INTRODUCTION

The N-hydroxysuccinimide (NHS, also known as HOSu) esters of carboxylic acids are the most widely used reactive esters for modifying amine-containing peptides and proteins.\(^1^4\) NHS esters are notorious for having a short, pH-dependent half-life in aqueous media.\(^5^7\) A fluorophenyl ester (e.g., 2,3,5,6-tetrafluorophenol) is a different type of reactive ester that is formed similarly to an NHS ester.\(^2^3\) The reactive ester formed with carboxylic acid and 2,3,5,6-tetrafluorophenol is known as the TFP ester, and it is reported by Hermanson\(^1\), Lockett, et al.\(^8\), and Wilbur\(^9\), to be more stable in slightly basic, aqueous media than NHS esters. See Figure 1 for a comparison of NHS and TFP esters.

Figure 1: Structures of the N-Hydroxysuccinimide (NHS) and 2,3,5,6-tetrafluorophenoxymethyl (TFP)

NHS Ester

TFP Ester

Table 1. Hydrolytic stability of TFP Ester compared to NHS Ester; shows the comparative stabilities.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Percent NHS Ester Remaining*</th>
<th>Percent Acid Formed from NHS ester</th>
<th>Percent TFP Ester Remaining</th>
<th>Percent Acid Formed From TFP Ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>87.6%</td>
<td>0.0%</td>
<td>100.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>60</td>
<td>81.3%</td>
<td>5.1%</td>
<td>100.0%</td>
<td>0.0%</td>
</tr>
<tr>
<td>120</td>
<td>70.6%</td>
<td>9.8%</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>180</td>
<td>58.4%</td>
<td>19.2%</td>
<td>97.6%</td>
<td>2.4%</td>
</tr>
<tr>
<td>240</td>
<td>49.5%</td>
<td>29.5%</td>
<td>96.3%</td>
<td>3.7%</td>
</tr>
<tr>
<td>480</td>
<td>22.9%</td>
<td>60.1%</td>
<td>87.4%</td>
<td>12.6%</td>
</tr>
<tr>
<td>1440</td>
<td>4.6%</td>
<td>44.1%</td>
<td>76.1%</td>
<td>23.9%</td>
</tr>
</tbody>
</table>

Figure 3: Stability of TFP Ester Compared to NHS Ester. The stability study was run as described in the text.

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Conjugation Reactions

BSA fraction V (Calbiochem) that had been purified on a Superdex 200 Hiloald 16/600 column (GE Healthcare) was conjugated to PN10284 or PN10553 in either TEAA buffer, pH 7.4, or 10 mM sodium phosphate, pH 7.2, containing 150 mM sodium chloride (phosphate buffer). The reaction progress was measured by removing aliquots at specified time intervals, quenching with TFA, and analyzing the progress of the reaction by (a) Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectroscopy (MALDI-TOF MS), and (b) SDS polyacrylamide gel electrophoresis (SDS-PAGE). The results of the conjugation experiments with phosphate buffer are shown in Figure 4.

Discussion and Conclusion

The results presented here demonstrate that TFP esters are much more stable in aqueous, slightly basic media than comparable NHS esters, and that TFP esters are more reactive to amines than comparable NHS esters. Both of these findings are of critical importance to bioconjugation. In addition, the results show that the TFP ester hydrolyzes cleanly in aqueous media to the corresponding acid, while NHS ester does not.

The NHS ester is currently the most commonly used reactive ester in bioconjugation. The results described here show that the NHS ester is not the most efficient or effective reactive ester that could be used. Typically, when conjugating a PEG or dPEG® crosslinker to a protein, the linker is used in huge excess relative to the protein to drive the reaction to completion. This is necessary because the NHS ester hydrolyzes rapidly in aqueous media, and because (as seen from these results) the NHS ester is not as efficient or effective at reacting with surface amines (e.g., lysine, arginine,) as the TFP ester. In stability studies, there was 16-fold more intact TFP ester than NHS ester at 24 hours, 76.1 % versus 4.6 %. In reactivity studies in phosphate buffer, at the same molar ratios, the TFP ester reacted with 70% more surface amine groups than the NHS ester 10.2 per BSA versus 6.0 per BSA). It is clear from the results presented that the TFP ester is superior to the NHS ester in terms of both stability and reactivity. We conclude that the TFP ester should become the active ester of choice for bioconjugation reactions in the future.

References

3. Han, S-Y. and Young-Ah, K. Tetrahedron (2004), 60, 2447-2467.