Modifications of the Self-Immolative Spacer PABOH in Antibody Drug Conjugates

A Major Qualifying Project Report

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Abstract

Antibody Drug Conjugates (ADCs) represent a rapidly growing and extensively potent class of pharmaceuticals that utilize antibodies to deliver toxic chemicals directly to tumors and other dangerous cells while avoiding healthy tissues. This technology is based on the controlled yet spontaneous release of a drug compound via the domino-like cascade effect of the self-immolative spacer system p-aminobenzyl alcohol (PABOH). This report seeks to investigate the effects that various PABOH analogues have on the kinetics of spacer degradation, in an effort to optimize the efficiency of the ADC in a therapeutic context.

Acknowledgements

I would like to express my sincerest gratitude to Dr. James P. Dittami for his guidance and patience throughout the entirety of this project. This appreciation must also be extended to Stacy Van Epps, Bryan Fiamengo, and the rest of the chemistry group at Abbott Bioresearch Center. Finally, a special thanks to Alicia Morgan for her constant moral and technical support.

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1. Introduction

Antibody Drug Conjugates (ADCs) are a promising new class of pharmaceuticals that unites the realms of powerful antibody technology and small molecule cytotoxic drugs. By linking a drug molecule to a carrier antibody, it is possible to combine the specificity and targeting power of antibodies with the high potency of otherwise toxic drug molecules. Antibodies are biomolecules that are highly targeted to specific proteins expressed on the surface of cells, called antigens. These protein targets can be carefully selected to guide antibodies in distinguishing between healthy and cancerous cells. With this technology, potent drugs with an established mechanism and therapeutical index are delivered directly to the target cell, resulting in not only increased efficacy, but also a marked decrease in harmful side effects.[1] The power and potential of this technology was validated in the 2009 phase I trials of brentuximab vedotin, the first FDA approved ADC, which boasted a "52% overall response rate in relapsed Hodgkin's lymphoma, with minimal toxicity."[2]

This promising study is a result of decades of research to circumvent the inherent problems in the design of immunoconjugate drugs. The entire ADC complex must be soluble in blood plasma while retaining its structure and covalent link to the drug toxin, until the antibody has bound a malignant cell. A premature release of the drug would confer no benefits over traditional drug

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[1] Teicher, B. A., & Chari, R. V. (2011). Antibody Conjugate Therapeutics: Challenges and Potential. *Clinical Cancer Research, 17*(20), 6389-6397.

[2] Beck, A., Haeuw, J. F., Wurch, T., Goetsch, L., Bally, C., Corvaia, N. (2010). The Next Generation of Antibody-drug Conjugates Comes of Age. *Discovery Medicine, 10*(53), 329-339.

approaches or antibody-only therapies. It is also imperative that this bond can only be cleaved upon internalization by the cell, or the ADC complex would render the drug inactive.[3] These concerns led to the design of synthetic linkers and self-immolative spacers that release the toxin in a controlled and predictable manner.[1,4] Such technologies have been used to create potent, robust medicines with half-lives spanning over days or weeks, resulting in less frequency of administration and an increased quality of life for patients.[5]

[3] Safavy, A., Georg, G. I., Vander Velde, D., Raisch, K. P., Safavy, K., Carpenter, M., Wang, W., Bonner, J. A., Khazaeli, M. B., Buchsbaum, D. J. (2004). Site-Specifically Traced Drug Release and Biodistribution of a Paclitaxel-Antibody Conjugate toward Improvement of the Linker Structure. *Bioconjugate Chemistry, 15*(6), 1264-1274.

[4] Senter, P. D., & Sievers, E. L. (2012). The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nature Biotechnology*, *30*(7), 631-637.

[5] Carter, P. J. (2006). Potent antibody therapeutics by design. *Nature Reviews Immunology*, *6*, 343-357.

2. Background

Interest in the targeted delivery of drugs to specific harmful cells can be traced back as far as a century to Paul Ehrlich, a German immunologist who proposed the idea of such a "magic bullet" therapy.[4] Animal models of the first ADC-like structures appeared in literature during the 1960's.[2] However, it wasn't until the development of the monoclonal, antigen-specific antibody in 1975 that clinical applications of ADCs became feasible.[4] Since then, much focus of ADC research has been concentrated on manipulating and optimizing the antibody itself and ensuring uniformity and reproducibility in the drug linkages.[5] We focus here, instead, on the development of a linker system with ideal kinetics, specifically at the point where the drug molecule is released from the complex.

The structure of our antibody drug conjugate system is based on that of brentuximab vedotin, the only ADC medication currently approved by the FDA, shown below.

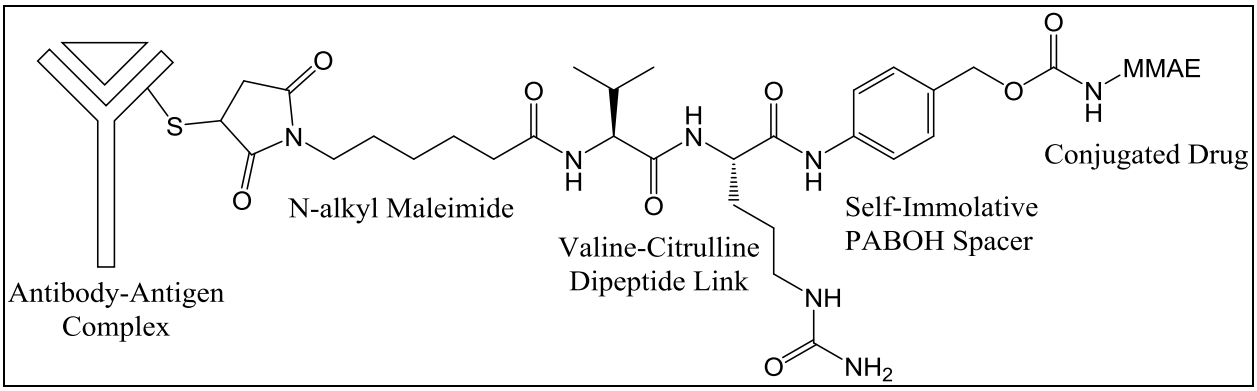

Figure 1: Structure of brentuximab vedotin (Adcetris)[4]

Created by Seattle Genetics and marketed as Adcetris, it was granted accelerated approval by the FDA in August 2011 for the treatment of relapsing Hodgkin lymphoma and anaplastic large cell lymphoma (ALCL). Adcetris quickly gained notoriety as the first treatment approved for Hodgkin lymphoma in over three decades. The structure of brentuximab vedotin consists of a monoclonal IgG antibody, followed by a stable yet self-degradative linkage, and finally the tubulin-inhibiting drug monomethyl auristatin E (MMAE). In a phase II clinical trial of this medicine, a 75% overall response rate (ORR) was observed in patients with Hodgkin lymphoma and 86% in ALCL , as compared to the unconjugated antibody which yielded 0% and 17% ORR, respectively. Remarkably, a reduction in tumor size was observed in 94% of Hodgkin lymphoma patients.[4]

Each antibody in a dose of Adcetris has an average of four drug molecules conjugated to its surface. A significant focus of ADC development has been on maximizing the amount of active drug delivered to the cell per antibody. The importance of this is demonstrated below in Figure 2, which outlines the six key steps in the ADC mechanism: reaching the target cell, binding it, internalization by the cell, cleavage of the drug molecule, transportation of the drug, and finally the desired drug delivery.

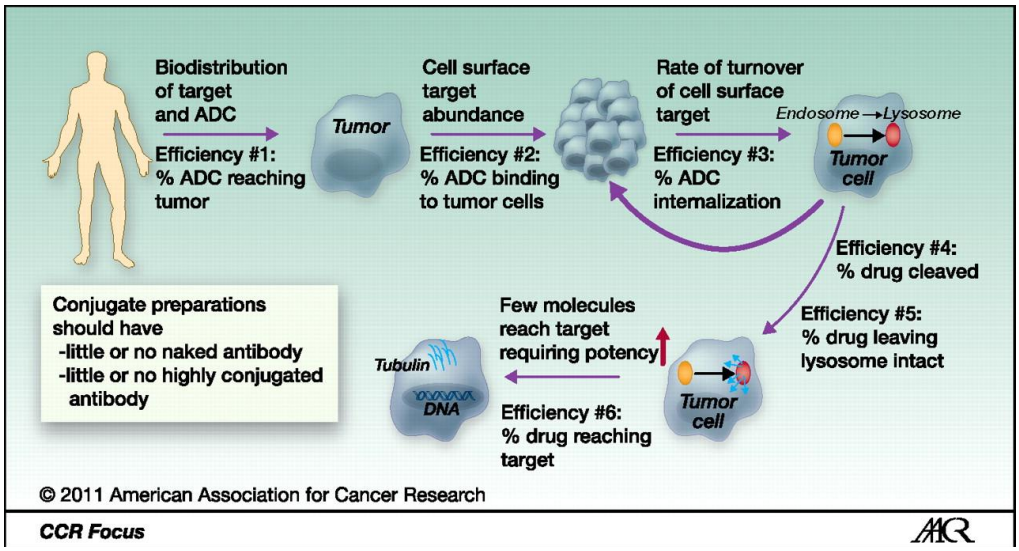

Figure 2: Overview of primary reactions in ADC delivery[1]

Assuming this to be a singly linked drug complex and estimating that each of these six events occurs at 50% efficiency, only 1.56% of the active drug will reach its target.[1]

ADCs with a larger number of drug linkages have also been investigated, but with a documented loss in potency and circulation time due to the likelihood of precipitation of the hydrophobic drug molecules.[3,4] Early ADC models employed abundant lysine residues on the surface of the IgG antibody to covalently link toxins, but this resulted in a lack of homogeneity and hyper-conjugated antibodies. With about 90 lysine molecules expressed on the antibody's surface, it was also difficult to prevent the drugs from binding to residues important in antigen recognition. Attention then turned to conjugation onto the antibody's external cysteine molecules. Treatment of the antibody with mild reducing conditions afforded only eight possible drug bond locations, none of which were crucial in antibody-antigen interactions.[2]

The linker system of brentuximab vedotin (Figure 2) is bound to the antibody's cysteine residue via a thiol conjugation to a maleimide ring.[4] This is a well characterized Michael-type addition

reaction generally known to be stable and spontaneous in blood plasma conditions.[6] Adjacent to the maleimide is a 5 carbon n-alkyl chain that serves as an inert spacer between the antibody and the self immolative linker-drug complex. This prevents steric and electronic interference by the conjugated drug with the antibody's ability to recognize and bind the desired antigen.[4]

Bound to the alkyl chain is a dipeptide linkage of valine to citrulline, which marks the essential starting point for the controlled drug release. When the ADC binds to its antigen, it is internalized into the cell by a lysosome in a process known as receptor-mediated endycytosis.[4] Inside the harsh environment of the lysosome, the ADC encounters the proteolytic enzyme Cathepsin B, which selectively cleaves peptide bonds. This specific protease is expressed almost exclusively in all mammalian lysosomes and high levels of cytosolic Cathepsin B have been correlated with poor cancer prognoses.[7,8] Earlier drug-conjugate techniques report using pH-sensitive linkers to initiate the self immolization, but the enzyme-mediated approach ensures that the complex is inside the lysosome, and thus the intended cell, before degradation.[1]

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[6] Baldwin, A. D., & Kiick, K. L. (2011). Tunable Degradation of Maleimide-Thiol Adducts in Reducing Environments. *Bioconjugate Chemistry, 22,* 1946-1953.

[7] Toki, B. E., Cerveny, C. G., Wahl, A. F., Senter, P. D. (2002). Protease-Mediated Fragmentation of *p*-Amidobenzyl Ethers: A New Strategy for the Activation of Anticancer Prodrugs. *Journal of Organic Chemistry, 67*(6), 1866-1872.

[8] Dubowchik, G. M., & Firestone, R. A. (1998). Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorganic & Medicinal Chemistry Letters, 8,* 3341-3346.

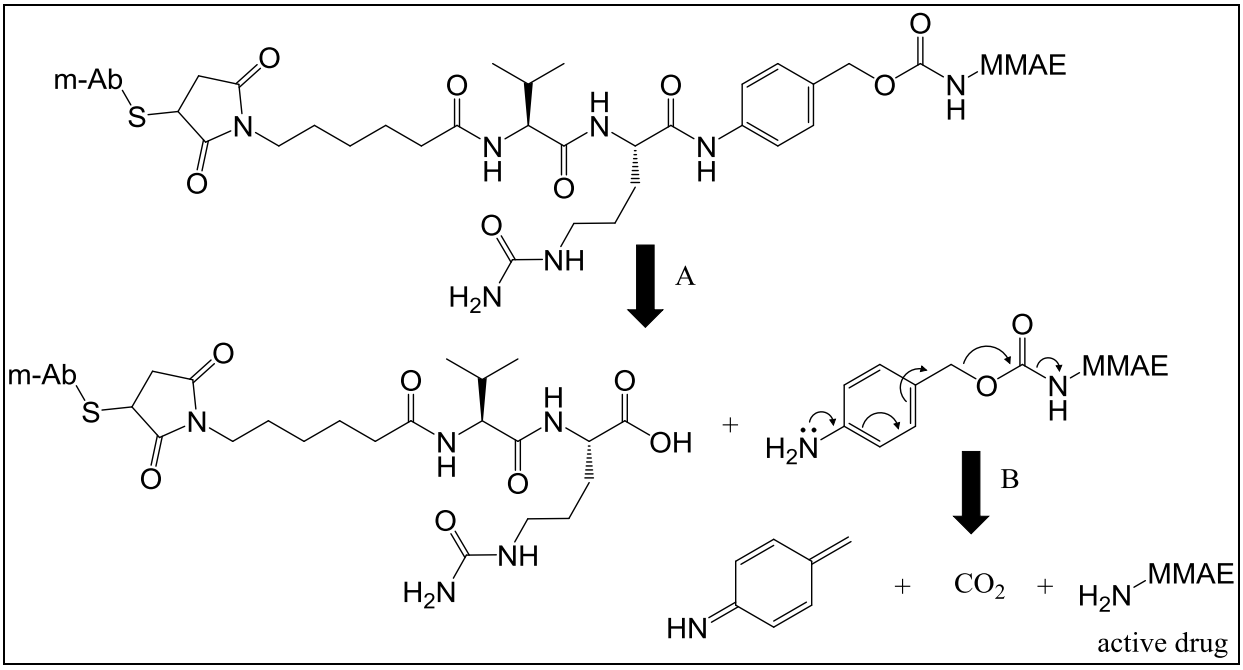


Figure 3: Cathepsin B mediated cleavage and 1,6-elimination of PABOH[4,9]

When Cathepsin B cleaves the dipeptide bond between valine and citrulline, electron density is forced into the subsequent benzylic derivative, driving the disassembly of the linker and the release of an active drug molecule (Figure 3A).[7] This cyclic spacer is para-aminobenzyl alcohol, commonly known as PABOH, and was developed in 1981 by the Katzenellenbogen group of the University of Illinois as an addition to traditional linkers in prodrug technology.[9,10] PABOH is one of the most frequently employed self immolative spacers.[7] When electron density enters the already conjugated system, PABOH decomposes through a 1,6-elimination reaction known as

[9] Carl, P. L., Chakravarty, P. K., Katzenellenbogen, J. A. (1981). A Novel Connector Linkage Applicable in Prodrug Design. *Journal of Medicinal Chemistry, 24*(5), 479-480.

[10] Erez, R., & Shabat, D. (2008). The azaquinone-methide elimination: comparison study of 1,6- and 1,4-eliminations under physiological conditions. *Organic & Biomolecular Chemistry, 6*, 2669-2672.

para-azaquinone-methide elimination (Figure 3B). This decomposition results in the spontaneous release of CO_2 and a quinone species for further catalysis, along with the active drug that can now function in the cell. There is also evidence that ortho analogues of PABOH can function similarly through a 1,4-elimination, though the 1,6-elimination may be slightly faster under physiological conditions[10]. Brief investigations into the kinetics of heterocyclic rings, such as pyridine, in an equivalent PABOH system have also been reported with similar success.[11]

[11] Perry-Feigenbaum, R., Baran, P. S., Shabat, D. (2009). The pyridinone-methide elimination. *Organic & Biomolecular Chemistry, 7*, 4825-4828.

3. Results and Discussion

The general procedure for the construction of our antibody drug conjugate was as follows:

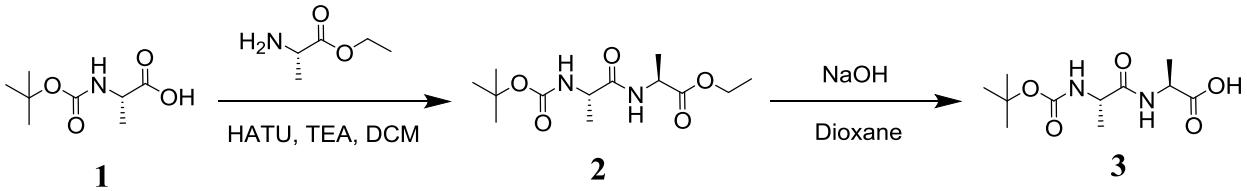


Figure 4: L-Alanine coupling and ethyl ester deprotection

Compound **1**, the N-terminal boc-protected L-Alanine, was coupled to a C-terminal ethyl ester-protected L-Alanine via the coupling reagent HATU. This reaction proceeded quickly and with excellent yield (93%), to afford compound **2**, the twice protected di-Alanine system, after silica gel chromatography. The presence of this product was determined by ^{1}H NMR via the disappearance of the carboxylic acid peak 1H singlet at δ=12.5 ppm and the presence of the corresponding peaks for the ethyl-protected Alanine: a 3H triplet at δ=1.16 ppm and 2H quadruplet at δ=4.05 ppm indicating the ethyl ester, a 3H triplet marking the methyl group (which is no longer a quadruplet) at δ=1.37 ppm, a 1H multiplet at δ=4.0 ppm indicating the adjacent C-H proton, and finally the amine doublet signal at δ= 8.15 ppm, which integrated to 1H instead of 2H. (NMR 1)

The twice protected di-Alanine system **2** then underwent saponification to remove the ethyl ester, via treatment with an excess of NaOH and then the subsequent adjustment to pH 4 with acetic acid. Product **3**, the deprotected carboxylic acid, was isolated by column chromatography in fair yield (76%). Its structure was confirmed by ^{1}H NMR by the disappearance of the ethyl ester

protons, the 3H triplet at δ=1.16 ppm and 2H quadruplet at δ=4.05 ppm, along with the reappearance of the carboxylic acid 1H singlet at δ=12.5 ppm.

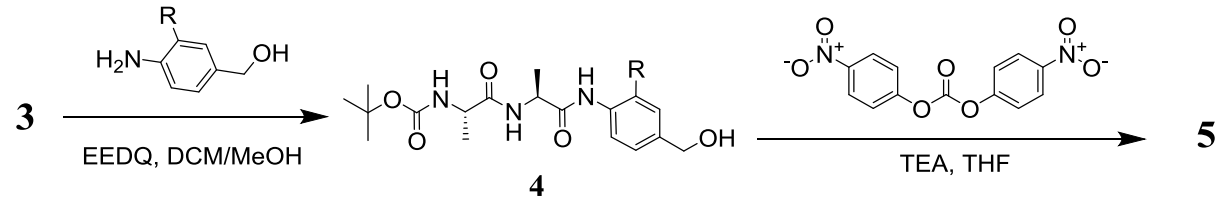


Figure 5: PABOH addition and carbamate introduction

The carboxylic acid deprotected di-Alanine compound **3** was then treated with (4-amino-phenyl)-methanol (PABOH) or a derivative thereof. In combination with the coupling reagent EEDQ, the amine group on PABOH added into the exposed carboxylic acid site of the dipeptide, yielding compound **4** after flash chromatography. This product was identified with ^{1}H NMR by the benzylic and methanol protons of each PABOH derivative, in the case of (4-amino-phenyl)-methanol as two 2H multiplets at 7.23 and 7.52 ppm, along with a 2H doublet at δ=4.43 ppm and an alcohol 1H triplet at δ=5.09 ppm, respectively, as well as ensuring the amine proton peak of PABOH at δ=9.87 integrated to 1H and not 2H.

The PABOH derivatives employed in this experiment are shown below:

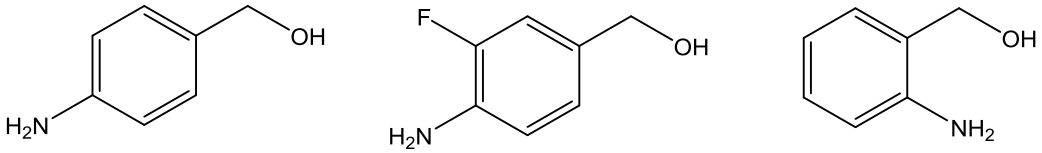


Figure 6: PABOH and other derivatives of interest

The final component of the self-immolative spacer, the carbamate functionality, was installed onto compound **4** by a triethylamine-mediated reaction with bis-(4-nitrophenyl) carbonate. After a flash column, this afforded compound **5** (below) in excellent yield (94%). ^{1}H NMR analysis confirmed the structure by the addition of two 2H benzylic multiplets at δ=7.57 and 8.31 ppm, as well as the disappearance of the alcohol triplet from PABOH at δ= 5.09. Consequentially, the adjacent methylene group 2H shifted from a doublet at δ=4.43 to a singlet at δ=5.25.

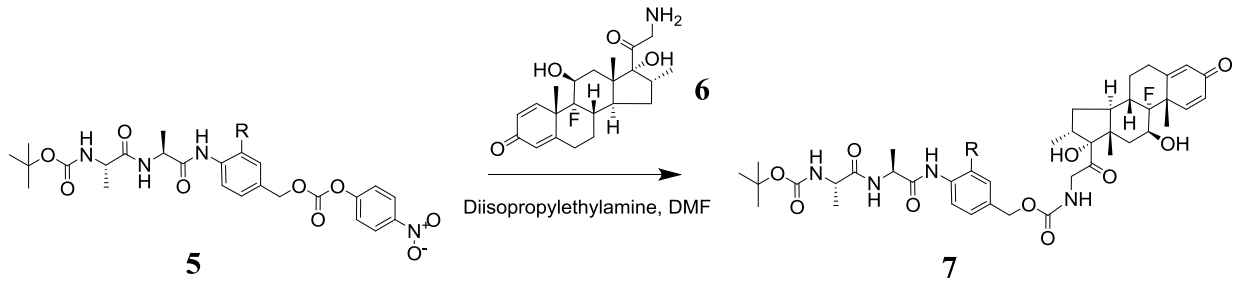


Figure 7: Attachment of the drug molecule dexamethamine

Commercial dexamethamine (**6**), the amine form of the common anti-inflammatory drug dexamethasone, was attached to compound **5** through a diisopropylethylamine-mediated addition. This resulted in compound **7** after silica gel chromatography, the structure of which was elucidated by ^{1}H NMR. The compound was identified by the disappearance of the benzylic 2H multiplets (δ=7.57 and 8.31 ppm) from the carbonate **5**, as well as the identification of the peaks corresponding with the drug molecule **6**, where the amine proton signal shifted from 2H at δ=8.20 to 1H at δ=7.99.

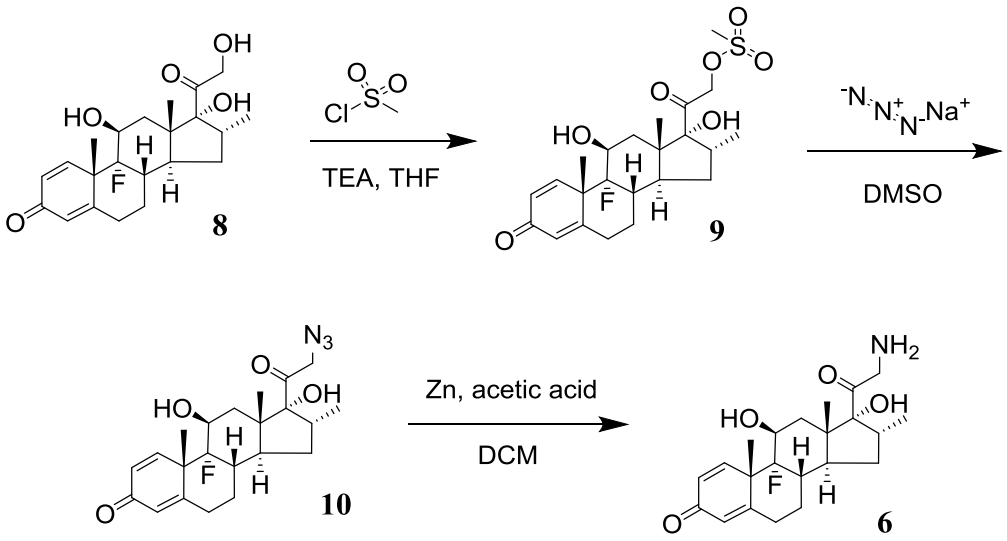
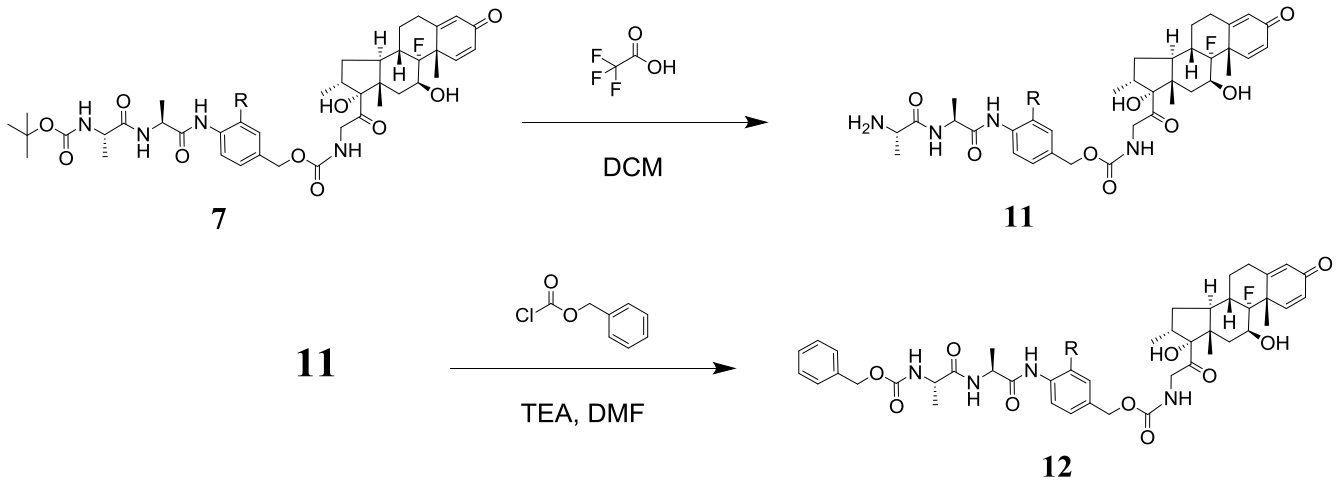


Figure 8: Amination of commercial dexamethasone

The amination of dexamethasone (**8**) to dexamethamine (**6**) was also performed in house according to the synthetic route above. A methylsulfonyl group was added to the terminal alcohol on compound **8** via a triethylamine-mediated reaction with methanesulfonyl chloride. This product **9** was attained via flash column separation and identified by ^{1}H NMR by the disappearance of the drug's alcohol triplet around δ=4.90 ppm, the doublet to singlet transformation of the 2H adjacent to the alcohol near δ=4.70 ppm, and the addition of a 3H singlet signal at δ=3.40 ppm indicating the methylsulfonyl group.

The introduction of the essential nitrogen molecule to compound **9** was performed by a substitution reaction using sodium azide to yield product **10**. This compound was recovered by flash chromatography and its structure was confirmed by ^{1}H NMR which indicated that the 3H methyl peak from the methylsulfonyl group at δ=3.40 ppm had vanished. The azide group on compound **10** was then reduced to the free amine by a reaction with solid zinc and acetic acid,

completing the amination process, in 97% yield. The synthetic dexamethamine was cleaned by flash column and its structure was confirmed by ^{1}H NMR via the appearance of a 2H broad singlet signal at δ=8.20 ppm, suggesting the terminal free amine.



7 11 11 12

Figure 9: Deprotection and addition of a new protecting group

Once the drug molecule had been attached, the final step was to cap the system in preparation for the assay. In a clinical setting, the molecule would be terminated with an n-alkyl maleimide as discussed earlier, to conjugate to the cysteine on the antibody. Here we experimented with different protecting groups in an effort to replicate the clinical setting without constructing the whole antibody linkage. Compound **7**, the boc-protected ADC system, was submitted for the assay and also used as a gateway to two other submissions.

Compound **7** was deprotected using trifluoroacetic acid, to yield the free amine form of the system **11** after treatment with sodium hydroxide. Acquired by column chromatography, its structure was confirmed by ^{1}H NMR by the lack of the t-butyl 9H singlet at δ=1.49 ppm. Compound **11** was submitted to the assay, and further treated with benzyl carbonochloridate in a triethylamine-mediated reaction to install the CBZ protecting group. This was run through a flash

column and the structure of the resulting product **12** was elucidated by ^{1}H NMR. This structure was identified by the benzylic protons of the CBZ, a broad 5H multiplet peak that appeared at 7.32 ppm.

This synthetic route produced nine compounds ready for a timed stability assay, shown below:

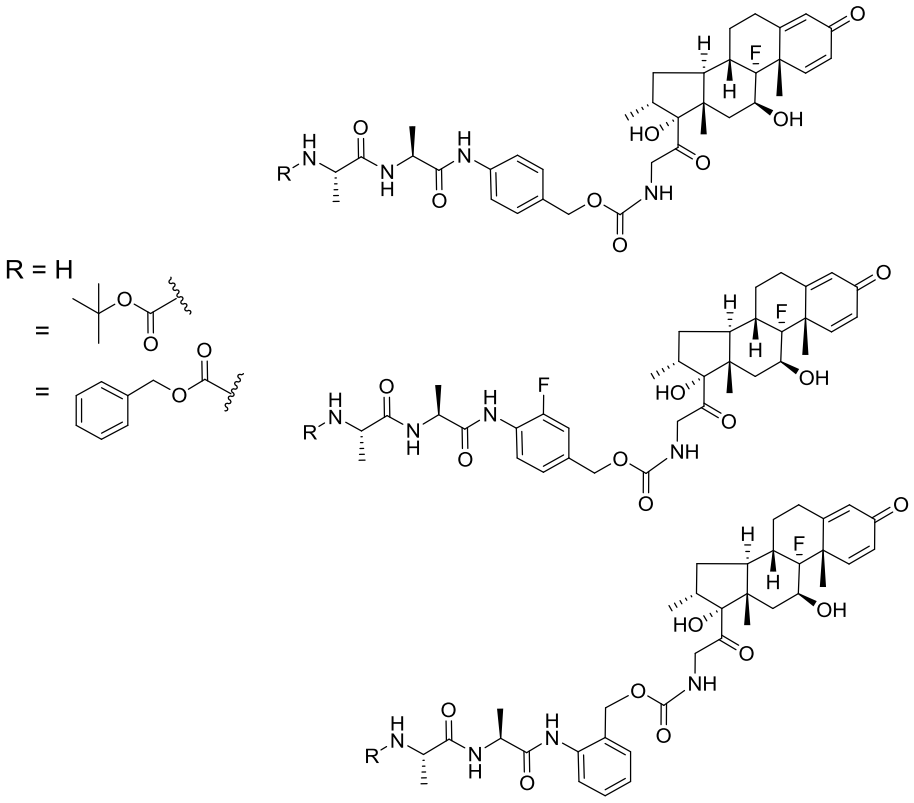


Figure 10: Summary of the ADCs submitted for assay

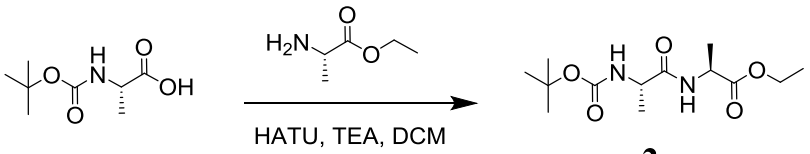
The basic idea of the assay is that a certain amount of the ADC molecule will be introduced to an environment of cell media, Cathepsin B, and a buffer to maintain the pH at physiological conditions. At specific time points, a sample will be taken and the amount of free drug in the mixture will be monitored. As a control, the ADC will also be exposed to either only the buffer

or cell culture media. Ideally, there will be no free drug in just the media or buffer environments, since it is important that the ADC remain intact until it reaches the the enzymatic lysosome of the cell. As time progresses in the Cathepsin B solution, we should see the amount of free drug increase and will be able to monitor the drug release kinetics of our novel ADC structures.

4. Experimental

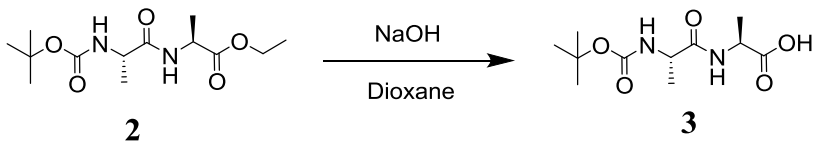
General Methods: The following procedures were based on experiments performed by chemists at Abbott Bioresearch Center. Reactions were carried out under nitrogen gas and monitored by thin-layer chromotography on silica coated glass plates and visualized under UV light at 254 nm as well as a potassium permanganate stain. Reactions were also monitored via LCMS in a gradient from 5 to 95% acetonitrile in ammonium acetate through a Halo C8 silica gel column. Removal of solvent was performed with a rotary evaporator under reduced pressure. Flash columns were run on pre-packed silica gel columns with an AnaLogix column machine, and the structures of the resulting products were elucidated via a 500 MHz Bruker NMR spectrometer. All chemical shifts in ppm are reported relative to TMS at 0.00 ppm and samples were scanned in deuterated DMSO.

The descriptions that follow depict various scales of reactions and describe only the original PABOH derivative. However, these reactions have successfully been run on many scales with comparable yields, and can be used interchangeably with other PABOH derivatives so long as the ratio of reagents is maintained.



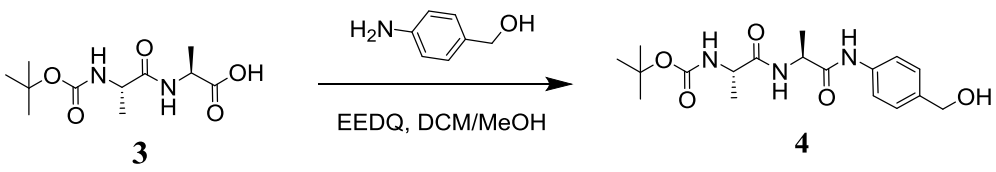
1 **2**

The coupling reagent HATU (2.211 g, 5.81 mmol) was combined with (**1**) N- terminal boc protected L-Alanine (1 g, 5.29 mmol) and C- terminal ethyl ester protected L-Alanine HCl (0.893 g, 5.81 mmol) in a 100 mL roundbottom flask. To this was added dichloromethane (DCM) (26.4 mL) and triethylamine (2.81 mL, 21.14 mmol), and the yellow solution was stirred at room temperature for one hour. It was then quenched with sodium bicarbonate and the product was extracted twice into DCM, dried over anhydrous sodium sulfate, and the solvent evaporated to give a yellow oil. This was dissolved in minimal DCM and loaded onto a silica flash column with a gradient from 0 to 60% ethyl acetate in heptane. This afforded compound **2**, the di-protected di-Alanine system (1.42 g, 93%).



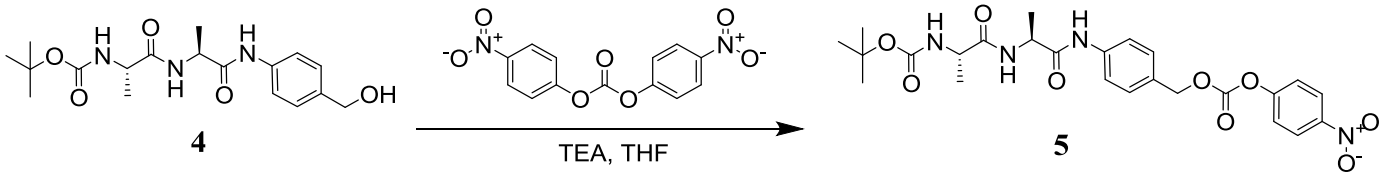
2 **3**

Compound **2** (1 g, 3.41 mmol) was added to a 100 mL roundbottom and dissolved in 1,4-dioxane (17.43 mL). To this was added 1M sodium hydroxide (13.87 mL, 13.87 mmol) and the homogenous solution was stirred at room temperature for 1.5 hours. The organic solvent was removed by vacuum, leaving the aqueous layer which was then acidified to pH 4 with 10% citric acid. The product was then extracted twice with ethyl acetate and washed with brine. After drying over sodium sulfate and removing the solvent, the white solid compound **3** was recovered (0.682 g, 76%)

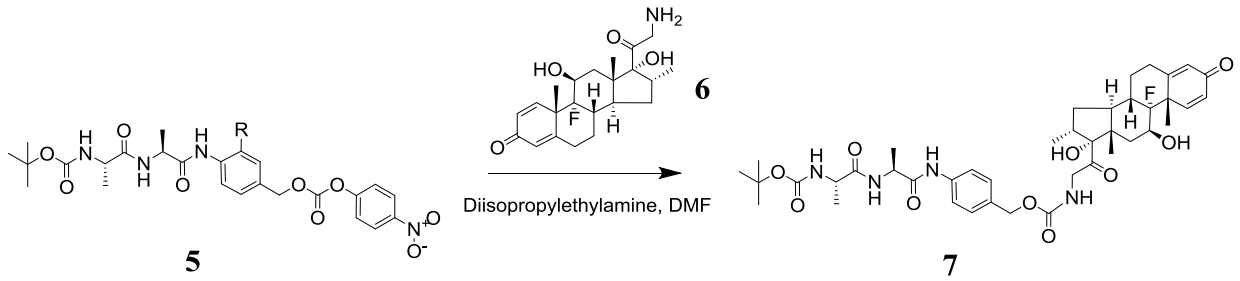


3 **4**

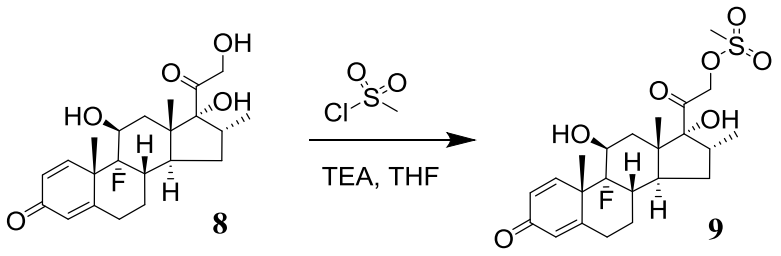
To a 150 mL roundbottom containing compound **3** (0.342 g, 1.314 mmol) was added equal parts DCM (3.28 mL) and methanol (3.28 mL) with stirring to dissolve the solid. PABOH (0.178 g, 1.445 mmol) was added to the flask, followed by the coupling reagent EEDQ (0.357 g, 1.445 mmol). The yellow homogenous solution was allowed to stir at room temperature overnight, after which the solvent was removed under vacuum to afford yellow crystals. These were dissolved in DCM, washed with water and brine, and the organic layer dried. The crude product was subject to a flash silica column in a gradient from 0 to 60% ethyl acetate in heptane to yield compound **4** (0.220 g, 46%).



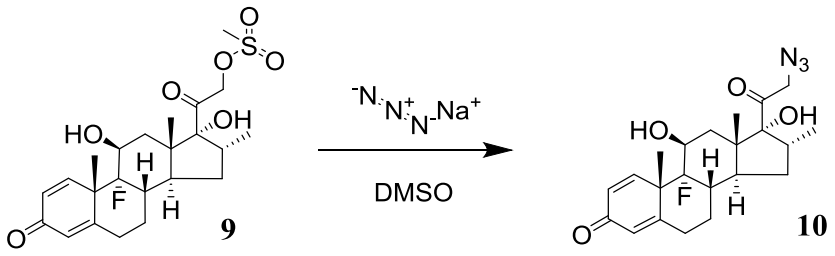
Compound **4** (.220 g, 0.602 mmol) was dissolved in THF (5 mL) in a 25 mL roundbottom flask. To this was added bis(4-nitrophenyl) carbonate (0.201 g, 0.662 mmol) with stirring and triethylamine (0.084 mL, 0.602 mmol) which afforded a yellow homogenous solution. The vessel was left stirring at room temperature overnight. It was then quenched with water and extracted twice into DCM. This was washed with brine and dried over anhydrous sodium sulfate, then subjected to a flash column with a gradient from 0 to 60% ethyl acetate in heptane. The organic solvent was removed under vacuum to yield compound **5** (0.299 g, 94%).



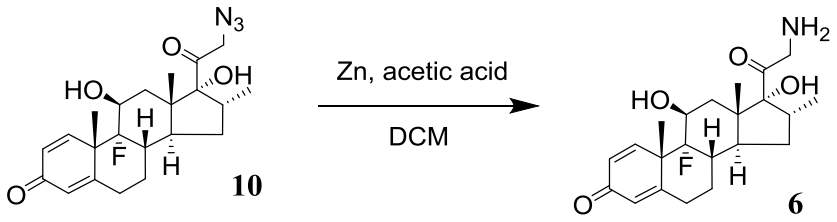
Compound **5** (1 g, 1.885 mmol) and commercial dexamethamine HCl (0.812 g, 2.073 mmol) were dissolved in DMF (15 mL) at room temperature. To this was added n,n-diisopropylethylamine (1.218 g, 9.42 mmol) and the reaction was allowed to stir. After one hour, another 0.120 g of the aminated drug were added to the solution to drive the reaction to completion. The solution was then partitioned between ethyl acetate and water, the organic layer washed with brine and dried over sodium sulfate, and solvent removed by vacuum. The crude solid was taken up in DCM and run through a flash silica column with a gradient over 0 to 10% methanol in DCM to give product **7** (1.348 g, 91%).



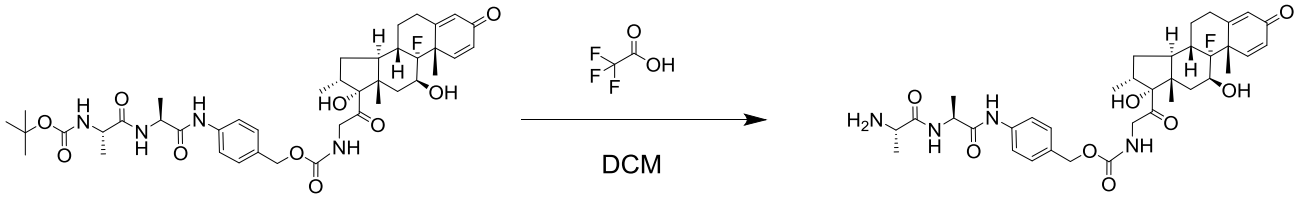
To aminate the commercially available drug, a solution of compound **8**, dexamethasone (2 g, 5.10 mmol) in THF (20 mL) was stirred and cooled to 0° C. To this was added triethylamine (1.065 mL, 7.64 mmol) and dropwise methanesulfonyl chloride (0.477 mL, 6.12 mmol). A white solid was constantly present in the solution and the vessel was allowed to stir at room temperature for 2.5 hours. The solids were filtered out and the THF removed by vacuum, leaving a tan solid which was partitioned between ethyl acetate and water. The organic layer was washed with brine and dried over sodium sulfate and the solvent removed by evaporation to yield compound **9** (1.56 g, 65 %).



Compound **9** (1.551 g, 3.30 mmol) was dissolved in DMSO (13 mL) and sodium azide (0.429 g, 6.59 mmol) was introduced. The solution was stirred at 50° C for 45 minutes as the solution progressed to a deep orange color. The solution was cooled to room temperature and poured into 50 mL of an ice and water mix to afford a bright yellow precipitate. The solid was filtered, dissolved in THF, washed with brine, dried over sodium sulfate, and concentrated by evaporation to yield 1.577 g of an amber oil. This is above 100% yield but the product, compound **10**, was clean by NMR except for DMSO.

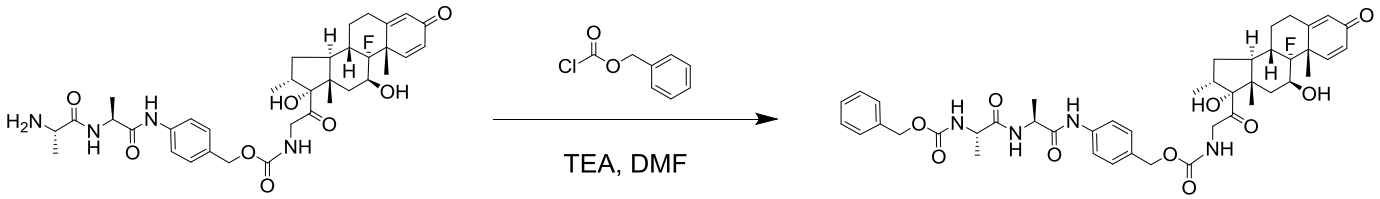


Compound **10** (1.376 g, 3.30 mmol) was dissolved in DCM (13.73 mL) and acetic acid (2.75 mL) and cooled to 0° C. To this was added flakes of solid zinc metal (2.156 g, 33.0 mmol) and the reaction was stirred for two hours. The solids were filtered through filter paper and rinsed with methanol, and the filtrate was dried under vacuum. The crude product was partitioned between ethyl acetate and water, and the aqueous layer was adjusted to pH 10 with 10% Na2CO3. The product was extracted from here into ethyl acetate, which was dried over anhydrous magnesium sulfate, and evaporated to yield compound **6**, the amine form of commercial dexamethasone (0.442 g, 33%).



7 **11**

Compound **7** (0.500 g, 0.639 mmol) was dissolved in DCM (15 mL) and cooled to 0° C. To this was added trifluoroacetic acid (0.492 mL, 6.39 mmol) dropwise. Upon full addition of the acid, the solution was allowed to equilibrate to room temperature and stir. This was then heated to 40° C and stirred for 5 hours. The solution was carefully quenched under proper ventilation with water, and the aqueous layer was basified dropwise with 1 N NaOH until no more solid production was observed. The solid was dissolved in diethyl ether, washed with brine, dried over sodium sulfate, and dried by vacuum. The crude product was treated with a flash column with a gradient of 50 to 100% of a 85:13.5:1.5 DCM:MeOH:NH4OH solution in DCM. This recovered compound **11** (.214 g, 49%).



11 **12**

Compound **11** (0.075 g, 0.099 mmol) was dissolved in DMF (1 mL). To this was added benzyl carbonochloridate (0.030 mL, 0.218 mmol) and triethylamine (0.053 mL, 0.395 mmol) and the reaction was allowed to stir as the vessel was heated to 45° C for three hours. The reaction was cooled to room temperature and quenched with sodium bicarbonate and extracted twice into DCM. This was washed with brine and dried over sodium sulfate with the solvent removed to yield a thick yellow oil. This was subjected to a flash column with a gradient of 60 to 100% ethyl acetate in heptane to yield Compound **12** (0.030 g, 37%).

References

Baldwin, A. D., & Kiick, K. L. (2011). Tunable Degradation of Maleimide-Thiol Adducts in Reducing Environments. *Bioconjugate Chemistry, 22,* 1946-1953.

Beck, A., Haeuw, J. F., Wurch, T., Goetsch, L., Bally, C., Corvaia, N. (2010). The Next Generation of Antibody-drug Conjugates Comes of Age. *Discovery Medicine, 10(53),* 329-339.

Carl, P. L., Chakravarty, P. K., Katzenellenbogen, J. A. (1981). A Novel Connector Linkage Applicable in Prodrug Design. *Journal of Medicinal Chemistry, 24(5),* 479-480.

Carter, P. J. (2006). Potent antibody therapeutics by design. *Nature Reviews Immunology, 6,* 343-357.

Dubowchik, G. M., & Firestone, R. A. (1998). Cathepsin B-sensitive dipeptide prodrugs. 1. A model study of structural requirements for efficient release of doxorubicin. *Bioorganic & Medicinal Chemistry Letters, 8,* 3341-3346.

Erez, R., & Shabat, D. (2008). The azaquinone-methide elimination: comparison study of 1,6- and 1,4-eliminations under physiological conditions. *Organic & Biomolecular Chemistry, 6,* 2669-2672.

Perry-Feigenbaum, R., Baran, P. S., Shabat, D. (2009). The pyridinone-methide elimination. *Organic & Biomolecular Chemistry, 7,* 4825-4828.

Safavy, A., Georg, G. I., Vander Velde, D., Raisch, K. P., Safavy, K., Carpenter, M., Wang, W., Bonner, J. A., Khazaeli, M. B., Buchsbaum, D. J. (2004). Site-Specifically Traced Drug Release and Biodistribution of a Paclitaxel-Antibody Conjugate toward Improvement of the Linker Structure. *Bioconjugate Chemistry, 15(6),* 1264-1274.

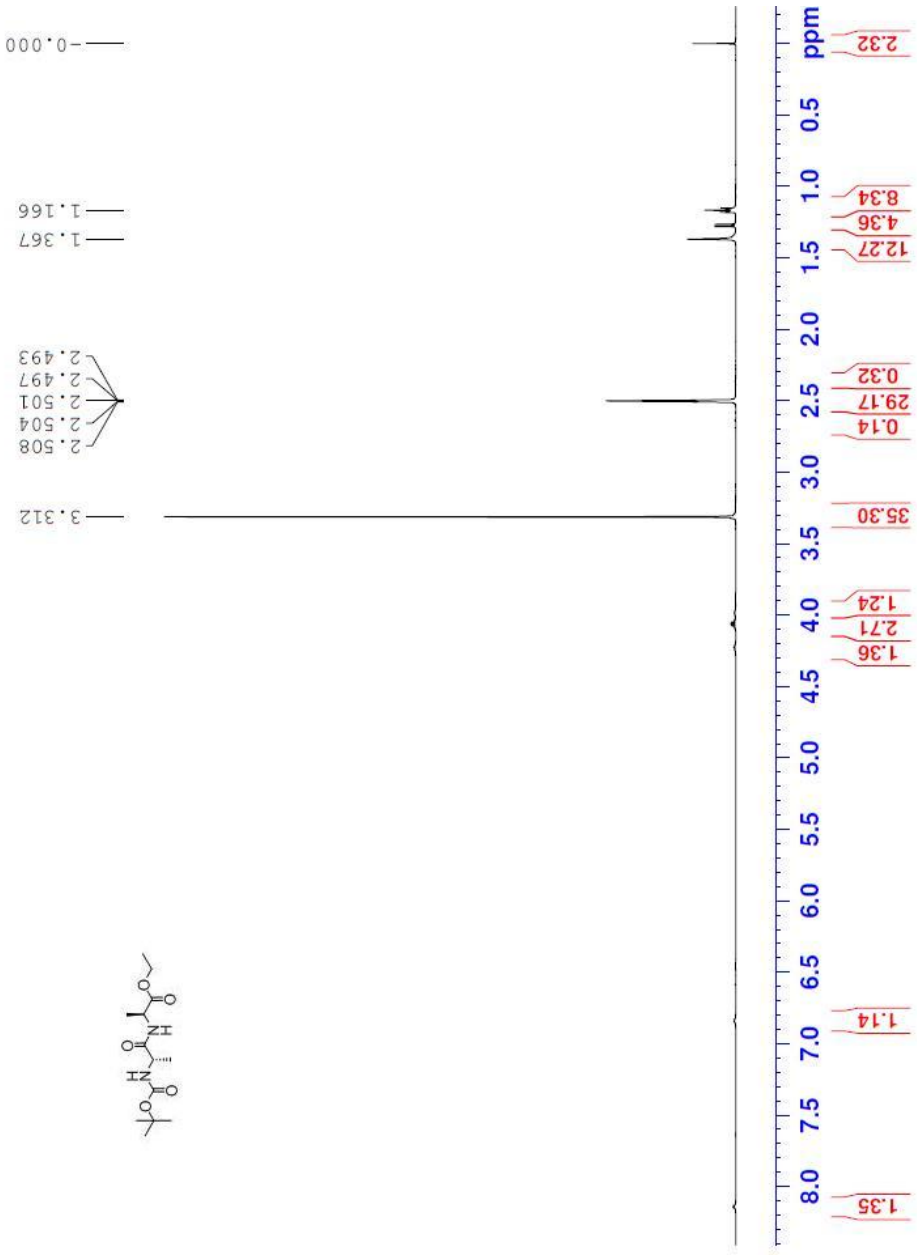
Senter, P. D., & Sievers, E. L. (2012). The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma. *Nature Biotechnology, 30(7),* 631-637.

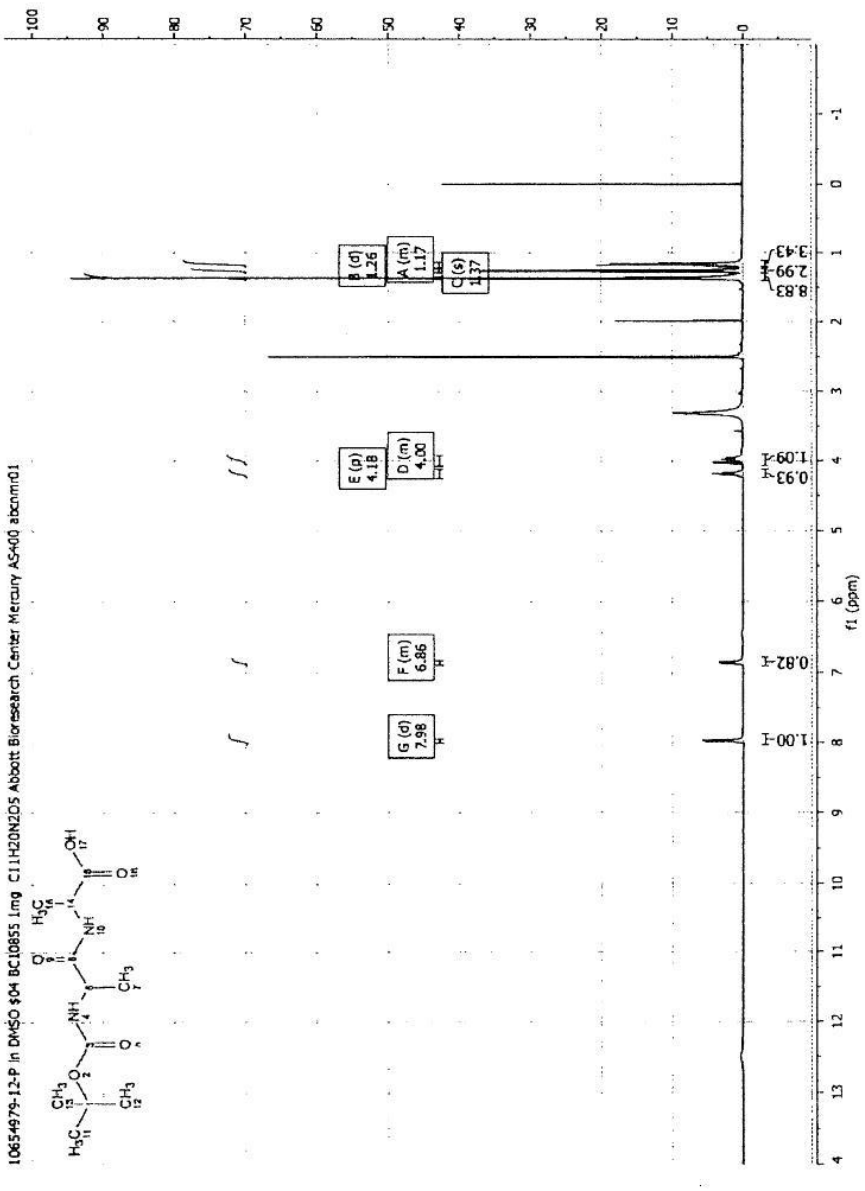
Teicher, B. A., & Chari, R. V. (2011). Antibody Conjugate Therapeutics: Challenges and Potential. *Clinical Cancer Research, 17(20),* 6389-6397.

Toki, B. E., Cerveny, C. G., Wahl, A. F., Senter, P. D. (2002). Protease-Mediated Fragmentation of p-Amidobenzyl Ethers: A New Strategy for the Activation of Anticancer Prodrugs. *Journal of Organic Chemistry, 67(6),* 1866-1872.

Appendices

Appendix A: NMR Data

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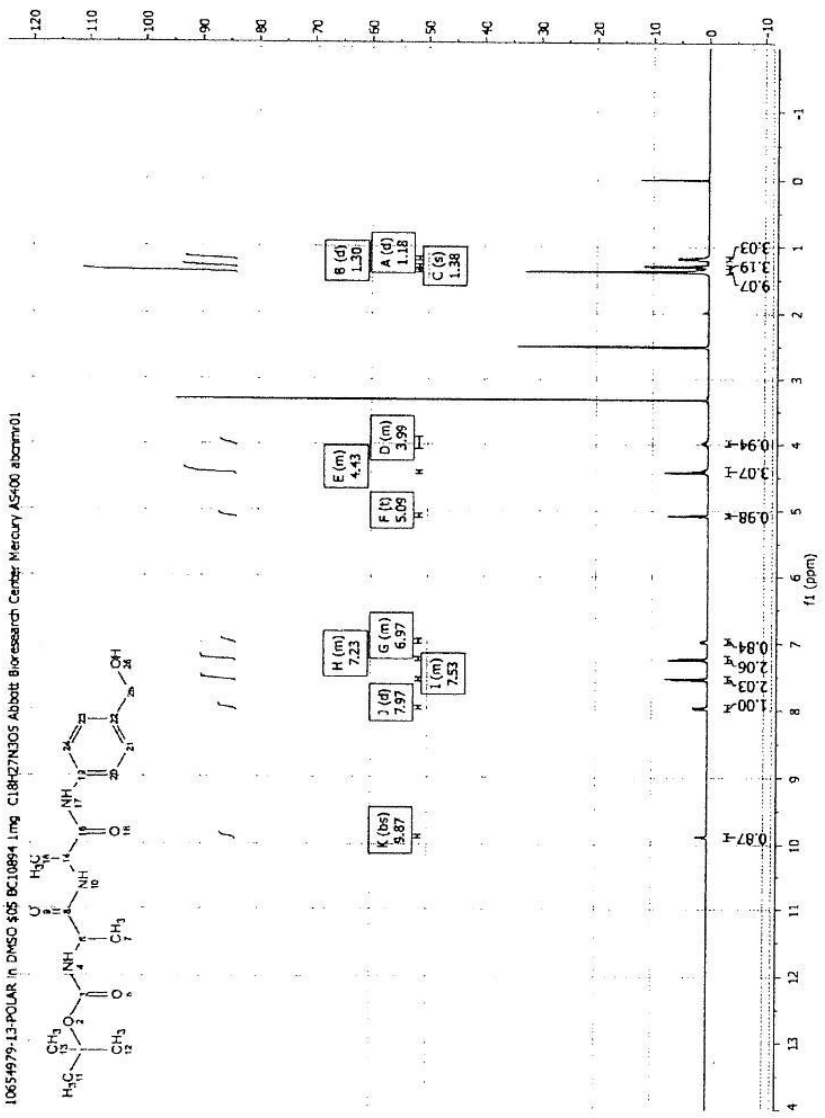
A (m) 1.17
C (s) 1.37
E (p) 4.18
D (m) 4.00
F (m) 6.86
G (d) 7.98
8.83
2.99
3.43
1.09
0.93
0.82
1.00
f1 (ppm)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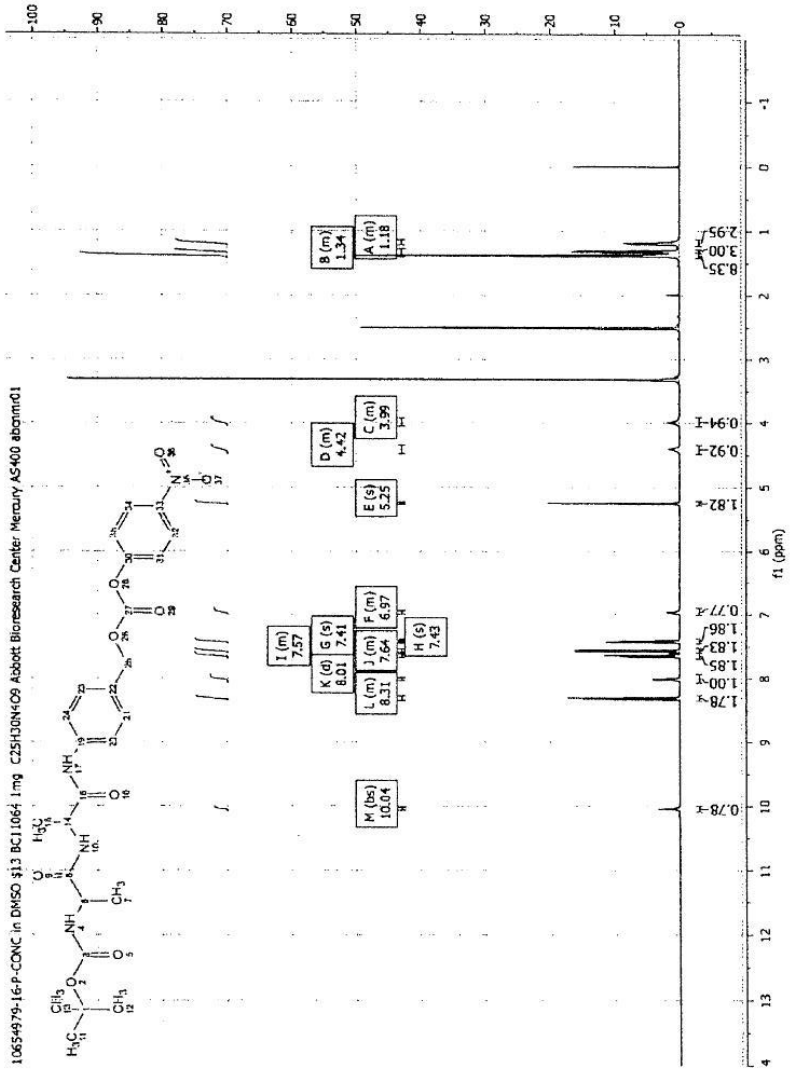
A (d) 1.18
C (s) 1.38
E (m) 4.43
D (m) 3.99
F (t) 5.09
H (m) 7.23
G (m) 6.97
J (d) 7.97
I (m) 7.53
K (bs) 9.87
9.07
3.19
3.03
0.94
3.07
0.98
0.84
2.06
2.03
1.00
0.87
f1 (ppm)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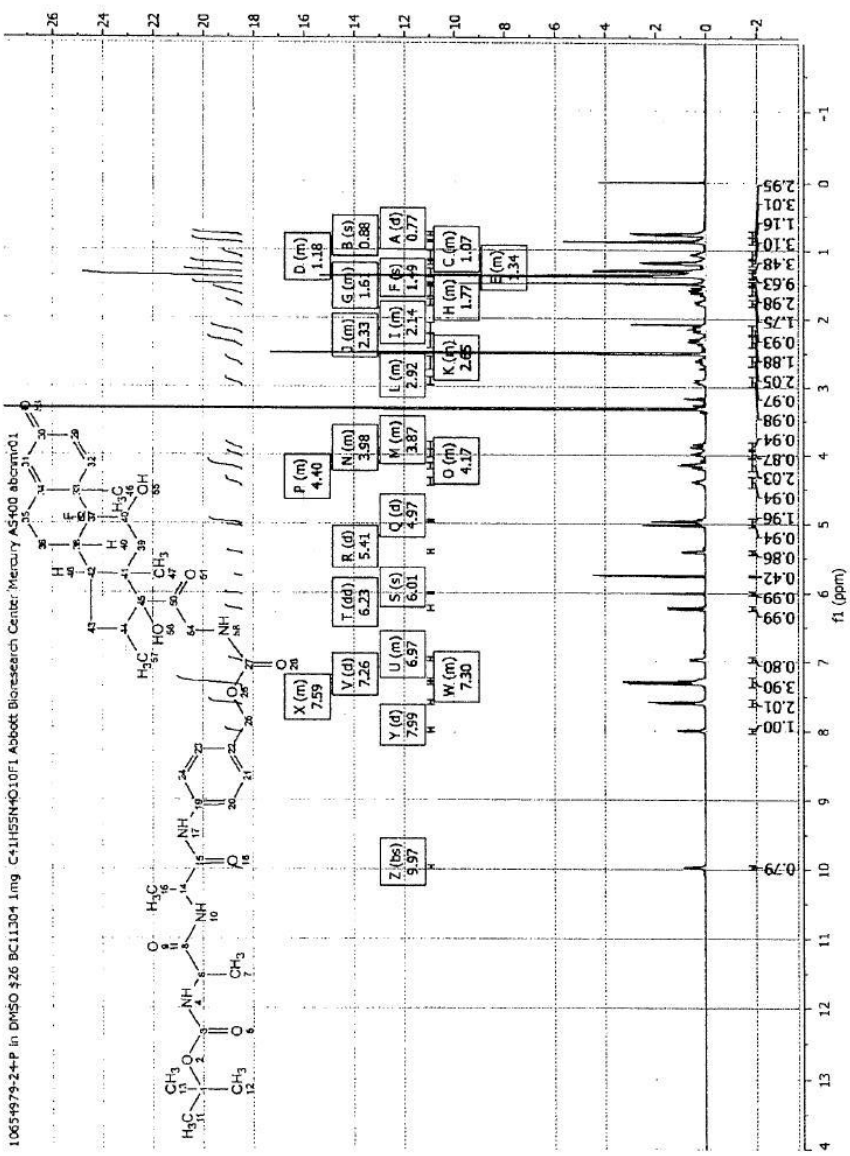
B (m) 1.34
C (m) 3.99
D (m) 4.42
E (s) 5.25
F (m) 6.97
G (s) 7.41
H (s) 7.43
I (m) 7.57
J (m) 7.64
K (d) 8.01
L (m) 8.31
M (bs) 10.04
8.35
3.00
2.95
0.94
0.92
1.82
0.77
1.86
1.83
1.85
1.00
1.78
0.78
f1 (ppm)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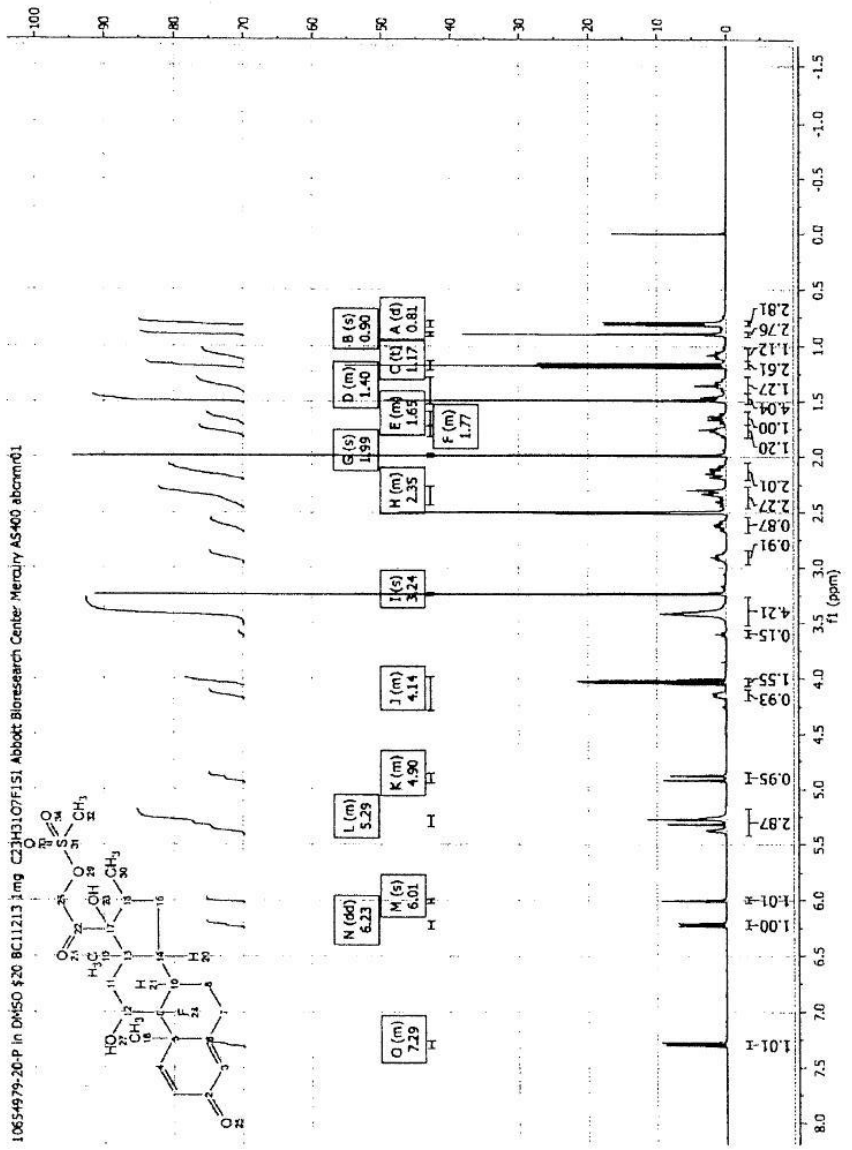
D (m) 1.18
B (s) 0.88
A (d) 0.77
G (m) 1.61
F (s) 1.49
C (m) 1.07
E (m) 1.34
D (m) 2.33
I (m) 2.14
H (m) 1.77
L (m) 2.92
K (t) 2.65
P (m) 4.40
N (m) 3.98
M (m) 3.87
O (m) 4.17
Q (d) 4.97
R (d) 5.41
S (s) 6.01
T (dd) 6.23
X (m) 7.59
Y (d) 7.26
U (m) 6.97
W (m) 7.30
Y (d) 7.99
Z (bs) 9.97
f1 (ppm)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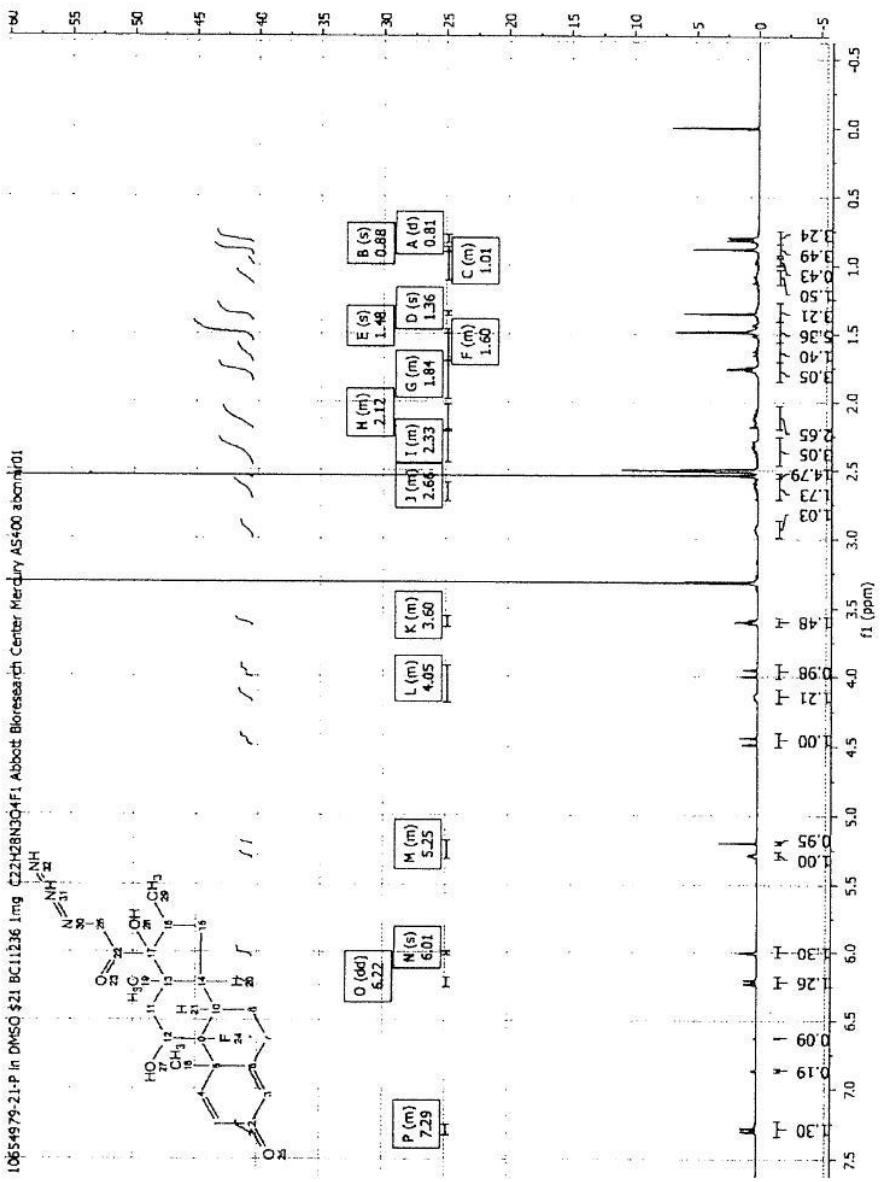
A (d) 0.81
B (s) 0.90
C (t) 1.17
D (m) 1.40
E (m) 1.65
F (m) 1.77
G (s) 1.99
H (m) 2.35
I (s) 3.24
J (m) 4.14
K (m) 4.90
L (m) 5.29
M (s) 6.01
N (dd) 6.23
O (m) 7.29
f1 (ppm)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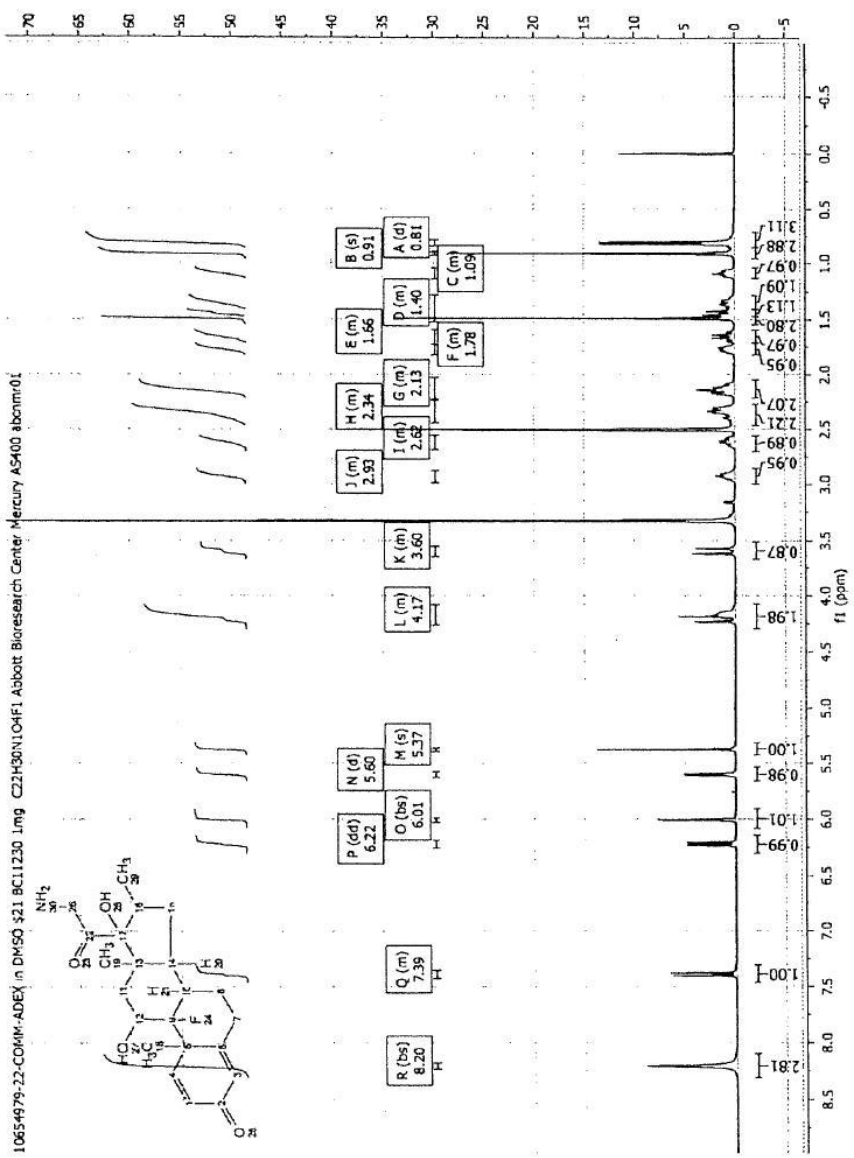
B (s) 0.88
A (d) 0.81
C (m) 1.01
E (s) 1.48
D (s) 1.36
F (m) 1.60
H (m) 2.12
G (m) 1.84
I (m) 2.33
J (m) 2.66
K (m) 3.60
L (m) 4.05
M (m) 5.25
O (dd) 6.22
N (s) 6.01
P (m) 7.29
f1 (ppm)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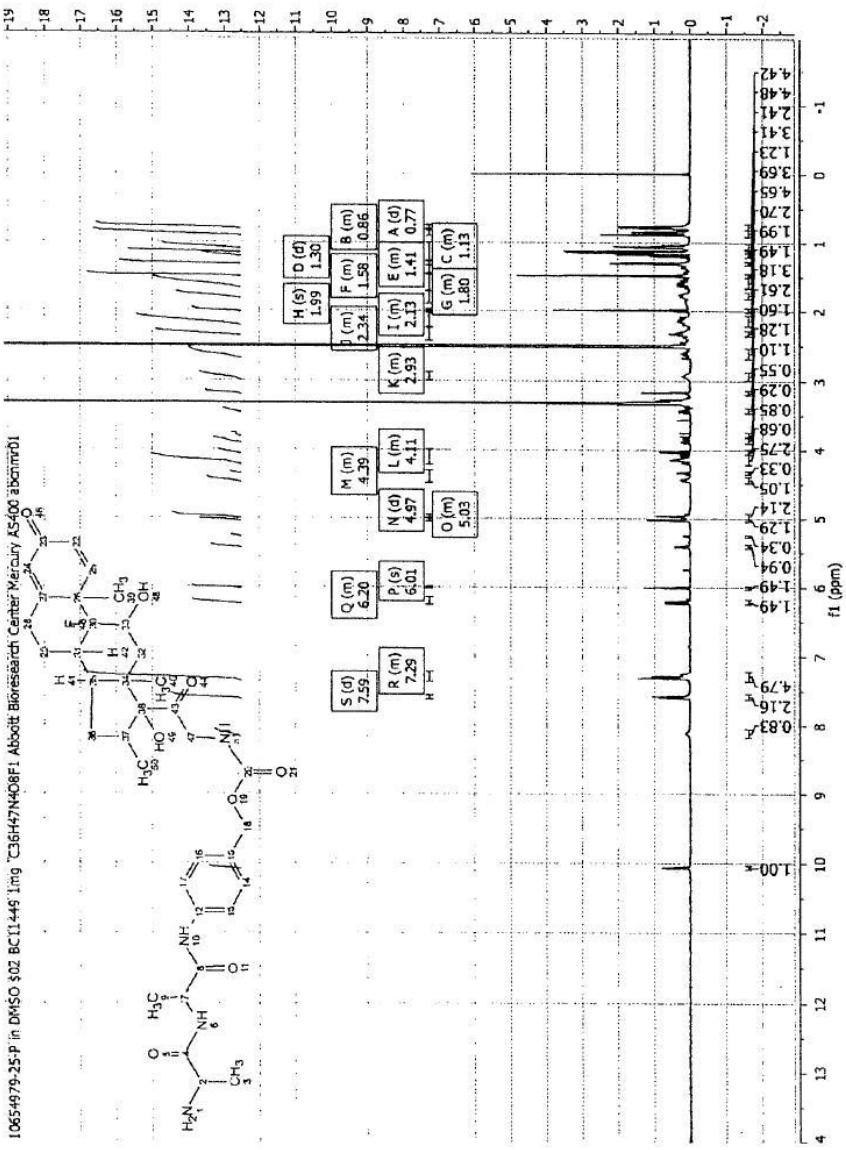
B (s) 0.91
C (m) 1.09
D (m) 1.40
E (m) 1.66
F (m) 1.78
G (m) 2.13
H (m) 2.34
I (m) 2.62
J (m) 2.93
K (m) 3.60
L (m) 4.17
M (s) 5.37
N (d) 5.60
O (bs) 6.01
P (dd) 6.22
Q (m) 7.39
R (bs) 8.20
f1 (ppm)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D (d) 1.30
B (m) 0.86
J (m) 2.34
F (m) 1.58
E (m) 1.41
A (d) 0.77
I (m) 2.13
G (m) 1.80
C (m) 1.13
K (m) 2.93
M (m) 4.39
L (m) 4.11
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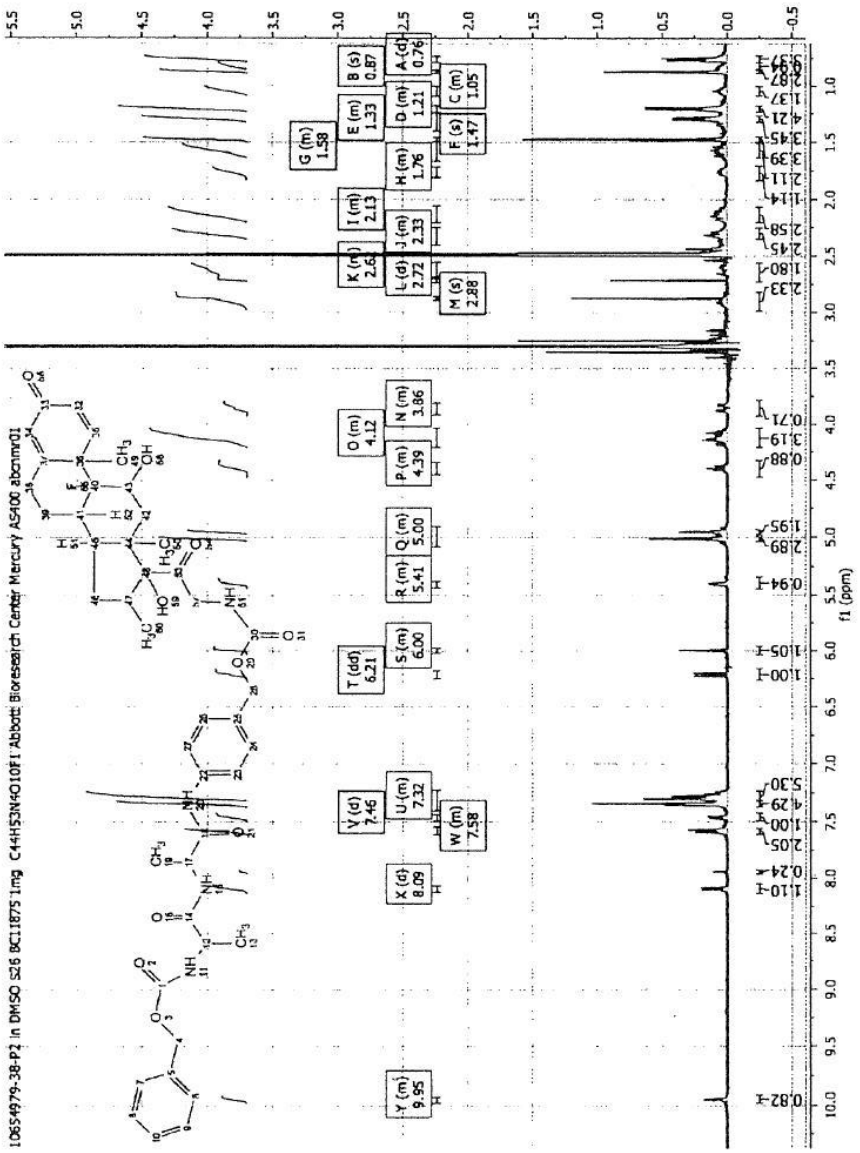
R (m) 7.29
f1 (ppm)


f1 (ppm)