The Development of a Leptin Receptor Antagonist Through Peptide and Peptoid Synthesis and Analysis via Surface Plasmon Resonance

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer characterized by its lack of estrogen receptors, progesterone receptors, and human epidermal growth factor 2 receptors. Because these receptors are not found within TNBC, a targeted therapeutic against TNBC has yet to be developed. We believe that there is sufficient evidence to suggest that targeting the leptin receptor (ObR) may elicit therapeutic affects against TNBC. Known antagonists to ObR include the peptide molecules LDFI and Allo-aca. A general problem facing peptide therapeutics is a characteristic 10-12 minute half-life in the human body due to non-specific proteases, not allowing enough time for the intended mechanism of action to have its desired effect. To address this issue, we created peptoid peptidomimetic versions of LDFI and Allo-aca in the forms of N-LDFI and N-Allo-aca-biotin. Peptoids are not subject to non-specific protease degradation and therefore have no known general half-life within the human body while also retaining very similar structures to their peptide counterparts. We used localized surface plasmon resonance, which detects binding affinities between molecules, to determine if N-LDFI and N-Allo-aca had similar binding affinities to ObR in comparison to LDFI and Allo-aca. Through this testing we were able to determine that LDFI has a binding affinity to ObR of K_d = 2.32×10^{-8} M and that N-LDFI does bind to ObR, though we are not confident of its binding affinity. We were unable to determine the binding affinities of Allo-aca and N-All-aca to ObR due to what we believe were either flaws in or methodology or unknown malfunctions with the instrument we used to perform localized surface plasmon resonance.

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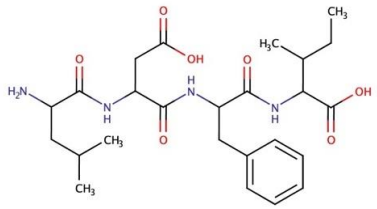
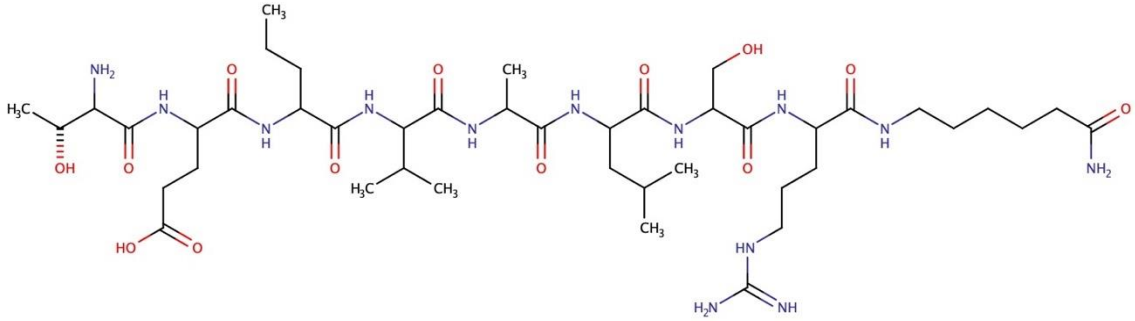
Introduction

Between 80-85% of all breast cancer produces at least one of three types of receptors: the estrogen receptor (ER), the progesterone receptor (PR), and the human epidermal growth factor 2 receptor (HER2)[1]. Breast cancer cells that are found to be positive for any of these receptors can be therapeutically treated by targeting the receptors due to the roles they are found to play in the growth of the cancer. The interaction of estrogen with ER is highly correlated with the propagation of breast cancer as well as the production of PR, though the mechanism of action behind these processes remains speculative (2). Breast cancer cells that are found to be positive for ER may be effectively treated with ER inhibitory drugs such as tamoxifen or raloxifene, or drugs that downregulate or stop the production of estrogen[2]. Similar to ER, cells that are found to have an excess presence of HER2 tend to exhibit aggressive growth tendencies, which may often play a major role in tumor development and metastasis (2). Treatment for cancer cells found with excessive amounts of HER2 are done with drugs such as trastuzumab or pertuzumab, which are monoclonal antibodies that target HER2 and induce an immune-mediated response, causing the downregulation and internalization of HER2[3]. Unfortunately, there are no targeted therapeutics for breast cancers that lack these receptors, which are classified as triple-negative breast cancer (TNBC). TNBC is a very aggressive form of breast cancer which characterizes between 15-20% of all mammary tumors[1,4]. During the first years of treatment, TNBC is characterized as being more likely to spread beyond the breast than hormone positive breast cancers and also has a higher rate of return after treatments are complete (2)[5]. The purpose of our research is to develop a hormone targeted therapeutic that can be used for the treatment of TNBC.

We believe targeting a receptor known as the leptin receptor (ObR) is a viable path for developing a targeted TNBC therapeutic[4]. ObR is a surface protein which binds to leptin and is weakly expressed in most normal tissues while being highly expressed in the hypothalamus and adipocytes [6]. Leptin is a product of the obese (*ob*) gene and an adipokine: a cell signaling protein secreted by adipose tissue [6]. Leptin plays a role in regulating energy, homeostasis, fertility, and the immune system, as well as most notably playing a major role in the regulation of body weight by altering feeding behaviors (1, 6, 11). While leptin is highly expressed in healthy adipose tissue, overexpression of leptin and ObR is clearly observed in adipocytes found in obese individuals[6]. This is significant because there is compelling evidence to suggest that obesity and the development of cancer are highly correlated[6]. Leptin and ObR are also found to be overexpressed in breast cancer, which makes sense seeing as 90% of human breast volume is composed of adipocytes[4,6]. This overexpression of leptin and ObR are highly correlated with the stimulation of breast cancer cell growth, transformation, and survival, while simultaneously enhancing the cancer cells' ability to resist chemotherapy[1,4]. It is apparent to us that inhibiting ObR has a strong potential for yielding success in limiting the growth of TNBC while enhancing the effects of traditional chemotherapies[1,4].



LDFI



Allo-aca

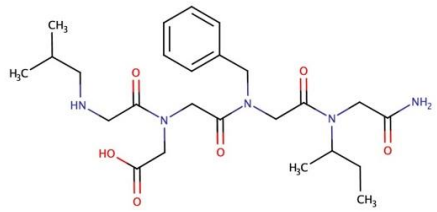
Figure 1. Structures of ObR anatagonist peptides LDFI and Allo-aca.

There are two peptide molecules that have demonstrated antagonistic activity towards ObR, which are known as LDFI and Allo-aca respectively (**Figure 1**). LDFI is small, 4 amino acid sequence within the structure of leptin which plays a crucial role in binding to ObR[7]. LDFI has been shown to inhibit leptin-induced cell growth and cell ability to mobilize as well as leptin signaling activation in ER negative and ER positive human breast cancer cells[7]. Leptin functions by binding to the leptin receptor, which in turn mediates a downstream signal, activating multiple signaling pathways[7]. Isolated LDFI is able to bind to one of the three binding domains located on ObR and inhibit the full leptin molecule from completely binding to ObR[7] Treatment with LDFI antagonized the leptin

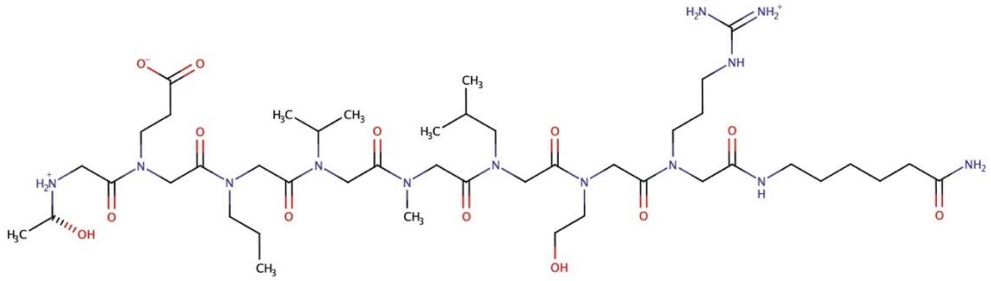
activation of these signaling pathways[7]. LDFI was also shown to counteract and reverse the upregulation of genes activated by leptin[7]. In the absence of leptin, there was no interference with regular cell growth and no cytotoxic effects were observed[7].

The peptide Allo-aca is the second potential leptin receptor antagonist that appears to be promising for inhibiting the effects that leptin has on TNBC cells. While LDFI consists of four amino acids and is thought to interact with the first binding site on ObR, Allo-aca consists of nine amino acids and is thought to interact with ObR's third binding site[1,7]. In mice, which were carriers of xenografted TNBC cells, survival time was increased by 80% [1].

A general problem facing peptide therapeutics is their short biological half-life, meaning they degrade within the body quickly, limiting the time they have to act on their targets[7]. The purpose of our project is to increase the stability of these potential therapeutics by synthesizing peptidomimetics of peptide ObR antagonists in the form of peptoids, which are referred to as N-LDFI and N-Allo-aca (**Figure 2**) [4].



N-LDFI



N-Allo-aca

Figure 2. Structures of LDFI and Allo-aca peptidomimetics, peptoids N-LDFI and N-Allo-aca.

Peptoids and peptides are very similar structurally. The distinction between the two is made by the location of the prosthetic group found on amino acid residues or residue mimics, which is located on the amide nitrogen found on the structure's backbone in peptoids and is found on the α-carbon in peptides (**Figure 3**).

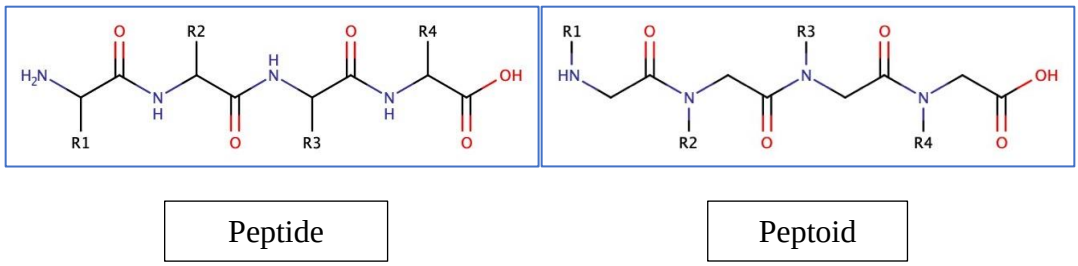


Figure 3. A comparison between the structural differences of peptides and peptoids.

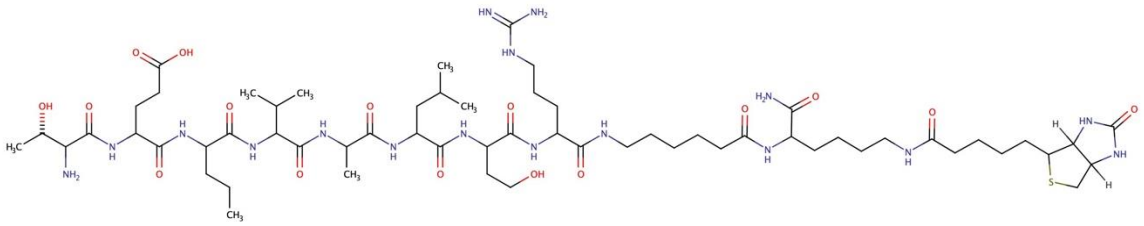
This subtle difference gives peptoids the characteristic of being highly resistant to proteolytic decay in the human body while retaining very similar structure to that of peptides, making them a particularly interesting candidate for peptidomimetic therapeutics[8].

We are able to test the quality of our peptide mimics using an instrument that utilizes surface plasmon resonance (SPR) to detect the binding affinities between molecules. The specific type of SPR that we have available to us at MTSU is known as localized SPR (LSPR). A plasmon is the collective oscillation of electrons in a noble metal, such as gold[9]. In noble metals, electrons move freely through the material as opposed to being stabilized in a particular location like one might find in a typical covalent bond. On the surface of such materials, surface plasmon resonance may be observed, which is the coherent oscillation of the surface conduction electrons excited by electromagnetic radiation[10]. The oscillation of these electrons may be thought of as the mechanical oscillations of electron gas, a state in which electrons are free to move in two dimensions, but tightly confined in the third, with respect to the fixed gold ionic cores[9]. When surface plasmons are confined to nanoparticles, such as gold ion nuclei, the particle's free electrons

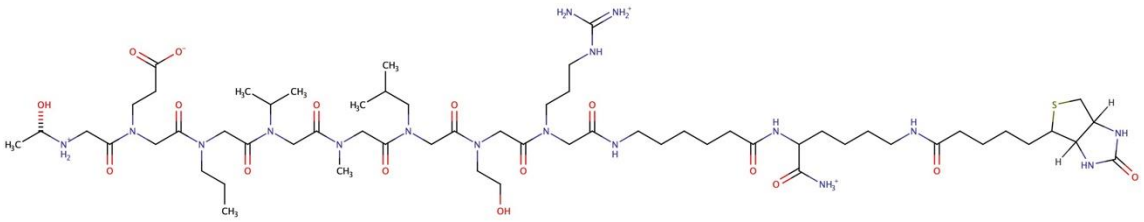
move in collective oscillation, also known as localized surface plasmon resonance[9]. These plasmons may be coupled with particular wavelengths of light to elicit particular surface plasmon modes[9]. To use this phenomenon to analyze binding affinities between molecules, we may examine the wavelength shift in surface plasmon absorbance that occurs when a molecule in solution binds to a molecule on the surface of the material[10]. In LSPR, a gold coated chip, which yields surface plasmon resonance, is present in between a flow cell and a white light emitting diode (LED), which emits a large array of wavelengths ranging from around 400-700 nm. The white light is emitted through the gold coated chip and measured by a detector behind the aforementioned chip. When a molecule binds to this chip, through access by the flow chamber, certain wavelengths emitted by the light source will be absorbed by the plamons, consequently altering the absorbance data gathered by the detector. It is these changes that allow us to determine specific binding affinities between molecules.

The work presented here aims to determine the binding affinities between the receptor (ObR) and several peptide and peptoid potential antagonists (Allo-aca, N-Allo-aca, LDFI, and N-LDFI. There are two methods that we have employed to do this using LSPR. The first is to couple ObR, which has a polyhistidine tag on it, to a gold sensor chip, which has a nitrilotriacetic acid (NTA) chelator complex attached to its surface, through use of the flow chamber in a pH-controlled solution. After ObR is coupled to the sensor chip, we are able to observe the binding affinities of Allo-aca, N-Allo-aca, LDFI, and N-LDFI by allowing them to run through the SPR's flow chamber at various concentrations and flow rates. Our second method, which may not be used with LDFI or N-LDFI due to their smaller sizes, reverses the roles used in the first method. Instead of Allo-aca and N-

Allo-aca acting as analytes (the molecules/molecular structures that flow through the flow chamber), they will act as the ligand (the molecules/ molecular structures attached to the sensor chip surface), while ObR will instead act as the analyte. To properly attach Allo-aca and N-Allo-aca to the surface of the sensor chip, each molecule was synthesized with biotin tags to form Allo-aca-biotin and N-Allo-aca-biotin (**Figure 4**), which bind with strong binding affinity to specialized sensor chips with streptavidin on the surface instead of NTA.



Allo-aca-biotin



N-Allo-aca-biotin

Figure 4. Structures of the Allo-aca and N-Allo-aca in their biotinylated forms.

Methods

Peptide synthesis

LDFI, Allo-aca, and Allo-aca-biotin were each synthesized by solid phase synthesis using traditional fluorenylmethyloxycarbonyl (Fmoc) protecting group strategies (**Fig 5**).

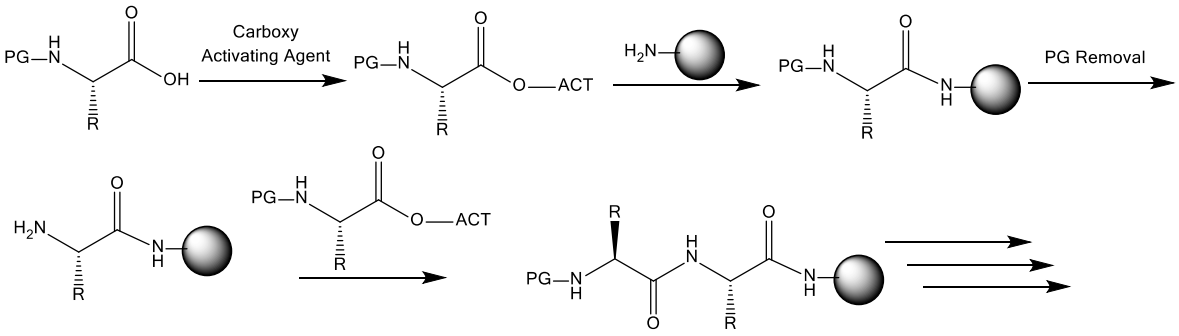


Figure 5. Illustration representing the traditional methodology for peptide synthesis.

To begin this synthesis, a solid support resin is needed to properly anchor the peptide. We choose to use Rink Amide polystyrene resin. To properly prepare the resin, two actions must be taken: the resin must be allowed to swell in the organic solvent dimethylformamide (DMF) for 30 minutes, and the Fmoc protecting group must be removed from the amide at the end of the linker using the base piperdine in a solution of DMF. This can be done by rocking the resin in a solution of 20% piperdine in DMF for 10 minutes twice. Once this is complete, steps may be taken to begin coupling the first amino acid to the resin. The first step is to activate the amino acid found at the C-terminus in the peptide sequence with the

common activating agent 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) in a solution of 5% N-methylmorpholine (NMM) in DMF. The OH group on the carboxcylic acid of the amino is deprotonated by NMM, allowing the newly formed carboxylate anion to attack the carbocation found on HBTU. As a result, a reactive intermediate is formed, which reacts with another newly formed molecule, 1-Hydroxy-7-azabenzotriazole (HOAt), to form an OAt activated ester that reacts spontaneously with free amines, which is what we find on our deprotected resin. Here nucleophilic substitution occurs and the reaction is complete. To effectively facilitate this process, we activate 4 equivalents of our selected amino acid with 4 equivalents of HBTU in 7 mL of a 5% NMM in DMF solution for 10 minutes. Once the activation is complete, we add the solution to the resin and allow it to gently rock for 1 hour at room temperature. After this is complete, the process is repeated. Deprotection of the Fmoc group on the new amino acid residue will be done with the same procedure for the resin. A new amino acid will be activated and coupled in the same way as previously described. Once all amino acid residues have been successfully coupled, the peptide may then be cleaved from the resin using a 7 mL solution composed of 95% trifluoroacetic acid (TFA), 2.5% deionized water, and 2.5% triisppropylsilane (TIS). The TFA is evaporated by gentle bubbling with air and the remaining peptide residue is put into a 50% acetonitrile 50% water solution, which is then purified using reverse phase high performance liquid chromatography (RP-HPLC). Once the compound is properly purified, the remaining acetonitirile and water can be removed using a rotary evaporator and lyophilizer respectivelyto yield the final product, which was confirmed by MS analysis. LDFI yield = 21.7% expected M+H 506.62 g/mol,

observed M+H 506.26 g/mol (**Appendix 1**). Allo-aca yield = 32.5% expected M+H 986.18 g/mol, observed M+H 986.65 g/mol (**Appendix 2**).

Allo-aca biotin

To biotinylate Allo-aca, we began the synthesis with a lysine (Lys) residue instead of amino hexanoic acid (aca), which was added next. Specifically, we used lysine that had a methyltrityl (MTT) protecting group protecting the free amine on lysine's prosthetic group so that when the overall synthesis of the molecule was complete this protecting group could be removed. Once the entire peptide was synthesized following the procedures outlined above, the MTT group was removed from lysine by treating 5x with 1% TFA in CH_2Cl_2 , allowing us to have access to the free amine. We were then able to activate biotin using (1-Cyano-2-ethoxy-2-oxoethylidene aminooxy) dimethylaminomorpholino carbenium hexafluorophosphate (COMU), a more efficient activator than HBTU, to allow the free amine on lysine's prosthetic group to attack the activated ester on biotin. We used 4 equivalents of biotin and 4 equivalents of COMU in 5% NMM in DMF. The final peptide was cleaved from the resin and purified as described above to yield pure Allo-aca-biotin yield = 9.5% expected M+H 1341.66 g/mol, observed M+H 1341.62 g/mol (**Appendix 3**).

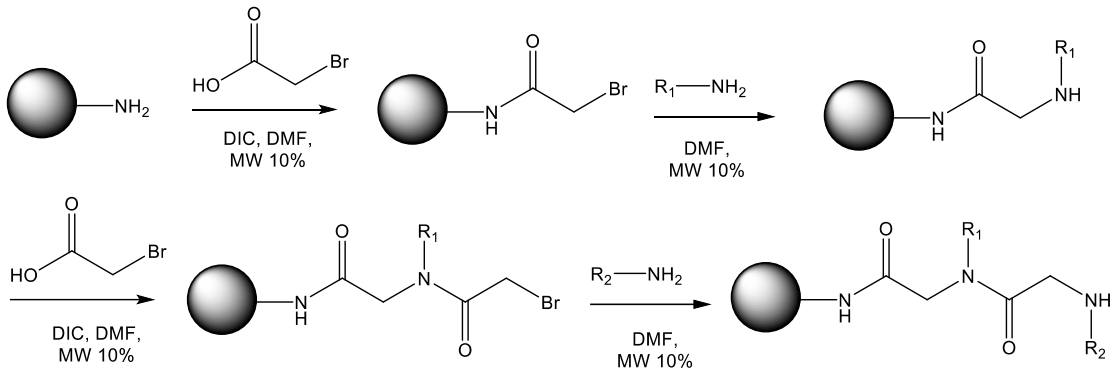


Figure 6. Illustration representing the traditional methodology for peptoid synthesis.

Peptoid synthesis

N-LDFI and N-Allo-aca-biotin were both synthesized using traditional solid phase peptoid synthesis strategies (**Figure 6**). Like peptide synthesis, traditional solid phase peptoid synthesis requires a solid support resin to anchor the product. Again, we choose to use to use Rink Amide polystyrene resin, which must also undergo the same initial procedure of peptide synthesis of resin swelling in DMF for 30 minutes and deprotection of the Fmoc group by gently rocking in 20% piperdine in DMF solution for 10 minutes two times. After the resin is prepared, bromoacetic acid will be used to construct what is essentially the backbone of the peptoid. To bind bromoacetic acid to the resin, we activate the OH group on the carboxcylic acid using N,N'-diisopropylcarbodiimide (DIC). To do this we prepare two solutions: one 2 M solution of bromacetic acid in 1.5 mL of anhydrous DMF and one solution consisting of 0.75 mL of DIC and 0.75 mL of anhydrous DMF (3.2 M final DIC solution). After the solutions have been given proper time to mix, they are each added to the column containing the resin and microwaved for 15 seconds at 10%

power two times. The solution is then gently rocked for 15 min. After this is done, nucleophilic substitution between the free amine on the resin and the activated ester on the bromoacetic acid has occurred assuming that everything was done properly. Next, we have whichever amine best mimics the prosthetic group of the amino acid that we are trying to imitate undergo an S_N2 reaction with the Br on the bromoacetic acid, with the amine acting as the nucleophile and the Br acting as the leaving group. To do this we add a 2 M solution of our selected amine in anhydrous DMF to the resin and follow the microwave procedure previously mentioned. We then gently rock the column for 15 min. From this point we may continue activating and coupling bromoacetic acid followed by the addition of specific amines until we have successfully completed the peptoid structure. Cleavage and purification of peptoids was done as previously described for peptides. The synthesis of N-LDFI was done completely using traditional peptoid synthesis methods; yield = 10.5% expected M+H 506.62 g/mol, observed M+H 506.35 g/mol (**Appendix 4**).

N-Allo-aca-biotin

The synthesis of N-Allo-aca-lys-biotin required a large variety of reactions that are not covered in the procedure for traditional peptoid synthesis. There will be a section dedicated to the explanation of each residue that was not synthesized using traditional methods, as well as residues that required the addition of protecting groups to the amines used for the synthesis of the respective residues. The final synthesis provided N-Allo-aca-biotin in 18.8% yield; expected M+H 1369.71 g/mol, observed M+H 1369.77 g/mol (**Appendix 5**).

Lys-biotin

Like Allo-aca-biotin, the first residue we added to N-Allo-aca-biotin was MTT protected lysine, so that we would have a residue on the optimal position of our molecule that could be properly biotinylated. The procedure for the addition of lysine, and later biotin to N-Allo-aca-biotin exactly mimic the procedure used for Allo-aca-biotin where traditional peptide chemistry was used for both the addition of lysine to the resin and biotin to the prosthetic group of lysine.

Amino-hexanoic acid

The addition of amino-hexanoic acid (aca) was done using traditional peptide chemistry due to the particular structure of aca, which is not that of a traditional amino acid.

Arginine mimic

Mimicking arginine required many steps. While traditional peptoid chemistry was used in this procedure, using a commercially available amine was not an option, meaning it needed to be synthesized in-house. To make our necessary amine, termed *N,N'*-(bis-Boc)-*N''*-(3-aminopropyl)guanidine , the synthesis of two precursors was necessary: 2-methylisothiouronium iodide and *N*,*N*'-bis(Boc)-*S*-methylisothiourea.

2-methylisothiouronium iodide

2-methylisothiouronium iodide was synthesized by Robert M. Green of the Bicker lab for a purpose unrelated to this project and used without further modification. However, it is worth recounting the procedure due to the compound's importance in the role of the arginine mimic synthesis and so that this synthesis may be more easily replicated. To begin, a mixture of 10.098 g (0.133 mol) of thiourea and 8.2 mL (0.133 mol) of iodomethane in

100 mL of methanol (MeOH) were heated to 65° C for 90 minutes. The MeOH was removed with the using of a rotary evaporator and the yellow solid product was moved to a filter where it was washed with 50 mL of diethyl ether (Et_2O) five times under a vacuum to produce 2-methylisothiouronium iodide as an amorphous white powder.

N,N'-Bis(Boc)-S-methylisothiourea

The process to synthesize *N*,*N*'-bis(Boc)-*S*-methylisothiourea begins by adding a solution of 19.668 g (90.12 mol) of di-tert-butyl dicarbonate (Boc_2O) in 25 mL of dichloromethane (CH_2Cl_2) to a stirring solution of 9.8 g (45.03 mmol) of 2-methyliosthiouronium iodide in 50 mL of saturated sodium bicarbonate ($NaHCO_3$) and 105 mL of CH_2Cl_2 for 48 hours. Once the reaction has been completed, a separating funnel was used to extract the organic phase. To dry the organic phase further, calcium chloride ($CaCl_2$) was used. The solvent was them removed using rotary evaporation, leaving a white, crude solid, which was then stirred in a solution of 100 mL of 9:1 ethanol (EtOH) and water for 1 hour. The solid was then removed from the solution by vacuum filtration to leave a white powder and complete the synthesis of the *N*,*N*'-bis(Boc)-*S*-methylisothiourea in a quantitative yield.

N,N'-(bis-Boc)-N''-(3-aminopropyl)guanidine and final addition of arginine mimic residue

To complete the synthesis of *N,N'*-(bis-Boc)-*N''*-(3-aminopropyl)guanidine and add the arginine mimic residue to our structure, we first made two separate solutions. First we composed a solution of 1.1 mL of 1,3-diaminopropane in 11.5 mL of DCM followed by the composition of a different solution which was made up of 1.503 g of the *N*,*N*'-Bis(Boc)-*S*-methylisothiourea previously made in 7.5 mL of CH_2Cl_2. We prepared the

solution containing 1,3-diaminopropane in an appropriately sized Erlenmeyer flask and began to stir it. Following this, we added our second solution containing *N,N*'-bis(Boc)-*S*-methylisothiourea to the stirring solution and allowed it to stir for 90 minutes. Once the reaction was complete we used 15 mL of CH_2Cl_2 to dilute the solution and then began to separate the product, *N,N'*-(bis-Boc)-*N"*-(3-aminopropyl)guanidine, from the sulfur based byproducts using a separatory funnel and 15 mL of water three times. We then did one more separation using 15 mL brine. We then removed the remaining CH_2Cl_2 from the solution using a rotary evaporator and were left with our final product of *N,N'*-(bis-Boc)-*N"*-(3-aminopropyl)guanidine, which appeared as a clear oily substance with white specks in it. This product could then be used to act as the amine in traditional peptoid chemistry to complete the addition of an arginine mimic. This must product must be used immediately upon synthesis to prevent degradation. The viability of this reagent was confirmed by MS of the NArg-Aca-Lys intermediate cleaved from the solid phase; expected M+H 415.56 g/mol, observed M+H 415.25 g/mol(**Appendix 6**).

Tert-Butyldimethylsilyl ether protected amines

Unlike in peptide synthesis, where the reactive prosthetic groups of amines come protected commercially, it is common to have to synthesize in-house protecting groups for the amines that mimic these reactive prosthetic groups so that unwanted reactions do not occur and ruin the product. For amines that mimic prosthetic groups with a reactive OH group, such as the *allo*-threonine and serine mimics found in N-Allo-aca-biotin, we use *tert*-butyldimethylsilyl ether (TBDMS) to protect these reactive OH groups. To start, we dissolved 0.021 mol of the respective amine (ethanolamine for the serine mimic and (S)-(+)-1-amino-2-propanol for the *allo*-threonine mimc) and 2.72 g (0.040 mol) of imidazole

in 20 mL of CH_2Cl_2. Separately, we then dissolved 3.17 g (0.021 mol) of *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in 10 mL of CH_2Cl_2. Once both mixtures were made, we began stirring the solution with our amine in it and slowly, over the course of 5 minutes, added the TBDMS-Cl solution. We allowed this newly mixed solution to stir at room temperature for 1 hour. Once the reaction was complete we added 20 mL of water to the solution and washed the solution two times with 20 mL of CH_2Cl_2 using a separatory funnel to collect the organic layer. The organic layer was then dried with $CaCl_2$ and rotary evaporation was used to remove the CH_2Cl_2, leaving behind the final product. This product could be used as the amine in traditional peptoid synthesis. Tbdms-ethanol amine was synthesized in 76.7% yield; expected M+H 176.35 g/mol, observed 176.09 M+H g/mol (**Appendix 7**). Tbdms-1-amino-2-propanol was synthesized in 39.7% yield; expected M+H 190.38 g/mol, observed 190.10 M+H g/mol (**Appendix 8**).

Alanine mimic

Because the amine that would be used to mimic alanine, methylamine, in traditional peptoid chemistry is much too volatile to remain as a liquid at room temperature and must be stored as a salt in solid form, we employed a different method for creating our alanine mimic. By using the amino acid sarcosine, we were able to perform traditional peptide chemistry and create the same structure as traditional peptoid chemistry with a hypothetical liquid phase methylamine would. For this reaction to successfully work, it is imperative that bromoacylation does not occur after the serine mimic has been added.

Glutamic acid mimic

The amine needed to generate the peptoid submonomer mimic of glutamic acid (β-alanine-OtBu) could only be purchased as the hydrochloride salt. A base was therefore

included during the amination step of traditional peptoid synthesis to facility *in situ* free-basing of the salt. Briefly, a solution of 1 M N,N-diispropylethylamine (DIPEA) and 1 M of β-alanine-(OtBu)•HCl in 3 mL of anhydrous DMF was prepared and given time for the solute to free-base and dissolve. When the solution was clear, it was added to the reaction and coupled under traditional peptoid synthesis procedures.

Localized surface plasmon resonance

All localized surface plasmon resonance data was collected using the Nicoya Lifesciences OpenSPR model: REV 3.0. Phosphate buffered saline (PBS) buffer with 1% bovine serum albumin (BSA) and 0.0005% tween 20 was used as the running buffer and for all tests. BSA and tween 20 additives were used to prevent non-specific binding during experiments.

Nitrilotriacetic Acid (NTA) sensor chip

To test find the binding affinities between LDFI and ObR and N-LDFI and ObR, we used NTA sensor chips to immobilize our polyhistidine-tagged ObR so that ObR would serve as the ligand in our experiments. To activate the NTA chelator complex bound to the sensor chip, we performed two injections of 40 mM $NiCl_2$ into the SPR flow cell at 20 μL/min. Successful activation was confirmed by the observation of binding curves that were only observed after the injection of $NiCl_2$, as well as an increase in the measured absorbance of the established baseline. Once the activation was complete, we then immobilized ObR to the sensor chip by injecting 10 μg/mL polyhistidine-tagged ObR into the flow chamber. Successful immobilization of ObR to the sensor chip was characterized by shifts in the absorbance spectra baseline ranging from 0.5 nm to 2 nm. Once a stable

baseline was established, injections of the analyte to test for binding affinities began. For both LDFI and N-LDFI, one injection into the flow chamber at 20 μM and 50 μM were performed, while two injections at 100 μM were performed. A stable baseline was observed before each injection was performed. To return the sensor chip to its original state after the experiment was complete, the ligand (ObR) was removed by performing two injections of 1 M imidazole and the Ni was removed by performing two injections of 10 mM HCl. Each analyte was tested individually, so that the general workflow was chip preparation, ligand loading, LDFI testing, chip stripping, chip preparation, ligand loading, N-LDFI testing.

Streptavidin sensor chip

To test the binding affinities between All-aca-biotin and ObR and N-Allo-aca-biotin and ObR, we used streptavidin sensor chips to immobilize the respective ligands. To immobilize Allo-aca-biotin and N-Allo-aca-biotin to their respective streptavidin sensor chips, two injections of the respective ligands were performed at 100 μg/mL for each test. Confirmation of immobilization was done by observations in the change in absorbance spectra baseline after injections. After successful ligand immobilization was complete, four injections of the ObR analyte into the flow cell were performed at the concentrations of 5 nM, 10 nM, 25 nM, and 50 nM. There is no procedure for to successfully return streptavidin sensor chips bound to biotin to their original state after testing.

Results and Discussion

Peptide and Peptoid Synthesis

A variety of molecules including LDFI, N-LDFI, Allo-aca, Allo-aca-biotin, and N-Allo-aca-biotin were synthesized for the purpose of the research conducted here. The synthesis of peptide molecules, LDFI, Allo-aca, and Allo-aca-biotin, was done using traditional fluorenylmethyloxycarbonyl (Fmoc) protecting group strategies and required no variation from this method of traditional solid phase peptide synthesis. Using this method, we were able garner a 21.7% yield of LDFI, a 32.5% yield of Allo-aca, and a 9.5% of Allo-aca-biotin.

The synthesis of the peptoid molecules, N-LDFI and N-Allo-aca-biotin, was done using traditional peptoid solid phase synthesis strategies. N-LDFI required no variation from these traditional methods and we were able to garner a 10.5% yield. To successfully synthesize N-Allo-aca-biotin, multiple methods which do not adhere to the traditional scheme of solid-phase peptoid synthesis were required. Overall, an estimated 30 steps were required to synthesize N-Allo-aca-biotin (**Figure 7**). Mimicking arginine, serine, *allo*-threonine, and glutamic acid each required methods with a high volume of steps relative to the typical addition of residues in traditional peptide and peptoid synthesis. Serine and *allo*-threonine peptoid mimics required *tert*-butyldimethylsilane (TBDMS) protections of ethanolamine and (S)-(+)-1-amino-2-propanol, respectively, to yield the requisite building blocks. The glutamic acid mimic, β-alanine, was purchased as a *tert*-butyl carboxy protected HCl salt. *In situ* free-basing of this salt was done using diiisopropylethylamine (DIPEA) to improve amine nucleophilicity.

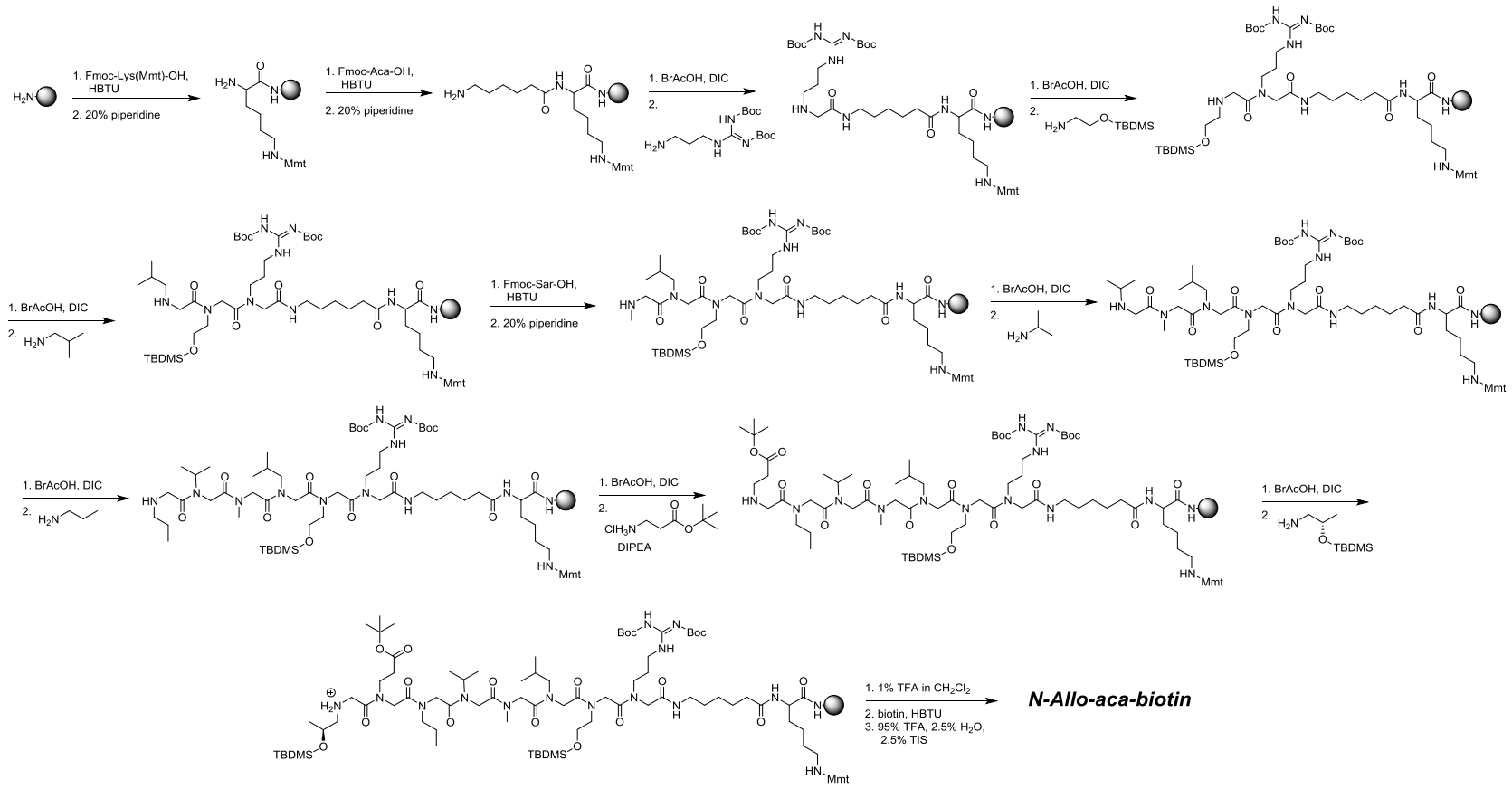


Figure 7. Synthetic scheme for N-Allo-aca-biotin

To successfully complete the addition of the arginine mimic residue to N-Allo-aca-biotin, the precursors *N,N'*-Bis(Boc)-S-methylisothiourea and *N,N'*-(bis-Boc)-N"-(3-aminopropyl)guanidine were both synthesized. *N,N'*-Bis(Boc)-S-methylisothiourea was synthesized from 2-methylisothouronium and Boc anhydride in a biphasic solution of $NaHCO_3$ and CH_2Cl_2.The compound was purified by liquid-liquid separation and dried *in vacuo* to yield the compound as a white powder.

To synthesize the amine needed to form the arginine mimic in traditional peptoid solid phase synthesis, *N,N'*-(bis-Boc)-N"-(3-aminopropyl)guanidine, diaminopropane was reacted with *N,N'*-Bis(Boc)-S-methylisothiourea in CH_2Cl_2. This final product was purified by liquid-liquid extraction and used quickly with traditional solid phase peptoid synthesis strategies to prevent cyclization that would occur if this molecule is stored for any period of time. Any researcher who has the intent of replicating this process should beware that a byproduct of this reaction is the strong, lingering smell of rotten eggs due to the release of sulfur.

ObR binding affinity to Allo-aca

Allo-aca-biotin successfully bound to our streptavidin sensor chip. After three injections of All-aca-biotin (100 μg/mL) the established baseline arbitrary units (AU) was shown to be greater than the established baseline before injections (**Figure 8**). The binding affinity between Allo-aca-biotin and the streptavidin attached to the sensor chip was not determined because Allo-aca-biotin was acting as a ligand, not an analyte.

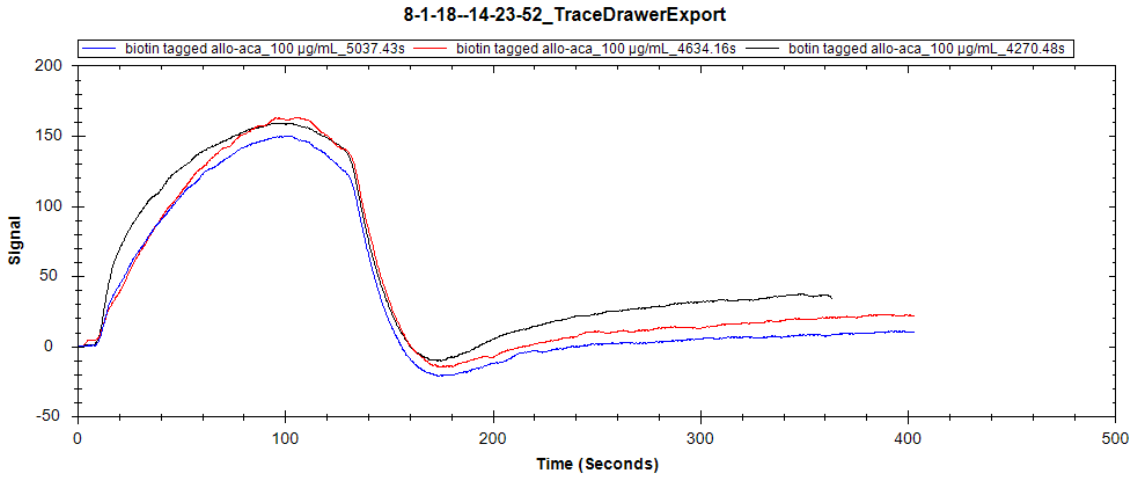


Figure 8. Binding curve of Allo-aca-biotin binding to streptavidin on the sensor chip.

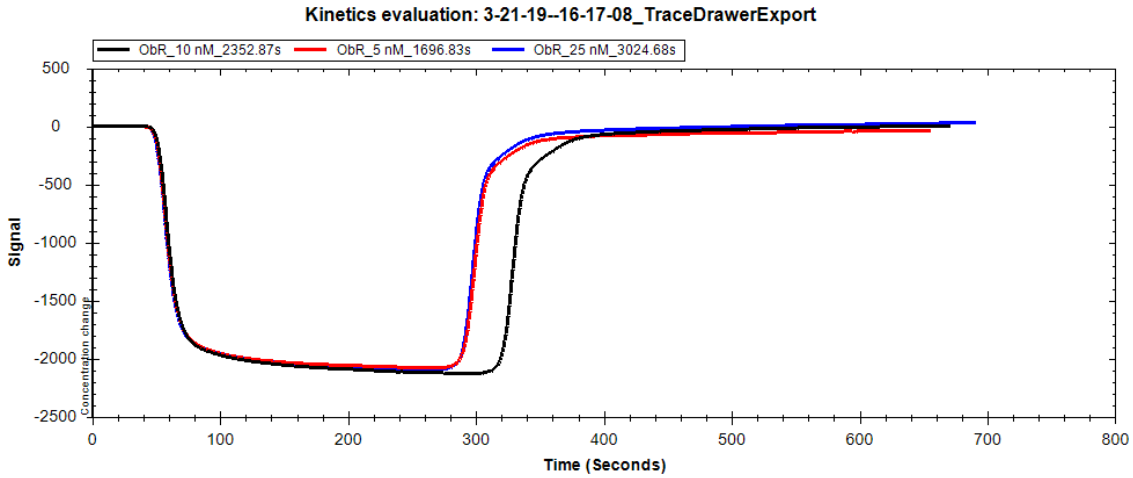


Figure 9. Binding curve after injection of ObR into the SPR system with Allo-aca bound sensor chip.

Injecting ObR as the analyte after Allo-aca-biotin was successfully bound to the streptavidin sensor chip did not yield a viable binding curve, but instead resulted in what is believed to be non-specific binding (**Figure 9**). It is not possible to successfully analyze the binding kinetics between an analyte and ligand when negative binding curves of this nature are the result of an experiment. The causes of negative binding signals being produced during SPR experiments are not well known, though nonspecific binding is thought to be a cause in certain instances. While it is possible that the lack of a viable binding curve produced by the injection of ObR as an analyte over the streptavidin sensor chip bound to Allo-aca acting as the ligand was due to a complete lack of binding affinity between Allo-aca and ObR, it is also possible that it was due to technical or instrumental issues not yet identified. Our method of biotinylating Allo-aca and binding it to the SPR sensor chip while ObR acted as the analyte has never been tried before and it is entirely within the realm of reason that we have discovered that this new method is not viable in this scenario. Another potential factor that lead to these results could be over saturation of the sensor chip with Allo-aca-biotin. It has been observed in separate studies that when linear ligands become oversaturated on SPR sensor chips their binding sites become inhibited by the close proximity of the other ligands[11].

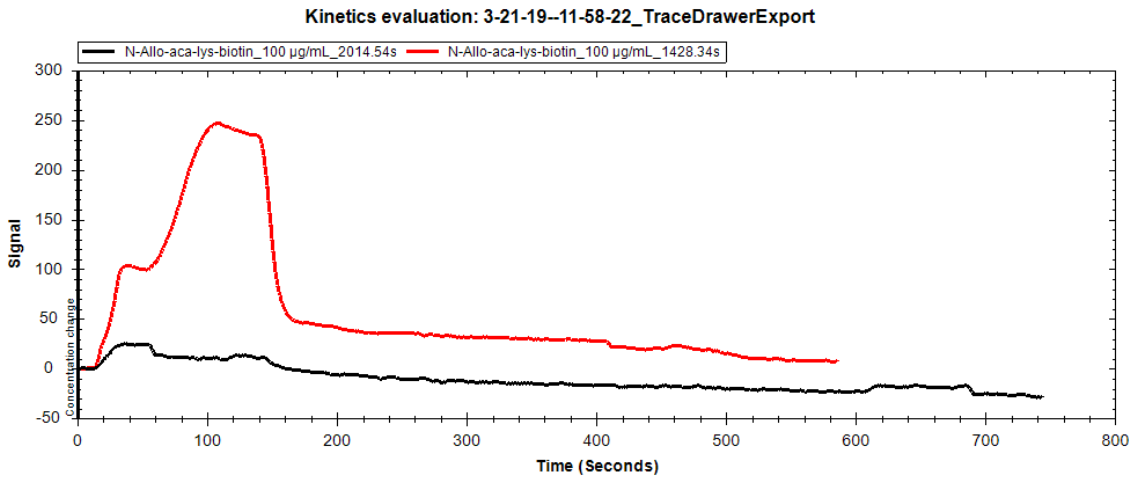


Figure 10. Binding curve of Allo-aca-biotin binding to streptavidin on the sensor chip.

ObR binding affinity to N-Allo-aca

N-Allo-aca-biotin successfully bound to our streptavidin sensor chip (**Figure 10**) with binding occuring after one injection. The ΔAU observed after the second injection of N-Allo-aca-biotin indicated that saturation of binding between N-Allo-aca-biotin and streptavidin occurred due to the lack of shift in the established baseline.

The results of our experiment concerning the binding affinity between ObR and N-Allo-aca are nearly identical to that of our experiment concerning the binding affinity between ObR and Allo-aca. Injecting ObR as the analyte after N-Allo-aca-biotin was successfully bound to the streptavidin sensor chip did not yield a viable binding curve, but instead resulted in what is believed to be non-specific binding (**Figure 11**). It is not possible to successfully analyze the binding kinetics between an analyte and ligand when negative binding curves of this nature are the result of an experiment. The conclusions drawn in

regards to the results for the experiments concerning N-Allo-aca and ObR are the same as those drawn for Allo-aca and ObR.

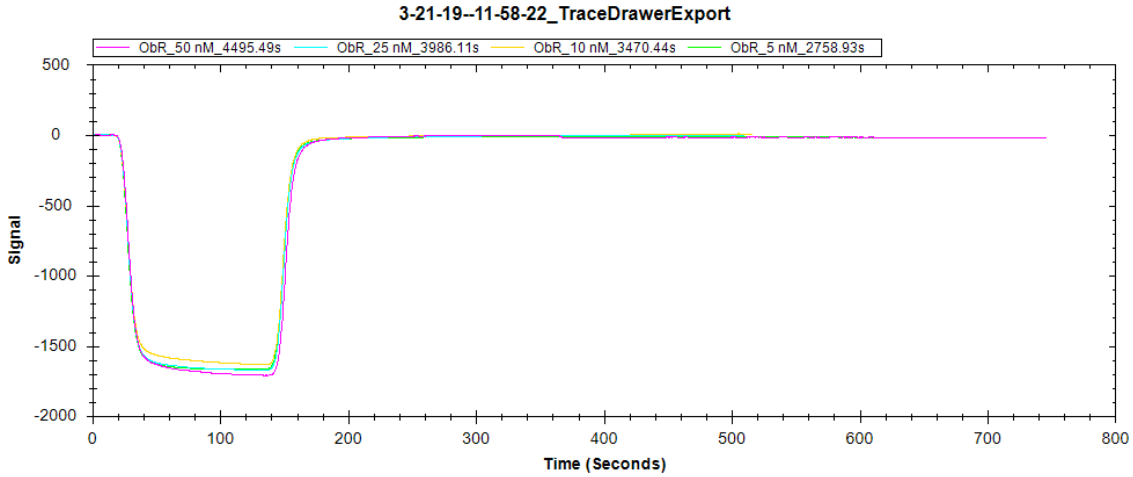


Figure 11. Binding curve after injection of ObR into the SPR system with N-Allo-aca bound sensor chip.

LDFI binding affinity to ObR

Nitrilotriacetic acid (NTA) was successfully activated which was made evident by the unique binding curve that occurs after the injection of $NiCl_2$ (**Figure 12**). ObR was able to successfully bind to the nickel modified NTA sensor chip which was evidenced by the large ΔAU and change in established baseline after the injection of ObR (**Figure 13**).

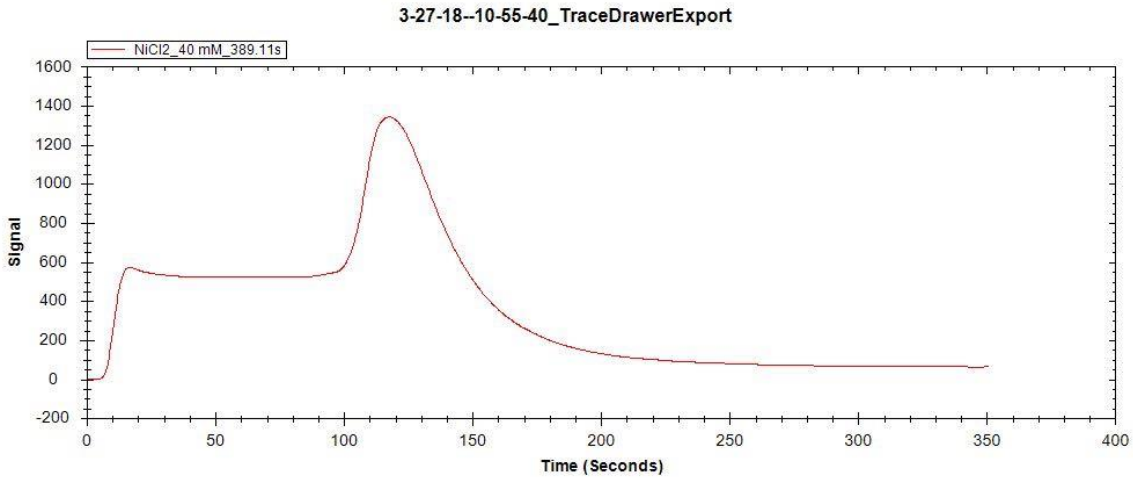


Figure 12. Binding curve after injection of $NiCl_2$ and successful activation of NTA.

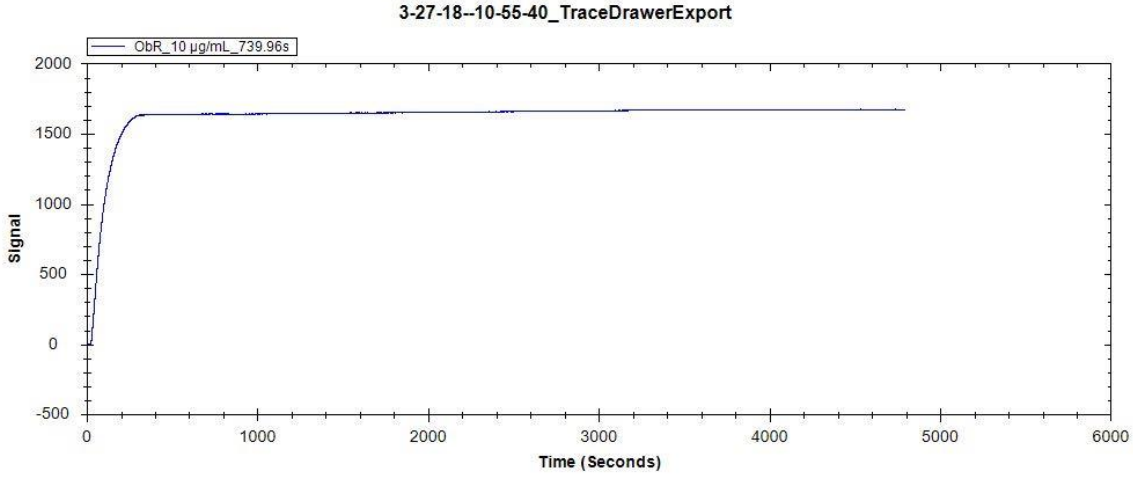


Figure 13. Binding curve of ObR to activated NTA sensor chip.

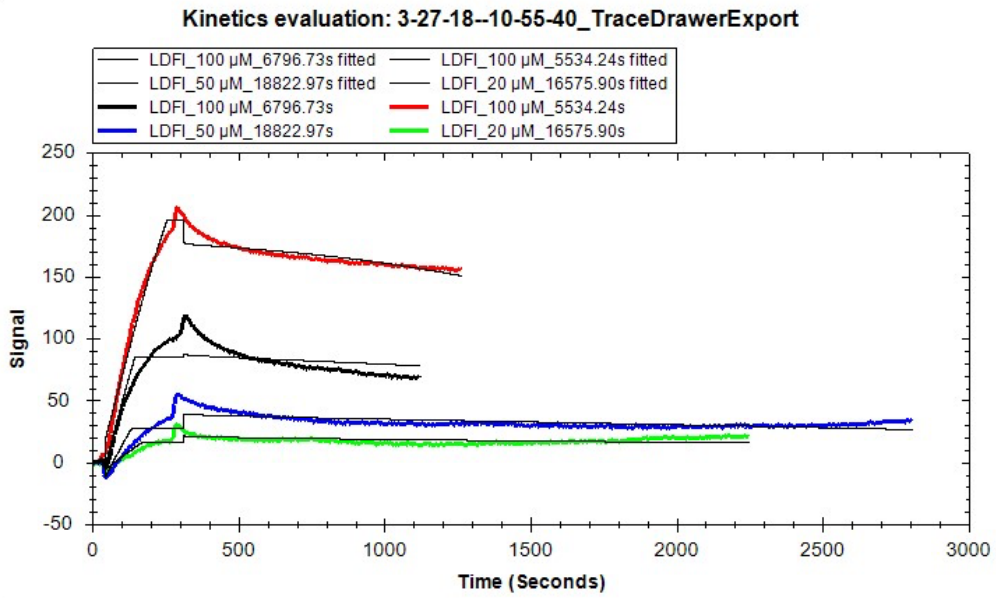


Figure 14. Binding curve fit after injection of LDFI at concentrations of 20 µM, 50 µM, and 100 µM into the SPR system with the ObR bound sensor chip.

The injections of LDFI at varying concentrations as an analyte yielded viable binding curves to analyze the binding strength and kinetics between LDFI and ObR (**Figure 14**). From the binding curve kinetics analysis, we determined the following values: $k_a = 7.44\times10^{5}$ $M^{-1}s^{-1}$, $k_d = 1.72\times10^{-2}$ s^{-1}, and $K_d = 2.32\times10^{-8}$ M. The Chi^2 value for these results is a value of 31.11. k_a represents the association constant, which is the rate at which association between the analyte and ligand occurs and is calculated in the initial phase of the curve following LDFI injection. k_d is the dissociation constant, which is the rate at which dissociation between the analyte and ligand occurs and is calculated in the later phase of the curve when LDFI is no longer flowing over the surface of the chip. By taking the value of k_d/k_a, we are able to calculate dissociation equilibrium constant, K_d. K_d is the

measure of concentration of analyte in units of molarity (M) that is needed to produce 50% binding between an analyte and ligand. The projected accuracy of these values is represented by the Chi^2 value, which measures the average deviation of experimental data. Although relative to the experimental setup, smaller Chi^2 numbers are indications of accurate data. The Chi^2 value of 31.11 collected from this data is significantly smaller than the Chi^2 values collected during our other experiments. This indicates to us that the k_d, k_a, and K_d values calculated from this experiment are accurate.

N-LDFI binding affinity to ObR

NTA was successfully activated which was made evident by the unique binding curves that occur after injections of $NiCl_2$ (**Figure 15**). ObR was successfully coupled to the NTA sensor chip as evidenced by the overall change in the stabilized baseline (**Figure 16**). Negative binding curves were observed during the injections of both $NiCl_2$ and ObR, which were not observed during the previous experiments with LDFI. The cause of these negative binding curves remain currently unknown to us, but we are still confident that successful activation of NTA and coupling of ObR occurred.

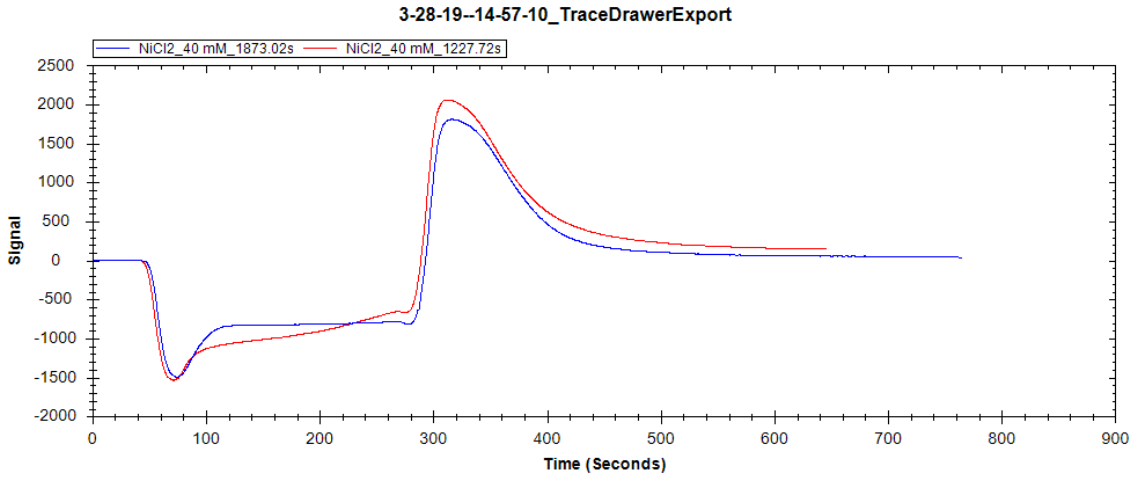


Figure 15. Binding curve after injection of $NiCl_2$ and successful activation NTA sensor chip.

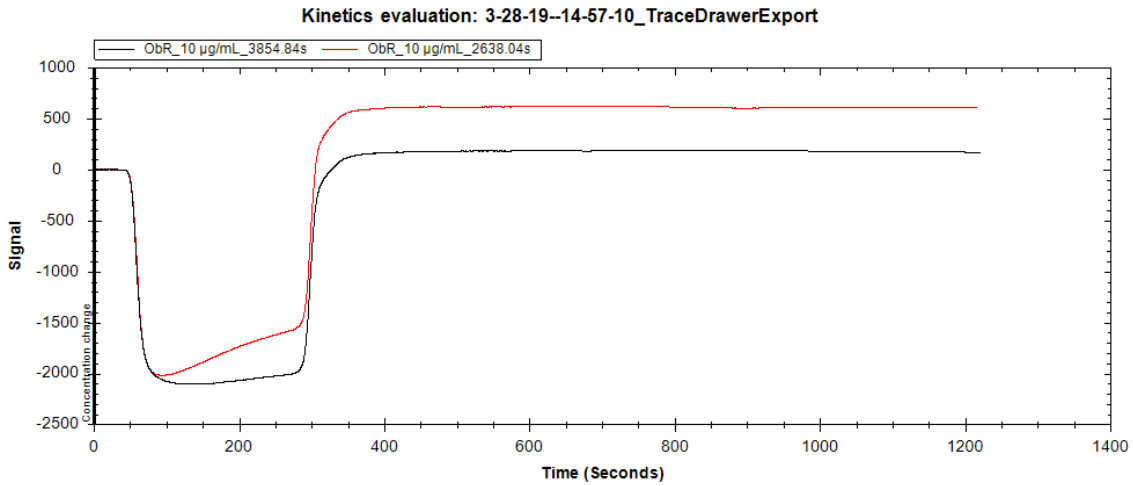


Figure 16. Binding curve of ObR to the activated NTA sensor chip.

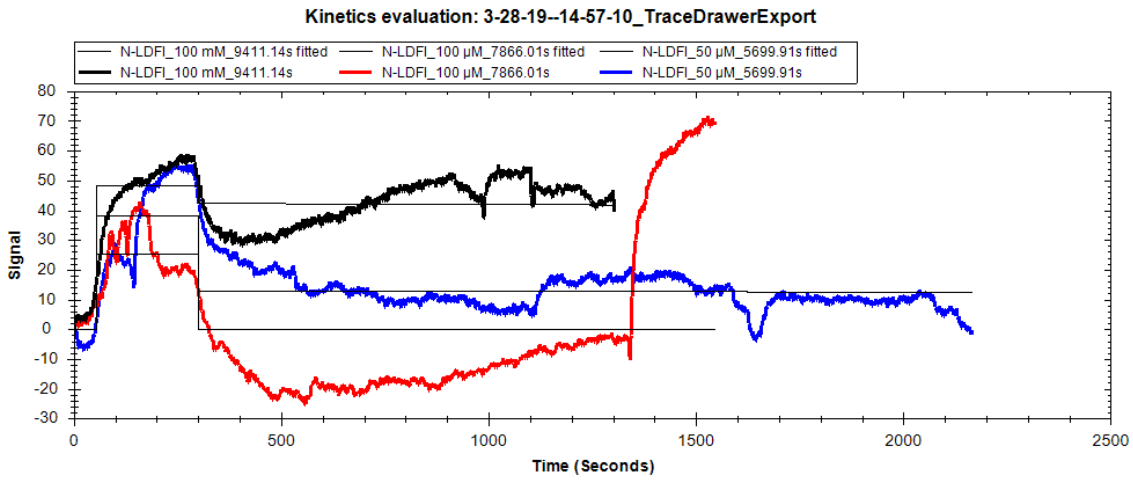


Figure 17. Binding curve fit after injection of N-LDFI at concentrations of 50 µM and 100 µM into the SPR system with ObR bound sensor chip.

N-LDFI was successfully bound to ObR (**Figure 17**). Analysis of the binding kinetics between N-LDFI was performed and the following values were obtained: k_a = 2.14×10^{5} $M^{-1}s^{-1}$), k_d = 1.88×10^{-5} s^{-1}, and K_d = 8.76×10^{-11} M. The Chi^2 value for these results is a value of 235.23. The data for N-LDFI at 20µM was not used in this analysis due to a large negative binding curve which would radically skew our data.

While we are confident that the data collected for the binding kinetics between LDFI and ObR is accurate, we are less confident in the binding kinetics data collected during experiments to determine the binding kinetics between N-LDFI and ObR. The Chi^2 value of 253.23 is significantly larger than that of LDFI/ObR (Chi^2 = 31.11). This is an indication that the average deviation of this experimental data is much larger than we would assume the average deviation would be in more accurate results. Alongside this, the

phenomena which caused the negative binding curves that were observed in each part of this experiment may have influenced the binding kinetics between N-LDFI and ObR in ways that we are currently unaware.

Considering these parameters that call into question the complete accuracy of this data, we may still confidently assess that binding between N-LDFI and ObR occurred, though the true binding affinity between N-LDFI and ObR may currently remain unknown. The questionable binding affinity of N-LDFI/ObR ($K_d = 8.76\times10^{-11}$ M) is much closer to the literature value binding affinity between ObR's natural ligand, leptin, and ObR ($K_d = 6.47\times10^{-11}$ M)[12] than that of LDFI/ObR ($K_d = 2.32\times10^{-8}$ M). We cannot accurately assess whether or not the binding affinity of N-LDFI with ObR is truly stronger that between LDFI and ObR, but we believe that it may potentially be.

Conclusions

Through the use of peptide and peptoid synthesis and localized surface plasmon resonance, we have found that the binding affinity between LDFI and the leptin binding domain is $K_d = 2.32 \times 10^{-8}$ M. We are confident that binding between N-LDFI and the leptin binding domain exists but cannot accurately deduce a specific value for K_d until more testing is done. We believe that the binding curves produced from the interactions between N-LDFI and ObR during our SPR experiments indicate that N-LDFI still has potential to be a therapeutic for TNBC.

A significant amount of the effort given to this research was focused on the synthesis of potential antagonist compounds. This includes the challenging and complex 30-step solution and solid phase synthesis of N-Allo-aca-biotin. Unfortunately, we cannot currently make any conclusions regarding the binding affinity between N-Allo-aca and ObR. Because binding did not occur between Allo-aca and ObR during our SPR experiments, we are led to believe that flaws in our methodology would have prevented N-Allo-aca from binding to ObR even if it were capable of doing so. To accurately compare the binding affinity of Allo-aca and ObR to N-Allo-aca and ObR, we will need to design an experiment that is capable to detect binding between Allo-aca and ObR.

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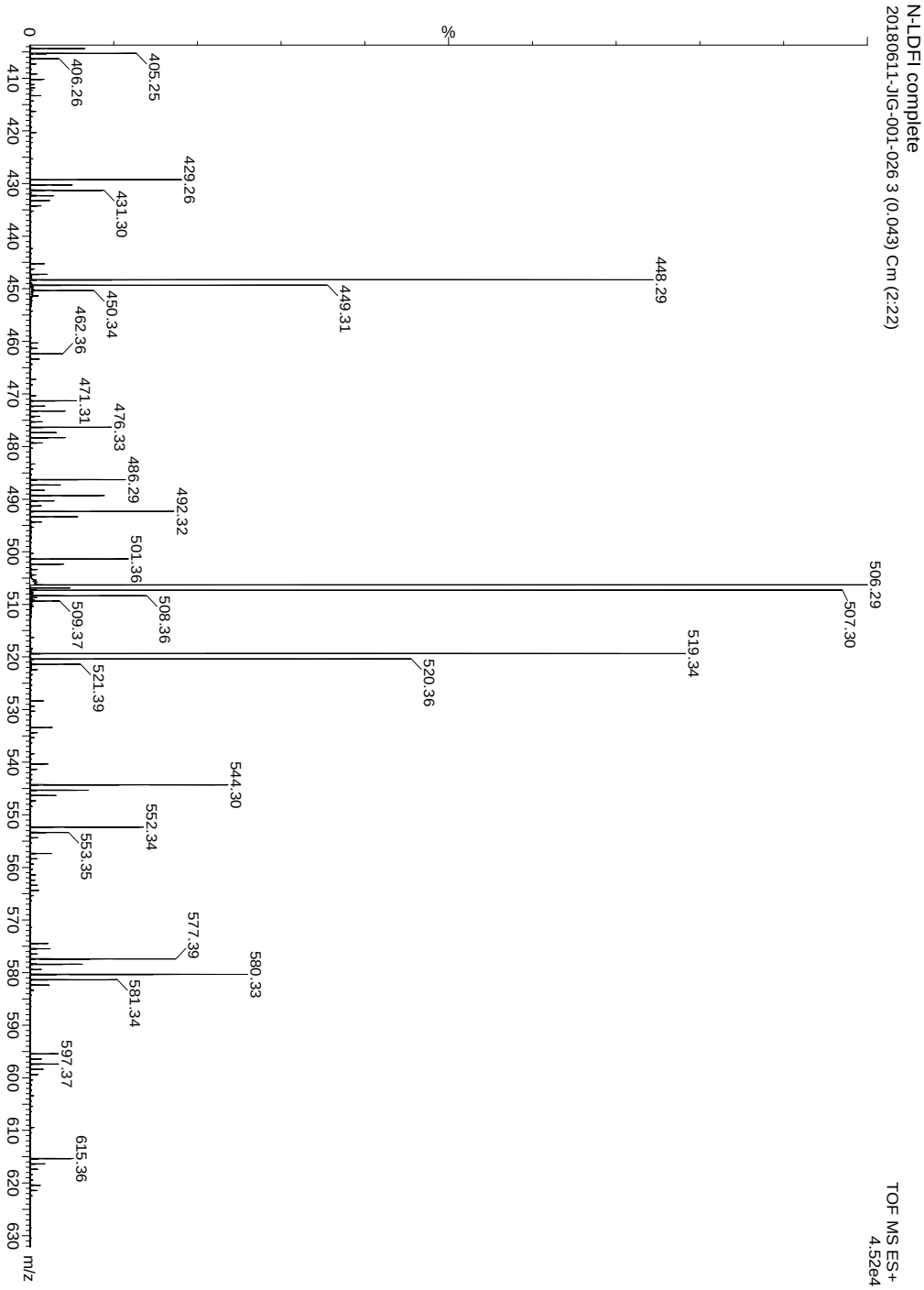
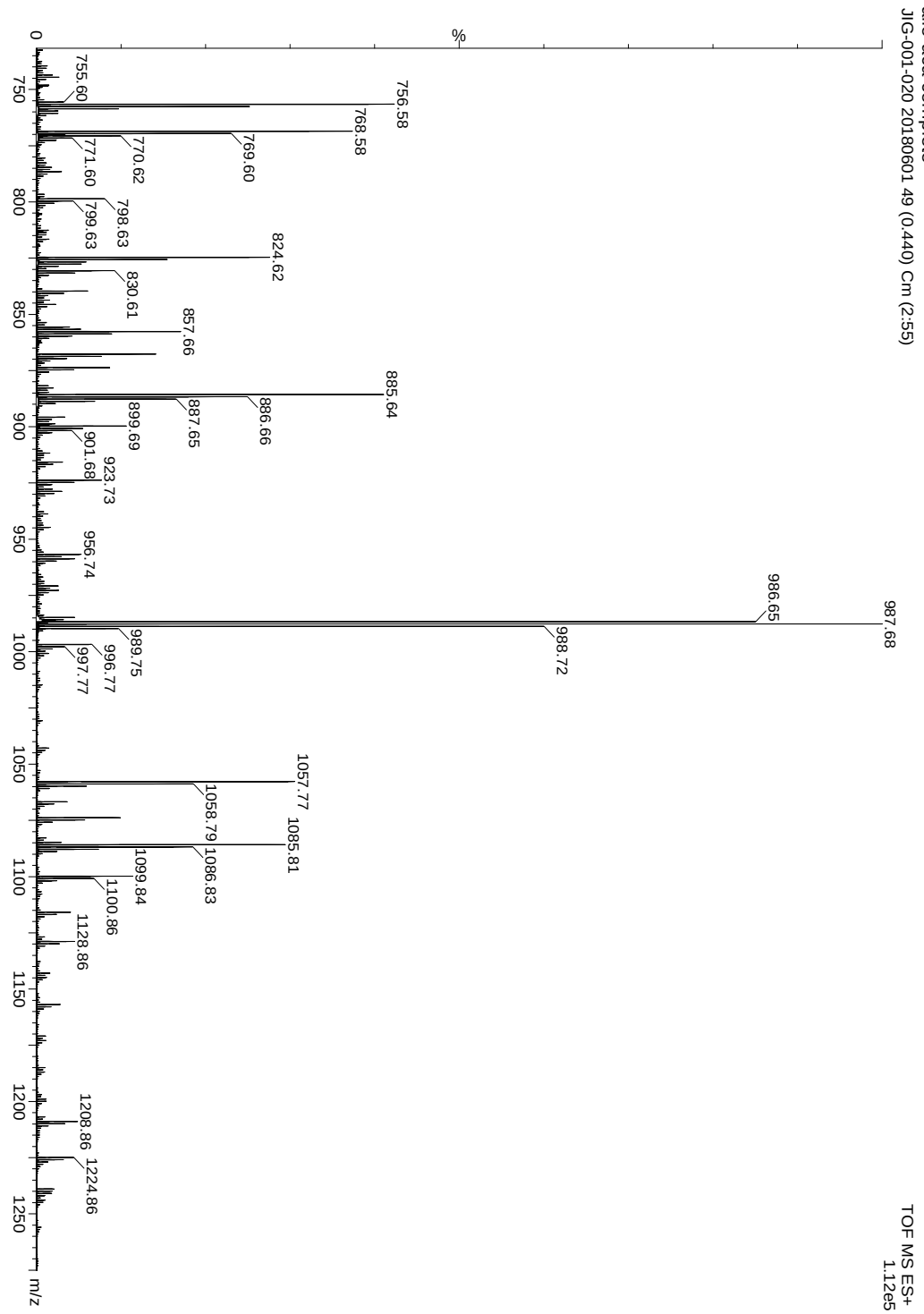
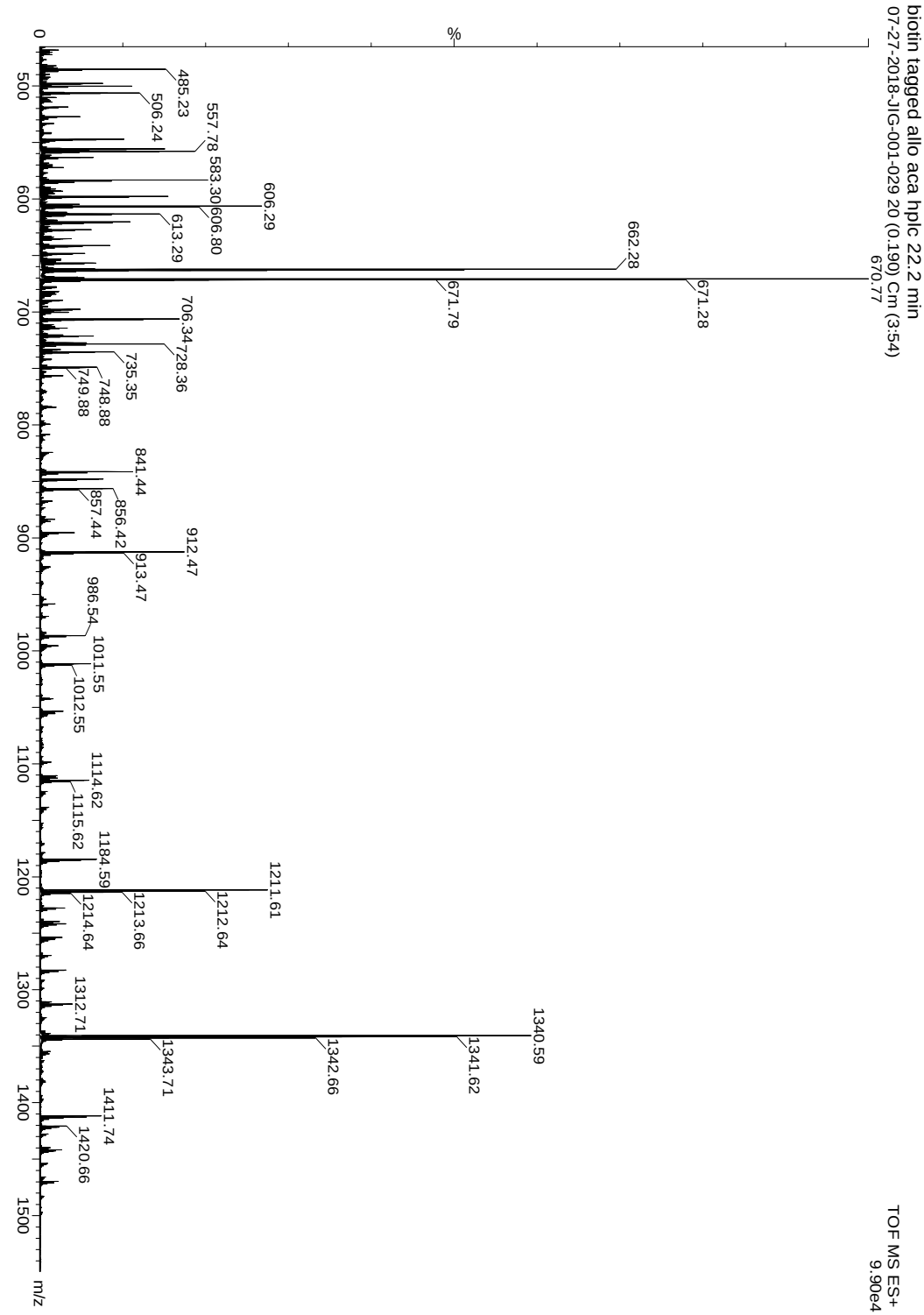
Appendix



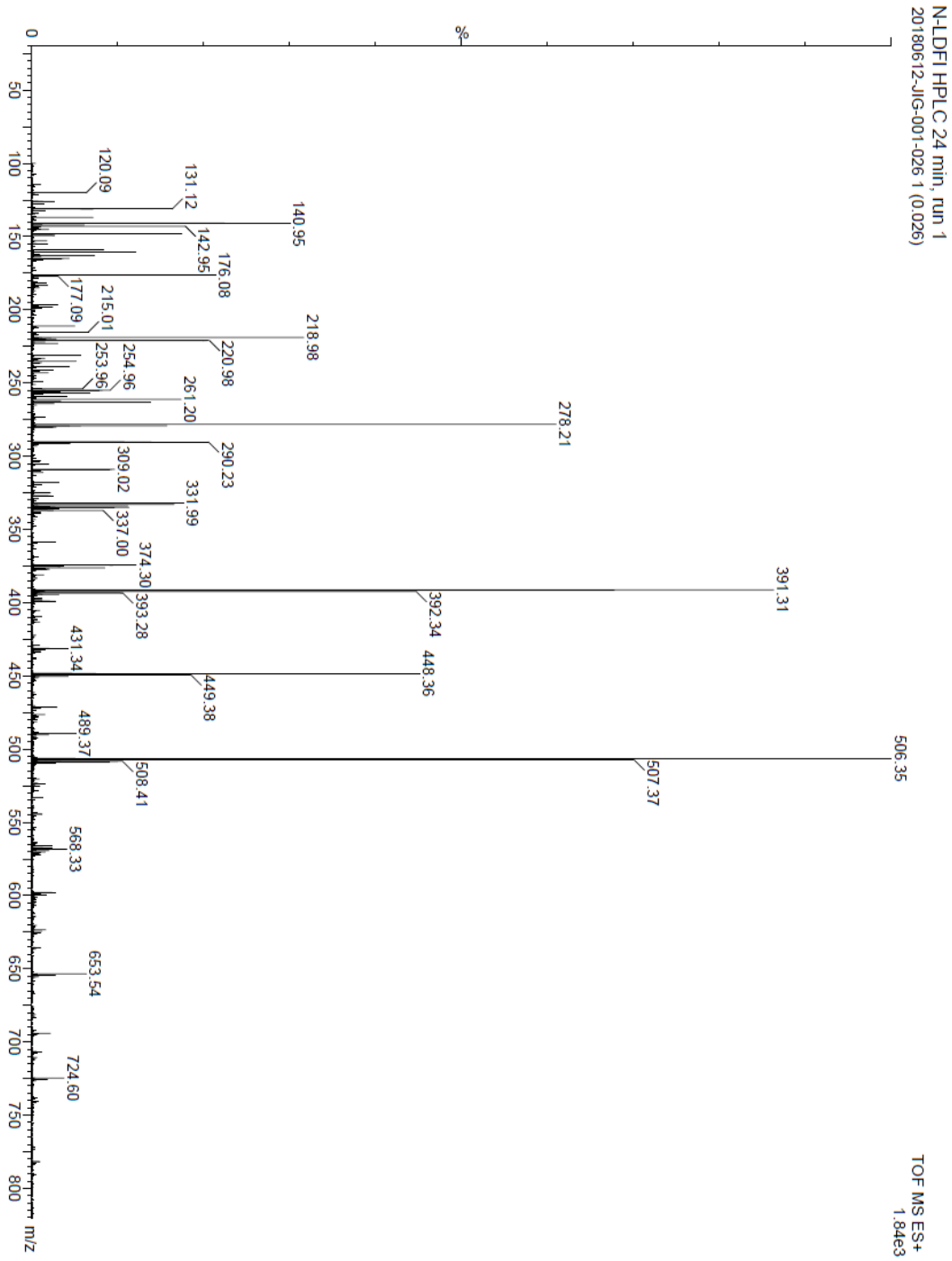
Appendix 1. LDFI expected M+H 506.62 g/mol, observed M+H 506.26 g/mol



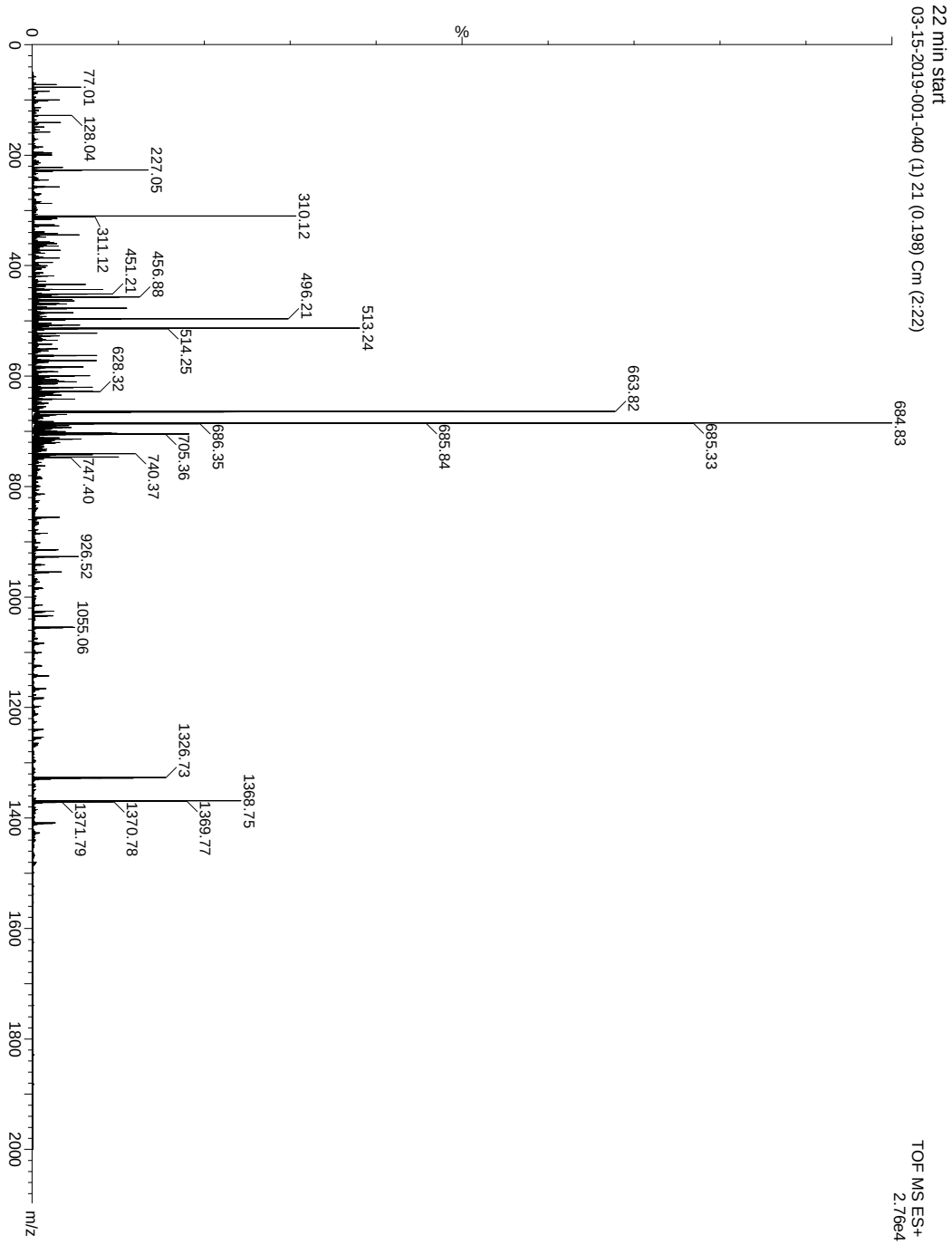
Appendix 2. Allo-aca expected M+H 986.18 g/mol, observed M+H 986.65 g/mol



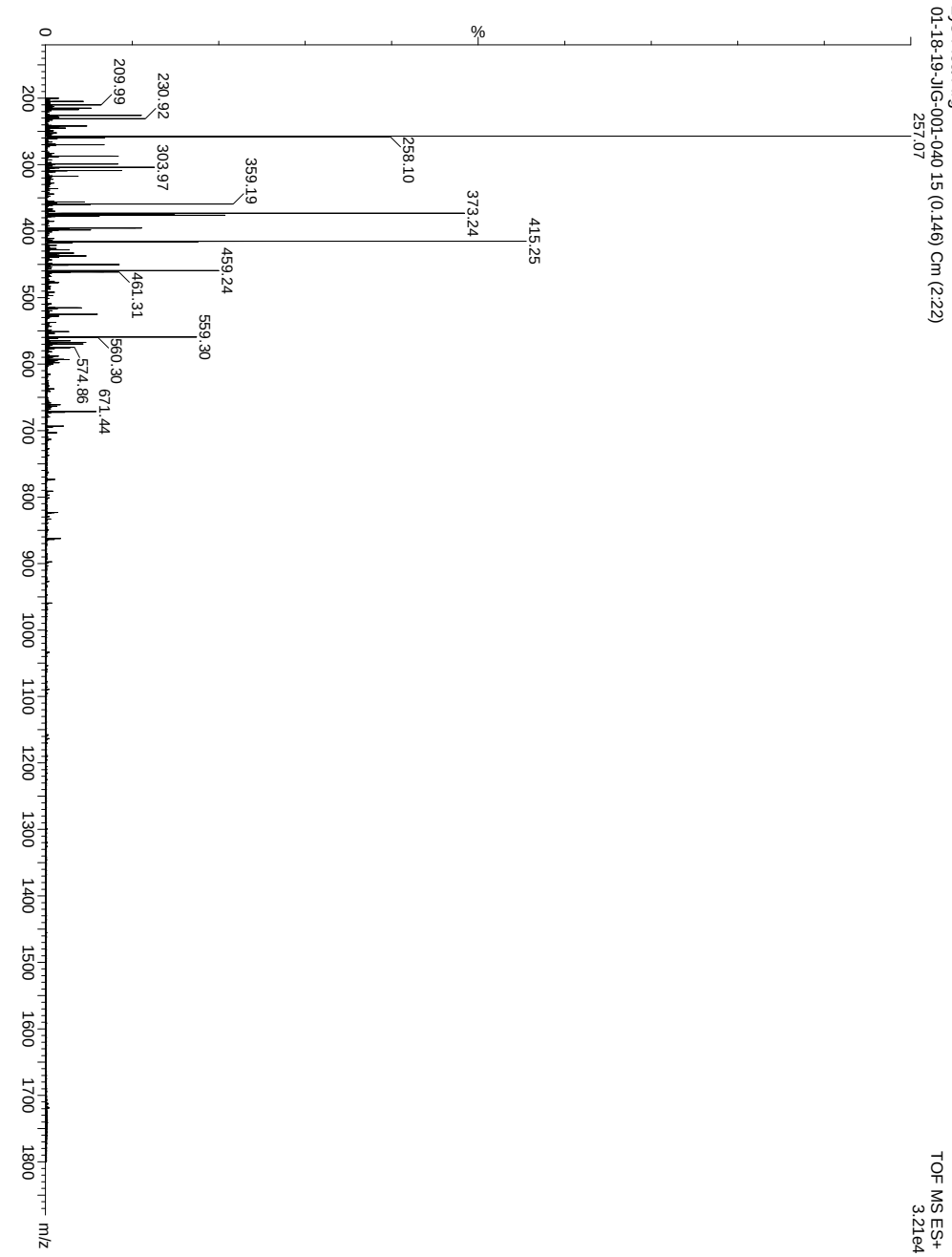
Appendix 3. Allo-aca-biotin expected M+H 1341.66 g/mol, observed M+H 1341.62 g/mol



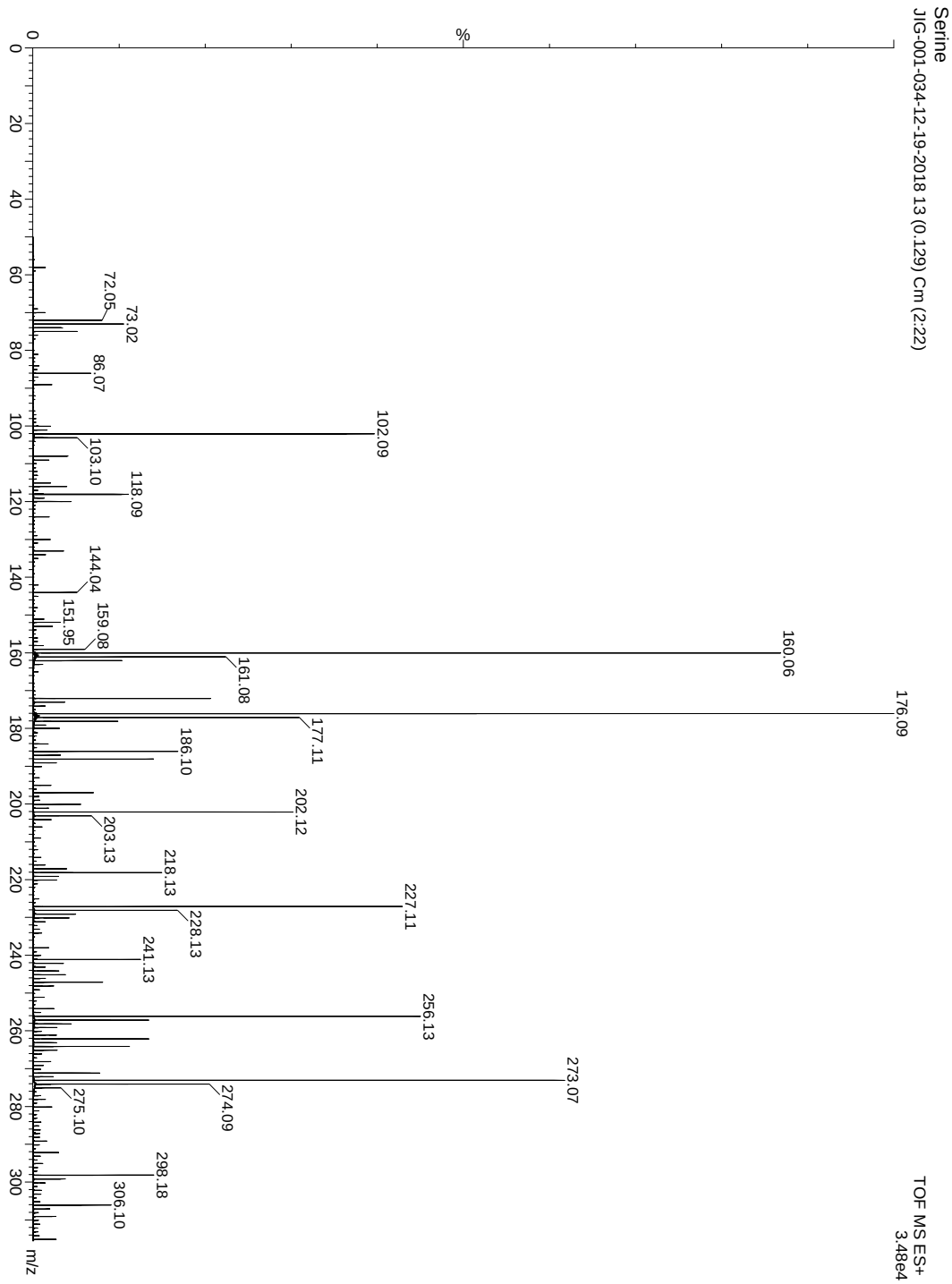
Appendix 4. N-LDFI expected M+H 506.62 g/mol, observed M+H 506.35 g/mol



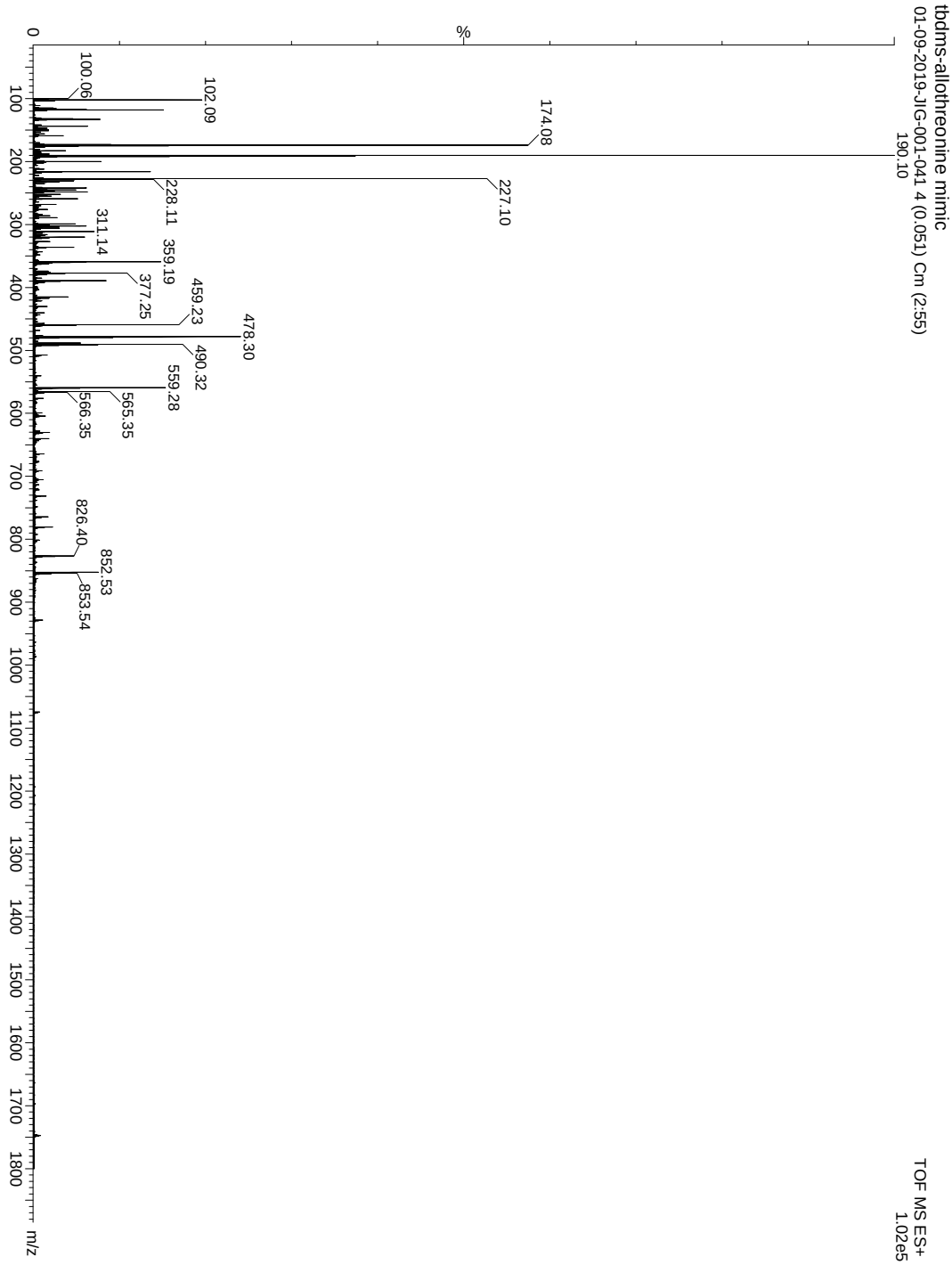
Appendix 5. N-Allo-aca-biotin in expected M+H 1369.71 g/mol, observed M+H 1369.77 g/mol



Appendix 6. N-lys-aca-arg expected M+H g/mol, observed M+H g/mol



Appendix 7. Tbdms-ethanol amine expected M+H 176.35 g/mol, observed 176.09 M+H g/mol



Appendix 8. Tbdms-1-amino-2-propanol expected M+H 190.38 g/mol, observed 190.10 M+H g/mol