A New Synthesis of a Potential Antiviral Prodrug Utilizing a 1,2-anhydrofuranose Intermediate

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Honors Thesis
Spring 2008

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Precis.

This research explores a new synthetic route to produce the potential anti-Hepatitis C drug Valopicitabine. Hepatitis C is caused by a virus, known as HCV, which can lead to cirrhosis of the liver and eventually liver failure. An estimated 120-170 million people are infected worldwide, including approximately 4.1 million Americans. HCV is a leading cause of liver transplants, accounting for about 35% of all liver transplants performed each year. Depending on the specific variation, or genotype, of virus, treatments have varying degrees of success. Type I HCV, the most common in the United States, shows sustained virologic response (SVR) in less than 50% of cases using current treatment regimens. SVR is a measure of treatment efficacy, refering to cases in which viral RNA is undetectable by sensitive tests at the end of treatment and remains undetectable 6 months later.

New drugs are currently being developed for HCV and other viral diseases, including HIV. Many of these drugs are from the class of compounds known as nucleoside analogs. Nucleoside analogs are variations on the natural genetic material, either DNA or RNA, which are used to treat some viral infections, cancer, and bacterial infections. Valopicitabine is one such nucleoside analog that is being considered as a Hepatitis C anti-viral treatment. This research develops a new synthesis of Valopicitabine, the 2'-methyl-3'-valyl analog of ribocytosine, an RNA analog that blocks the viral enzyme RNA-dependent RNA polymerase (RdRp).

The new synthesis uses an unusual method to couple to modified ribose sugar to the cytosine base. Traditionally, nucleoside analogs are produced by the Vorbrüggen protocol, which performs the coupling reaction with the help of strong Lewis acids. These catalysts are incompatible with acid-sensitive chemical protecting groups, as well as the necessary valyl moiety on the 3'-OH. In order to synthesize Valopicitabine via the Vorbrüggen protocol, the

glycosylation must be performed prior to addition of the 3'-valyl group. This results in extra protecting and deprotecting steps in order to specify the location of the valyl modification, essentially blocking all other reactive sites on the ribose and cytosine. The synthesis explored here uses an unusual coupling via a 1,2-anhydrofuranose to afford glycosylation without the use of Lewis acids, therefore allowing the valyl modification to be made prior to addition of the cytosine base. Only two chemical protecting groups are needed, reducing the number of protecting and deprotecting steps and simplifying the synthesis.

The methods used in this research are standard organic synthesis protocols. Reactions were performed under inert argon atmosphere in organic and sometimes aqueous solvents. Purification was by medium pressure liquid chromatography (MPLC) and/or crystallization, and analysis was performed by thin layer chromatography (TLC), nuclear magnetic resonance (NMR) imaging, and infrared (IR) spectroscopy.

The series of reactions results in Valopicitabine in six steps, providing a complete synthetic route to produce 2'-C-methyl nucleosides and other analogs. The protocol begins with D-fructose, an inexpensive and readily available sugar. This work is important to the future development, design, and manufacture of anti-viral nucleoside analogs, as well as other applications of nucleoside analogs. Reducing the manufacturing steps, and therefore cost, of medications helps make drugs more affordable to patients, increasing the scope of access to treatment.

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I. Introduction.

The hepatitis C virus, or HCV, is estimated to infect 4.1 million Americans (1). Seventy-four to 80% of those infected with HCV develop a chronic infection, which may lead to cirrhosis and end-stage liver disease (2) (3). HCV infections are the most common cause for liver transplants, accounting for over 35% of liver transplants performed each year (2) (3) (4), and some studies suggest that 15% of infected persons will develop cirrhosis within 15 years (5).

The virus previously referred to as non-A, non-B hepatitis (NANBH) was discovered in 1989. Choo and colleagues isolated and amplified a gene, derived from RNA, coding for an HCV antigen, demonstrating the identity of one of the causative agents of NANBH and coining the name Hepatitis C (6). Although NANBH may be caused by other forms of hepatitis, including Hepatitis D, HCV remains the most common source of NANBH (7).

Hepatitis C has multiple strains, known as genotype variants, which have unique mutations and RNA sequences. The different genotypes may influence the course of the disease and the effectiveness of treatments (4). It is not completely understood how the genotypes compare to each other because studies use different criteria to determine the effectiveness of treatment and progression of disease. Genotype I is the most common in the United States. Genotypes II and III seem to respond best to the current treatments, while little is understood about the rare genotypes IV, V, and VI (1) (4).

Worldwide, estimates range from 120 to 170 million people living with chronic HCV (3). Transmission occurs primarily by blood-to-blood contact, including blood transfusions and shared injection needles (1). Sexual transmission is possible but much less common. For developed countries, including the US, injection drug users are at the highest risk (1) (2). The

U.S. blood supply has been screened by a sensitive test since 1992, decreasing the risk of acquiring HCV by blood transfusion to virtually zero (1). However, undeveloped countries may have fewer regulations on their blood supplies, causing a higher rate of transmission from transfusions (3). Injection drug use also remains a common means of transmission in developing countries.

Treatments for HCV include a combination drug therapy, utilizing an immune protein called interferon and the nucleoside analog class of antiviral drugs (1) (3). The naturally occurring interferon protein is produced in response to infection by viruses and other pathogens, limiting the spread of the virus to nearby cells (8). The use of interferon injections for the treatment of HCV enhances the normal immune response to help reduce the total viral load (1) (8). Used in combination with Ribavirin (fig. 1) (9), a nucleoside analog, the treatment's overall effectiveness depends greatly upon the genotype of the virus (1) (8) (10). Sustained virologic response, defined as "the absence HCV RNA in serum by a sensitive test at the end of treatment and six months later" (1), can be achieved in 75% – 80% of patients infected with genotype II and III HCV, but less than 50% of those infected with genotype I HCV (1). Patients who fail to reduce their viral load from these treatments may opt to try the therapy again, but the success rate for a second treatment is low and there are no viable alternatives to these drugs (1) (3).

Figure 1

Chemical Structure of Ribavirin (8) The sugar ribose is coupled to an unnatural base that mimics either adenine or guanine, depending on its orientation.

The antiviral properties of nucleoside analogs such as Ribavirin come from their ability to inhibit various enzymes needed for viral replication. Nucleoside analogs mimic the natural nucleosides that make up the genetic material, either

RNA or DNA, with specific modifications that allow them to block replication (11) (12). Natural nucleosides contain two moieties: a sugar group and a nitrogen-containing base, usually thymine, cytosine, adenine, guanine, or uracil. The sugar can be ribose, as in RNA, or deoxyribose, found in DNA. Either moiety can be chemically modified to create an analog. Once in the cell, nucleoside analogs are phosphorylated the same way as natural nucleosides, forming the functional nucleotide that acts as the building block of DNA and RNA. In order for the compound to be active, it must be able to bind functionally to the enzyme kinase *in vivo,* converting it from the prodrug nucleoside to the active nucleotide. Studies have shown nucleoside analogs to be effective against viruses, bacteria, and cancers, as all of these require active replication of genetic material (12) (13) (14). Other major nucleoside analogs include AZT, the primary treatment for HIV infection, Ribavirin, currently used for multiple types of viral infections, and several cancer chemotherapy agents.

Valopicitabine, or NM283, is a nucleoside analog target being considered as an HCV therapy. It is thought to block the viral enzyme RNA-dependent RNA polymerase (RdRp), also known as RNA polymerase NS5B (3) (10) (13). Polymerases are enzymes used to copy genetic material for replication. HCV is an RNA-based virus, therefore its polymerase uses ribonucleotide building blocks to produce new copies of its genome. When RdRp incorporates a nucleoside analog into the growing RNA strand, the next nucleotide is unable to

Figure 2

Chemical Structure of Valopicitabine. Valopicitabine is an analog of the natural nucleoside cytosine, modified by the addition of a methyl group at the 2' position and a valyl group at the 3' position.

bind normally, terminating replication (13). These drugs can also be incorporated into the host's healthy replicating cells, which results in the toxicity associated with most nucleoside antivirals (1). Selective toxicity can be achieved by increasing the specificity of the drug for unique viral enzymes, limiting the degree to which normal cellular polymerases incorporate the drug molecule. The more specific the molecule, the less it is able to inhibit normal cellular enzymes and cause toxicity to the patient. Valopicitabine was tolerated well in early trials and appears to inhibit viral replication effectively (2).

Valopicitabine is an RNA nucleoside analog, containing the sugar ribose and the nitrogenous base cytosine (fig. 2). It has a 2'-C-methyl modification, providing the basis for its chemical activity *in vivo*, as well as a 3'-valyl group that is cleaved once the drug has entered the cell. This modification is important to the bioavailability of the drug, making it easier for the compound to be absorbed in the digestive tract and to cross cell membranes (13). Because the valyl group is a natural amino acid, it may also help increase the half-life of the drug in the blood stream by slowing the compound's reaction with deaminases, which convert the cytosine base to uracil, rendering the drug inactive (fig. 3) (13).

Figure 3

Reaction Scheme for Cytidine Deaminase. This reaction can occur the similarly for cytidine analogs, including Valopicitabine. The 3'-valyl group may help protect the drug from binding to cytidine deaminase *in vivo*.

Because it is a prodrug, Valopicitabine is modified intracellularly to produce the active compound. Prodrugs are common for the treatment of many diseases because cellular metabolism often modifies the chemical structure of drugs. These changes can be destructive,

inactivating the drug and decreasing its efficacy, or they can be utilized to produce the active compound in a specific fashion. For Valopicitabine, the 3'-valyl group is cleaved by an unknown mechanism after it enters the cell. Like other nucleoside analogs, it is then activated by cellular kinases to produce the 5'-triphosphate nucleoside analog (fig. 4). The final active molecule is 5'-triphosphate-2'-C-methylcytosine. Without the phosphate modifications, the molecule will not be incorporated into the viral genome by RdRp.

Figure 4
Reaction scheme for activation of Valopicitabine intracellularly. The valyl moiety facilitates crossing of the membrane, after which it is likely cleaved. Cellular kinases add phosphate groups to the 5'-OH, generating 5'-triphosphate-2'-C-methylcytosine.

Although clinical trials of Valopicitabine are currently on hold, this research has provided a new synthesis for nucleoside analogs, a much-needed step to make drug discovery, clinical trials, and production more efficient and cost effective for the treatment of many viral diseases. Current synthesis methodology used for Valopicitabine and other nucleoside analogs is known as the Vorbrüggen protocol (fig. 5) (12) (15) (16) (17). The method requires harsh Lewis acids, such as $SnCl_4$ or $(CH_3)_3SiOSO2CF_3$, to achieve glycosylation (16). Lewis acids are incompatible with many common chemical protecting groups, including the N-*tert*-butoxycarbonyl (Boc) and the important valyl substituent used for Valopicitabine synthesis (15). To circumvent this problem, the Vorbrüggen protocol condenses the ribose and cytosine moieties

in a glycosylation reaction prior to adding the valyl modification. The protected sugar and silylated base are combined in the presence of Lewis acid catalyst. A 1,2-acyloxonium ion is formed on the sugar via reaction with the catalyst. The silylated base attacks at the 1'-carbon preferentially, while the silyl group simultaneously cleaves and regenerates the acid catalyst (18). Following glycosylation, a protecting group must be specifically removed and replaced with the modification of interest. Although this method is viable, it requires the protection of all free hydroxyl and amine groups on both the sugar and the base in order to have regiospecific addition of the valyl. In addition, Vorbrüggen glycosylations are not highly stereospecific unless a 2' ester is present. A mixture of the α and β anomers, in which the base is oriented below or above the plane of the sugar, respectively, is produced. Since only the β anomer is active, the protocol produces some amount of the incorrect product.

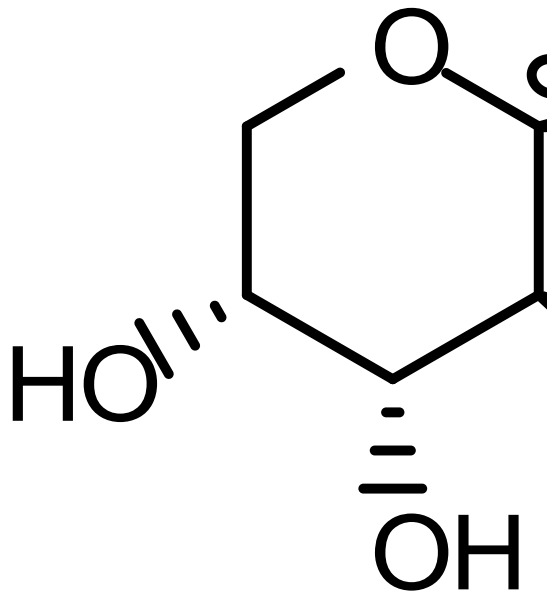
Figure 5
Typical Vorbrüggen Synthesis. The reaction scheme shows the synthesis of unmodified cytosine via the Vorbrüggen protocol. Bz – Benzoyl. TMS- Trimethylsilane

This research simplifies the synthesis of nucleosides, specifically Valopicitabine, using an unusual 1,2-anhydrofuranose intermediate for glycosylation of the nitrogenous base cytosine, rather than a Lewis acid (19) (20) (21). The use of a 1,2-anhydrofuranose in this synthesis affords greater control over the addition reaction, directing the base to the 1' carbon in the β position. Addition occurs to the 1' carbon because it is less crowded than the tertiary 2' carbon;

additionaly, the presence of the adjacent oxygen makes the 1' carbon more electrophilic, causing it to react more readily with the nucleophilic cytosine base. Due to the orientation of the C-2-OH, the epoxide forms in the α position allowing backside attack from the activated nucleophile, forming the β anomer exclusively. This method is compatible with most protecting groups and the valyl substituent, allowing the modification of the sugar prior to addition of the cytosine base. Starting with the inexpensive and readily available sugar D-fructose, the method is stereospecific and requires fewer protecting groups, resulting in a complete synthesis in six steps.

II. Central Question.

The development of a new synthetic route to produce the nucleoside analog Valopicitabine is explored via an unusual 1,2-anhydrofuranose reaction to couple the ribose sugar with the base cytosine.



III. Experimental.

General methods. Reactions were run in a 10mL round-bottomed flask fitted with a septum, a magnetic stirring bar, and vented with a needle, unless otherwise noted. Some reactions were performed under an inert argon

Figure 6

Compounds a through g. a: D-fructose. b: 2-C-methyl-D-ribonolactone. c: 3-Boc-valyl-2-C-methyl-5-O-tritylribonolactone. d: 3-Boc-valyl-2-C-methyl-5-O-tritylribose. e: 3-Boc-valyl-2-C-methyl-5-O-trityl-1,2-anhydrofuranose (not isolated). f: 3'-Boc-valyl-2'-C-methyl-2'-OTMS-5'-trityl-ribocytosine. g: Valopicitabine HCl

atmosphere, while others were run in ambient conditions. Solvents were from Sigma-Aldrich, and reagents were the highest purity available. Workup procedures were performed according to standard organic chemistry protocols, usually by extracting the reaction mixture into an organic solvent and washing this extract with aqueous solutions of dilute HCl, sodium bicarbonate, and brine. Thin layer chromatography (TLC) to monitor reaction progress was performed using silica gel on glass plates. The plates were visualized by UV, iodine, or charring with ninhydrin. Purification was by column chromatography through silica gel on a medium-pressure pumped system (MPLC) or by gravity flow. Nuclear Magnetic Resonance (NMR) imaging was used to determine the purity and identity of compounds. Samples, on the order of 5 – 10 mg, were analyzed in deuterated chloroform, with a relaxation delay of 4.0 s for ^{1}H and 1.0 s for ^{13}C. ^{13}C Distortionless Enhancement by Polarization Transfer (DEPT) was also used under the same conditions as ^{13}C NMR.

D-Fructose to Ribonolactone. This reaction was performed previously by members of the Rob Ronald group, as described by Whistler and BeMiller (22). D-fructose (fig. 6a) with aqueous $Ca(OH)_2$, followed by treatment with H_2SO_4, yielded 2-C-methyl-D-ribonolactone (fig. 6b).

Figure 7

Protection of ribonolactone. The reaction scheme to add 5-trityl and 3-valyl protecting groups to compound b. Ph_3CCl – triphenyl chloride. DMAP - Dimethylamino pyridine. Py - pyridine

Protection of Ribonolactone.

Purified 2-C-methyl-D-ribonolactone was first protected with a trityl group on the 5-OH. The reaction was carried out in a 3-necked, 100mL round bottomed flask, fitted with a thermometer, a stopper, a

condenser with a septum, a magnetic stirring bar, and heated to 70^{o}C. Under constant stirring, the ribonolactone was dissolved in five parts anhydrous pyridine and 1 mole equivalent of triphenylchloride was added in 1g aliquots, spaced 20 minutes apart for a total of 140 minutes. The reaction was transferred to dichloromethane and worked up under aqueous conditions (Fig 7a).

The 3-Boc-valyl group was added to the crude 5-tritylated product without further purification. A mixed anhydride was first prepared to facilitate the addition reaction. N^{a}-Boc-valine was reacted with di-t-butyl dicarbonate in seven parts dichloromethane to form (Boc)val-O-Boc, the mixed anhydride that serves to donate Boc-valyl to the 3-OH. A solution of the 5-O-trityl-ribonolactone with a catalytic amount of dimethylamino pyridine (DMAP) was prepared, and then the (Boc)val-O-Boc was added slowly over 10 minutes. The resulting product was 5-O-trityl-3-Boc-valyl-2-C-methyl-D-ribonolactone, a protected ribonolactone (fig. 6c, fig. 7b, c). Purification by MPLC, eluted with 2% methanol, 28% ethyl acetate, 70% hexanes, afforded the pure product, the structure of which was confirmed by NMR.

Figure 8

Reduction of protected ribonolactone. Sodium borohydride reduction under controlled temperature and pH produced compound 1d. $NaBH_4$ – sodium borohydride. THF – Tetrahydrofuran.

Reduction of ribonolactone. The protected ribonolactone product was subjected to sodium borohydride reduction under carefully controlled pH and temperature conditions (fig. 8). The reaction was run in a 250mL 3-necked, round-bottomed flask fitted with a magnetic stirring bar, a thermometer, two addition funnels, a pH probe, and cooled in an ice bath. Water, 2.0 M KOH, and THF (15mL: 2mL: 20mL) were used as solvents and 2.0 M H_3PO_4 was added throughout the reaction to maintain the pH below 10.0. The borohydride was dissolved in 5.0 ml 0.01 M KOH, 10 ml water and 10 ml THF over ice. This $NaBH_4$ solution was added drop wise to the stirred solution of ribonolactone on an ice bath, maintaining the temperature at 5°C (+/- 2°C). Three and a third mole equivalents of $NaBH_4$ were needed to complete the reaction, forming 5-O-trityl-3-Boc-valyl-2-C-methyl-D-ribose (fig. 6d). After aqueous workup, the product was purified by MPLC using 4% methanol, 28% ethyl acetate, 68% hexanes. The NMR of the protected 2-C-methyl-ribose was inconclusive due to a mixture of anomers. To simplify the structural analysis, a small amount of the product was treated with 1,1-carbonyldiimidizole to form a cyclic carbonate ester (fig. 9), which was confirmed via NMR and IR.

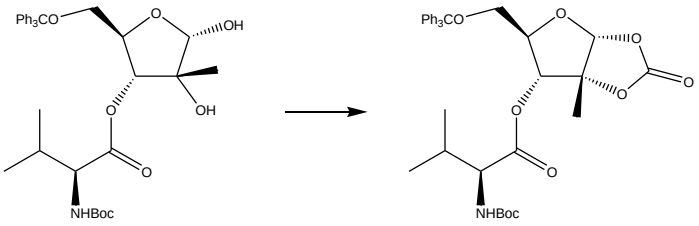


Figure 9

Cyclic carbonate confirmation of compound 1d. To determine conclusively if the reduction reaction occurred, a derivative was produced that requires the presence of the free 1 hydroxyl. NMR and IR confirmed product formation.

Condensation with Nitrogenous Base. Having produced the fully protected 2-C-methylribose, the crucial step in the synthesis was activation of the sugar to couple it with the cytosine base and thus form the nucleoside. In order to do this it was necessary to prepare the

anomeric hydroxyl to react with the C-2-OH, forming the α epoxide. A mesylate leaving group was used to activate the C-1-OH, allowing the C-2-OH to act as a nucleophile. The purified protected ribose from the previous step was reacted with mesyl chloride in triethylamine and dichloromethane (fig. 10). The lactol was weighed into the flask and dissolved in 10 parts dichloromethane. Eight parts triethylamine was added and the reaction flask was flushed with argon gas. Three and a half mole equivalents of mesyl chloride was measured, diluted three times in dichloromethane, and added drop-wise to the reaction over a period of 60 minutes. After careful workup in pH 7 buffered aqueous solution and extraction into t-butylether, the mixture was transferred into toluene and trimethylsilyl-cytosine (TMS-cytosine) was added to the mixture (fig. 11). The reaction was allowed to stir at room temperature for 45 minutes, then it was transferred to a heat bath at 60-65^{o}C. After 26 hours, a small yield of the protected 2'-methyl-cytosine nucleoside was produced (fig. 6f, fig. 11). During coupling, the C-2-OH reformed and reacted with TMS from the silylated cytosine, forming the TMS derivative of the protected nucleoside. Aqueous workup and flash chromatography, eluted with 5% methanol, 95% dichloromethane, followed by NMR and IR, gave the desired product.

Figure 10

Reaction to produce the 1,2-anhydro compound. Although not isolated, the reaction to produce the epoxide is presumed to occur and provide stereoselective addition of the cytosine base. MsCl – Mesyl chloride. Et_3N – triethylamine. CH_2Cl_2 - dichloromethane

Figure 11

Glycosylation using the epoxide intermediate. Activated TMS-cytosine is added to the 1,2-andhydro-sugar to form protected Valopicitabine. Rt – room temperature.

Deprotection by methanolysis. The fully protected Valopicitabine was methanolyzed under acidic conditions to remove the acid labile protecting groups all in one step. The cytosine derivative was dissolved in five parts methanol, to which was added a solution of 5 parts methanol and 8 mole equivalents of acetyl chloride to generate anhydrous HCl. The solution stirred at room temperature overnight. After aqueous workup and recrystallization, an NMR determined that 2'-methyl-3'-valyl-cytosine, or Valopicitabine, was the final product (fig. 6g).

IV. Results.

Beginning with the ribonolactone, protection was successful over two steps with a 78% crude yield, or 66% purified. The TLC plate (fig. 12) of the reaction progress showed that addition of the trityl protecting group progressed over a series of trityl chloride additions, about 140 minutes total. The ^{1}H NMR showed strong peaks in the range of aromatic rings,

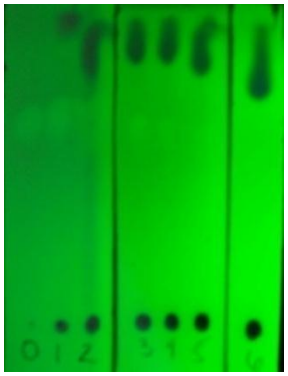


Figure 12

Tritylation of ribonolactone. TLC visualized with UV, developed in 10% isopropanol – 90% ethyl acetate. Lane 0 contains the starting ribonolactone, not visualized because it does not contain a UV chromophore. Lanes 1-5 are each after an addition of trityl chloride. The increase in appearance of a dark spot near the top of the plate indicates the formation of 5'-trityl-ribonolactone.

corresponding to the trityl group. After addition of Boc-valyl, further peaks in the methyl range indicated the presence of the three identical methyl groups on the Boc protecting moiety and two diasteromer isopropyl methyl groups from the valyl group (Fig 13). NMR indicated that the product was pure except some residual solvent.

Reduction of the ribonolactone to the ribose required 3 to 15 mole equivalents of sodium borohydride and an extension of the pH range to nearly 10.0 from the original range of 7.1 to 7.3. A TLC of the reaction showed formation of product within 20 min. (Fig. 14). The purified product, when analyzed by NMR, was inconclusive to the identity of the compound. A separate reaction with 1,1-dicarbonatediimidizole produced a cyclic carbonate derivative, only possible if

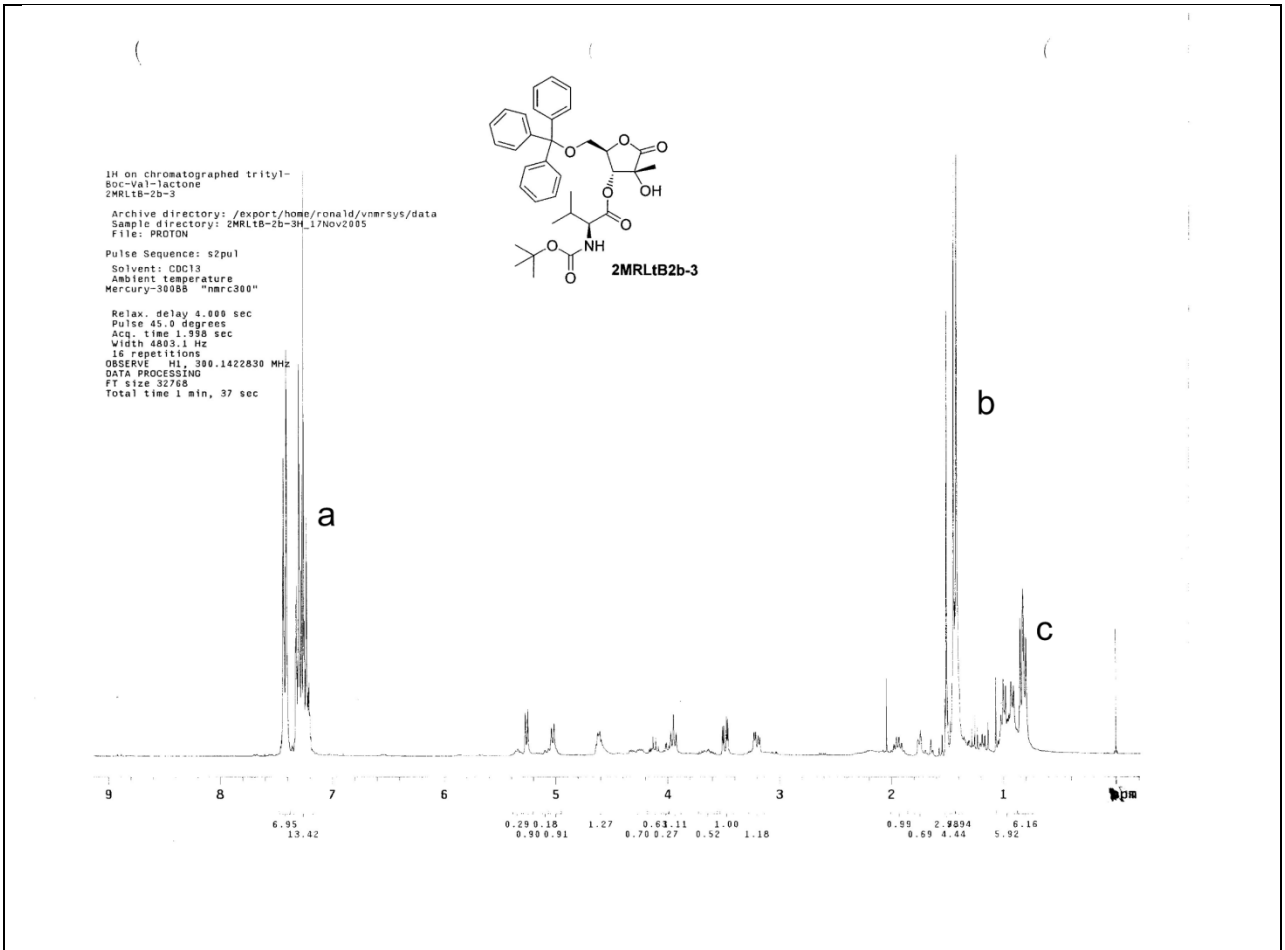


Figure 13

^{1}H NMR of protected ribonolactone. The peaks at **a** correspond to the aromatic hydrogens found on the trityl protecting group. The nine identical hydrogens at **b** are from the Boc protecting group, and **c** are the six hydrogens on the valyl substituent. All other peaks correspond to those seen in the starting material.

the free 2'-OH was produced in the reduction reaction. This derivative was analyzed by IR and NMR, confirming the presence and identity of the compound (Fig. 15). The anomeric carbon at the site of the reduction was identified (23) (24), as well as the corresponding peaks that were common with the ribonolactone.

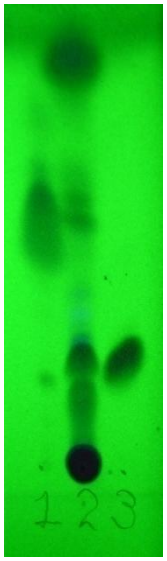


Figure 14

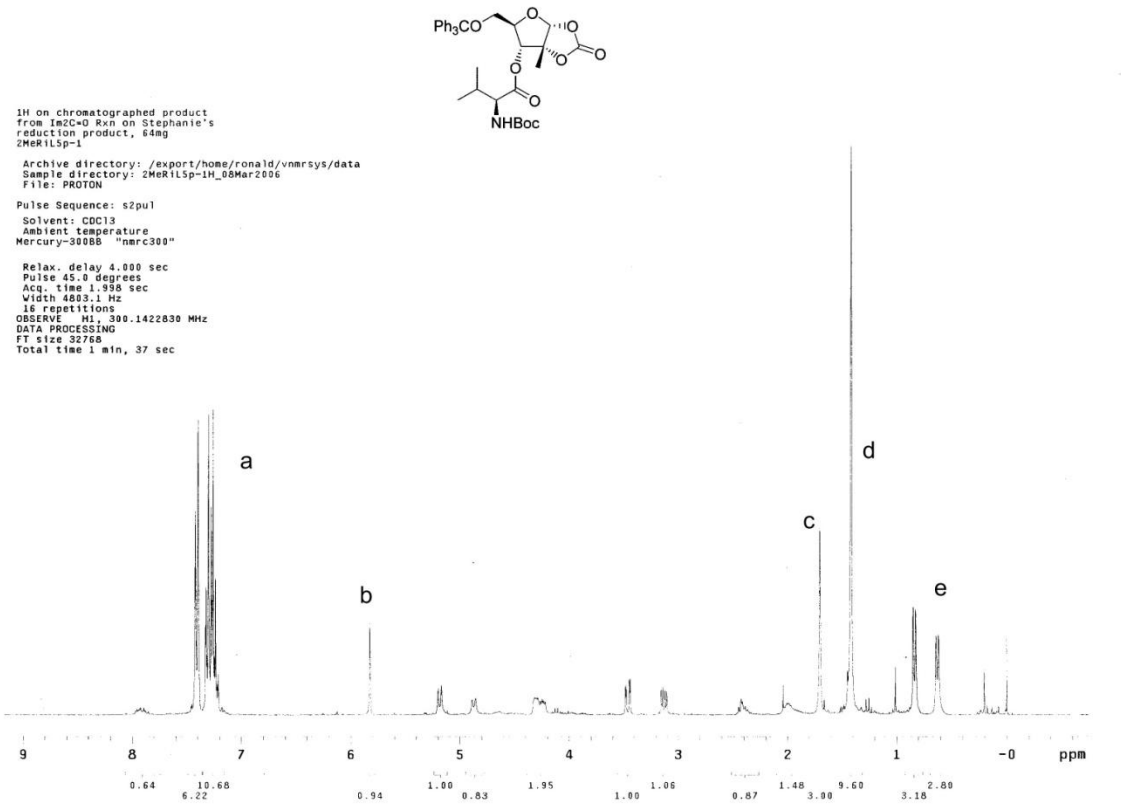
Reduction reaction. TLC visualized with UV, developed in 5% methanol – 95% dichloromethane. Lane 1 shows the ribonolactone starting material, lane 2 is after 20 minutes of reaction, and lane 3 is the desired product, obtained from Microbiologica. The TLC shows a reduction in starting material and formation of the desired product, along with several side products.

Condensation with the nitrogenous base resulted in low yields, as well as a large number of side products. The epoxide product was unable to be isolated after the initial reaction with mesyl chloride, suggesting that it is relatively unstable. TMS-cytosine, prepared in a separate reaction, was used as an activated form of cytosine, encouraging the glycosylation reaction to occur more efficiently (25). The protected ribocytosine nucleoside was produced in 7.1% yield after purification. The IR and NMR were consistent with the desired product (fig. 16 and 17).

Methanolysis removed all protecting groups cleanly, resulting in nearly quantitative yield of the final product, Valopicitabine.

Figure 15

^{1}H NMR on the cyclic carbonate confirmation of reduction reaction. The peaks at (a) correspond to the trityl aromatic hydrogens, (b) is the anomeric C-1', (c) is the 2-C-methyl substituent, (d) is the 9 methyl hydrogens from the Boc group, and the two doublets at (e) are the diastereotopic isopropyl methyl groups on the valyl..



V. Discussion.

The overall series of reactions resulted in the desired product in fewer steps than the current protocol. Formation and protection of the ribonolactone occurred as expected in high yield using common, inexpensive reagents and relatively simple procedures. The trityl protecting group is ideal for the first protection step because it reacts most efficiently with primary alcohols, making it specific for the 5-hydroxyl on the ribose sugar. This reaction

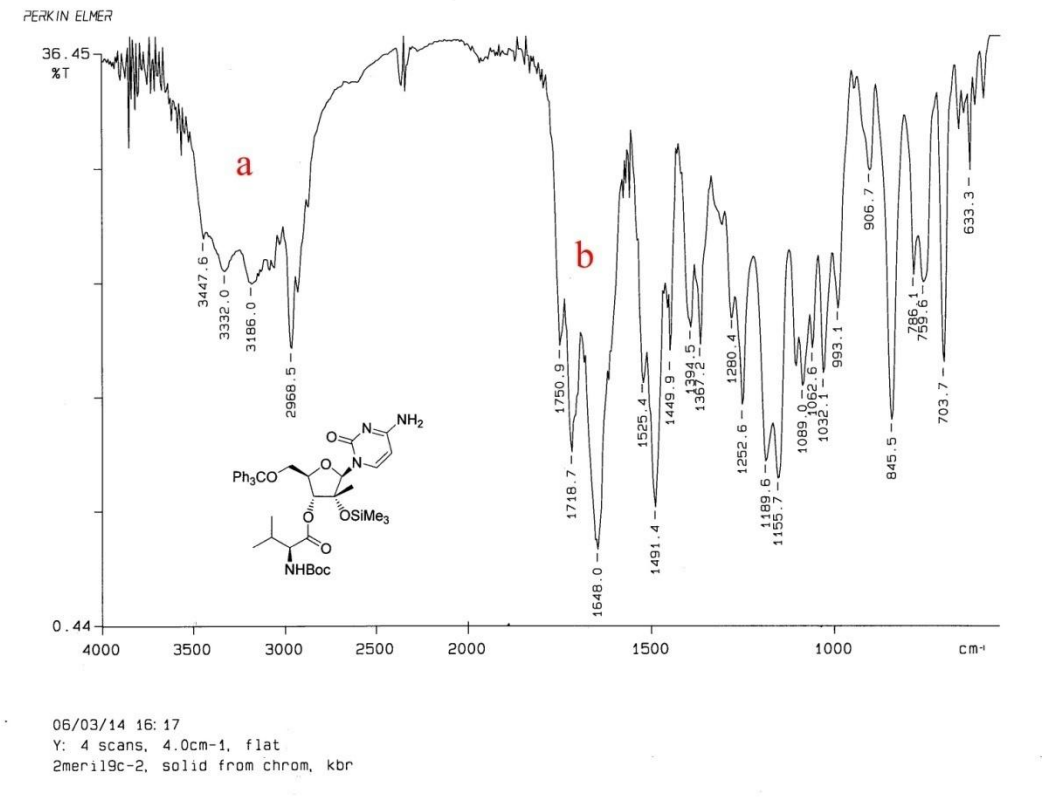
appears to be quantitative based on the TLC plates from the reactions, and the crude product from the tritylation reaction was Boc-valylated directly without isolation.

Addition of Boc-valyl at the 3' position presented some difficulty as not all of the starting material converted to product. (Boc)val-O-Boc reacts readily with alcohols, primary and secondary reacting most readily, thus the 3' position was expected to be more reactive than the 2' position. The incomplete reaction may be due to impurities from the tritylation reaction. The problem may be remedied by purifying the tritylated compound before proceeding to add the Boc-valine, affording better control over the reaction and providing higher yields. However, fewer purification steps help make a synthesis affordable and practical for large-scale manufacturing. Sacrificing some yield to reduce the amount of chromatography may be beneficial in the overall synthesis.

The presence of the valyl group is important to the bioavailability of the drug, helping it to cross membranes into cells where its action occurs (2) (10). The valyl group, once added, has a reactive amine that can interfere with further reactions in the synthesis, so it is blocked by a Boc group before the addition occurs. The Boc-protected valyl, however, is sensitive to Lewis acids; because of its reactivity, normal Vorbrüggen chemistry cannot be carried out in the presence of a Boc group (15). The valyl group would normally be added after completion of Vorbrüggen glycosylation (12), however, this adds steps to the synthesis and increases the number of sites that must be chemically protected, and then later deprotected. The synthesis developed here allows Boc-valine to be added prior to condensation with the cytosine base, reducing the number of steps and simplifying the procedure.

Figure 16

IR of protected Valopicitabine. The four peaks at (a) correspond to the four nitrogens present in the molecule (3 in the cytosine ring and one on the valyl group). The three peaks at b correspond to the three carbonyls present in the molecule (1 in the cytosine ring, 1 in the valyl group, and 1 in the Boc group).

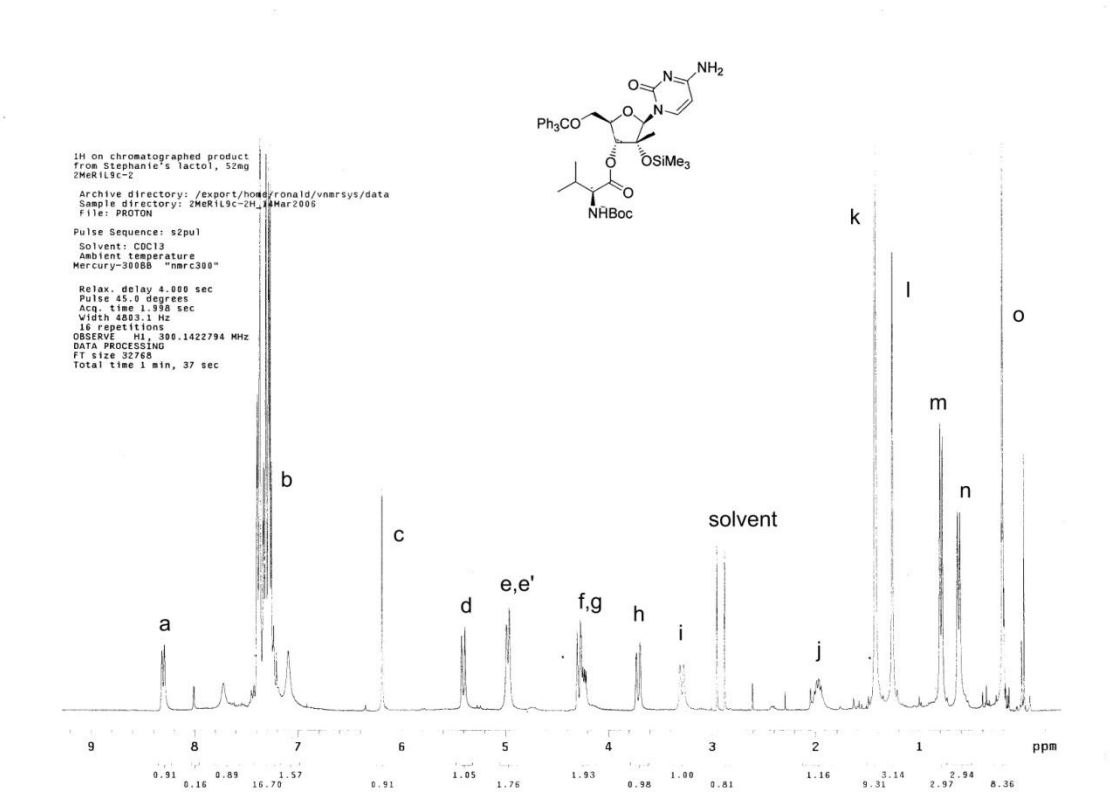


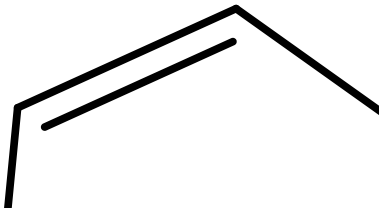
Reduction of the protected ribonolactone required sensitive methods to keep the rest of the molecule intact. Esters, such as the lactone in question, are readily hydrolyzed under basic conditions. Normally, borohydride reductions result in an increase in pH during the course of the reaction. In addition, lactones, which are somewhat more reactive than ordinary esters, can be selectively reduced to lactols. To control the pH of the reaction, phosphate buffer was used and H_3PO_4 was added to the reaction mixture as needed. It was discovered that no reaction

Figure 17

NMR of protected product. Spectral assignments as follows: (a) C-6 CH on Cytosine ring (b) aromatic CH from trityl group (c) anomeric CH at C-1', showing β orientation. (d) CH at C-3' (e) NH on Boc group (e') C-5 on Cytosine ring (f) C-4' CH (g) C-alpha on valyl substituent (h) C-5' CH2 (i) C-5' CH2 (j) CH on valyl side chain (k) 3 CH3 from Boc protecting group (l) CH3 connected to C-2' (m) one CH3 from valyl side chain (n) one CH3 from valyl side chain (o) Trimethyl silane on 2'-OH

Below: Complete structure of protected Valopicitabine with spectral assignments shown.





occurred at neutral or slightly acidic pH ranges. However, borohydride reacts rapidly with water, evolving hydrogen gas in the pH range in which lactols are stable and not reduced to diols; this allowed the pH to climb up to 9.5 until the reaction proceeded. The ice-cold conditions also helped slow the reaction, protecting the borohydride from reacting with solvent, allowing greater specificity for the lactone, and preventing over-reduction to open the ring. The addition must be done slowly to prevent exothermic acid-base reactions from heating the mixture. The crude reaction had a yield of 75.3%; however, chromatography resulted in only 36.6% pure product. Several more equivalents of $NaBH_4$ may be needed to complete the reaction and increase yields. Chromatography also failed to purify and recover all of the product; therefore, changes in chromatography conditions could also improve the yields.

Another potential problem with the reduction reaction is related to the type of protecting group at the 5' carbon. It has been shown that electronic effects from a protecting group can influence chemistry across the ring, even at the 1' position (26). As an alternative to the trityl group, the use of a second Boc protecting group at the 5' position was explored. However, it proved to be difficult to prevent the addition of excess Boc-groups at the 3' and 2'-OH position. Such an over-reaction would prevent the subsequent chemistry that is crucial to this synthesis. Further explorations are needed to determine the best overall 5' protecting group to encourage reduction at the 1' position without imposing unnecessary difficulty on the synthesis.

The key step in this synthesis is the formation of the nucleoside by condensation without Lewis acids and with correct β stereochemistry. To accomplish this, it was necessary to convert the diol to an expoxide with α-stereochemistry. Epoxides are opened by backside attack of nucleophiles using a pathway similar to the classical bimolecular substitution observed for alkyl halides (21). For asymmetric epoxides, as in this case, attack is generally specific for the least

crowded carbon and/or the most electrophilic carbon. The incoming nucleophile, for this reaction, the silylated cytosine, was expected to selectively attack the C-1 position because it is less sterically hindered than C-2; this position is also activated by the neighboring oxygen (21).

Addition of mesyl chloride to the ribose formed a mesylate on the 1' carbon, most likely as a mixture of α and β anomers. This compound is suspected to have cyclized to form an epoxide intermediate (fig 6e), although this intermediate was not isolated in this series of reactions. The mesylate is used because it makes an excellent leaving group, allowing the C-2 hydroxyl to attack and form a strained 3-member ring. The α-configuration is expected to form due to the stereochemistry of the C-2-OH, ideally converting the anomeric mixture to a single anomer. Once formed, the epoxide is highly reactive in ring opening reactions caused by nucleophilic attack because of ring strain; epoxides cause the normal bond angles for carbon to be reduced from 104.5^{o} (the normal tetrahedral geometry), to 60^{o} (an equilateral triangle).

Cytosine was activated by trimethylsilane (TMS) in a separate reaction. Silylated bases are commonly used in glycosylation reactions, including the Vorbrüggen protocol and the older silyl-Hilbert-Johnson reactions, to increase the nucleophilicity of the base (27) (28) (29). The addition of TMS also helps solubilize cytosine, which is often too polar to dissolve under the necessary organic solvent reaction conditions. Silylation is performed by hexamethyldisilizane (HMDS) according to the protocol developed by Nishimura and colleagues (27). Addition of the activated cytosine to an epoxide intermediate is expected to result in an attack from the side opposite the epoxide, resulting in the β-anomer cytosine exclusively. This product was indeed observed and characterized by NMR. A single peak from the anomeric position in the ^{1}H NMR indicates the presence of only 1 stereo configuration around the anomeric center (23) (30).

Comparison to NMR of known product manufactured by Microbiologica revealed correct stereo- and regio- chemistry of the protected nucleoside product. This step ordinarily requires harsh Lewis acids to join the cytosine base to the sugar; the success of this method utilizing a 1,2-anhydro intermediate allows for acid sensitive groups, in particular the 3'-valyl, to remain intact during the synthesis. The low yield from these two combined steps could be due to incomplete conversion to the epoxide, poor reactivity with the addition of cytosine, too many undesired cytosine reactions, or a combination. Further studies were done using the 5'-O-tol, 3'-O-tol-2'-methylribose derivative to learn more about epoxide formation. Using these alternate protecting groups, the 1,2-anhydrosugar was isolated and characterized by liquid chromatography - mass spectrometry (LCMS). From this experiment, it appears that the epoxide forms readily and completely and is slightly more stable with toluyl protecting groups rather than trityl and Boc-valyl. If the trityl and Boc-valyl protected ribose forms the epoxide similarly, than the problem lies with the epoxide-opening reaction with the cytosine base. Experimentation is needed to determine exactly what conditions are limiting the yield and to optimize the reaction procedure.

Although the yield of this reaction was small, giving only 7.1% chromatographed product, this research shows that a 6-step synthesis from D-fructose results in the prodrug Valopicitabine. More research is needed to improve the yields and purification processes before it will be practical as a large-scale synthetic method. Alternate protecting groups may allow for product crystallization rather than chromatography for purification, making the method more feasible for large-scale preparation and manufacturing. It is also necessary to experiment with multiple variables, including solvent, temperature, reagents, and pH, that could be manipulated to increase the yield. Despite its limitations, this new synthesis of Valopicitabine may apply to

numerous nucleoside analogs used as anti-viral and anti-cancer drugs, as well as many yet to be discovered.

VI. Conclusions.

The nucleoside analog Valopicitabine is formed in six steps; fructose is converted to 2-C-methylribonolactone, which is protected with a 5-trityl and 3-Boc-val. This protected compound is reduced to the protected lactol, which can be used to form an epoxide intermediate and condense with cytosine. The final 2'-methyl-3'-valyl-cytosine was obtained by deprotection.

VII. Acknowledgements.

Thanks to Dr. Robert C. Ronald for his mentorship and oversight on this project, and to Dr. Elizabete Cruz and Dr. Pat Meier for advice and ideas along the way. We thank the Microbiologica research group for funding of this project.

VIII. Appendix.

IR: Infrared spectroscopy is used to identify specific types of functional groups on a molecule for identification purposes. Infrared radiation is absorbed by some of the bonds in a molecule in characteristic stretching and bending vibrations. Each type of bond absorbs in a specific range of wavelengths with a distinctive pattern. When used in combination with other analytical techniques, it is possible to identify conclusively the structure of an organic compound. See Introduction to Spectroscopy by Pavia, Lampman, and Kriz (31).

NMR: NMR is a technique that uses a small amount of sample in organic solvent to produce characteristic patterns that can be used to identify the compound of interest. For ^{1}H NMR, the sample is placed in a magnetic field and a specific frequency of radio wave is pulsed through, knocking the natural spin of hydrogen molecules out of alignment with the magnetic field. As the hydrogen atoms bounce back, they give off energy, which is detected by the NMR spectrometer. Depending on the environment of the hydrogen atom due to its bonds and surrounding atoms, a unique frequency is observed for each hydrogen, making it possible to determine what types of hydrogen atoms are present and how they are bonded. ^{13}C NMR uses similar technology; however, ordinary ^{12}C has no natural spin and therefore cannot be detected. Since all compounds have a natural abundance of ^{13}C, the spectrometer uses the spin of the carbon isotope in a magnetic field to determine the frequency of resonance. See Introduction to Spectroscopy by Pavia, Lampman, and Kriz (31).

TLC: Thin layer chromatography is a technique used to visualize organic molecules. A compound in solution is spotted near the bottom of a silica-coated plate. When placed in a jar containing a small amount of organic solvent, the solvent travels up the plate and carries the organic molecules with it. The molecules are separated based on their polarity; the more polar the molecule, the more it is attracted to the silica on the plate, slowing its travel up the plate. Less polar molecules are carried with the solvent, traveling farther up the plate. Once separated, the molecules can be visualized under UV light. Compounds containing multiple conjugated double bonds quench the background fluorescence on the plate, appearing as dark spots. They

can also be visualized by other techniques specific to the type of molecule, such as ninhydrin spray, which detects nitrogen-containing compounds.

Ring Numbering: Nucleosides are numbered according to the ring position, with 3- referring to the third member of the base ring and 3'- referring to the third position on the sugar (fig. 18) (28). When the sugar is isolated from the base, the numbers are not followed by the prime designation.

Figure 18

Ring numbering convention for unmodified cytosine.

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