The Convergent Synthesis
Of the
Dexamethasone-C8-Cephem-Methotrexate Substrate

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I. Abstract

Dexamethasone-C7-cephem-methotrexate (11) is a heterodimeric small molecule that is used in a yeast three-hybrid system to screen for enzymatic activity within a pool of proteins. The two terminal positions of the small molecule are ligands for proteins in vivo and are linked by a cephem derivative, which is a cephalosporinase substrate. Dexamethasone (Dex) and methotrexate (Mtx) reside on either side of the commercially available cephem core necessitating their individual synthesis prior to production of the target molecule. The molecule is synthesized convergently so that the cephem core can be replaced with other substrates in order to produce dexamethasone-methotrexate small molecule variations that can be used for further screening.

![Structure of D8CM, the small molecule CID.](image)

II. Introduction:

Dexamethasone-C8-Cephem-Methotrexate is a synthetically made Chemical Inducer of Dimerization (CID) used in a yeast three-hybrid system. As opposed to the previously constructed yeast three-hybrid system created in the Cornish lab by Hening Lin and Wassim Abida, this new yeast three-hybrid system incorporates 2 new entities that would allow the system to screen for in vivo enzymatic catalysis. Cephem, a cleavable linker, was added between the methotrexate and dexamethasone ligands and an enzyme was also added to the system. The methotrexate ligand binds to a DNA-binding domain-dihydrofolate reductase while the dexamethasone ligand binds to an activation domain-glucocorticoid receptor protein and the Cephalosporinase substrate links the two ligands and therefore fusion proteins together. This system has two main parts, one being the chemistry involved with synthesizing this small molecule as well as the biological task of creating the yeast and screening for enzymatic catalysis. The focus of this paper
is the synthesis of this small molecule and its characterization. Most of this synthesis has been previously attempted in the Cornish Laboratory but addition of a C8 linker opposed to a C7 linker to dexamethasone is new to the synthesis because of its potential for making purification of the end product more straightforward. The small molecule has been synthesized in its more stable protected form so that it remains as pure as possible until it is used. All characterization throughout the synthesis of the small molecule was done with $^1$H NMR and MS.

A functional group on the methotrexate ligand of the small molecule is key for its ability to bind to DHFR. Although bromo pteridine can be purchased commercially, other individuals in the Cornish group have found that the commercial bromo pteridine is not successful in its coupling to methotrexate. As a result, 2, 4-diamino-6-bromomethyl pteridine is synthesized from 2,4,5,6-Tetraaminopyrimidine.

Peptide coupling involves the protection of either the N-terminus or C-terminus in order to specify the site of the coupling. Amino groups can be protected with Di-tert-butyl dicarbonate through a nucleophilic acyl substitution and carboxylic acids are usually protected through a Fisher Esterfication that converts them into methyl or benzyl esters. Protected amino acid can be coupled with other amino acids at the carboxylic acid site with the help of coupling reagents such as 1,3-dicyclohexylcarbodiimide or 1-hydroxybenzotriazole to activate the carboxylic acid as well as a base to deprotonate the amine. In some cases it has been found that using both DCC and HOBt in conjugation works better than either one independently. Esters of amino acids can be hydrolyzed or deprotected using aqueous base or aqueous acid to yield carboxylic acids in a saponification reaction. Oxidation can occur with either ozone and zinc or an oxidizing reagent such as sodium periodate. Disulfides can be converted into free thiols with tributylphosphine and water. Such methods are characteristic of those used in the convergent synthesis of protected Dexamethasone-C8-Cephem-Methotrexate (11), which will be discussed in this paper.

I previously completed the synthesis of Methotrexate on a small scale during Spring 2002 and have completed synthesis of the entire small molecule this summer on a large scale. Performing this reaction on a large scale was new to both me as well as the Cornish lab and has caused some problems that were not prevalent when performed on a
small scale. In addition, the synthesis of bromo pteridine was an extensive synthesis that I had attempted for the first time. In fact, the synthesis of D8CM would not have been completed without the availability of bromo pteridine in its mostly pure form.

III. Results

The synthesis of D8CM is convergent because it consists of joining 3 parts, the dex and mtx ligands and the cephem core. The methotrexate ligand (6) was first synthesized starting with L-Glutamic Acid γ-Methyl Ester and protecting it at the C-terminus with t-butyl acetate, which is a good protecting group because it sterically hinders the carboxylic acid from interfering in the peptide coupling. After 3 hours, the product showed an Rf of .3 in hexanes: EtOAc, 1:1. Once product was evident, the reaction was placed in an ice bath in order to avoid interaction between t-butyl acetate and the amine by overreacting. It was extracted with .5N hydrochloric acid so that the product would move into the aqueous layer and any organic impurities could be removed. It was then basified and extracted in ethyl acetate and back into the organic phase so that any water-soluble impurities could be removed. 77 % and 78 % were the yields achieved in this reaction. ¹H NMR was performed with deuterated chloroform and showed evidence of product.

The solid reagents for peptide coupling of glutamic acid to PABA were pumped on the high vacuum prior to being dissolved in DMF because the DMF is air and moisture sensitive. The reaction was also run under nitrogen gas to further prevent any moisture problems. The product turned a yellow color indicating the formation of a urea byproduct from DCC. A TLC was taken after the reaction ran overnight and showed an Rf that matched that of a sample of Colleen Bleczinski’s product in addition to an impurity with an Rf close to that of the product’s. Aqueous sodium bicarbonate and brine were added to the product to neutralize it and it was consequently extracted with ethyl acetate in order to separate it from any water-soluble impurities. An emulsion formed but was left in the organic later since it would eventually be passed through sodium sulfate, which would eliminate any water. Column chromatography was performed in order to try to separate the product from its impurities. The product, however, did not separate from the higher Rf impurity that may have resulted from delaying the addition of the
glutamic acid to the already activated PABA. Since the product was difficult to purify it was reacted with 1, 10-diaminodecane to eliminate the activated PABA. TLC in 1:1, Hexanes: EtOAc, showed the product as the highest Rf spot and made the product easier to purify by column chromatography. There was a detrimental effect on the yield of this step of the synthesis because the diamine was added to the product in two separate instances and 3 columns were run on the product. The overall yield of this step was 38%. The product was characterized by $^1$H NMR in deuterated methanol.

In the saponification of the glutamic-PABA in order to deprotect the carboxylic acid, aqueous lithium hydroxide was added to form a white precipitate upon addition. The reaction was initially run in an insulated ice bath in order to ensure that the reaction did not proceed too quickly. A TLC indicated an Rf of .6 for the product in 5:1, dichloromethane-methanol, which matched a previous trial (Bleczinski) as well as slight evidence of starting material. 1N hydrochloric acid was added to the reaction in order to neutralize it and then extracted into ethyl acetate and impurities dissolved in the aqueous were removed. The highest yield achieved with this reaction was 89 %. $^1$H NMR was done with deuterated methanol showed evidence of product.

The deprotected glutamic-PABA and cystamine were pumped on high vacuum to eliminate moisture and then dissolved in DMF and stirred under nitrogen gas to further eliminate moisture. The TLC indicated product with an Rf of .4 in 1:10, methanol: dichloromethane as well as some starting material that did not disappear even after running the reaction for additional time. The product was a deep yellow indicating the urea byproduct from DIC. The product was purified by column chromatography and the product eluted in 5% methanol/dichloromethane after eliminating the impurity in 2% methanol/dichloromethane. The yield accompanied with this reaction was 27 %. $^1$H NMR was taken in deuterated methanol and showed evidence of product.

Bromo pteridine was the last addition to the methotrexate ligand. The bromo pteridine was synthesized on both a 5-gram and 25-gram scale. The 5-gram scale showed the characteristic rust precipitate in the second step of the synthesis when 1,3-dihydroxyacetone, cysteine hydrochloride monohydrate and sodium acetate (aq) were added to 2, 4, 5, 6-diamino-6-bromomethyl pteridine and bubbled for 72 hours. After 2 recrystallizations, bromination and a final recrystallization, the product was cocoa brown
color. $^1$H NMR in deuterated methanol and MS showed mostly pure product, about 85% pure. The impurity found in small percentage is the methyl pteridine that forms during the bubbling step. On a 5-gram scale, 2.32 grams of bromo pteridine was obtained. The bromo pteridine, assumed to be pure, was coupled to the methotrexate but only yielded a mono-substituted disulfide by $^1$H NMR because the protons around the aromatic ring showed 4 distinct peaks rather than 2. One reason for this may be that 2 equivalents of the bromo-pteridine were not enough for the reaction. The bromo pteridine, however, had a distinct powerful odor of acetic acid and it was thought that perhaps the acetic acid was protonating the amine and affecting the reaction. The bromo pteridine was then pumped on the high vacuum in a desiccator for 3 days. The reaction was attempted again and some was purified by prep TLC in 10 % methanol/dichloromethane. The reaction was successful by H NMR in deuterated methanol so the rest of the product was purified by column chromatography in which the product elutes in 10 % methanol/dichloromethane. When column chromatography was performed TLCs showed impurities under the long-wave UV lamp that cannot be viewed under the short-wave UV lamp. The yield of this step was 55 %. The synthesis of bromo pteridine on a 25-gram scale did not yield the rust precipitate that should form after during the bubbling step. That synthesis was not continued because it is still unclear what formed instead of the pteridine alcohol during that step.

The final part of the synthesis of the methotrexate ligand is its reduction to a free thiol with tri-n-butylphosphine. This reaction was run under nitrogen to ensure that the free thiol would not oxidize during the reaction. A TLC was taken in 5:1, dichloromethane: methanol and the product showed an Rf of .6. The product was purified by column chromatography and the product eluted at 7% methanol/dichloromethane. The percent yield of this step was 145 % since the disulfide yields two equivalents of product for every product.

Commercially available Dexamethasone was oxidized with sodium periodate, a strong oxidizing agent, and sulfuric acid. A TLC in 1:10, methanol: dichloromethane showed an Rf of 0.3 for the product and showed no evidence of starting material. It was then basified with brine and sodium hydroxide and washed with dichloromethane to rid of any impurities soluble in the organic phase and then acidified and extracted with ethyl
acetate. A second extraction was done with dichloromethane: methanol, 1:1 but may not have been necessary. $^1$H NMR in deuterated methanol showed evidence of product with a few impurities. The product was washed with methanol several times to eliminate any insoluble material such as salts from sodium periodate and consequent $^1$H NMR showed purer product. The final yield for this step was 98%.

The oxidized dexamethasone was then coupled with a C8 linker using PyBOP as a coupling reagent as well as DIEA as a base. When all of the reagents were combined the reaction turned a golden color. After stirring overnight under nitrogen gas to avoid moisture contact, the reaction turns a dark brown color. There was still evidence of starting material in addition to the product with an Rf of 0.2 according to TLC in 10% methanol/dichloromethane. Consequently an extra equivalent of DIEA was added and the reaction stirred for an additional hour. After one hour there was no change in amount of starting material so the product was washed with a solution of brine and sodium bisulfate in order to remove any impurities such as salts. $^1$H NMR in deuterated methanol showed evidence of product. The yield of this product was 82%.

The Dexamethasone was then saponified with lithium hydroxide in the same manner that the glutamic-PABA was saponified. TLC after 1 hour in 10% methanol/dichloromethane showed evidence of product with an Rf of 0.32 in addition to starting material. The reaction ran for an additional 8 hours and there still seemed to be no change in the amount of starting material. The crude was acidified so that a white precipitate formed and then was extracted with ethyl acetate. An emulsion occurs during the extraction and is included in the organic phase because if it holds any water it will be eliminated through the sodium sulfate. $^1$H NMR in deuterated methanol showed evidence of product. This step’s final yield was 91%.

The last addition to the dexamethasone ligand before its addition to the methotrexate ligand is the cephem core. The reaction was first attempted and by TLC in 2% methanol/dichloromethane showed an Rf of 0.5, which matched that of Colleen Bleczinski. That product was purified by column chromatography. The assumed product showed evidence of the Dexamethasone compound by $^1$H NMR in deuterated methanol but the cephem peaks were not resolved enough. A prep TLC was then attempted in 2% methanol/dichloromethane but did not resolve the cephem peaks anymore. A
characteristic doublet, however, at 5.8 ppm does give evidence that the cephem compound is in fact there but the purity of the compound is still questionable. The reaction was performed again yielding an $^1$H NMR spectrum identical to the previously attempted reaction prior to TLC.

Since the purity of the synthesized Dexamethasone-C8-Cephem chloride is questionable, the previously synthesized methotrexate was joined to dexamethasone-C8-cephem chloride made by Colleen Bleczinski in the Cornish lab. Sodium iodide and DIEA aid the SN2 reaction that is involved in joining the two ligands together via cephem chloride. The reaction ran under nitrogen gas due to the presence of a free thiol capable of being oxidized. The product was purified by column chromatography and eluted at 10 % methanol/dichloromethane. $^1$H NMR shows evidence of product but the cephem peaks are not as resolved as they should be. A MS showed relatively pure product. Yield of protected D8CM was 87 %.

**IV. Discussion**

The hurdles that must be overcome in synthesizing D8CM make its synthesis a challenge. The impurity associated with the coupling of PABA to glutamic acid seemed only to be an issue on a large scale. Since there is potential for this impurity, it would be wise to run the reaction and always add 0.5 equivalents of diamine before working up the reaction. The addition of the diamine also makes purification by Silica column much simpler. Besides this step of the synthesis, purification does not pose a problem but both the PABA coupling and the cystamine coupling steps with yields under 40% do have detrimental effects on the final percent yield. Perhaps there is room for optimization in these two instances. In the case of the PABA coupling, there seems to be HOBr-activated PABA but for some reason it is not reacting with the glutamic acid. Perhaps it is the amount of equivalents or rather the coupling reagents used. The reaction was more successful on a small scale implying that perhaps special care must be taken when the reaction is run on a larger scale. I attempted to optimize the reaction by limiting the amount of HOBr in the reaction. The yield of this reaction was only 41 %.

The synthesis of bromo pteridine is also an extremely challenging part of this synthesis because its outcome is unpredictable. For instance on a 5-gram scale, approximately 85% pure product was obtained but on a 25-gram scale the pteridine
alcohol needed for the bromination step never formed. The same protocol was used in both instances but only the smaller scale was successful. The competing compound that forms during the synthesis of bromo-pteridine is methyl-pteridine. As long as this is in low ratio to the bromo pteridine, it can be used in the D8CM synthesis. The success of bromo pteridine, however, does not always only depend on the purity because Eric Althoff and Hening Lin have used 90% pure bromo pteridine and have been unsuccessful in attaching it to methotrexate. When I initially coupled the 85% bromo-pteridine that I had synthesized to methotrexate, a mono-substituted disulfide was all that was obtained. The bromo pteridine did, however, have a distinct odor of acetic acid, which was used during recrystallization. Although acetic acid is a weak acid it was hypothesized that perhaps it was still having an effect in the reaction by protonating the amine. After pumping on the bromo pteridine for 3 days in a desiccator, the prevalent smell of acetic acid disappeared and the bromo pteridine reacted successfully yielding the disulfide. Colleen Bleczinski faced the same problem, which may imply that the pteridine she used also contained acetic acid. It is advisable that all bromo pteridine be pumped on the vacuum pump prior to use in a reaction.

Due to the complicity of producing pure bromo pteridine or bromo pteridine that works well, it would be a great help in the synthesis of D8CM if bromo pteridine could be replaced with a molecule more simple to synthesize. The problem, however, is that bromo pteridine is the functional part of the methotrexate ligand that binds to DHFR. Therefore, a replacement for bromo-pteridine would have to have the binding capabilities that were available in bromo pteridine. A replacement for bromo pteridine would also be helpful to the biologists, such as Debleena Sengupta, using the molecule in their assays. Since pteridine absorbs at the same wavelength used for screening in vivo, when the pteridine is attached to the molecule, screening cannot be performed. Perhaps D8CM can be synthesized without the pteridine for screening in vitro and correlated to screening of D8CM with pteridine in vivo. If a correlation can be found between the binding of these two molecules this is a promising idea.

The cephem chemistry is the most complicated part of the synthesis of D8CM. Purification of Dexamethasone-C8-Cephem Chloride is done by column chromatography but is difficult to separate from impurities. Although every individual in the Cornish lab
who has purified a cephem product by column chromatography has run the column slowly with a very nonpolar solvent gradient perhaps an even slower gradient would work better. Future synthesis of this compound should involve column chromatography run even more slowly to optimize separation, which I plan to attempt in the fall. But it also seems that in some cases purification is not the primary reason that the product is difficult to acquire. It seems that something may affect the cephem core either when reacting or during purification. This was evident by $^1$H NMR because although there was a broad peak where the cephem peaks should be found, they were unresolved. Perhaps the cephem chloride decomposes after it is coupled to dexamethasone. This may be true since after 2 weeks, previously synthesized Dex-C8-Cephem chloride showed two spots by TLC while it showed only 1 spot immediately after purification. Another idea is that it may decompose in its original form even though it is stored in the -80°C freezer. Ways to avoid interaction with the cephem core in this synthesis may be to flame dry all glassware that is used as well as pumping on all reagents on the high vacuum for a least a day and storing everything involving cephem in a -80°C freezer.

Since there was much difficulty in obtaining the Dex-C8-Cephem, I used some that had been made previously by Colleen Bleczinski in the Cornish Lab. With her material and my free thiol, 122 mgs of the final protected Dexamethasone-C8-Cephem-Methotrexate was made and is available for use. The $^1$H NMR of the compound showed evidence of product but exact peaks of the Cephem core are unclear. The characteristic doublet at 5.7 ppm, however, is evident. A MS shows that the product is almost pure. Hening Lin in the Cornish Lab is going to repurify the compound and then deprotect it for use by Debleena Sengupta. 132 mgs of the unprotected Dexamethasone-C8 is left in stock (JP-01-46). 115 mgs of the disulfide without the bromo pteridine is also in stock (JP-01-30) as well as 1.2 grams of bromo pteridine. There are 4.23 grams of the t-butyl protected glutamic acid (JP-01-33) and 665 mgs of the glutamic-PABA (JP-01-45). With these stocks I plan on retrying the Dex-C8-Cephem synthesis and using it to synthesize more of the protected D8CM compound in the fall.
HO

O

NH₂

O

O

HClO₄

OO

CH₃

O

NH₂

O

3 hrs

77 %

1

DCC, HOBt, DIEA

DMF, 12 hrs

38 %

DMF, 12 hrs

77 %

H₂O, 1 hr

89 %

3

DMAP, DIC

0.35 DMF, 12 hrs

27 %

4

85% pure according to H¹ NMR

DMA, 55 C

5

PBu₃

H₂O/DMF, 12 hrs

145 %
Dexamethasone Synthesis

6

EtOH/H2O, 12 hrs
NaIO₄, H₂SO₄
98 %

7

PyBOP, DIEA
0.25M DMF, 12 hrs
82 %

8

LiOH
H₂O/MeOH, 12 hrs
91 %

9

DIC, DIEA
0.1M DMF, 12 hrs

10
Synthesis of Dexamethasone-C8-Cephem-Methotrexate

Synthesis of Bromo Pteridine

V. Experimental
Methods and Materials:
Nitrogen gas was used in all coupling reactions since both DMF and DMA solvents are air and moisture sensitive. Silica gel was the choice of absorbent for the column chromatographies performed. A solvent gradient was used for all column
chromatographies. This was done so that better separation was achieved initially to remove high Rf impurities with a low polarity solvent system and then a consequently more polar solvent system that would ultimately elute the product more quickly. All TLCs and prep TLCs were performed on glass silica plates. After extractions, all products were dried with sodium sulfate in order to remove water. All final products are pumped on the high vacuum overnight and then stored in the –20°C freezer. Verification of all products was obtained using a 400 MHz \(^1\)H NMR machine and a mass spectroscopy machine.

**Synthesis of Dexamethasone-C8-Cephem-Methotrexate:**

**General Procedure for Protecting Glutamic Acid (Product 1).**

Tert-Butyl Acetate (192 ml, 1426 mmol) and Perchloric Acid (3.7 ml, 62 mmol) were added to L-Glutamic Acid-\(\gamma\)-methyl (5 g, 31 mmol) and stirred for 2 hours at room temperature. A TLC was taken of the starting material and the product in 1% Methanol/Dichloromethane solution and then stained with ninhydrin and heated. The reaction was then cooled in an ice bath. It was then extracted four times with cold 0.5 N hydrochloric acid (175 ml). Sodium bicarbonate was then added until pH 7 was reached. It was then extracted again 4 times with ethyl acetate (738 ml). The organics were collected and combined and passed through sodium sulfate. The solvents were rotavapped off and the product was put on the vacuum pump overnight. The product (5.2 g, 24 mmol) with 77 % yield was stored in a -20°C freezer. 

**Product 1.** (Rf 0.31, hexane-EtOAc 1:1) \(^1\)H NMR (400 MHz): 1.41 ppm (s, 9H), 3.59 ppm (s, 3H), 3.39 ppm (t, 1H), 3.45 ppm (t, 2H), 3.3 ppm (q, 2H).

**General Procedure for Peptide Coupling with 4-Amino Benzoic Acid (Product 2).**

4-Amino Benzoic Acid (3.81 g, 25.2 mmol) was combined with 1-Hydroxybenzotriazole (5.8g, 43.2 mmol), dicyclohexylcarbodiimide (7.4 g, 36 mmol), product 1 (5.2 g, 24 mmol) and pumped on the high vacuum for one hour. The solids were dissolved in DMF (40 ml, 0.6M) and DIEA (4.1 ml, 24 mmol) was added to the stirring solution that ran under nitrogen gas overnight at room temperature. TLC of the actual product and this product was prepared and pumped on for 10 minutes and then run in a 1:1, hexanes: ethyl acetate mixture. 1:2:1, distilled water: saturated sodium bicarbonate: brine, (1 L) was
added to the product. It was then extracted 4 times with ethyl acetate (450 ml). It was passed over sodium sulfate and rotavapped and pumped on overnight. A column was packed with Silica gel (600 ml) and a solvent system of 2:1 hexanes: ethyl acetate. The crude product was dry-loaded. Samples were collected during the chromatography and progress was checked with TLC in a 1:1, hexanes: ethyl Acetate mixture. Samples showing evidence of product (lower Rf) were combined and rotavapped and put on the vacuum pump overnight. The impure product and 1,10-diaminododecane (885 mg, 4.9 mmol) was dissolved in DMF (33ml, 0.3M) and DIEA (1.72ml, 10 mmol) was added and the reaction ran under nitrogen overnight at room temperature. A column was packed in 2:1 hexanes: EtOAc and the product was dry-loaded. The solvent system was then changed to 1:1, hexanes: EtOAc. The product (3.16 g, 9 mmol), with a percent yield of 38%, was stored in a -20° Freezer.

**Product 2.** 1H NMR (400 MHz): 3.59 ppm (s, 2H), 4.39 ppm (t, 1H), 6.55 ppm (d, 2H), 7.55 ppm (d, 2H), 1.45 ppm (s, 9H), 2.73 ppm (s, 3H), 2.29 ppm (t, 2H), 2.15 ppm (q, 1H), 1.95 (q, 1H).

**General Procedure For Saponification with Lithium Hydroxide (3).**
A 0.4M solution was made with product 2 (3.2 g, 9 mmol) and methanol. It was placed on an insulated ice bath and stirred for 10 minutes. A solution of lithium hydroxide (.75 mg, 118 mmol) and water (4.5 ml) was made and added all at once to the stirring solution to form a white precipitate. The ice bath was removed and the reaction proceeded for an addition 50 minutes. A TLC of the starting material and product was performed in a 5:1, dichloromethane: methanol solution. The methanol was then rotavapped off and water was added (180 ml). The solution was then acidified with 1N hydrochloric acid to a pH of 2. It was then extracted 5 times with ethyl acetate (195 ml) and the organics were combined and passed over sodium sulfate. The ethyl acetate was rotavapped off and the product was put on the vacuum pump over night. The dried product (2.66 g, 7.9 mmol), with 89% yield, was then stored in the -20° Freezer.
Product 3. (Rf 0.67, dimethyl chloride: methanol 5:1). 1H NMR (400 MHz): no singlet at 3.59 ppm.

General Procedure for Coupling Product 3 with Cystamine. (Product 4)
Cystamine (0.89 g, 3.96 mmol), DMAP (.965 g, 7.9 mmol) and product 3 (2.66 g, 7.9 mmol) were pumped on for a 2 hours. The reagents were dissolved in DMF (22.6 ml, 0.35M) so that the reaction was purged with nitrogen gas and then DIC (2.47ml, 15.8 mmol) was added and the reaction stirred overnight. A TLC was prepared with the starting material and the product and eluted with 10% methanol-dichloromethane. The DMF was rotavapped off from the product. A column was packed with Silica gel (250 ml) and 2% methanol/dichloromethane. The crude product was dry-loaded. TLCs were taken throughout chromatography with 10% methanol/dichloromethane. When there was no more evidence of high Rf material, the solvent was changed to a 5% methanol/dichloromethane solution. Samples indicating product were combined and the solvents were rotavapped and the product was pumped on vacuum pump overnight. The product (1.65g, 2.09 mmol), with a 27% yield, was stored in the -20°C freezer.

Product 4. (Rf 0.4, methanol: dichloromethane 1:10) 1H NMR (400 MHz): 1.34 ppm (s, 9H), 1.99 ppm (q, 1H), 2.15 ppm (q, 1H), 2.8 ppm (t, 2H), 3.72 ppm (s, 3H), 3.35 ppm (t, 1H), 6.49 ppm (d, 2H), 7.6 ppm (d, 2H).

General Procedure for Coupling Product 4 with Bromo Pteridine. (Product 5)
Product 4 (250 mg, .317 mmol) and bromo pteridine (.59 g, 1.49 mmol) were pumped on the high vacuum for 1 hour. The reagents were the dissolved in DMA (.8 ml) and stirred overnight under nitrogen gas in a 55°C oil bath. TLC of product and bromo pteridine was taken in 10 % methanol/dichloromethane. A column was loaded in 10% methanol/dichloromethane and the product was dry-loaded. The solvent system was then changed to 20 % methanol/dichloromethane. The product was rotavapped dry and put on the high vacuum overnight. The product (.199 g, .175 mmol) with a yield of 55% was stored in the -20°C freezer.
**Product 6.** (Rf 0.3, 10% methanol/dichloromethane) $^1$H NMR (400 MHz): 7.7 ppm (d, 2H), 6.7 ppm (d, 2H), 8.6 ppm (s, 1H), 3.2 ppm (s, 3H), 1.4 ppm (2, 9H).

**General Procedure Reducing Product 5. (Product 6)**

Product 5 was dissolved in DMF (5.8 ml, .03M) and the reaction was purged with nitrogen gas. Tri-n-butylphosphine (.13 ml, .53 mmol) and water (.58 ml) were added and the reaction was purged again and ran overnight under nitrogen gas at room temperature. TLC was taken of the crude product in 5:1, dichloromethane: methanol. A column was loaded in 2% methanol/dichloromethane and the product was dry-loaded. The solvent system moved from 5% and then 7% methanol/dichloromethane. Product was collected, rotavapped, put on high vacuum overnight and thereafter stored in a –20°C freezer. The yield of product 6 (.144 g, .25 mmol) was 145 %.

**Product 6.** (Rf 0.6, 5:1, dichloromethane: methanol) $^1$H NMR (400 MHz): 7.7 ppm (d, 2H), 6.7 ppm (d, 2H), 8.6 ppm (s, 1H), 3.2 ppm (s, 3H), 1.4 ppm (2, 9H).

**General Procedure of Oxidation of Dexamethasone (Product 7).**

Dexamethasone (235mg, .599 mmol) was dissolved in ethanol (46ml, .013M) and stirred. Water (18.5ml) and sodium periodate (153.6mg, 0.718 mmol) were added followed by sulfuric Acid (1.2ml, 2.395 mmol) and stirred overnight. A TLC with starting material and product in 10% Methanol/Dichloromethane was taken and then ethanol was rotavapped off of the product. Water (18ml, .033M) and brine (9.6ml, .0625M) were added to the remaining solution and sodium hydroxide was added until a pH of 12 was reached. The solution was then washed 3 times with dichloromethane (50 ml) and TLC was taken in 10% methanol/dichloromethane. The solution was then adjusted to a pH of 3 with 1M Sodium Bisulfate. It was extracted 2 times with ethyl acetate (50 ml) and a TLC was taken. It was then extracted 2 times with 1:1 dichloromethane: ethyl acetate (50ml). The organic layers were combined and dried with sodium sulfate. The solution was then rotavapped and put on the vacuum pump overnight. The dried product (.2144g, .576 mmol), with a yield of 94%, was stored in a -20°C freezer.
**Product 7.** (Rf 0.2, methanol: dichloromethane 1:10). 1H NMR: 6.3 ppm (d, 1H), 7.45 ppm (d, 1H), 4.25 ppm (d, 1H).

**General Procedure of Product 6 coupled to C8 Linker. **(Product 8)

Oxidized Dexamethasone (1.9 g, 5 mmol), the C8 Linker and PyBOP (2.86 g, 520 mmol) were pumped on the high vacuum overnight. The solids were dissolved in DMF (20 ml, 0.25M) and DIEA (4.35 ml, 25 mmol) was added. A TLC was taken in 10 % methanol/dichloromethane. The crude was redissolved in dichloromethane (300 ml) and washed twice with 1:1 brine: 0.1N sodium bicarbonate (200 ml). The organics were dried through sodium sulfate and rotavapped dry. A column was loaded in 2 % methanol/dichloromethane and the product was dry-loaded. The entire column was run in 2% methanol/dichloromethane. The product (2.19 g, 4.1 mmol), which is stored in the -20°C freezer, gave a yield of 82%.

**Product 8.** (Rf: 0.2, 10% methanol/dichloromethane). 1H NMR (400 MHz): 6.3 ppm (d, 1H), 7.45 ppm (d, 1H), 4.25 ppm (d, 1H), 1.4 ppm (s, 6H), 3.3 ppm (m, 1H), 3.6 ppm (s, 3H).

**Oxidation of Product 8. **(Product 9)

Product 8 was dissolved in methanol (10.25 ml, .4M) and stirred on ice. Lithium hydroxide (.5 g, 12 mmol) was dissolved in water (3 ml, 1.4 M) and added to the reaction and stirred on ice for 10 minutes. The ice bath was removed and the reaction was left to stir at room temperature for 9 hours. TLCs were taken in 10 % methanol/dichloromethane. The solvent was pumped off and the crude was redissolved in water (88 ml) and acidified to a pH of 1 with 1N hydrochloric acid. It was then extracted 4 times with ethyl acetate (88 ml) and then washed twice with brine (229 ml). The organics were combined and dried through sodium sulfate. The solvents were rotavapped off and the product was put on the high vacuum overnight. The product (1.94 g, 3.73 mmol) had a yield of 91 % and was stored in the -20°C freezer.
**Product 9.** (Rf: 0.3, 10% methanol/ dichloromethane). \(^1\)H NMR (400 MHz): 6.3 ppm (d, 1H), 7.45 ppm (d, 1H), 4.25 ppm (d, 1H), 1.4 ppm (s, 6H), 3.3 ppm (m, 1H), no singlet at 3.6 ppm.

**General Procedure for Coupling Product 9 to Cephem Chloride.** (Product 10)

Product 7 (.5 g, .96 mmol) was combined with cephem chloride (.967 g, 2.4 mmol) and pumped on for 5 hours. The reagents were then dissolved in DMF (9.67 ml, 0.1M) and DIEA (.42 ml, 2.4 mmol) and DIC (.37 ml, 2.4 mmol) were added. The reaction was purged with nitrogen gas and left to stir overnight under nitrogen at room temperature. TLCs were run in 5:1, dichloromethane: methanol. The DMF was pumped off and the crude was dissolved in dichloromethane (43 ml). It was then washed twice with 1:1, 0.1N sodium bicarbonate: brine. The organics were dried over sodium sulfate and rotavapped dry. The product was then redissolved twice in dichloromethane (3 ml). A column was packed in 1% methanol/dichloromethane and the product was dry-loaded. The solvent gradient was then changed to 2% methanol/dichloromethane and finally 3% methanol/dichloromethane.

**Product 10.** (Rf: 0.5, 3 % methanol/ dichloromethane). \(^1\)H NMR (400 MHz): 6.3 ppm (d, 1H), 7.45 ppm (d, 1H), 4.25 ppm (d, 1H), 1.4 ppm (s, 6H), 3.3 ppm (m, 1H), 5.7 ppm (d,.6H), 5.2 ppm (q, 2H).

**General Procedure joining Product 6 to Product 10.** (Product 11)

Product 6 (.143 g, .25 mmol) and 9 (.217 g, .25 mmol) were combined and pumped on high vacuum for 2 hours. They were then dissolved in DMF (.84 ml, 0.3M) and sodium iodide (.185 mg, 1.23 mmol) and DIEA (.087ml, .5 mmol) was added. The reaction was purged with nitrogen gas and stirred at room temperature overnight. A TLC was taken in 5:1, dichloromethane: methanol. The DMF was pumped off and the crude was dissolved in dichloromethane (3 ml). A column was packed in 1% methanol/dichloromethane and the product was dry-loaded. The solvent gradient was changed to 3%, 5%, 7% and finally 10% methanol/dichloromethane. Product 10 (.122g, .09mmols) had a yield of 87% and was stored in the -20°C freezer.
Dexamethasone-C8-Cephem-Methotrexate. (Rf: 0.6, 5:1, dichloromethane: methanol).

$^1$H NMR (400 MHz): 7.8 ppm (d, 2H), 6.1 ppm (s, 1H), 5.8 ppm (d, 0.6 H), 4.5 ppm (m, 1 H), 2.7 ppm (m, 1H), 1.7 ppm (q, 1 H), 1.1 ppm (s, 3H).

General Procedure for Synthesizing 2,4-diamino-6-bromomethyl pteridine. (Product 12)

2, 4, 5, 6-tetraaminopyrimidine sulfate (5 g, 21 mmol) was added to water (84 ml) and stirred for 10 minutes on a heat plate to give a clear yellow solution. Barium chloride (5.1 g) in water (21 ml) was added to the solution and stirred for an additional 10 minutes on heat and then cooled overnight. The solution was chilled in the -20°C freezer for 5 minutes. A clear yellow solution was filtered out and the precipitate was cleaned with cold water. The solution was brought to a volume of 300 ml with water. 1, 3-dihydroxyacetone (10.8 g, 60 mmol) and cysteine hydrochloride (2.34 g, 20 mmols) were added to a 4M sodium acetate solution (98.4 g). The solution was then brought to a pH of 9 with 1N sodium hydroxide and the 2, 4, 5, 6-tetraaminopyrimidine solution was added to it. The reaction was bubbled with air for 72 hours. The material was filtered and rinsed with water (400 ml) and ether (50 ml) and left to dry overnight. Ethanol (100 ml) was added to the precipitate and brought to a boil. 48 % HBr/ethanol was added to the solution until all solid was dissolved and filtered while hot. It was left in the freezer overnight. The solid was filtered out and dried and then rinsed with ethanol and left on the high vacuum overnight. Dimethylacetamide (300 ml) was added to the precipitate and pumped on for 1 minute until bubbling and put under argon and immediately added triphenylphosphine dibromide (11.3 g, 28.67) and more DMA (30 ml). Ethanol (1 ml) was added to stirring reaction and left to stir for 2 hours. The DMA was rotavapped to a third of its volume (100 ml). Benzene was added and the reaction was left overnight. The benzene was removed and acetic acid (75 ml) was added and stirred at 100°C for 15 minutes. When the solution was semi-dissolved, the reaction was removed from the heat and left to sit overnight. The precipitate was then filtered out and dried. Propanol (250 ml) was then added to the precipitate and heated. The solution was then brought to room
temperature and placed in a -20°C freezer overnight. The solution was filtered and dried. After several days, the solid was pumped on the high vacuum in a desiccator for 3 hours.

Product 12. (Rf: 0.6, 20% methanol, dichloromethane). $^1$H NMR (400 MHz): 9.5 ppm (s, 1H), 4.8 ppm (s, 2H).

VI. Summary/Goals:

- The synthesis of Dexamethasone-C8-Cephem Methotrexate in its protective form has been completed and characterized. The product is evident but may have to be purified further. During the next few months, the compound will be repurified and deprotection by Hening Lin so that it can be used by Debleena Sengupta.
- Addition of cephem chloride to Dexamethasone will be attempted again. In this instance the glassware will be flame-dried the reagents will be pumped on the high vacuum for several day. Column Chromatography will be done using a solvent gradient that changes by 0.2% of methanol to dichloromethane. This will hopefully separate the product from any impurities and a yield a more pure final product.
- There is about 2 grams of pteridine alcohol that must be synthesized into bromo pteridine. 25 grams of unsuccessful bromo pteridine still resides in the lab and I hope determine what must be done to salvage it.
- There are stocks of glutamic-PABA, dexamethasone-C8 linker and bromo pteridine that I hope to use in continuing to synthesize this small molecule independently.
- Due to the difficulty of synthesizing bromo pteridine and the problems that incur when screening for it in vivo, I would like to start designing a new methotrexate derivative in the fall.
- The potential of the yeast three-hybrid system may lead to introducing new substrates in place of the cephem core of D8CM. Other cleavable linkers may prove to be helpful in both the synthesis and the purification of D8CM.
References: