The Synthesis of FGAR (N-Formylglycinamide Ribonucleotide) and the Isolation and Amplification of the Human FGAR Amidotransferase cDNA.

Presented to the Faculty of Lycoming College
For Departmental Honors in Biology

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Abstract:

The enzyme FGAR (N-formylglycinamide-ß-D-ribofuranosyl-5-O-phosphate) amidotransferase catalyzes the fourth step in the purine biosynthetic pathway. To synthesize FGAR, the substrate for this enzyme, we have modified previously published procedures plagued by low yields, expensive purifications, and the lack of stereo- and regio- selectivity. We first protected the 2′ and 3′ hydroxyls of D-ribose with benzaldehyde and converted the 1′ hydroxyl to an allyl substituent. The next step was to phosphorylate at the 5′ position using dibenzyl phosphate in a Mitsunobu reaction. Deprotection at C1 provided the lactol, which by ^{1}H-NMR appears to have the 1′ hydroxyl in the ß position. The human FGAR amidotransferase enzyme encoded by *purL* is needed to catalyze FGAR to FGAM. Another goal of this project is to ligate the human *purL* cDNA into an expression vector. The human purL cDNA was obtained from a reverse transcription reaction from mRNA, this ensures the absence of introns. The mRNA was obtained from human white blood cells that are expressing the *purL* gene.

Introduction:

A major concern today is the rise of antibiotic resistant bacteria, especially *Staphylococcus aureus*, bacteria found in normal flora on the skin. Researchers at universities and pharmaceutical companies are aggressively looking for new antibiotics that will be effective against pathogenic bacteria such as *S. aureus*. In determining how to proceed in producing an antimicrobial drug, researchers look for and then attempt to exploit differences in vital pathways or functions between the bacterium and humans. Recently an article was published in the journal Science which states that researchers are comparing the genomes of bacteria vs. humans looking for possible new drug targets (Rosamond and Allsop, 2000). Humans and most bacteria have different forms of an enzyme in the

purine biosynthetic pathway, suggesting that this could be a useful target.

Different types of FGAR amidotransferase are found in most prokaryotes (bacteria) when compared to eukaryotes. The exception to this statement is beta and gamma-proteobacteria, which have the same enzyme as eukaryotes. The enzyme FGAR amidotransferase catalyzes the fourth step in the purine biosynthetic pathway. In the fourth step the amide nitrogen from glutamine is transferred to FGAR (formylglycinamide ribonucleotide) to form FGAM and glutamate. ATP provides the energy for the reaction. The purine biosynthetic pathway is essential for all organisms to survive since it produces adenine monophosphate (AMP) and guanine monophosphate (GMP), two of the four nucleotides in DNA and RNA.

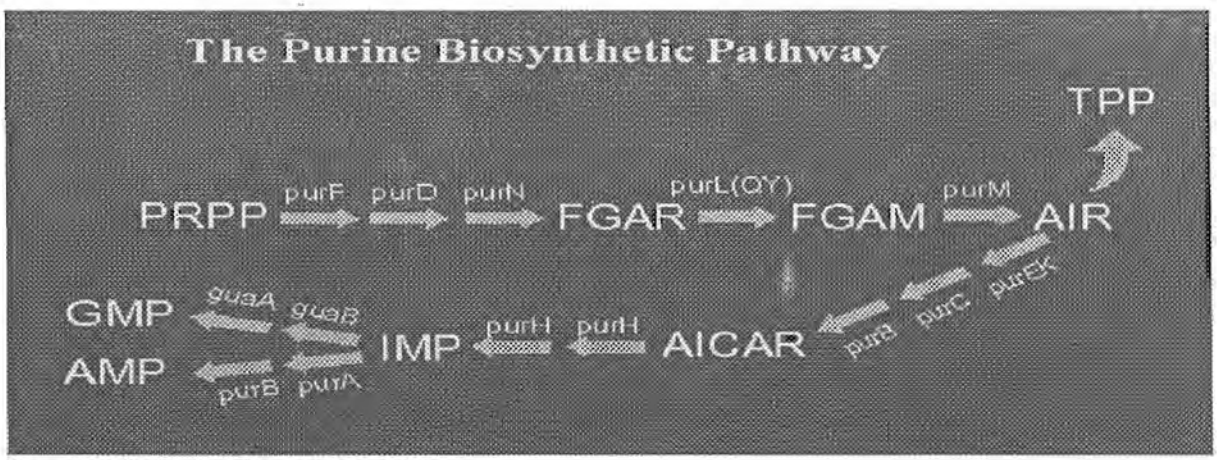


Figure 1: The Purine Biosynthetic Pathway

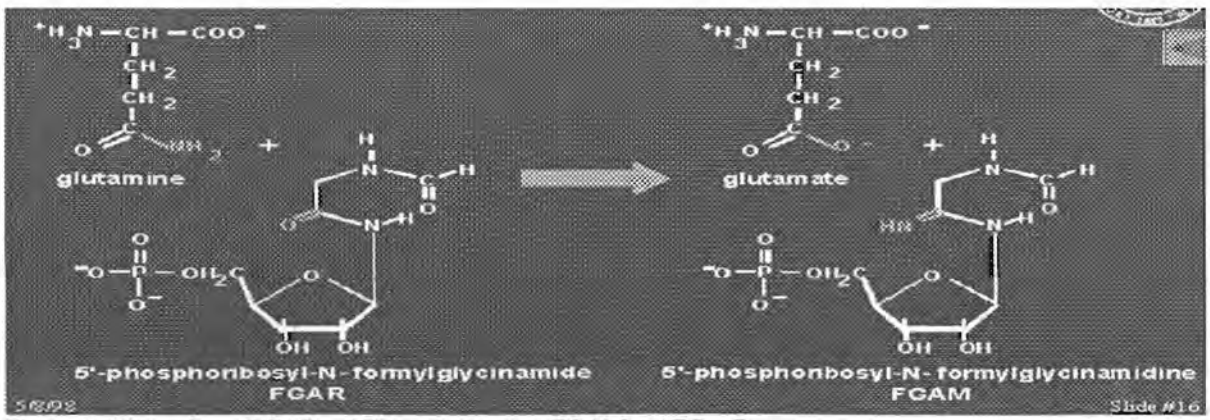
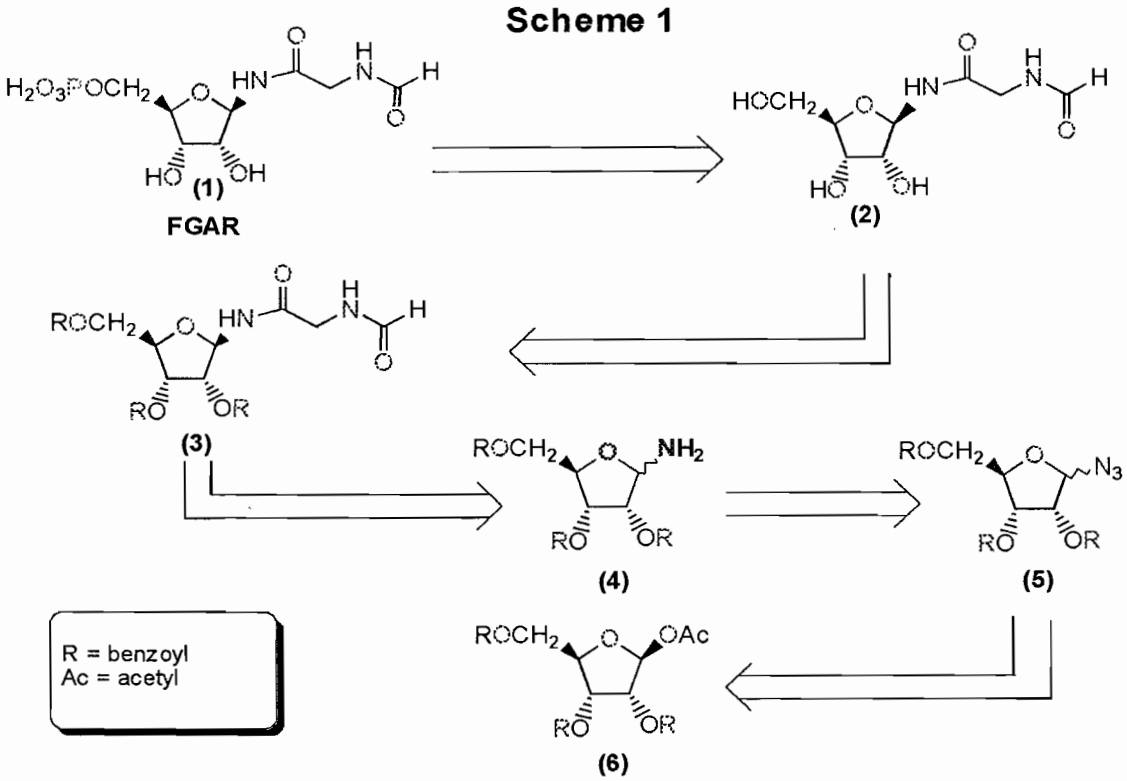


Figure 2: Glutamine + FGAR + ATP = glutamate + FGAM + ADP + P_i.

There are two types of FGAR amidotransferase, type I which is a single subunit version found in eukaryotes (and beta and gamma proteobacteria), coded by the *purL* gene, and type II, the multi-subunit version found in prokaryotes, coded by the genes *purL, purY,* and *purQ* (Newman, Ferguson, Cook, unpublished data). Now that a key difference has been found, the next step in search for an inhibitor is to do a series of enzyme assays to identify a chemical compound that inhibits the type II version of the enzyme without interfering with the type I enzyme. My research was focused on the synthesis of the substrate FGAR (formylglycinamide ribonucleotide) since FGAR can not be purchased and the amplification and cloning of the cDNA for the human *purL*.

The published procedure for the synthesis of FGAR had some problems, such as low yields, expensive purifications, and the lack of stereo- and regio- selectivity. Schendel and Stubbe's (1986) synthesis for the reduction of the azide to the amine called for the use of PtO_2 and to be hydrogenated at 10 psi. Nagarajan and Ganem (1987) published an easier and less expensive procedure for the reduction of an azide to an amine that will be used to replace the previous procedure. A lack of stereocontrol at C1 was a problem noted in Schendel and Stubbe's procedure, they obtained a 1:1 mixture of alpha / beta anomers. The synthesis was modified in attempt to alleviate some or all of the problems. Looking at the new synthesis retrosynthetically, FGAR (**1**) can be envisioned being made from the selective phosphorylation of the triol, compound **2**. The triol can be made from the protected ribose derivative (**3**) (Schendel and Stubbe, 1986). The protected ribose derivative is made with the coupling of N-formylglycine to the hemiaminal (**4**) in a condensation reaction (Schendel and Stubbe, 1986). The hemiaminal (**4**) is made from the reduction of an azide (**5**) (Nagarajan and Ganem, 1987). The azide is made from an S_N1 reaction with a fully esterified D-

ribose, which was purchased from Aldrich (Camarasa et al. 1980). The fully esterified D-ribose used as the starting compound was 1-acetyl-2,3,5-tri-O-benzoyl-β-ribofuranose (**6**).



One problem in the published synthesis by Yoshikawa et al. (1967) was with the lack of regiocontrol in the C5 phosphorylation reaction. Reaction also presumably occurred at C2 and C3. To increase the regioselectivity of the phosphorylation and to produce a possible higher yield, the use of trimethylborate to selectively protect the C2, C3 cis-diol, thus only allowing the C5 hydroxyl to be free for phosphorylation, was incorporated in the synthesis (Danlhoff et al. 1980). The boronate-protecting group will then be removed after phosphorylation by the addition of ethylene glycol (Danlhoff et al. 1980).

The other part of the project is to amplify the cDNA of the human *purL* by PCR (polymerase chain reaction) and ligate it into an expression vector. Ultimately, the goal is to

transform the plasmid containing the human *purL* cDNA into *E. coli*, then induce the expression of fusion protein that includes FGAR amidotransferase at the N-terminus. The protein can then be collected and purified for use in enzyme assays at a later date. Synthesis of the human FGAR amidotransferase protein begins with the transcription of DNA into RNA. For eukaryotes the introns are spliced-out of this primary transcript leaving just the exons before the mRNA leaves the nucleus of the cell. This mRNA is then translated by the ribosomes into a polypeptide (protein). In eukaryotes the presence of introns in DNA prevents the direct amplification of the purL gene. To combat the intron problem, mRNA is reverse transcribed into cDNA (cDNA is DNA with the introns removed).

The following approach will be utilized to obtain the cDNA, amplify, and ligate it into a plasmid. The first step is to collect white blood cells and isolate the RNA using the RNAqueous system™, by Ambion. The isolated RNA will be reverse transcribed using reverse transcriptase, thus producing cDNA of all the mRNA currently being expressed in the white blood cell. The DNA sequence for the human *purL* cDNA was found on the National Institute of Health's Genbank database. This sequence was used to design primers to isolate just the desired cDNA and to amplify it by PCR. The primers were designed with Nde1 and EcoRV restriction sites to facilitate cloning into a vector. Once the desired cDNA is PCR amplified, the next step would be to ligate the cDNA into the plasmid vector pTYB2, which is part of the T7 IMPACT system™ (Chong et al. 1997). This plasmid vector was chosen because it contains an intein domain and a chitin-binding domain just after the insertion site for the *purL* gene (Guan et al. 1988). The intein and chitin binding domains are useful for the purification of the protein. Upon expression of the plasmid, the intein and chitin binding domains are also expressed forming a fusion protein.

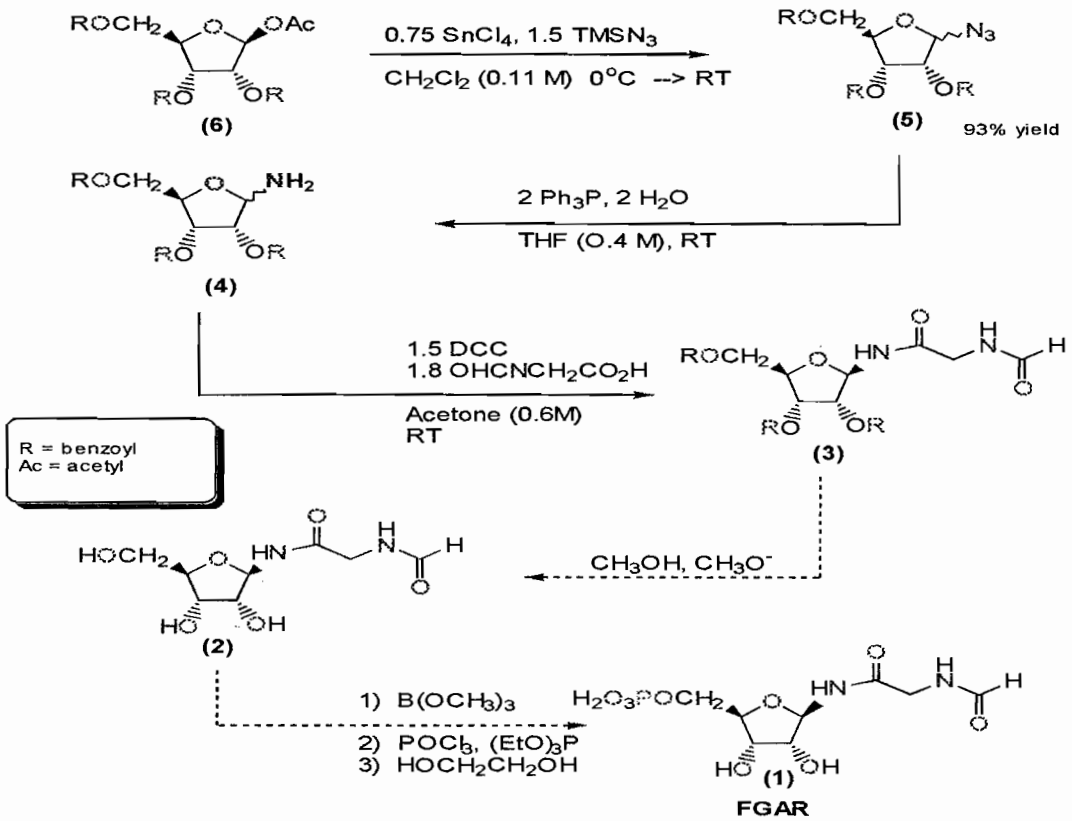
Proteins are extracted and passed through a chitin column such that only proteins with a chitin binding domain will bind to the column while the other protein are washed away. Then the concentration of dithiothreitol (DTT) is altered and the intein domain on the fusion protein self-cleaves allowing the desired protein to be collected. The goal of this part of the research is to insert the cDNA into the pTYB2 plasmid vector.

The overall goal of this project is to obtain purified FGAR amidotransferase proteins for the human PurL, *E. coli* PurL, and the PurL, PurY, and PurQ proteins from the bacteria *Staph. aureus*, to use in enzyme assays. Student researchers are currently working on the *E. coli* PurL, and the three sub-units from *Staph. aureus.* Enzyme assays can be started once the substrate FGAR is synthesized and all the proteins have been purified.

Results and Discussion:

The first step (Scheme 1) in the synthesis of FGAR was to convert the C1 acetoxy of 1-acetyl-2, 3, 5-tri-O-benzyl-beta-ribofuranose to an azide using trimethylsilyl azide and tin (IV) chloride in an S_N1 reaction to form compound **5**. This was accomplished in a 93% yield with a mixture of anomers. Compound **5** was then reduced to an amine using triphenylphosphine and water. The resulting hemiaminal (**4**) was not purified because the chromatographic stability of the molecule was in question. Without purification, N-formylglycine was coupled to the hemiaminal using DCC (N, N-dicyclohexylcarbodiimide) in a condensation reaction (Schendel and Stubbe, 1986). The resultant protected ribose derivative (**3**) was a mixture of anomers. However after column chromatography the alpha anomer was isolated, the beta anomer could not be isolated because it was in a mixture with the alpha anomer.

Scheme 2

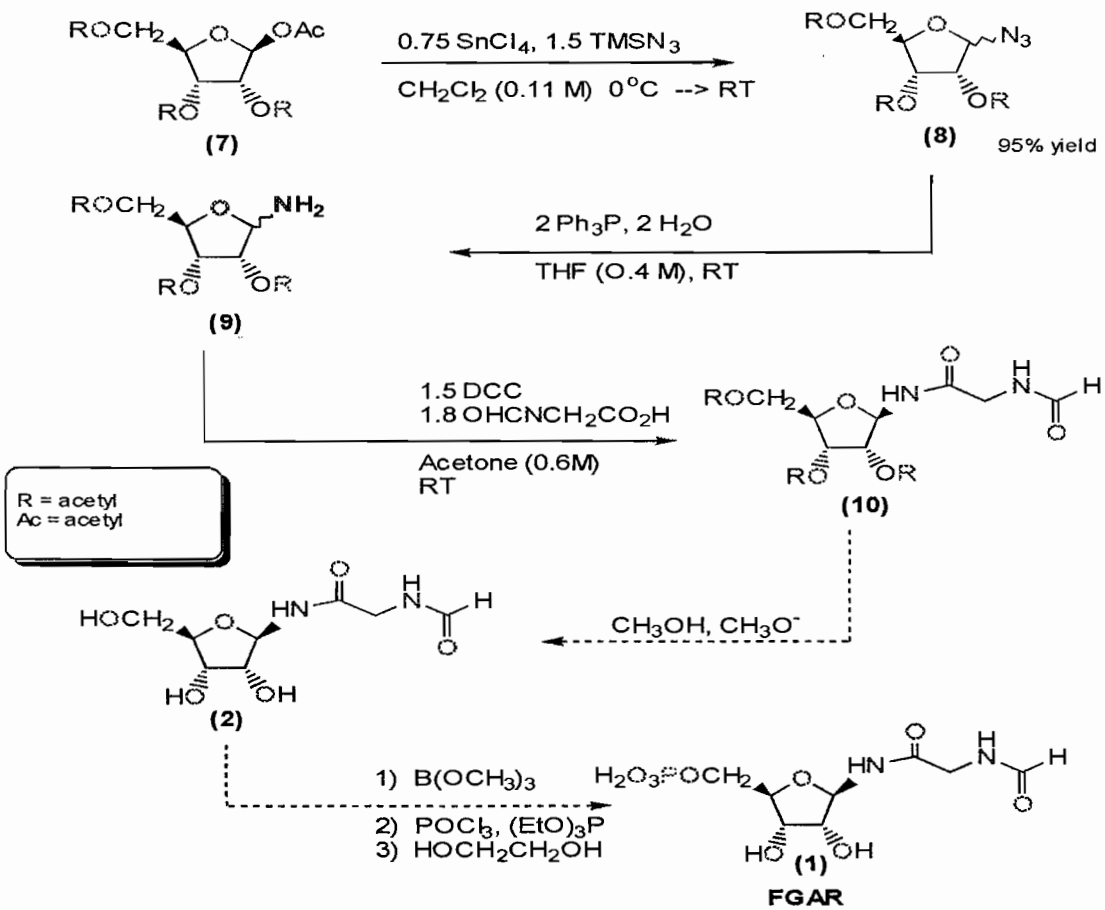


Dashed lines are proposed reactions

Determination that the isolated compound was not exclusively in the beta conformation was done by ^{1}H-NMR. The coupling pattern that would be expected if the hydrogen was in the alpha position would be a singlet due to the Karplus relationship and a dihedral angle with the C2 hydrogen being approximately 90^{0} (Silverstein and Webster, 1998). The ^{1}H-NMR of this compound has a doublet at 6.4 ppm with a coupling constant of 6.0 Hz. This indicates that compound (**3**) is in the alpha conformation. If the N-formylglycine moiety were in the beta position then there would only be a singlet due to the Karplus relationship. This singlet is observed at 5.7 ppm in a mixture of anomers between alpha and beta. So the beta conformation can be synthesized, but with the same problems other published literature had in that a mixture of

anomers was obtained. This synthesis was altered at this point because the belief was that the benzoyl protecting group at the C5 position could be sterically hindering the beta attack and favoring the alpha conformation. So the idea was to use the same scheme but instead change all the benzoyl protecting groups to the less sterically demanding acetyl protecting groups.

Scheme 3

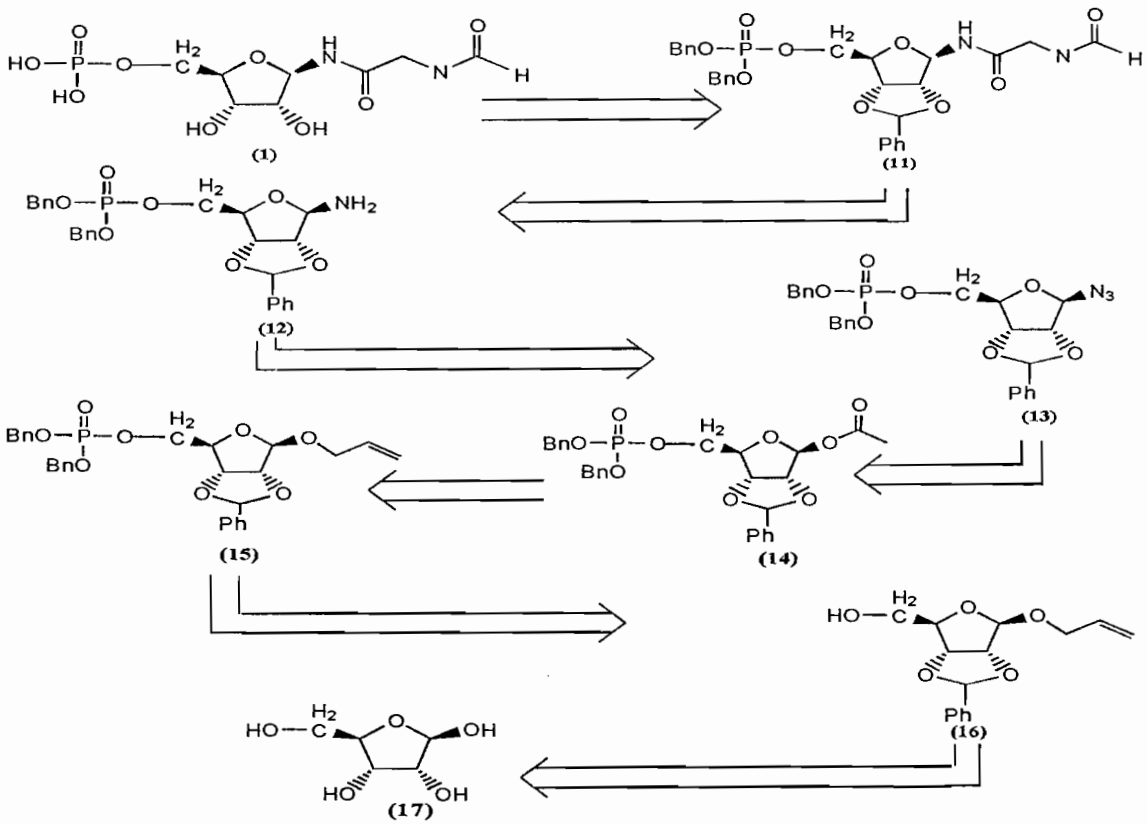


Dashed lines are proposed reactions

The first step in the second synthesis of FGAR was to convert the C1 acetoxy of 1,2,3,5-tetraacetate –beta- D- ribofuranose to an azide using trimethylsilyl azide and tin (IV) chloride (as a Lewis acid) in an S_N1 reaction to form compound **8**. This was accomplished in a 95% yield as a mixture of anomers. Compound **8** was then reduced to an amine using triphenylphosphine and

water. The resulting hemiaminal (**9**) was not purified as in the benzoyl case. N-formylglycine was coupled to the hemiaminal using DCC (N, N-dicyclohexylcarbodiimide) in a condensation reaction. The product could not be unambiguously assigned as compound **10**, by ^{1}H-NMR. The NMR data did not compare with expected chemical shifts and coupling patterns. The peak that is believed to be the C1 hydrogen should be a singlet if the hydrogen is in the alpha conformation or it should be a doublet if it is in the beta conformation. What appears is a doublet of doublets. To remove any possible coupling with the amide hydrogen, the amide hydrogen was exchanged for a deuterium using D_2O. The spectrum after the deuterium exchange changed the doublet of doublets to a quartet. This was not the result expected, so a new synthetic plan was created.

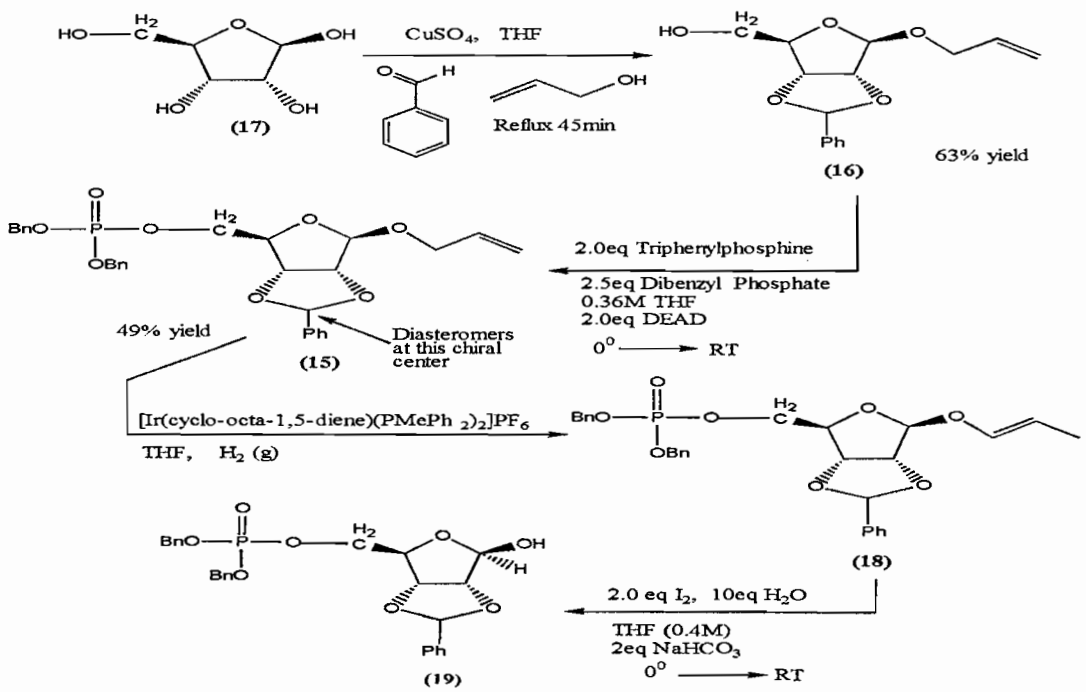
Scheme 4



In the synthesis outlined in Schemes 2 and 3 the problem that was persisting was the lack

of control over the stereochemistry at C1, so a new synthetic plan needed to be adopted in attempt to control the stereochemistry at C1. Looking at this retrosynthetically, FGAR (**1**) can be envisioned being made by deprotecting a protected FGAR derivative (**11**). Compound (**11**) can be made by coupling N-formylglycine to the amine at C1 in a condensation reaction with compound **12**. The hemiaminal can be formed through a reduction of an azide (**13**) (Nagarajan and Ganem, 1987). The azide can be made from a S_N1 reaction from 1-acetyl -2,3,5 - protected ribose derivative (**14**) (Camarasa et al. 1980). The C1 acetoxy is formed from the isomerization, hydrolysis, and acetylation of the allyl acetal at the C1 position (**15**) (Gigg, 1979). To construct compound **15**, a 1,2,3 – protected ribofuranoside (**16**) can be phosphorylated at C5 via a Mitsunobu reaction (Mitsunobu et al. 1970). Compound **16** can be synthesized from D-ribose using a benzylidene protecting group for C2 and C3 and an allyl acetal to protect C1 (Levene and Stiller, 1933).

Scheme 5



Using D-ribose (**17**) as the starting material, the goal was to protect the C2 and C3 hydroxyls using a benzylidene-protecting group and to convert the C1 hydroxyl to an allyl acetal. This was accomplished with a yield of 63%. It was learned that this reaction's optimum reaction time at reflux was 45 minutes. The next step was to do a Mitsunobu reaction, phosphorylating at C5. Compound (**16**) was reacted with triphenylphosphine, diethyl azodicarboxylate and dibenzyl phosphate to form compound (**15**); a 49% yield was obtained. During column chromatography it was apparent that the two diastereomers (diastereomeric at the benzylidene acetal C) could be separated. A ^{31}P-NMR was performed and one peak was discernible in the first diastereomer to elute from the column. A mixture of diastereomers eluted next off the column and a ^{31}P-NMR was performed with two phosphorus signals. The next eluant off the column was the other diastereomer and this had one phosphorus signal present in the ^{31}P-NMR. This proved that the diastereomers were separated. Both diastereomers would be used to ascertain if they performed different chemistry.

After phosphorylation, the double bond in the allyl acetate needed to be isomerized to eventually hydrolyze and then acetylate at C1. Wilkinson catalyst [Rh $(PPh_3)_3Cl$] was the initial choice to isomerize the double bond using the conditions in the paper published by Gigg, 1979. The reaction did not work, starting material was recovered and a different approach was investigated. Thus [Rh $(PPh_3)_3Cl$] was used in conjunction with DABCO (diazabicyclo [2.2.2] octane), ethanol, water, and benzene (Corey and Suggs, 1973). This procedure worked in isomerizing the double bond but it removed the benzyl protecting groups in the process. Ethanol was probably the source of hydrogen needed to remove the benzyl groups. Several more attempts were made using rhodium as the catalyst in isomerizing the double bond without much success, so

a different catalyst was sought. The new choice was an iridium complex [Ir (cycloocta- 1,5 - diene)$(PMePh_2)_2]PF_6$ which is red in color. The isomerization would be done under milder conditions as compared to the Wilkinson catalyst. The procedure for this isomerization is to use THF as the solvent and to degas with N_2. After the mixture is degassed, the iridium complex is "activated" by adding H_2 (Baudry et al. 1978). As the iridium complex was being "activated" the reaction mixture turned from blood red to colorless, a good indicator that a reaction had taken place. This reaction worked in isomerizing the double bond to produce the enol ether (**18**) as a mixture of geometric isomers.

After the C1 enol ether was formed, it was oxidatively hydrolyzed at C1 using I_2, water, and sodium bicarbonate to form the hemiacetal (**19**). The idea was to determine the stereochemistry of the resulting hemiacetal and if it is predominately in the alpha conformation (due to hydrogen bonding) then it may be possible to do the N-formylglycine coupling via the Mitsunobu reaction, which will save two steps in the synthesis. The hemiacetal that was formed was in the beta conformation and not in the alpha conformation.

The conformation of the hemiacetal was determined by ^{1}H-NMR spectroscopy. If the hemiacetal was in the alpha conformation then the signal for the C1 hydrogen should appear as a doublet in the spectrum, due to coupling with the C2 hydrogen. However, the ^{1}H-NMR spectrum obtained had a doublet at 5.7 ppm with a coupling constant of 2.0 Hz. The coupling that was appearing could be from the C1 hydrogen coupling with the alcohol hydrogen. To eliminate this coupling, D_2O was added to exchange out the alcohol hydrogen for a deuterium (^{1}H-NMR invisible). This resulted in a singlet at 5.7 ppm, which is consistent with the hemiacetal being in the beta conformation due to the Karplus relationship (dihedral angle approx. 90^0) mentioned

previously (Silverstein and Webster, 1998).

The envisioned future of this synthesis will involve a bulky protecting group at C2 and C3 which will hopefully provide more steric hinderance on the alpha face of the molecule. The synthetic approach will use a protected N-formylglycine group and react it with the sterically hindered ribose derivative in a hindered S_N1 reaction. Since the protected N- formylglycine is a very bulky molecule, the steric hinderance on the alpha face should cause the major product to be in the beta conformation.

Isolation and Amplification of purL

To attack the problem of finding and amplifying the mRNA that codes for the purL protein (FGAR amidotransferase) in the species *Homo sapiens,* the nucleotide sequence was needed. The sequence was found on the National Institute of Health's database on the web. The sequence for purL can be viewed in appendix A. To obtain the clone, RNA had to be isolated. This was done by using the RNAqueaous system by Ambion. To verify that RNA was successfully isolated a sample of the isolate was run on a RNA gel. RNA was present and this was determined by the presence of the 28S and 18S sub-units of ribosomal RNA appear as two bands in the smear at the bottom of the left lane.

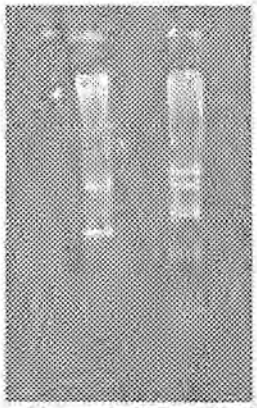


Figure 3: RNA gel. The left lane the bands of human 28S and 18S sub-units of rRNA can be seen towards the bottom. The band at the top is presumably high molecular weight RNA.

cDNA can be produced from the RNA by adding the enzyme reverse transcriptase (RT), which will take the RNA templates and produce cDNA.

The RT reaction was done with the Moloney Murine Leukemia Virus (M-MLV) and Avian Myeloblastosis Virus (AMV) reverse transcriptase enzymes, which bind to the oligo dT primer. Since mRNA has a poly A tail, the oligo dT primer is incorporated into the reaction. cDNA was used as a template in PCR with human *purL* primers to amplify the human cDNA. The only enzyme that produced cDNA was the M-MLV RT. The lane that corresponds to the M-MLV reaction mixture was the only lane with a smear in the below gel photo.

Figure 4: PCR reaction. The smeared lane is presumably cDNA.

cDNA is produced from the RT reaction, but the method that is being used will produce a cDNA for every RNA template being expressed in the white blood cell at time of harvesting. To select just the desired cDNA to be amplified during PCR, primers were made using the purL sequence that will only bind to the purL cDNA. cDNA was obtained and PCR amplified as seen in the gel photo with the appearance of the lower marked band, however , the band is at approx. 1.1 kilobases (kb) and purL is approximately 4 kb. The product does not match with the expected result, concluding that the PCR reaction did not successfully purL.

There are several possible reasons why the PCR did not work and a short PCR product was produced; one reason could be since the gene is so large that the Taq polymerase enzyme did not stay on the gene and so only a small segment of the gene was copied. Another possible reason was provided by Dr. Stephen Benkovic of Pennsylvania State University, who stated that the human purL gene is very GC rich (high GC content in gene around 67%) and that causes a very high melting temperature, due to more hydrogen bonding. With the gene having a high GC content, it requires more energy to separate the two strands of cDNA, which would prevent PCR amplification. He suggested the use of Advantage® - GC 2 PCR kit from Clonetech, due to his successful PCR amplification of the human purL gene by using this kit. The Advantage® - GC 2 PCR kit was purchased and tried in the PCR reaction. This unfortunately did not provide any PCR product. However, the S. aureus purL gene is GC rich and that gene was PCR amplified by researchers, so there might be another contributing factor to this problem.

Varying the conditions of the PCR reactions with the Advantage® - GC 2 PCR kit would be the next step, however more cDNA would be needed. The problem with this is that more reverse transcriptase would also be needed, but the budget did not allow for this expenditure at this time.

The other contributing factor might be the reverse transcription reaction. Even though the purL gene is only four kilobases. The mRNA strand that needs to be reverse transcribed has another 1.1 kilobases at 3′ untranslated region that is added to the length. This gives the total mRNA to be transcribed to be over 5 kilobases in size. This size issue can cause the problems that were encountered. To solve the possible problem with the 3′ untranslated region, a stop primer instead of the oligo dT can be used. The stop primer will attach at the end of the coding

region during the RT reaction, thus eliminating the involvement of the extra 1.1 kilobases. After removing the extra 1.1 kb mentioned above, the *purL* gene is still very large and thus will be PCR amplified in two pieces. The two sections would be amplified by PCR, ligated separately into a plasmid vector, and transformed into *E. coli*, and cloned. The two halves of the gene can then be ligated into the full length *purL* gene.

Conclusion:

The synthesis of FGAR can be accomplished at this time, however it will likely encounter some of the same problems as seen in previous synthesis, mainly the stereocontrol at C1. Since FGAR is not required at this time, the problem with stereocontrol at C1 can be investigated. Stereochemical control in organic chemistry is an important consideration in organic synthesis, and development of a method to control the sterochemistry of a molecule would be useful. The next step in the synthesis of FGAR is to attempt Scheme 6 mentioned in the Results and Discussion.

The ligation of the human purL gene has not been accomplished at this time, the problem that was encountered was either the reverse transcription reaction or the PCR reaction. One thing that should have been attempted was running the putative cDNA on a cDNA gel to determine if high molecular weight cDNA was present. This would have shown if the RT reaction was the problem or if the PCR reaction. If it is the RT reaction, future work will involve removing the 3′ untranslated region as desrcibed in the Results and Discussion section.

Experimental:

General lab procedures – All glassware used in air and water sensitive reactions were oven dried for at least three hours before starting the experiment. Also air and water sensitive reactions

were run under an inert atmosphere (N_2). All solvents that were used in air and water sensitive reactions were distilled from an appropriate drying agent prior to being utilized. Thin- layer chromatography was performed using glass backed silica plates (purchased from Analtech) and mixtures of ethyl acetate in hexane. All column chromatography were performed using silica gel (70-270 mesh) purchased from Aldrich. Infrared spectra were taken with a Mattson Polaris spectrophotomer. Trimethylsilane was used as an internal standard for all NMR spectra, and deuteroform ($CDCl_3$) was the solvent for all NMR spectra. All NMR spectra were performed on a Bruker 300 MHz NMR. All reactants were added in the order as they appear in the experimental description for each compound.

Synthesis of compound 5

1-Acetyl-2,3,5-tri-O-benzoyl-β-ribofuranose (200 mg, 0.390 mmol), dichloromethane (3.6 ml, 0.11 M), trimethylsilyl azide (0.079 ml, 0.59 mmol), and tin (IV) chloride (0.30 ml, 0.29 mmol) were added to a round bottom flask equipped with a stir bar. The flask was closed with a rubber septa and stirred overnight starting at 0^0 C and going to room temperature. After reacting for 16 hours, TLC analyses in 15% ethyl acetate in hexane indicated the reaction had gone to completion. The reaction was treated 8 ml saturated sodium bicarbonate solution to neutralize any remaining acid. The product was extracted with 18 ml (3 x 6 ml) dichloromethane and the organic layers were combined. The solvent was removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a full 14/20 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 20 – 25 ml. The mobile phase regimen used was 5% ethyl acetate/ hexane (100 ml), 10% ethyl acetate/ hexane (100 ml), and 15% ethyl acetate/ hexane (250 ml). Fractions 6- 12 were collected and determined to contain the desired product by ^{1}H-NMR. Yield of oily white compound was 93.4%. ^{1}H-NMR (300 MHz, $CDCl_3$) beta conformation δ 8.0 (6H, m) 7.5 (9H, m) 5.85 (1H, dd, J = 6.4, 4.8 Hz) 5.69 (1H, d, J = 1.7 Hz) 5.6 (1H, dd, J = 4.8, 1.6 Hz) 4.8 (1H, m) 4.58 (2H, m). The discernible alpha conformation signals δ 5.9 (1H, d, J = 5 Hz) 5.79 (1H, dd, J = 6.5, 3.4 Hz) 5.53 (1H, dd, J = 6.4, 5.0 Hz). IR ($CDCl_3$) 3019, 2956, 2117, 1728, 1266, 1121 cm^{-1}.

Synthesis of Compound 4

Azide compound 5 (180 mg, 0.37 mmol), tetrahydrofuran (10.5 ml, 0.035 M), water (0.013 ml, 0.74 mmol), and triphenylphosphine (194 mg, 0.74 mmol) were added to a round bottom flask equipped with a stir bar. The reaction was stirred overnight at room temperature. After reacting for 13 hours, TLC analyses in 15% ethyl acetate in hexane indicated the reaction had gone to completion. The reaction was treated with 10 ml sodium chloride and the product

was extracted with 20 ml (2 x 10 ml) $CHCl_3$. The organic layers were combined and the solvent removed under reduced pressure. The product was not characterized or purified.

Synthesis of Compound 3

Amine compound 4 (180 mg, 0.39 mmol), dry acetone (0.63 ml, 0.61 M), N-formylglycine (72 mg, 0.70 mmol), and DCC (N, N-dicyclohexylcarbodiimide) (120 mg, 0.59 mmol) were added to a round bottom flask equipped with a stir bar. The reaction was stirred for 48 hours at room temperature. TLC analyses in 20% ethyl acetate in hexane indicated that product was formed but the reaction did not go to completion. The reaction was stopped by vacuum filtering through sand using chloroform to quantitatively transfer product. The solvent was removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a full 14/20 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 20 – 25 ml. The mobile phase regimen used was 10% ethyl acetate/ hexane (100 ml), 20% ethyl acetate/ hexane (100 ml), 40% ethyl acetate/ hexane (100 ml), and 100% ethyl acetate (150 ml).
Fractions 6 and 7 were collected in one flask and fractions 8 and 9 were collected in a separate flask. It was determined by ^{1}H-NMR that the product in fractions 6 and 7 is the alpha anomer and the product in fractions 8 and 9 are a mixture of alpha and beta anomer with beta being the major product. Yield of both anomers was approximately 40%. ^{1}H-NMR (300 MHz, $CDCl_3$) alpha anomer δ 8.1 (6H, m) 7.6 (9H, m) 6.4 (1H, d, J = 5.5 Hz) 5.4 (1H, t, J = 5.7 Hz) 5.3 (1H, dd, J = 10.1, 4.2 Hz) 4.7 (1H, dd, J = 7.7, 3.7 Hz) 4.55 (1H, dd, J = 12.1, 5.0 Hz) 4.25 (1H, dt, m). ^{13}C-NMR ($CDCl_3$) δ 168, 167, 166, 133.7, 133.5, 133.3, 132.5, 130.3, 129.8, 129.6, 129.5, 129.4, 129, 128.8, 128.6, 128.5, 128.4, 125.8, 78.5, 74.3, 74.1, 63, 14.7, 14.6. The discernable beta anomer signals ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.9 (1H, dd, J = 6.4, 4.9 Hz) 5.8 (1H, m), 5.7 (1H, dd, J = 4.9, 1.0 Hz) 5.65 (1H, s).

Synthesis of Compound 8

1,2,3,5 – Tetraacetate-beta-D-ribofuranose (200 mg, 0.63 mmol), dichloromethane (5.7 ml, 0.11 M), trimethylsilyl azide (0.13 ml, 0.94 mmol), and tin (IV) chloride (0.47 ml, 0.47 mmol) were added to a round bottom flask equipped with a stir bar. The flask was closed with a rubber septa and stirred overnight starting at 0^0 C and going to room temperature. After reacting for 18 hours, TLC analyses in 25% ethyl acetate in hexane indicated the reaction had gone to completion. The reaction was treated 12 ml saturated sodium bicarbonate solution to neutralize any remaining acid. The product was extracted with 30 ml (3 x 10 ml) dichloromethane and the organic layers were combined. The solvent was removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a full 14/20 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 20 – 25 ml. The mobile phase regimen used was 10% ethyl acetate/ hexane (250 ml), 15% ethyl acetate/ hexane (100 ml), 20% ethyl acetate/ hexane (200 ml), 25% ethyl acetate/ hexane (200 ml), 30% ethyl acetate/ hexane (200 ml), 40% ethyl acetate/ hexane (150 ml), 50% ethyl acetate/ hexane (150 ml), and 100% ethyl acetate (50 ml). It was determined by TLC and ^{1}H-NMR that fractions 12 through 16 contained the desired product. Yield was 95% of white oily compound. ^{1}H-NMR (300 MHz, $CDCl_3$) beta anomer δ 5.4 (1H, d, J = 2.0 Hz) 5.36 (1H, dd, J = 4.9, 6.7 Hz) 5.15

(1H, m) 4.4 (2H, m) 4.19 (1H, m) 2.13 (3H, s) 2.12 (3H, s) 2.08 (3H, s). The discernible alpha conformation signals δ 5.6 (1H, d, J = 5 Hz), 5.25 (1H, dd, J = 3.7, 6.6 Hz) 2.15 (1H, s) 2.14 (1H, s) 2.10 (1H, s). ^{13}C ($CDCl_3$) beta anomer δ 171, 170, 169, 93, 79, 74, 70.5, 63, 20.65, 20.5, 20.45. The discernable alpha anomer peaks δ 90, 20.7, 20.55, 20.4.

Synthesis of Compound 9

The azide compound 8 (500 mg, 1.7 mmol), tetrahydrofuran (47.4 ml, 0.035 M), water (0.06 ml, 3.4 mmol), and triphenylphosphine (870 mg, 3.4 mmol) were added to a reaction flask equipped with a stir bar. The reaction was stirred overnight at room temperature. After reacting for 15 hours, TLC analyses in 50% ethyl acetate in hexane indicated the reaction had gone to completion. The reaction was treated with 10 ml sodium chloride and the product was extracted with 20 ml (2 x 10 ml) $CHCl_3$. The organic layers were combined and the solvent removed under reduced pressure. The product was not characterized or purified.

Synthesis of Compound 10

Amine compound 4 (500 mg, 1.8 mmol), dry acetone (2.98 ml, 0.61 M), N-formylglycine (340 mg, 3.2 mmol), and DCC (N, N-dicyclohexylcarbodiimide) (560 mg, 2.7 mmol) were added to a round bottom flask equipped with a stir bar. The reaction was stirred for 18 hours at room temperature. TLC analyses in 50% ethyl acetate in hexane indicated that product was formed but the reaction did not go to completion. The reaction was stopped by vacuum filtering through sand using chloroform to quantitatively transfer product. The solvent was removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a full 19/22 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 40 – 50 ml. The mobile phase regimen used was 15% ethyl acetate/ hexane (300 ml), 20% ethyl acetate/ hexane (300 ml), 25% ethyl acetate/ hexane (450 ml), 30% ethyl acetate/ hexane (250 ml), 35% ethyl acetate/ hexane (250 ml), 40% ethyl acetate/ hexane (300 ml), and 100% ethyl acetate (250 ml). The product could not be unambiguously be assigned from spectral data.

Synthesis of Compound 16

D- ribose (2.0 g, 13 mmol), tetrahydrofuran (10 ml, 1.3 M), benzaldehyde (2.7 ml, 26 mmol), allyl alcohol (10 ml, 0.15 mol) copper (II) sulfate (8 g, 5 mmol), and 4 drops from a pasteur pipet of sulfuric acid were added to the reaction flask equipped with a stir bar. The reaction was then refluxed for 45 minutes. The reaction was monitored by TLC in 15% ethyl acetate in hexane. TLC analysis revealed that the reaction had gone to completion. The reaction was stopped and the reaction mixture was vacuumed filtered through sand. The filtrate was collected in a filter flask containing 10 ml of saturated sodium bicarbonate to neutralize the acid. The product was extracted with 3 x 10 ml of ether. The organic layers were combined and the solvent removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a full 24/40 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 75 - 100 ml. The mobile phase regimen used was 9% ethyl acetate/ hexane (600 ml), 14% ethyl acetate/ hexane (400 ml), 19% ethyl acetate/ hexane (400 ml), and 34% ethyl acetate/ hexane (250 ml). The

product eluted in fractions 7- 9. The fractions were collected and the solvent was removed under reduced pressure. The product is a colorless oil, produced with a 63.2% yield. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.5 (2H, m) 7.4 (3H, m) 6.0 (1H, s) 5.9 (1H, m) 5.4 (1H, m) 5.3 (3H, m) 5.0 (1H, d, J = 5.2 Hz) 4.78 (1H, d, J = 6.1 Hz) 4.57 (1H, t, J = 2.9 Hz) 4.3 (1H, dd, J = 9.4, 6.0 Hz) 4.1 (1H, m) 3.75 (2H, m). The other discernable diastereomer signals, δ 5.8 (1H, s) 4.96 (1H, d, J = 6.1 Hz) 4.77 (1H, d, J = 6.6 Hz) 4.63 (1H, t, J = 2.7 Hz).

Synthesis of Compound 15

The protected ribose derivative (100 mg, 0.36 mmol), tetrahydrofuran (1.0 ml, 0.36 M), dibenzylphosphate (25 mg, 0.9 mmol), DEAD (diethyl azodicarboxylate) (114 mg, 0.72 mmol), and triphenylphosphine (189 mg, 0.72 mmol) were added to a round bottom flask equipped with a stirbar. With the addition of DEAD the reaction turned from clear to a pale yellow in color. The reaction was started at 0^0 C and stirred overnight to room temperature. After reacting for 16 hours, TLC analysis displayed that the reaction had gone to completion. Saturated sodium bicarbonate 8 ml was added to neutralize the reaction mixture. The product was extracted with 15 ml (3 x 5 ml) ether. The organic layers were combined and the solvent was removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a full 14/20 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 20 - 25 ml. The mobile phase regimen used was 10% ethyl acetate/ hexane (150 ml), 20% ethyl acetate/ hexane (250 ml), and 30% ethyl acetate/ hexane (300 ml). One diastereomer of the product eluted on fractions 25 – 30, and the second diastereomer eluted on fractions 32 – 34. These fractions were collected and the solvent was removed under reduced pressure. The yield was 49% of a white oily compound. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.5 (15H, m) 5.92 (1H, s) 5.8 (1H, m) 5.25 (1H, dd, J = 1.5, 18.8 Hz) 5.2 (1H, s), 5.18 (1H, m) 5.08 (2H, d, J = 2.5 Hz) 5.05 (1H, d, J = 2.5 Hz) 4.8 (1H, d, J = 5.6 Hz) 4.7 (1H, d, J = 5.6 Hz), 4.4 (1H, t, m) 4.0 (4H, m). The second diastereomer was also characterized by ^{1}H-NMR. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.5 (15H, m) 5.8 (1H, m) 5.7 (1H, s) 5.25 (1H, dd, J = 1.6, 17.3 Hz) 5.23 (1H, s), 5.18 (1H, dd, J = 1.5, 10.6 Hz) 5.08 (2H, d, J = 2.9 Hz) 5.05 (1H, d, J = 3.0 Hz) 4.75 (1H, d, J = 6.2 Hz) 4.7 (1H, d, J = 6.1 Hz), 4.45 (1H, t, m) 4.15 (2H, m) 4.0 (2H, m).

Synthesis of Compound 18

Compound 17 (210 mg, 0.39 mmol), tetrahydrofuran (7 ml, 0.56 M), and iridium catalyst ([Ir (cyclo-octa-1,5- diene) $(PMePh_2)_2$] PF_6) (10 mg, 0.01 mmol) were added to a reaction flask with stir bar. The reaction solution was degassed with N_2 (g) for five minutes. After the nitrogen flush, the iridium catalyst was activated by bubbling H_2 (g) into the solution the color should turned from red to clear. After the catalyst activation, N_2 (g) was bubbled into the flask for 15 minutes to displace the H_2 (g). The reaction flask was sealed and the reaction stirred for overnight. The reaction was stopped by adding 10 ml water into the flask. The product was then extracted using 30 (3 x 10 ml) ether, and the organic layers were combined. The solvent was removed under reduced pressure. The product was not purified.

Synthesis of Compound 19

Compound 18 (210 mg, 0.39 mmol), tetrahydrofuran (0.98 ml, 0.4 M), water (0.2 ml, 0.78 mmol), sodium bicarbonate (66 mg, 0.78 mmol), and iodine (199 mg, 0.78 mmol) were added to a round bottom flask with stir bar. The reaction was started at 0^0 C and reacted for 30 minutes. Sodium thiosulfate (5 ml) was added to the reaction flask to reduce iodine to iodide. The product was extracted with 30 ml (3 x 10 ml) ether. The organic layers were combined and the solvent was removed under reduced pressure.

Purification and characterization of the product

The product was chromatographed on a 2/3 full 14/20 column. The product was placed on the column with dichloromethane. The quantity of the fractions collected were 20 - 25 ml. The mobile phase regimen used was 20% ethyl acetate/ hexane (150 ml), 35% ethyl acetate/ hexane (150 ml), 50% ethyl acetate/ hexane (150 ml), 65% ethyl acetate/ hexane (150 ml), and 80% ethyl acetate/ hexane (100 ml). Fractions 16 – 23 appear to contain the desired product by TLC analysis in 45% ethyl acetate/ hexane. These fractions were collected and the solvent was removed under reduced pressure. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 7.4 (15H, m), 5.8 (1H, s), 5.62 (1H, d, J = 2.1 Hz) 5.1 (2H, d, J = 8.2 Hz) 5.0 (2H, dd, J = 4.3, 8.6 Hz) 4.7 (1H, m) 4.5 (1H,m) 4.3 (1H, m) 4.05 (1H, dt, J = 11.7, 5.2 Hz). ^{1}H-NMR (300 MHz, $CDCl_3$, D_2O (99.9% D) δ 7.4 (15H, m) 5.75 (1H, s) 5.62 (1H, s) 5.1 (2H, d, J = 8.2 Hz) 5.05 (2H, dd, J = 3.9, 8.7 Hz) 4.68 (1H, s) 4.5 (1H, dd, J = 5.8, 8.0 Hz) 4.3 (1H, m) 4.05 (1H, dt, J = 10.8, 5.7 Hz).

Primer Design

The human purL sequence was found on Genebank web site. Using the sequence the primers could be constructed. This was done with the insertion of an NdeI restriction site at the start primer (5′ end) and a EcoRV restriction site at the stop primer (3′ end). The primers were purchased from Sigma Genosys. The full length sequnce of the start primer is 5′ - CCGCCGCATATGTCCCCAGTCCTTCACTTC – 3′. The stop primer sequnce is 5′ - GCGCGCGATATCGCAGCTTCCCTCCAGGGTCCAG – 3′. The stop primer has a moderate risk of forming secondary structures.

Isolation of RNA

White blood cells were harvested form a willing lab assistant and washed with PBS. RNA will be isolated using the RNAqueous kit procedure (Ambion). Lysis/ binding solution was added to the white blood cells. This causes the white blood cells to lyse and ethanol is added to this mixture in equal volumes to prevent the RNA from precipataing out of solution. The mixture is then placed on a special filter that allows the RNA to bind to the frit. The filter is then centrifuged to allow all impurities to pass throught the filter. A regimen of wash solutions and centifugations purify the RNA that is bound to the frit. An elution solution is added to the frit and the filter tube is incubated at 70^0 C for 10 minutes, this allows the RNA to come off the frit. Then the tube is centrifuged and a clean microfuge tube collects the elutant which contains the RNA. The conformation of RNA was performed by using a RNA agarose gel from Reliant®. Ribosomal RNA was detected on gel.

Reverse Transcription Reaction

The RNA that was isolated was used as the template to synthesize cDNA. The Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase enzyme was added to transcribe the RNA into cDNA. An Oligo dT primer was added to the mixture to attach at the poly A tail to facilitate reverse transcription. Also added to the reaction mixture were dNTP's to provide nucleotides for synthesis, RNasin to eliminate any RNAses, 10x RT buffer solution, BSA, and water to give a total volume of 50 µl. The reaction was incubated at 37^0 C for one hour. The cDNA was extracted using a phenol/ chloroform extraction procedure. Rinsed precipitated cDNA with 70% ethanol/ water, and then removed solvent under reduced pressure. White pellet of presumably cDNA at visualized at the bottom of microfuge tube. Resuspended cDNA in 30 µl of dH_2O.

PCR amplification of cDNA

The cDNA that was synthesized in the RT reaction was added to a microfuge tube to be PCR amplified. The start and stop primers listed previously were added to facilitate the binding of Ex-Taq polymerase enzyme to the cDNA. Deoxynucleotide triphosphates (dNTPs) were added to provide nucleotides for the synthesis of DNA. To bring the volume of the mixture to 50 µl, water, Taq buffer, and the Ex-Taq polymerase enzyme were added. Mineral oil (50 µl) was added to the top of the reaction mixture to prevent evaporation of the reaction mixture while in the thermal cycler. The PCR reaction was placed in the thermocycler. The PCR product was analyzed by gel electrophoresis. A sample was run on a 1% agarose gel for 1 hour and visualized under UV. The human purL band was not present.

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Appendix

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GGCCGCGAGTGCATCTTCCACGAACCTAATTCATCTCTCCAGCAAAGGACACATCTCTCCAGCAAAGGACACCTCTCTCCAGCAAAGGAC
                                                                                          90

ACCTGCAGAGATGTCCCCAGTCCTTCACTTCTATGTTCGTCCCTCTGGCCATGAGGGGGCAGCCTCTGGACACACTCGGAGGAAACTGCA
                                                                                         180
          M  S  P  V  L  H  F  Y  V  R  P  S  G  H  E  G  A  A  S  G  H  T  R  R  K  L  Q
          |-------------------------------------- fgarat --------------------------------|

AGGGAAACTGCCAGAGCTGCAGGGCGTCGAGACTGAACTGTGCTACAACGTGAACTGGACAGCTGAGGCCCTCCCCAGTGCTGAGGAGAC
                                                                                         270
  G  K  L  P  E  L  Q  G  V  E  T  E  L  C  Y  N  V  N  W  T  A  E  A  L  P  S  A  E  E  T
  |-------------------------------------- fgarat --------------------------------|

AAAGAAGCTGATGTGGCTGTTTGGTTGCCCCTTACTGCTGGATGATGTTGCTCGGGAGTCCTGGCTCCTTCCTGGCTCCAATGACCTGCT
                                                                                         360
  K  K  L  M  W  L  F  G  C  P  L  L  L  D  D  V  A  R  E  S  W  L  L  P  G  S  N  D  L  L
  |-------------------------------------- fgarat --------------------------------|

GCTGGAGGTCGGGCCCAGGCTGAACTTCTCCACCCCAACATCCACCAACATCGTGTCAGTGTGCCGCGCCACTGGGCTGGGGCCTGTGGA
                                                                                         450
  L  E  V  G  P  R  L  N  F  S  T  P  T  S  T  N  I  V  S  V  C  R  A  T  G  L  G  P  V  D
  |-------------------------------------- fgarat --------------------------------|

TCGTGTGGAGACCACCCGGCGCTACCGGCTCTCGTTTGCCCACCCCCCGTCAGCTGAGGTGGAAGCCATTGCTCTGGCTACCCTGCACGA
                                                                                         540
  R  V  E  T  T  R  R  Y  R  L  S  F  A  H  P  P  S  A  E  V  E  A  I  A  L  A  T  L  H  D
  |-------------------------------------- fgarat --------------------------------|

CCGGATGACAGAGCAGCACTTCCCCCATCCCATCCAGAGTTTCTCCCCTGAGAGCATGCCGGAACCCCTCAATGGCCCTATCAATATACT
                                                                                         630
  R  M  T  E  Q  H  F  P  H  P  I  Q  S  F  S  P  E  S  M  P  E  P  L  N  G  P  I  N  I  L
  |-------------------------------------- fgarat --------------------------------|

GGGTGAGGGCCGGCTTGCGCTGGAGAAGGCCAACCAGGAGCTTGGTCTGGCTTTAGACTCTTGGGACCTAGACTTCTACACCAAGCGCTT
                                                                                         720
  G  E  G  R  L  A  L  E  K  A  N  Q  E  L  G  L  A  L  D  S  W  D  L  D  F  Y  T  K  R  F
  |-------------------------------------- fgarat --------------------------------|

CCAGGAGCTACAGCGGAACCCGAGCACTGTGGAGGCCTTTGACTTGGCGCAGTCCAATAGCGAGCACAGCCGACACTGGTTCTTCAAGGG
                                                                                         810
  Q  E  L  Q  R  N  P  S  T  V  E  A  F  D  L  A  Q  S  N  S  E  H  S  R  H  W  F  F  K  G
  |-------------------------------------- fgarat --------------------------------|

CCAGCTCCACGTGGATGGGCAGAAGCTGGTGCACTCACTGTTTGAGTCCATCATGAGCACCCAGGAATCCTCGAACCCCAACAACGTCCT
                                                                                         900
  Q  L  H  V  D  G  Q  K  L  V  H  S  L  F  E  S  I  M  S  T  Q  E  S  S  N  P  N  N  V  L
  |-------------------------------------- fgarat --------------------------------|

CAAATTCTGTGATAACAGCAGTGCAATCCAGGGAAAGGAAGTCCGATTCCTACGGCCTGAGGACCCCACACGGCCAAGCCGCTTCCAGCA
                                                                                         990
  K  F  C  D  N  S  S  A  I  Q  G  K  E  V  R  F  L  R  P  E  D  P  T  R  P  S  R  F  Q  Q
  |-------------------------------------- fgarat --------------------------------|

ACAGCAAGGGCTGAGACATGTTGTCTTCACAGCAGAGACTCACAACTTTCCCACAGGAGTATGCCCCTTTAGTGGTGCAACCACTGGCAC
                                                                                        1080
  Q  Q  G  L  R  H  V  V  F  T  A  E  T  H  N  F  P  T  G  V  C  P  F  S  G  A  T  T  G  T
  |-------------------------------------- fgarat --------------------------------|
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AGGGGGCCGGATTCGAGATGTCCAGTGCACAGGCCGCGGGGCCCACGTGGTGGCTGGCACTGCCGGCTATTGCTTTGGAAATCTGCATAT  1170
  G  G  R  I  R  D  V  Q  C  T  G  R  G  A  H  V  V  A  G  T  A  G  Y  C  F  G  N  L  H  I
                                                fgarat

TCCAGGTTACAATCTGCCCTGGGAGGATCTAAGCTTCCAGTATCCTGGGAATTTTGCCCGGCCCCTGGAGGTTGCCATTGAAGCCAGTAA  1260
  P  G  Y  N  L  P  W  E  D  L  S  F  Q  Y  P  G  N  F  A  R  P  L  E  V  A  I  E  A  S  N
                                                fgarat

TGGAGCTTCTGACTATGGCAACAAGTTTGGGGAACCAGTGCTGGCTGGCTTCGCCCGCTCCTTGGGCCTCCAGCTCCCAGACGGCCAGCG  1350
  G  A  S  D  Y  G  N  K  F  G  E  P  V  L  A  G  F  A  R  S  L  G  L  Q  L  P  D  G  Q  R
                                                fgarat

GCGTGAGTGGATCAAGCCCATCATGTTTAGTGGGGGCATTGGGTCCATGGAAGCTGACCACATAAGCAAGGAGGCCCCAGAGCCAGGCAT  1440
  R  E  W  I  K  P  I  M  F  S  G  G  I  G  S  M  E  A  D  H  I  S  K  E  A  P  E  P  G  M
                                                fgarat

GGAAGTTGTAAAGGTTGGAGGTCCCGTCTACAGGATTGGAGTTGGAGGTGGAGCTGCTTCATCTGTGCAGGTGCAGGGAGATAACACCAG  1530
  E  V  V  K  V  G  G  P  V  Y  R  I  G  V  G  G  G  A  A  S  S  V  Q  V  Q  G  D  N  T  S
                                                fgarat

TGACCTGGACTTTGGGGCTGTGCAGCGAGGAGACCCGGAGATGGAACAGAAGATGAACCGTGTGATCAGGGCTTGTGTGGAGGCCCCCAA  1620
  D  L  D  F  G  A  V  Q  R  G  D  P  E  M  E  Q  K  M  N  R  V  I  R  A  C  V  E  A  P  K
                                                fgarat

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  G  N  P  I  C  S  L  H  D  Q  G  A  G  G  N  G  N  V  L  K  E  L  S  D  P  A  G  A  I  I
                                                fgarat

TTACACCAGCCGCTTCCAGCTTGGGGACCCAACCCTGAATGCCCTGGAAATCTGGGGGGCTGAGTACCAGGAATCAAATGCTCTTCTGCT  1800
  Y  T  S  R  F  Q  L  G  D  P  T  L  N  A  L  E  I  W  G  A  E  Y  Q  E  S  N  A  L  L  L
                                                fgarat

GAGGTCCCCCAACCGGGACTTCCTGACTCATGTCAGTGCCCGTGAACGTTGCCCGGCTTGCTTCGTGGGCACCATCACTGGAGACCGGAG  1890
  R  S  P  N  R  D  F  L  T  H  V  S  A  R  E  R  C  P  A  C  F  V  G  T  I  T  G  D  R  R
                                                fgarat

AATAGTGCTGGTGGACGATCGGGAGTGTCCTGTCAGAAGAAATGGCCAGGGGGATGCCCCCCCGACACCCCCGCCAACCCCTGTGGACCT  1980
  I  V  L  V  D  D  R  E  C  P  V  R  R  N  G  Q  G  D  A  P  P  T  P  P  P  T  P  V  D  L
                                                fgarat

GGAGCTCGAATGGGTGCTGGGCAAGATGCCTCGGAAGGAGTTCTTCCTGCAGAGGAAGCCCCCCATGCTGCAGCCTCTGGCCTTGCCCCC  2070
  E  L  E  W  V  L  G  K  M  P  R  K  E  F  F  L  Q  R  K  P  P  M  L  Q  P  L  A  L  P  P
                                                fgarat
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AGGGCTGAGCGTGCACCAGGCTCTGGAGAGGGTTCTGAGGCTGCCCGCCGTGGCCAGCAAGCGCTACCTCACCAATAAGGTGGACCGCTC                2160
  G  L  S  V  H  Q  A  L  E  R  V  L  R  L  P  A  V  A  S  K  R  Y  L  T  N  K  V  D  R  S
                                                    fgarat

CGTGGGAGGCCTGGTGGCCCAGCAGCAGTGCGTGGGGCCCCTGCAAACTCCTCTGGCAGATGTAGCGGTTGTGGCACTGAGCCATGAGGA                2250
  V  G  G  L  V  A  Q  Q  Q  C  V  G  P  L  Q  T  P  L  A  D  V  A  V  V  A  L  S  H  E  E
                                                    fgarat

GCTCATAGGGGCTGCCACAGCCTTGGGAGAACAGCCAGTCAAGAGCCTGCTGGACCCAAAAGTCGCCGCCCGGCTGGCCGTGGCCGAAGC              2340
  L  I  G  A  A  T  A  L  G  E  Q  P  V  K  S  L  L  D  P  K  V  A  A  R  L  A  V  A  E  A
                                                    fgarat

CCTCACCAACCTGGTGTTTGCTCTGGTCACTGACCTCCGGGATGTGAAGTGTAGCGGGAACTGGATGTGGGCAGCCAAGCTCCCAGGGGA              2430
  L  T  N  L  V  F  A  L  V  T  D  L  R  D  V  K  C  S  G  N  W  M  W  A  A  K  L  P  G  E
                                                    fgarat

GGGCGCAGCTTTGGCGGATGCCTGTGAGGCTATGGTGGCAGTGATGGCAGCCCTGGGTGTGGCAGTGGATGGTGGCAAGGACTCCCTCAG              2520
  G  A  A  L  A  D  A  C  E  A  M  V  A  V  M  A  A  L  G  V  A  V  D  G  G  K  D  S  L  S
                                                    fgarat

CATGGCTGCTCGGGTTGGCACTGAGACCGTGCGGGCTCCTGGGTCACTGGTCATCTCAGCCTATGCCGTCTGCCCAGACATCACAGCCAC              2610
  M  A  A  R  V  G  T  E  T  V  R  A  P  G  S  L  V  I  S  A  Y  A  V  C  P  D  I  T  A  T
                                                    fgarat

TGTGACCCCAGACCTCAAGCATCCTGAAGGGAGAGGCCATCTGCTCTATGTGGCTCTGAGCCCTGGGCAGCACCGGCTCGGGGGCACAGC              2700
  V  T  P  D  L  K  H  P  E  G  R  G  H  L  L  Y  V  A  L  S  P  G  Q  H  R  L  G  G  T  A
                                                    fgarat

TCTGGCCCAGTGCTTCTCCCAGCTTGGGGAACACCCTCCAGACCTGGACCTTCCTGAGAACTTGGTGCGGGCCTTCAGCATCACTCAGGG              2790
  L  A  Q  C  F  S  Q  L  G  E  H  P  P  D  L  D  L  P  E  N  L  V  R  A  F  S  I  T  Q  G
                                                    fgarat

GCTGCTGAAAGACCGCCTCCTCTGCTCAGGCCACGATGTCAGTGACGGAGGCCTCGTCACATGCCTGCTGGAGATGGCCTTTGCTGGAAA              2880
  L  L  K  D  R  L  L  C  S  G  H  D  V  S  D  G  G  L  V  T  C  L  L  E  M  A  F  A  G  N
                                                    fgarat

TTGCGGGCTACAGGTGGATGTGCCTGTCCCCAGGGTTGATGTCCTGTCTGTGCTGTTCGCTGAGGAGCCAGGCCTCGTGCTGGAGGTGCA              2970
  C  G  L  Q  V  D  V  P  V  P  R  V  D  V  L  S  V  L  F  A  E  E  P  G  L  V  L  E  V  Q
                                                    fgarat

GGAGCCAGACCTGGCCCAGGTGCTGAAGCGTTACCGGGATGCTGGCCTCCATTGCCTGGAGCTGGGCCACACAGGCGAGGCCGGGCCCCA              3060
  E  P  D  L  A  Q  V  L  K  R  Y  R  D  A  G  L  H  C  L  E  L  G  H  T  G  E  A  G  P  H
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CGCCATGGTCCGGGTGTCAGTGAACGGGGCTGTGGTTCTGGAGGAGCCTGTTGGGGAGCTGCGAGCCCTCTGGGAGGAGACGAGTTTCCA                3150
  A  M  V  R  V  S  V  N  G  A  V  V  L  E  E  P  V  G  E  L  R  A  L  W  E  E  T  S  F  Q
                                                  fgarat

GCTGGACCGGCTACAGGCAGAGCCTCGCTGTGTGGCAGAGGAGGAACGGGGCCTGAGGGAGCGGATGGGGCCCAGCTATTGCCTGCCCCC              3240
  L  D  R  L  Q  A  E  P  R  C  V  A  E  E  E  R  G  L  R  E  R  M  G  P  S  Y  C  L  P  P
                                                  fgarat

CACCTTTCCCAAAGCCTCCGTGCCCCGTGAGCCTGGTGGTCCCAGCCCCCGAGTCGCCATCTTGCGAGAGGAGGGCAGTAATGGAGACCG              3330
  T  F  P  K  A  S  V  P  R  E  P  G  G  P  S  P  R  V  A  I  L  R  E  E  G  S  N  G  D  R
                                                  fgarat

GGAGATGGCCGATGCCTTCCACTTAGCTGGGTTTGAGGTATGGGACGTGACCATGCAGGACCTCTGCTCTGGGGCAATTGGGCTGGACAC              3420
  E  M  A  D  A  F  H  L  A  G  F  E  V  W  D  V  T  M  Q  D  L  C  S  G  A  I  G  L  D  T
                                                  fgarat

TTTCCGTGGCGTGGCCTTCGTGGGCGGCTTCAGCTATGCAGATGTCCTGGGCTCTGCCAAAGGGTGGGCAGCTGCTGTGACCTTTCATCC              3510
  F  R  G  V  A  F  V  G  G  F  S  Y  A  D  V  L  G  S  A  K  G  W  A  A  A  V  T  F  H  P
                                                  fgarat

CAGGGCTGGGGCTGAGCTGAGGCGCTTCCGGAAGCGGCCAGACACCTTCAGCCTGGGCGTGTGTAATGGCTGTCAACTGCTGGCTCTGCT              3600
  R  A  G  A  E  L  R  R  F  R  K  R  P  D  T  F  S  L  G  V  C  N  G  C  Q  L  L  A  L  L
                                                  fgarat

CGGCTGGGTGGGAGGCGACCCCAATGAGGATGCTGCAGAGATGGGCCCTGACTCCCAGCCAGCCCGGCCAGGCCTTCTGCTACGCCACAA              3690
  G  W  V  G  G  D  P  N  E  D  A  A  E  M  G  P  D  S  Q  P  A  R  P  G  L  L  L  R  H  N
                                                  fgarat

CCTGTCTGGGCGCTACGAGTCTCGCTGGGCCAGCGTGCGTGTGGGGCCTGGGCCAGCCCTGATGCTGCGAGGGATGGAGGGCGCCGTGCT              3780
  L  S  G  R  Y  E  S  R  W  A  S  V  R  V  G  P  G  P  A  L  M  L  R  G  M  E  G  A  V  L
                                                  fgarat

GCCCGTGTGGAGTGCGCACGGGGAAGGTTACGTAGCATTTTCTTCTCCGGAACTCCAAGCTCAGATTGAGGCCAGGGGCTTGGCTCCACT              3870
  P  V  W  S  A  H  G  E  G  Y  V  A  F  S  S  P  E  L  Q  A  Q  I  E  A  R  G  L  A  P  L
                                                  fgarat

GCACTGGGCTGATGATGACGGGAACCCCACAGAGCAGTACCCTCTGAATCCCAATGGGTCCCCAGGGGGCGTGGCTGGCATCTGCTCCTG              3960
  H  W  A  D  D  D  G  N  P  T  E  Q  Y  P  L  N  P  N  G  S  P  G  G  V  A  G  I  C  S  C
                                                  fgarat

TGATGGCCGCCACCTGGCTGTCATGCCTCACCCTGAGCGGGCCGTTAGGCCTTGGCAGTGGGCATGGCGACCCCCTCCATTTGATACTCT              4050
  D  G  R  H  L  A  V  M  P  H  P  E  R  A  V  R  P  W  Q  W  A  W  R  P  P  P  F  D  T  L
                                                  fgarat
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GACCACCTCCCCCTGGCTCCAGCTCTCTATCAATGCCCGAAACTGGACCCTGGAAGGGAGCTGCTGACTGGCCACAGGGGCTCACCTGGG 4140

T T S P W L Q L S I N A R N W T L E G S C .
fgarat

CCCCATGGCTTTTCACCTAAGTGGGTCCTGCCCCCTCCCCCATGACCTTCAGGAGCACCCCATATTATTTCCAAAAATATCTTGGACAGA 4230

CAAGGACCAAAATGCCAAAATCTCAGCGGACTCGATGATCTGCCTGCTGATGTTCCTTCTGTGGCTGTGTCTATTTTCAGTTCTGCTCTA 4320

ACATGGCATGCCCTTTCTCAGCCCAGGAAACAGCATGTGGTTCAGAGAAAAGAGCGACAAGGAAAAGTTAGGACTCCTGAGGTCCGAACA 4410

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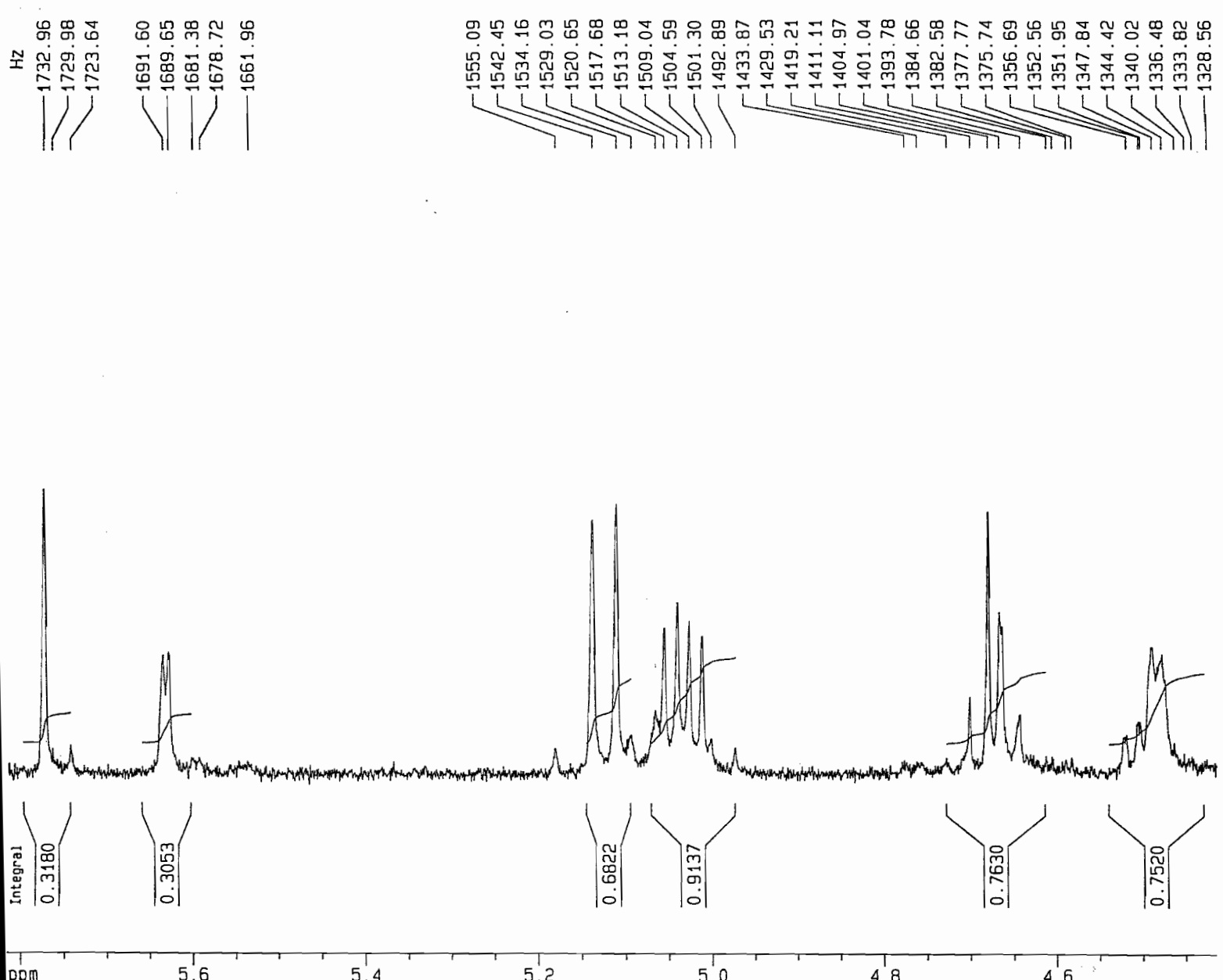


Current Data Parameters

NAME	jam28Hcr
EXPNO	1
PROCNO	1

F2 - Acquisition Parameters

Date_	20000307
Time	15.00
INSTRUM	spect
PROBHD	5 mm BBO BB-
PULPROG	zg30
TD	65536
SOLVENT	CDCl3
NS	16
DS	2
SWH	6172.839 Hz
FIDRES	0.094190 Hz
AQ	5.3084660 sec
RG	724.1
DW	81.000 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 ========

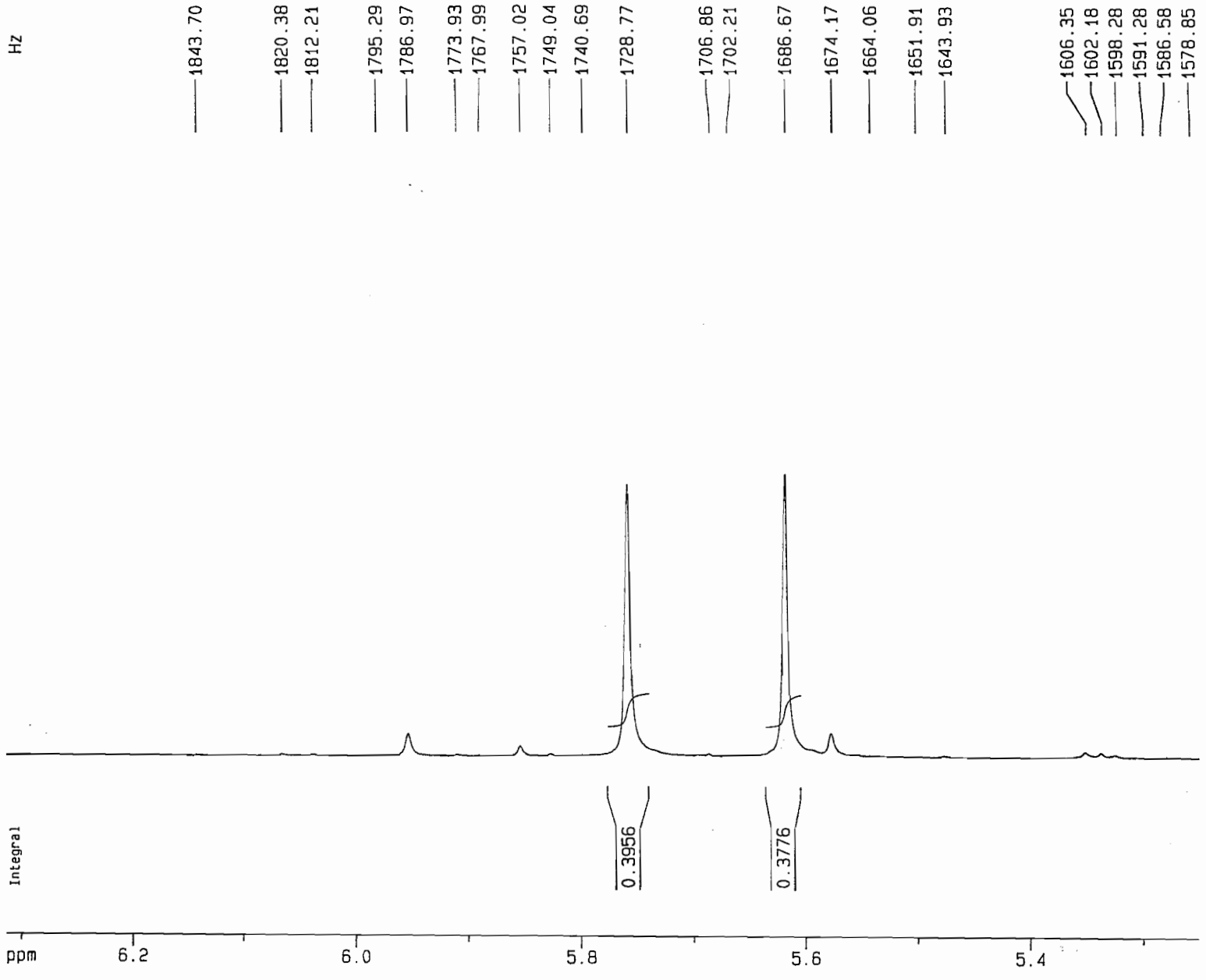
NUC1	1H
P1	11.00 usec
PL1	0.00 dB
SFO1	300.1318534 MHz

F2 - Processing parameters

SI	32768
SF	300.1300057 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
F1P	5.816 ppm
F1	1745.43 Hz
F2P	4.415 ppm
F2	1325.05 Hz
PPMCM	0.07003 ppm/cm
HZCM	21.01896 Hz/cm



Current Data Parameters

NAME	jam28H16_23D20
EXPNO	5
PROCNO	1

F2 - Acquisition Parameters

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Time	16.58
INSTRUM	spect
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PULPROG	zg30
TD	65536
SOLVENT	CDCl3
NS	16
DS	2
SWH	6172.839 Hz
FIDRES	0.094190 Hz
AQ	5.3084660 sec
RG	143.7
DW	81.000 usec
DE	6.00 usec
TE	300.0 K
D1	1.00000000 sec

========== CHANNEL f1 ========

NUC1	1H
P1	11.00 usec
PL1	0.00 dB
SFO1	300.1318534 MHz

F2 - Processing parameters

SI	32768
SF	300.1300090 MHz
WDW	no
SSB	0
LB	0.00 Hz
GB	0
PC	1.00

1D NMR plot parameters

CX	20.00 cm
F1P	6.313 ppm
F1	1894.77 Hz
F2P	5.251 ppm
F2	1576.06 Hz
PPMCM	0.05309 ppm/cm
HZCM	15.93532 Hz/cm