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# A Highly Efficient *in situ* N-Acetylation Approach for Solid Phase Synthesis

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**Abstract**

We describe a new general N-acetylation method for solid phase synthesis. Malonic acid is used as precursor and the reaction proceeds by *in situ* formation of a reactive ketene intermediate at room temperature. We have successfully applied this methodology to peptides and non-peptidic molecules containing a variety of functional groups. The reaction gave high yields compared to known acetylation methods, irrespective of the structure, conformation and sequence of the acetylated molecule. Computational studies revealed that the concerted mechanism via the ketene intermediate is kinetically favorable and leads to a thermodynamically stable acetylated product. In conclusion, our method can be easily applied for acetylation in a wide variety of chemical reactions performed on the solid phase.

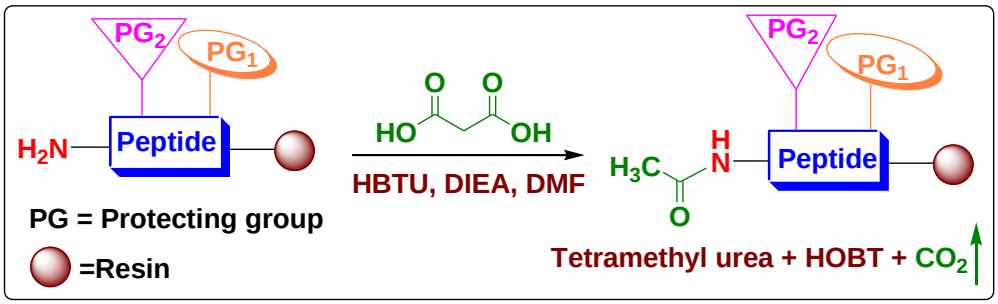
## Introduction

Acetylation of amines or alcohols is a widely used organic transformation[1,2]. It is extremely useful in solid-phase synthesis of peptides (SPPS), peptidomimetics and organic molecules. Acetylation is used to determine the degree of coupling in SPPS[2] and is widely used for capping at the end of each coupling cycle[3]. It is used for mimicking the peptide bond in the native protein by eliminating the positive charge from the N-termini of peptides[4]. Lysine acetylation is a naturally occurring post-translational modification that needs to be introduced into peptides in many cases[5,6].

Acetylation in SPPS is typically carried out by acetic anhydride ($AC_2O$) under basic conditions[7]. However, this conventional acetylation method sometimes fails to provide adequate yields[8] and its yields highly depend on the nature of the target peptide[9]. A variety of both homogeneous and heterogeneous catalysts are routinely used to improve the yields of acetylation reactions. These include 4-dimethyl amino pyridine (DMAP), iodine, p-toluene sulfonic acid, zeolite HSZ-360, various metal oxides, silicate, chlorides, nitrate, triflate salts, montmorillonit K-10 and KSF, ferric perchlorate adsorbed on silica-gel, mixed metal oxides, Nafion-H, NBS, [TMBSA][$HSO_4$] ionic liquid and potassium dodecatungstocobaltate trihydrate[10]. O-acylation via a ketene intermediate using strong electron withdrawing partners[11] such as pre-synthesized phosphate and malonate ester derivatives was also reported. Using these catalysts results in high yields in cases of relatively simple, less sterically hindered small organic molecules containing amines or alcohols. The serious limitation of these methods is when employed for rigid, more complex molecules especially during SPPS. This results in low yields, prolonged reaction times and the use of excess reagents or catalysts. This, in turn, might lead to possible side reactions such as unspecific surface binding of catalysts, early deprotection of orthogonal protecting groups, truncation of peptides or undesired peptide epimerization[12].

Here we report a novel, cost-effective, mild and highly efficient approach for the N-acetylation of peptides containing multiple functional groups in solid phase synthesis. The reaction proceeds via a highly reactive *in situ* ketene intermediate along with the formation of $CO_2$ at room temperature (scheme 1). Malonic acid serves as the starting material for the acetylation, replacing the conventionally used $Ac_2O$. The methodology is highly favorable both kinetically and thermodynamically.

**Scheme 1. The general protocol for peptide N-acetylation**




**Results and discussion**

The N-terminal free amine of a resin bound protected peptide was treated with malonic acid that was pre-activated using O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and diisopropyl ethyl amine (DIPEA) in DMF at room temperature. The acetylation typically took 10-30 minutes. The completion of the reaction was monitored by the Kaiser-ninhydrin[13] and chloranil tests[13]. The peptide was cleaved from the resin using standard TFA mediated cleavage [14]. MALDI-TOF MS of the resulting peptides confirmed the successful incorporation of the acetyl group (Table 1). All the acetylated peptides exhibited a higher retention time compared to the corresponding the non-acetylated peptides in the reverse phase HPLC, indicating increased hydrophobicity due to the acetylation. The addition of the acetyl group was confirmed by the appearance of a sharp singlet around 1.9-2.2 ppm in $^{1}$H NMR (Fig. 1 and supplementary). The progress of the reaction was also reflected by the observation that the ratio between the peak at 1.86 ppm (representing the acetyl group) and the peak at 2.7 ppm (representing the formation of tetramethyl urea) versus the reaction time formed a sigmoidal curve (figure 2). DIPEA and $Et_3N$ in DMF or DMSO were the optimal conditions to obtain maximum efficiency in terms of yield and reaction time (see supplementary).

We have applied the methodology to numerous peptides (1-26) derived from various proteins studied in our laboratory and from other sources (Table 1). The peptides were different in their sequence, length, MW, conformation (cyclic vs. linear) and hydrophobicity. In spite of their different properties, all peptides (1-26) were chemoselectively N-acetylated with high yields.

In cases of hydrophobic peptides (17, 19, 22, 23), the amine is less exposed to the solvent and thus the conventional acetylation fails to provide reasonable yields in many cases. Similarly, the

secondary amine of proline residues in peptides (10, 12, 13) is usually difficult to acetylate using conventional approaches[16]. All these limitations were successfully tackled using the technique presented herein. Even a long chain, high MW, rigid and polyfunctionalized linear peptide (25) was acetylated under the same conditions. The yield was not affected by the stereochemistry (D or L) of the acetylated residues. Our acetylation technique was applicable for various coupling agents including BOP, PyBOP, HATU, HCTU, TBTU and TSTU. The acetylation was also not influenced by altering the resin from rink amide to Wang, polystyrene or PEG based resins. For example, a single phenylalanine (24) underwent successful acetylation irrespective of the nature of the resin.

To estimate the efficiency of our acetylation protocol, our acetylation technique was quantitatively compared with the conventional $AC_2O$-mediated technique using peptide 13 as a model. Peptide 13 was selected for N-acetylation since it contains the less reactive amine of the proline residue at its N-terminal position. In both cases, we used the same number of equivalents of the starting materials and same reaction time. The formation of the acetylated peptide was analyzed using MALDI-TOF MS. The initial mass of the starting peptide (13) was found at the calculated value of 1626 Da. Peptide 13 was acetylated using equivalent amounts of $AC_2O$ and $Et_3N$. The MALDI-MS taken after 30 min. showed that the acetylation was incomplete. Both the starting peptide 13 (1626 Da) and the acetylated peptide 39 (1668 Da) were observed at roughly 5:1 intensity ratio, indicating that conversion was far from complete. The yield did not improve significantly when the reaction was performed overnight and in presence of 20 equivalents (excess) of $AC_2O$. Therefore, the conventional acetylation technique was not suitable for the peptide 13.When performing the acetylation of same peptide 13 for 30 minutes using our malonic-acid mediated protocol, we observed in MALDI-TOF MS that the aetylation reaction was successfully completed. The peak of the acetylated peptide (39) at 1668 Da was the only one observed, while no peak corresponding to the starting peptide (13) at 1626 Da was found. This shows that our methodology can be applied to peptides with hindered amines where acetylation is difficult to achieve by conventional methods. In general, no epimerization, truncation or aggregation was evident in the isolated peptides.

**Mechanism of acetylation:** We performed NMR based kinetics study on the acetylation of the simple molecule anisidine as a model (53) to understand the mechanism of *in situ* acetylation at room temperature. Stoichiometric amount of malonic acid (55), HBTU (57) and DIPEA were

mixed in $d_6$-DMF in an NMR tube. The spectra were recorded for 3 minutes each. Initially, a malonate-TMU complex (58, $\delta_H$ 3.1ppm) and a ketene (61, $\delta_H$ 2.6 ppm) were formed due to the removal of HOBt (62) (Fig. 1 and scheme 2). The intensity of the ketene peak gradually decreased with time and the peak completely disappeared at 24 mins, immediately after adding the anisidine (53). At 27 minutes, a distinct peak at $\delta_H$ 2.2 ppm began to appear, indicating the N-acetylation of anisidine (54). A similar phenomenon was also observed in $d_6$-DMSO where anisidine was added in the beginning of the reaction. The spectra were recorded every 3 minutes at room temperature. The N-acetylated anisidine (54) was formed after 9 minutes and the reaction completed immediately within 12 minutes. After that, there was practically no change in intensity of the acetylated peak (supplementary Fig. S3). These two experiments provide a direct evidence for the spontaneous *in situ* formation of a ketene intermediate (61).

**Scheme 2. Suggested mechanisms for N-acetylation**

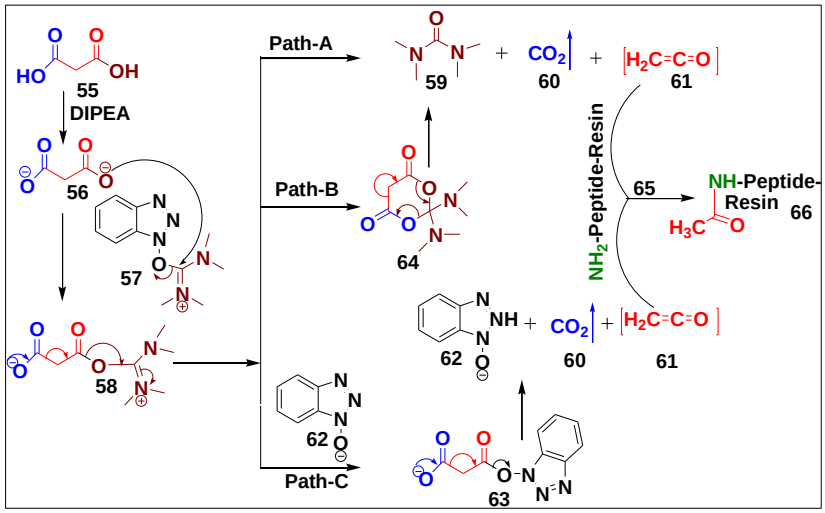


**Table 1. The peptides acetylated in this study***

| Non-acetylated peptide Sequence | .MW[a] | Acetylated product sequence | Obs. MW[b] | Reaction Time (mins) | Yield (%) |
|---|---|---|---|---|---|
| WNSLKIDNLDV[15], 1 | 1316 | (Ac)-WNSLKIDNLDV-$CONH_2$, 27 | 1358 | 10 | 98 |
| LSNWKIDNLDV, 2 | 1316 | (Ac)-LSNWKIDNLDV-$CONH_2$, 28 | 1358 | 14 | 95 |
| LMLSPDDIEQWFTED, 3 | 1838 | (Ac)-LMLSPDDIEQWFTED-$CONH_2$, 29 | 1882 | 20 | 96 |
| KILNPEEIEKYVAEI, 4 | 1789 | (Ac)-KILNPEEIEKYVAEI-$CONH_2$, 30 | 1832 | 16 | 98 |
| $NH_2$-(Succinyl)GWSLKIDNLDV, 5 | 1356 | (Ac)-(Succinyl)GWSLKIDNLDV-$CONH_2$, 31 | 1398 | 22 | 94 |
| $NH_2$-W(succinyl)DGSLKIDNLDV, 6 | 1356 | (Ac)-W(succinyl)DGSLKIDNLDV-$CONH_2$, 32 | 1398 | 25 | 96 |
| WHKQEVRRLKSLHEAE, 7 | 2046 | (Ac)-WHKQEVRRLKSLHEAE-$CONH_2$, 33 | 2089 | 19 | 93 |
| RAEREQDPRVAPQQCN, 8 | 1969 | (Ac)-RAEREQDPRVAPQQCN-$CONH_2$, 34 | 2060[c] | 16 | 92 |
| CYCCGLRSFRELTYQ, 9 | 1842 | (Ac)-CYCCGLRSFRELTYQ-$CONH_2$, 35 | 1884 | 22 | 94 |
| PDCYWGRWCRTQVKAHHAMKF 10 | 2799 | (Ac)- PDCYWGRWCRTQVKAHHAMKF -$CONH_2$, 36 | 2841 | 30 | 93 |
| WVARPLSPTRLQPALP, 11 | 1802 | (Ac)-WVARPLSPTRLQPALP-$CONH_2$, 37 | 1844 | 15 | 96 |
| QAGPPSRPPRYSSSS, 12 | 1573 | (Ac)-QAGPPSRPPRYSSSS-$CONH_2$, 38 | 1616 | 20 | 91 |
| PYSPLSPKGRPSSPR, 13 | 1626 | (Ac)-PYSPLSPKGRPSSPR- $CONH_2$, 39 | 1668 | 30 | 92 |
| RQAGDDFSRRYRRDF, 14 | 1945 | (Ac)-RQAGDDFSRRYRRDF-$CONH_2$, 40 | 1987 | 30 | 90 |
| NSLKIDNLDV, 15 | 1130 | (Ac)-NSLKIDNLDV-$CONH_2$, 41 | 1172 | 20 | 99 |
| FmocNH-WNSLK( $NH_2$)IDNLDV, 16 | 1539 | WNSLK(Ac)IDNLDV-$CONH_2$, 42 | 1581 | 10 | 98 |
| FIVLK, 17 | 619 | (Ac)-FIVLK-$CONH_2$, 43 | 661 | 20 | 97 |
| WIDNLD, 18 | 775 | (Ac)- WIDNLD-$CONH_2$, 44 | 839[c] | 28 | 96 |
| MLVVLIL, 19 | 801 | (Ac)- MLVVLIL-$CONH_2$, 45 | 843 | 25 | 96 |
| GGGGK, 20 | 375 | (Ac)-GGGGK-$CONH_2$, 46 | 417 | 12 | 97 |
| WNSLK, 21 | 647 | (Ac)-WNSLK-$CONH_2$, 47 | 689 | 15 | 98 |
| GFFRWG, 22 | 769 | (Ac)- GFFRWG -$CONH_2$, 48 | 811 | 20 | 92 |
| FGRFWG, 23 | 769 | (Ac)- FGRFWG -$CONH_2$, 49 | 811 | 25 | 93 |
| F, 24 | 166 | (Ac)-F-$CONH_2$, 50 | 208 | 14 | 99 |
| WQHPEKENEGDITIFPESLQPSETLK QMNSMNSVGTFLDVKRLRQLPKLF, 25 | 5857 | (AC)-WQHPEKENEGDITIFPESLQPSETLK QMNSMNSVGTFLDVKRLRQLPKLF- $CONH_2$, 51 | 5899 | 30 | 92 |

*For the MS and HPLC data of all peptides see supplementary figures S1 and S2. [a] molecular weight of the unmodified peptides; [b] Molecular weight of the acetylated peptides as measured by MALDI-TOF; [c]Molecular weight with the addition of sodium or potassium

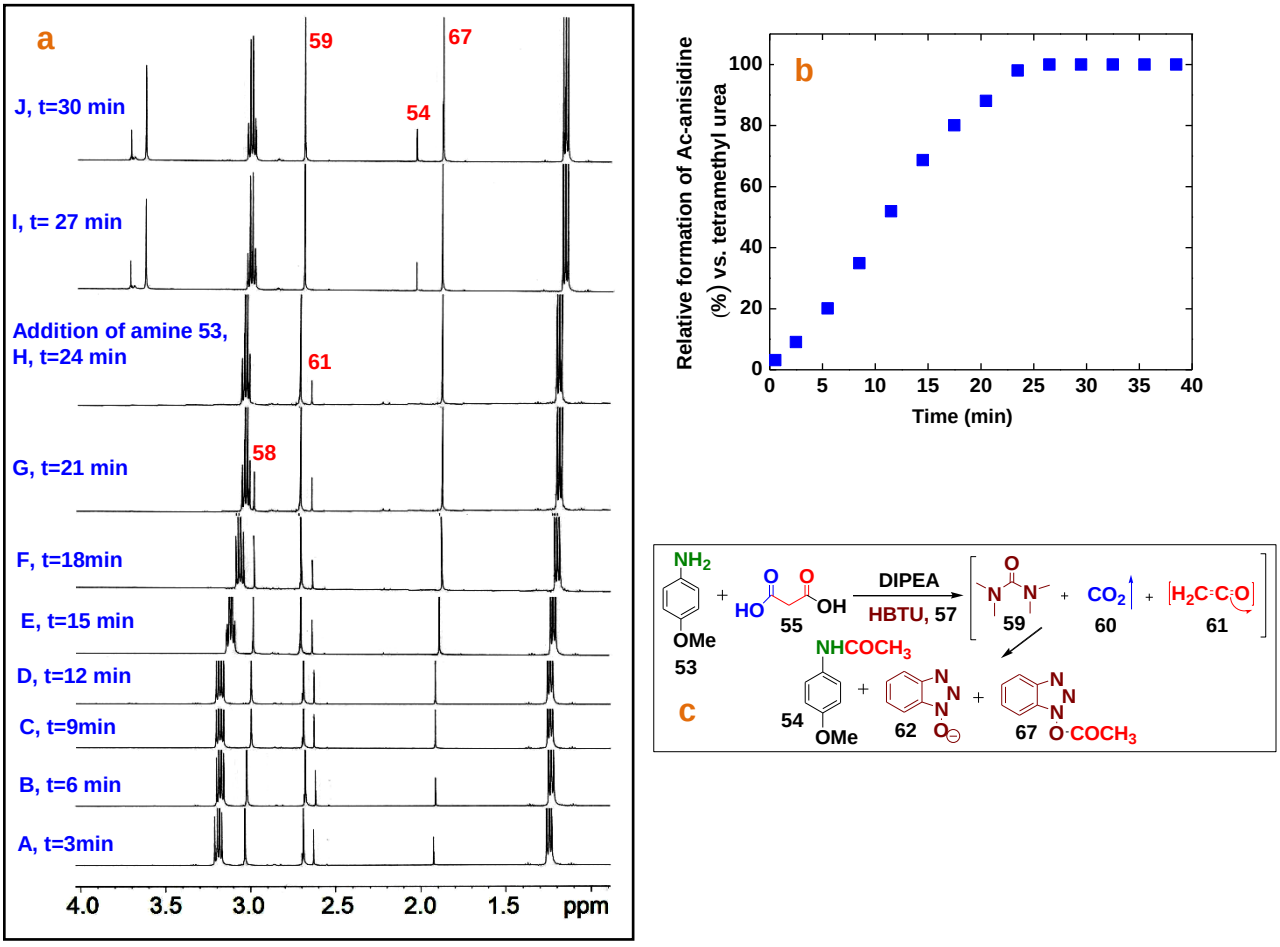



**Figure 1. Monitoring the *in situ* acetylation kinetics of anisidine as a model compound by $^{1}$H NMR.** (a) **A-G** represents the ketene formation; **H** shows the disappearance of the ketene intermediate; **I-J** depicts the attack by an external amine of anisidine, 53; (b) Relative formation of N-acetyl anisidine (54) vs. tetramethyl urea with time; (c) Scheme of N-acetylation with anisidine.

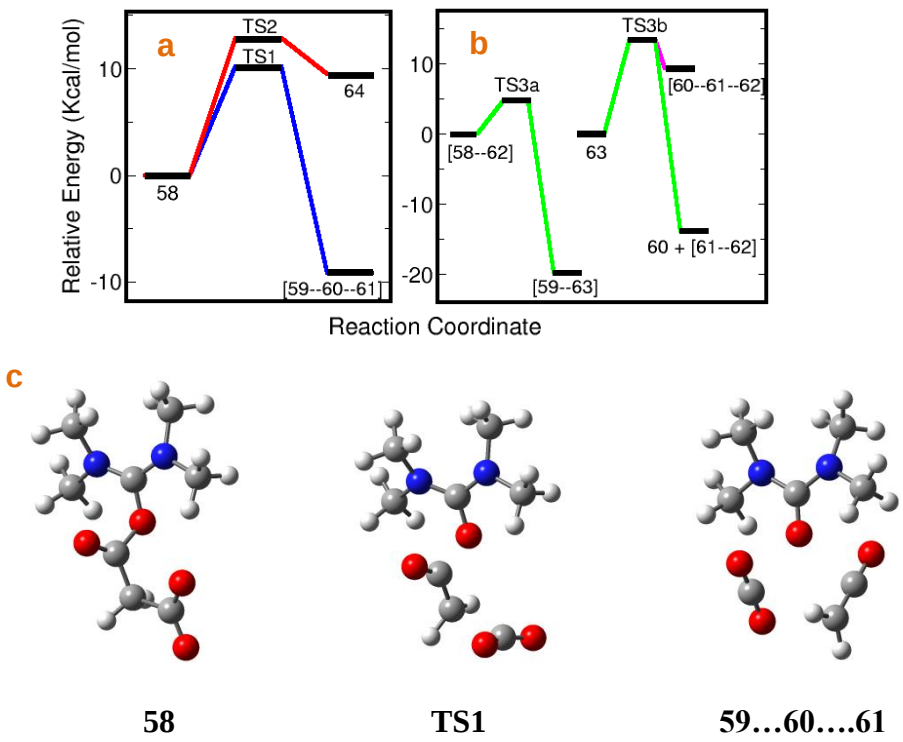



**Figure 2. Energy profiles for the three proposed reaction pathways show that path A is favourable:** (a) Path A (blue) represents the self-dissociation of the intermediate complex 58 to form tetramethyl urea (59), $CO_2$ and the reactive ketene; path B (red) represents the formation of the cyclic intermediate 64. The corresponding reaction is indicated in scheme 2; (b) Path C (green and purple) represents the formation of 63 followed by its self-dissociation and is also shown in scheme 2; (c) The optimized structure for the most favorable path A. Shown are the reactant (58), transition state (TS1) and the products (59--60--61).

Based on the NMR results, three different possible pathways for the acetylation mechanism could be suggested (scheme 2). Initially, the malonate reacts with the HBTU to form an intermediate complex (58) following the leaving of the HOBt (62). In path A, the complex 58 undergoes self-dissociation to form $CO_2$ (60) and a reactive ketene (61). In path B, 58 forms a cyclic intermediate 64, followed by self-dissociation to $CO_2$ and ketene. In path C, the HOBt reacts with intermediate 58 to form an HOBt - malonate complex (63) followed by similar self-dissociation to form the ketene and $CO_2$. The feasibility of the three different mechanisms was investigated by theoretical calculations using the density functional theory based hybrid B3LYP[17] method with the 6-311++g** basis set[18] applying the Gaussian 09[19] program suit. Based on the experimental conditions, we have considered DMF as the solvent and the polarization continuum model (PCM) with integral equation formalism variant (IEFPCM)[20] implemented in Gaussian 09 as the solvation method. Geometry optimization and subsequent

frequency calculations were performed to determine the extrema for all the reactants, intermediates, transition states and products. The zero point energy was considered for all the species in evaluating energy profiles of the reaction mechanisms (Fig. 2) shown in scheme 2. All the transition states were determined by a single imaginary frequency characterized by the corresponding reaction coordinates. The calculated energy barrier for the self-dissociation of 58 (path A) is only 10.2 kcal/mol (TS1) and the reaction is exothermic by -9.2 kcal/mol (Fig. 2a). This shows that this reaction pathway is kinetically and thermodynamically favorable. For path B, the TS energy for the formation of the cyclic intermediate 64 is 12.8 kcal/mol (TS2) but endothermic in nature (Fig. 2a). We could not detect any subsequent self-dissociation of 64. Rather, it readily went back to 58 and followed Path A. The energy profile indicates that the backward reaction is highly favorable with a barrier of 3.3 kcal/mol. The NMR studies also support this observation. If the cyclic intermediate 64 would be formed from the linear 58, we would expect a significant shift in the position of the methylene group peak towards a more shielding region. However, no change in the chemical shift of the methylene peak was observed (Fig. 1). We conclude that Path-B is not feasible. For Path C, the attack by the HOBt anion (62) to generate 63 is an exothermic and almost instantaneous with a small barrier of only 4.6 kcal/mol (TS3a, Fig 2b). However, the subsequent self-dissociation is endothermic in nature and has a barrier of 13.6 kcal/mol (TS3b, Fig 2b), which is higher than Path A. The main driving force for this reaction could be the formation of the $CO_2$ gas. When considering the $CO_2$ as a separate species that readily leaves the system and the HOBt - ketene complex [61---62] as the only remaining product in the reaction medium, the reaction is found to be exothermic by -13.8 kcal/mol. The overall energy profiles lead to path A that is energetically more favorable than path C. To quantify this, we have further calculated the rate of the corresponding two self-dissociation reactions. The calculated rate for path A is $3.7\times10^{6}$ $s^{-1}$ and for path C is $5.7\times10^{3}$ $s^{-1}$. This shows that path A is approximately 650 times faster than path C and thus the self-dissociation process follows explicitly path A.

## Conclusions

We have demonstrated a highly efficient, cost effective approach for peptide acetylation via an *in situ* formation of a ketene intermediate. Activated malonic acid is shown to be a more potent acetylating precursor compared to $Ac_2O$. We have established a reaction mechanism of

simultaneous *in situ* ketene formation and decarboxylation at room temperature. The process is highly favorable both kinetically and thermodynamically. A combination of NMR studies and theoretical analysis revealed the acetylation mechanism. This novel acetylation reaction should be highly applicable for solid phase peptide and organic synthesis.

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