Purification of modified amino acids: a student perspective of the ACCQPrep® preparative HPLC system

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Chromatography Application Note AN129

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Abstract

This application note explores the purification of a modified threonine amino acid using the ACCQ*Prep* preparative HPLC system. The amino acid was successfully purified using the scout runs and 'Focus Gradient' function and will be used for future solid phase peptide synthesis steps.

Background

Human cell surfaces are decorated with a large variety of complex carbohydrate molecules, or glycans, which play important roles in the regulation of various biological processes. Glycans are frequently linked to proteins in a process called protein glycosylation. One form of human protein glycosylation involves the modification of serine and threonine residues with mannose, which is further extended into several different glycan structures of varying complexity and length. Despite growing interest in this area due to the glycans' involvement in both congenital muscular dystrophies and Lassa virus infections, they remain relatively poorly understood. In particular, there are important questions about their biosynthesis which need to be addressed if we are to fully understand the roles of the glycans in cell physiology and in human disease. In this project, we aim to chemically synthesise a series of glycopeptides, derived from a naturally occurring mannosylated glycoprotein, as chemical tools to study the activity and substrate specificity of a set of glycosyltransferases that are involved in the biosynthesis of these glycans. The approach to be taken involves the synthesis of peptides of different length modified with an azide at the site where glycosylation normally takes place, followed by click-chemistry mediated coupling to various synthetic alkyne-tagged oligosaccharide constructs.

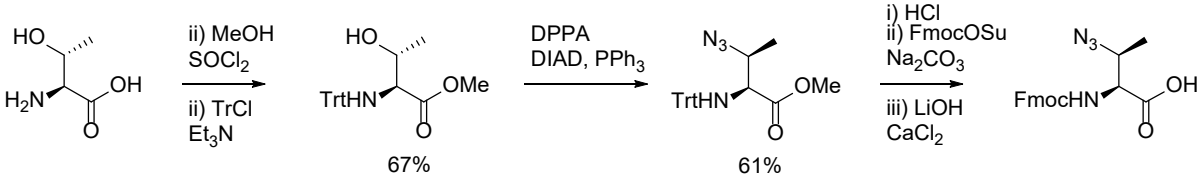
Experimental

At this time, only the synthesised azide-tagged amino acid was purified using the ACCQ*Prep* HPLC system (see Scheme 1).

The final stage was done on a small scale, due to problems deprotecting the carboxylic acid terminus, which was protected with a methyl ester. Solid phase peptide synthesis using this amino acid post-purification revealed that the amino acid was still protected with the methyl ester.

Method Development and Purification

A sample was prepared by dissolving 80.1mg of the crude product in 3mL 1:1 acetonitrile/water to use for the same solvent system on the Prep HPLC. (This gave a crude solution with 26.7mg/mL.) The initial scouting run used a 0.20mL (5.34mg) injection, with the ELSD detector and both UV wavelengths on at 220nm and 280nm. A 20 mm x 150 mm 5 µm C18 AQ column was used for both the scouting run and the 'Focus Gradient' runs.



Scheme 1: Synthesis scheme of azide-tagged threonine. Unfortunately, after analysis of SPPS proteins, we found that the last deprotection step was unsuccesful.

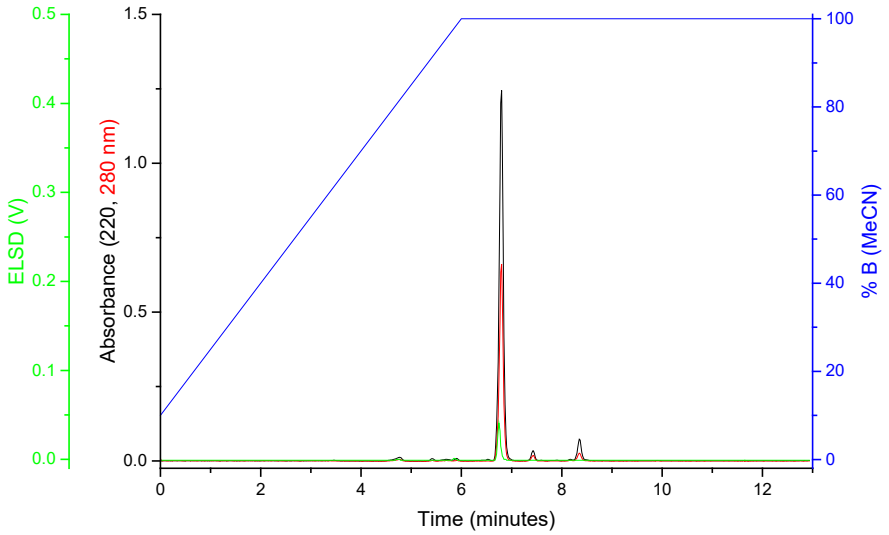


Figure 1: A scouting gradient run with C18 AQ column on the ACCQ*Prep* HPLC system. The focused gradients in Figures 2 and 3 were generated using this run.

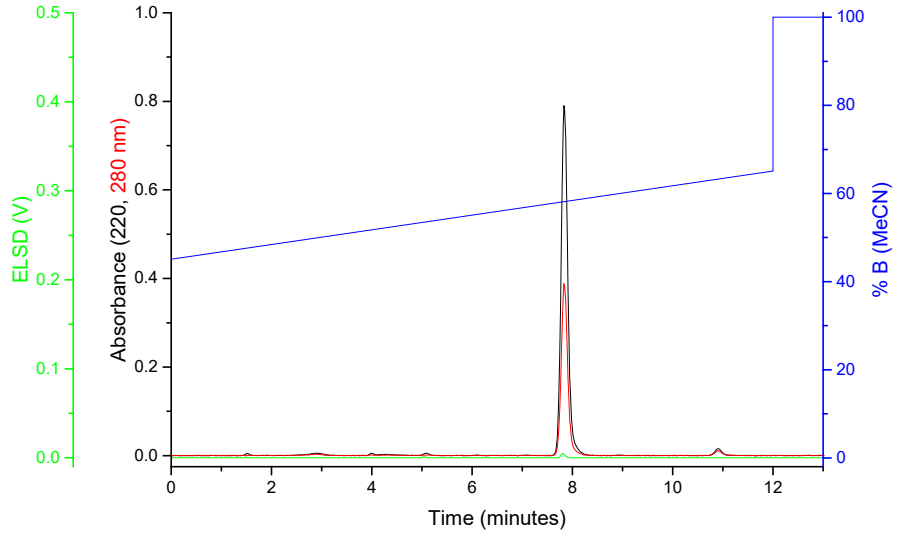


Figure 2: A focused gradient created from the scouting run in Figure 1 using a C18 AQ column.

The scouting run itself proved effective in separating the product from the impurities, and from this the 'Focus Gradient' function can be used, focusing on the peak that eluted at ~6.5mins. The same sample and injection volume (0.20mL/5.34mg) was used for these runs, and for subsequent runs to separate the rest of the crude sample. This gave a gradient from 45% to 65% acetonitrile over 12 minutes, then up to 100% acetonitrile for 5.3 minutes to wash out any remaining compound(s) on the column. The target compound eluted at 7.6 minutes as a narrow peak, with sharp UV absorptions due to the Fmoc-protecting group present on the amino acid. However, there is a lack of intensity in the ELSD signal; this could be rectified by using larger injection volumes or increasing the ELSD gain. The melting point of the compound is estimated to be close to 90 °C. This is significant, because melting point has been shown to affect ELSD detection.[1] Using a water -acetonitrile gradient, decreases in sensitivity were found for compounds with melting points lower than 90 °C.

1. Webster, G.K.; Jensen, J.S.; Diaz, A.R. An investigation into Detector Limitations Using Evaporative Light-Scattering Detectors for Pharmaceutical Applications. J. *Chromatogr. Sci.*, **42**, 2004, 485

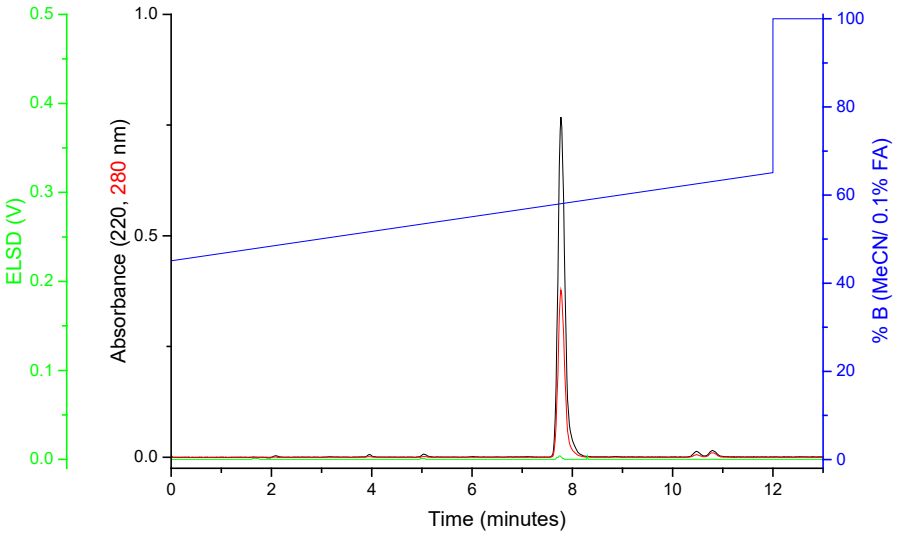


Figure 3: The same focused gradient and sample size, but ran with MeCN/H_2O doped with 0.1% formic acid. The second peak has split into two peaks, which suggests a better separation of the impurities.

The focused gradient run was also used with the same solvent system, with both acetonitrile and water doped with 0.1% formic acid. The chromatogram shows the same successful separation of the desired compound, but the second impurity peak at around 10.8 minutes seems to have been better separated, shown by the presence of a new peak. However, because of the small sample load the system did not register the two peaks to be recovered into test tubes. Again, with more sample injected, this issue could be rectified, and the impurities could be recovered for identification. The UV detector gain could also be increased, or the threshold lowered to collect these peaks. The use of the focused gradient generator limits the run time so that "collect-all" can be used to collect everything while still cutting the larger peaks.

Conclusion

Using the C18 AQ column to reverse-phase purify the amino acid proved successful, with the UV detector helping to identify the UV-active Fmoc-protecting group. The lack of intensity from the ELSD detector could be rectified by injecting more crude sample for purification, which would have saved time in purifying the whole sample.

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December 8, 2022