

Supplementary Information

Synthesis and characterization of novel bio-chiral dopants derived from bio-betulin obtained from fermentation process

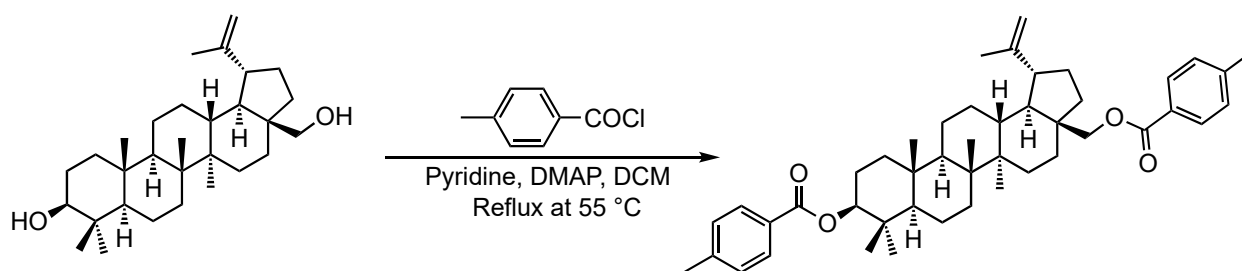
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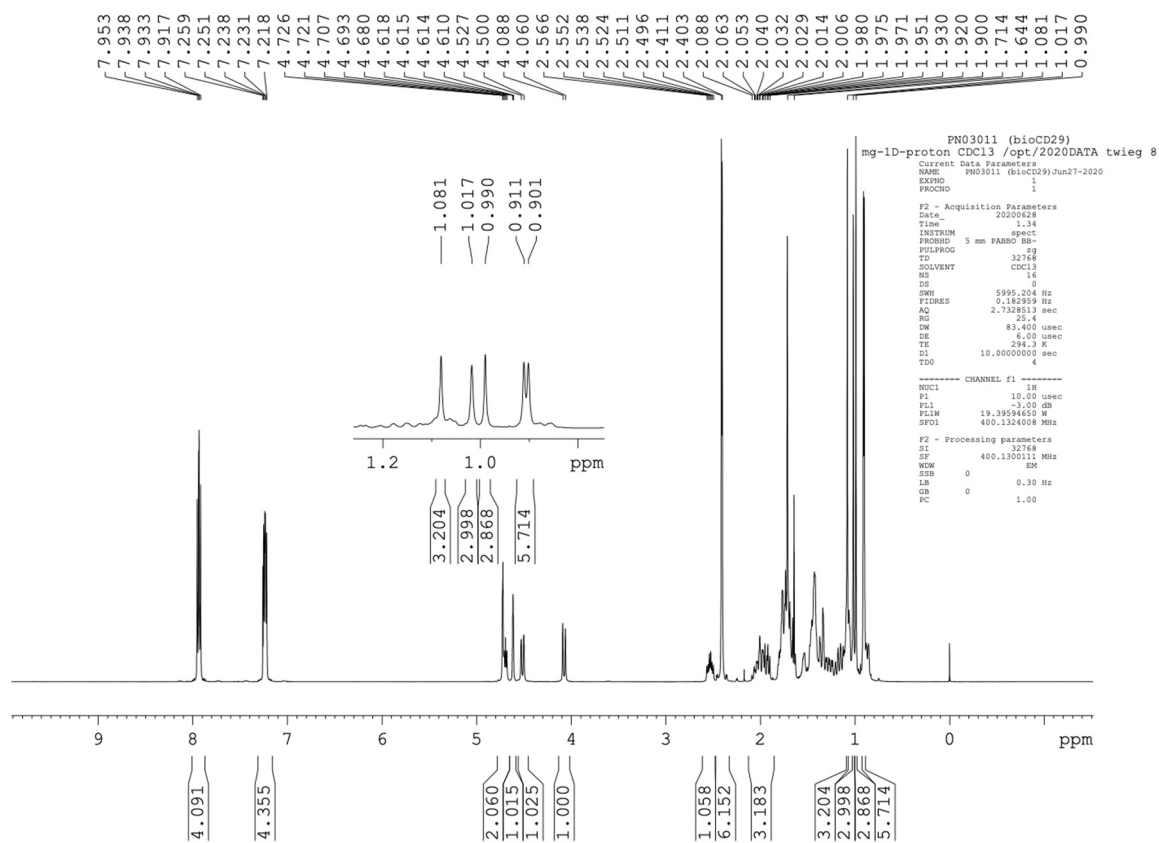
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Kent State University, Kent, OH 44242, USA.*

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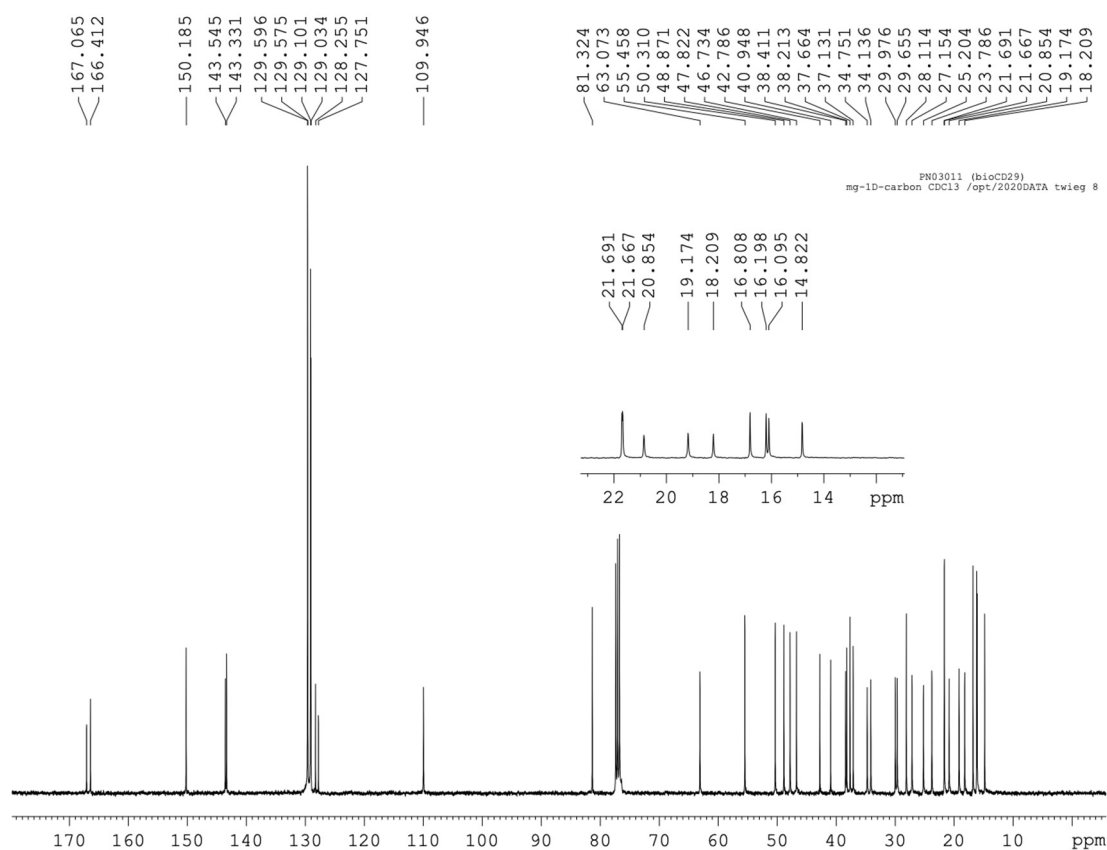
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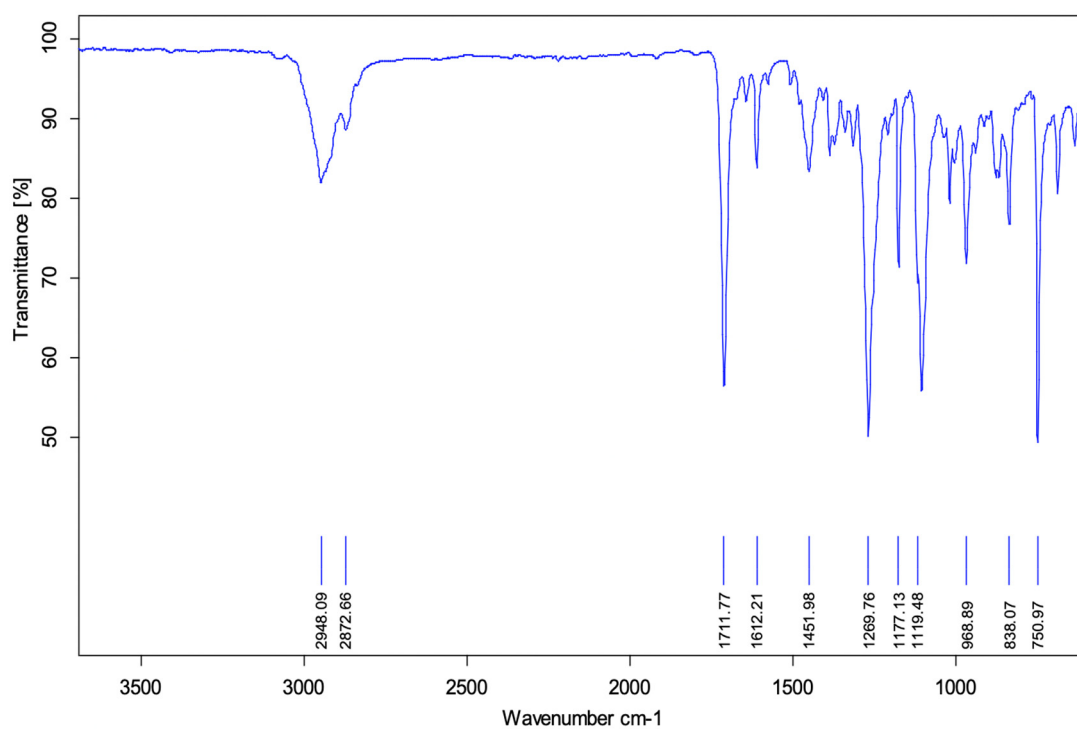
Preparative Route for Bio-CD29 (using fermentation derived betulin)



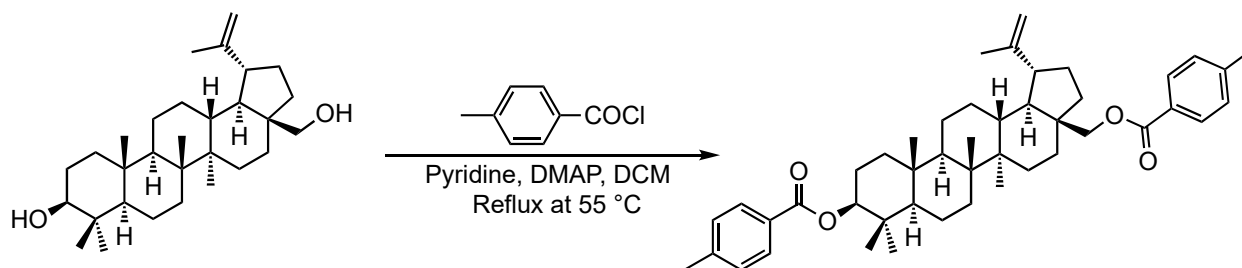
^1H NMR of Bio-CD29 (prepared from fermentation derived betulin)



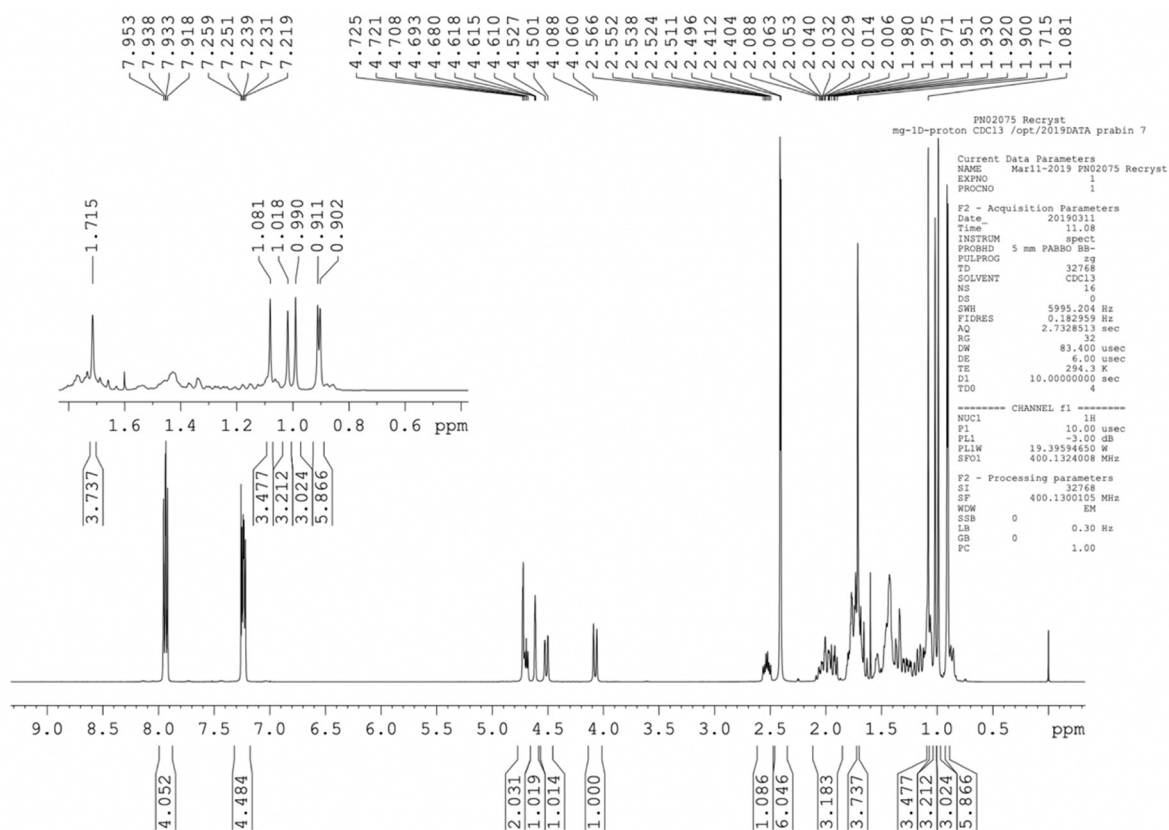
^{13}C NMR of Bio-CD29 (prepared from fermentation derived betulin)



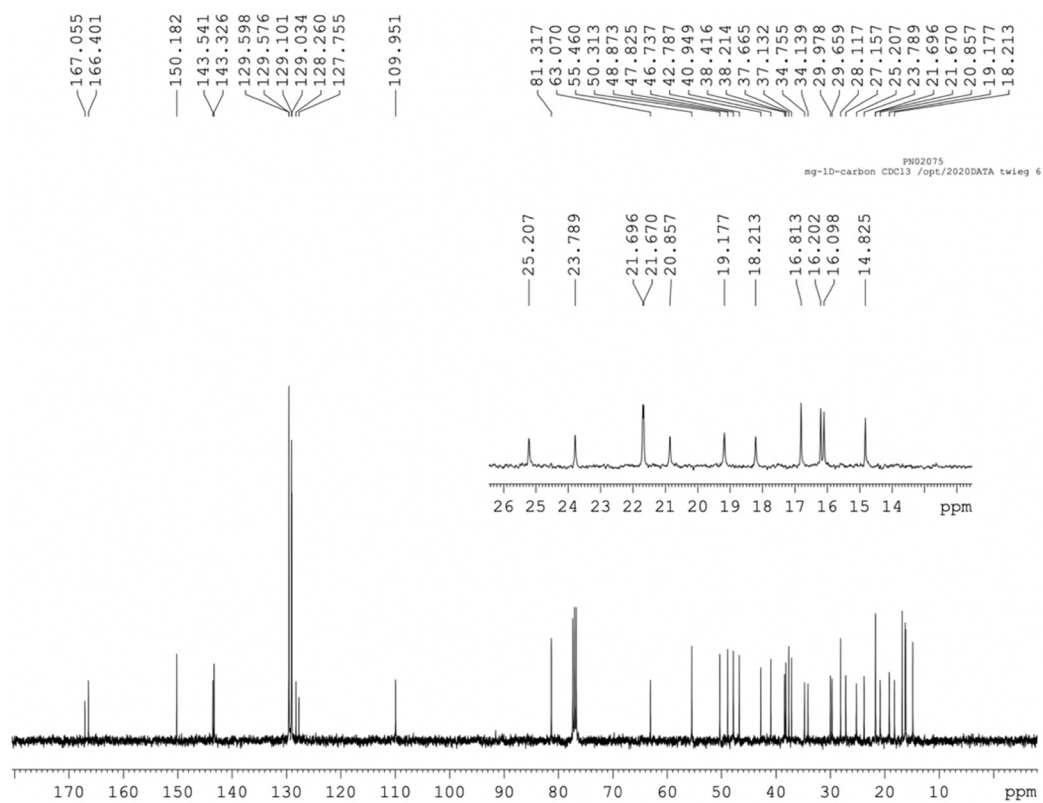
IR Spectrum of Bio-CD29 (prepared from fermentation derived betulin)



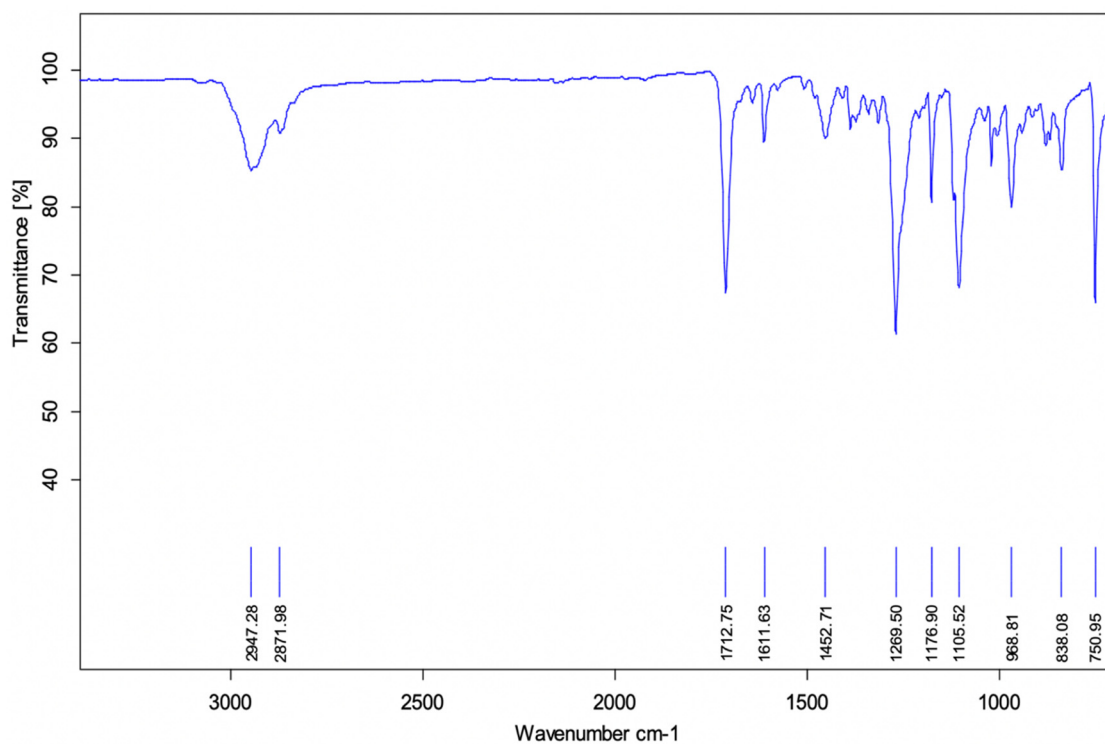
Preparative Route for CD29 (using commercial betulin)



¹H NMR of CD29 (prepared from commercial betulin)

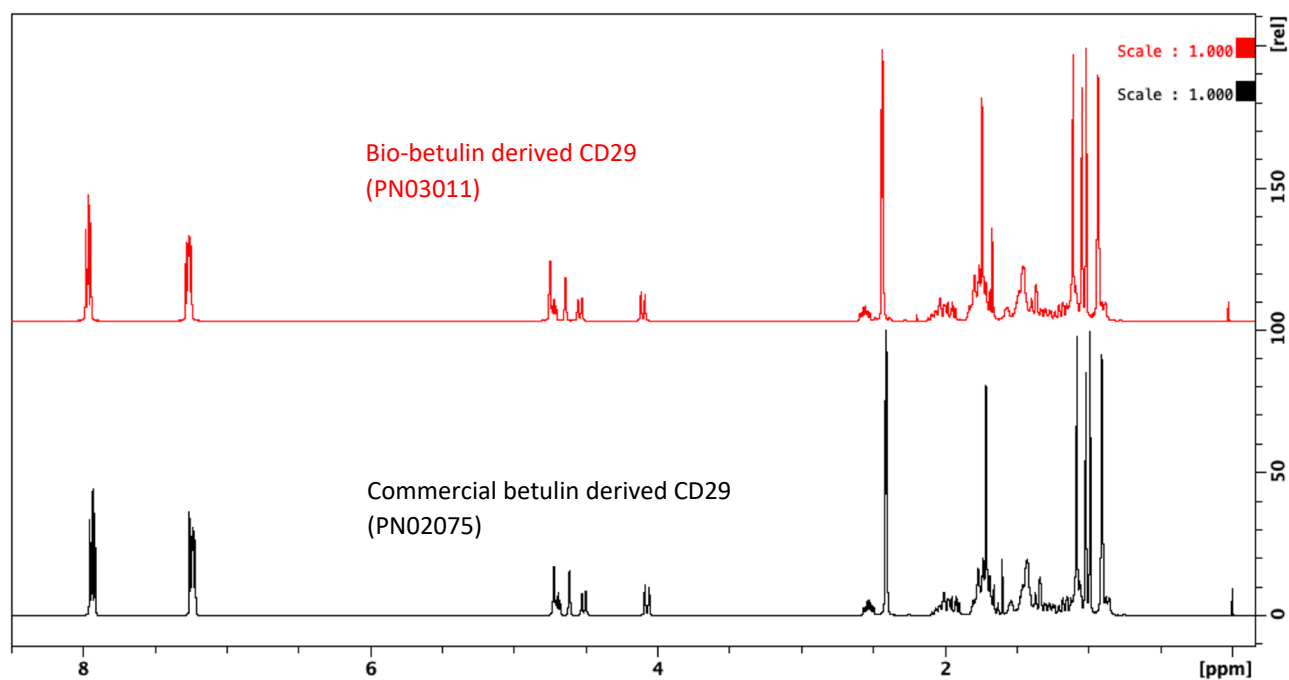


^{13}C NMR of CD29 (prepared from commercial betulin)

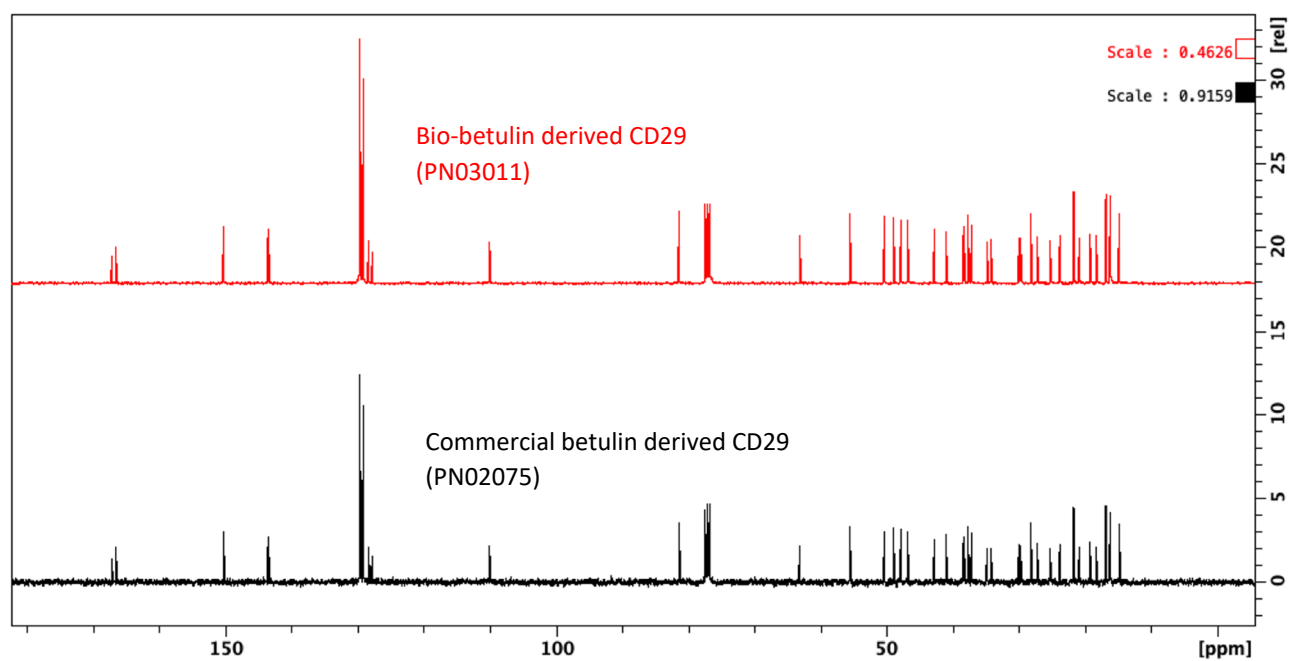


IR Spectrum of CD29 (prepared from commercial betulin)

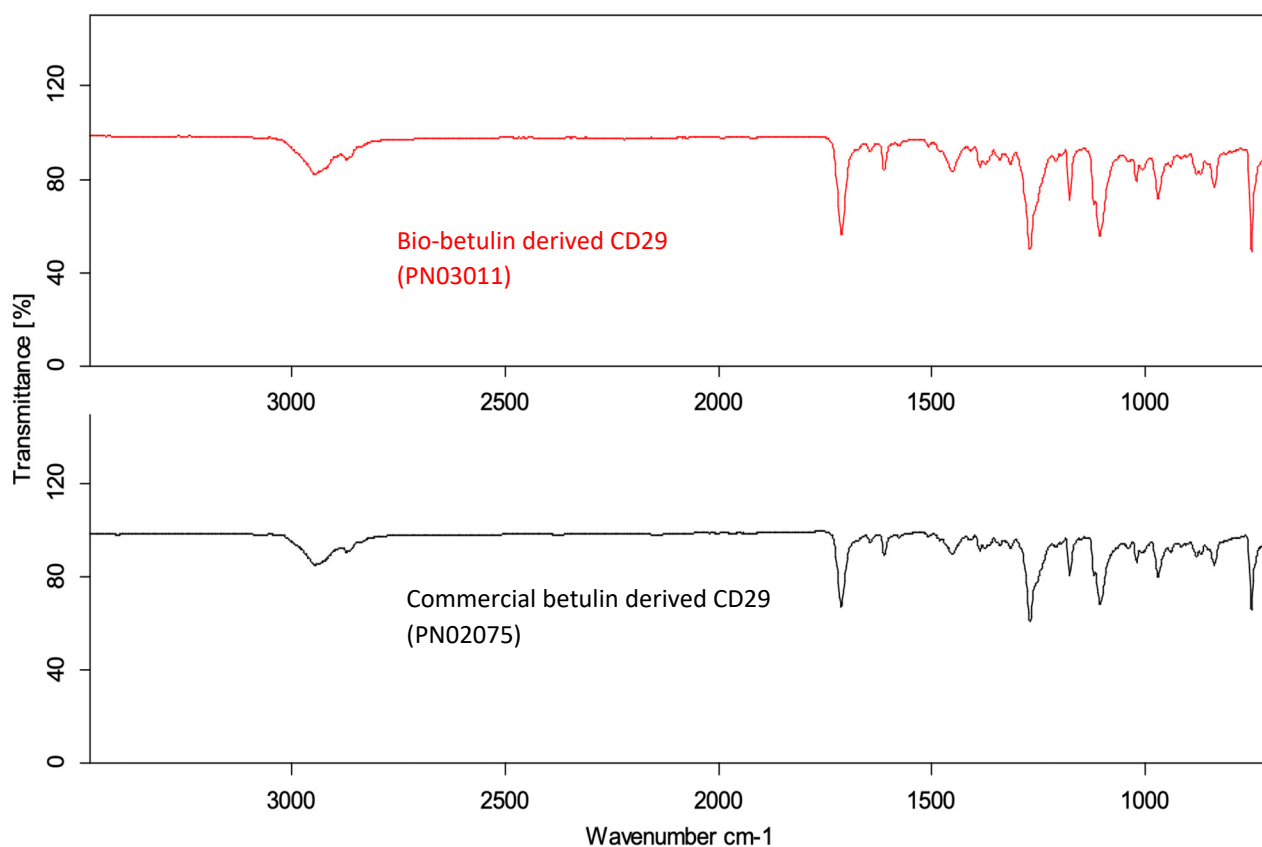
Spectral comparisons of bio-betulin and commercial betulin derived CD-29:



^1H NMR comparison of CD-29 obtained from fermentation derived and commercial betulin.



^{13}C NMR comparison of CD-29 obtained from fermentation derived and commercial betulin.

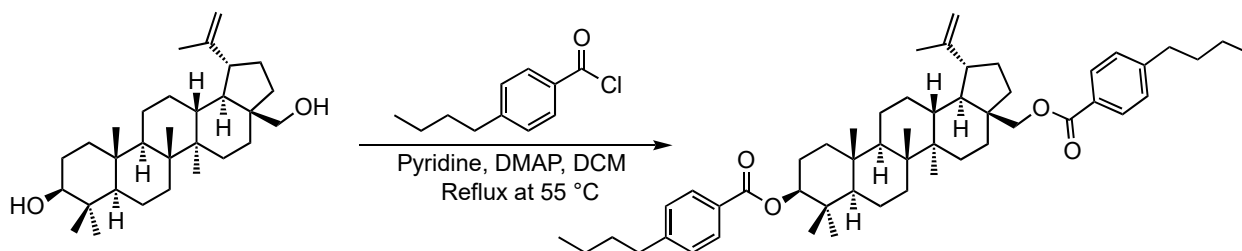


IR comparison of CD-29 obtained from fermentation derived and commercial betulin.

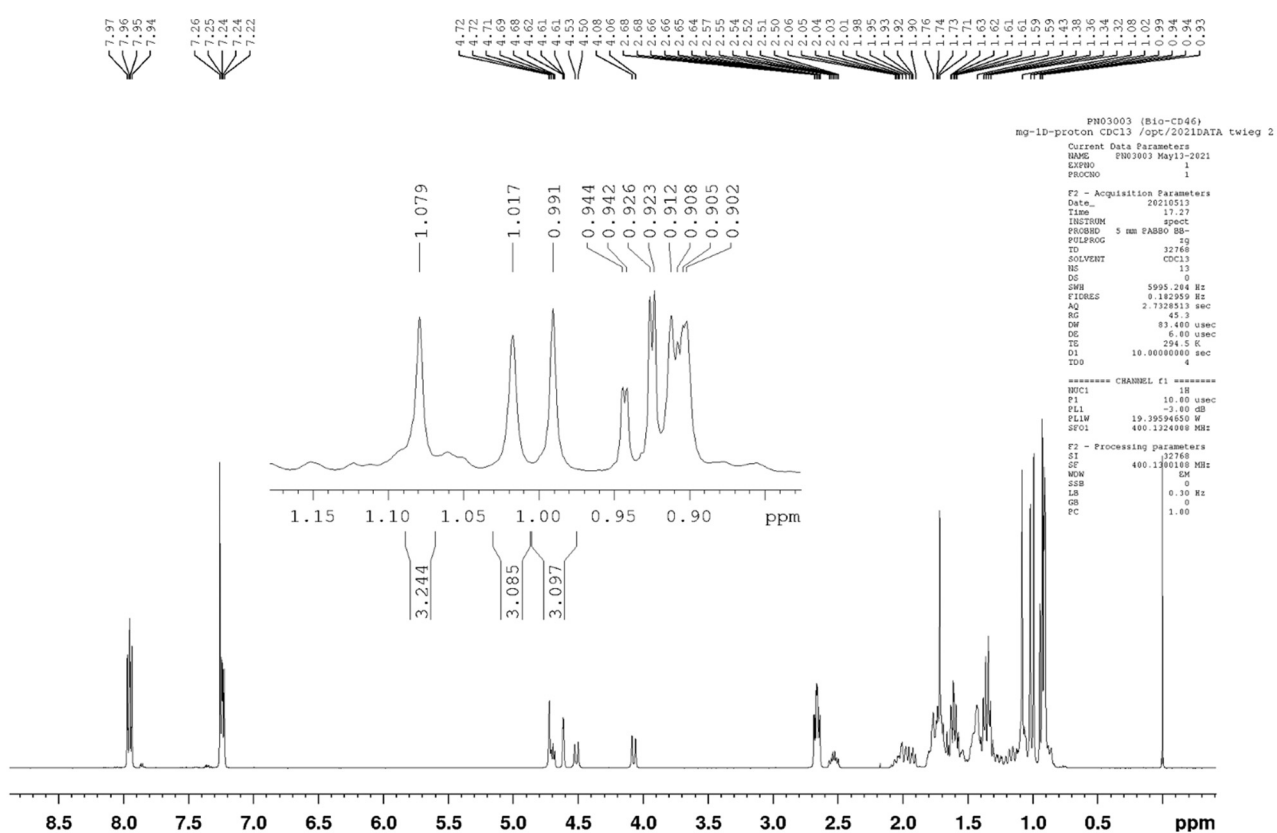
Comment:

The ¹H NMRs of **CD29** prepared from commercial betulin and fermentation derived betulin are identical. Likewise, the respective ¹³C NMRs and infrared spectra are identical.

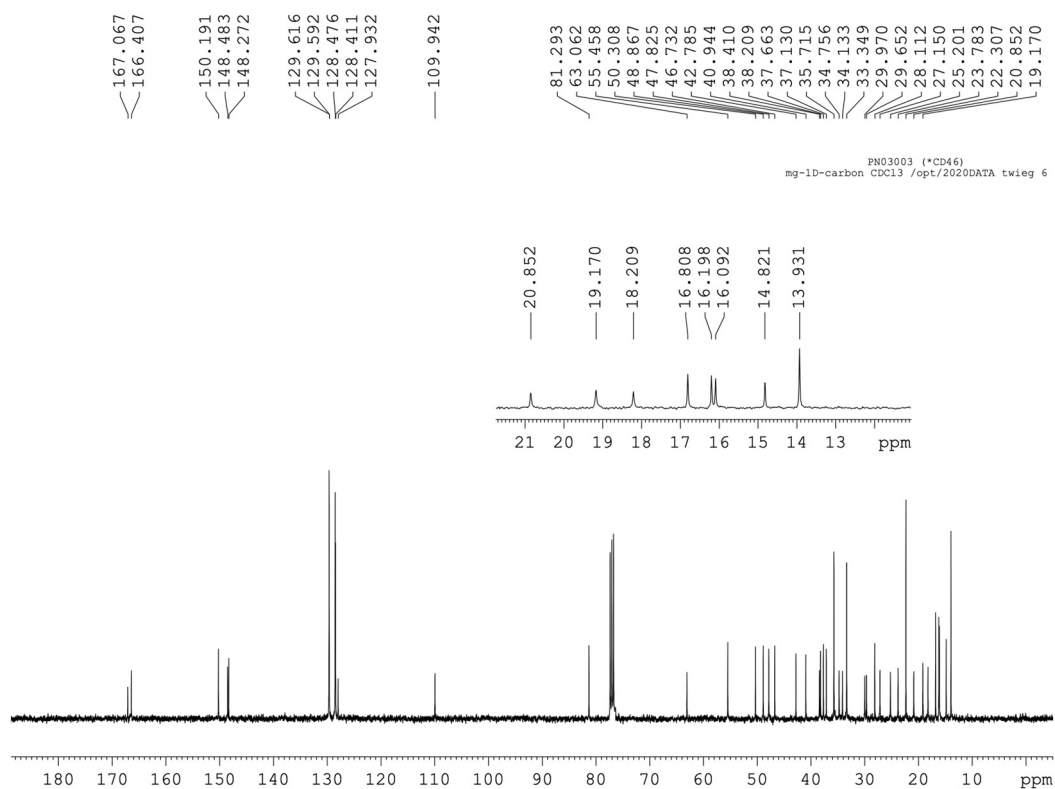
This is all evidence that the **CD29** prepared from the two betulin sources are identical.



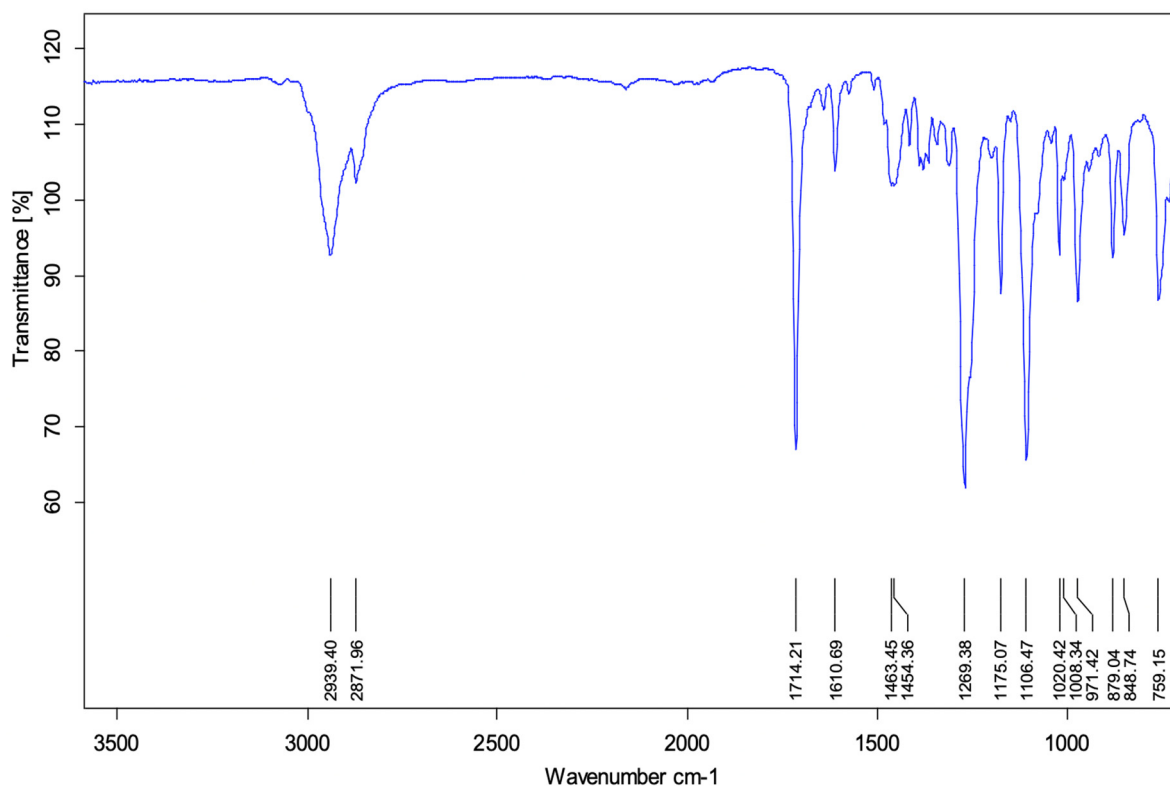
Preparative Route for Bio-CD46 (using fermentation derived betulin supplied by Zymergen)



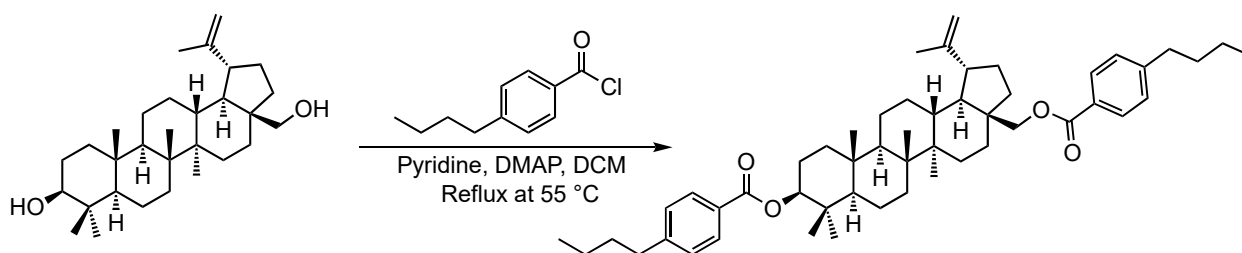
¹H NMR of Bio-CD46 (using fermentation derived betulin supplied by Zymergen)



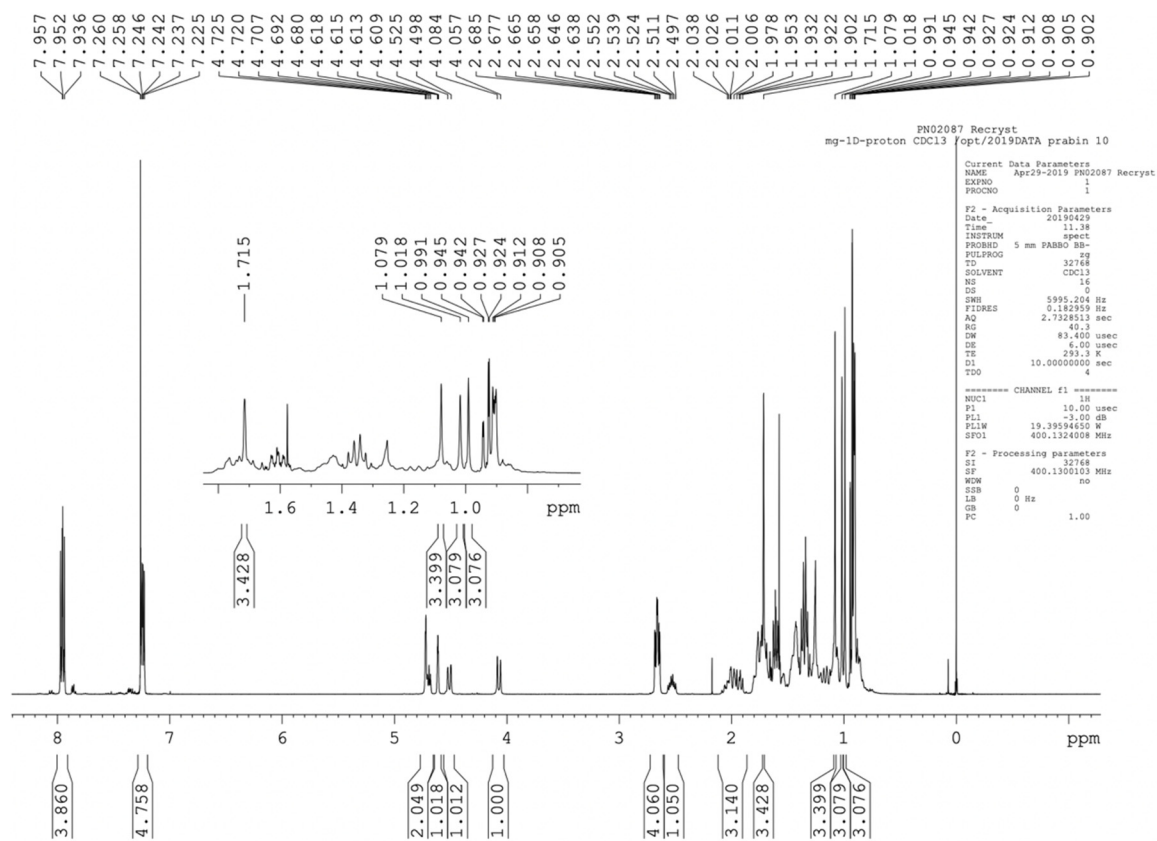
¹³C NMR of Bio-CD46 (using fermentation derived betulin)



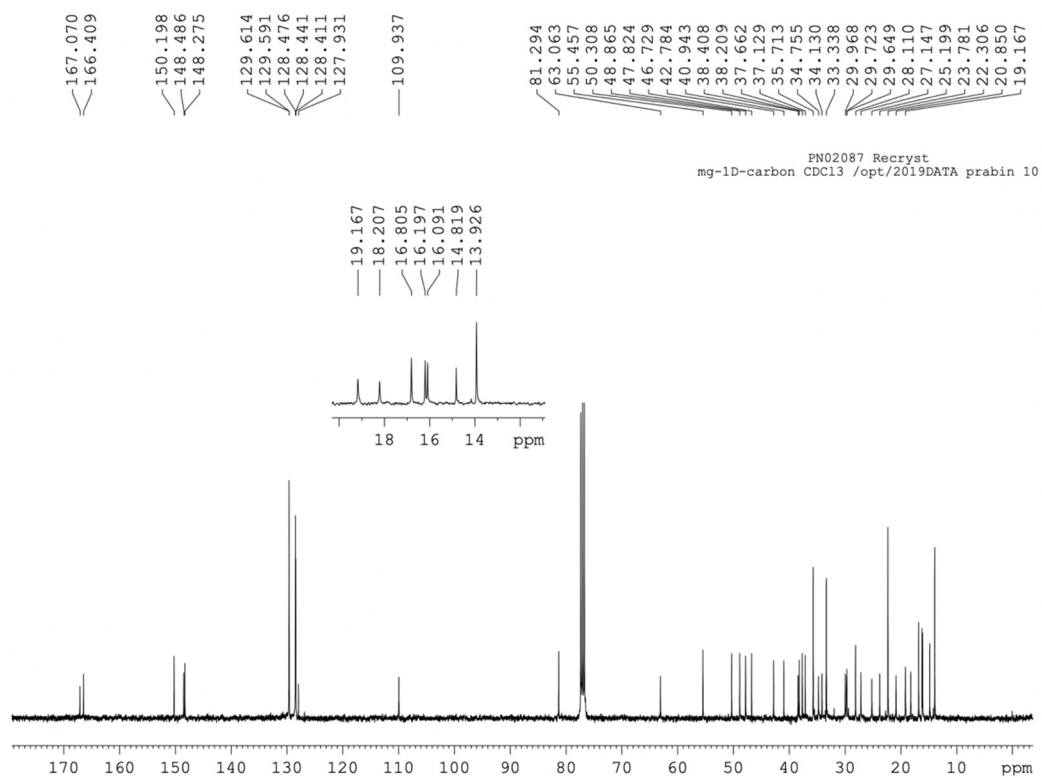
IR Spectrum of Bio-CD46 (using fermentation derived betulin)



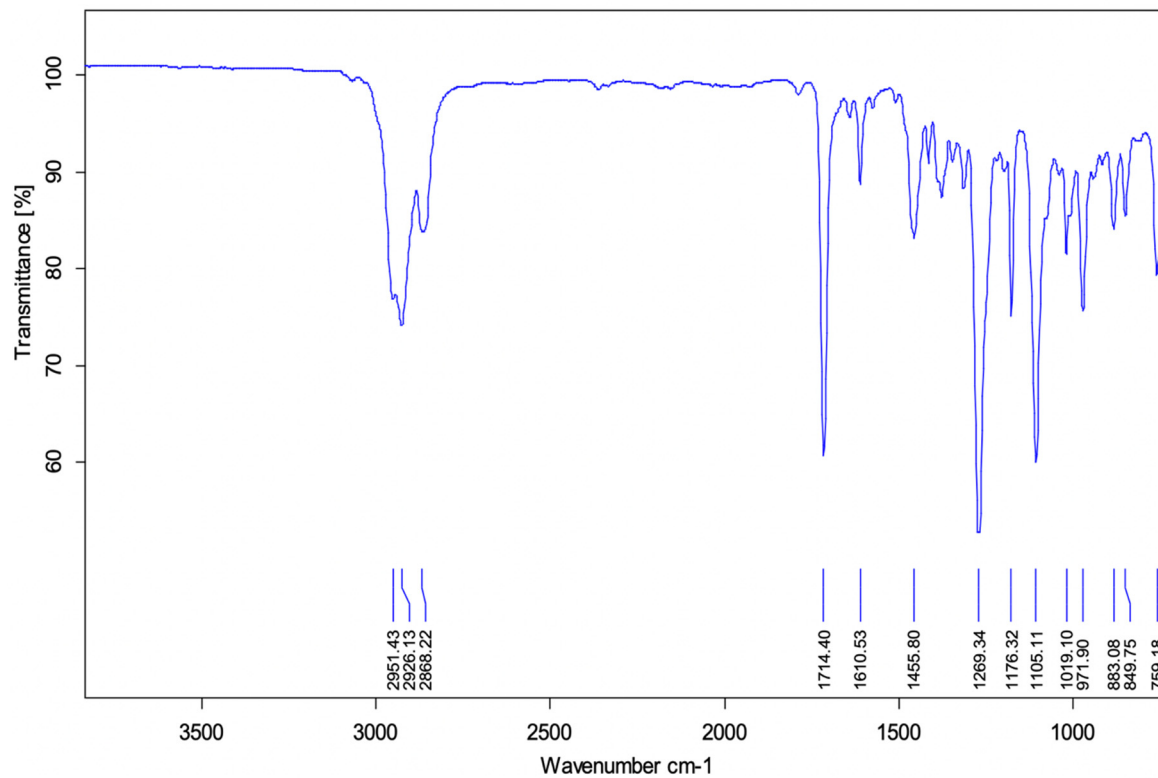
Preparative Route for CD46 (using commercial betulin)



¹HNMR of CD46 (using commercial betulin)

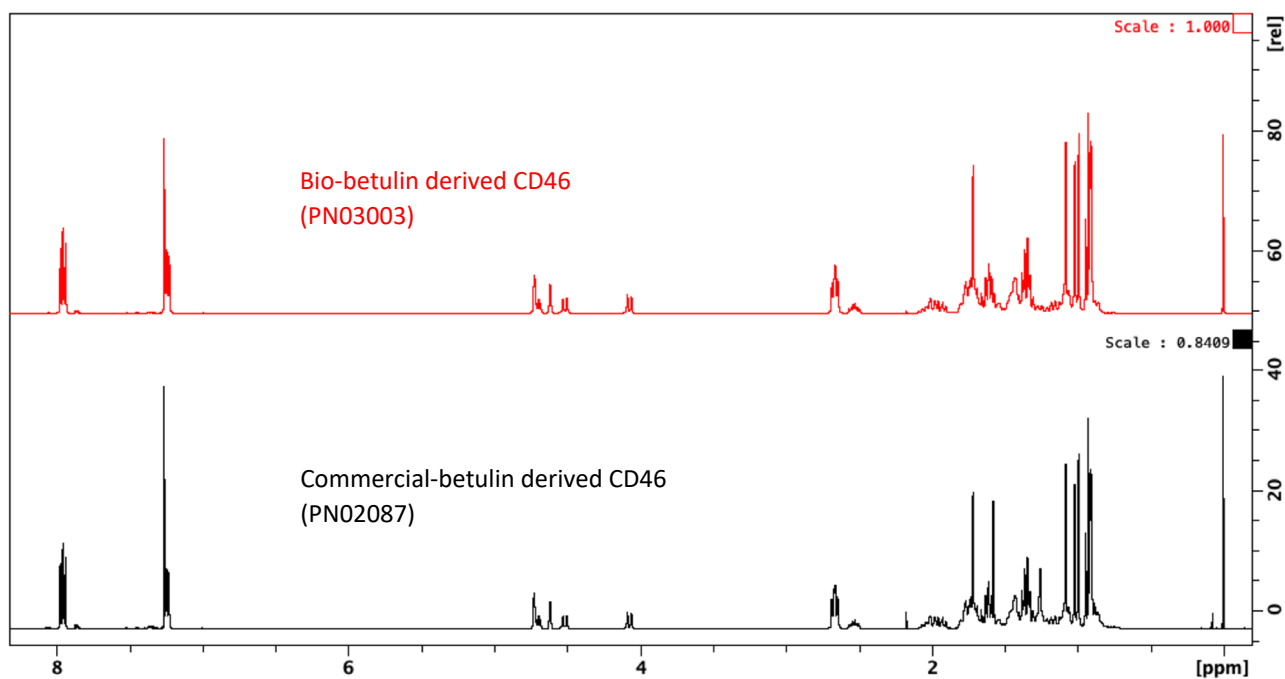


¹³CNMR of CD46 (using commercial betulin) PN02087

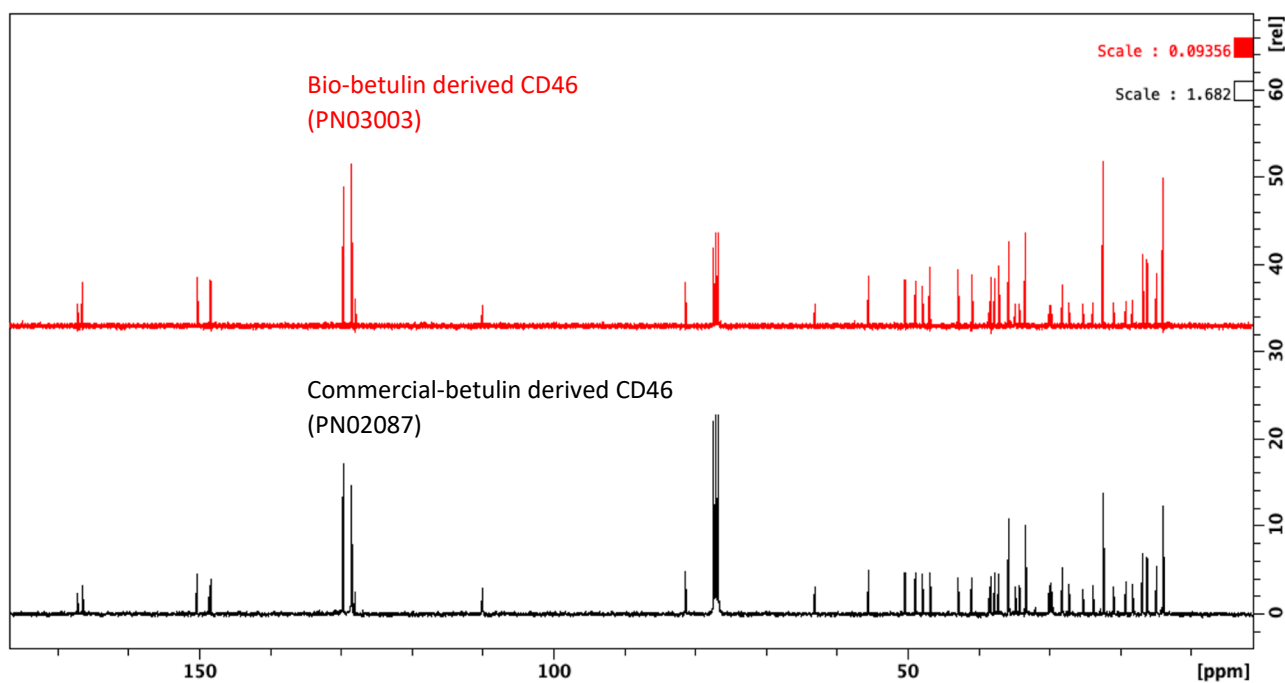


IR Spectra of CD46 (using commercial betulin) PN02087

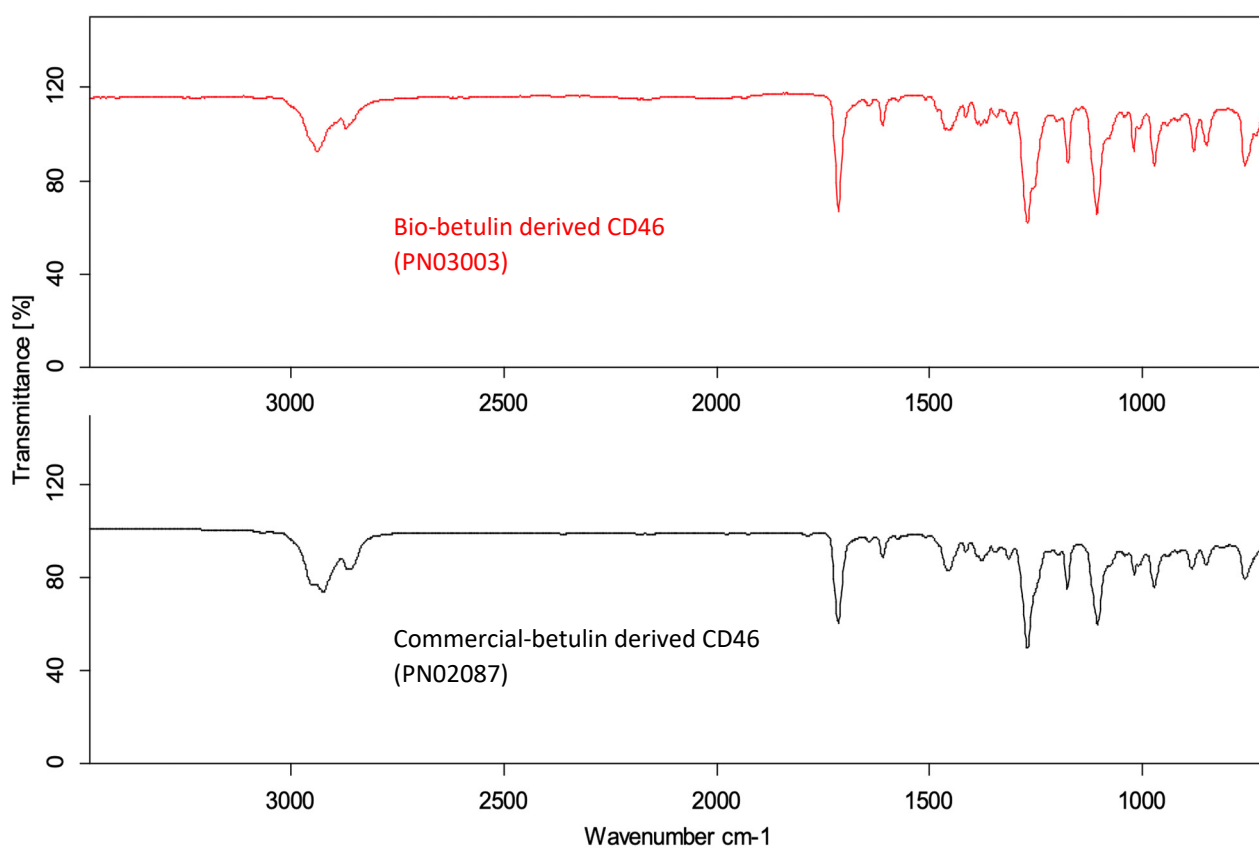
Spectral comparisons of bio-betulin and commercial betulin derived CD-46:



^1H NMR comparison of CD-46 obtained from fermentation derived and commercial betulin.



^{13}C NMR comparison of CD-46 obtained from fermentation derived and commercial betulin.



IR comparison of CD-46 obtained from fermentation derived and commercial betulin.

Comment:

The ¹H NMRs of **CD46** prepared from commercial betulin and fermentation derived betulin are identical. Likewise, the respective ¹³C NMRs and infrared spectra are identical.

This is all evidence that the **CD46** prepared from the two betulin sources are identical.

HPLC Analysis Protocol:

Betulin or its diester derivative (~6.000 mg) was weighed out and dissolved in 1,4-dioxane solvent (2.0 ml) to make ~3 mg/ml solution. This solution was vortexed and then sonicated for 10 minutes. A cholesterol solution in 1,4-dioxane (~2 mg/ml) as a standard solution was also prepared in the same manner. The betulin and cholesterol solutions prepared in this fashion (0.1 ml each) were transferred into a 2 ml vial and diluted to 1.0 ml by adding acetonitrile-water (90:10 v/v) solvent. This mixture was injected to the HPLC and the column was eluted with acetonitrile/water mixture. The chromatographic separation was achieved using a Restek Ultra BiPh 3 μ m 150 x 3.0 mm. The flow rate was maintained 0.5 ml/min and the detection wavelength was set to 210 nm.

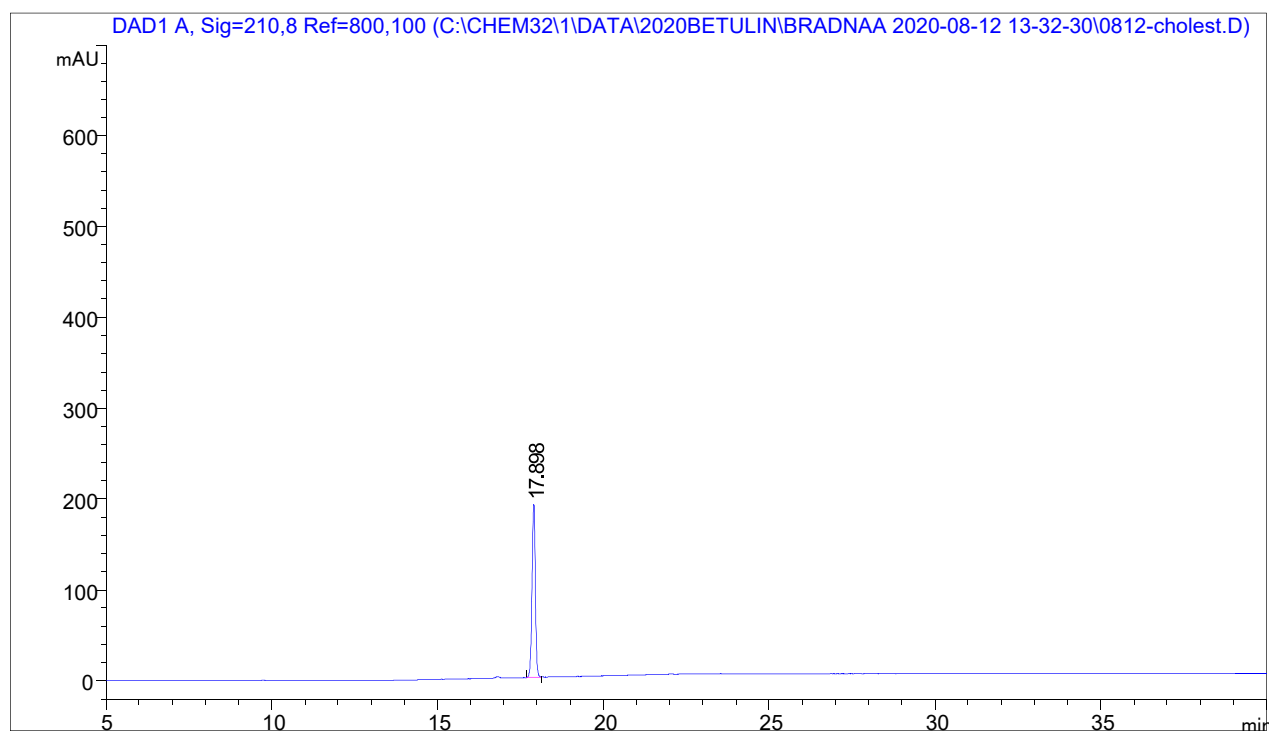
Sample Info: Dioxane (Blank test).



Comment:

The HPLC of a dioxane only sample was performed here as a blank test to observe any signal that may appear due to contaminants in the dioxane solvent used for preparation of the betulin or betulin derivative samples. In the range from 5 minutes to 40 minutes no peaks due to any contaminant (that absorbs at 210 nm) were observed.

Sample Info: Cholesterol, 0.207 mg/ml (Blank test).



Signal 1: DAD1 A, Sig=210,8 Ref=800,100

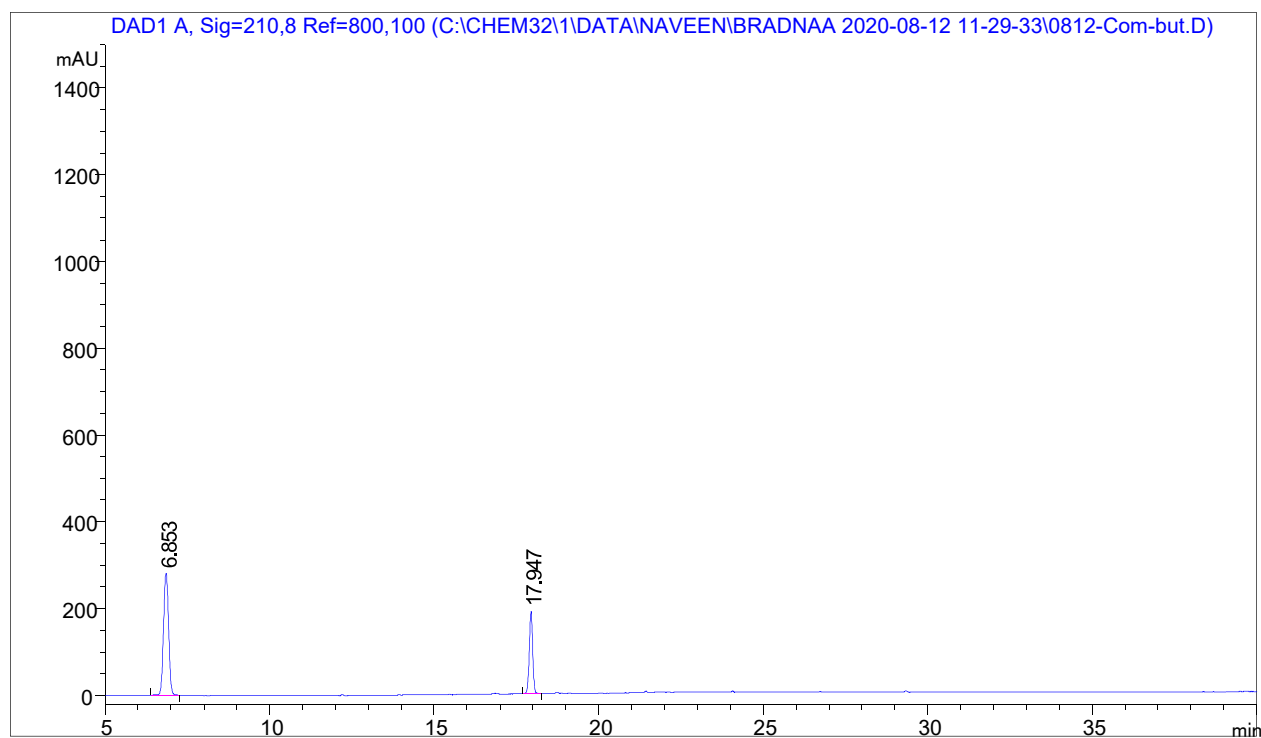
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.898	BB	0.1072	1324.38367	190.31343	24.8020

Comment:

The HPLC of a cholesterol only sample was performed to determine the cholesterol peak position in the HPLC. Cholesterol is a steroid and an aliphatic polycyclic substance similar to betulin. Cholesterol (Sigma Grade, $\geq 99\%$ C8667) was used as an internal standard for every HPLC run and its concentration was kept constant (0.207 mg/ml) in all experiments. From the HPLC it was observed that the cholesterol peak appeared at ~ 17.9 minutes. A single peak in the HPLC indicated this commercial standard as a pure compound.

In the HPLC runs the cholesterol standard (and other peaks) may not appear consistently at the exact same time as there may be some small discontinuity between sample injection and initiation of the HPLC run.

Sample Info: Commercial Betulin (sample = 0.312 mg/ml, cholesterol = 0.207 mg/ml).



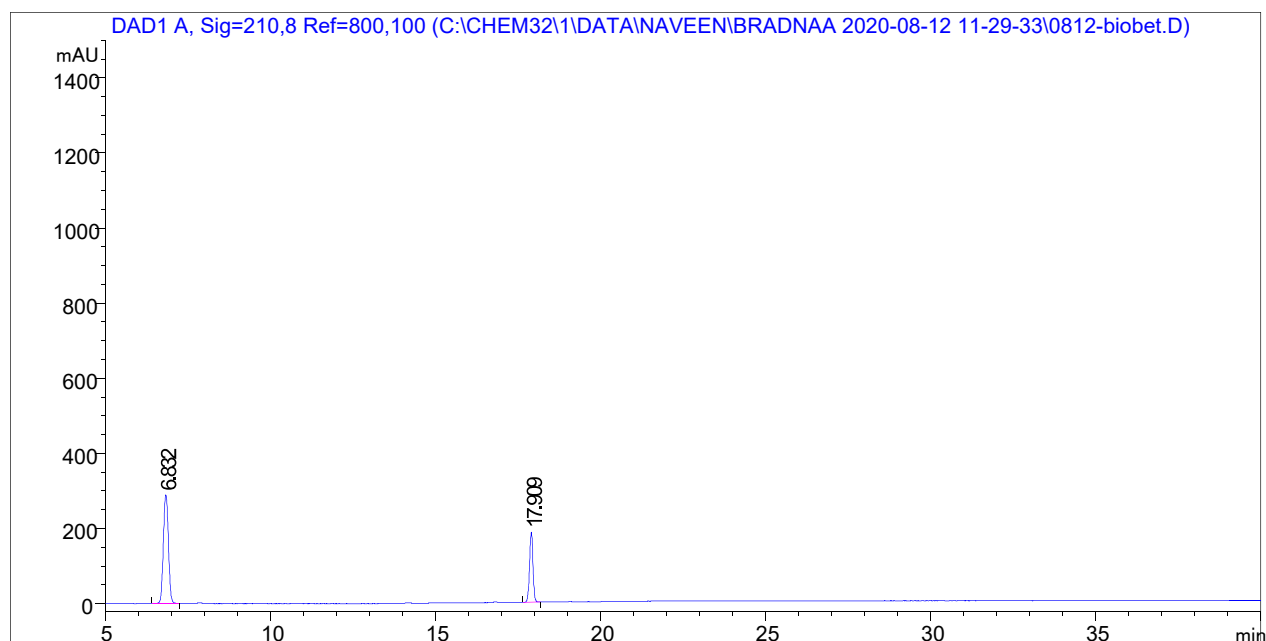
Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.853	BB	0.1693	3016.38110	280.94537	25.6396
2	17.947	BB	0.1128	1376.94995	189.53867	11.7042

Comment:

This HPLC of commercial betulin (Ark Pharm Inc., USA) was run to learn the purity and peak position of this compound. This HPLC was also acquired to compare the HPLC spectrum of the bio-betulin sample received from Zymergen. From the HPLC it was observed that the peak at ~6.85 minutes was due to the pure betulin compound. The cholesterol standard appears at ~17.95 (similar to the retention time in the cholesterol blank test).

Sample Info: Bio-betulin as received from Zymergen (sample = 0.334 mg/ml, cholesterol = 0.207 mg/ml).



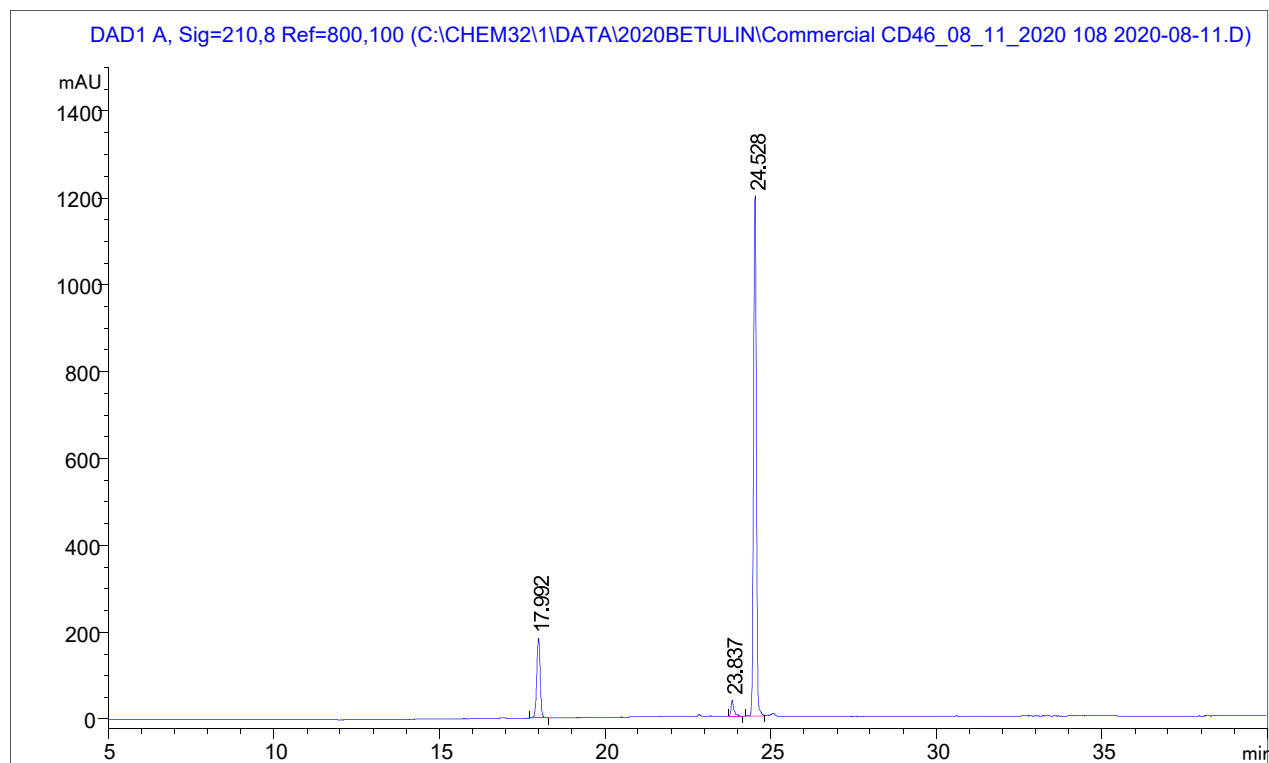
Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.832	BB	0.1679	3070.93701	289.11407	26.1176
2	17.909	VB	0.1123	1350.91174	186.99074	11.4892

Comment:

This HPLC of bio-betulin (Zymergen, Emeryville, CA) was performed to analyze the compound's purity and to compare it with commercial bio-betulin. The HPLC retention time found for the bio-betulin (~6.83 min) is the same as was found with that of commercial betulin (~6.85 minute). The cholesterol standard appears at ~17.91 minute. Note that there are no additional detectable peaks in the HPLC run and thus the bio-betulin is found to contain no contaminants (that absorb at 210 nm).

Sample Info: CD46 derived from commercial betulin (sample = 0.306 mg/ml, cholesterol = 0.207 mg/m).



Signal 1: DAD1 A, Sig=210,8 Ref=800,100

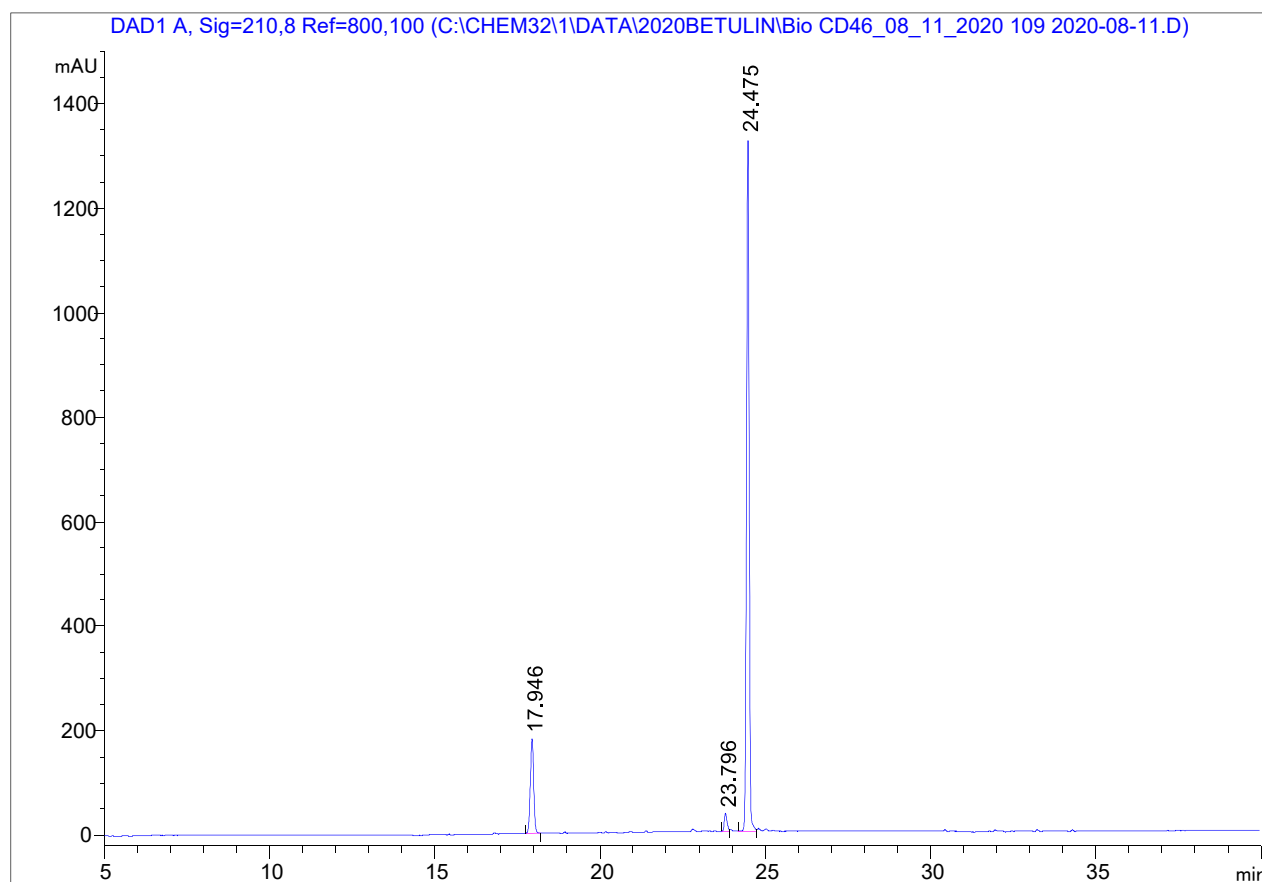
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.992	BB	0.1106	1303.26868	184.18884	8.8089
2	23.837	BB	0.0882	226.40718	37.63148	1.5303
3	24.528	VV	0.0919	6981.00195	1198.66296	47.1853

Comment:

The HPLC of **CD46** (bis-*p*-butylbenzoylbetulin diester) obtained from commercial betulin was run to analyze the purity of the compound. It was observed that the **CD46** peak appeared at 24.53 minutes. The cholesterol standard appears at ~17.99 minutes.

A small peak at 23.84 minutes due to some unidentified impurity was also observed.

Sample Info: Bio-CD46 derived from bio-betulin (sample = 0.318 mg/ml, cholesterol = 0.207 mg/ml).



Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.946	BB	0.1105	1281.51440	181.26723	8.2514
2	23.796	BV	0.0886	207.57158	35.30722	1.3365
3	24.475	VV	0.0863	7315.06299	1327.54871	47.1003

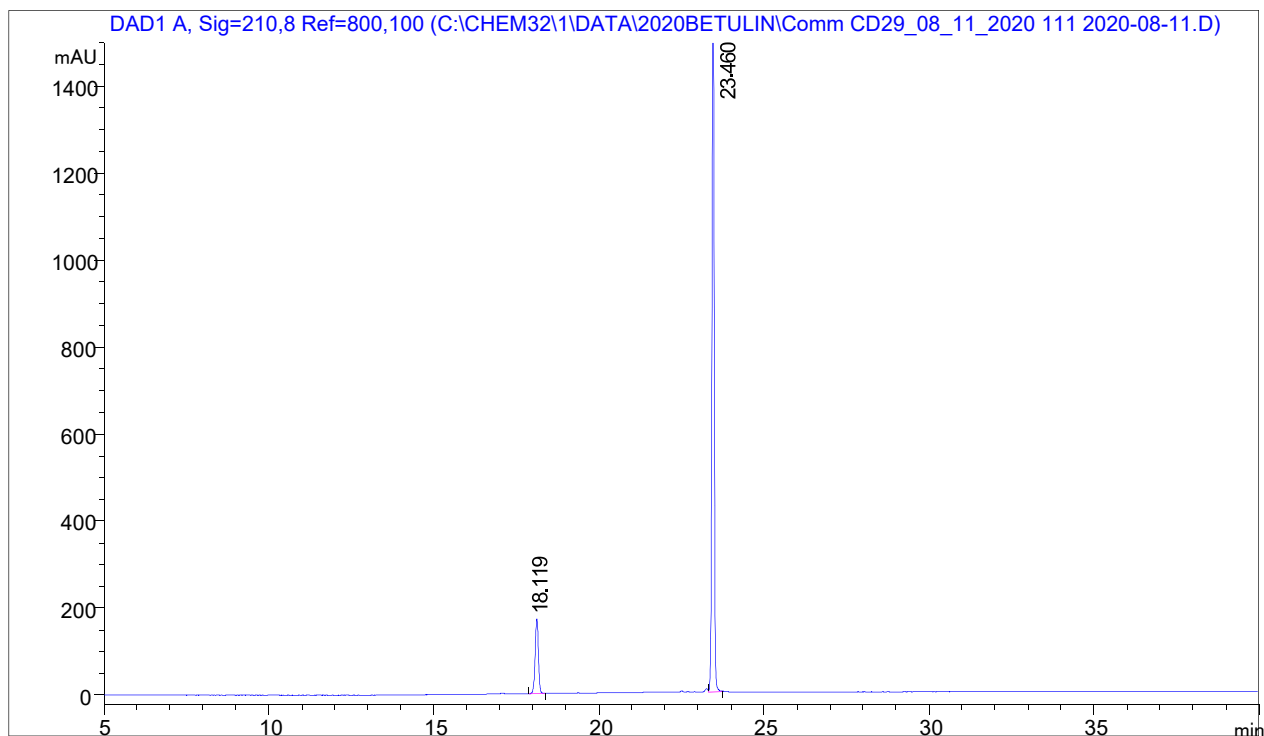
Comment:

The HPLC of **Bio-CD46** (bis-*p*-butylbenzoylbetulin diester) obtained from bio-betulin was run to analyze the purity of the compound. It was observed that the bio-CD46 peak appeared at 24.75 minutes. The cholesterol standard appears at ~17.95 minutes.

The HPLC traces of **Bio-CD46** prepared from commercial betulin and betulin supplied by Zymergen are the same (small time difference due to injection issues or temperature control).

A small peak at 23.80 minutes due to some unidentified impurity is also observed here. This same impurity peak was previously seen in the commercial **CD46** sample.

Sample Info: CD29 derived from Commercial betulin (sample = 0.350 mg/ml, cholesterol = 0.207 mg/ml).



Signal 1: DAD1 A, Sig=210,8 Ref=800,100

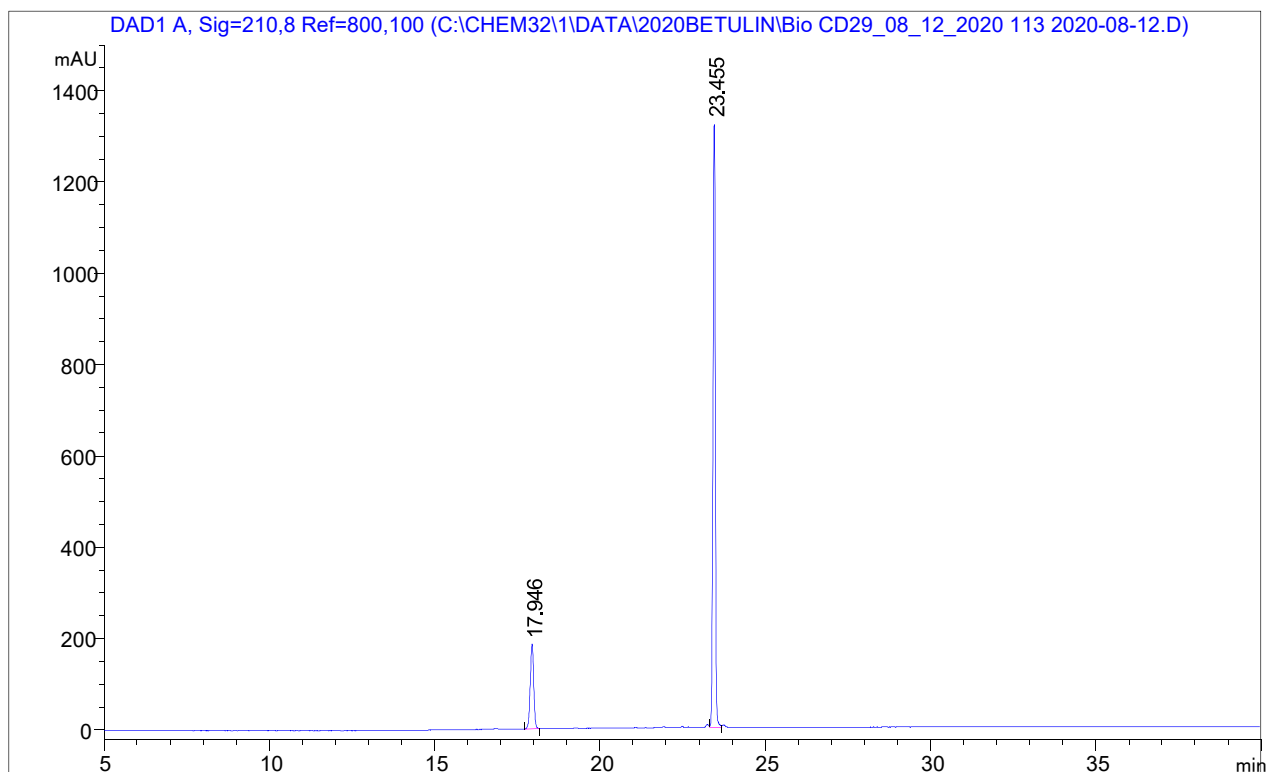
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	18.119	BB	0.1063	1184.28467	172.04953	8.0741
2	23.460	VB	0.0755	7123.36377	1498.60925	48.5651

Comment:

The HPLC of **CD29** (bis-*p*-toluylbetulin diester) obtained from commercial betulin was run to evaluate the purity of the compound. It was observed that the CD29 peak appeared at ~23.46 minutes. The cholesterol standard appears at ~18.1 minutes.

In this case no minor peaks were seen in the HPLC (only cholesterol and **CD29** were detected).

Sample Info: Bio-CD29 obtained from Bio-betulin (sample = 0.301 mg/ml, cholesterol = 0.207 mg/ml).



Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.946	BB	0.1130	1357.90381	186.38481	9.5740
2	23.455	VV	0.0776	6540.34473	1324.98071	46.1133

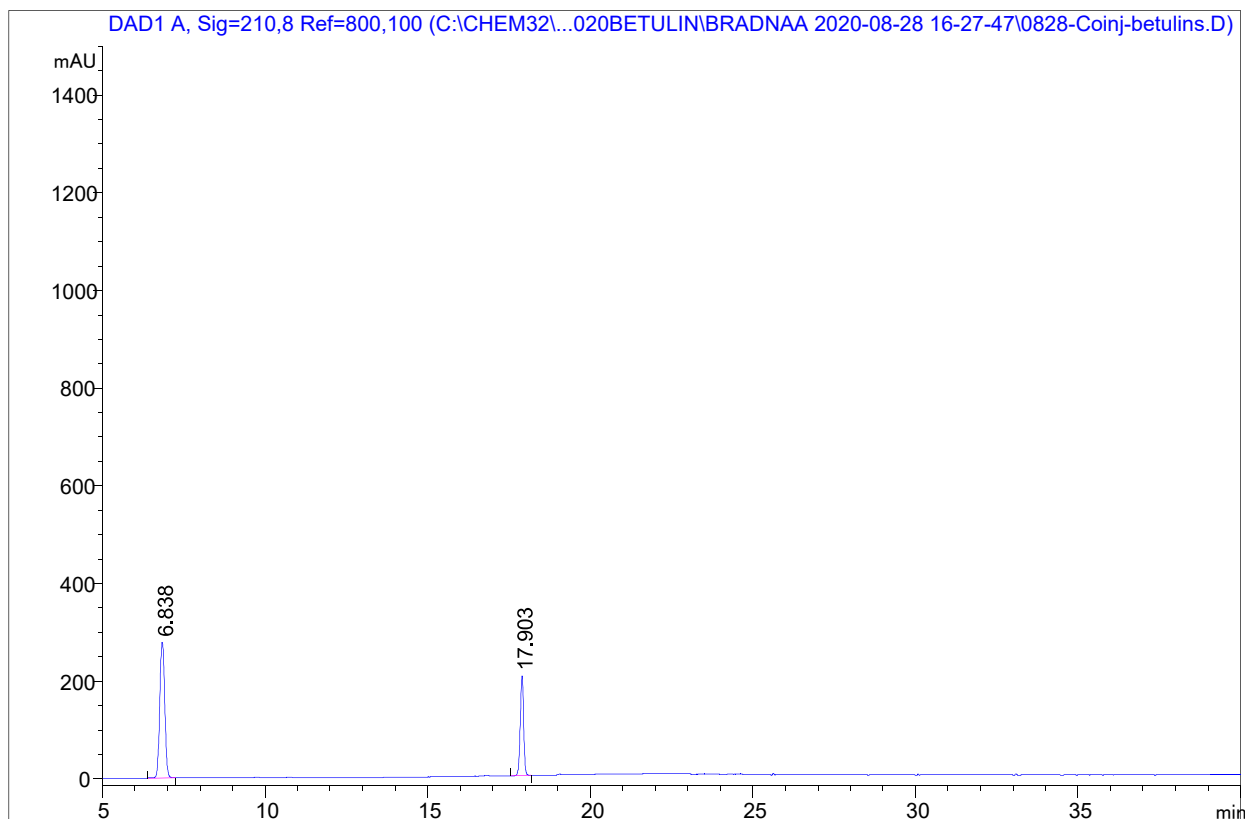
Comment:

The HPLC of **Bio-CD29** (bis-*p*-butylbenzoyl betulin diester) obtained from bio-betulin was run to analyze the purity of the compound. It was observed that the bio-CD46 peak appeared at 24.75 minutes. The cholesterol standard appears at ~17.95 minutes.

The HPLC traces of **CD29** prepared from commercial betulin and the **Bio-CD29** made from bio-betulin supplied by Zymergen are the same (small time difference due to injection issues or temperature control).

In this case no minor peaks were seen in the HPLC (only cholesterol and **Bio-CD29** were detected).

Sample Info: Commercial Betulin, Bio-betulin & cholesterol. (Betulin = (0.156 + 0.167) mg/ml, Cholesterol = 0.207 mg/ml). Co-injection.



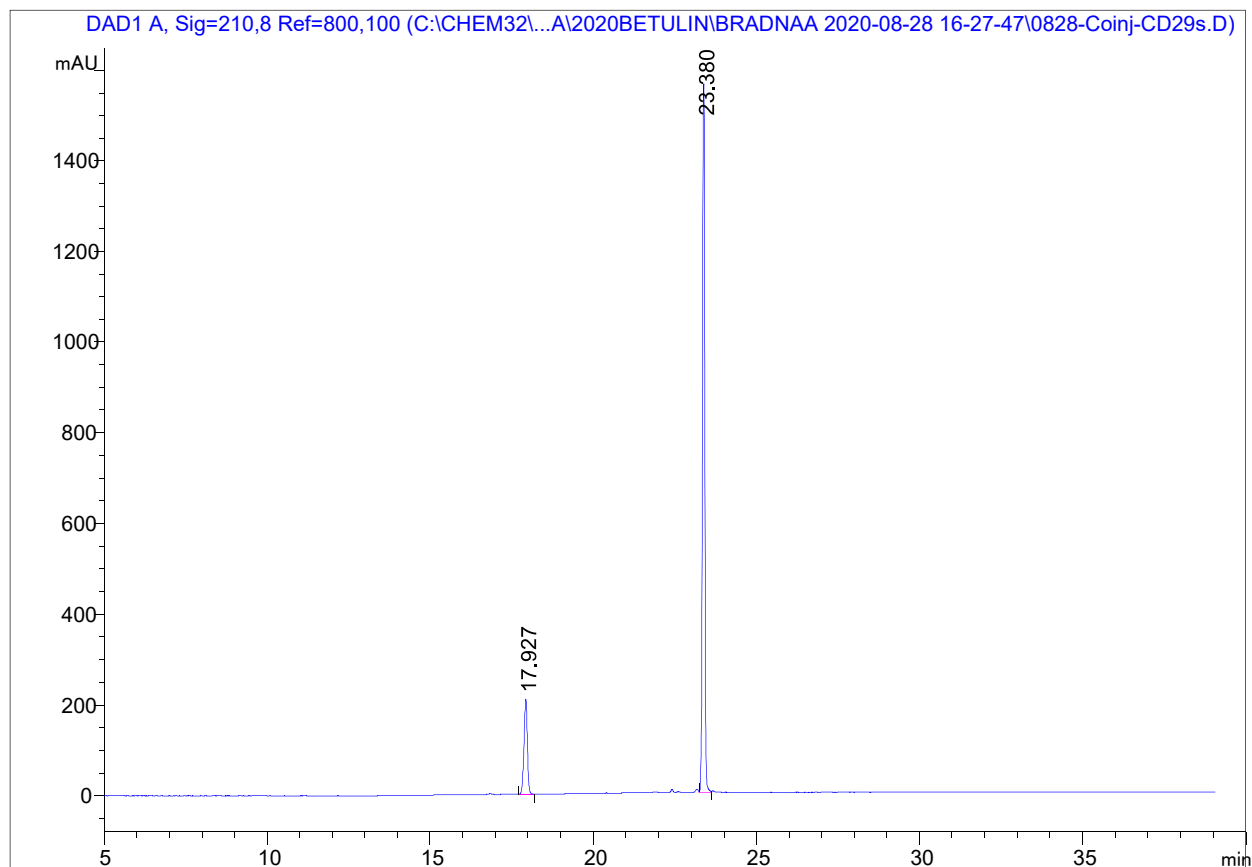
Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	6.838	BB	0.1705	2969.91821	278.28247	24.6395
2	17.903	BB	0.1122	1471.87048	203.86864	12.2111

Comment:

The HPLC co-injection of a commercial betulin and bio-betulin mixture was performed to compare the compound obtained commercially and from bio source (Zymergen). This co-injection analysis is performed to back up the analysis of the individual materials seen earlier. The co-injection HPLC trace obtained here has a single peak at ~6.84 minutes retention time indicating commercial and bio-betulin were identical. The cholesterol standard appears at ~17.90 minutes.

Sample Info: Commercial betulin derived CD29, bio-betulin derived CD29 & Cholesterol (CD29 = (0.175 + 0.150) mg/ml, Cholesterol = 0.207 mg/ml). Co-injection.



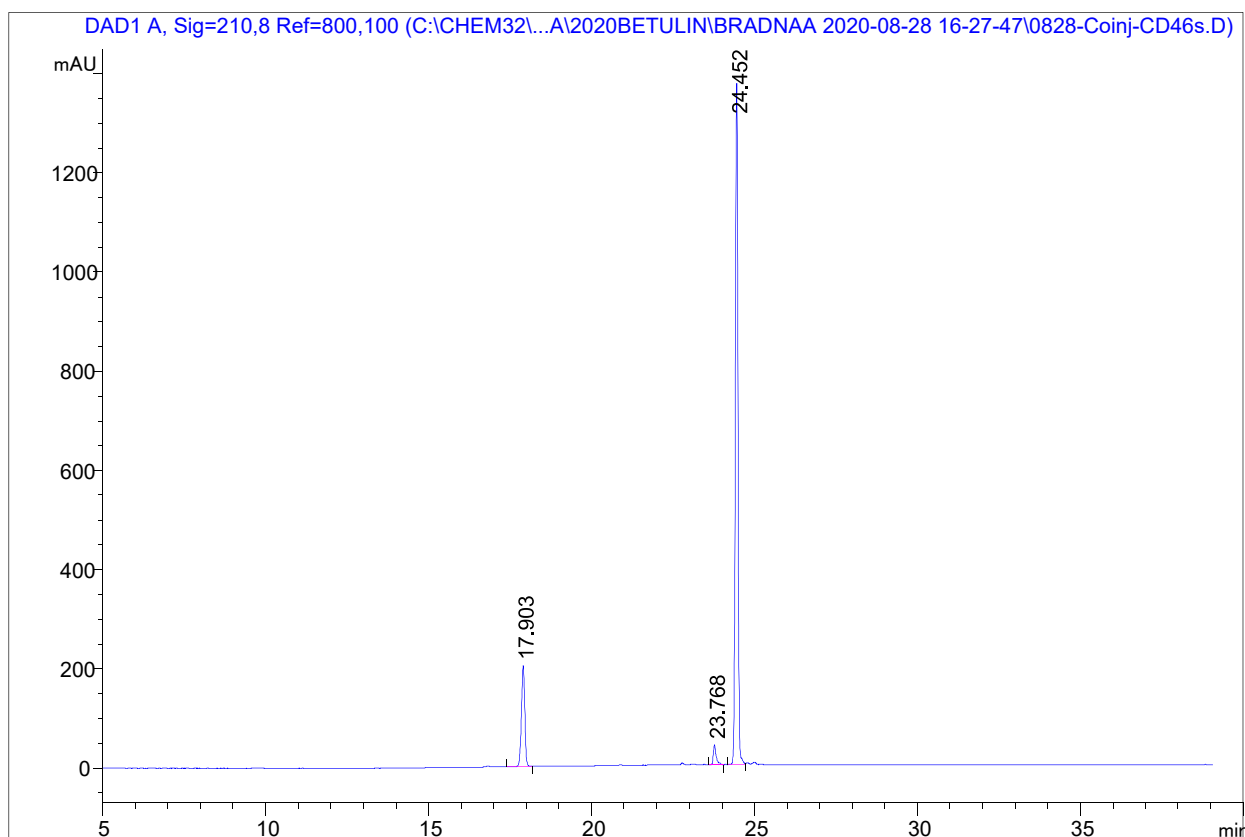
Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.927	BB	0.1120	1518.04675	210.71484	9.0503
2	23.380	VV	0.0764	7575.38477	1567.08862	45.1627

Comment:

The HPLC co-injection of commercial betulin derived **CD29** and bio-betulin derived **Bio-CD29** was performed to confirm the compound derived from the commercial source and the compound obtained from the bio source are identical. This co-injection analysis is performed to back up the analysis of the individual materials. The HPLC showed a single peak at 23.38 minutes retention time confirming the two ester derivatives are identical. The cholesterol standard appears at ~17.9 minutes.

Sample Info: Commercial betulin derived CD46, bio-betulin derived CD46 and cholesterol (CD46 = 0.153 + 0.159) mg/ml, Cholesterol = 0.207 mg/ml). Co-injection.



Signal 1: DAD1 A, Sig=210,8 Ref=800,100

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	17.903	VB	0.1123	1471.73401	203.57829	8.6928
2	23.768	VV	0.0909	255.27625	40.82912	1.5078
3	24.452	VV	0.0860	7541.87695	1374.66516	44.5462

Comment:

The analysis involves an HPLC co-injection of commercial betulin derived **CD46** and bio-betulin derived **bio-CD46**. This co-injection analysis is performed to back up the analysis of the individual materials. The HPLC shows a sharp peak at ~24.45 minutes retention time confirming both CD46 derivatives were same. The cholesterol standard appears at ~17.9 minutes. The minor peak at ~23.77 is due to some impurity. Notably, the impurity produced from CD46 derived from commercial betulin is the same as the impurity derived from the bio-betulin.

The minor product in the **CD46** preps was further investigated by intentional synthesis with samples removed at intervals, isolation of chromatography fractions and HPLC experiments. Experiments run thus far indicate that the minor product is not a monoester of betulin and is also not 4-*n*-butylbenzoic acid. The impurity likely arises from contamination of the 4-*n*-butylbenzoyl chloride (not the betulins).

Melting point table for CD29 and CD46 derived from fermentation derived and commercial betulin

#	Ref. Code	Melting Point
1	PN03011 (Bio-CD29)	207-209 °C
2	PN02075 (Commercial-CD29)	207-209 °C
3	PN03003 (Bio-CD46)	160-162 °C
4	PN02087 (Commercial-CD46)	159-161 °C

Comment:

Melting points were determined by microscopy.

The similar melting points for the **bio-CD29** derived from fermentation derived betulin and **CD29** derived from commercial betulin indicate the compounds are identical (and thus the commercial betulin and fermentation derived betulin precursors are identical).

Likewise, the similar melting points for **bio-CD46** derived from fermentation derived betulin and **CD46** derived from commercial betulin indicate the compounds are identical (and thus the commercial betulin and fermentation derived betulin precursors are identical).