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

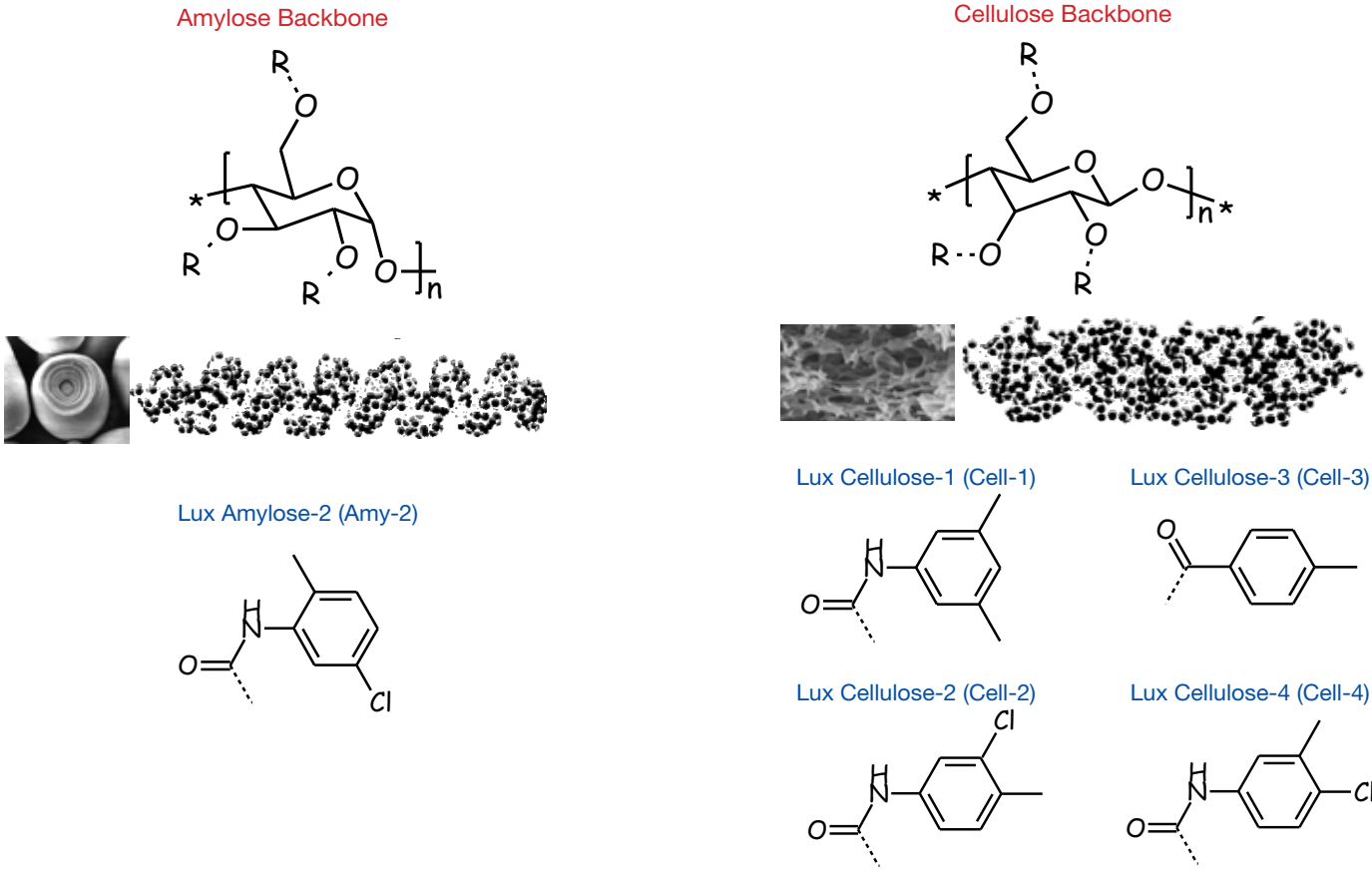
Marc Jacob, Michael Klein, Liming Peng, and Tivadar Farkas
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA

In this technical note, we report the chiral separation of the most common 19 FMOC protected α-amino acids derivatives under reversed phase separation mode using Lux polysaccharide-based chiral stationary phases. All FMOC α-amino acids analyzed in this study are baseline resolved with an analysis time below 25 min in isocratic conditions. The order of elution as well as the enantiomer identification are also reported.

Introduction

N-Fluorenylmethoxycarbonyl (FMOC) α-amino acids are important building blocks for the solid phase synthesis of peptides.[1] After the development of FMOC/tBu strategy[2] 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 acids 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 the peptide chemists to analyze purified fractions as well as peptide purity. In this application, 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**.[3]

Figure 1.
Structures of Polysaccharide-Based CSPs



Materials and Methods

All analyses were performed using an Agilent® 1100 series LC system (Agilent Technologies, Inc., Palo Alto, CA, USA) equipped with quaternary pump, in-line degasser, multi-wavelength UV detector, and autosampler. Lux® columns used for analysis were obtained from Phenomenex (Torrance, CA, USA). The HPLC column dimensions were 250 x 4.6 mm ID and all columns were packed with 5 µm particles. FMOC protected L and D amino acids used in this study were provided by Bachem® (Bubendorf, Switzerland). All solvents were purchased from EMD (San Diego, CA, USA).

HPLC Conditions:

Columns:	Lux 5 µm Cellulose-1	250 x 4.6 mm	00G-4459-E0
	Lux 5 µm Cellulose-2	250 x 4.6 mm	00G-4457-E0
	Lux 5 µm Cellulose-3	250 x 4.6 mm	00G-4493-E0
	Lux 5 µm Cellulose-4	250 x 4.6 mm	00G-4491-E0
	Lux 5 µm Amylose-2	250 x 4.6 mm	00G-4472-E0
Flow Rate:	1 mL/min		
Temperature:	Ambient		
Detection:	UV @ 220 nm		
Injection Volume:	5 µL		
Sample concentration:	2 mg/mL in Methanol (MeOH) or Acetonitrile (ACN) (pure FMOC amino acids enantiomer L and D were mixed in a ratio of 2:1 (L:D))		

Results and Discussion

Five different polysaccharide-based chiral stationary phases (CSPs) Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, Lux Cellulose-4, and Lux Amylose-2 (**Figure 1**) 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,[4] it was decided to use trifluoroacetic acid (TFA) or formic acid (FA) as acidic additives with acetonitrile (ACN) or methanol (MeOH) as organic modifier (see experimental conditions). 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 % TFA/ACN in a volume ratio of 40:60. For retention time (Rt) < 6 min and resolution (Rs) < 1.5 (no baseline resolution), the amount of ACN was decreased in order to improve retention and chiral recognition. If no chiral separation was obtained with ACN as modifier, columns were screened with 0.1 % FA/MeOH in a volume ratio of 20:80. In general, we observed more retention with TFA as an additive than with FA when using ACN as modifier and as expected ACN elution power is stronger than MeOH. Quite a few FMOC amino acids can be separated with either ACN or MeOH 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 five Lux polysaccharide-based CSPs. In the case of Ile, Leu, Met, Phe, and Val FMOC derivatives, baseline resolution was achieved on the five CSPs.

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

Baseline resolution | Chiral separation | No resolution

FMOC-AA-OH	Cell-1	Cell-2	Cell-3	Cell-4	Amy-2
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					

Under our RP screening protocol, Cellulose-2 was the most successful phase with 18 chiral recognitions followed by Cellulose-3 as represented in **Figure 2**.

Figure 2.
Enantioselectivity Comparison Between Polysaccharide-Based CSPs

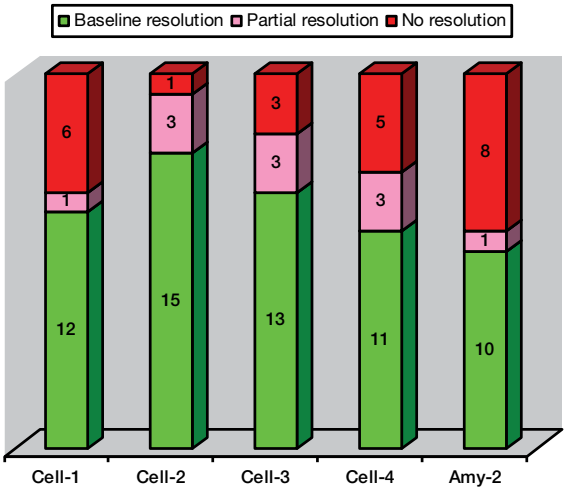


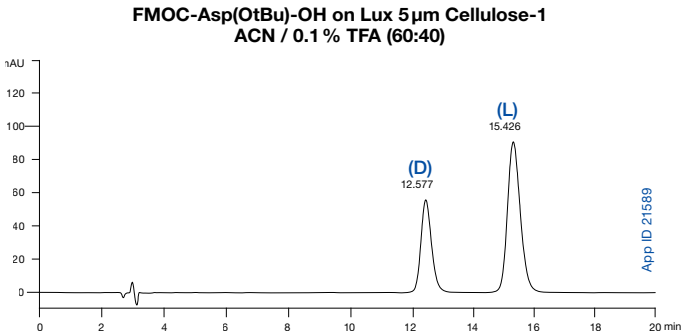
Table 2 describes some of the best separation observed for each FMOC amino acid screened. Retention time for both enantiomers, alpha value, resolution achieved, and order of elution are provided. All the separation reported are baseline resolved and the run time is less than 25 min. Interestingly, Trityl (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**.

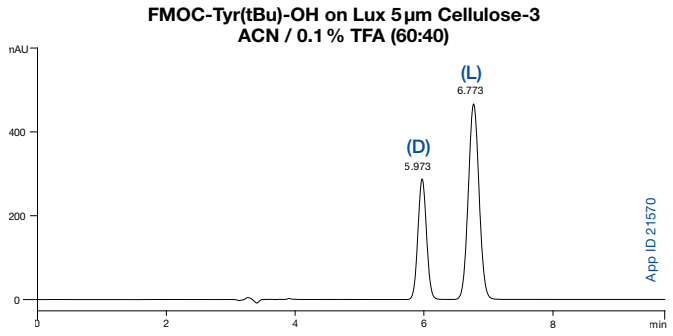
Table 2.
Optimal Reversed Phase HPLC Enantioseparation of the 19 Most Common FMOC Protected α-Amino Acids

FMOC-AA-OH	CSP	Mobile Phase	Rt_1 [a]	Rt_2 [a]	Alpha	Rs	App ID [b]
FMOC-Ala-OH	Cell-3	MeOH / 0.1 % TFA (80:20)	7.165	9.551	1.55	5.63	21550
FMOC-Arg(Pbf)-OH	Cell-1	ACN / 0.1 % TFA (70:30)	8.547	9.991	1.24	2.71	21580
FMOC-Asn(Trt)-OH	Cell-2	ACN / 0.1 % TFA (55:45)	20.825	23.124	1.10	1.60	21873
FMOC-Asp(OtBu)-OH	Cell-1	ACN / 0.1 % TFA (60:40)	12.577	15.426	1.28	4.18	21589
FMOC-Cys-(Trt)-OH	Cell-4	MeOH / 0.1 % TFA (90:10)	9.969	11.375	1.20	1.79	21641
FMOC-Gln(Trt)-OH	Cell-4	ACN / 0.1 % TFA (70:30)	7.184	8.866	1.39	4.47	21631
FMOC-Glu(OtBu)-OH	Cell-1	ACN / 0.1 % TFA (60:40)	13.979	16.652	1.23	3.55	21590
FMOC-His(Trt)-OH	Cell-1	ACN/ 0.1 % FA (60:40)	4.865	5.783	1.39	2.33	21582
FMOC-Ile-OH	Cell-3	ACN / 0.1 % TFA (40:60)	12.22	13.64	1.15	2.86	21553
FMOC-Leu-OH	Cell-3	MeOH / 0.1 % TFA (90:10)	4.56	5.654	1.64	3.60	21647
FMOC-Lys-(Boc)-OH	Cell-3	ACN / 0.1 % TFA (50:50)	5.615	6.52	1.33	3.59	21546
FMOC-Met-OH	Cell-1	ACN / 0.1 % TFA (60:40)	11.423	13.064	1.18	2.96	21559
FMOC-Phe-OH	Cell-1	ACN / 0.1 % TFA (60:40)	18.965	21.963	1.18	2.80	21585
FMOC-Pro-OH	Cell-4	ACN / 0.1 % TFA (60:40)	5.865	6.818	1.32	3.31	21643
FMOC-Ser(tBu)-OH	Cell-3	ACN / 0.1 % TFA (40:60)	8.654	9.599	1.16	2.87	21549
FMOC-Thr(tBu)-OH	Cell-4	ACN / 0.1 % TFA (60:40)	7.69	8.92	1.26	3.78	21629
FMOC-Trp(Boc)-OH	Cell-1	ACN / 0.1 % TFA (80:20)	8.179	9.576	1.25	3.28	21586
FMOC-Tyr(tBu)-OH	Cell-3	ACN / 0.1 % TFA (60:40)	5.973	6.773	1.26	2.89	21570
FMOC-Val-OH	Cell-1	ACN / 0.1 % TFA (60:40)	11.669	15.052	1.37	3.90	21579

[a] Highlighted in blue is the retention time for the D enantiomer
[b] To view the full application enter the App ID into the search field on our website at www.phenomenex.com/ChiralAppSearch

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





Conclusion

Five 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.

All FMOC amino acids evaluated were fully resolved (Rs>1.5) in less than 25 min analysis time by RP separation mode. TFA 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 five polysaccharide-based chiral stationary phase used in this study.

APPLICATIONS

Lux® Ordering Information

3 µm Analytical Columns (mm)							SecurityGuard™ Cartridges (mm)	
Phases	50 x 2.0	150 x 2.0	50 x 4.6	100 x 4.6	150 x 4.6	250 x 4.6	4 x 2.0* /10pk	4 x 3.0* /10pk
Cellulose-1	00B-4458-B0	00F-4458-B0	00B-4458-E0	00D-4458-E0	00F-4458-E0	00G-4458-E0	AJ0-8402	AJ0-8403
Cellulose-2	00B-4456-B0	00F-4456-B0	00B-4456-E0	00D-4456-E0	00F-4456-E0	00G-4456-E0	AJ0-8398	AJ0-8366
Cellulose-3	00B-4492-B0	00F-4492-B0	00B-4492-E0	00D-4492-E0	00F-4492-E0	00G-4492-E0	AJ0-8621	AJ0-8622
Cellulose-4	00B-4490-B0	00F-4490-B0	00B-4490-E0	00D-4490-E0	00F-4490-E0	00G-4490-E0	AJ0-8626	AJ0-8627
Amylose-2	00B-4471-B0	00F-4471-B0	00B-4471-E0	00D-4471-E0	00F-4471-E0	00G-4471-E0	AJ0-8471	AJ0-8470
						for ID:	2.0–3.0 mm	3.2–8.0 mm

5 µm 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* /10pk	4 x 3.0* /10pk
Cellulose-1	00B-4459-B0	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-B0	00B-4493-E0	00D-4493-E0	00F-4493-E0	00G-4493-E0	AJ0-8621	AJ0-8622
Cellulose-4	00B-4491-B0	00B-4491-E0	00D-4491-E0	00F-4491-E0	00G-4491-E0	AJ0-8626	AJ0-8627
Amylose-2	00B-4472-B0	00B-4472-E0	00D-4472-E0	00F-4472-E0	00G-4472-E0	AJ0-8471	AJ0-8470
					for ID:	2.0–3.0 mm	3.2–8.0 mm

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

For scale-up preparative and bulk part numbers please visit www.phenomenex.com

References

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Acknowledgements

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guarantee

If Lux analytical columns (≤ 4.6 mm ID) do not provide at least an equivalent or better separation as compared to a competing column of the same particle size, similar phase and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

Australia
t: 02-9428-6444
f: 02-9428-6445
auinfo@phenomenex.com

Austria
t: 01-319-1301
f: 01-319-1300
anfrage@phenomenex.com

Belgium
t: +31 (0)30-2418700
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: (800) 543-3681
f: (310) 328-7768
info@phenomenex.com

Denmark
t: 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: 09 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: 01 30 09 21 10
f: 01 30 09 21 11
franceinfo@phenomenex.com

Germany
t: 06021-58830-0
f: 06021-58830-11
anfrage@phenomenex.com

India
t: 040-3012 2400
f: 040-3012 2411
indiainfo@phenomenex.com

Ireland
t: 01 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: 051 6327511
f: 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 001-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: 030-2418700
f: 030-2383749
nlinfo@phenomenex.com

New Zealand
t: 09-4780951
f: 09-4780952
nzinfo@phenomenex.com

Norway
t: 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: (800) 541-HPLC
f: (310) 328-7768
info@phenomenex.com

Sweden
t: 08 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: 01625-501367
f: 01625-501796
ukinfo@phenomenex.com

All other countries: Corporate Office USA
t: (310) 212-0555
f: (310) 328-7768
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