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# Allenone-Mediated Racemization/Epimerization-Free Peptide Bond Formation and Its Application in Peptide Synthesis

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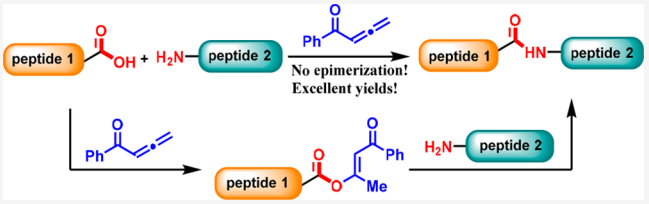
**ABSTRACT:** Allenone has been identified as a highly effective peptide coupling reagent for the first time. The peptide bond was formed with an $\alpha$-carbonyl vinyl ester as the key intermediate, the formation and subsequent aminolysis of which proceed spontaneously in a racemization-/epimerization-free manner. The allenone coupling reagent not only is effective for the synthesis of simple amides and dipeptides but is also amenable to peptide fragment condensation and solid-phase peptide synthesis (SPPS). The robustness of the allenone-mediated peptide bond formation was showcased incisively by the synthesis of carfilzomib, which involved a rare racemization-/epimerization-free N to C peptide elongation strategy. Furthermore, the successful synthesis of the model difficult peptide ACP (65−74) on a solid support suggested that this method was compatible with SPPS. This method combines the advantages of conventional active esters and coupling reagents, while overcoming the disadvantages of both strategies. Thus, this allenone-mediated peptide bond formation strategy represents a disruptive innovation in peptide synthesis.






## INTRODUCTION

Peptide synthesis has fascinated chemists for more than 100 years, and it remains a crucial focus of research because of the importance of peptides in drug discovery, biomedical studies, and materials science, as well as in other fine chemicals.[1] The fundamental chemistry of peptide synthesis is amide bond formation, which in itself is an important research topic.[2] Consequently, various amide bond formation strategies have been developed.[3] However, except for the most reliable and widely used coupling-reagent-mediated condensation of proteinogenic $\alpha$-amino acids, only a few other amide bond formation strategies are applicable to peptide synthesis because of the limited availability of the chiral starting materials[4] and the frequent loss of chiral integrity at the C$\alpha$ position.[5] Despite being of high standard, the current peptide synthesis methods involving iterative amide bond formation between proteinogenic $\alpha$-amino acids are reaching their inherent limits. In particular, the poor atom economy of the solid-phase peptide synthesis (SPPS) has led to sustainability and environmental concerns.[6] In addition, the formation of peptide epimers and other impurities due to the overactivation of conventional coupling reagents is a primary concern in peptide drug manufacturing. Unfortunately, these limitations cannot be completely overcome by simply optimizing the existing peptide synthesis methods, which heavily rely on the reagents and techniques developed between 1950 and 1980, when little focus was put on green chemistry. On the other hand, the immensely growing demand for peptides in biomedical studies and the large-scale manufacturing of peptide-based drugs necessitate the development of efficient, low-cost strategies for peptide synthesis. Notable coupling reagents[7] and innovations[8] in both new technology[9] and catalytic peptide bond formation strategies[10] have also been developed over the past two decades. However, most of them are still in their infancy. In such a context, novel coupling reagents and efficient peptide bond formation strategies are in great demand.

Dicyclohexylcarbodiimide reported by Sheehan in 1955 is the first and most widely used coupling reagent for amide bond formation (Scheme 1, eq 1).[11] Later, a diphenylketenimine coupling reagent was designed by replacing one of the imine functionalities with a C−C double bond (Scheme 1, eq 2).[12] A similar activation mechanism was observed for Woodward's reagent K, which acts as a masked ketenimine.[13] However, in addition to racemization induced by the oxazolone pathway, the basic centers in these coupling reagents cause serious racemization via an intramolecular $\alpha$-proton abstraction during peptide synthesis (Scheme 1).[14] To suppress the racemization, 1-hydroxybenzotriazole (HOBt), 1-hydroxy-7-azabenzotriazole (HOAt), and other racemization suppressors that could transfer the highly reactive acyl species into the less reactive esters *in situ* were developed.[5a] In combination with a racemization suppressor, especially the recently developed cyano(hydroxyimino)acetate (Oxyma),[15] carbodiimides, among other coupling reagents such as phosphonium[16] and

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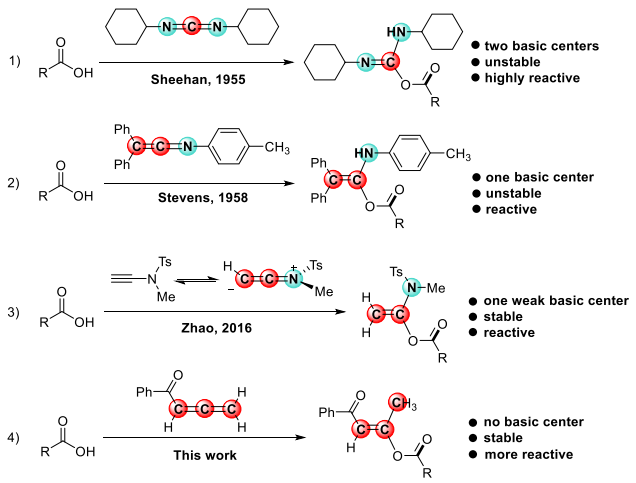
**Scheme 1. Design Concept for Coupling Reagents Containing an Electrophilic sp Carbon Center**

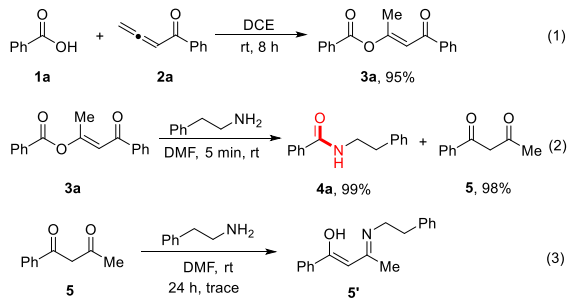



uronium/aminium salts,[17] are the mainstay in peptide synthesis. Our group has recently disclosed that ynamides could be used as peptide coupling reagents (Scheme 1, eq 3).[18] The electron-withdrawing group on the nitrogen atom plays a crucial role in attenuating the basicity of the ynamide,[19] thus enabling the ynamide coupling reagent to facilitate peptide bond formation in a racemization-free manner (Scheme 1, eq 3). Although ynamide coupling reagents are effective for liquid-phase peptide synthesis, they are not attractive for SPPS because of their moderate reactivity. A common feature of carbodiimide, ketenimine, and the 1,3-dipole resonance structure of ynamide coupling reagents is that all of them activate carboxylic acids via an addition reaction of the carboxyl group to the vicinal double-bond systems containing an electrophilic sp carbon atom[20] (Scheme 1). We thus hypothesized that allene derivatives, which also contain an electrophilic sp carbon center, would be potential coupling reagents. More importantly, the absence of a basic center in the reactive adduct intermediate would be beneficial for avoiding the racemization caused by the intramolecular $\alpha$-proton abstraction as well as the oxazolone pathway. After extensive studies, we disclose that allenones are promising coupling reagents for amide/peptide bond formation with $\alpha$-carbonyl vinyl ester intermediates, which are prepared via an unprecedented spontaneous 1,4-addition/isomerization cascade reaction of carboxylic acids and allenones, as robust active esters (Scheme 1, eq 4).

## RESULTS AND DISCUSSION

**Discovery of Allenone-Mediated Amide Bond Formation (AMABF).** At the outset of our study, we discovered that benzoic acid **1a** reacted smoothly with allenone **2a** via an unprecedented 1,4-addition/isomerization cascade reaction to furnish $\alpha$-carbonyl vinyl ester **3a** under mild conditions (Scheme 2, eq 1). Most importantly, aminolysis of **3a** furnished the target amide **4a** in quantitative yield (Scheme 2, eq 2). It is noted that the application of vinyl esters for amide synthesis had been excluded because of the side condensation reaction between the free amino group and the 1,3-dicarbonyl byproduct liberated during amide bond formation.[21] To our delight, control experiments demonstrated that this side reaction was rather sluggish in our case and had no detrimental effect on the amide bond formation efficiency

**Scheme 2. Allenone-Mediated Two-Step Amide Bond Formation Strategy**




(Scheme 2, eq 3). The formation of **3a** and **4a** proceeded spontaneously in the absence of any additive or catalyst. Further optimization of the reaction conditions suggested that the solvent played a crucial role in both steps. Generally, nonpolar solvents were beneficial for activation, while polar aprotic solvents favored the subsequent aminolysis. Dichloroethane (DCE) was identified as the best solvent for the 1,4-addition/isomerization cascade reaction, while the optimal yield for the aminolysis reaction was obtained in *N,N*-dimethylformamide (DMF). It should be noted that both activation and aminolysis proceeded smoothly in 2-methyltetrahydrofuran (2-MeTHF), a green solvent, thereby illustrating the compliance of this method with the principles of green chemistry (see the Supporting Information for details). Our systematic study suggested that allenone **2a** was a promising coupling reagent for facilitating the condensation of a carboxylic acid and an amine to furnish an amide bond.

The efficiency of the allenone-mediated two-step amide bond formation reaction was evaluated using a diverse range of carboxylic acids and amines. A broad substrate scope was observed for the electrophilic carboxyl and nucleophilic amine partners (Scheme 3). The 1,4-addition/isomerization cascade reaction of aliphatic, aryl, and $\alpha,\beta$-unsaturated carboxylic acids proceeded smoothly to furnish the target $\alpha$-carbonyl vinyl esters in excellent yields. Usually, more acidic carboxylic acids resulted in higher reaction rates. Owing to the excellent activation efficiency, the $\alpha$-carbonyl vinyl esters could be obtained in quantitative yields and used directly for the subsequent aminolysis reaction without further purification, thus offering a convenient one-pot protocol for the synthesis of the target products. The aminolysis reaction proceeded rapidly to furnish the desired amide within a few minutes in good to excellent yields, thereby illustrating the high reactivity of the $\alpha$-carbonyl vinyl esters. Both bulky amines and carboxylic acids were applicable to this reaction, giving a desired amide such as **4g** in excellent yield. Notably, challenging aryl amines with lower nucleophilicity also gave the corresponding target amides (**4h,i**) in excellent yields. For these challenging substrates, the use of 10 mol % of HOBt as a catalyst dramatically accelerated the reaction (**4g–i**).

**Application of Allenone-Mediated Amide Bond Formation (AMABF) for Dipeptide Synthesis.** Encouraged by the successful proof of concept for the allenone-mediated amide bond formation, we attempted to apply such an efficient strategy to peptide bond formation, which is usually plagued by racemization/epimerization and other side reactions caused by the overactivation of conventional coupling reagents. First, the $\alpha$-carbonyl vinyl esters of 20 proteinogenic amino acids and various non-natural amino acids were prepared using allenone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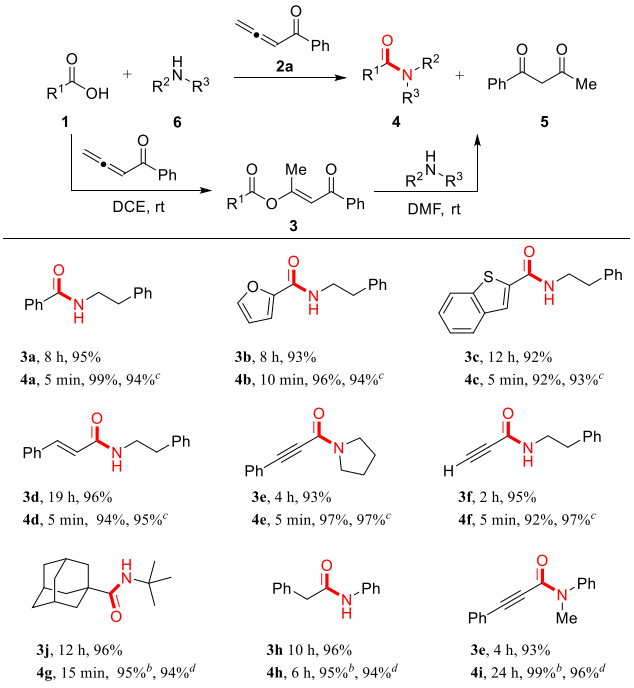
**Scheme 3. Allenone-Mediated Amide Bond Formation**[a]




[a]First step: allenone **2a** (0.2 mmol), carboxyl acid (0.22 mmol), DCE (2 mL). Second step: α-carbonyl vinyl ester (0.2 mmol), amine (0.22 mmol), DMF (1 mL), isolated yield. [b]10 mol % of HOBt as catalyst. [c]Two-step one-pot reaction. [d]Two-step one-pot reaction with 10 mol % of HOBt as the catalyst for the second step.

**2a** as the coupling reagent under the optimal reaction conditions (Scheme 4). All of the proteinogenic amino acids, except His, gave the corresponding target α-carbonyl vinyl esters in excellent yields. The low yield with His might be attributed to its imidazole side chain functional group (**9p**). The 1,4-addition/isomerization reaction was highly efficient, and no excess reactant was required, although 1.1 equiv of the amino acid was typically used for the reactions. Conventional urethane-based amine protecting groups such as carboxybenzoyl (Cbz), *tert*-butylcarboxyl (Boc), and 9-fluorenylmethylene carboxyl (Fmoc) groups were compatible under the reaction conditions employed. Moreover, steric hindrance had little influence on the activation efficiency (**9c,g**). Even the activation of the most sterically hindered, non-natural, α,α-disubstituted amino acid α-aminoisobutyric acid (Aib) proceeded smoothly to give the target active ester **9h** in 91% yield. In the next step, all of the proteinogenic amino acid based α-carbonyl vinyl esters underwent aminolysis smoothly at room temperature to furnish the target peptides in excellent yields. Sterically hindered natural and non-natural amino acids were well tolerated in both the electrophilic carboxyl and nucleophilic amino coupling partners. The steric hindrance of the carboxyl partner imposed a greater effect on the reaction in comparison to that of the amino partner (**10j** vs **10k**). In general, the aminolysis reactions proceeded to completion within a few minutes, while longer reaction times were required for α-carbonyl vinyl esters of sterically hindered amino acids such as Ile, Val, and Aib (**10c,h,i,k,l**). Even the highly hindered dipeptide of *N*-methyl amino acid such as Fmoc-MeLeu-MeLeu-OBn (**10o**) could be obtained in excellent yield. As was observed for simple amides, the reaction time for sterically hindered dipeptides could be shortened dramatically by using 10 mol % HOBt as a catalyst (for a comparative study of the HOBt catalysis effect, see the Supporting Information). Importantly, no racemization/epimerization occurred in the activation or aminolysis reaction. Indeed, phenylglycine (Phg), which is extremely prone to loss of chiral integrity upon activation (60 times more prone to racemization than Ala), could be employed as either the carboxylic acid or the amine coupling partner (**10u,v**). Although no racemization occurred during the activation of Phg (**9o**), it underwent 10% epimerization in the subsequent aminolysis reaction at room temperature. Fortunately, the epimerization could be completely suppressed by performing the aminolysis reaction at low temperature (**10u**). The side chains of Ser and Thr (−OH) and Trp (NH) were tolerated in both the activation and the aminolysis reaction, and hence, their protection was not necessary. Although His did not act as a good carboxyl partner (**10w**), it worked well as an amine partner (**10x**). While the peptide bond was formed in two steps, allowing the efficiency of each step to be systematically investigated, all of the dipeptides could be synthesized through a one-pot reaction, with results comparable to or better than those of the two-step strategy (Scheme 4). Even though free amines were employed as the nucleophile, the HCl salt of amines also worked well for this reaction in the presence of a tertiary amine, such as *N,N*-diisopropylethylamine (see the Supporting Information for details).

**Allenone-Mediated Peptide Fragment Condensation.** Urethane-based amine protecting groups are known to be beneficial for suppressing racemization during peptide bond formation. To further demonstrate the robustness of the allenone coupling reagent, peptide fragment condensation involving the activation of a peptide acid, which is more prone to racemization than the urethane-protected amino acid, was studied. As shown in Scheme 5, dipeptide, tripeptide, and tetrapeptide acids that are highly prone to epimerization are viable substrates for the allenone-mediated peptide fragment condensation. Although the time required for activation increased with increasing peptide length, the aminolysis reaction proceeded rapidly, with the reaction time ranging from minutes to hours. The activation of peptide acids and the subsequent aminolysis proceeded smoothly to furnish the target tri-, tetra-, penta-, and hexapeptides in excellent yields (**14a−k**). Importantly, no epimerization was observed for the entire peptide fragment condensation process, thus providing an attractive convergent peptide synthesis strategy.

**Synthesis of Carfilzomib with Allenone 2a as the Coupling Reagent.** To illustrate the synthetic utility of this method, carfilzomib, an anticancer peptide drug used for treating refractory multiple myeloma, was synthesized using allenone **2a** as the coupling reagent for constructing the four peptide bonds. It has been reported that the chiral keto-epoxide warhead is unstable under the conditions of peptide elongation (i.e., conditions for both coupling and deprotection) and hence must be introduced at the final stage of the peptide synthesis.[22] Although an N to C peptide elongation strategy can fulfill this requirement, this strategy is rarely used for peptide synthesis because of the serious epimerization. Considering the remarkable superiority of allenone **2a** in suppressing the epimerization of peptide acids, we speculated that allenone **2a** could be an ideal coupling reagent for carfilzomib synthesis via an N to C peptide elongation strategy. To monitor the entire process carefully, all of the intermediates were isolated and characterized. As shown in Scheme 6, all of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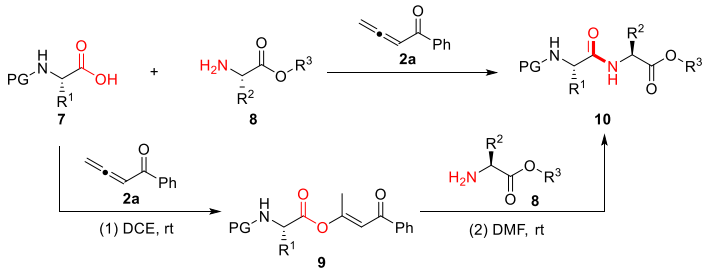
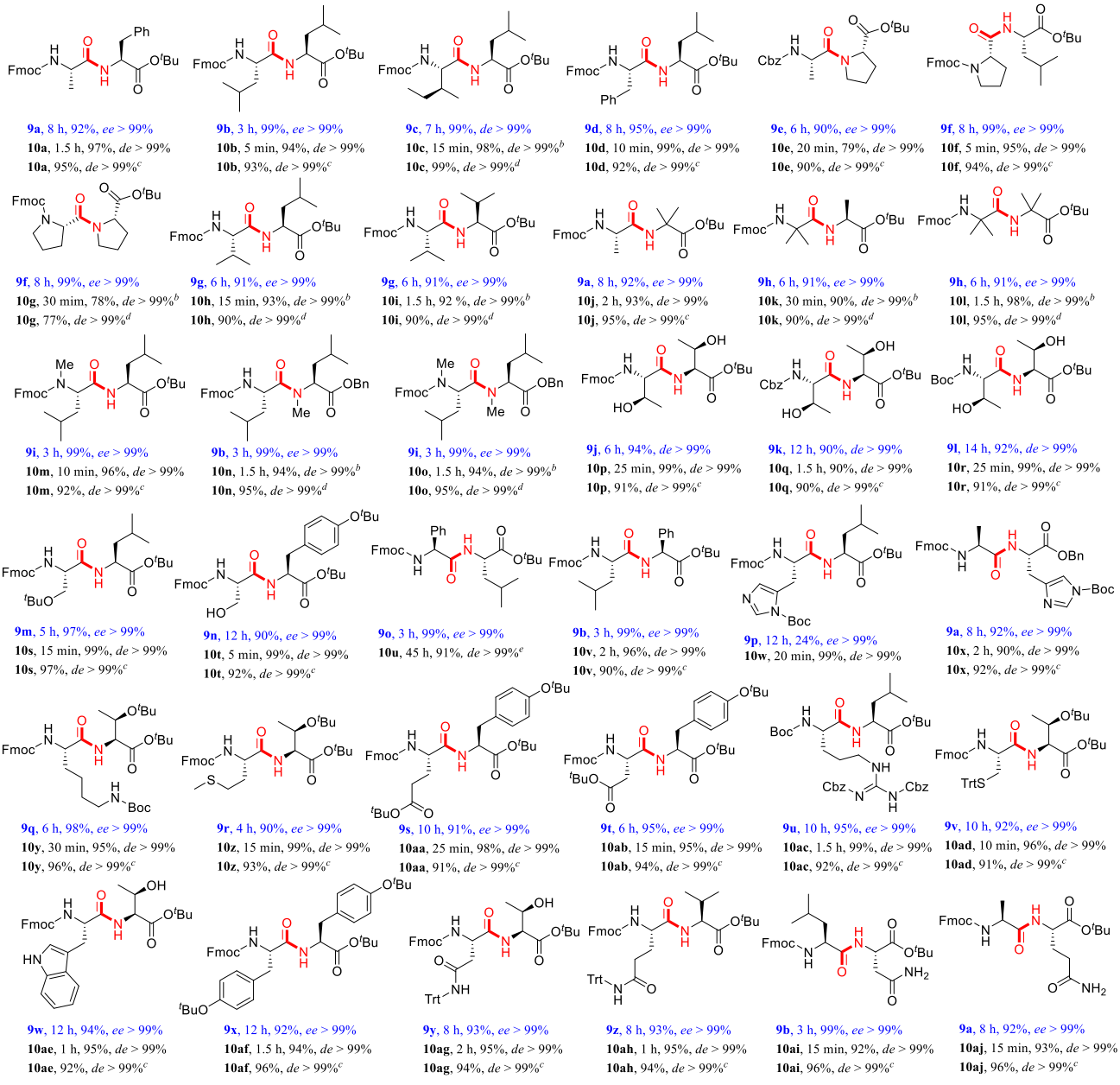
**Scheme 4. Allenone-Mediated Peptide Bond Formation**[a]




[a]Reaction conditions: (1) allenone **2a** (0.2 mmol), amino acid (0.22 mmol), DCE (2 mL), isolated yield; (2) $\alpha$-carbonyl vinyl ester (0.2 mmol), amine (0.22 mmol), DMF (1.5 mL), isolated yield. The ee and de were determined by HPLC analysis. [b]10 mol % of HOBt was used in the second step. [c]Two-step one-pot strategy. [d]Two-step one-pot strategy with 10 mol % of HOBt was used in the second step. [e]−55 °C.

the activation and aminolysis steps occurred spontaneously without any racemization/epimerization to provide the target peptide intermediates in excellent yields. The total synthesis of carfilzomib was accomplished using a nine-step linear protocol with an overall yield of 68%, which is significantly higher than those of the reported procedures (26−38% yields).[22] In addition, the excellent efficiency of *tert*-butyl ester deprotection, carboxylic acid activation, and aminolysis enabled each peptide bond to be constructed via a one-pot three-step



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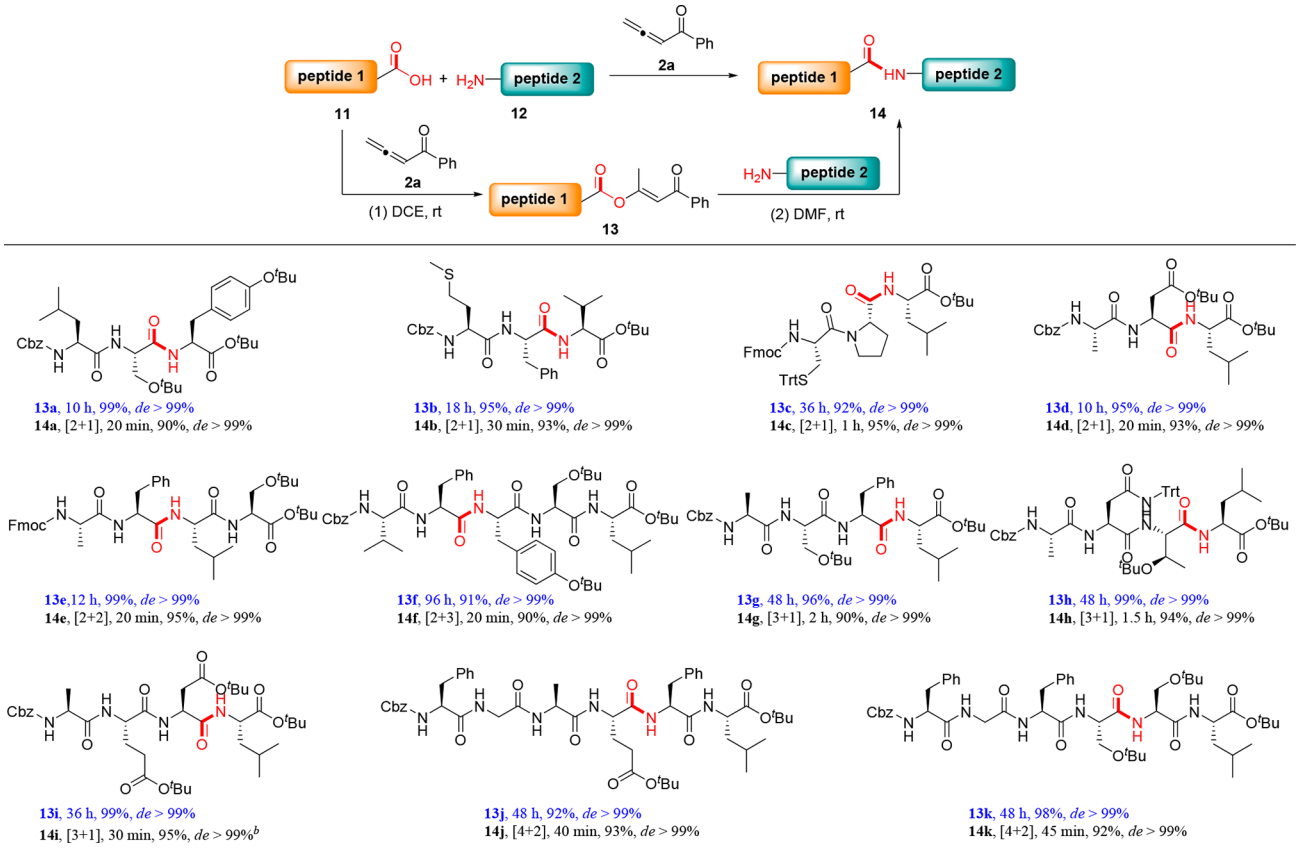
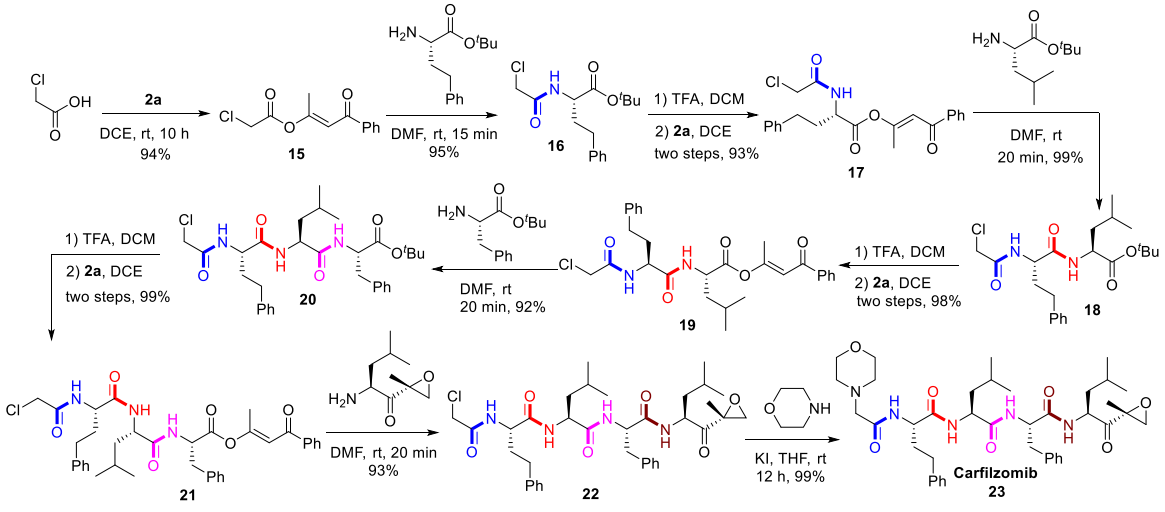
**Scheme 5. Allenone-Mediated Peptide Fragment Condensation**[a]




[a]Reaction conditions: (1) allenone **2a** (0.2 mmol), peptide acid **11** (0.1 mmol), DCE (2 mL), isolated yield; (2) $\alpha$-carbonyl vinyl ester **13** (0.1 mmol), amine (0.11 mmol), DMF (1 mL), isolated yield. The de was determined by HPLC analysis. [b]0 °C.

**Scheme 6. Synthesis of Carfilzomib with Allenone 2a as the Coupling Reagent**




protocol, thus shortening the carfilzomib synthesis to six steps in total.

**Application of AMABF in Solid-Phase Peptide Synthesis.** Before the advent of coupling reagents, the aminolysis of active esters played an important role in peptide synthesis.[21b,23] Active esters such as nitrophenyl,[24] pentafluorophenyl,[25] 2,4,5-trichlorophenyl,[26] *N*-hydroxysuccinimide,[27] *N*-(Cbz-$\alpha$-aminoacyl)benzotriazole,[28] and triazine[7b,29] esters have been frequently used in peptide synthesis. However, except for the occasional application of the *p*-nitrophenyl esters of asparagine and glutamine to prevent the dehydration side reactions of their carboxamide side chains, the enthusiasm for using active esters has faded with the development of coupling reagents. This might be attributed to the moderate reactivity of the active esters, which results in a long coupling time ranging from hours to days, thereby affecting their application in SPPS. Additionally, the prior preparation of active esters necessitates the use of stoichiometric coupling reagents, hydroxyl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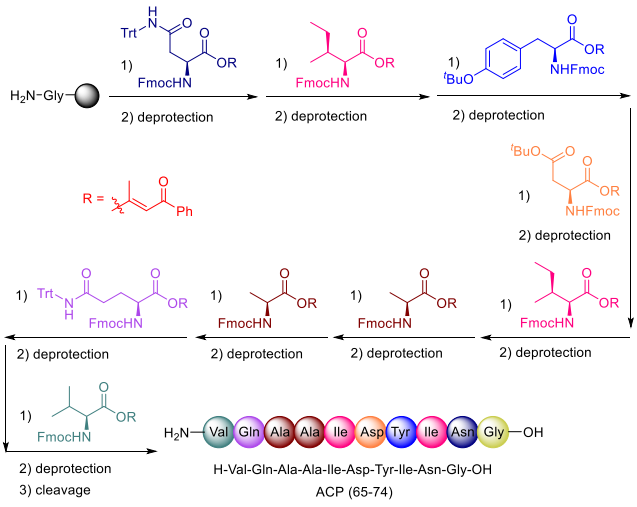
nucleophiles, and even racemization suppressors, rendering them less attractive in terms of step and atom economies. Addressing these disadvantages will make the active ester strategy an attractive alternative method for conventional peptide syntheses. The aforementioned α-carbonyl vinyl esters could be easily prepared on a gram scale from allenone **2a** and amino acids in the absence of any other reagent. More importantly, they underwent aminolysis rapidly to afford the peptide bond in a racemization-free manner. In addition, the α-carbonyl vinyl esters were stable; hence, they could be isolated, purified, characterized, and stored for a long time. Both the allenone coupling reagent and the α-carbonyl vinyl esters of proteinogenic amino acids could be stored in a refrigerator for 12 months without any deterioration. These features offer α-carbonyl vinyl esters an advantage to avoid the drawbacks associated with the conventional active esters and coupling reagents while retaining their advantages. H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH is a peptide fragment (65−74) of the acyl carrier protein (ACP). ACP (65−74) is frequently employed as a model difficult peptide for testing the efficiency of novel SPPS strategies because it not only contains sterically hindered amino acid residues but is also highly prone to interchain aggregation and alteration in the internal secondary structures, both of which hamper peptide bond formation. Therefore, ACP (65−74) would be an ideal model to evaluate the feasibility of our new strategy for SPPS. The synthesis of ACP (65−74) was initiated using a Fmoc-Gly-CTC resin (0.77 mmol/g resin loading), with the α-carbonyl vinyl esters of the desired proteinogenic amino acids as the building blocks (Scheme 7). The progress of SPPS coupling was monitored by

**Scheme 7. Solid-Phase Synthesis of ACP (65-74) with α-Carbonyl Vinyl Esters as the Building Blocks**[a]




[a]Reaction conditions for SPPS of ACP(65−74): coupling, 2 equiv of vinyl ester, 0.1 equiv of HOBt, DMF; Fmoc removal, piperidine/DMF (1/4); TFA cleavage, cleavage cocktail (TFA/TIS/$H_2O$ = 95/2.5/2.5). Abbreviations: TFA, trifluoroacetic acid; TIS, triisopropylsilane.

HPLC as well as the Kaiser test. Unlike the conventional coupling reagent-based SPPS method, which requires a large excess of amino acids, coupling reagents, and additives,[6a] our method required only 2 equiv of the α-carbonyl vinyl ester to realize excellent coupling efficiency. Although 10 mol % of the HOBt catalyst was employed to increase the aminolysis reaction rate, peptide bond formation proceeded smoothly in the absence of any additive or catalyst. Generally, the coupling reactions proceeded quickly and reached completion in 20−30 min with excellent conversions. Even sterically hindered amino acids such as Val and Ile could be incorporated into the growing peptide chain, albeit requiring slightly longer reaction times (1−2 h). Thus, using proteinogenic amino acid based α-carbonyl vinyl esters as the building blocks, ACP (65−74) was obtained with an excellent purity of 98% after cleavage from the resin (Figure 1). However, with the same amount of amino

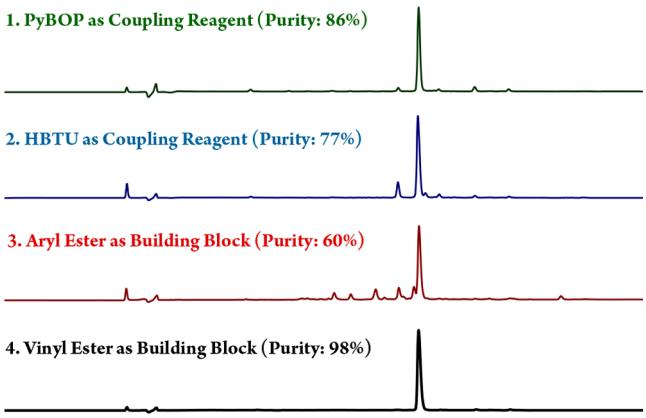



**Figure 1.** HPLC profiles of crude ACP (65−74) obtained using different SPPS methods.

acids, ACP (65−74) was obtained with moderate purities of 86%, 77%, or 60% when PyBOP, HBTU, or pentafluorophenyl ester was used as the coupling reagent or active ester, respectively. This result unambiguously illustrated that α-carbonyl vinyl esters originating from allenone are superior active esters for SPPS because they can prevent the racemization/epimerization and other side reactions resulting from the overactivation of conventional coupling reagents. Additionally, the stock solution of the α-carbonyl vinyl esters of proteinogenic amino acids remained intact in DMF at room temperature for 5 days, thus rendering them suitable for automated SPPS.

## CONCLUSION

In conclusion, we have developed the first allenone-mediated peptide bond formation strategy involving the condensation of proteinogenic amino acids. The carboxylic acids are activated by allenone via an unprecedented 1,4-addition reaction, followed by C−C double-bond isomerization, to afford α-carbonyl vinyl esters in excellent yields. These α-carbonyl vinyl esters are potent active esters that undergo aminolysis smoothly and rapidly to furnish amides and peptides in quantitative yields. Both activation and the subsequent aminolysis proceed spontaneously in the absence of any additive or catalyst. This synthetic method is not only compatible with simple amides and dipeptides but also applicable to peptide fragment condensation and SPPS. Racemization/epimerization, which is a prime concern in conventional peptide bond formation strategies, can be eliminated by adopting this method. Notably, the α-carbonyl vinyl esters derived from the 20 proteinogenic amino acids are stable and can be stored for 12 months without degradation. The optimal balance between the reactivity and stability of the α-carbonyl vinyl esters of the proteinogenic amino acids makes them ideal building blocks for SPPS. Furthermore, the α-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carbonyl vinyl esters can be easily prepared by the treatment of amino acids with allenone without the use of an extra nucleophilic hydroxyl partner, which is indispensable in the preparation of conventional active esters. Our method combines the advantages of the conventional active esters and coupling reagents, while overcoming their disadvantages, and thus opens a new avenue for the development of peptide coupling reagents. It is foreseeable that such an allenone-mediated amide formation strategy will be attractive for peptide synthesis, particularly for peptide drug manufacturing, wherein the impurities associated with conventional peptide synthesis strategies are a major concern.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site at DOI: The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c04614.

Experimental procedures, characterization data, $^{1}H$ and $^{13}C$ NMR spectra, HPLC data, and LC-MS data (PDF)

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### Author Contributions

‖Z.W. and X.W. contributed equally.


### Notes

The authors declare no competing financial interest.

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