Application of 2D-Liquid Chromatography for the Separation of a Mixture of Isomeric and Structurally Related Azatryptophan Derivatives

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CRediT author statement

Parinita Shaw: data collection, visualization, investigation, and writing experiments; Pandidurai Sekar: data collection; Balasaheb Chavan: data investigation, validation, and manuscript drafting; Sharad Duche: conceptualization, methodology, validation, supervision, reviewing, and editing; Amrita Roy: supervision, draft reviewing, and editing; Arvind Mathur: supervision, reviewing, and funding acquisition.

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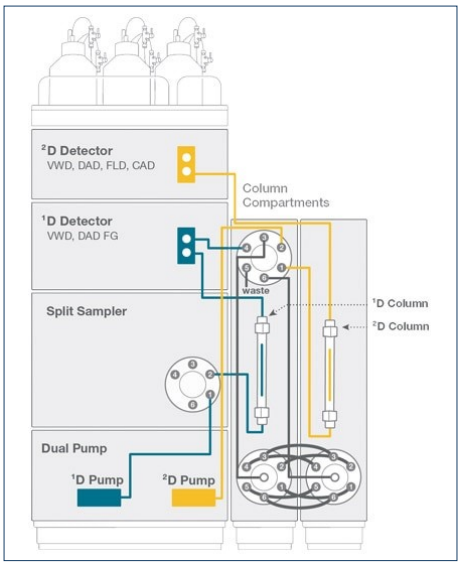

Figure S1. Schematic diagram of the Thermo Scientific Vanquish Online 2D-LC system.

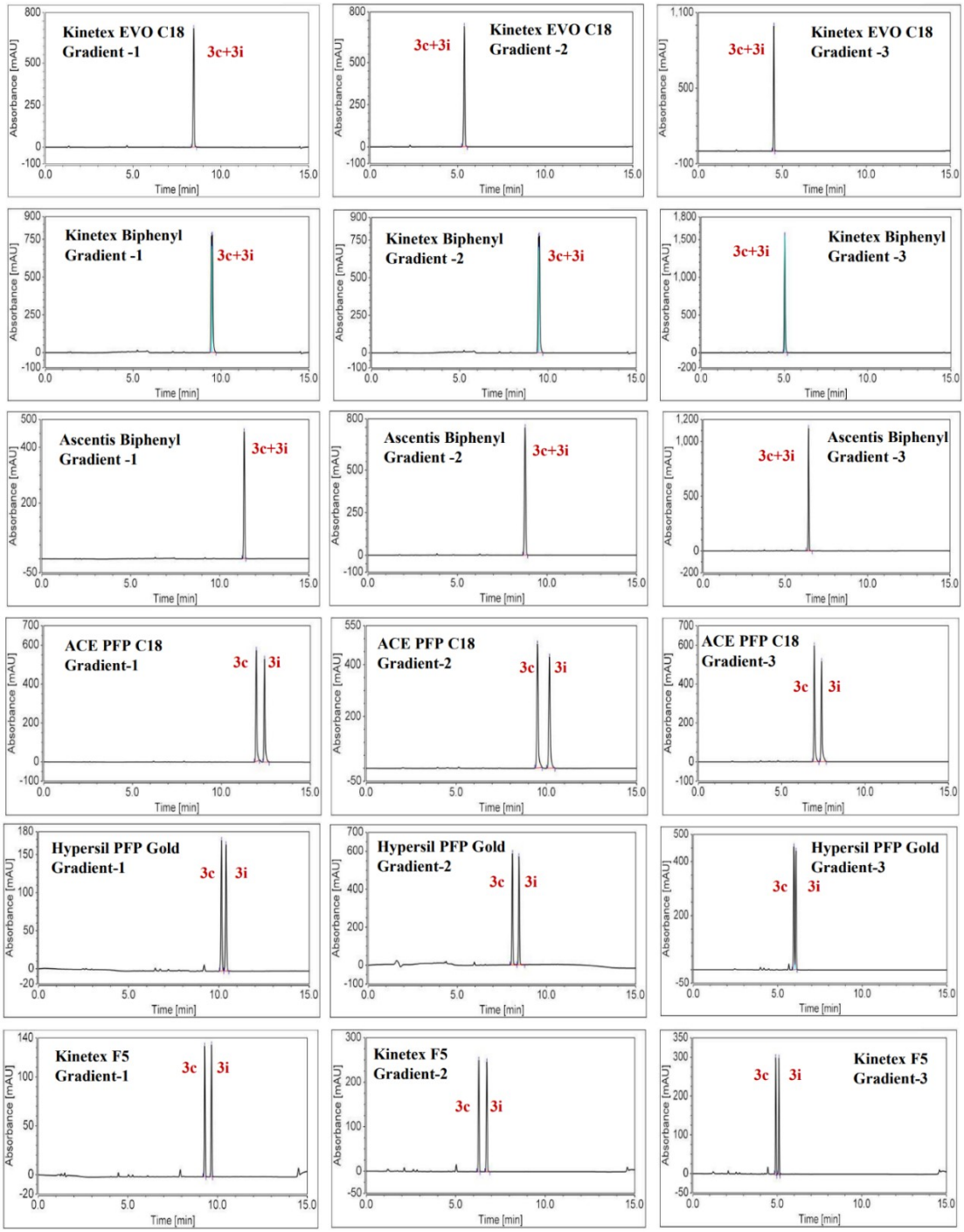

Figure S2: Chromatograms of separation of racemate 3c and 3i with mobile phase A as 0.05% TFA in water: ACN (95:5, v/v) and mobile phase B as water: ACN (5:95, v/v) with different stationary phases.

The chromatogram in above figure shows that, racemate 3c and 3i were coeluted with C18 and Ascentis biphenyl stationary phases, whereas split peak was observed with the Kinetex biphenyl column. The separation was observed only with the PFP stationary phases. The racemates were well separated using ACE PFP C18 column whereas slightly broad peak shapes were obtained with the Hypersil PFP Gold column. Best separation and peak shapes were observed with the Kinetex F5 column. (peak asymmetry is 0.98 and 0.99 for 3c and 3i, respectively).

Table S1: Chromatograms of separation of racemate 3c and 3i with mobile phase A as 0.05% TFA in water: ACN (95:5, v/v) and mobile phase B as water: ACN (5:95, v/v) with different stationary phases.

Column used in first dimension	Gradient	Peak Name	Retention time (min)	Peak asymmetry	Resolution	Remarks
Kinetex EVO C18 (100x4.6mm), 2.6µm	Gradient - 1	3c	8.46	0.92	No	No separation
		3i				
	Gradient – 2	3c	5.38	0.92	No	
		3i				
	Gradient - 3	3c	4.50	0.93	No	
		3i				
Kinetex BPH (100x4.6mm), 2.6µm	Gradient - 1	3c	9.47	NA	No	Split peak
		3i	9.51			
	Gradient – 2	3c	6.49	NA	No	
		3i	6.55			
	Gradient - 3	3c	5.03	NA	No	
		3i	5.04			
Ascentis® Express Biphenyl (100x2.1mm), 2.7µm	Gradient - 1	3c	11.37	1.07	No	No separation
		3i				
	Gradient – 2	3c	8.78	1.00	No	
		3i				
	Gradient - 3	3c	6.41	1.00	No	
		3i				
ACE PFP C18 (150x4.6mm), 3µm	Gradient - 1	3c	11.87	0.78	2.5	Peaks were well separated
		3i	12.32	0.84		
	Gradient – 2	3c	7.14	0.93	2.2	
		3i	7.41	0.95		
	Gradient - 3	3c	6.88	0.85	2.5	
		3i	5.10	1.07		
Thermo Hypersil gold PFP (150x4.6mm), 1.9µm)	Gradient - 1	3c	18.59	0.96	3.3	Peaks were well separated but peak shape is broad
		3i	19.05	0.95		
	Gradient - 2	3c	14.38	1.01	3.5	
		3i	15.03	1.04		
	Gradient - 3	3c	9.58	0.97	2.9	
		3i	10.05	0.96		
Kinetex F5 (150x4.6mm), 2.6µm	Gradient - 1	3c	9.28	1.04	4.4	Peaks were well separated and best resolution and peak shape within all column's trials
		3i	9.67	1.01		
	Gradient – 2	3c	6.29	0.98	4.8	
		3i	6.74	0.99		
	Gradient - 3	3c	4.91	1.08	2.6	
		3i	5.10	1.07		

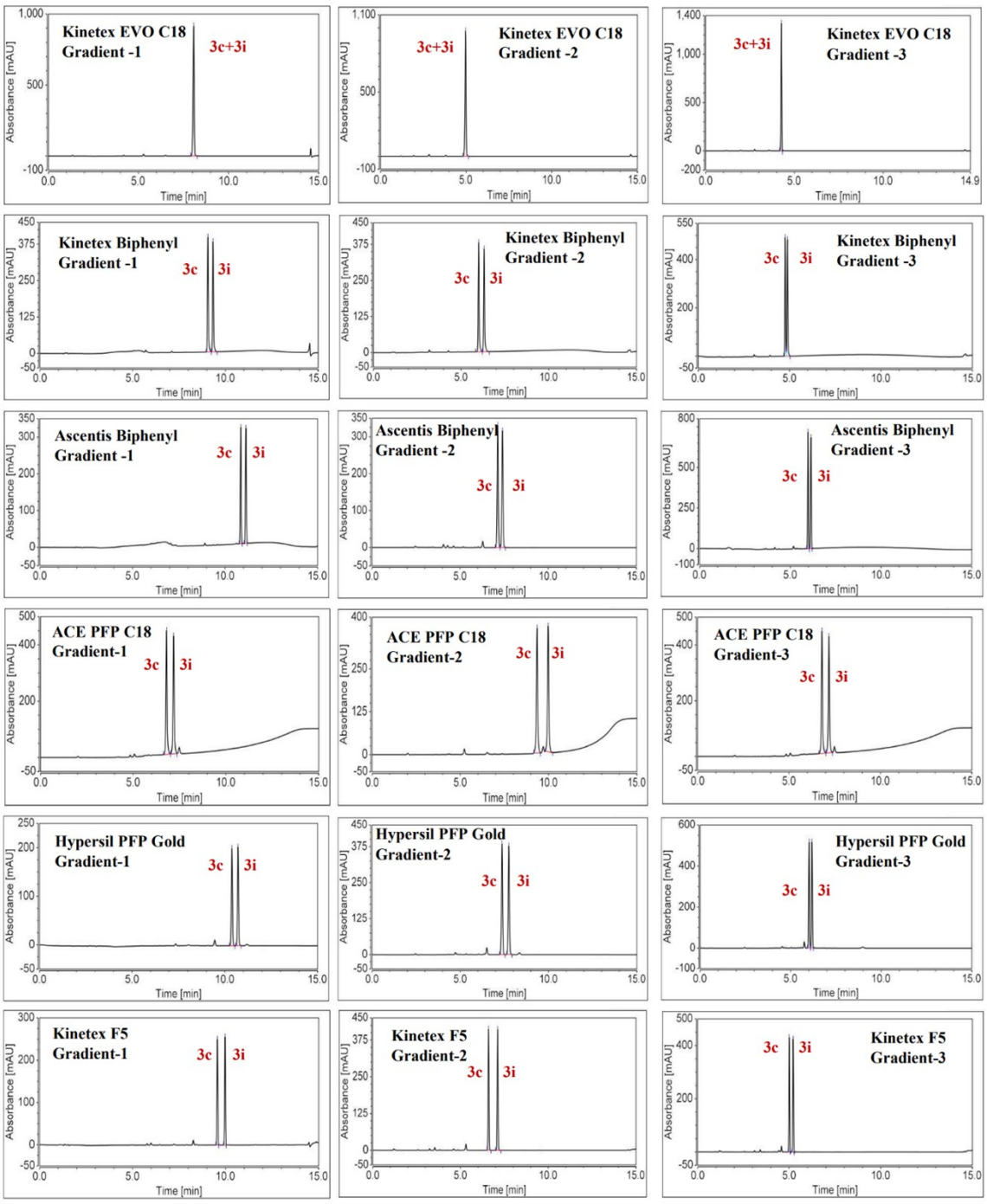

Figure S3: Chromatograms of separation of racemate 3c and 3i with mobile phase A as 10 mM ammonium acetate in water (pH 4.5) and mobile phase B as 100% ACN with different stationary phases.

The chromatogram in above figure shows that, racemates 3c and 3i were not separated using the C18 stationary phase and less separation was observed with Kinetex biphenyl as well as Ascentis biphenyl stationary phases. The racemates were found to be well separated with all PFP columns whereas Kinetex F5 column gives a maximum resolution of 5.5 with good peak shape.

Table S2: Chromatograms of separation of racemate 3c and 3i with mobile phase A as 10 mM ammonium acetate in water (pH 4.5) and mobile phase B as 100% ACN with different stationary phases.

Column-ID	Gradient	Peak Name	Retention time (min)	Peak asymmetry	Resolution	Remarks
Kinetex EVO C18 (100x4.6mm), 2.6µm	Gradient - 1	3c	8.61	0.86	No	No separation between 3c and 3i
		3i				
	Gradient – 2	3c	4.97	0.90	No	
		3i				
	Gradient - 3	3c	4.27	0.93	No	
		3i				
Kinetex BPH (100x4.6mm), 2.6µm	Gradient - 1	3c	9.07	1.14	3.0	Separation observed between 3c and 3i
		3i	9.34	1.18		
	Gradient – 2	3c	6.00	1.20	3.3	
		3i	6.32	1.16		
	Gradient - 3	3c	4.76	1.13	1.7	
		3i	4.88	1.00		
Ascentis® Express Biphenyl (100x2.1mm), 2.7µm	Gradient - 1	3c	10.85	0.97	3.2	Separation observed between 3c and 3i
		3i	11.13	0.98		
	Gradient – 2	3c	8.09	1.00	3.8	
		3i	8.47	0.96		
	Gradient - 3	3c	5.98	1.03	2.1	
		3i	6.14	0.95		
ACE PFP C18 (150x4.6mm), 3µm	Gradient - 1	3c	11.82	1.00	3.7	Better resolution of 3c and 3i
		3i	12.26	0.93		
	Gradient – 2	3c	9.34	1.02	5.1	
		3i	9.98	1.01		
	Gradient - 3	3c	6.80	1.09	3.5	
		3i	7.18	1.02		
Thermo Hypersil gold PFP (150x4.6mm), 1.9µm)	Gradient - 1	3c	10.38	0.93	3.0	Separation observed between 3c and 3i
		3i	10.71	1.03		
	Gradient – 2	3c	7.37	0.97	3.2	
		3i	7.75	1.02		
	Gradient - 3	3c	6.03	0.93	1.7	
		3i	6.18	1.05		
Kinetex F5 (150x4.6mm), 2.6 µm	Gradient - 1	3c	9.56	1.05	4.9	Best Separation
		3i	9.98	1.01		
	Gradient – 2	3c	6.60	0.99	5.5	
		3i	7.12	1.04		
	Gradient - 3	3c	5.00	1.03	2.9	
		3i	5.22	0.94		

Table S3: Chromatograms of separation of racemate 3c and 3i with mobile phase A as 10mM ammonium acetate in water (pH 7.0) and mobile phase B as ACN with different stationary phases.

Column used in first dimension	Gradient	Peak Name	Retention time (min)	Peak asymmetry	Resolution	Remarks
Kinetex EVO C18 (100x4.6mm), 2.6µm	Gradient - 1	3c	8.06	1.16	No	No separation of 3c and 3i
		3i				
	Gradient – 2	3c	4.96	1.47	No	
		3i				
	Gradient - 3	3c	4.26	1.75	No	
		3i				
Kinetex BPH (100x4.6mm), 2.6µm	Gradient - 1	3c	9.07	1.14	3.0	Separation of 3c and 3i observed
		3i	9.34	1.18		
	Gradient – 2	3c	6.00	1.20	3.3	
		3i	6.32	1.16		
	Gradient - 3	3c	4.76	1.13	1.7	
		3i	4.88	1.00		
Ascentis® Express Biphenyl (100x2.1mm), 2.7µm	Gradient - 1	3c	10.85	0.97	3.2	Baseline peak separation obtained
		3i	11.13	0.98		
	Gradient – 2	3c	8.09	1.00	3.8	
		3i	8.47	0.96		
	Gradient - 3	3c	5.98	1.03	2.1	
		3i	6.14	0.95		
ACE PFP C18 (150x4.6mm), 3µm	Gradient - 1	3c	11.87	1.04	4.1	Better resolution of 3c and 3i as compared with other columns
		3i	12.30	1.12		
	Gradient – 2	3c	9.60	0.95	5.1	
		3i	10.24	1.01		
	Gradient - 3	3c	6.96	0.98	3.7	
		3i	7.38	0.92		
Thermo Hypersil gold PFP (150x4.6mm), 1.9µm)	Gradient - 1	3c	10.4	0.95	3.2	Baseline peak separation obtained
		3i	10.74	0.94		
	Gradient – 2	3c	7.39	0.93	3.3	
		3i	7.78	1.01		
	Gradient - 3	3c	6.02	0.97	1.8	
		3i	6.18	1.06		
Kinetex F5 (150x4.6mm), 2.6 µm	Gradient - 1	3c	9.57	0.94	4.9	Highest resolution of 3c and 3i as compared with other columns
		3i	9.98	0.97		
	Gradient – 2	3c	8.58	1.01	6.6	
		3i	9.20	0.98		
	Gradient - 3	3c	5.00	1.02	2.9	
		3i	5.22	0.93		

The coelution of the racemate 3c and 3i were observed using the C18 stationary phase. The racemates were separated with both biphenyl columns and Thermo Hypersil Gold PFP column but with low resolution. Better resolution was obtained with the ACE PFP C18 column whereas Kinetex F5 column gives a maximum resolution of 6.6 with good peak shape.

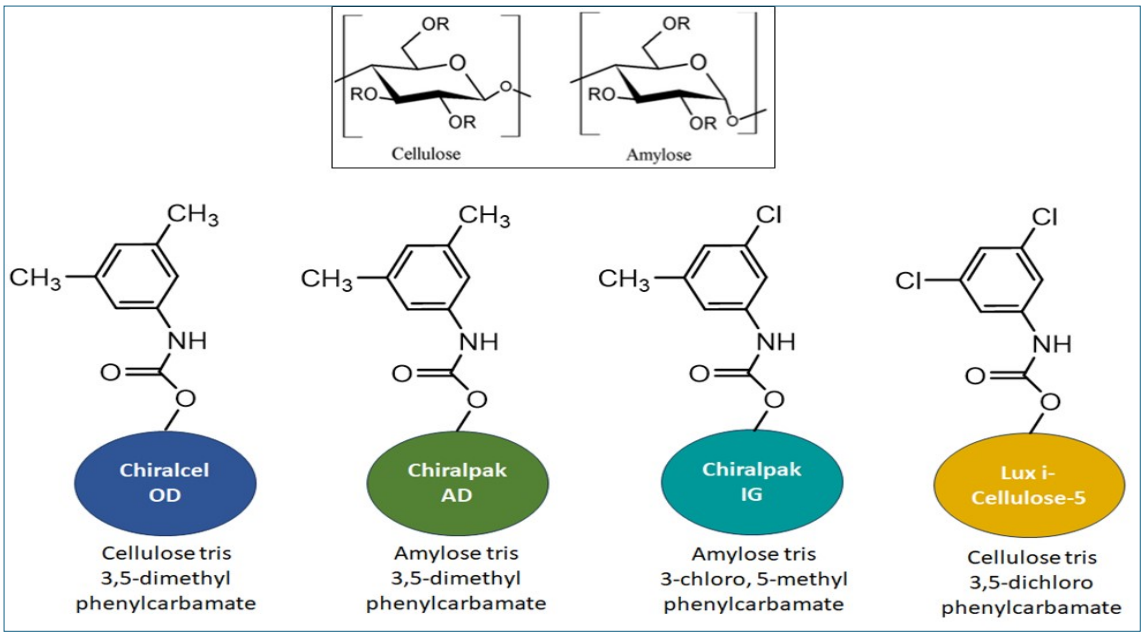

Figure S4: Structures of chiral stationary phases.

Table S4. Chromatograms of separation of 3c (R and S) and 3i (R and S) enantiomers using the Chiralcel-OD-H column with the mobile phase as different combination of 0.1% DEA in MeOH and ACN.

Stationary Phase	**Chiralcel-OD-H**				
Chromatographic conditions	**Compound Name**	**Retention time (min)**	**Peak asymmetry**	**Resolution**	**Remarks**
0.1% DEA in MeOH (100%)	3c-(R+S)	12.53	1.37	NA	Single peak was observed for 3c and separation was observed for 3i
		NA	NA		
	3i- (R+S)	11.17	1.25	2.17	
		12.63	1.15		
0.1% DEA in MeOH: ACN (90: 10 v/v)	3c-(R+S)	8.23	1.41	6.00	Sharp peaks with maximum resolution
		11.30	1.08		
	3i- (R+S)	7.98	1.25	2.84	
		9.05	1.15		
0.1% DEA in MeOH: ACN (70: 30 v/v)	3c-(R+S)	6.20	1.21	5.84	Both peak separated but more resolution required
		7.44	1.12		
	3i- (R+S)	5.87	1.31	2.77	
		6.44	1.22		
0.1% DEA in MeOH: ACN (50: 50 v/v)	3c-(R+S)	5.68	1.23	4.31	Sharp peaks with less separation
		6.38	1.20		
	3i- (R+S)	5.21	1.29	2.38	
		5.60	1.18		
0.1% DEA in MeOH: ACN (30: 70 v/v)	3c-(R+S)	5.43	1.21	3.62	Sharp peaks with less resolution comparatively
		6.03	1.25		
	3i- (R+S)	5.07	1.24	2.50	
		5.46	1.17		

With 0.1% DEA in methanol (100%) as a mobile phase, only one peak was observed for 3c, and two peaks were observed for 3i. All other combinations have provided the separation for the 3c and 3i enantiomers with best peak shapes and separation observed with the 0.1% DEA in methanol:ACN (90:10 v/v) ratio.

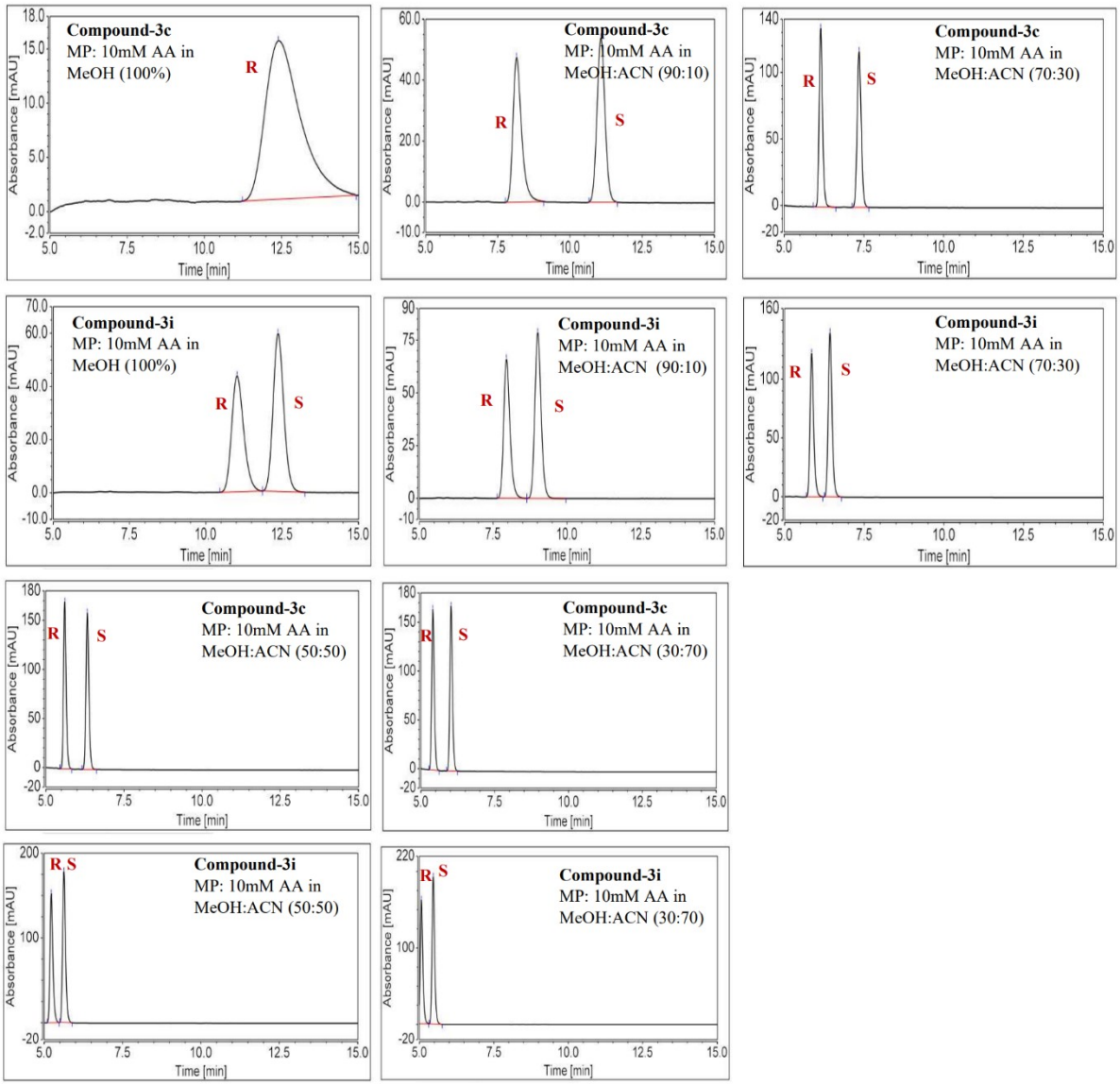

Figure S5: Chromatograms of separation of 3c (R and S) and 3i (R and S) enantiomers using the Chiralcel-OD-H column with the mobile phase as a different combination of 10 mM ammonium acetate in MeOH and ACN.

The above chromatograms showed that single peak was obtained for the 3c and two peaks for 3i (R and S) enantiomers. Best separation for 3c (R and S) and 3i (R and S) enantiomers with good peak shape were observed with 10% ACN. In other combinations of ammonium acetate and ACN, 3c and 3i enantiomers were eluted with good peak shape but with less resolution.

Table S5: Chromatograms of separation of 3c (R and S) and 3i (R and S) enantiomers using the Chiralcel-OD-H column with the mobile phase as a different combination of 10 mM ammonium acetate in MeOH and ACN.

Stationary Phase	**Chiralcel-OD-H**				
Chromatographic conditions	**Compound Name**	**Retention time (min)**	**Peak asymmetry**	**Resolution**	**Remarks**
10 mM Ammonium Acetate in MeOH (100%)	3c-(R+S)	12.40	1.60	NA	Single peak was observed for 3c, and separation was observed for 3i
		NA	NA		
	3i- (R+S)	11.03	1.22	1.99	
		12.37	1.15		
10 mM Ammonium Acetate in MeOH: ACN (90: 10 v/v)	3c-(R+S)	8.15	1.43	5.66	We observed best separation
		11.08	1.07		
	3i- (R+S)	7.95	1.27	2.77	
		9.01	1.16		
10 mM Ammonium Acetate in MeOH: ACN (70: 30 v/v)	3c-(R+S)	6.15	1.19	5.65	We observed good separation
		7.35	1.13		
	3i- (R+S)	5.85	1.26	2.77	
		6.43	1.19		
10 mM Ammonium Acetate in MeOH: ACN (50: 50 v/v)	3c-(R+S)	5.61	1.15	4.44	Both isomers separated
		6.33	1.20		
	3i- (R+S)	5.23	1.30	2.47	
		5.63	1.18		
10 mM Ammonium Acetate in MeOH: ACN (30: 70 v/v)	3c-(R+S)	5.41	1.17	3.85	Both isomers separated but baseline needs to improve
		6.03	1.16		
	3i- (R+S)	5.07	1.28	2.57	
		5.47	1.17		

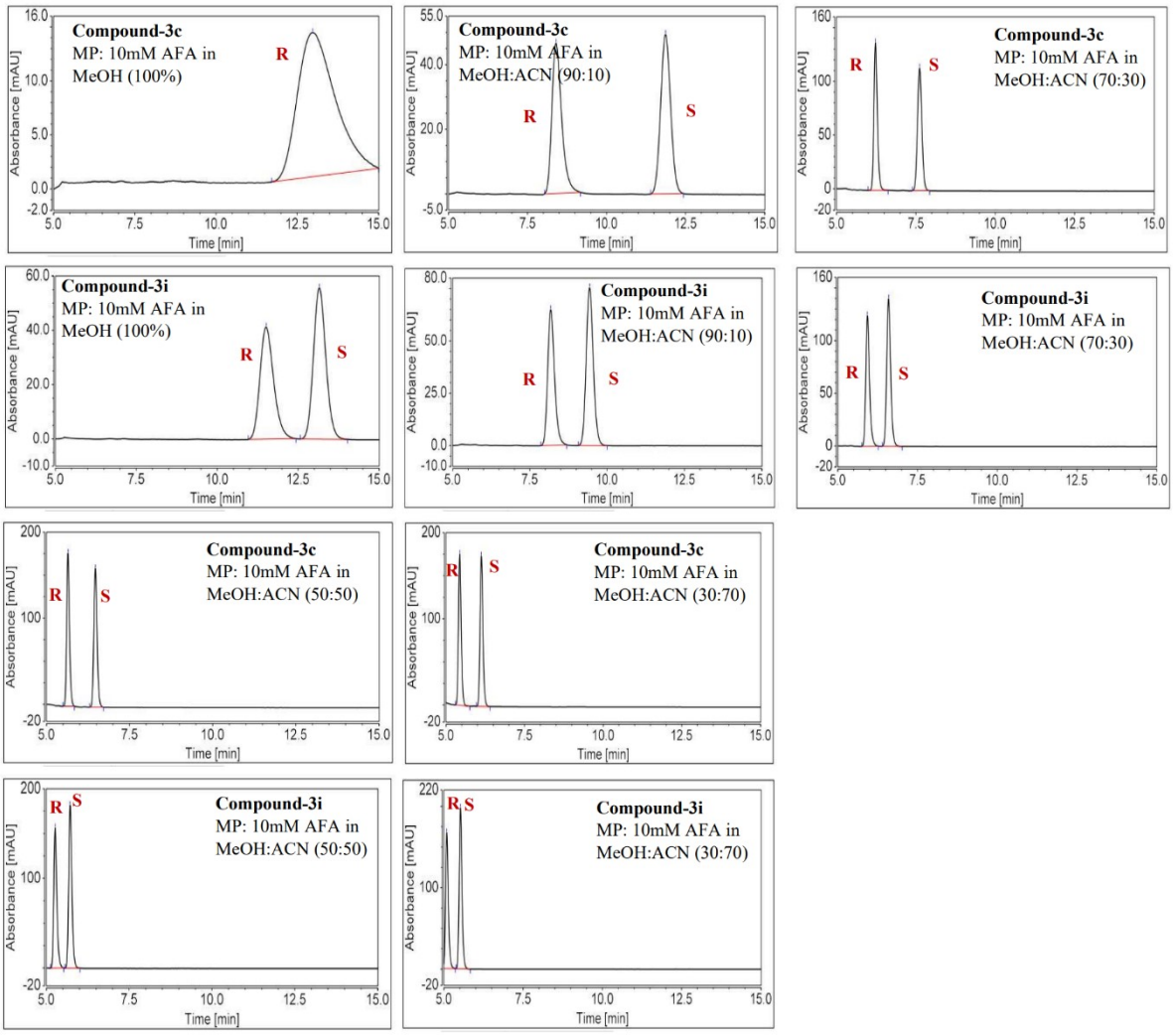

Figure S6: Chromatograms of the separation of 3c (R and S) and 3i (R and S) enantiomers using the Chiralcel-OD-H column with the mobile phase as a different combination of 10 mM ammonium formate in MeOH and ACN.

Similar findings were observed with the ammonium formate buffer as with the ammonium acetate buffer. Above chromatograms showed that single peak was obtained for the 3c and two peaks for 3i (R and S) enantiomers with 100% ammonium formate in methanol. Best separation for 3c (R and S) and 3i (R and S) enantiomers with good peak shape were observed with 10% ACN. In other combinations of ammonium formate and ACN, 3c and 3i enantiomers were eluted with good peak shape but with less resolution.

Table S6: Chromatograms of the separation of 3c (R and S) and 3i (R and S) enantiomers using the Chiralcel-OD-H column with the mobile phase as a different combination of 10 mM ammonium formate in MeOH and ACN.

Stationary Phase	Chiralcel-OD-H				
Chromatographic conditions	**Compound Name**	**Retention time (min)**	**Peak asymmetry**	**Resolution**	**Remarks**
10 mM Ammonium formate in MeOH (100%)	3c-(R+S)	12.98	1.41	NA	Single peak was observed for 3c, and separation was observed for 3i
		NA	NA		
	3i- (R+S)	11.52	1.23	2.24	
		13.16	1.12		
10 mM Ammonium formate in MeOH: ACN (90:10 v/v)	3c-(R+S)	8.40	1.38	6.15	Sharp peaks with maximum resolution
		11.87	1.06		
	3i- (R+S)	8.18	1.24	3.09	
		9.43	1.11		
10 mM Ammonium formate in MeOH: ACN (70:30 v/v)	3c-(R+S)	6.23	1.22	6.21	Both peak separated but more resolution required
		7.62	1.08		
	3i- (R+S)	5.93	1.32	3.09	
		6.59	1.15		
10 mM Ammonium formate in MeOH: ACN (50:50 v/v)	3c-(R+S)	5.65	1.16	5.00	Both the peaks merged with impurity
		6.48	1.11		
	3i- (R+S)	5.27	1.28	2.67	
		5.72	1.24		
10 mM Ammonium formate in MeOH: ACN (30:70 v/v)	3c-(R+S)	5.43	1.23	4.32	Sharp peaks with less resolution comparatively
		6.13	1.17		
	3i- (R+S)	5.09	1.24	2.75	
		5.53	1.15		

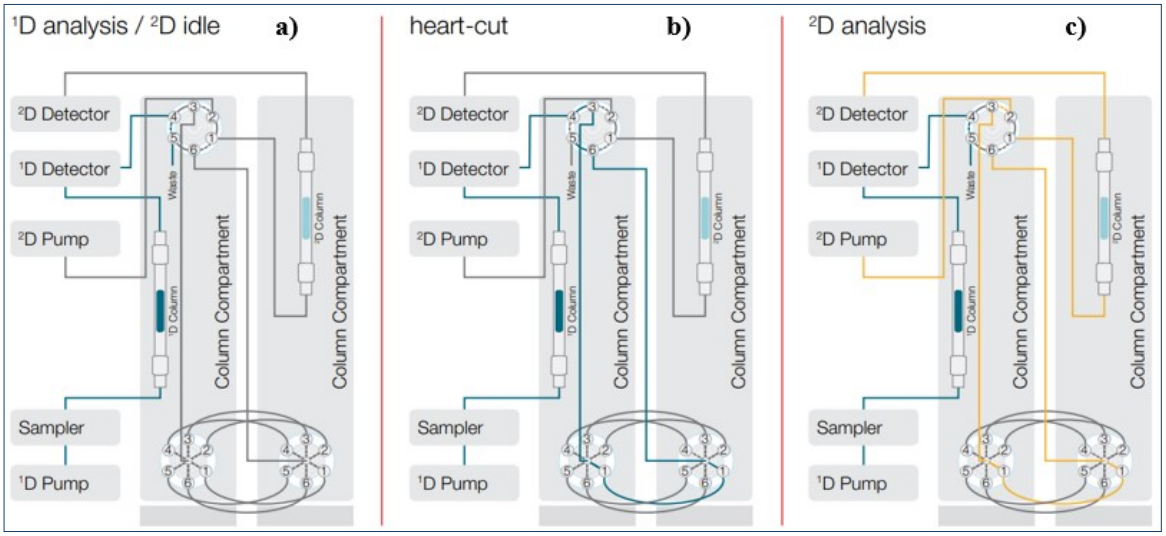

Figure S7. Flow schemes of the different steps in multi-heart-cut 2D-LC (example for loop 1)