Solid Phase Peptide Synthesis Methodology with Integrin α5 and Ligand Ac-RGDNP-NH2

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Abstract

Peptides are short chains of amino acids that biologically function as enzymes, hormones, receptors, antibiotics, and others. Thus, scientists have been very interested in the process of peptide synthesis, especially when comparing the differences between the methods of peptide synthesis, attempts at utilizing peptides to a degree of medicinal significance and studying the structure and chemical composition of peptides.

Solid phase peptide synthesis methodology was used to synthesize Integrin α5 and Ligand Ac-RGDNP-NH2 for future use in testing the binding affinity between the two peptides. Synthesis, final deprotection, cleavage, analytical high phase liquid chromatography (HPLC), were finished and purification begun for Ac-RGDNP-NH2. Synthesis of the first five amino acids of Integrin α5 were completed and a sample was cleaved and run under the analytical HPLC to show a chromatogram with a distinct peak with few side reactions despite problems during synthesis. Results from mass spectrometry and HPLC chromatograms indicate successful synthesis.

Solid Phase Peptide Synthesis Methodology with Integrin α5 and Ligand Ac-RGDNP-NH2

Introduction

Peptide synthesis is the process of making peptides, which are short chains of amino acids, chemically. There also exist biological methods to extract peptides from living organisms. Synthesis, however, occurs either by solid phase peptide synthesis (SPPS), founded by Robert Merrifield, where amino acids are attached one by one to a solid support matrix called a resin, or through solution phase peptide synthesis where synthesis occurs in a liquid. Solid phase peptide synthesis also employs washing and filtration to remove remaining reagent.

In SPPS, reactive –OH groups in the resin are reacted and bound with the carboxyl group of a amino acid; the amino acid protecting group (in many cases, 9-fluorenylmethyl carbamate (Fmoc) protecting groups) is then removed, thus deprotecting the amino acid and exposing its reactive groups so that it may be coupled with the next amino acid in the sequence. Synthesis proceeds from the C (carboxyl) to N (amine) terminal. At the end, the peptide is cleaved from the resin. Fmoc protection groups are often used instead of the tert-Butyloxycarbonyl **(**tBoc) protecting groups mostly because Fmoc can be easily removed with a weak base such as piperidine as well as its ability to have the peptide cleaved by the use of an acid such as trifluoroacetic acid (TFA), which is preferential to the hydrofluoric acid (HF) that the tBoc cleavage requires.

Since peptides have many biological roles in the body, including acting as hormones, enzymes or antibiotics, they are synthesized often for medical use, especially in lieu of relatively recent research that reveals that peptides may be used for the treatment of Type 2 diabetes.

Two types of peptides are integrins, which were originally described as cell-surface receptors that integrate the extracellular matrix and the intracellular cytoskeleton for cell migration and adhesion, as first described in the 1987 article “Cell” by R.O. Hynes, and ligands, which are typically an extracellular membrane protein that bind to the integrin (1). The ligand is also often a RGD peptide. Thus, both ligands and segments of integrins are synthesized so that the binding may be evaluated. After the synthesis of both the ligand and segments of the integrin, the attempts are made to bind the ligand to each segment of the integrin so as to find the location of the active site; then, the binding strength is measured. With strong enough binding affinities, peptides may eventually used for therapeutics in diseases that have been shown to result when peptides are not binding or functioning properly, such as cancer.

The focus of this paper will be on the process of peptide synthesis of both an integrin segment, Integrin α5, and a RGD peptide that operates as a ligand to Integrin α5, Ac-RGDNP-NH2. This synthesis is carried out with solid phase peptide synthesis (SPPS) methodology where amino acids are attached to a solid support matrix, called a resin, one

by one while removing soluble reagents during the synthesis process by filtration and washing and then after the desired sequence is achieved, the peptide is cleaved from the resin. 9-Fluorenylmethyl carbamate (Fmoc) protecting groups were used on all of the amino acids to protect amine functional group of the growing peptide as each new amino acid was coupled.

Procedure

Chemicals.
Resin used for both peptides was Rink Resin SS, 100-200 mesh, 1% DVB purchased from Advanced Chemtech in Louisville, KY. Fmoc-Pro-OH, Fmoc-Asn(Trt)-OH, Fmoc-Leu-OH and Fmoc-Gly-OH were purchased from Benn Chemicals in Dielsdorf, Switzerland. Fmoc-Arg(Pbf)-OH was purchased from Peptides International. Fmoc-Tyr(tBu)-OH, Fmoc-Ser(tBu)-OH and Fmoc-Asp(OtBu)-OH from Novabiochem in San Diego, CA.
All other chemicals and solvents were of the highest purity available (>98%) and were used as received.

Resin Preparation.
For both the synthesis of Integrin α5 and Ac-RGDNP-NH2, rink amide resin (0.75 mmol/g) was weighed out to a measure of 0.333g with a possible excess of up to 0.005g (exact mass used in shown in Table 1 for Integrin α5 and Table 2 for Ac-RGDNP-NH2 in the Results section of this paper) and placed in a plastic reaction vessel (SPE Accessories 12mL polypropylene cartridges with 20μ PE frit). Since the resin out of the bottle is very dry and the couplings occur within the resin, the resin must be swelled up with dimethylformamide (DMF). Roughly 6mL of DMF was added into the reaction vessel and stirred with a glass stirring rod over 1 minute. The resin was then left in the DMF for 10 minutes, where the resin is stirred in the vessel approximately every three minutes for at least 20 seconds per stirring.

Ninhydrin and Trinitrobenzenesulfonic Acid (TNBS) Tests.
Ninhydrin and TNBS tests were then performed to firstly confirm a negative result and also to show what a perfectly negative result looks like. This was done by taking a 9" disposable glass Pasteur pipette, and two disposable glass culture tubes. The Pasteur pipette was inserted into the reaction vessel to collect some beads of resin and the tip was dipped into the bottom of each culture tube. The culture tube with more beads in it was be used for the ninhydrin test, which consists of adding one drop of each ninhydrin A (ninhydrin with ethanol), B (phenol with ethanol), and C (KCN in pyridine) solution and heating the tube for 3 minutes at 100 degrees Celsius in the Thermolyne Dri-Bath. It is negative if the beads are clear and the solution is yellow. It is positive if the solution turns blue and there is color on the beads.

The TNBS test was be conducted with the remaining culture tube. One drop of TNBS A (dilution of DIEA in DMF) and B solutions (TNBS) (which are kept refrigerated) are added to the tube and let sit for 10 minutes. After ten minutes, if the beads are clear, the

result is negative. If the beads are red, the result is positive. In this case, both tests turned out negative for both resins.

Deprotection.
Since the resin is protected with Fmoc protecting groups, it must be deprotected so that the first amino acid has something to bind to. Since all amino acids are protected with Fmoc deprotecting groups, deprotection was done before each coupling.

Deprotection began with draining DMF in the reaction vessel and treating the resin with roughly 6 mL of 20% piperidine in DMF over 5 minutes, placing the cap on the reaction vessel (note: when the cap is removed, rinse the cap with DMF over the reaction vessel so that any resin stuck to the cap will by rinsed back into the vessel), turning the reaction vessel to mix the DMF with the resin, releasing the pressure by opening the valve that is attached to the reaction vessel, and then placing it on the stirrer until the time is up. Then, the solution was drained and a fresh 6mL of 20% piperidine/DMF was added, the vessel was capped and turned, pressure released, and placed on the shaker (Barnstead Thermolyne LabQuake shaker) for 30 minutes.

During the 30 minute deprotecting, the appropriate mass of the first amino acid was weighed out (for Ac-RGDNP-NH2, that was Proline, and for Integrin α5, that was Glycine), using the scale into a glass vial, and then 2ml of 0.5M HBTU/DMF, 1mL of 1.0M HOBt/DMF, and 0.348mL of diisopropylethylamine (DIEA) was added into the glass vial containing the dry amino acid with separate syringes. The vial was then vortexed until the amino acid was dissolved completely.

When the 30 minute piperidine treatment is over, remove the cap, drain the solution, and wash the resin by adding enough DMF into the vessel to cover the resin and stir with a stirring rod for 1 minute. Repeat three times and deprotection is over.

Coupling.
Coupling is then the process of adding each amino acid to the growing peptide chain (or to the resin, if it is the first amino acid). The solution in the reaction vessel was drained, and the solution containing the amino acid was added into the reaction vessel. The vessel was capped and turned, pressure released, and placed on the stirrer for 2 hours. After the two hours of coupling, the amino acid solution was drained and the resin was washed with DMF three times for 1 minute each.

Conduct a ninhydrin and TNBS test – the result should be negative if coupling was successful. If there was a positive result in the test, a fresh solution in half scale should be prepared (half the mass of amino acid 1mL 0.5M HBTU/DMF, 0.5 mL 1.0M HOBt/DMF, 0.174 mL DIEA) and used to treat the resin over another 2 hours. Then wash the resin and check the ninhydrin test gain.

If there is a slightly positive ninhydrin test, the resin should be treated with 0.475 mL acetic anhydride in DMF over 10 minutes, and then washed (3 times, 1 minute each) and ninhydrin test redone.

The process for deprotection and coupling was then repeated until the last amino acid in the sequence was successfully coupled.

Chloranil Test.
For Ac-RGDNP-NH2, after coupling Asparagine, a chloranil test was conducted by using a pipette to transfer a small sample of resin to a glass culture tube and then adding one drop of Chloranil A (dilution of 2% acetaldehyde in DMF) and Chloranil B (dilution of 2% chloranil in DMF) to the culture tube. If it is negative, it will look the same as a negative ninhydrin test with a slight green tinge in the solution.

Capping.
After coupling the last amino acid, Ac-RGDNP-NH2 was first deprotected and then "capped" with acetic anhydride in DMF. This was done by adding approximately 0.5 mL of acetic anhydride into the reaction vessel and adding DMF until the resin was covered. It was then put on the stirrer over 13 minutes. Ninhydrin and TNBS test were conducted to assure a negative result.

Cleavage During Synthesis.
Integrin α5 was cleaved after the coupling of the first five amino acids. The process for this was: Label a SPE Accessories 3mL polypropylene cartridges with 20μ PE frit and use a pipet to transfer roughly 10 mg of resin into the small cartridge. Wash the resin five times with dichloromethylene (DCM) and dry the washed resin under the dessicator over 20 minutes. Add one drop of triisopropylsilane, 1 drop dimethyl sulfide, 2 drops anisole, 1 drop ethanedithiol, and 2 mL of TFA to the vessel. Cap it with the cap plug and cover it with aluminum foil. Shake over 90 minutes. Drain the TFA solution into a 15ml polypropylene tube (Falcon brand) after first weighing the weight of the empty polypropylene tube with its cap. Rinse the resin in the 3mL polypropylene cartridge with TFA and filter into the 15mL tube. Evaporate the TFA solution in the 15mL tube to a volume of 0.5mL with a gentle stream of nitrogen gas. Add 10 mL cold ethyl ether to the concentrated TFA solution, immediately vortex it for 20 seconds and release the pressure. Weigh the tube with the peptide and TFA in it and match the weight of it with another 15 mL tube filled with water. Centrifuge the tube using the water-filled tube as a balance over 10 minutes. Decant the ether solution slowly away while not disturbing the precipitate on the bottom and add 10mL cold ether to the precipitate and vortex it over 1 minute and release the pressure. Balance another tube to match the weight of the ether and peptide and centrifuge for 10 minutes again. Decant the ether solution away and dry the peptide precipitate under vacuum over 3 hours and then analyze using the analytical HPLC.

Final Cleavage.
The resin was washed with dichloromethylene (DCM) three times for one minute each (same procedure as with DMF washings, only with DCM) and placed under the desiccator overnight.

A 50 mL polypropylene tube (Falcon brand) was weighed while dry and empty with its cap (coming out, in this case to a measure of 13.0103 g). The weight was recorded on the tube, and the dried resin was transferred using a spatula from the reaction vessel to the tube. A small magnetic stir bar was also added. After attaching the tube to a clamp under the fume hook, 1mL of anisole, 0.5mL of dimethyl sulfide, 0.5 mL of ethanedithiol, and 18 mL of
trifluoroacetic acid (TFA) were added to the resin. The tube was capped and covered with aluminum foil and placed on a magnetic stirrer over 90 minutes.

A second 50 mL polypropylene tube with its cap on was weighed and its mass recorded (in this case, 13.0103 g).

The second tube was placed on a lower clamp, and the old reaction vessel was placed on a higher clamp with the lower opening over the top opening of the tube. The reaction vessel was used to filter out the solution in the first tube so that the peptide would drip into the second (lower clamp) flacon tube. This is done by transferring the solution in the first tube into the reaction vessel using a Pasteur Pipet and rubber bulb and then allowing the solution to drip through the filter paper on the bottom of the reaction vessel.

The resin remaining in the first tube was then rinsed with 2 mL of TFA and 2 mL of DCM and then drained into the second tube using the reaction vessel still. Rinse the reaction vessel used for filtration with bleach and leave it more than one day before disposal. Also rinse all pipettes used during cleavage with bleach and leave them for more than one day before disposal.

Using a gentle stream of nitrogen gas, the TFA solution in the tube in the lower clamp was evaporated to a volume of approximately 3 mL. Forty mL of cold ether was added to the concentrated TFA solution and immediately vortexed for over 30 seconds. The pressure was released and the cap was replaced onto the tube. Weigh the tube now filled with peptide precipitate and ether and acquire another tube and fill that tube with water until the weight of the second tube matches the tube with ether in it. This is used to balance the centrifuge. Centrifuge the precipitate for 10 minutes, making sure to have the balancing tube directly across from the tube with the peptide and ether in it. Decant the ether solution slowly away into an ether disposal bottle while trying not to disturb the precipitate on the bottom by just pouring out the liquid in the tube. Add cold ether again to the precipitate, vortex over 1 minute until the precipitate is mixed into the ether, and release the pressure. Weigh the tube with ether in it, match that weight with a balancing tube, and centrifuge again for 10 minutes. Decant the ether solution away again, trying to not disturb the precipitate on the bottom. Open the cap of the tube and replace without screwing it on tightly and place under the desiccator over more than six hours. Weigh the tube with the peptide and write on both the tube and the cap of the tube. Calculate the weight of the crude peptide by subtracting away the mass of the empty tube and cap. Record this number in a notebook or on the tube. Wrap the cap to the tube with parafilm after screwing it on tightly and store in the freezer before analysis and purification

HPLC Analysis.
To prepare solutions of the peptide for purification and analysis, the dried peptide was dissolved in approximately 1mL of AcOH/Water (1:1) in the tube and vortexed until thoroughly dissolved. Three 1.5 mL tubes (eppendorf) were labeled and the solution in the tube was divided equally among the three tubes using a Pasteur pipet and rubber bulb to transfer the solution. All three tubes were centrifuged for 10 minutes, and then the clear solution was transferred into three new 1.5 mL tubes.

The insert (National Scientific C4011-631 Target Micro-Serts) was placed inside a Target DP Vial and the peptide solution from one of the centrifuged tubes was transferred using a pipette into the insert until it was about half full. The Agilent technology 1100 Series HPLC system with the Vydac C18 analytical HPLC Columns was set up and run with a gradient of 0% to 50% in 25 minutes with injection volumes of 5 µl at a wavelength of 230 nm and was also run under isocratic conditions over 20 minutes. Chromatograms are shown in the results section under figure 1.1 and 1.2.

Mass spectrometry.
Because two peaks resulted in the analytical chromatograms, both peaks were collected and sent to mass spectrometry analysis to determine which peak is the peptide so that purification of the proper peak may result. Results from mass spec are shown in figure 2.1 and 2.2. It was revealed that the second peak from the chromatogram contained the peptide.

Purification.
Purification of Ac-RGDNP-NH2 should occur under the isocratic conditions of 3% over a period of 9 minutes, observed at a wavelength of 230 nm using a C4 column on the prep HPLC with injection volumes of 25 µL. The retention time of the second peak should be approximately 6 min. A Vydac C4 semi-preparational HPLC Column was used with an Agilent technology 1100 Series HPLC system. The peak was collected into three test tubes, the first test tube containing part "A" of the peak, which is the initial rise, part "B" of the peak, which is the middle and highest point of the peak, and part "C" which is the descending portion of the peak. After being collected into test tubes, the liquid in each of the test tubes should be transferred into respective A, B, and C 50mL polypropylene tubes that have been weighed when empty and with a cap.

Results and Conclusions

Ac-RGDNP-NH2 was synthesized with little problem, requiring no 2nd couplings or special treatment. Couplings were done each in two hours, with ninhydrin and TNBS tests yielding negative results, confirming that coupling was successful with each amino acid. Details are given in Table 1. During synthesis, after glycine was coupled, black impurities were noticed floating in the reaction vessel. Analytical chromatograms, shown in figure 1.1 and 1.2, however, showed two peaks, requiring the peptide to be sent to mass spec analysis (form shown in table 2) to determine which peak contained the peptide. The peptide was run under both a gradient and under isocratic conditions, as described in the Procedure of this paper.

Synthesis of Integrin α5 had several problems, mostly requiring either second couplings or capping after the first coupling. Ninhydrin tests usually came out with no color on beads but with a slightly brownish tinge in the solution whereas TNBS tests came out either slightly positive or positive. Details are listed in Table 3. Black impurities were noticed in the reaction vessel after the coupling of glycine, just like it was in Ac-RGDNP-NH2. Additionally, problems with coupling started to arise after the coupling of glycine. After the capping of tyrosine, capping with acetic anhydride stopped being as effective as TNBS tests after capping still came out slightly positive instead of negative. No second capping was done so as to avoid excessive side reactions. Chromatogram of the peptide after coupling of the first Aspartic Acid is shown in Figure 3.1

Synthesis of Integrin α5 was not completed due to time constraints and will be a future work.
Other future works include: another trial of the synthesis of Integrin α5 to fix the coupling problem, purification of both Ac-RGDNP-NH2 and Integrin α5, testing the binding of Integrin α5 to Ac-RGDNP-NH2, attempting larger-scale synthesis of both Integrin α5 and Ac-RGDNP-NH2.

Works Cited

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3. Knudsen, L. B., Journal of Medical Chemistry, **2004.** vol. 47.

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Table 1

Ac-RGDNP-NH2

No.	Amino Acid	Mass	1st Coupling
	NH2	0.3347 g	
5	Pro Fmoc-Pro	0.3439 g	2 Hours Ninhydrin: (−) TNBS: (−)
4	Asn Fmoc-Asn(Trt)	0.6011 g	2 Hours Chloranil: (−)
3	Asp Fmoc-Asp(OtBu)	0.4177 g	2.5 Hours Ninhydrin: (−) TNBS: (−)
2	Gly Fmoc-Gly	0.3028 g	2 Hours Ninhydrin: (−) TNBS: (−)
1	Arg Fmoc-Arg(Pbf)	0.6538 g	2 Hours Ninhydrin: (−) TNBS: (−)
	Ac	0.5 mL + DMF	13 minutes Ninhydrin: (−) TNBS: (−)

Table 2

Mass Spectrometry of Ac-RGDNP-NH2

Sequence	Ac-Arg-Gly-Asp-Asn-Pro-NH2											
	Ac-RGDNP-NH2											
		C	H	N	O	S	Exact	Avg			Exact	Avg
Ala	0	3	7	1	2		89.048	89.095		C	12.0000	12.0112
Arg	1	6	14	4	2		174.112	174.204		H	1.0078	1.0079
Asn	1	4	8	2	3		132.054	132.120		N	14.0031	14.0067
Asp	1	4	7	1	4		133.038	133.104		O	15.9949	15.9994
Cys	0	3	7	1	2	1	121.020	121.161		S	31.9721	32.0660
Glu	0	5	9	1	4		147.053	147.132		F	18.9984	18.9984
Gln	0	5	10	2	3		146.069	146.147		Cl	34.9689	35.4527
Gly	1	2	5	1	2		75.032	75.068		Br	78.9183	79.9094
His	0	6	9	3	2		155.070	155.157		I	126.9045	126.9045
Ile	0	6	13	1	2		131.095	131.176		P	30.9738	30.9738
Leu	0	6	13	1	2		131.095	131.176		Si	27.9769	28.0855
Lys	0	6	14	2	2		146.106	146.190		Na		
Met	0	5	11	1	2	1	149.051	149.215		K		
Phe	0	9	11	1	2		165.079	165.193				
Pro	1	5	9	1	2		115.063	115.133				
Ser	0	3	7	1	3		105.043	105.094				
Thr	0	4	9	1	3		119.058	119.121				
Trp	0	11	12	2	2		204.090	204.230			Samples:	
Tyr	0	9	11	1	3		181.074	181.193			AH1 P1	
Val	0	5	11	1	2		117.079	117.149			AH1 P2	
No. of AA	5											
P		21	35	9	9	0	557.256	557.567				
C-NH2	1		1	1	-1		-0.984	-0.985				
Ac	1	2	2		1		42.011	42.038				
Biotin		10	14	2	2	1	226.078	226.301				
Fmoc	0	15	10		2		222.068	222.245				
Aloc	0	4	4		2		84.021	84.075				
OAll		3	4				40.031	40.065				
tBu		4	8				56.063	56.108				
Boc		5	8		2		100.052	100.118				
Trt		19	14				242.110	242.323				
Pbf		13	16		3	1	252.082	252.336				
Lactam			-2		-1		-18.011	-18.015				
Disulfide			-2				-2.016	-2.016				

Table 2 continued

							Exact	Average				
Peptide		23	38	10	9	0	598.283	598.620				
[M+H]+							599.283	599.620				
[M+2H]2+							300.141	300.310				
[M+3H]3+							200.428	200.540				
[M+4H]4+							150.571	150.655				
[M+5H]5+							120.657	120.724				

Order form sent to mass spectrometry analysis shows that the exact peptide mass should be 598.283 grams with a average mass of 598.620 g. Using this information, Results from mass spectrometry analysis revealed that the second peak in the chromatogram in figure 1.2 contained the peptide, thus giving the peptide an approximate retention time of 6 minutes.

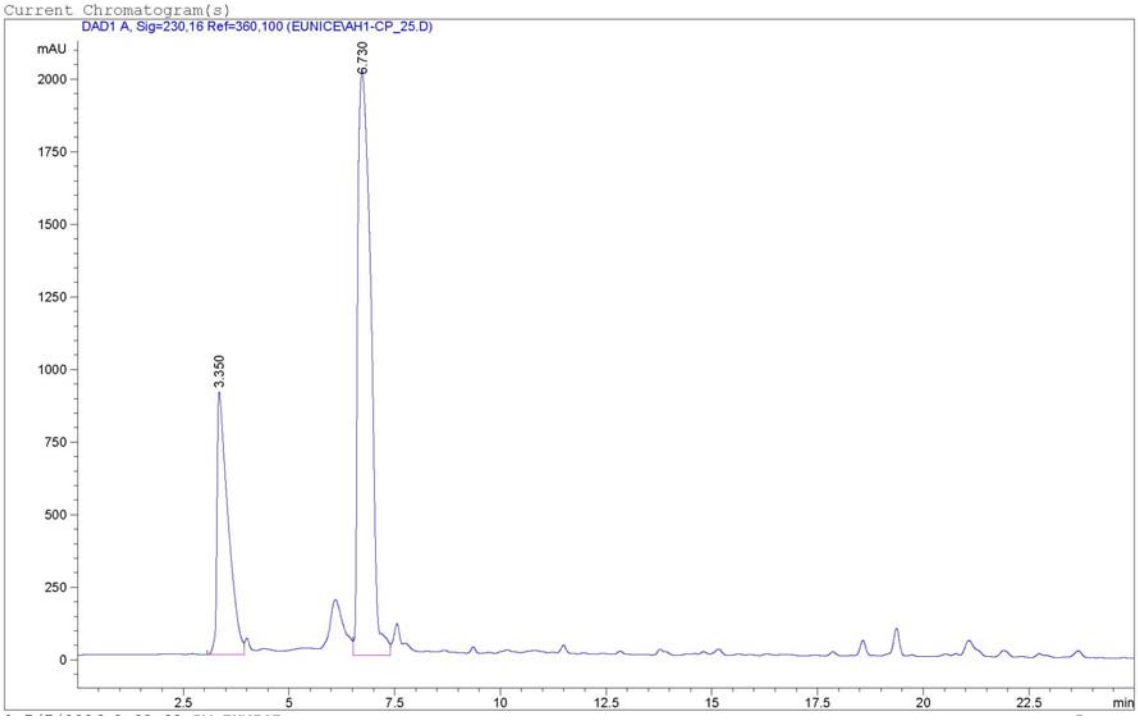
Table 3

Integrin α5

No.	Amino Acid	Mass	1st Coupling Ninhydrin/TNBS	2nd Coupling Ninhydrin/TNBS	3rd coupling or capping Time/TNBS
	NH2	0.3390 g			
11	Fmoc-Gly-OH	0.3017 g	2 Hours (−)/ (−)		
10	Fmoc-Leu-OH	0.3585 g	2 Hours (−)b/ (+)	2 Hours (−)/(s +)	Cap 10 min/(−)
9	Fmoc-Tyr(tBu)-OH		2.5 Hours (−)b/(s+)		Cap 10 min/(−)
8	Fmoc-Ser(tBu)-OH		2 Hours (−)b/(s+)		Cap 10 min/ (s+)
7	Fmoc-Asp(OtBu)-OH		2 Hours (−)b/(s+)		Cap 10 min/ (s+)
	CLEAVE	For	Analytical	HPLC	
6	Fmoc-Asp(OtBu)-OH				
5	Fmoc-Phe-OH				
4	Fmoc-Ile-OH				
3	Fmoc-Ala-OH				
2	Fmoc-Gln(Trt)-OH				
1	Fmoc-Ala-OH				
	Ac				

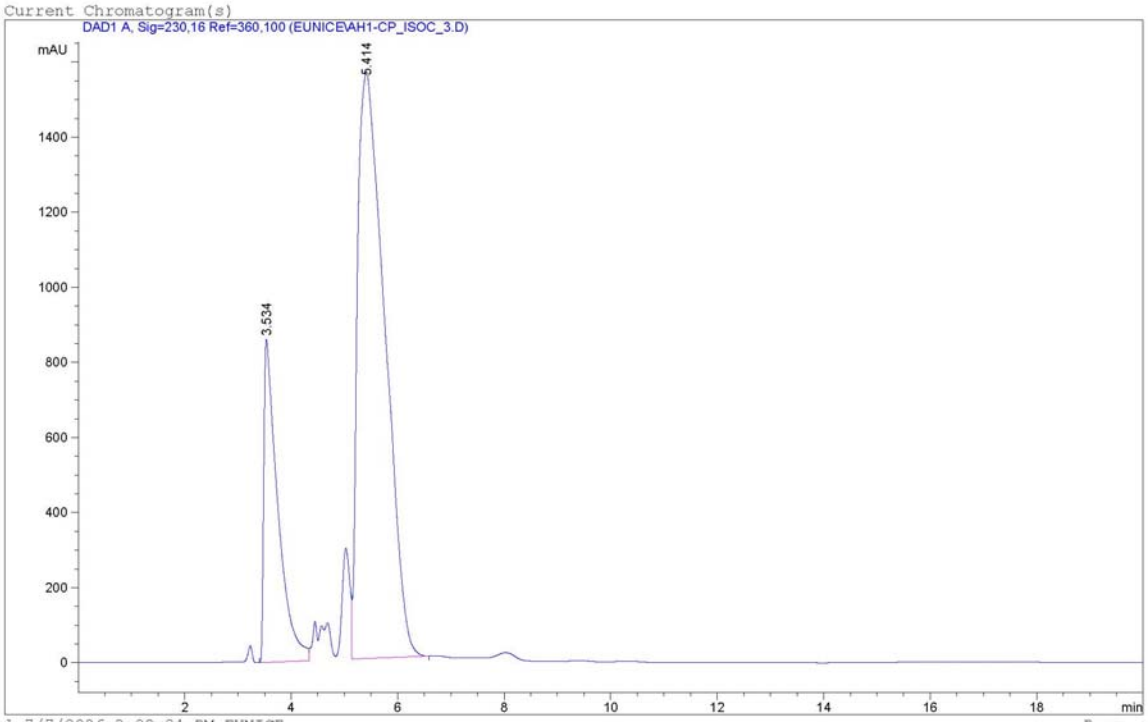
A "(−)b" after a Ninhydrin test indicates that the test was negative, with no color on the beads, but a light brownish tinge in the solution. A "(s+)" after a TNBS test means that the test came out slightly positive with a light orange color appearing on the beads of resin.

Figure 1.1
Chromatogram of Ac-RGDNP-NH2 after removal of Fmoc deprotection groups and final cleavage



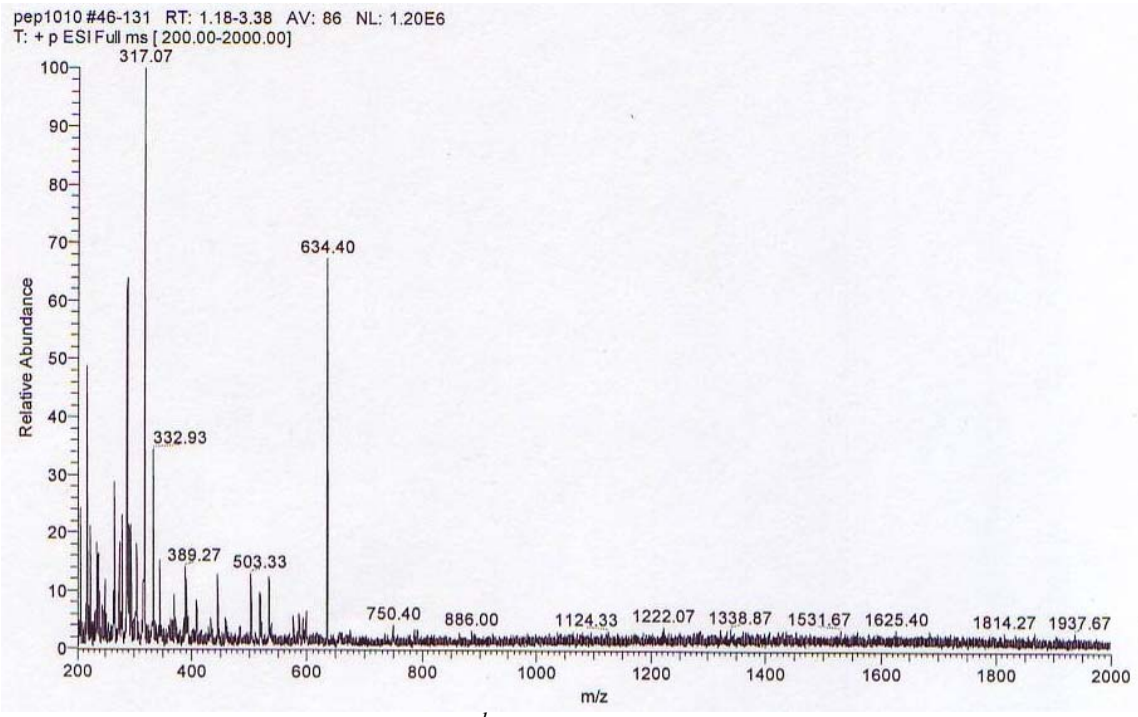
Method: Gradient from 0% to 50% over a period of 25 minutes, with a 2% change per minute; Injection volume: 5 µl; Column used:C18; Wavelength: 230 nm

Figure 1.2
Chromatogram of Ac-RGDNP-NH2 after removal of Fmoc deprotection groups and final cleavage



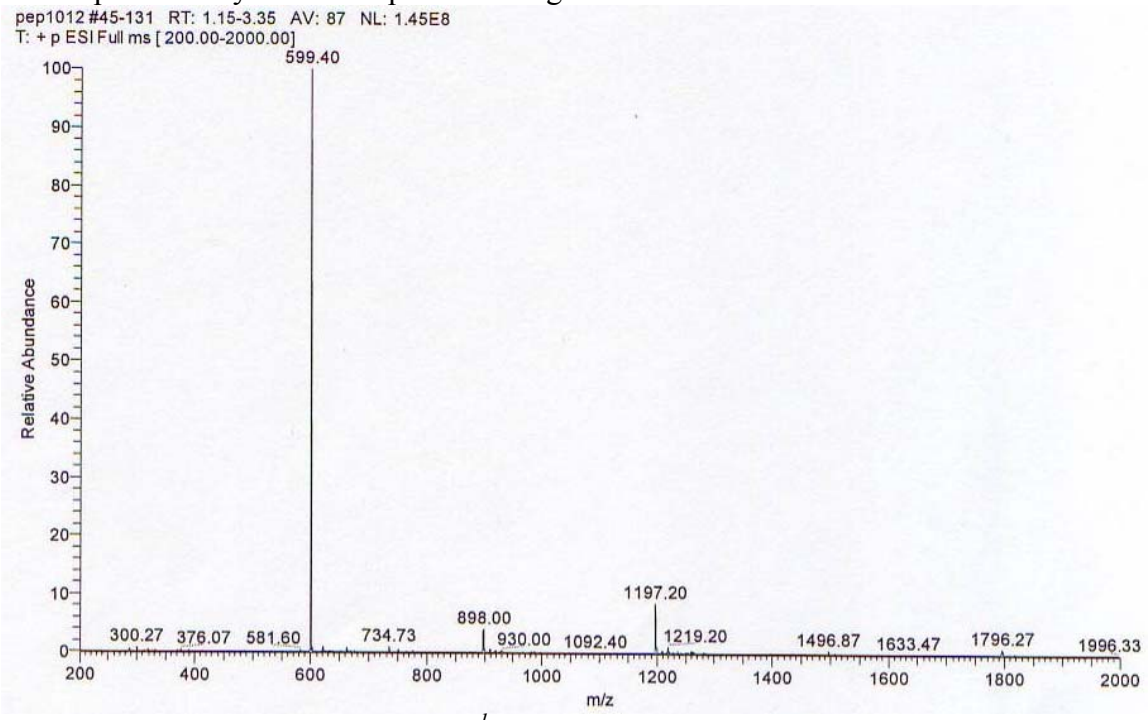
Method used: Isocratic over 20 minutes; Injection volume: 5 µl; Column used: C18; Wavelength: 230 nm

Figure 2.1
Mass Spectrometry of first peak from figure 1.2 in Ac-RGDNP-NH2



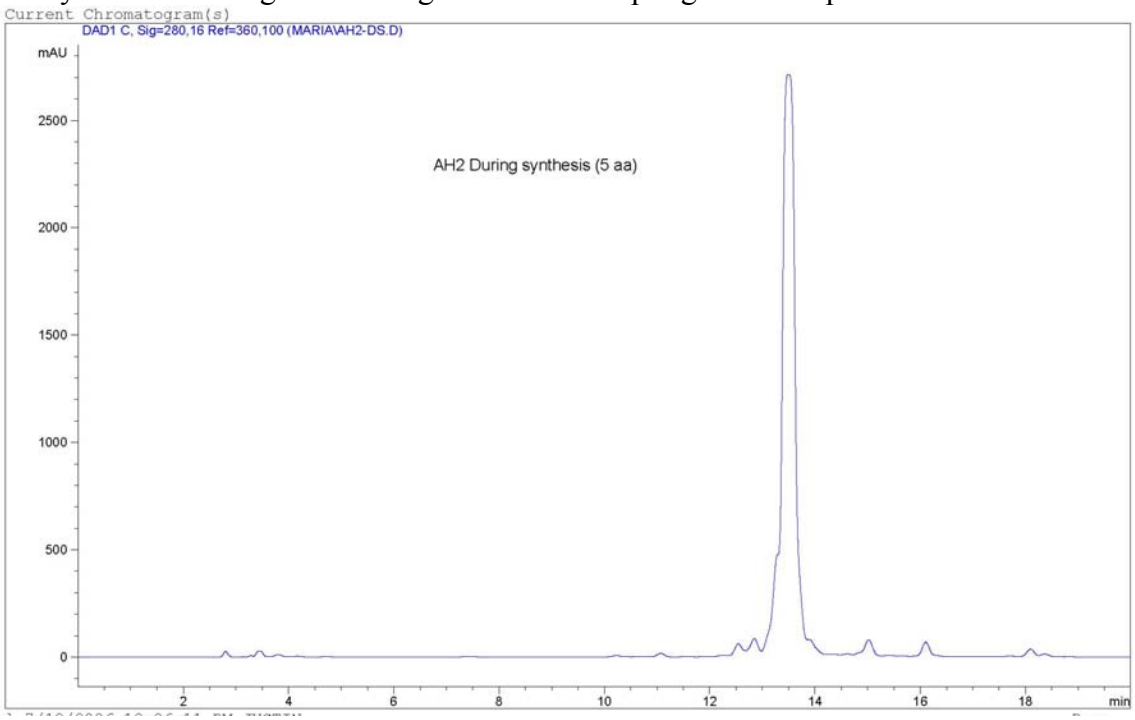
No dilution, direct infusion 10µl.min^{-1}. The spectrum does not contain peaks of a species with the expected mass 598.3 g. (The 634.4 peak is present most probably because of carryover from the previous run.)

Figure 2.2
Mass Spectrometry of second peak from figure 1.2 in Ac-RGDNP-NH2



No dilution, direct infusion 10µl.min^{-1}. The Spectrum contains peaks of a species with the expected mass 598.3g:
$[M+H]^+$ 599.4g

Figure 3.1
Analytical chromatogram of Integrin α5 after coupling of first aspartic acid



Injection volume:40µl ; Column used:C18 ; Wavelength:280nm
Despite problems during synthesis, chromatogram shows one defined peak with few side reactions.