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Research Article

Immobilized Carbodiimide Assisted Flow Combinatorial Protocol to Facilitate Amide Coupling and Lactamization

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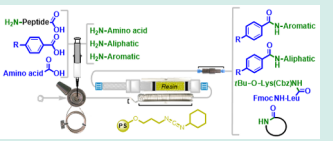
ABSTRACT: Through a screen of over one hundred and 30 permutations of reaction temperatures, solvents, carbodiimide resins, and carbodiimide molar equivalences, in the presence, absence, or combination of diisopropylamine and benzotriazole additives, a convenient and first reported carbodiimide polymer-assisted flow approach to effect amide coupling and lactamization was developed. The protocol entails injecting a single solution (1:9 dimethylformamide: dichloromethane) containing a carboxylic acid and an amine or linear peptide sequence into a continuous stream of dichloromethane. The protocol remained viable in the absence of base, did not require carboxylate preactivation which, and in concert with minimal workup requirements, enabled the isolation of products in high yields. Compared to the utilization of untethered carbodiimide reagents, the flow procedure was also observed to provide a degree of racemization safety.



KEYWORDS: *polymer-assisted solution-phase synthesis, immobilized carbodiimide, flow amide coupling, flow lactamization*

INTRODUCTION

While the concept of solid-phase-assisted synthesis was initially devised for the on-resin assembly of peptide sequences,[1] the technique has since been widely adopted throughout the chemical industry with immobilized reagents employed as anchors, catalysts, reagents, and scavengers. Further, it is becoming increasingly evident that the advantages provided by immobilized species have only been partially realized through their application in batch-based chemistries. Indeed, it is becoming apparent that additional gains are attainable when these agents are integrated into flow-based synthetic protocols.[2−45]

With respect to employing immobilized carbodiimide reagents to mediate the coupling of solution-phase reactants, the concept of employing immobilized coupling reagents to promote amide bond formation was first described over half a century ago.[10] In its original guise, a suspension of poly-(hexamethylenecarbodiimide) (Figure 1.1) was utilized to condense a handful of N^{α}-protected amino acids with amino acid ester protected hydrochlorides from which a series of dipeptides were afforded in nearly quantitative yields.[10] Since this preliminary account, subsequent iterations of the carbodiimide "capture-and-release" synthesis, microporous heterogeneous polystyrene-based matrices have been most often utilized. Nevertheless, a handful of soluble PS-carbodiimide reagents have examined. For these, ring-opening metathesis (ROMP)-based and polyethylene glycol-based (PEG)-based polymers were employed as immobilization matrices (i.e., **2** and **3**, Figure 1, respectively); however, these have received minimal attention[11−15]

At present, a handful of PS-carbodiimide resins are commercially available, and of these, the most commonly utilized are resin-**4**[16−22] and resin-**5**,[23−30] and the ethyl-carbodiimide functionalized resin-**6**[31−37] (Figure 2). These resins have been successfully employed in a myriad of synthetic programs ranging from combinatorial synthesis,[22,23,26,28,38] diversity orientated synthesis,[16,27,33,36] peptide macrocyclization,[39,40] and within the synthesis of an approved pharmaceutical agent.[19]

While there exists some anecdotal evidence that immobilization can enhance the reactivity of a carbodiimide reagent through a "site-isolation" or a "pseudodilution-effect",[41,42] the primary advantages of utilizing solid-supported carbodiimide reagents are realized during reaction workup and purification.[3,38,43,44] Typically, a single filtration-evaporation sequence is often sufficient to afford the desired product in good yield and purity. Similarly, as unreacted carbodiimide can be simply removed from the reaction solution, coupling yields and reaction rates can be enhanced with the employment of a significant excess of polymeric reagent without causing separation issues. Nonetheless, despite the potential synergies between PS-carbodiimide mediated coupling and flow chemistry, to date, no such protocols have been reported. Accordingly, we were curious to ascertain whether the

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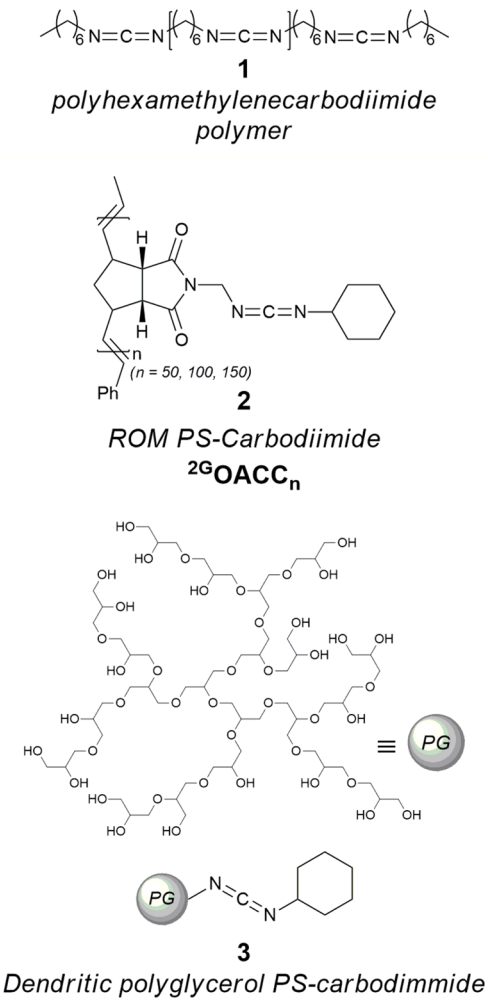

Figure 1. (**1**) Schematic of the polyhexamethylenecarbodiimide polymer, (**2**) ring-opening metathesis polymer (ROMP)-based, and (**3**) a fragment of dendritic polyglycerol-based carbodiimide reagents.

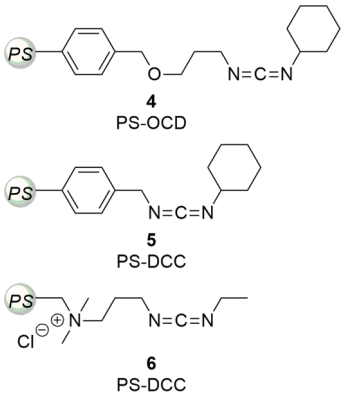

Figure 2. Commercially available polystyrene-based (PS) resins appended with cyclohexyl (resin-**4** and -**5**) or 1-ethyl-3-(3-(dimethylamino)propyl)-based carbodiimide moieties (resin-**6**).

remarkable convenience offered by solid-supported carbodiimide-based reagents could be further enhanced with the development of a continuous flow "catch-and-release" procedure.

RESULTS AND DISCUSSION

Appraisal of Currently Reported Batch Protocols Employed to Effect PS-Carbodiimide Couplings. To ascertain suitable parameters upon which to develop a flow protocol, a survey of several previously reported batch protocols which utilized either resins-**4**, -**5**, or -**6** was conducted. Throughout this assessment, a high degree of conformity was observed across reagent and reactant stoichiometry, reaction temperature, and reagent concentrations. Irrespective of the resin utilized, most utilized near two molar equivalents of carbodiimide, 1.1−1.2 equiv of carboxylic acid, along with one equivalent of amine (solution concentration 0.05−0.2 M), under ambient conditions.[16,18−20,23,24,26−30,32−34,36,37]

Regards alterations, it has been reported that increasing amine equivalences imparts detrimental effects, which was postulated to be a result of the formation of a guanidine adduct on the carbodiimide moiety.[20] Additionally, the efficiency of the PS-carbodiimide "catch-and-release" sequence appears to be independent of reagent concentration.[20] However, for solvent, the necessity for benzotriazole additives, and reaction tolerance to non-nucleophilic bases, a significant level of contradiction is observed. For solvent dichloromethane (DCM) and chloroform ($CHCl_3$) have been traditionally favored because of superior resin swelling capabilities.[16,19,20,22,24,25,28,31,32,34−37] Furthermore, it has been proposed that solvent polarity endows significant impacts.[20] For example, polar aprotic solvents, including acetonitrile, dimethyl sulfoxide, 1,2-dichloroethane, and tetrahydrofuran (THF), have been reported to impart deleterious effects.[20] Nonetheless, where starting material solubility is limited within $CHCl_3$ or DCM; it has been demonstrated that the inclusion of *N*,*N*-dimethylformamide (DMF) can be tolerated in concentrations ranging from 10 to 50%.[16,20,24,26,27]

However, in contrast, in other instances, near quantitative couplings within neat DMF[23] and other polar aprotic solvents, such as *N*-methylpyrrolidinone (NMP),[17,24] dimethylacetamide (DMA),[17,21] and acetonitrile,[29] have been reported. Furthermore, in a handful of examples, the inclusion of polar protic solvents, including *tert*-butanol[33] and methanol[29] have been utilized.

For benzotriazole additives, in addition to reducing epimerization, in several instances, the inclusion of either 1-hydroxy-7-azabenzotriazole (HOAt) or hydroxybenzotriazole (HOBt) has been reported to be essential is for amide couplings performed at both ambient and elevated temperatures.[17,29] For example, in the absence of HOBt, Sauer et al. reported <5% generation of the desired amide.[17] However, upon employing identical reaction conditions, along with one-equivalent of HOBt under microwave irradiation, reaction conversion increased to >95%.[17] Conversely, numerous examples of near quantitative conversion have been reported in the absence of a benzotriazole additive, although perhaps of significance, these protocols were performed within DCM[19,20,22,31,35,36] or $CHCl_3$,[20,32,34] whereas the Sauer protocol employed DMA as a solvent.

Batch Experiments to Assess Correlations between Parameter Variation S and Coupling Efficiency. Because of the above-outlined discrepancies, we initially opted to perform a sequence of batch-based couplings to establish favorable reaction parameters upon which to develop a continuous flow protocol. In this way, correlations between

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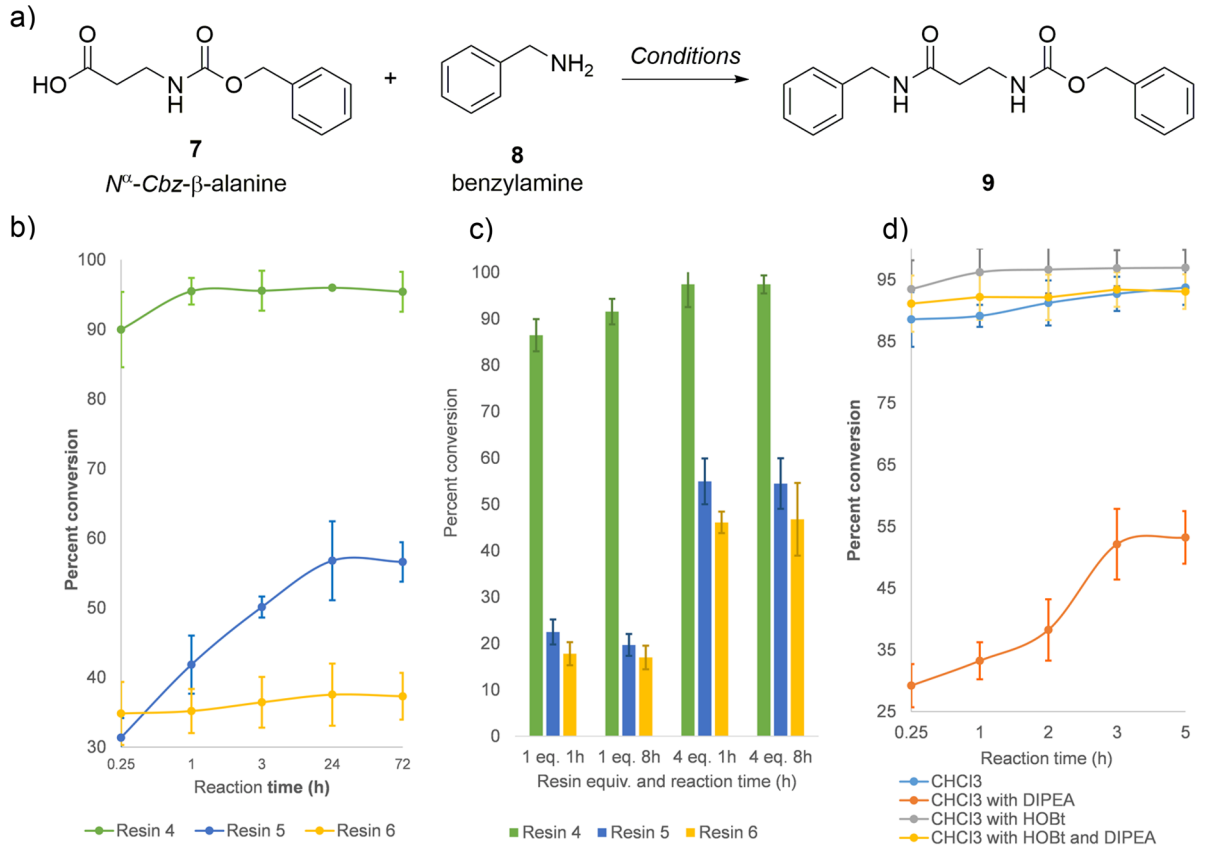

Figure 3. (a) Coupling of Cbz-*β*-alanine (**7**) with benzylamine (**8**) to afford the amide-based derivative (**9**). (b) Percent conversion as a function of time for the coupling of Cbz-*β*-alanine with benzylamine using the carbodiimide resins **4**–**6**. (c) Percent conversion as a function of time for the coupling of Cbz-*β*-alanine with benzylamine afforded by resins **4**–**6** to afford **9** using 1-mol equiv or 8-equivalences of resin. (d) Percent conversion as a function of time for the coupling of Cbz-*β*-alanine with benzylamine afforded by the carbodiimide resin-**4** using 2-molar equivalences of immobilized carbodiimide in the presence or absence or combination of 2-molar equivalences of DIPEA or HOBt.

reaction parameters and outcomes could be determined without introducing any potential nuances associated with flow processing. Within these studies, we aimed to assess the effects of solvent and immobilized carbodiimide variations in addition to exploring the results of adding, excluding, or combining non-nucleophilic based and HOBt.

As detailed in Figure 3, the initial focus turned to carbodiimide variations. Here, the three commercial available polystyrene resins, resin-**4** (PS-OCD), resin-**5** (PS-DCC), and resin-**6** (PS-EDCI) were individually assessed (Figure 3a). For each of these examinations, a chloroform solution containing **7** (1.0 equiv, 0.0635 M) and 2 mol equiv of immobilized carbodiimide was placed in a sample vial, which was lightly agitated for 15 min using a standard microplate shaker. Following this activation period, 1.0 equiv of benzylamine was added, and aliquots were collected at 15 min, 1, 3, and 24 h.

For the first batch couplings, the ether tethered cyclohexyl-based carbodiimide resin-**4** proved to be the most effective, affording near quantitative conversion within 15 min. By contrast, both resin-**5** and -**6** were ineffectual, affording maximum conversions of ∼60% and 40%, respectively, after 24 h. However, the low coupling conversion observed for the EDCI resin is not entirely unexpected, as the presence of the tertiary nitrogen is known to impart deleterious effects on coupling efficiencies.[45] In contrast, less certainty can be provided for the low conversion provided by resin-**5**. Nonetheless, here, we postulate that the truncated linker results in the polystyrene matrix sterically hindering access of the carboxylic acid to the carbodiimide moiety, thus limiting the formation of the *O*-acylurea intermediate. Nevertheless, as detailed in Figure 3b, for both resin-**5** and -**6**, a doubling of carbodiimide molar equivalents provided a minimal enhancement on the coupling productivity. Similarly, for resin-**4**, a doubling of molar equivalences provided a negligible improvement on coupling efficiency, whereas halving the quantity (e.g., 1 mol equiv) reduced the formation of **9** by near 10%.

To examine whether the addition of HOBt and a non-nucleophilic base, which would be a requirement if using amine salts, imparted impacts on the coupling efficiency, a subsequent series of reactions were performed using resin-**4**. As detailed in Figure 3c, the inclusion of HOBt delivered a minimal yet observable enhancement on coupling efficiency, whereas the addition of the non-nucleophilic base diisopropylethylamine (DIPEA) proved highly detrimental, near halving the overall conversion of **7** to **9**.

Lastly, the effects of solvent variations were assessed (Figure 4). Here, once again, 1 equiv of **7** and **8**, along with 2 mol equiv of resin-**4** were reacted in an assortment of organic solvents. As charted in Figure 3, the combination of 2 equiv of HOBt with $CHCl_3$ once again proved most effectual, affording near 85% conversion within 15 min under ambient conditions. Additionally, the singular utilization of DCM or $CHCl_3$ both promoted ∼80% conversion within 15 min and ∼90% conversion within 30 min. Further, in accordance with previous reports, utilization of a 10% DMF: $CHCl_3$ solution imparted a marginal decrease in reaction efficiency.[16,20,22]

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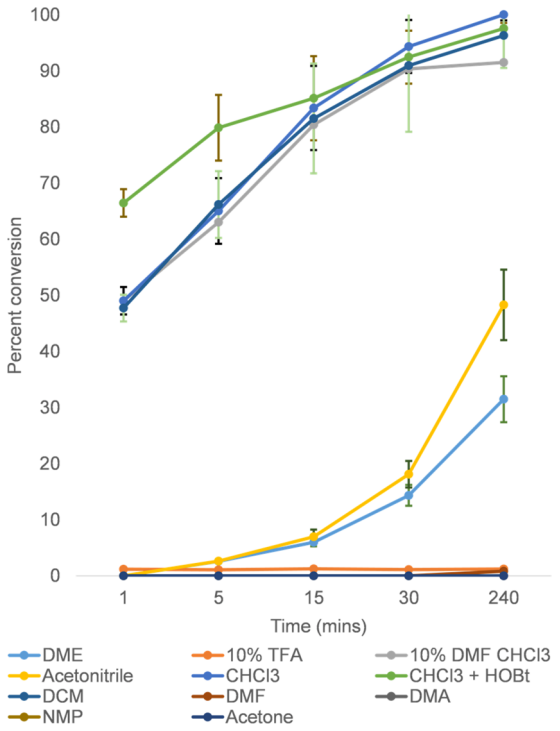

Figure 4. Percent conversion as a function of time for the coupling of Cbz-*β*-alanine with benzylamine afforded by the carbodiimide resin-**4** using 2 molar equivalences of immobilized carbodiimide with various solvents.

However, as previously reported, solvents significantly dissimilar to chloroform and DCM afforded appreciably lower levels of conversion.[20] For example, the utilization of both acetonitrile and 1,2-dimethoxyethane (DME) afforded less than 20% conversion within 30 min. Furthermore, with the employment of DMF, the maximum conversion for the desired product was ∼3%, which was only observable after 18 h. To expand upon the utilization of a TFA-based mixture, we were interested in assessing the plausibility of performing couplings within a peptidyl resin cleavage solution with an overarching aim of potentially conducting postsynthesis modifications on peptides within crude peptidyl-cleavage solutions.

Application of Batch Results to the Development of a Flow Protocol. With further insights into the primary determinants which affect coupling efficiency, attention turned to devising a polymer-assisted solution-phase flow protocol using a previously reported flow reactor.[46,47] As detailed in Figure 5, this rudimentary system was comprised of a semipreparative HPLC pump, a Rheodyne injector fitted with a 2 mL injection loop, a commercially available glass column in which to accommodate the PS-carbodiimide resin, a thermostat-controlled heat block, a 1.5 m heating coil, and a back pressure regulator (BPR, 100 psi).

The initial flow trials were performed in the absence of HOBt and utilized DCM as the reaction solvent. For experimental protocols, in accordance with the proposed "catch-and-release" mechanism, the DCM solution of N^{α}-Cbz-*β*-alanine was initially injected into a stream of DCM which was flowed through the resin bed at 1.0 mL/min. Following this injection, to wash the resin, the DCM stream flowed through the resin bead for an additional 10 min. After this washing period, the injection loop was loaded with a benzylamine-DCM solution, and upon injection, the eluting solution was collected. Once ∼20 mL of eluent had been collected, the crude DCM solution was concentrated in vacuo prior to being subjected to NMR analysis, which revealed the presence of only benzylamine.

While initially discouraging, we were cognizant that the desired product **9** could potentially be afforded through two pathways (i.e., PATH-A and PATH-B, Figure 6). Further, with the introduction of a benzotriazole reagent, PATH-C and PATH-D would also be possible. For the initial flow trial reaction (i.e., Figure 5), it was presumed that the "catch-and-release" sequence would proceed via PATH-A (Figure 6). However, as the initial reaction sequence failed to afford the desired product, as opposed the being captured as the *O*-acylurea intermediate we were of the opinion that in the absence of an amine nucleophile, the carboxylate was rapidly transformed and released as the corresponding symmetrical anhydride (i.e., initial steps of PATH-B).

To ascertain whether the "captured" carboxylate could be released as the symmetrical anhydride, a further trial was performed in which a DCM solution of **7** (0.0635 mmol, 1 equiv) flowed through a bed of resin-**4** (0.1 g, 0.127 mmol, 2 equiv) at 1.0 mL/min (Table 1, entry 1). As detailed in Table 1, entry 1, ^{1}H NMR analyses of the column eluent indicated that the symmetrical anhydride was indeed afforded in quantitative conversion hence demonstrating the captured carboxylate could indeed be released as the symmetrical anhydride. Nevertheless, it was apparent that the formation of a benzotriazole ester remained readily achievable (i.e., Figure 7-4). Moreover, as charted in Figure 7-5, **9** could be afforded with the addition of an equivalent of benzylamine to the eluent collected from the second trial hence indicating that both the *O*-HOBt and *N*-HOBt adducts functioned as reactive intermediates. Lastly, through the utilization of a mixed resin

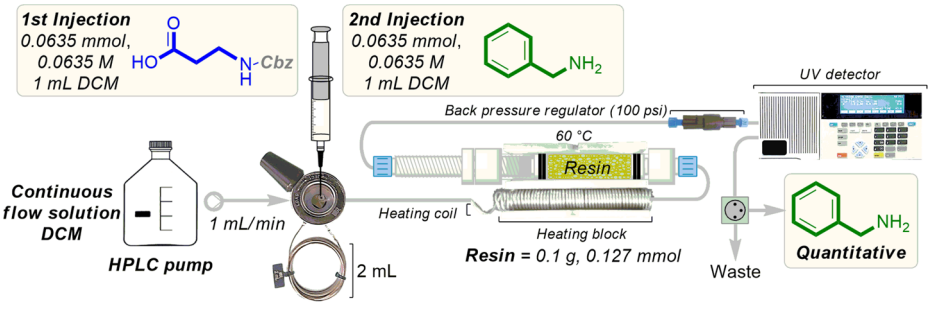

Figure 5. Flow reactor configuration and the reaction parameters used to attempt the "catch-and-release" synthesis of compound **9** through the sequential injection of Cbz-*β*-alanine and benzylamine.

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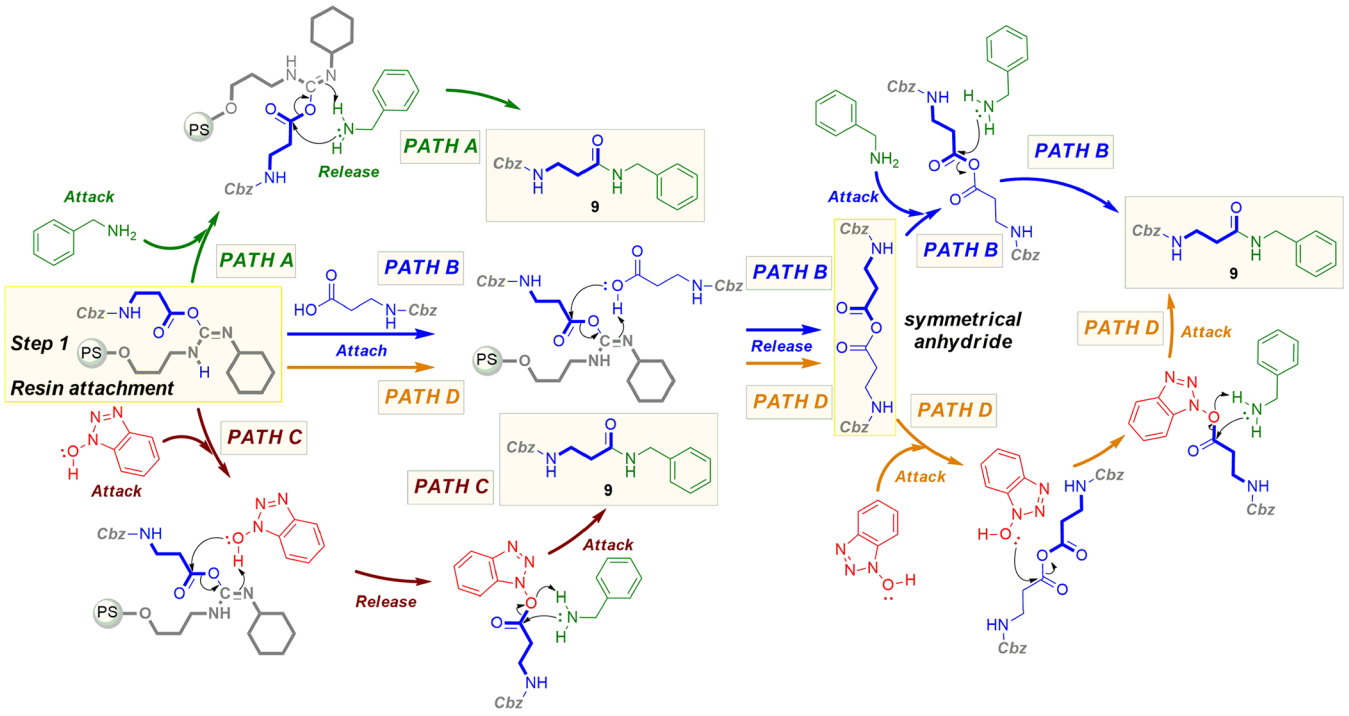
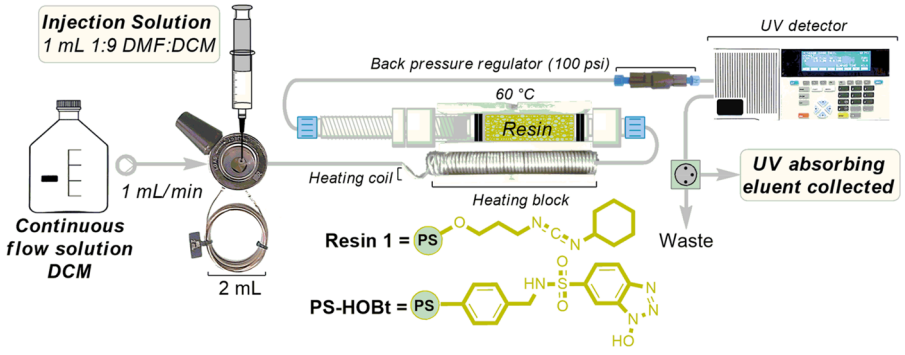
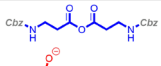
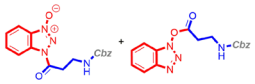


Figure 6. Schematic representation of the potential reaction sequences through which compound **9** could be formed in both the presence and absence of HOBt. (Path-A) Release of the captured *O*-acylurea intermediate as the amide product via direct amine attack. (Path-B) Release of the captured *O*-acylurea intermediate as the symmetrical anhydride, which is subsequently attacked by the amine to afford **9**. (Path-C) Release of the captured *O*-acylurea intermediate as the activated HOBt-ester, which is then attacked by the amine to afford compound **9**. (Path-D) Release of the captured *O*-acylurea intermediate as the symmetrical anhydride which is subsequently attacked by HOBt and the resulting activated HOBt-ester is attacked by benzylamine to afford the amide product **9**.

Table 1. Flow Reactor Configuration and Parameters Used to Conduct the Continuous Flow Injection-Based Couplings to Examine the Coupling Sequence by Which Compound 9 Was Afforded



Injection Solution	Resin	Eluent Content	Conversion[a]
9 (0.0635 mmol, 0.0635 M)	Resin-**4** (0.127 mmol, 2 eq.)		> 95%
9 (0.0635 mmol, 0.0635 M) **HOBt** (0.0635 mmol, 0.0635 M)	Resin-**4** (0.127 mmol, 2 eq.)		> 95%
9 (0.0635 mmol, 0.0635 M)	Resin-**4** (0.127 mmol, 2 eq.) PS-HOBt (0.127 mmol, 2 eq.)	Only solvent associated signal observed in the ^{1}H NMR	0%

[a]Percent conversions determined via ^{1}H NMR analysis.

bed composed of resin-**4** (0.1 g, 0.127 mmol, 2 equiv) and PS-HOBt resin (0.070 mg, 0.0635 mmol, 1 equiv), the carboxylate was captured as within the resin matrix (i.e., Figure 7-6), the amide product could be obtained through flowing a solution of the corresponding amine through the mixed resin bed (i.e., Figure 7-7).

Assessing a Continuous Infusion Approach. In light of the above-mentioned trials, it was assumed that a simplified strategy in which a solution containing both **7** and **8** were continuously infused through a bed of the resin-**4** could afford an improved approach. Further, although the previous trials indicated that the inclusion of a benzotriazole as an activator

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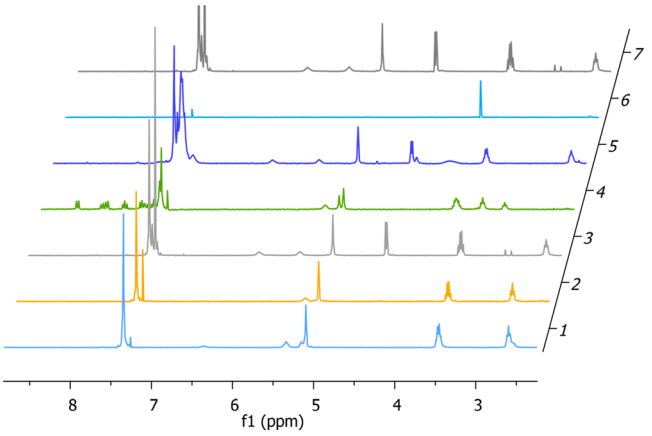


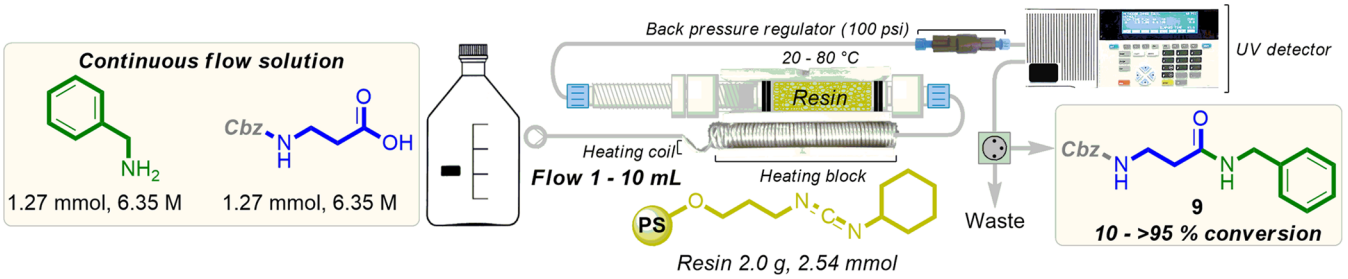
Figure 7. (1) ^{1}H NMR spectrum of Cbz-β-alanine. (2) ^{1}H NMR spectrum of column eluent obtained from flowing 1 equiv of Cbz-β-alanine through 2-molar equiv of resin-**4**. (3) ^{1}H NMR spectrum of column eluent obtained from flowing 1 equiv of Cbz-β-alanine through 2-mol equiv of resin-**4**, which was subsequently treated with 1 equiv of benzylamine. (4) ^{1}H NMR spectrum of column eluent obtained from flowing 1 equiv of Cbz-β-alanine and HOBt through 2-mol equiv of resin-**4**. (5) ^{1}H NMR spectrum of column eluent obtained from flowing 1 equiv of Cbz-β-alanine and HOBt through 2 mol equiv of resin-**4** following treatment with 1 equiv of benzylamine. (6) ^{1}H NMR spectrum of column eluent obtained from flowing 1 equiv of Cbz-β-alanine through a mixed bed containing 2-molar equiv of resin-**4** and 2-molar equiv HOBt-resin. (7) ^{1}H NMR spectrum of column eluent obtained from flowing 1 equiv of benzylamine through a mixed bed containing 2-molar equiv of resin-**4** and 2-molar equiv HOBt-resin through which had Cbz-β-alanine flowed through prior.

could broaden both substrate and solvent scope, to limit the likelihood of precipitate formation, and to expedite reaction workup, we opted to employ additive-free conditions. In addition to assessing the viability of this approach, the potential scope for optimization was also explored. Here, both flow rate and temperature were sequentially altered. Initially, the stock solution was pass through the resin bed at 25 °C and 1 mL/min before the flow rate was incrementally increased to 2, 5, and 10 mL/min, respectively. At each flow rate, an aliquot of the column eluent was collected and analyzed via HPLC. The flow rate was then reduced to 2 mL/min, and the column temperature was increased to 40 °C before being increased at 20 °C increments up to 80 °C. As detailed in Table 2, while an increase in temperature afforded increased conversions, varying the flow rate from 1−5 mL/min imparted minimal impacts on overall starting material turnover.

Assessing Single Injection Addition. With proof-of-principle in hand, the focus turned to the implementation of a single reagent injection protocol. Accordingly, the resin column was loaded with 400 mg of resin-**4** (0.536 mmol, 2.0 equiv) through which was passed a continuous stream of DCM at 2 mL/min. Upon complete resin swelling, the column was heated to 60 °C, the U.V. detector was zeroed (320 nm), and the Rheodyne injection loop was loaded with a 1 mL of 10% DMF: DCM solution containing **7** (0.27 mmol, 0.27 M, 1 equiv) and **8** (0.27 mmol, 0.27 M, 1 equiv). Following injection, the reaction stream eluting from the column was monitored via UV, and upon absorbance detection, which occurred in ∼1.5 min, the column eluent was collected until UV absorbance returned to baseline (this transpired within ∼0.5 min). On the basis of both HPLC and ^{1}H NMR analysis, it appeared that the aforementioned ∼90 s retention time was sufficient to promote quantitative conversion to the desired amide **9**.

As a note, it appears that it is carbodiimide molar equivalents rather than reagent residence time, which is the predominant determinate governing reaction efficiency. For example, with a reduction in resin loading to 100 mg, along with a corresponding reduction of reagent molar equivalences, **9** was again afforded in a quantitative conversion at a flow rate of 2 mL/min. Here, the retention time associated with using 100 mg was ∼20-s (resin volume approximately 0.8 cm^3), yet **9** was again afforded in quantitative conversion. However, we note

Table 2. Flow Reactor Configuration and the Reaction Parameters Used to Conduct the Continuous Flow Infusion Trial Reactions for the Coupling of Benzylamine with Cbz-β-alanine to Afford the Amide Product 9



temperature (°C)	flow rate (mL/min)	conversion[a] (%)
25	1	75
25	2	80
25	5	93
25	10	79
40	2	>98
60	2	>98
80	2	>98
80	8	>98

[a]Conversions were derived via HPLC analysis at a wavelength of 214 nm and were based on the consumption of Cbz-β-alanine relative to product formation.

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that for both the 400 and 100 mg resin quantities, increases in flow rate beyond 5 mL min appear to reduce reaction yields.

Multiple Coupling Cycles Using a Single Resin Batch. In theory with the utilization of 10-mM equivalents of resin-**4**, one should be able to prepare at least five discrete batches of amide on a 1 mmol scale or ten discrete amides on a 0.5 mmol scale and so forth. To explore the viability of such a strategy, the resin column was initially loaded with 800 mg of resin-**4** (1.072 mmol, 4.0 equiv), through which was successively pass six 1 mL injections, each containing benzoic acid (**10**) (0.27 mmol, 0.27 M, 1.0 equiv) and benzylamine (**8**) (0.27 mmol, 0.27 M, 1.0 equiv) (Figure 8). Here, as each injection volume

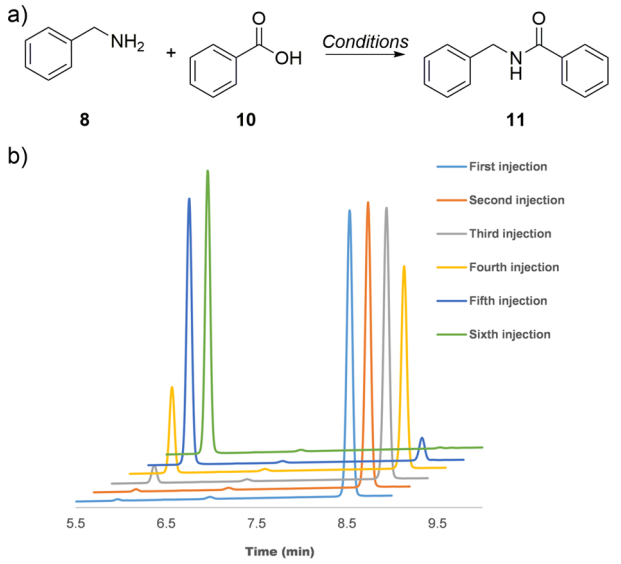

Figure 8. (a) Condensation reaction between benzylamine **8** and benzoic acid **10** using PS-DCC to afford amide **11**. (b) HPLC chromatographs of column eluents obtained from flowing successive injections of 1 mL solutions containing benzylamine and benzoic acid through a single batch of resin-**4**.

equated with 0.25 mol equiv relative to the resin-**4**, it was proposed that quantitative conversion to the amide **11** should be afforded from the first two injections and that this would incrementally decrease across subsequent injections. Indeed, as detailed in Figure 8, it was apparent that there was a certain amount of credence to the supposition mentioned above. For example, analysis of the column eluent emerging from injections one and two indicated that **11** was afforded in quantitative conversion (t_R = 8.5 min). With regards to injections three and four, conversion to **11** reduced to 80% and 60% respectively, whereas, for the fifth and six doses, trace and no observable quantities of **11** were observed, respectively.

Hence, to remain viable, the above trials suggest that at least twice the molar equivalences of resin-**4** are required to effect quantitative amide formation. While theory dictates that 1-equivalent should be sufficient, it is suspected that the relatively high resin loading (1.27 mmol/g) may impose steric hindrance. Nonetheless, under the proviso that carbodiimide excess is maintained, successive couplings could be cleanly effected using a single batch of resin.

Assessing Analogue Library Synthesis and Substrate Amenability. With an eye to compound library synthesis, the subsequent reaction trials explored synthesizing a small library of amide derivatives based upon either 4-nitro- or 4-methylbenzoic acid. As detailed in Tables 3 and 4, the sequential injection protocol proved to be a viable strategy for analog library synthesis. With regards to substrate amenability, it appears that carboxylate electron density perturbation imparts no adverse impacts on coupling efficiency as similar conversion observed with the employment of 4-nitro- or 4-methylbenzoic acid. Concerning amine amenability, for primary amine-based analogs, the desired amide products were afforded in >95% conversion. Furthermore, coupling with aniline-based amines afforded a series of aromatic derivatives in >90% conversion. However, while the protocol proved to be both rapid and convenient, it does not appear to circumvent the inherent coupling limitations of solution-phase carbodiimide couplings. For example, the strategy proved ineffectual for the coupling of both hindered and deactivated amines (e.g., entries 2 and 3, Table 3 and entries 2 and 3, Table 4).

Thus, in summary, using the injection-based flow protocol, two discrete series of amides were synthesized from a relatively diverse pool of achiral amines. However, given the propensity of additive-free carbodiimide based couplings to induce epimerization, it was assumed that the practicability of the flow protocol would not extend beyond achiral substrates. Nonetheless, to the best of our knowledge, no studies examining the effects that PS-carbodiimide-mediated imparts on epimerization have been reported. Consequently, to gain insight into the propensity for immobilized carbodiimide reagents to induce epimerization and to ascertain whether the inclusion of additives or solvent choice could influence diastereomeric ratios, our focus turned to amino acid couplings.

To initially ensure the flow protocol was amenable to coupling protected amino acid residues, a preliminary trial was performed, which employed Fmoc-Leu-OH (**32**) and the ethyl ester protected lysine HCl derivative (**33**). Here the desired dipeptide **34** was afforded in quantitative yield with no epimerization observed (Figure 9). Further, this result demonstrated HCl-amine salts could be coupled without the inclusion of base. While this is currently under further examination, at this point, we suspect that the excess carbodiimide may sequester the HCl.

With this result, our attention then turned to the coupling of residues that are vulnerable to direct epimerization, such as serine.[48−50] Hence, as detailed in Table 5, Fmoc-(*t*-Bu)Ser-OH (**35**) and the ethyl ester protected lysine derivative (**33**) served as coupling partners for the initial epimerization assessments. To assess the epimerization vulnerability of this coupling, two solution-phase batch couplings were initially performed. Both of these couplings were conducted in a 10% DMF DCM using 2 equiv of solution-phase DCC under ambient conditions. For the initial additive-free experiment, the coupling of **33** and **35** was effected with an overall conversion of ∼80% and a diastereomeric ratio (d.r.) of 19:1 (Figure 10). For the second reaction, which incorporated a catalytic quantity of DMAP, near-complete chiral inversion (i.e., d.r. 1:15.7, Figure 10) was observed.

Upon establishing vulnerability to stereomutation, focused switched to assessing the coupling of **33** to **35** under flow conditions. As detailed in Table 5, across the 14 trials, which examined additive and solvents variation, no epimerization was observed (Table 5). The additive-free approach also afforded the histidine-based dipeptide **37** and the cysteine-based dipeptide **38** with >80% conversions, with no evidence of stereomutation (Table 6). Hence, it appears the utilization of

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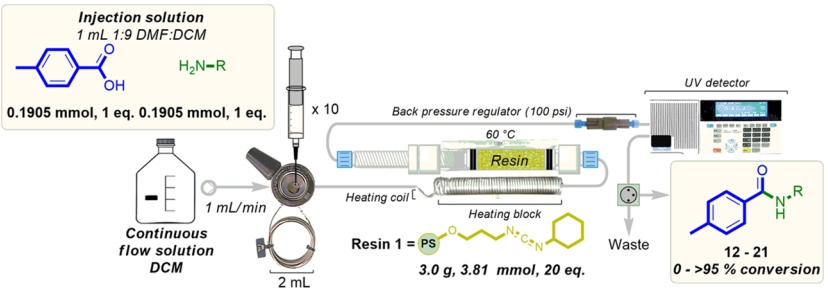
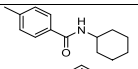
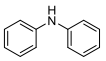
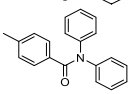
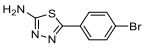
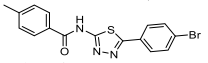
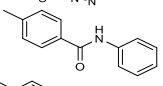
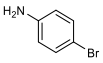
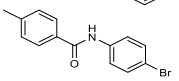
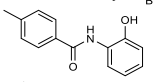
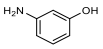
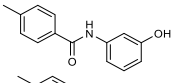
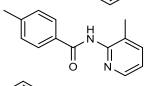
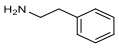
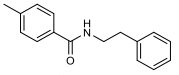
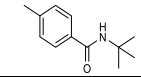
Table 3. Flow Reactor Configuration and the Reaction Parameters Used to Conduct the Continuous Flow Injection-Based Coupling of Various Amines with 4-Methylbenzoic Acid to Afford Compounds 12−21



Injection	Amine	Compound	Amide	Conversion[a]
1		**12**		98%
2		**13**		0%
3		**14**		0%
4		**15**		96%
5		**16**		98%
6		**17**		89%
7		**18**		91%
8		**19**		78%
9		**20**		99%
10		**21**		96%

[a]Conversions were derived via HPLC analysis at a wavelength of 214 nm and were based on the consumption of 4-methylbenzoic acid relative to product formation.

an immobilized carbodiimide presents a degree of racemization protection over their nontethered solution-phase counterparts.

In light of the above-discussed epimerization studies, our efforts turned toward examining intramolecular peptide-based cyclization. At present, solution-phase approaches are predominately employed to effect peptide macrocyclization. Nevertheless, irrespective of technique, the overarching effectiveness of a cyclization procedure is largely based on the degree to which intermolecular interactions can be mitigated as these lead to the formation of in dimers and oligomers. For solution-phase cyclization, this can be achieved through employing high dilution conditions and, by using partially protected linear sequences. However, paradoxically solution-phase cyclization is, in part, limited by the often poor solubility profiles of protected peptides and by the steric hindrance provided by the side-chain protecting groups. However, from a scale-up perspective, it is perhaps the necessity for high dilution (i.e., ∼1−3 L g^{-1}), which presents the most significant impediment.[51]

The advantage of utilizing immobilized carbodiimide reagents is 2-fold. First, the post coupling urea byproduct remains bound to the immobilized support and is thus readily removable from the reaction solution. Second, immobilized reagents are known to afford a pseudodilution effect which has been observed to limit the formation of byproducts which result from intermolecular couplings.[39,42,52,53]

While both resin-**4** and resin-**5** have been previously employed under batch conditions to effect peptide macrothiolactonization,[39,40] we were eager to examine the practicalities of the protocol extended to lactamization. To this end, the intramolecular cyclization of five dipeptides and four penta-peptides was explored. Each of these dipeptides were initially constructed on polystyrene 2-chlorotrityl chloride using previously reported protocols.[46,47] With respect to the peptides containing Asp, Ser, or Tyr residues, linear sequence cyclization was performed with side-chain protecting groups in place. The cyclization trails were performed in two separate batches (i.e., table entries 1−5 and entries 6−9 Table 7). Each dipeptide was dissolved in 2 mL of a 10% DMF:DCM solution (0.127 mmol, 0.0635 M, 1.0 equiv), which was injected and subsequently passaged through a single batch of PS-DCC resin-1 (0.8 g, 1.016 mmol, 8.0 equiv) at the reduced flow rate

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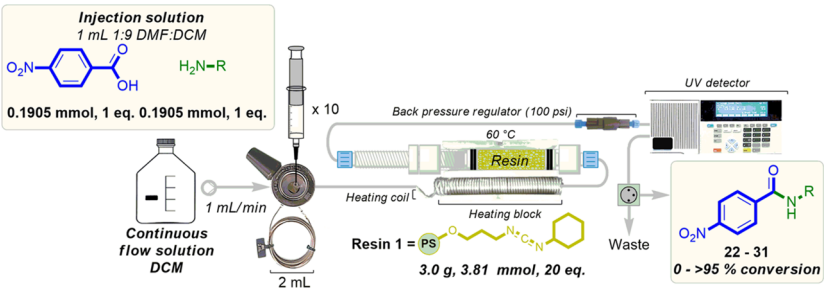
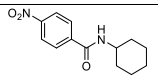
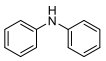
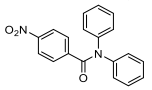
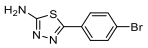
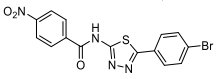
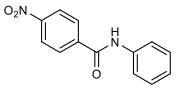
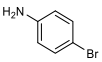
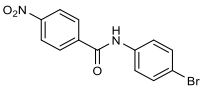
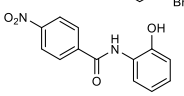
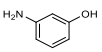
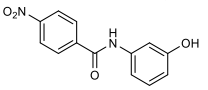
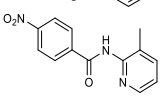
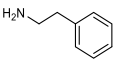
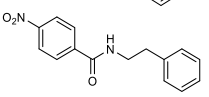
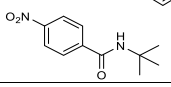
Table 4. Flow Reactor Configuration and the Reaction Parameters Used to Conduct the Continuous Flow Injection-Based Coupling of Various Amines with 4-Nitrobenzoic Acid to Afford Compounds 22−31



Injection	Amine	Amide	Conversion[a]
1		22 	99%
2		23 	0%
3		24 	0%
4		25 	99%
5		26 	99%
6		27 	91%
7		28 	79%
8		29 	76%
9		30 	97%
10		31 	99%

[a]Conversions were derived via HPLC analysis at a wavelength of 214 nm and were based on the consumption of amine relative to product formation.

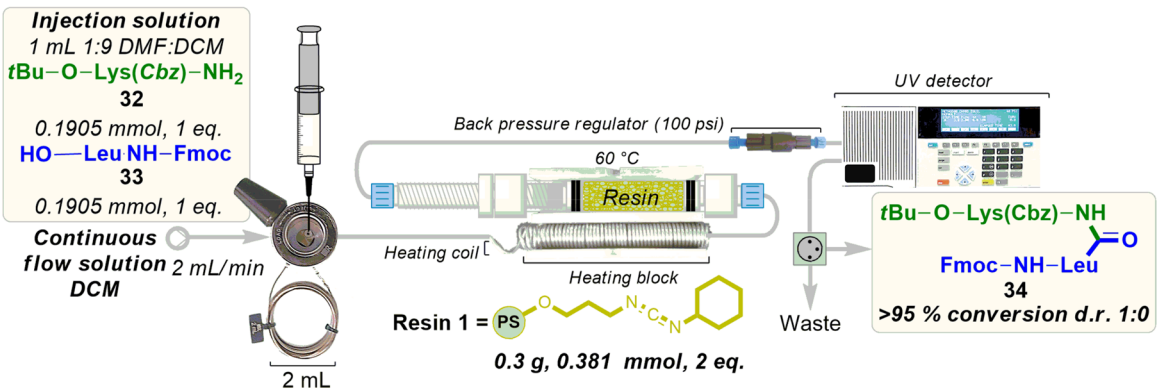


Figure 9. Flow coupling of H_2N(Cbz)Lys-*O*-*t*Bu with Fmoc-Leu-OH under flow conditions using 2 equiv of immobilized carbodiimide resin in the absence, presence, or combination of DIPEA and HOBt. Percent conversion determined via HPLC analysis based on the free acid amino-acid consumption, with d.r. values derived from the utilization of an L-leucine functionalized chiral column.

https://dx.doi.org/10.1021/acscombsci.0c00001
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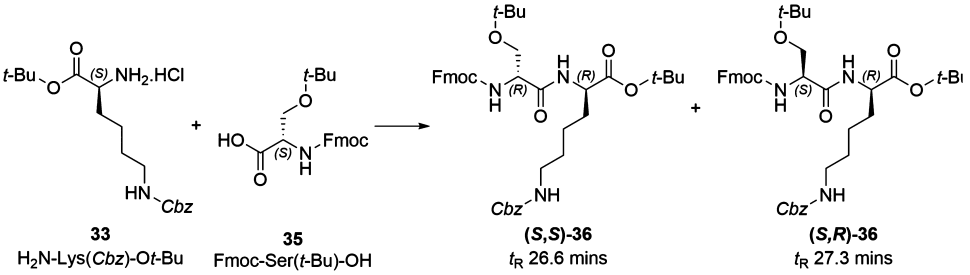
Table 5. Flow Coupling of H_2N-Lys(Cbz)-*O*-*t*Bu with Fmoc-(*t*Bu)Ser-OH under Flow Conditions Using 2 equiv of Immobilized Carbodiimide Resin in the Absence, Presence, or Combination of DIPEA and HOBt[a]



additives	continuous flow solution	conversion (%)[b]	d.r.[c]
none	DCM	87	1:0
DIPEA	DCM	78	1:0
HOBt	DCM	97	1:0
DIPEA, HOBt	DCM	96	1:0
none	NMP	73	1:0
DIPEA	NMP	78	1:0
HOBt	NMP	72	1:0
DIPEA, HOBt	NMP	74	1:0
none	DMA	67	1:0
DIPEA	DMA	77	1:0
HOBt	DMA	82	1:0
DIPEA, HOBt	DMA	78	1:0

[a]Flow rate = 2 mL/min. Injection solution contained 1 equiv of H_2N-Lys(Cbz)-*O*-*t*Bu and 1 equiv of Fmoc-(*t*Bu)Ser-OH dissolved in 2 mL of solution of indicated stock flowing solution. [b]Percent conversion determined from HPLC analysis based on free acid amino-acid consumption. [c]d.r. values determined using an L-leucine functionalized column.

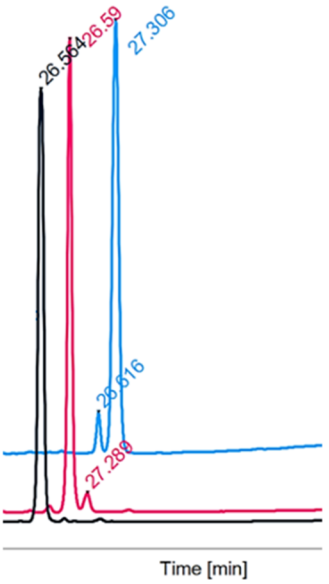
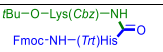
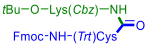


Figure 10. (a) Black: Chiral HPLC analysis of the crude reaction material afforded from the flow coupling of H_2N-Lys(Cbz)-*O*-*t*Bu with Fmoc-(*t*Bu)Ser-OH under flow conditions using 2 equiv of immobilized carbodiimide in the absence of DIPEA and/or HOBt. Flow rate = 2 mL/min. (b) Pink: Chiral HPLC analysis of the crude reaction material afforded from the batch coupling of H_2N-Lys(Cbz)-*O*-*t*Bu with Fmoc-(*t*Bu)Ser-OH using solution-phase DCC. (c) Blue: Chiral HPLC analysis of the crude reaction material afforded from the batch coupling of H_2N-Lys(Cbz)-*O*-*t*Bu with Fmoc-(*t*Bu)Ser-OH using solution-phase DCC with a catalytic quantity of DMAP.

Table 6. Flow Coupling of H_2N-Lys(Cbz)-*O*-*t*Bu with Fmoc-His(Trt)-OH or Fmoc-Cys(Trt)-OH Using Immobilized Carbodiimide Resin in the Absence of DIPEA and HOBt[c]

Entry	Additive	Product	Conversion[a]	d.r.[b]
1	None	**37** 	> 95%	1:0
2	None	**38** 	> 95%	1:0

[a]Percent conversion determined from HPLC analysis based on free acid amino acid consumption. [b]d.r. values determined using an L-leucine functionalized column. [c]Reagents and conditions: Couplings were mediated with 2 equiv of resin-**4** at 60°C, 2 mL/min with a continuous DCM flow. The injection solution was composed of a 10% DMF: DCM (2 mL) solution containing the carboxylate (0.127 mmol, 0.0635 M, 1. equiv) and H_2N-Lys(Cbz)-*O*-*t*Bu (0.127 mmol, 0.0635 M, 1 equiv).

of 0.5 mL/min. Pleasingly, as outlined in Table 6, through ^{1}H NMR and chiral HPLC analysis of each injection elution, it was deduced that each linear sequence was cleanly converted to the desired cyclic product within half an hour. In a bid to further reduce this coupling period, a series of trials that examined increased flow rates were performed; however, however, flow rates above 0.5 mL/min proved to be detrimental. Nonetheless, this 0.5 h coupling period compares favorably to previous reports. For example, under batch conditions, resin-**5** was observed to effect side-chain-to-tail thiolactonisation ring closing of 5-amino acid sequence within 3−5 h.[39,40,51]

CONCLUSION

While several batch, combinatorial, and microwave catch-and-release protocols employing carbodiimide-functionalized resins have been reported, this study describes the first appraisal of

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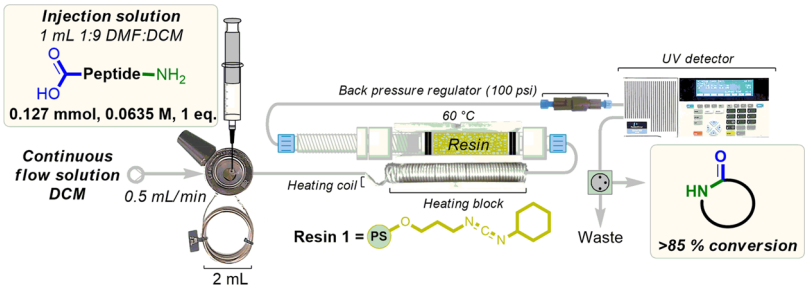
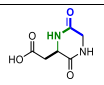
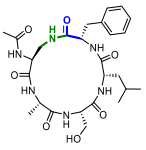
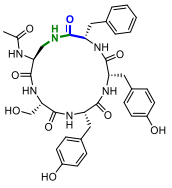
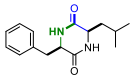
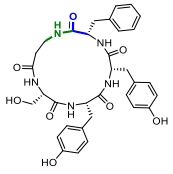
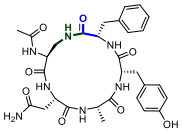
Table 7. Immobilized Carbodiimide-Mediated Lactamization of Linear Peptides Using Resin-4 under Flow Conditions in the Absence of DIPEA and HOBt[b]



Compound	Cyclic Peptide Sequence	Conversion[a]	Compound	Cyclic Peptide Sequence	Conversion[a]
39		> 95%	45		> 95%
40		> 95%			
41		> 95%	46		> 95%
42		> 95%			
43		> 95%			
44		> 95%	47		> 95%

[a]Percent conversion determined via HPLC analysis based on the consumption of the corresponding linear peptide sequence. [b]Reagents and conditions: Couplings were mediated with 2 equiv of resin-**4** at 60 °C, 0.5 mL/min with a continuous DCM flow. The injection solution was composed of a 10% DMF: DCM (2 mL) solution containing the linear peptide sequences (0.127 mmol, 0.0635 M). The linear sequences were constructed using previously reported protocols.

this class of resins under flow chemistry conditions. With the employment of an iterative screening process, a convenient and rapid polymer-assisted flow protocol was developed to mediate amide bond formation and lactamization. The optimized protocols entailed injecting a single solution (1:9 DMF: DCM 1−2 mL) containing 1 equiv of a carboxylic acid and 1 equiv of an amine, or linear peptide sequence, into a continuous stream of dichloromethane. The continuous stream is infused through a fixed bed of the ether tethered carbodiimide resin-**4** (2 mol equiv) heated to 60 °C. For amide formation, a flow rate of 2.0 mL/min was utilized, whereas a flow rate of 0.5 mL/min was used to effect "head-to-tail" lactamization of a series of linear dipeptides in addition to "side-chain-to-tail" lactamization of a series of penta-peptides.

Throughout this screening process, it was apparent that the characteristics of the solvent and the resin matrix impart a significant influence on reaction efficiency. For the continuous flowing solution, DCM and $CHCl_3$ proved optimal; however, with the inclusion of HOBt, both DMA and NMP proved to be viable alternatives. With regards to the resin matrix, although only three commercially available resins were assessed, the substantial disparity in coupling efficiency promoted by resin-**5** and resin-**6** relative to resin-**4** indicates that an extended nonionic polymer-to-carbodiimide linking tether is advantageous.

The primary advantages afforded by the flow protocol relate to enhanced convenience. As detailed in Tables 3 and 4, a single batch of resin can be utilized to construct a series of amide-based analogs. The coupling time frame is dependent on swollen resin matrix volume. For example, with the utilization of 400 mg of resin-**4**, ∼100 mg of compound **9** was afforded in quantitative conversion in ∼2 min, whereas on a reduced reaction scale using 100 mg of resin, compound **9** was afforded in quantitative conversion in ∼0.5 min. This time frame compares favorably with the 5 min coupling time frame reported under microwave irradiation.[17] Furthermore, the protocol displayed practicability with a variety of amine, aniline, and carboxylates of differing solubility profiles and appears to offer a degree of epimerization protection over solution-phase carbodiimide coupling.

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Concerning limitations, it appears that the protocol is ineffectual for the coupling of hindered and deactivated amines (i.e., entries **2** and **3**, Table 2). Further, from both practical and economic perspectives, the viability of the protocol is limited to subgram reaction scales. However, while the limitations associated with amine reactivity are most likely inherent to carbodiimide coupling, the economic viability issues could be addressed with the development of a facile resin recycling protocol. A handful of urea dehydration protocols for the generation, or recycling, of carbodiimide moieties from urea adducts, have been described.[18,54] However, at present in our hands, each of these methodologies has proven to be ineffectual. Nevertheless, efforts to devise an effective resin regeneration strategy remain ongoing, and it is hoped will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscombsci.0c00001.

Experimental procedures, ^{1}H NMR, ^{13}C NMR, and LR-MS spectra and HPLC chromatograms for purified compounds, HPLC chromatograms of each trial reaction, and high-resolution ESI MS spectra for previously unreported compounds (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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