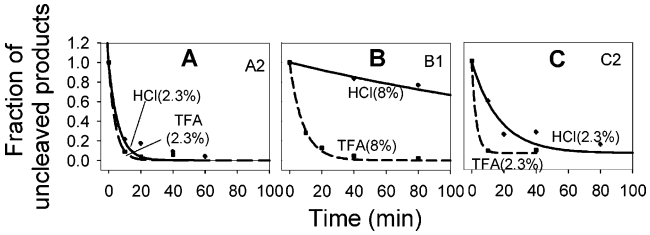


Figure 4. Comparison of cleavage reactions using TFA and HCl. Compounds on linkers (A) **A**, (B) **B**, and (C) **C** were studied. The lines represent the best fit to a pseudo-first-order reaction rate equation.

To replace TFA with HCl in acid cleavage reactions, we were also interested in comparing the effectiveness of HCl to TFA. Cleavage reactions in TFA have been extensively studied.[9] Various resin-bound compounds could be cleaved from 10 min to 5 h using 0.5−5% TFA solutions. In this work, most compounds studied can be easily cleaved using a low concentration of HCl (0.9−2.3%) and less time (60−90 min). Even in the most difficult case, a moderate 8% HCl and an extended time (10−15 h) are enough to cleave the product. One example is shown in Figure 4. The difference between the two acids is only evident on linkers that are more difficult to cleave. On the most acid-labile linker, **A**, there is no difference between TFA and HCl. On the relatively inert linker, **B**, there is a notable difference in cleavage reaction rates. We noticed that this difference may be partially because these two cleavage reactions used different solvents (dioxane and DCM). DCM has been shown to be a better swelling solvent for polystyrene resins. Even in very slow cleavage cases, using a practical, but longer reaction time (20−30 h) can properly cleave products from the solid phase.

Conclusion

The role of a good cleavage reagent is to cleave the product from solid-phase support quickly and completely without degradation. The reagent should be removed easily and completely, and the possible residual reagent should cause no harmful effect in biological assays. TFA, although a reactive cleaving reagent, does not meet the requirements because of its toxicity and the difficulty associated with its removal. In this study, we studied the cleavage reaction rates using HCl as a cleaving reagent. We carried out a thorough kinetic study of cleavage reactions of 18 resin-bound compounds including pyrimidine, sulfonamide, urea, amide, and carbamide from three commonly used acid-labile linkers. Two urea compounds were cleaved faster than the other compounds on all linkers. The indole linker **A** and benzyl linker **C** have been found to be more acid labile than the benzhydryl linker **B** in terms of the cleavage reaction rate. Pyrimidine, sulfonamide, amides, and carbamides were cleaved faster on **A** and **C** than on **B**. Most compounds can be easily cleaved using low concentrations of HCl (0.9−2.3%) and less time (60−90 min). Even in the most difficult case, such as **B2**, a moderate 8% HCl and an extended time (10−15 h) are enough to cleave the product. Therefore, our

kinetic studies established HCl as a biologically compatible, removable, and effective substitute for TFA when the final compounds are used for biological screening and drug discovery.

Experimental Section

Materials. Chemicals used in the solid-phase synthesis and cleavage reactions were purchased from Aldrich. HCl solutions in dioxane were made in house. The HCl gas was introduced into a flask containing distilled dioxane for about 3 h. The concentration of HCl in dioxane was then determined by NaOH titration using phenolphthalein as an indicator. HCl solutions of different concentrations (0.1, 0.6, 0.9, 2.%, and 8%) were then prepared by dilution of this solution with dioxane.

General Procedure for the Synthesis of Resins A1−A6, B1−B6, and C1−C6. Step 1. Reductive Amination. The aldehyde resin used as the starting material for the synthesis of resins **A1−A6** was prepared by the procedure described elsewhere,[9] whereas the aldehyde resins used as staring materials for resins **B1−B6** and **C1−C6** were obtained from commercial sources. The appropriate aldehyde resin (22 g, 26.4 mmol, 1.0 equiv each) and anhydrous THF (220 mL) was added to a 1 L wide-mouth Nalgene bottle. Ethyl 3-aminobutyrate (158.4 mmol, 6.0 equiv) was added to this suspension, and the bottle was capped, sealed with silicone tape, and shaken horizontally for 2 h. Sodium triacetoxy borohydride (17.0 g, 80.0 mmol, 3 equiv) and glacial acetic acid (15 mL, 264 mmol, 10 equiv) were added to the reaction mixture, and the bottle was purged with nitrogen for 2 min. The bottle was capped, vented with a needle, and shaken for 17 h at room temperature. The resin was collected in a coarse-fritted funnel attached to a jointed Erlenmeyer flask connected to vacuum. The resin was subsequently washed with 500 mL of each of the following solvents: THF, MeOH, CH_2Cl_2, 15% DIEA in CH_2Cl_2, MeOH, and alternating CH_2Cl_2 and MeOH (2×). The resin was dried under high vacuum and analyzed as described in the Results and Discussion section.

Step 2. Alkylation or Acylation. Resins A1−A6, B1−B6, and C1−C6. 1-(6-Chloro-5-nitro-pyrimidin-4-yl)-piperidine-3-carboxylic acid ethyl ester was prepared by the general procedure as described elsewhere.[13] The acylators were obtained from commercial sources.

The appropriate amine-substituted resin from step 1 (1 g, 1.1 mmol, 1.0 equiv each) was added to a 50 mL wide-mouth Nalgene bottle, followed by dichloromethane (10 mL) and triethylamine (3.3 mmol, 3 equiv). The bottle was capped, and the reaction mixture was shaken to mix the reagents. The appropriate alkylator or acylator (2.2 mmol, 2 equiv) was added to this suspension. The capped bottle was vented with a needle and shaken for 17 h at room temperature. The resin was collected in a coarse-fritted funnel attached to an Erlenmeyer flask connected to vacuum. The resin was subsequently washed with 50 mL of each of the following solvents: THF, MeOH, CH_2Cl_2, 15% DIEA in CH_2Cl_2, MeOH, and alternating CH_2Cl_2 and MeOH (2×). The resin was dried under high vacuum and analyzed as described in the Results and Discussion section.

Cleavage of Resin-Bound Synthetic Products. A1−A6, **B1−B6**, and **C1−C6** (∼30 mg each) were cleaved using 0.1, 0.6, 0.9, 2.3, or 8% HCl in dioxane (2 mL) at room temperature. First, dry resins were swollen in a 3 mL bottom-capped syringe tube with a frit in DCM (2 mL) for 10 min. After the DCM was removed, an HCl solution was added. The tubes were capped at both ends and shaken horizontally on an orbital shaker. A droplet of the suspension was taken from the reaction vessel at various time intervals. The resins were washed with DCM (5×, 1 mL for each wash) and analyzed by single-bead FT-IR.

Single-Bead FT-IR Microspectroscopy. All spectra were collected on a Nicolet 380 FT-IR spectrophotometer (Thermo Fisher Scientific, Waltham, MA) coupled with a continuum microscope. The system was operated by the OMNIC software. The microscope is equipped with a 100× Cassegrain objective and a liquid nitrogen-cooled mercury−cadmium−telluride (MCT) detector. The resin beads were flattened in a diamond window (SpectraTech, Shelton, CT). The view mode aided in the location of a single flattened bead, and the transmission mode was used for the measurement. A clean area on the diamond window next to the bead was used to collect the background spectrum. Data were collected at 4 cm^{-1} resolution, and 128 scans were averaged.

Data Analysis. To correct peak area variations cause by factors such as variations in bead size, the thickness of flattened bead, and the random fluctuation of the instrument, we used an IR band of polystyrene at 1947 cm^{-1} as an internal reference peak. The ratio of the integrated peak area to the internal reference peak area in the same measurement was used to quantify the reaction conversion and plotted against time for kinetic analysis. For overlapping IR bands, the PeakFit program (copyright 1989, 1990 by AISN Software) was used to fit bands with Gaussian functions. Areas of the deconvoluted peaks were used for quantitative analysis. The integrated values were normalized by assignment of the peak area of the compounds at 0 min as 1. These data points were fitted to a pseudo-first-order rate equation[14] by using nonlinear regression analysis with SigmaPlot for Windows, version 10.0 (Systat Software Inc., Richmond, CA), on a personal computer.

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Supporting Information Available. Spectroscopic and kinetic data for resin-bound compounds and LC/MS data for cleaved compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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