Cycloaddition of N-Acylenamines

Reaction Rates of Various N-Acylenamines in the Inverse-Electron-Demand Diels–Alder Reaction


Abstract: In light of the bioorthogonal inverse-electron-demand Diels–Alder strategy, an extended investigation into the effects of ring strain and electron inductive effects on the reactivity of the N-acylaniline core towards tetrazine has been carried out. Through a comparative study between N-acylazetines, N-vinylcarbamates and an N-vinylamide it was shown that ring strain has a more significant effect on reaction rate than electron donation. A significantly improved synthetic route is reported for the preparation of an N-acylazetine biorthogonal tag we have invented previously.

Introduction

The inverse-electron-demand Diels–Alder (IEDDA) reaction between (cyclic) alkenes and tetrazines has been well-studied for several decades.[1–7] Renewed interest arose in 2008, due to the breakthrough of tetrazine ligation in the field of bioorthogonal chemistry.[8,9] Bioorthogonal ligation handles require an intricate balance between several physical properties; preferably the handle should be sterically small in size and be stable under physiological conditions, while maintaining high reactivity towards tetrazines. An additional beneficial property is the moderate-to-high hydrophilicity to assist the water solubility of the functionalized chemical probe. By now, a strong positive correlation had been established between the ring strain within cyclic alkenes and the enhanced reactivity towards tetrazines.[5] Contrary, significantly less effort has been directed towards the study on the effects of the alkene electron density on reactivity. Recently we reported[10] a new ligation handle featuring an N-acylazetine as the reactive IEDDA partner (e.g. 1 in Figure 1), which was designed to utilize both ring strain and electron-donating properties to enhance reactivity towards tetrazines. An N-acylazetine tag functionalized as a p-nitrophenyl active ester was synthesized and successfully applied in an activity-based protein profiling experiment. These advancements warranted further investigation into the structure–reactivity relationship of the N-alkene structure.

We here describe the synthesis of two N-acylazetines (1, 2, Figure 1) and two N-vinylcarbamates (3, 4) and the assessment of their stability in water and reactivity towards tetrazines. By comparing the reaction-rate constants of the IEDDA reactions of N-acylazetines 1 and 2 with N-vinyl derivatives (3–5) insight can be gained about the contribution of both ring strain and electron-donating effects on their reactivity towards tetrazines. Ultimately this knowledge may lead to improved probes for the tetrazine ligation strategy.

N-vinylcarbamates (3 and 4) and vinylamide (5) can be considered as linear analogues of the original cyclic N-acylazetine (1) and thereby potentially suitable for IEDDA reactions with tetrazine derivatives. A possible tautomerization equilibrium between the enamine and imine forms, as in 3, makes N-vinyl groups both electrophilic at the α-carbon and nucleophilic at the β-carbon.[11] This property make vinylamides susceptible to hydrolysis, or polymerization in either acid-catalyzed or radical-mediated processes.[12,13] N-methylation of N-vinyl derivatives suppresses tautomerization and makes the corresponding N-methyl-N-vinylamides more stable (i.e. 4). In addition, disconnection of the N-acylazetine core between the N-CH₂ and the N-vinyl-CH₂ leads to commercially available N-methyl-N-vinylamide (5). To ensure the necessary water solubility in the kinetic experiments, morpholine was incorporated in the N-acylazetines (1–2) and N-vinylcarbamates (3–4).

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Figure 1. The N-acylazetines 1 and 2 and N-vinyl derivatives 3–5.
Results and Discussion

The earlier described synthesis of \( N \)-acylazetines 1 consists of six reaction steps and lacks overall efficiency and scalability.[10] Therefore, another method to synthesize 1 was explored (Scheme 1). Commercially available 3-hydroxyazetidine 6 was selected as the starting compound, and the tosyl group was used instead of the mesyl group as a more potent and UV-detectable leaving group. Boc-protection of the amine followed by tosylation of the hydroxyl group in 6 provided protected azetidine 7 in 72 % yield over two steps. To prevent potential displacement of the tosyl group the Boc-group in 7 was cleaved using \( p \)-toluenesulfonic acid.

\[
\begin{align*}
\text{HCl} \quad \text{HN} \quad \text{OH} \\
\text{6} \quad \text{a, b} \quad \text{BocOH} \quad \text{OTs} \\
\end{align*}
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Scheme 1. Synthesis of \( N \)-acylazetines 1, 2 and \( N \)-vinylcarbamates 3, 4. Reagents and conditions: [a] i: Boc\(_2\)O, TEA, MeOH, 0 °C, 2 h, used crude. [b] TsCl, TEA, DCM, 2 h, 72 % over 2 steps. \[c\] \( p \)-nitrophenol was introduced using DIC, K\(_2\)CO\(_3\), MeCN, reflux, 16 h, 80 %. [d] Morpholine, DCM, 1 h, 84 %. [e] KO\(_t\)Bu, DMF, 2 h. \[f\] morpholine, DCM, 1 h, 84 %, 100 % \[g\] Na\(_t\), H\(_t\)O/toluene (1:1), 6 h. \[h\] 2-morpholinoethanol, pyridine, hydroquinone, toluene, 100 °C, 29 %. \[i\] Na\(_t\), THF, MeI, 0 °C, 1.5 h, 75 %.

Subsequent purification by crystallization from MeOH yielded azetidine 8 in 80 %. Treatment of 8 with both succinic and glutaric anhydride was carried out in the presence of potassium carbonate in acetonitrile at reflux temperature, to give the four- and five-carbon spacer 9a, and 9b respectively. Next, the key elimination was initiated by the addition of a solution of KO\(_t\)Bu in THF. The presence of the tosyl group in 9a and 9b improved solubility while the reaction proceeded readily at room temperature. The respective \( N \)-acylazetidines intermediates were treated in situ with bis(\( p \)-nitrophenyl)carbodine to provide linkable handles 10a (80 %) and 10b (69 %) in good yields over two steps. Initially, the \( p \)-nitrophenol was introduced using DIC or EDC as coupling reagents. However, switching to bis(\( p \)-nitrophenyl)carbonate resulted in cleaner and more consistent versions. Treatment of \( p \)-nitrophenyl esters 10a and b with morpholine gave target \( N \)-acylazetines 1 and 2 in high yield. Alternatively, one could consider converting the \( N \)-acylazetidine-containing carboxylic acids to the amides in a “one-pot” procedure. We considered this approach to be impractical and did not investigate it.

The route of synthesis toward \( N \)-vinylcarbamates 3 and 4 makes use of the Curtius rearrangement of acryl azide (12), generated in situ from acryloyl chloride (11) and sodium azide. Upon heating, azide 12 rearranges into vinyl isocyanate (13), which can be transformed into \( N \)-vinylcarbamates by coupling with an alcohol of choice. A small amount of base and radical scavenger, such as hydroquinone or phenothiazine, is needed to suppress dimerization and radical polymerization. In practice, a solution of acryl azide 12 in toluene was prepared and slowly added to a reaction mixture containing 2-morpholinoethanol, pyridine and hydroquinone in toluene at 100 °C, providing \( N \)-vinylcarbamate 3 in 29 % isolated yield. The vinyl nitrogen atom was methylated with sodium hydride and methyl iodide in THF at 0 °C, to give \( N \)-methyl-\( N \)-vinylcarbamate 4.

The instability of \( N \)-vinylcarbamates brings about the question of whether these compounds would survive the conditions of the kinetic experiments and future bioorthogonal tagging reactions. The aqueous stability of \( N \)-acylazetines 2 and \( N \)-vinylcarbamates 3 and 4 was evaluated by dissolving the respective compounds in deuterated water and monitoring the solution over a period of 13 hours at 37 °C with \( ^1 \)H NMR spectroscopy. After the full duration, secondary \( N \)-vinylcarbamate 3 showed 4 % hydrolysis of the vinyl group, as determined by integration of the formyl hydrogen (\( \delta = 9.74 \) ppm) resulting from the formed acetaldehyde. Fortunately, this rate of decomposition proved to be insignificant within the 30 minutes timeframe required for the kinetic experiments (Table 1). \( N \)-acylazetine 2 and \( N \)-methyl-\( N \)-vinylcarbamate 4 proved to be stable during the experiment. In addition, the \( N \)-acylazetine group stability was evaluated in the presence of 100 mM of ethanethiol and 2-aminoethanol, to mimic the physiological conditions used during in vivo activity-based protein profiling experiments. Again, no decomposition occurred.

With the \( N \)-acylazetines 1, 2 and \( N \)-vinyl derivatives 3, 4, 5 in hand, the stage was set for determining the rates of IEDDA reactions with tetrazine 14 (Table 1). The conditions we opted to apply for the kinetic experiments were similar to those used by Devaraj et al. in their work featuring the 1-methylcyclopropene ligation handle.[14] Pseudo-first-order and second-order reaction rates were assessed by reacting a 20-fold excess of the respective dienophile (1−5) with tetrazine 14 in 12 % aqueous DMSO. The rate of tetrazine consumption was measured by monitoring the characteristic tetrazine absorption at 517 nm. Each experiment was conducted thrice both at room temperature (20 °C) and at body temperature (37 °C). The results are summarized in Table 1. The availability of \( N \)-acylazetine 1 and 2 allows the determination of the influence of the spacer length on the IEDDA reaction rate. The outcome indicates that shortening the spacer length by one carbon as in 1 results in a minor decrease of the reaction rate. This result could be explained by the increased proximity of the electron-withdrawing carbonyl
Table 1. Reaction rates for addition of dienophiles 1–5 to tetrazine 14. The first-order reaction rates were calculated from three data sets each using all measurement intervals up until $t_{1/2}$. The error ranges are the standard deviations derived from the generated first-order rate data. Half-life values are calculated from first-order rate constant: $t_{1/2} = \ln(2)/k$.

<table>
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<th>Dienophile</th>
<th>T [°C]</th>
<th>1st Order [s$^{-1}$]</th>
<th>2nd Order [M$^{-1}$s$^{-1}$]</th>
<th>Half-life [s]</th>
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<td>1</td>
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<td>4.10 · 10$^{-3}$ ± 3.71 · 10$^{-4}$</td>
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<td>7.50 · 10$^{-1}$ ± 2.62 · 10$^{-2}$</td>
<td>157</td>
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<td>37</td>
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<td>1.32 ± 7.40 · 10$^{-3}$</td>
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</tr>
<tr>
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<td>37</td>
<td>1.97 · 10$^{-3}$ ± 9.34 · 10$^{-5}$</td>
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<td>5.05 · 10$^{-4}$ ± 1.68 · 10$^{-6}$</td>
<td>8.54 · 10$^{-2}$ ± 2.88 · 10$^{-4}$</td>
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</table>

Conclusions

An investigation into the contribution of ring strain and electron-donating effects upon the IEDDA reactivity of N-acylenamines towards tetrazines is described. To this end, an improved synthesis toward N-acylazetines 1, 2 is presented while N-vinylcarbamates 3, 4 could be accessed through the Curtius rearrangement of acryl azide. The reaction rate constants were determined for N-acylazetine 1 and 2 and N-vinyl compounds 3–5 at 20 °C and 37 °C. Comparison between the reaction rates of N-acylazetine 2 and N-vinylamide 5 shows a 15-fold higher reaction rate for the four-membered ring. The influence of the differences in electron donation between a N-vinylamide and a N-vinylcarbamate was significantly less, resulting in a 2.8-fold increase in reaction rate for the latter.

Experimental Section

All solvents and reagents were obtained commercially and used as received. Reactions were executed at ambient temperatures unless stated otherwise. Reactions were monitored by TLC analysis, spraying with varying stains; an aqueous solution of cerium molybdate [(NH$_4$)$_6$Mo$_7$O$_{24}$·4H$_2$O 25 g/L], an aqueous solution of potassium permanganate (5 g of KMnO$_4$, 2 5 g of K$_2$CO$_3$ per L) or an ethanolic solution of bromocresol [0.4 g in 1 L, addition of 0.1 M NaOH(aq) until the solution turns blue]. Column chromatography was performed on silica gel (40–63 μm). $^1$H and $^{13}$C-APT spectra were recorded on a Bruker AV-400 (400 MHz), Bruker DMX-600 (600 MHz) or Bruker BioSpin (850 MHz). All present $^{13}$C-APT spectra are proton decoupled. High-resolution mass spectrometry was recorded by direct injection (2 μL of a 2 μM solution in water/acetonitrile; 50:50; v/v and 0.1 % formic acid) on a mass spectrometer (Thermo Finnigan LTQ Orbitrap) equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 250 °C) with resolution R = 600000 at $m/z$ 400 (mass range $m/z = 120–400$).

tert-Butyl 3-(Tosyloxy)azetidine-1-carboxylate (7): A solution of 3-hydroxyazetidine hydrochloride (115 mmol, 10.55 g, 1 equiv) and Et$_3$N (161 mmol, 22.5 mL, 1.4 equiv) in MeOH (115 mL) was pre-
pared at 0 °C. Boc₂O (126.5 mmol, 27.6 g, 1.1 equiv.) was added and the ice bath was removed. After 5 h of stirring, the reaction mixture was concentrated in vacuo, redissolved in DCM and washed twice with water. The water layers were combined and extracted twice with DCM. The organic layers were combined, dried with magnesium sulfate, filtered and concentrated in vacuo. The intermediate Boc-hydroxyazetidine was used without further purification. An ice-cooled solution of the Boc-protected intermediate and Et₃N (172.5 mmol, 24 mL, 1.5 equiv.) in dry DCM (100 mL) was prepared under argon atmosphere. p-Toluenesulfonyl chloride (138 mmol, 26.3 g, 1.2 equiv.) was added in eight portions over 2 h and the reaction mixture was stirred overnight. The reaction mixture was washed with water twice and the combined aqueous layers were extracted thrice with DCM. The organic layers were combined, dried with magnesium sulfate, filtered and concentrated in vacuo. For the crude product was purified by column chromatography (5 % → 10 % EtOAc in pentane), yielding tosylate 7 as a yellow oil. (82.6 mmol, 27.7 g, 72 % over two steps). ¹H NMR (300 MHz, CDCl₃): δ = 7.75 (d, J = 8.1 Hz, 2 H), 7.34 (d, J = 8.1 Hz, 2 H), 4.97 (dd, J = 10.8, 6.6, 4.3 Hz, 1 H), 4.14–4.01 (m, 2 H), 3.97–3.82 (m, 2 H, 2.43 (s, 3 H), 1.38 (s, 9 H) ppm. ¹³C NMR (75 MHz, CDCl₃): δ = 155.86, 145.61, 132.91, 130.38, 129.39, 128.17, 125.92, 67.85, 53.37, 21.86, 21.55 ppm. HRMS: Calculated for C₁₁H₁₀NO₃S⁺ = 261.02816, found 261.0278 ppm.

Azetidin-3-yl 4-Methylbenzenesulfonate (8): A solution of compound 7 (82.6 mmol, 27.1 g, 1 equiv.) in DCE (165 mL) was charged with p-toluenesulfonic acid (90.9 mmol, 173.3 g, 1.1 equiv.) and refluxed for 20 h. The reaction mixture was concentrated in vacuo. The crude product was crystallized from MeOH, yielding compound 8 as a white crystalline substance (65 mmol, 25.9 g, 79 %). ¹H NMR (400 MHz, CDCl₃): δ = 9.05 (br. s, 1 H), 8.94 (br. s, 1 H), 7.70 (d, J = 8.2 Hz, 4 H), 7.29 (d, J = 8.1 Hz, 2 H), 7.19 (d, J = 7.8 Hz, 2 H), 5.06 (t, J = 6.2 Hz, 1 H), 4.25 (br. s, 1 H), 4.13 (br. s, 1 H), 2.42 (s, 3 H, 2.39 (s, 3 H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 146.07, 141.26, 132.55, 114.19, 113.93, 77.16, 58.67, 56.91, 29.13, 29.03, 26.74, 25.76 ppm. HRMS: Calculated for C₁₁H₁₀NO₃S⁺ = 261.02816, found 261.0278 ppm.

5-Oxo-4-[3-(tosyloxy)azetidin-1-yl]butanoic Acid (9a): Compound 8 (20.0 mmol, 5.58 g, 1.1 equiv.) was co-evaporated with dioxane, redissolved in MeCN (200 mL), and put under argon atmosphere. Succinic anhydride (18.2 mmol, 1.82 g, 1 equiv.) was added to the reaction mixture, followed by potassium carbonate (31.8 mmol, 4.48 g, 2.5 equiv.) and the reaction mixture was refluxed for 6 h. Reaction progression was monitored by TLC, using a bromocresol green/MeOH (10 % EtOAc in pentane), yielding tosylate 9a as a white crystalline substance (40.0 mmol, 6.82 g, 72 % over two steps). ¹H NMR (400 MHz, CDCl₃): δ = 8.2 (d, J = 9.2 Hz, 2 H), 7.31 (d, J = 9.1 Hz, 2 H), 6.91 (s, 0.5 H), 6.71 (s, 0.5 H), 5.75 (d, J = 5.3 Hz, 1 H, 4.61 (s, 1 H), 4.48 (s, 1 H), 2.97 (t, J = 6.6 Hz, 2 H), 2.75 (t, J = 6.5 Hz, 1 H), 2.66 (t, J = 6.5 Hz, 1 H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 171.99, 169.76, 165.26, 164.88, 154.32, 137.43, 136.64, 125.22, 122.55, 114.19, 113.93, 77.16, 58.67, 56.91, 29.13, 29.03, 26.74, 25.76 ppm. HRMS: Calculated for C₁₂H₁₀NO₅S⁺ = 328.08245 [M + H]⁺, found 328.08245.

5-Oxo-5-[3-(tosyloxy)azetidin-1-yl]pentanoate Acid (9b): Compound 8 (20.0 mmol, 5.58 g, 1.1 equiv.) was co-evaporated with dioxane, redissolved in MeCN (200 mL) and put under argon atmosphere. Glutaric anhydride (12.7 mmol, 1.45 g, 1 equiv.) was added to the reaction mixture, followed by potassium carbonate (31.8 mmol, 4.48 g, 2.5 equiv.) and the reaction mixture was refluxed for 6 h. Reaction progression was monitored by TLC, using a bromocresol green stain to visualize the produced carboxylic acid. The reaction mixture was diluted with water (200 mL) and Amberlite-H⁺ (IR120, ±50 g) was added until the pH fell below 3. The solution was filtered and the residual MeCN was removed in vacuo. The water layer was extracted twice with EtOAc. The organic layers were combined, dried with magnesium sulfate, filtered and concentrated in vacuo. For the crude product was purified by column chromatography (3 % → 5 % EtOAc in DCM), yielding compound 9b as a white crystalline substance (8.9 mmol, 3.06 g, 70 %). ¹H NMR (400 MHz, CDCl₃): δ = 7.81 (d, J = 8.3 Hz, 2 H), 7.40 (d, J = 8.1 Hz, 2 H), 5.08 (tt, J = 6.8, 4.2 Hz, 1 H), 4.45–4.33 (m, 1 H), 4.26–4.13 (m, 2 H), 3.93 (dd, J = 11.5, 4.3 Hz, 1 H), 2.49 (s, 3 H, 2.42 (s, 3 H, 2.17 (t, J = 7.3 Hz, 2 H), 1.92 (p, J = 7.2 Hz, 2 H) ppm. ¹³C NMR (101 MHz, CDCl₃): δ = 177.76, 172.54, 154.84, 132.58, 130.24, 127.93, 67.11, 57.23, 54.93, 32.93, 30.29, 21.78, 19.57 ppm. HRMS: Calculated for C₁₃H₁₂NO₅S⁺ = 342.10058 [M + H]⁺, found 342.10045.
left stirring for 1 hour. Subsequently the reaction mixture was charged with bis(p-nitrophenol)carbonate (9.8 mmol, 2.95 g, 1.1 equiv.) and left stirring for another 3 hours. The reaction mixture was diluted with EtOAc and washed twice with 10% aqueous sodium hydrogen carbonate, twice with water and once with brine. The combined organic layers were dried with magnesium sulfate, filtered and concentrated in vacuo. The crude product was purified by column chromatography (50% → 100% EtOAc in pentane), yielding compound 10b as a yellow crystalline substance (6.1 mmol, 1.78 g, 69%). 1H NMR (400 MHz, CDCl3): δ = 6.87 (d, J = 9.2 Hz, 2 H), 7.31 (d, J = 9.1 Hz, 2 H), 6.91 (s, 0.5 H), 6.71 (s, 0.5 H), 5.75 (d, J = 5.1 Hz, 1 H), 4.61 (s, 1 H), 4.48 (s, 1 H), 2.97 (t, J = 6.6 Hz, 2 H), 2.75 (t, J = 6.5 Hz, 1 H), 2.66 (t, J = 6.5 Hz, 1 H) ppm. 13C NMR (101 MHz, CDCl3): δ = 170.76, 165.26, 164.88, 155.48, 145.32, 137.43, 136.64, 125.22, 122.55, 114.19, 113.93, 77.16, 58.67, 56.91, 29.13, 29.03, 26.74, 25.76 ppm.

1-(5-Morpholino-5-oxopentanoyl)azetidin-3-yl 4-Methylbenzenesulfonate (2): A solution of compound 10b (0.68 mmol, 198 mg, 1 equiv.) in DCM (1.7 mL) was charged with morpholine (2.04 mmol, 0.18 mL, 3 equiv.) and left stirring for 2 hours. The reaction mixture was directly purified by column chromatography (50%/5% acetone/EtOH in DCM), yielding compound 2 (683 mg, 10.5 mmol, 1.05 equiv.) in water (2.5 mL). The ice bath was removed and the two-layered reaction mixture was stirring vigorously for 1 hour. After complete addition, the reaction mixture was sonicated until hydrogen evolution was complete. The reaction mixture was cooled to 0 °C and methyl iodide (2.52 mmol, 0.16 mL, 1.05 equiv.) was added. The reaction mixture was stirred for 1.5 h, quenched with Et3N-HCl and concentrated in vacuo. The crude product was purified by column chromatography (2% → 5% EtOH in DCM), yielding compound 4 as a yellow liquid. (1.81 mmol, 387 mg, 75%). 1H NMR (400 MHz, CDCl3): δ = 7.24–7.01 (m, 1 H), 6.47–6.24 (m, 1 H), 3.76–3.59 (m, 4 H), 3.01 (s, 2 H), 2.54–2.38 (m, 4 H) ppm. 13C NMR (101 MHz, CDCl3): δ = 134.20, 133.51, 91.95, 66.86, 63.42, 57.25, 53.80, 30.23 ppm. HRMS: Calculated for C17H19N2O4 215.13975 [M + H]+, found 215.13911.

Acknowledgments

This work was supported by an ECHO-grant from the Netherlands Organisation for Scientific Research (NWO) (711.011.015).

Keywords: Diels–Alder reaction • Tetrazine • Reaction rates • Cycloaddition • N-Acylanilines


Received: January 18, 2018