Synthesis and Analysis of 2-Hydroxy-Cyclohexylhydroxylamine as a Model Compound

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INTRODUCTION

The calicheamicins contain enediynes that have the potential to cleave double stranded DNA. Most naturally occurring calicheamicins show DNA sequence selectivity but the sequence is short and occurs frequently in the human genome. Consequently, these compounds cannot be used selectively to cleave a foreign DNA such as that of an invading virus. This work is part of a project to enhance the selectivity of calicheamicin by replacing its short recognition sequence with a synthetic oligodeoxynucleotide. The synthetic antisense oligonucleotide should confer high specificity to the calicheamicin.

A plan to conjugate a synthetic antisense oligonucleotide to a calicheamicin γ_1^I fragment was determined. This work entails the synthesis of a model compound. The model compound will be used to optimize the conjugation conditions of the oligonucleotide to the calicheamicin γ_1^I fragment. In this report, the antisense strategy and calicheamicin will be reviewed, the model compound and the synthetic route will be described, and the experimental methods, results, and discussion will be presented.

ANTISENSE

Antisense oligonucleotide therapy has often been referred to as the "Magic Bullet." The bullet image comes from the therapy's presumed ability to kill or otherwise attack the target. It is referred to as magical because of the specificity of the method. Once "fired," the ideal antisense oligonucleotide seeks out only the desired target, ignoring all other potential targets, somewhat like a guided missile. This high specificity is expected to result in low toxicity. This

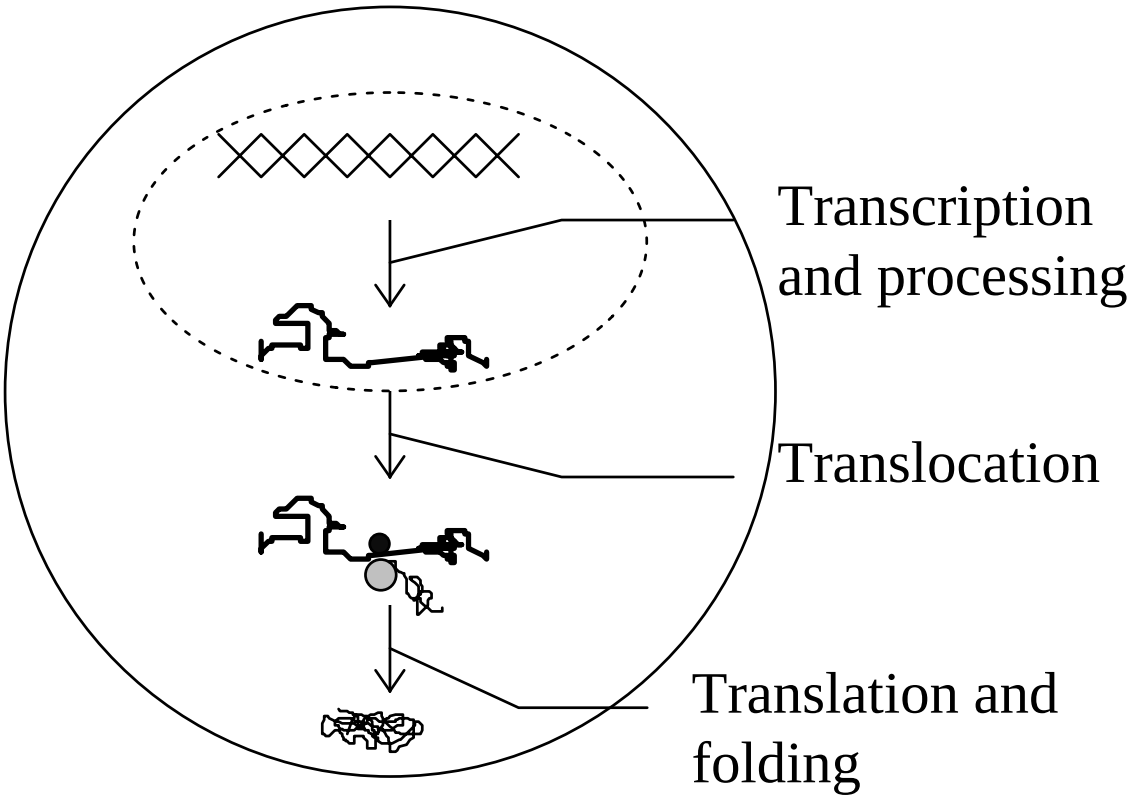
represents a significant advantage over current antiviral/anticancer agents.

The major targets of antisense oligonucleotide therapy occur in the production of proteins. The goal of antisense therapy normally is to reduce or shut off production of a particular protein (other uses of antisense are also being explored). Figure A shows the major steps in protein production. The first step is transcription of the DNA to give a pre-mRNA; the m-RNA contains the code to produce a protein. This occurs when RNA polymerase binds to a gene on the DNA duplex. At this stage, the DNA is most likely in the closed form (corresponding to a double strand). The closed form is converted into an open complex by the unwinding of the DNA duplex. The pre-mRNA is then transcribed. The RNA polymerase reads the template or antisense DNA strand and produces a pre-mRNA that has the same sequence as the coding or sense DNA strand.[aa]

The second step in protein production is processing the pre-mRNA which yields a mRNA. Processing includes splicing out introns, capping the 5' end to increase stability, poly-adenylation of the 3' end, and sometimes editing. In the next step, the mRNA is translocated out of the nucleus into the cytoplasm. It is generally accepted that pre-mRNA's cannot be translocated out of the nucleus. Processing allows the mRNA to be transported into the cytoplasm where it can be translated into a protein.[aa]

Once the mRNA is in the cytoplasm, it can be translated into a protein by a ribosome (shown as two circles on the mRNA in the center of Figure A). The ribosome begins translation at the start sequence (AUG) and continues to translate until one of the stop sequences is reached. The protein (seen at the bottom of the cell in Figure A) requires folding and sometimes processing to become active.[aa]

Figure A. Major steps in the protein production pathway.



Theoretically, any part of the protein synthesis process could be targeted to prevent the final production of proteins. The main target is the mRNA (the different mechanisms are discussed below). In a cell, many different mRNAs are present, most contain codes for the cell's natural proteins but a few contain the code for the proteins the virus is producing. The antisense oligonucleotides must be able to differeciate between the many mRNAs and find and complex to the target mRNA. This is accomplished by Watson-Crick base pairing between the bases on the oligonucleotide and mRNA. In order for the two DNA or RNA strands to complex, the

sequences must be complementary. Two complementary strands form Watson-Crick base pairs consisting of hydrogen bonds between the complementary bases of the nucleotides. DNA consists of four nucleotides, namely: guanine, cytosine, thymine, and adenine, which are abbreviated G, C, T, and A respectively. In RNA, uracil, U, replaces all T's and the sugar ring has an extra hydroxyl group. G and C form three hydrogen bonds and A and T (or U) form two hydrogen bonds. Care must be used when planning a target sequence since U can pair with G in duplexes involving RNA. Statistically, a sequence of 15-18 nucleotides occurs only once in the human genome.[a-c] Thus, a ~5,000 D (15 nucleotides) oligonucleotide should be able to target a unique DNA or RNA target.

Another target of antisense therapy is the DNA duplex in the nucleus. The antisense oligonucleotide binds in the major grove of the duplex by Hoogskin pairing to for a triple helix. Chemical difficulties require that the target sequence contain almost exclusively either purines or pryrimadines.

Figure F shows seven hypothesized mechanisms of antisense action. The first target in the protein synthesis pathway is the inhibition of transcription.[b] An antisense oligonucleotide sequence can be engineered to attack the open DNA complex and prevent transcription.[b] Such a sequence must be able to compete with the RNA polymerase or possibly other molecules for binding.

A newer approach to inhibit transcription involves a triple helix agent. It blocks transcription by binding to the double stranded DNA duplex.[c,bb,cc] This has the possibility of preventing both transcription and replication. There is evidence of high specificity, but the selected target must have a high purine or pyrimidine concentration. Limited binding affinity can also be a problem that is overcome by adding alkylating agents that destroy the cell's DNA.

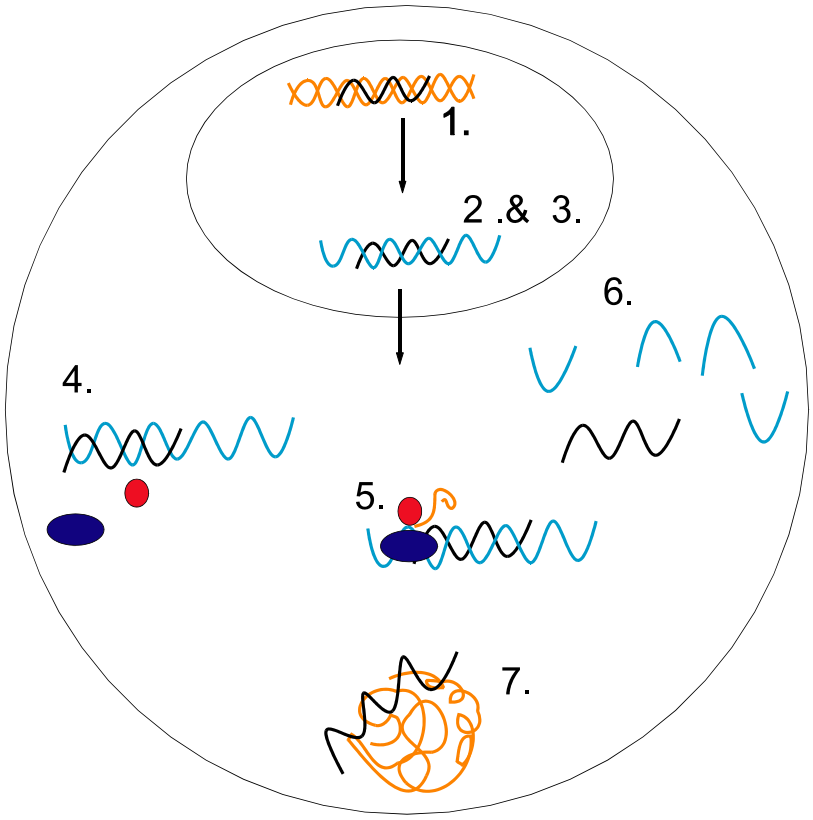
The major advantage to the transcription inhibition is the relatively small number of targets. In protein synthesis, one DNA duplex produces hundreds or thousands of mRNA's. The mRNA's are normally translated many times producing many thousands of proteins. The number of gene targets is therefore orders of magnitude smaller than the number of mRNAs. The major disadvantages of this therapy are the difficulty in delivering the antisense sequence to the nucleus and maintaining high specificity.

The second and third mechanisms occur in the post-transcriptional processes.[b] The pre-mRNA undergoes processing before being translocated from the nucleus to the cytoplasm. In the second mechanism, the antisense oligonucleotide prevents processing and subsequently translocation out of the nucleus. In the third mechanism, translocation is directly blocked by the oligonucleotide. It has already been demonstrated that preventing these steps reduces the amount of protein produced by cells.[b]

Mechanisms 4-6 involve preventing translation.[b] Once the mRNA is in the cytoplasm, it must be translated into a polypeptide. Several initiation factors and a ribosome must first bind to the mRNA before the translation can begin. An antisense oligonucleotide targeted to the correct region could prevent the binding of the initiation factors or ribosome (mechanism 4 in Figure F). Once translation has begun, a tightly bound antisense oligonucleotide could stop the progress of the ribosome until the ribosome falls off (mechanism 5 in Figure F). The incomplete protein will not be functional and will be degraded. Mechanism 6 involves degrading the mRNA. RNase H is a fairly ubiquitous compound in cells that recognizes DNA/RNA duplexes. It binds to the duplex and cleaves the RNA, which is then quickly degraded by exonucleases. This mechanism has the advantage of recycling the antisense oligonucleotide. Once the mRNA has been degraded, the oligonucleotide is free to seek out another target. Inhibition of translation is

currently the most tested method of antisense therapy. There are numerous examples of its successes as well as failures.[a-f]

Figure F. Hypothesized mechanisms of protein inhibition by antisense therapy. The six short black squiggles represent the antisense oligonucleotide, the light double stranded lines at the top of the nucleus represent the cell's DNA, the medium dark squiggles near numbers 2-6 represent the mRNA and the light squiggle in 5 and 7 represents a polypeptide.



In mechanism 7, the protein is deactivated by the binding of a molecule. This is technically not an antisense therapy, but it is discussed here, since there is evidence that oligonucleotides can bind to proteins and other objects, especially when the oligonucleotides are in high concentrations. (Oligonucleotide modification can also affect the binding of

oligonucleotides to their targets and other objects with which it comes in contact.) The binding of the oligonucleotide to something besides its target is a non-specific effect and is typically not desired. One desires the antisense oligonucleotide to seek out and bind to its complimentary DNA or RNA. However, if the desired therapeutic effect is obtained with insignificant side effects, then the therapy is beneficial regardless of its mechanism.

Determining if one of the above mechanism or another one is operating is often difficult. Scientists, designing a new antisense strategy, decide which step of protein production they wish to attack. They then design the therapy accordingly. A positive result does not indicate the planned mechanism worked. Inactivation of the ribosome, RNA polymerase or another important molecule often yields the desired result, and would fall under the non-specific effects discussed above. Often other studies are needed to determine the mechanism.

CALICHEAMICINS

The calicheamicins are potent antitumor antibiotics that specifically bind and cleave double stranded DNA. The sequences recognized by calicheamicin γ_1^I, the most prominent member of the calicheamicins, are common in genomic DNA, which make it extremely toxic. Calicheamicin γ_1^I (**1**), shown in Figure B, was isolated from *Micromonospora echinospora* ssp. *calichensis*.[j] Calicheamicin γ_1^I has remarkable properties, including activity in the biochemical prophage induction assay at concentrations < 1 pM, high antibacterial activity, and extreme potency against murine tumors and solid neoplasms with optimal doses of 0.15-5 μg/kg.[j] It is generally regarded that the carbohydrate tail of calicheamicin γ_1^I is responsible for its specificity.[k] Calicheamicin T (**2** in Figure B) is a derivative of calicheamicin γ_1^I, which is readily obtainable by cleaving off the BCD sugars in the tail.[k] Walker et. al.[k] have shown that

calicheamicin T cleaves DNA nonspecifically, although the activity is lower. This could be due to weaker binding. Several attempts at improving calicheamicin's DNA-cleaving properties have been made through various methods without obtaining the desired effects.[j] It is hoped that calicheamicin T can be given high specificity by conjugating it to synthetic DNA.

Figure B. Calicheamicin γ_1^I (**1**) and the daughter fragment, calicheamicin T (**2**).

1 **2**

The calicheamicins contains the reactive enediyne group directly responsible for DNA cleavage. The mechanism of cleavage has been well established in the literature and is shown in Figure C.[j,k,l] The trisulfide group, on the upper part of the molecule, acts as the trigger for the reaction. A nucleophile attacks the central sulfur atom causing a conjugate addition. The strain

caused by the addition induces the enediyne to undergo a Bergman-type cycloaromatization that generates the highly reactive benzenoid diradical. The diradical abstracts a hydrogen atom from each DNA strand which results in cleavage of both DNA strands.

Figure C. The mechanism by which calicheamicin cleaves a DNA duplex.

No reports on the ability of calicheamicin to cleave a DNA/RNA hybrid were found in the literature. Considering the above mechanism, cleavage of the hybrid should occur. This can be envisaged to occur as illustrated in Figure J. First, the oligo-calicheamicin conjugate binds to the target RNA to form an RNA-DNA duplex. Second, the calicheamicin T fragment, which contains the non-specific binding domain, folds back and binds in the minor grove of the duplex. Finally, the calicheamicin T fragment cleaves the duplex. Rapid degradation of the cleaved RNA and oligonucleotide follows.

STRATEGY

As mentioned above, the project goal was to increase the specificity of calicheamicin γ_1^I by conjugating it to a synthetic oligonucleotide. Figure D shows the conjugation plan. The carbohydrate tail of calicheamicin γ_1^I is cleaved off to give calicheamicin T.[k] The calicheamicin T fragment is conjugated to an oligonucleotide by a bifunctional group such as *p*-maleimidophenyl isocyanate (PMPI).[r] Calicheamicin T contains a hydroxyl amine that will react with the isocyanate group of PMPI. The oligonucleotide would be synthesized with a sulfhydryl group on the 5' end allowing conjugation to the maleimido group on PMPI.

Although calicheamicin γ_1^I has already been totally synthesized,[o,p] the procedure is not at all simple. Reaction conditions for the conjugation needed to be worked out with a model compound so that the calicheamicin γ_1^I available could be most effectively used. This work entailed the synthesis of the model compound.

Figure J. Cleavage of a DNA/RNA duplex by calicheamicin.

Figure D. The plan to conjugate an oligonucleotide to calicheamicin.

The model compound needed similar functional groups as those found on calicheamicin T. No suitable compound was commercially available. Drs. Todd Capson and Duane Ruffner chose a suitable compound, 2-hydroxy-cyclohexylhydroxylamine (HCHA) (**3** in Figure E). HCHA lacks an oxygen in the ring and some sterically hindering groups found in calicheamicin T, but it does contain a hydroxyl amine and a hydroxyl on an adjacent carbon similar to the calicheamicin T. The hydroxylamine is the most important part of the molecule since calicheamicin T will be conjugated to PMPI through it. It was thought that although lacking some features, the model compound would be sufficiently similar to the reaction site of calicheamicin T to use to determine optimum conjugating conditions.

The major disadvantage of HCHA was lack of information and availability. It was not found in *Chem Abstracts* using the molecular formula and several derivatives of the name. A CAS number was also not found. It apparently has never been studied.

A two step synthesis of HCHA was proposed and attempted; first, generation of an oxime[u] from 2-hydroxycyclohexanone, and second, reduction of the oxime to a hydroxyl amine. Problems in the first step led to further literature searches and yielded a "one-pot" reductive amination.[v] It was hypothesized that HCHA could be synthesized by reductive amination of 2-hydroxycyclohexanone with hydroxylamine in the presence of cyanohydridoborate as shown in Figure E. 2-hydroxy-cyclohexanone is commercially available as a dimer from Aldrich. Aldrich[s] reported that NMR and FT-IR show that there is a small amount of monomer in all dimer samples which cannot be purified. This suggests an equilibrium exists between the two species. Although the equilibrium greatly favors the dimer, reaction with the monomer is possible by taking advantage of Le Chatelier's principle.

Figure E. The synthesis plan for the model compound, 2-hydroxy-cyclohexylhydroxylamine (HCHA).

The synthesis of HCHA was achieved faster than expected. The next step in the plan was the conjugation of HCHA to PMPI as seen in Figure K. It is hypothesized that the nitrogen of the hydroxylamine will react with the isocyanate group on PMPI to give **4.**[t,x] The best order of conjugation in connecting calicheamicin T, PMPI, and the oligonucleotide together was not known. Since we had two of the three model reactants we decided to start by coupling HCHA and PMPI. In practice, the conjugation of PMPI and the oligonucleotide would probably be done first because of the limited quantity of calicheamicin T. Further studies are required to

Figure K. Plan to conjugate HCHA to PMPI through the hydroxylamine.

METHODS

SYNTHESIS AND ANALYSIS OF HCHA

HCHA was synthesized as described above. The 2-hydroxy-cyclohexanone dimer and hydroxylamine hydrochloride were obtained from Aldrich and the sodium cyanohydridoborate was obtained from Sigma. The stationary phase of the liquid chromatography was Baxter SšP Brand Silica Gel 60 C (230-400 mesh).

The 2-hydroxy-cyclohexanone dimer (0.228 g = 2.00 mmol of ketone monomer) was partially dissolved in 1.0 mL of methanol. The hydroxylamine hydrochloride (0.175 g = 2.52

mmol) was dissolved in 0.5 mL of water and added to the reaction. A trace of bromocresol green was added to the reaction and the pH was raised to ≈ 6 with NaOH. Sodium cyanohydridoborate (0.0650 g) was dissolved in 1 mL methanol, the solution was added to start the reaction, and a pH ≈ 4 was maintained throughout the reaction with methanolic hydrochloric acid. The reaction was stirred for 4 hours at room temperature. The pH was raised to 10 and excess NaCl was added. The HCHA was extracted out with 4 X 5 mL methylene chloride and dried over powdered $MgSO_4$. After removing the solvent, approximately 85 mg (0.66 mmol = 33%) of crude HCHA was obtained.

The crude product was analyzed by TLC. Several solvent systems were attempted until a clean separation of the starting material and product was achieved. The TLC revealed that the crude product was contaminated and would have to be purified. TLC was also used to determine the solvent system for purification. The HCHA was purified by flash chromatography[w] using a flowrate of 2 cm/min of ethyl acetate:methanol (9:1) (for TLC: R_f (HCHA) = 0.3, R_f (ketone) = 0.6). After purification, approximately 20 mg (0.15 mmol = 8%) was recovered. The purified product was analyzed by mass spectrometry and proton NMR.

The purpose of this work was qualitative not quantitative; therefore, determination of the amount of product yielded was not carefully determined. The goal was to find a viable synthetic route to produce usable amounts (mgs) of the model compound. Efficiency of the route would be worked on only if inadequate amounts were produced. The weights of products were determined using relatively heavy glassware, which gave significant errors in the measurements; thus, the values given above are not highly accurate.

COUPLING OF HCHA TO PMPI

HCHA was coupled to PMPI according to the reaction scheme described above. PMPI was obtained from Calbiochem. The HCHA (2 mg = 0.015 mmol) and PMPI (6.5 mg = 0.03 mmol) were added to the reaction vessel under dry N_2 and 190 L of freshly distilled DMSO was added. The reaction was stirred overnight and extracted with ether and water at pH = 14 following the same procedure as described above. The crude product was purified by flash chromatography as described above using chloroform:methanol (98:2). The complex was analyzed by proton NMR. TLC proved as useful as above in determining the purity of the product, separation solvent system, and compound detection of the separation.

A Bruker 200 MHz NMR was used to analyze the compounds. Deuterated chloroform was the solvent for both samples. A mass spectrophotometer was used to determine the molecular weight of the first product, HCHA.

RESULTS AND DISCUSSION

HCHA was synthesized first. As described above, the reaction was first attempted as a two step reaction. TLC gave no indication that the intermediate product had been formed. The one step reaction eliminated a step and more importantly eventually yielded the desired product. TLC proved invaluable in this project. The reaction was monitored by TLC to determine if a product was obtained and to monitor the disappearance of the starting compound. TLC was also used to determine that the purity of the isolated crude product. The LC separating solvents were chosen by TLC analysis and the separation resulted in a fairly clean compound.

The proton NMR spectrum of HCHA is shown in Figure G. A collection of the NMR spectra of several similar compounds is contained in the appendix. Tentative peak assignments were made based on the NMR of similar compounds and from reference books (see the Appensix for several spectra).[y,z] The peaks from 1.2 to 2.2 ppm correspond to the hydrogens on the cyclohexane ring excluding those assigned next. The peak from 2.8 to 2.9 ppm most likely corresponds to the hydrogen on the carbon bearing the hydroxylamine. The peak from 4.1 to 4.2 ppm most likely corresponds to the hydrogen on the carbon bearing the hydroxyl. Assignment of the peak at 6.2 ppm is more difficult and will be discussed further below. The other peaks in the spectrum are contaminates and the peak at 7.24 ppm corresponds to the solvent, chloroform. The contaminate peaks are much smaller than the HCHA peaks, which confirms the purity seen by TLC.

Integration of the peaks revealed more problems in interpreting the NMR spectrum. It appears that ~41 AU^2 corresponds to 1 hydrogen. Using this value, one obtains approximately 15 hydrogens for the four peaks mentioned above. HCHA only has 13 hydrogens. The abnormally large integration value is most likely due to contaminants. The peak from 1.2 to 2.2 ppm integrates to 10 hydrogens but it most likely corresponds to 8 protons on the cyclohexane ring. If 82 AU^2 are due to contaminants then the correct value of 13 hydrogen is obtained. The broad peak at 6.2 ppm integrates to 3.25 protons, which would correspond to the amino's hydrogen, the two hydroxyls' hydrogens, and exchanging protons. The broadness of the peak indicates that it belongs to hydrogens which are easily exchanged, which is the case for both the hydroxyl and the amino hydrogens. Considering the literature spectra and apparently different environment of each proton, it was not expected that the amino’s and both hydroxyls’ hydrogens would all overlap. If the three proton peaks were very close together, they could merge into one

Figure G. Proton NMR for the model compound, HCHA.

peak, but larger differences were expected. Merging peaks are often detected by an asymmetrical peak which does not seem to be present. More NMR studies using other solvents and techniques are necessary to confirm the identity by NMR. A more cost effective and faster determination of the product was molecular weight determination.

The molecular weight of HCHA was determined by mass spectrometry to confirm the synthesis of the correct compound. The spectrum is seen in Figure H. The predominate peak at 131.38 most likely corresponds to HCHA (MW = 131.174). This strongly supports the synthesis of the correct compound. Only small amounts of contaminates were observed again confirming the purity of the product.

Good results allowed us to continue working on the next step of the project, conjugating HCHA to PMPI. The reaction proceeded as described above with only a few difficulties. TLC proved as invaluable in this reaction as in the previous one. It was used to determine if the reaction worked, to find the optimum separation solvents, and to detect the correct compound and its purity.

The purified product was analyzed by proton NMR and the result is seen in Figure I. Unfortunately, only a very small amount of product was obtained < 5 mg. It is also apparent that all the diethyl ether was not removed. Very good peaks for ether are observed at 1.11 - 1.23 ppm and at 3.4 - 3.5 ppm. The chloroform peak at 7.24 ppm is also very prominent. Few peaks were observed in the expected place. Time prevented further analysis. Proof of conjugation of HCHA and PMPI was therefore not obtained during this work.

Figure H. Mass spectrum for the model compound, HCHA (MW = 131.174).

Figure I. Proton NMR spectrum for the HCHA/PMPI conjugate.

CONCLUSION

In this report, the antisense therapy and calicheamicins have been reviewed and the rationale, strategy, and results of the synthesis of HCHA were presented. The synthesis of HCHA was apparently successful as shown by the NMR and mass spectroscopy analysis. Although these methods did not give irrefutable conformation of the desired product, they strongly support the synthesis of HCHA. The coupling of HCHA to PMPI was attempted but proof of conjugation was not obtained.

REFERENCES

a. C. A. Stein, Y.-C. Cheng. *Science.* **261,** 1004-1012 (1993).

b. E. Uhlmann, A Peyman. *Chem. Rev.* **90,** 543-584 (1990).

c. M. L. Riordan, J. C. Martin. *Nature.* **350,** 442-443 (1991).

d. G. Zon. *Pharm. Res.* **5**, 539-549 (1988).

e. G. Y. Wu, C. H. Wu. *J. of Biol. Chem.*. **267**, 12436-12439 (1992).

f. J. Frese, Jr., C. H. Wu, G. Y. Wu. *Advanced Drug Delivery Reviews*. **14**, 137-152 (1994).

j. K. C. Nicolaou, A. L. Smith, E. W. Yue. *Pro. Natl. Acad. Sci. USA.* **90,** 5881-5888 (1993).

k. S. Walker, R. Landovitz, W. D. Ding, G. A. Ellestad, D. Kahne. *Pro. Natl. Acad. Sci. USA.* **89,** 4608-4612 (1992).

l. N. Zein, M. Poncin, R. Nilakantan, G. A. Ellestad. *Science.* **244,** 697-699 (1989).

m. K. C. Nicolaou, W.-M. Dai, S.-C. Tsay, V. A Estevez, W. Wrasidlo. *Science.* **256,** 1172-1178 (1992).

n. T. L. Z. Zeng, V. A. Estevez, K. U. Baldenius, K. C. Nicolaou, G. F. Joyce. *J. Am. Chem. Soc.* **116,** 3709-3715 (1994).

o. K. C. Nicolaou, C. W. Hummel, M. Nakada, E. N. Pitsinos, A. L. Smith, K. Shibayama, H. Saimoto. *J. Am. Chem. Soc.* **115,** 7593-7635 (1993).

p. K. C. Nicolaou. *Angew. Chem. Int. Ed. Engl.* **32**, 1377-1385 (1993).

r. M. E. Annunziato, U. S. Patel, M. Ranade, P. S. Palumbo. *Bioconj. Chem.* **4**, 212-218 (1993).

s. Personal communication with Aldrich Chemical Co.

t. Isocyanate ref.

u. J. M. Trochet, N. Bizzozero, M. Geoffroy. *Carbohydr. Res.* **191**, 138-143 (1989).

v. R. F. Borch, M. D. Bernstein, H. D. Durst. *J. Am. Chem. Soc.* **93,** 2897-2904 (1971).

w. W. C. Still, M. Kahn, A. Mitra. *J. Org. Chem.* **43**, 2923-2925 (1978).

x. Personal communication with Calbiochem, commercial provider of PMPI.

y. R. M. Silverstein, G. C. Bassler, T. C. Morrill. *Spectrophotometric Determination of Organic Compounds*. Fifth edition, John Wiley & Sons, Inc., New York, 1991.

z. CRC Handbook of Chemistry and Physics. 71st edition. D. R. Lide Ed. CRC Press, Inc., Boca Raton, p. **9**-131, 1990.

aa. B. Alberts, D. Bray, J. Lewis, M Raff, K Roberts, J. D. Watson. *Molecular Biology of the Cell.* Third edition, Garland Publishing, Inc., New York, 1994.

bb. Nilabh Chaudhary, Jeffery S. Bishop, Krishna Jayaraman, and Judith K. Guy-Caffey. *Delivery Strategies for Antisense Oligonucleotides Therapeutics.* Chapter 3. Saghir Akhtar, Ed. CRC Press, Boca Raton, 1995, 39-60.

cc. Eric T. Kool. *Delivery Strategies for Antisense Oligonucleotides Therapeutics.* Chapter 8. Saghir Akhtar, Ed. CRC Press, Boca Raton, 1995, 123-149.