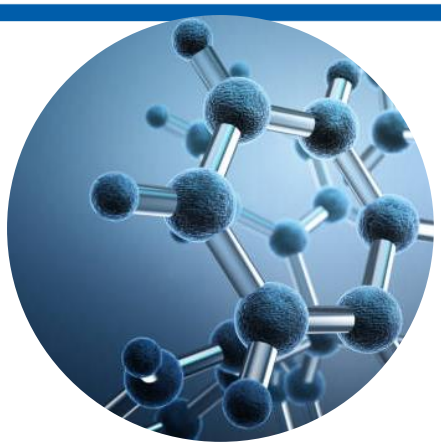

TN-1148

HPLC Enantioseparation of N-FMOC α-Amino Acids Using Lux™ Polysaccharide-Based Chiral Stationary Phases Under Reversed Phase Conditions

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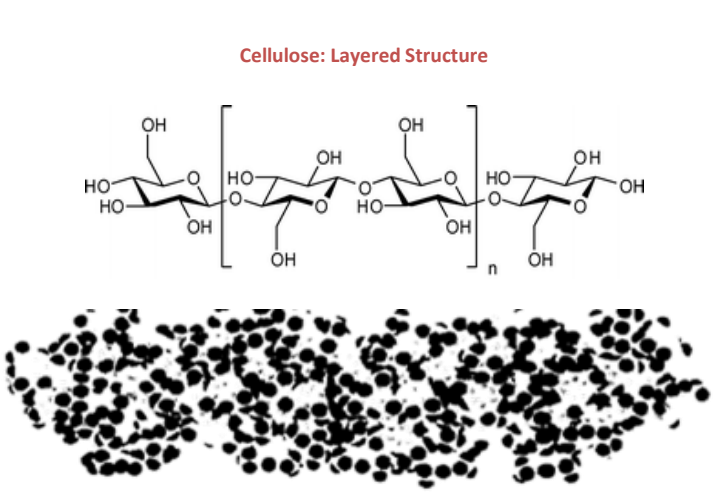
Introduction

N-Fluorenylmethoxycarbonyl (FMOC) α-amino acids are important building blocks for the solid phase synthesis of peptides. After the development of FMOC/tBu strategy for solid phase peptide syntheses, FMOC α-amino acids have become the raw materials of choice for the preparation of synthetic peptides. Using this methodology, long peptides (up to 100 residues) can be prepared in a few days with high yield from micro molar (g) up to molar scale (kg). As the number of amino acid residues increases, the final purity and overall yield of the peptide produced is directly affected by the chemical and chiral purity of the protected amino acids used. Currently, for the most common commercially available FMOC protected α-amino acids (19 natural amino acids), the expected enantiomeric purity is > 99.0 % enantiomeric excess (ee) for the L form and sometimes the purity required must be ≥99.8 % ee. This level of precision can only be achieved by very few analytical techniques, chiral HPLC being one of them. The main advantages of chiral HPLC analysis over other techniques are speed, detection level, and ease of use. HPLC is also used on a regular basis by peptide chemists to analyze purified fractions as well as peptide purity. In this technical application note, we report for the first time, the chiral separation of the most common commercially available FMOC protected α-amino acids under reversed phase conditions using polysaccharide-based chiral stationary phases (CSPs) depicted in **Figure 1**.

LC Conditions

Columns: Lux 5 µm Cellulose-1 (00G-4459-E0)
Lux 5 µm Cellulose-2 (00G-4457-E0)
Lux 5 µm Cellulose-3 (00G-4493-E0)
Lux 5 µm Cellulose-4 (00G-4491-E0)
Dimensions: 250 x 4.6 mm
Mobile Phase: See **Table 2**
Flow Rate: 1.0 mL/min (Isocratic)
Injection Volume: 5 µL
Temperature: Ambient
LC System: Agilent® 1100
Detection: UV @ 220 nm

Figure 1. Structures of Polysaccharide-Based CSPs.



Results and Discussion

Four different polysaccharide-based chiral stationary phases (CSPs) Lux™ Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, and Lux Cellulose-4, were explored in the reversed phase (RP) HPLC enantioseparation of the 19 most common FMOC protected α-amino acids.

Due to the acidic nature of FMOC amino acid derivatives and based on our previous extensive screening work in RP mode, it was decided to use Trifluoroacetic Acid or Formic Acid as acidic additives with Acetonitrile or Methanol as organic modifier. Those mobile phases are arguably the most used in RP mode. All the analysis were performed in isocratic mode with run time below 25 min.

Initial screening of the Lux CSPs was performed with 0.1 % Trifluoroacetic Acid / Acetonitrile in a volume ratio of 40:60. For retention time (Rt) < 6 min and resolution (Rs) < 1.5 (no baseline resolution), the amount of Acetonitrile was decreased to improve retention and chiral recognition. If no chiral separation was obtained with Acetonitrile as modifier, columns were screened with 0.1 % Formic Acid / Methanol in a volume ratio of 20:80. In general, we observed more retention with Trifluoroacetic Acid as an additive than with Formic Acid when using Acetonitrile as modifier and as expected Acetonitrile elution power was stronger than Methanol. Quite a few FMOC amino acids can be separated with either Acetonitrile or Methanol as modifier.

Table 1 summarizes all the separations and chiral recognition observed after performing RP screening using the protocol described above. As shown in **Table 1**, all the amino acids tested were successfully resolved on at least one of the four Lux polysaccharide-based CSPs. Under our RP screening protocol, Cellulose-2 was the most successful phase with 18 chiral recognitions followed by Cellulose-3 with 16 chiral recognitions as represented in **Figure 2**.

Table 2 describes some of the best separations, with conditions, observed for each FMOC amino acid screened. Retention time for both enantiomers, alpha value, resolution achieved, and order of elution are provided. All the separation reported are baseline resolved, and the run time is less than 25 min. Interestingly, Trityl (Trt) side chain-protected FMOC amino acids such as His, Asn, and Cys derivatives are more challenging to separate and baseline resolution is only achieved using Cellulose-2, Cellulose-3, and Cellulose-1, respectively. Selected chiral separation of FMOC-Asp(OtBu)-OH and FMOC-Tyr(tBu)-OH are shown in **Figure 3**.

Table 1. Chiral Recognition of the 19 Most Common FMOC Protected α-Amin Acids.

Baseline Resolution | Chiral Separation | No Resolution

FMOCC-AA-OH	Cellulose-1	Cellulose-2	Cellulose-3	Cellulose-4
FMOC-Ala-OH				
FMOC-Arg(Pbf)-OH				
FMOC-Asn(Trt)-OH				
FMOC-Asp(OtBu)-OH				
FMOC-Cys-(Trt)-OH				
FMOC-Gln(Trt)-OH				
FMOC-Glu(OtBu)-OH				
FMOC-His(Trt)-OH				
FMOC-Ile-OH				
FMOC-Leu-OH				
FMOC-Lys-(Boc)-OH				
FMOC-Met-OH				
FMOC-Phe-OH				
FMOC-Pro-OH				
FMOC-Ser(tBu)-OH				
FMOC-Thr(tBu)-OH				
FMOC-Trp(Boc)-OH				
FMOC-Tyr(tBu)-OH				
FMOC-Val-OH				

Figure 2. Enantioselectivity Comparison Between Polysaccharide-Based CSPs.

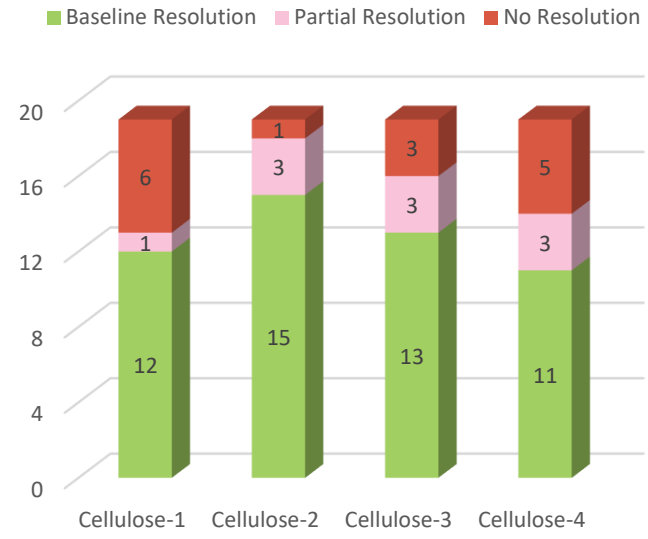
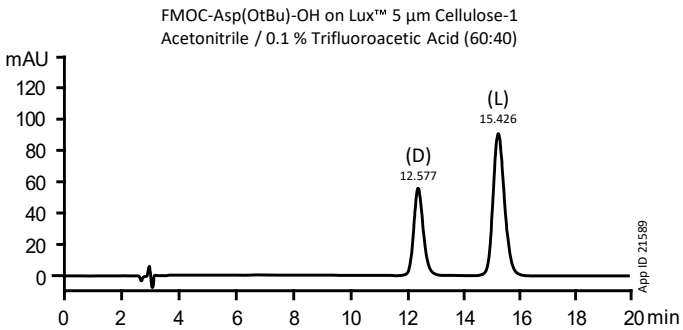


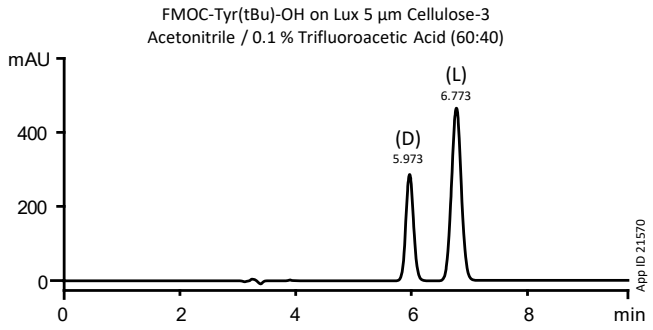
Table 2. Optimal Reversed Phase HPLC Enantioseparation of the 19 Most Common FMOC Protected α-Amino Acids.

FMOC-AA-OH	CSP	Mobile Phase	Rt_1[a]	Rt_2[a]	Alpha	Rs	App ID
FMOC-Ala-OH	Cellulose-3	Methanol / 0.1 % Trifluoroacetic Acid (80:20)	7.165	9.551	1.55	5.63	21550
FMOC-Arg(Pbf)-OH	Cellulose-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (70:30)	8.547	9.991	1.24	2.71	21580
FMOC-Asn(Trt)-OH	Cellulose-2	Acetonitrile / 0.1 % Trifluoroacetic Acid (55:45)	20.825	23.124	1.10	1.60	21873
FMOC-Asp(OtBu)-OH	Cellulose-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	12.577	15.426	1.28	4.18	21589
FMOC-Cys-(Trt)-OH	Cellulose-4	Methanol / 0.1 Trifluoroacetic Acid (90:10)	9.969	11.375	1.20	1.79	21641
FMOC-Gln(Trt)-OH	Cellulose-4	Acetonitrile / 0.1 % Trifluoroacetic Acid (70:30)	7.184	8.866	1.39	4.47	21631
FMOC-Glu(OtBu)-OH	Celloluse-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	13.979	16.652	1.23	3.55	21590
FMOC-His(Trt)-OH	Cellulose-1	Acetonitrile / 0.1 % Formic Acid (60:40)	4.865	5.783	1.39	2.33	21582
FMOC-Ile-OH	Cellulose-3	Acetonitrile / 0.1 % Trifluoroacetic Acid (40:60)	12.220	13.640	1.15	2.86	21553
FMOC-Leu-OH	Cellulose-3	Methanol / 0.1 % Trifluoroacetic Acid (90:10)	4.560	5.654	1.64	3.60	21647
FMOC-Lys-(Boc)-OH	Cellulose-3	Acetonitrile / 0.1 % Trifluoroacetic Acid (50:50)	5.615	6.520	1.33	3.59	21546
FMOC-Met-OH	Cellulose-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	11.423	13.064	1.18	2.96	21559
FMOC-Phe-OH	Cellulose-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	18.965	21.963	1.18	2.80	21585
FMOC-Pro-OH	Cellulose-4	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	5.865	6.818	1.32	3.31	21643
FMOC-Ser(tBu)-OH	Cellulose-3	Acetonitrile / 0.1 % Trifluoroacetic Acid (40:60)	8.654	9.599	1.16	2.87	21549
FMOC-Thr(tBu)-OH	Cellulose-4	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	7.690	8.920	1.26	3.78	21629
FMOC-Trp(Boc)-OH	Cellulose-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (80:20)	8.179	9.576	1.25	3.28	21586
FMOC-Tyr(tBu)-OH	Cellulose-3	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	5.973	6.773	1.26	2.89	21570
FMOC-Val-OH	Cellulose-1	Acetonitrile / 0.1 % Trifluoroacetic Acid (60:40)	11.669	15.052	1.37	3.90	21579

[a]Highlighted in blue is the retention time for the D enantiomer.

Figure 3. RP HPLC Enantioseparations of FMOC-Asp(OtBu)-OH and FMOC-Tyr(tBu)-OH.





Conclusions

Four different polysaccharide-based chiral stationary phases were explored in reversed phase HPLC for the separation of the 19 most common FMOC protected α-amino acids. Under our RP screening protocol, Lux™ Cellulose-2 was the most successful phase with 18 chiral recognitions (15 baseline resolved) followed by Lux Cellulose-3 with 16 chiral recognitions (13 baseline resolved).

All FMOC amino acids evaluated were fully resolved (Rs>1.5) in less than 25 min analysis time by RP separation mode. Trifluoroacetic Acid as acidic additive and Acetonitrile as organic modifier are the best choice combination for successful chiral separation of FMOC α-amino acids derivatives.

Based on this study, we feel confident that with a proper screening protocol most of the FMOC protected amino acids can be resolved with the four polysaccharide-based chiral stationary phase used in this study.

Lux Ordering Information

5 µm Minibore and Analytical Columns (mm)						SecurityGuard™ Cartridges (mm)	
Phases	**50 x 2.0**	**50 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**4 x 2.0***	**4 x 3.0***
						/10pk	/10pk
i-Amylose-1	00B-4762-B0	00B-4762-E0	00D-4762-E0	00F-4762-E0	00G-4762-E0	AJ0-8640	AJ0-8641
i-Amylose-3	—	00B-4779-E0	00D-4779-E0	00F-4779-E0	00G-4779-E0	AJ0-8651	AJ0-8650
i-Cellulose-5	—	00B-4756-E0	00D-4756-E0	00F-4756-E0	00G-4756-E0	AJ0-8631	AJ0-8632
Cellulose-1	—	00B-4459-E0	00D-4459-E0	00F-4459-E0	00G-4459-E0	AJ0-8402	AJ0-8403
Cellulose-2	00B-4457-B0	00B-4457-E0	00D-4457-E0	00F-4457-E0	00G-4457-E0	AJ0-8398	AJ0-8366
Cellulose-3	—	00B-4493-E0	00D-4493-E0	00F-4493-E0	00G-4493-E0	AJ0-8621	AJ0-8622
Cellulose-4	—	—	00D-4491-E0	00F-4491-E0	00G-4491-E0	AJ0-8626	AJ0-8627
Amylose-1	00B-4732-B0	—	00D-4732-E0	00F-4732-E0	00G-4732-E0	AJ0-9337	AJ0-9336
					for ID:	2.0-3.0 mm	3.2-8.0 mm

*SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

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