Alkaline Protease from *Bacillus subtilis* Catalyzed Michael Addition of Pyrimidine Derivatives to α,β-Ethylenic Compounds in Organic Media

Ying Cai, Xiao-Feng Sun, Na Wang, Xian-Fu Lin*

Department of Chemistry, Zhejiang University, Hangzhou, 310027, P. R. China
Fax +86(0571)87952618; E-mail: llc123@css.zju.edu.cn

Received 17 November 2003; revised 6 January 2004

**Abstract:** Michael addition reactions of pyrimidine derivatives to α,β-ethylenic compounds were catalyzed by an alkaline protease from *Bacillus subtilis* in DMSO at 50 ºC. The structure of adducts were determined by IR, NMR and MS. The yields were from 20% to 84%.

**Key words:** enzymes, nucleobases, Michael additions, catalysis, alkaline protease

In recent years, there has been a growing interest in the synthesis of bioactive compounds in the field of organic chemistry. Most nucleosides analogues or purine and pyrimidine derivatives have high anti-viral activity, e.g. adenine based 9-(S)-(3-hydroxy-2-phosphonylmethoxypropyl)adenine (HPMPA) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA), and guanine based penciclovir, acyclovir, famiciclovir and ganciclovir. Some nucleoside derivatives also can be used as reference samples for the evaluation of mutagenicity and/or carcinogenicity via GC-MS analysis. These compounds can be prepared primarily by alkylation or Michael addition reactions of nucleic bases to α,β-ethylenic compounds.

Michael addition has been used in the formation of CC, CS, CO and CN bonds over the years. Aza-Michael reaction has been used in synthesis of nucleoside analogues or derivatives as antiviral and anticancer agents in the past decade. This type of reaction normally requires a strong base or a Lewis acid to activate either the nucleophile or the acceptor. For example, K2CO3 catalyzed regiospecific Michael additions of various Michael acceptors with derivatives of purines and pyrimidines, Lewis acid catalyzed Michael additions of pyrimidine bases, and other catalyst such as t-BuOK and crown ethers have also been used in this type reaction.

The development of new synthetic strategies continues to attract attention, as there are drawbacks to many of the existing catalysts. Recently, new catalysts such as basic clay, montmorillonite and guanidinium have been used to accelerate Michael reaction. Even biomacromolecules such as RNA and antibody have been used to catalyze the addition reactions.

Compared with chemical catalysts, enzymes have a high catalytic activity and high selectivities under mild reaction conditions. These features have brought about an exponential increase in interest in the area of biotransformations. Much research on enzyme catalyzed reaction in non-aqueous media has been reported. Hydrolyses (mainly lipase and protease), as novel catalysts, can catalyze the synthesis of esters, polyesters, lactones, amides and peptides in a chemo-, regio- and enantioselective manner.

To our best knowledge, enzymatic Michael addition reactions have been only rarely reported. Kitazume and co-workers used baker’s yeast and hydrolytic enzymes to catalyze the Michael addition of fluorne-containing compounds such as 2-(trifluoromethy)propenoic acid with several nucleophiles in buffer solution (Na2HPO4 and KH2PO4 solution), and asymmetric fluorinated bioactive molecules were synthesized successfully.

**Scheme 1** Enzymatic Michael addition of uracil (1) and its derivatives 2–4 to acrylates

In this paper, we investigate the Michael addition reactions of six pyrimidine derivatives to acrylates (methyl acrylate, ethyl acrylate and n-butyl acrylate) catalyzed by an alkaline protease from *Bacillus subtilis* in DMSO at 50 ºC, and all adducts were N-1 alkylated (Scheme 1, Table 1).

The structures of adducts were determined by IR, 1H NMR, 13C NMR and ESI-MS. The results show that the Michael addition reactions were achieved. Results of 1H NMR showed that all adducts were N-1 alkylated.
Comparing the $^1$H NMR spectra with the data of N-1 adduct of uracil and thymine, showed that peaks corresponding to the active proton of N-3 at $\delta = 8.3$–$9.3$ were present at the same time as the peaks corresponding to the alkyl group. This result shows that alkaline protease from *Bacillus subtilis* has a regioselectivity for Michael addition reaction of uracil derivatives with acrylates. When the reactant had two kinds of NH (compounds 5a, 6a) (Figure 1), the $^1$H NMR of two adducts 5b, 6b of cytosine derivatives showed that peaks corresponding to 4-N-H remained unchanged. This result implies that the enzyme also produced regioselectivity in the Michael addition reaction of cytosine derivatives with acrylates.

![Figure 1](image)

Table 1 Michael Addition of Pyrimidines to Acrylates

<table>
<thead>
<tr>
<th>Entry</th>
<th>Base</th>
<th>Product</th>
<th>Reaction Time (h)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1a</td>
<td>12</td>
<td>58.7</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1b</td>
<td>12</td>
<td>47.0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1c</td>
<td>12</td>
<td>44.0</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>2b</td>
<td>24</td>
<td>84.0</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3b</td>
<td>24</td>
<td>76.4</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>4a</td>
<td>48</td>
<td>28.7</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>4b</td>
<td>48</td>
<td>22.0</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>4c</td>
<td>48</td>
<td>20.0</td>
</tr>
<tr>
<td>9</td>
<td>5</td>
<td>5b</td>
<td>24</td>
<td>44.1</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>6b</td>
<td>24</td>
<td>32.0</td>
</tr>
</tbody>
</table>

The enzyme source is one of the main factors influencing enzymatic Michael addition. When the acrylates were used as Michael acceptors, both alkaline protease from *Bacillus subtilis* and protease from *Aspergillus oryzae* (EC 3.4.24.39, 1.7 U/mg, Fluka Co.) can catalyze the reaction with pyrimidine derivatives. No product was obtained even after 5 days in the absence of enzyme. When the alkaline protease from *Bacillus subtilis* was used to catalyze the addition of uracil with methyl methacrylate, no adduct was obtained after 72 hours. Under the same conditions, protease from *Aspergillus oryzae* resulted in less than 5% yield after 72 hours.

We also investigated the effect of organic media on enzymatic Michael addition. Initial studies were undertaken using uracil and methyl acrylate as a model system. Eight organic media from polar to non-polar, e.g., DMSO, DMF, dioxane, acetone, THF, pyridine, chloroform and n-hexane were screened. The conversion of uracil was 58.7% after 12 hours in DMSO. In hydrophilic organic media like DMF and pyridine, Michael additions were achieved after 24 hours, but the conversion of uracil was lower than 10% by HPLC. The Michael addition was not effective in dioxane, acetone, THF, chloroform and n-hexane.

The structure of the Michael donor and acceptor also affected the results of the enzymatic Michael reaction. When thymine was used as a Michael donor, yields of the adducts decreased if the acrylate had a longer alcohol chain (Figure 2). When ethyl acrylate was used as the Michael acceptor, the main influence was caused by electron withdrawing groups, which can reduce the electron cloud density of N-1 in uracil derivatives. The steric effect of 5-substitution can also affect the result: a larger group caused a lower yield (Figure 3). If the uracil derivatives have a better electron-donating group and a smaller 5-substituent such as 5-fluorouracil and 5-bromouracil, the yield was high. In contrast, when the Michael donor, which had an electron-donating group and a larger 5-substituent group, such as thymine, was reacted with the acceptor which has a longer alcohol chain, such as n-butyl acrylate, the adduct was obtained in a lower yield. The detailed results are listed in Table 1.

![Figure 2](image)

In conclusion, the alkaline protease from *Bacillus subtilis* can catalyze the Michael addition reaction of pyrimidine derivatives with acrylates in organic media. Results of Michael additions were affected by the source of the enzyme, organic media, the structure of the Michael donor and acceptor. Michael additions of imidazole, purine, amine and other nitrogen nucleophiles to $\alpha,\beta$-ethylenic compounds catalyzed by hydrolase are in progress.
TLC (silica gel; MeOH-CH2Cl2-EtOAc, 1:9:10) was used to monitor the reaction. The position of addition was confirmed by 1H NMR (Bruker AMX-500 MHz) using CDCl3 as solvent and chemical shifts are expressed in ppm with reference to Me4Si. IR spectra were measured with a Nicolet Nexus FTIR 670 spectrophotometer. The conversion degrees during processes were analyzed by HPLC with a reversed-phase Shim-Pack VP-ODS column (150 × 4.6 mm) and a UV detector (265 nm). MeOH–H2O (40:60) was used as eluent at 1.0 mL min–1. Mass spectrometry data were obtained on a LCQ Advantage spectrometer (Thermo Finnigan, San Jose, CA) using electrospray ionization (ESI) with a reversed-phase Supelcosil LC-18 column (150 × 4.6 mm). ESI-MS: m/z = 213 (M + 1).

n-Butyl 3-(1-Uracil)propionate (1c)
Isolated yield: 43.3%; white crystals; mp 64–66 °C.
IR (KBr): 1736 (OC=O), 1677 cm–1.
1H NMR (500 MHz, CDCl3): δ = 0.92 (t, 3 H, J = 6.0, OCH3), 2.78 (t, 2 H, J = 5.9, NCH2), 3.97 (t, 2 H, J = 5.7, NCH3), 4.18 (q, 2 H, J = 7.1, OCH2), 7.27 (d, 1 H, J = 7.1, O=C=CH2), 7.96 (s, 1 H, O=CNH2=O).
ESI-MS: m/z = 241 (M + 1).

Ethyl 3-(1'-Fluouracil)propionate (2b)
Buf crystals; mp 143–150 °C (decomp).
IR (KBr): 1729 (OC=O), 1698, 1659 cm–1.
1H NMR (500 MHz, CDCl3): δ = 1.27 (t, 3 H, J = 7.1, CH3), 2.78 (t, 2 H, J = 5.8, O=C=CH2), 3.91 (q, 2 H, J = 6.0, O=CNCH=), 7.78 (s, 1 H, Br=C=CH2), 8.81 (s, 1 H, O=CNHC=O).

Ethyl 3-(1'-Bromouracil)propionate (3b)
Isolated yield: 68.5%; yellow crystals; mp 120–121 °C. IR (KBr): 1735 (OC=O), 1698, 1655 cm–1.
1H NMR (500 MHz, CDCl3): δ = 1.91 (s, 3 H, =C=CH2), 2.78 (t, 2 H, J = 5.8, O=C=CH2), 3.96 (t, 2 H, J = 6.0, NCH3), 4.23 (q, 2 H, J = 6.0, CH2=O), 7.20 (s, 1 H, O=CNHC=O).

Methyl 3-(1'-Thymine)propionate (4a)
Isolated yield: 29.1%; white crystals; mp 120–121 °C. IR (KBr): 1735 (OC=O), 1698, 1655 cm–1.
1H NMR (500 MHz, CDCl3): δ = 2.26 (s, 3 H, =C=CH2), 2.76 (t, 2 H, J = 6.0, O=C=CH2), 3.96 (t, 2 H, J = 6.0, NCH3), 7.20 (s, 1 H, O=CN=CH2), 8.87 (s, 1 H, O=CNHC=O).
ESI-MS: m/z = 213 (M + 1).

Ethyl 3-(1'-Thymine)propionate (4b)
Isolated yield: 24.8%; white crystals; mp 149–150 °C.
IR (KBr): 1707 (OC=O), 1692, 1655 cm–1.
1H NMR (500 MHz, CDCl3): δ = 1.26 (t, 3 H, J = 7.1, CH2=O), 1.91 (s, 3 H, =C=CH2), 2.76 (t, 2 H, J = 6.0, O=C=CH2), 3.96 (t, 2 H, J = 6.0, NCH3), 4.15 (q, 2 H, J = 7.1, OCH2), 7.20 (s, 1 H, O=CNHC=O).

Butyl 3-(1'-Thymine)propionate (4c)
White crystals; mp 85–86 °C.
IR (KBr): 1723 (OC=O), 1697, 1648 cm–1.
1H NMR (500 MHz, CDCl3): δ = 0.93 (t, 3 H, J = 7.4, CH2=CH2), 1.36 (m, 2 H, CH2=CH2), 1.60 (m, 2 H, O=C=CH2), 1.91 (s, 3 H, =C=CH2), 2.76 (t, 2 H, J = 6.0, O=C=CH2), 3.96 (t, 2 H, J = 6.0, NCH3), 4.10 (t, 3 H, J = 6.7, OCH2), 7.19 (s, 1 H, O=CN=CH2), 8.31 (s, 1 H, O=CNHC=O).
ESI-MS: m/z = 255 (M + 1).

Ethyl 3-(1'-fluocytosine)propionate (5b)
Isolated yield: 30.6%; white crystals; mp 186–195 °C (decomp).
IR (KBr): 1731 (OC=O), 1680, 1619, 1520 cm–1.
1H NMR (500 MHz, CDCl3): δ = 1.25 (t, 3 H, J = 7.1, CH3), 2.83 (t, 2 H, O=CH2), 3.97 (t, 2 H, J = 5.6, NCH2), 4.13 (q, 2 H, J = 7.1, OCH2), 7.54 (d, 1 H, J = 5.7, FC=CHN).

ESI-MS: m/z = 230 (M + 1).

Ethyl 3-(1'-N-Acetylcytosine)propionate (6b)
Isolated yield: 28.7%; white crystals; mp 130–131 °C.

IR (KBr): 3327 (NH), 1726 (OC=O), 1707, 1655, 1622 cm–1.

1H NMR (500 MHz, CDCl3): δ = 1.24 (t, 3 H, J = 7.1, CH3), 2.86 (t, 2 H, J = 5.8, O=CH2), 4.12 (m, 4 H, OCH2, NCH2), 7.36 (d, 1 H, J = 7.20, NCH=CH), 7.81 (d, 1 H, J = 7.28, NCH=CH), 9.34 (s, 1 H, NH).

13C NMR (500 MHz, CDCl3): δ = 14.37 (CH2), 25.15 (O=CH2), 32.57 (O=CH2), 47.52 (NCH2), 61.33 (OCH2), 96.59 (C-5), 150.73 (C-6), 154.90 (C-2), 163.18 (C-4), 171.01 (NHC=O), 171.68 (O=CO).

ESI-MS: m/z = 253 (M + 1).

References