Preparation of Penicillins by Acylation of 6-Aminopenicillanic acid with Acyl Chlorides

Week One: Synthesis

Wear gloves during this experiment. Dissolve 1.05g of sodium bicarbonate in 12mL of acetone: water, 1:3 ratio in a 50mL round-bottom flask. Stir with magnetic stirrer until sodium bicarbonate is dissolved. Add 0.540g of 6-aminopenicillanic acid to mixture while it is stirring. Separately, prepare a solution of X g (0.00250 moles) of the acyl chloride in 1mL of acetone. Add a little more acetone if the acyl chloride does not dissolve completely after vigorous mixing. To the 6-APA solution, add dropwise the acyl chloride/acetone solution over a period of 5 minutes. By use of a magnetic stirrer, stir the resulting mixture vigorously for 40 minutes. Place dI H₂O, 5M sulfuric acid, and 6mL n-butyl acetate in separately labeled test tubes and cool in ice bath. After 40 minutes, place reaction mixture in a 30mL separatory funnel. Extract the reaction mixture twice with 6mL portions of room temperature n-butyl acetate, the organic n-butyl acetate extracts being set aside. Place the remaining aqueous solution into a 50mL beaker and cover with the 6mL of cold n-butyl acetate. Cautiously acidify to pH of 2.0, using the cold 5M sulfuric acid. Separate the n-butyl acetate and wash it with cold water. Remove most of the water with a glass pipette, and dry the remaining solution for 10 minutes over anhydrous sodium sulfate. Remove drying agent by filtration. Place filtrate in a 50mL beaker and add 1mL of a 50% solution of potassium 2-ethylhexanoate in butanol. If crystallization of the potassium salt does not spontaneously occur within a few minutes, cool the solution in an ice bath. By filtration, collect the crystalline material and wash it on the filter with a little dry acetone. If crystallization does not occur after
cooling in an ice bath, label a beaker with the lab section and your name. Place the beaker carefully in a desiccator for inspection next week. If crystallization did occur, press between filter paper to dry. Place in a vial labeled with lab section letter, your initials, and the acyl chloride number.

Questions

1. Write out a mechanism for the acylation of 6-APA with your acyl chloride.

2. During the first two extractions, what is it that is being removed from the solution? Explain.

3. What is the purpose of the acidification step?

4. What is in each phase after the 3rd extraction? Explain.

Hand in this lab report at the beginning of next week, before you start the second part of this lab.
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Week Two: Biological Assay

Before coming to lab, decide on concentrations to test between the ranges of 1000µg/mL to 10µg/mL. A minimum of three concentrations (high, medium, and low) must be selected, but feel free to test more concentrations to get a better range. Email your lab TA how many concentrations you are planning on doing two days prior to your lab. Calculate how to prepare these concentrations (even if your reaction did not produce crystals) before coming to the lab, and have your calculations ready.

Preparing bacteria and antibiotic solutions
Retrieve crystals synthesized from last week. If your experiment did not result in initial crystal formation, but crystals have now formed, then perform filtration and dry crystals between filter paper, then follow this week’s procedure. If your synthesis resulted in crystals last week, determine the percent yield and melting point range. Obtain a vial and label it with lab section and initials. Make a 100mg/mL stock solution by dissolving 0.1000g of the crystal in 1mL of dI water. Dilute this to your selected concentrations, remember that the penicillins will be further diluted when mixed with the bacteria; therefore you need to adjust your dilutions accordingly (see below for calculation assistance). Prepare one culture tube for each concentration you are planning to test. Label culture test tubes with the concentrations, the lab section, your initials, and the acyl chloride number. Dilute stock bacteria one to ten in bacterial broth before use. Pipet 1mL of bacterial broth in each culture tube, and subsequently add 100µL of your penicillin to each tube. Lastly, add 300µL of diluted stock bacteria. One group needs to create a blank. This test tube will have 1mL of bacterial broth, 100µL dI water, and 300µL of stock bacteria. Place your tubes in an incubator at 37ºC overnight. Tomorrow’s lab section will test the optical density for you.

Testing Optical Density
You will be responsible for testing the optical density of the lab section that is held the day before your lab. To do this, follow the procedure for the Ocean Optics USB2000 Spectrometer with Vernier Logger Pro. If this is not available, use a UV/Vis spectrometer or a Spec 20. Use the bacterial broth as your blank when calibrating the instrument. Set the instrument to measure absorption at 600 nm. Measure the optical density of the bacterial broth at each concentration of antibiotic three times and use the average of these results to make a graph. Remember to also measure the bacterial broth that did not contain any penicillin, only dl H2O. Calculate the IC50 for your penicillin. Submit your results.

**Preparing a Bacterial Stock**

You will be responsible for preparing a bacterial stock for the lab section that is held the day after yours. To create a stock, label a culture test tube as stock with the date and type of bacteria and fill it with 3mL of the bacterial broth. Work close to the flame from a Bunsen burner when handling the bacteria. This will prevent the bacteria you are working with to create aerosols as well as preventing your bacterial stock from being contaminated by other bacteria. Use bacteria from the stock plate and fill the yellow loop with bacteria. Place the loop with the bacteria in the test tube with the broth and stir gently until the bacterial matter is completely released from the loop. Make sure the bacteria does not end up on the walls of the culture tube. Place loop in an autoclave bag. Put stock bacterial broth in an incubator at 37°C and leave over night. Each lab group will need 300 µL of diluted bacterial broth for each of the concentrations they are planning to test. Your TA will know how many concentrations that will be tested, and how many group there are in the next lab section. If more than 30 mL of diluted stock solution is needed for the lab section; prepare two (or more) culture tubes.
Guidelines:

*Calculating penicillin Concentrations.*

(highest concentration in µg/mL) * 14 = \( C_2 \)

\[ C_1V_1 = C_2V_2 \]

where \( C_1 \) is the stock concentration (100,000 µg/mL)

Example:

Say you want your highest concentration to be 1000 µg/mL and you want 2mL of it. The antibiotic solution is diluted 1:14 when added to the bacterial broth, therefore you need to make a solution that is 14 times more concentrated (14 000 µg/mL)

\[ (1000 \, \mu g/mL)(14) = 14000 \, \mu g/mL \]

\[ V_1 = \frac{(14000 \, \mu g/mL)(2mL)}{100,000 \, \mu g/mL} = 0.28mL \]

\[ 2mL - 0.28mL = 1.72mL \]

The calculations show that you need to add 280µL of your 100,000 µg/mL stock solution to 1720µL of dI water to get 2mL of a 14000 µg/mL solution. When this antibiotic solution is added to the broth and bacteria, it will have a final concentration of 1000µg/mL.

*Calculating the IC\(_{50}\) from Optical densities (OD)*

To calculate the IC\(_{50}\), subtract the lowest OD from the highest OD (should be the non-inhibited blank) and divide by 2. This gives you the OD at which 50% of the bacteria are inhibited from growing (the IC\(_{50}\) OD). Look on your graph and find the two concentrations that resulted in ODS closest to the IC\(_{50}\) OD. There should be one concentration with OD above the IC\(_{50}\) OD, and one OD below the IC\(_{50}\) OD. As an example, these concentrations could be 100 µg/mL and 20 µg/mL. Subtract the optical densities of these two and divide by the increment between these numbers (100-20 = 80 in this case). This number is the increase between each single concentration between 20 and 100 assuming the values are linear. In excel, make a list from 100 to 20. Then next
to each calculate the theoretical OD at that concentration. For example, you know the optical density for 100 µg/mL. The optical density for 99 µg/mL would be that of 100 µg/mL + the incremental increase you calculated above. The optical density for 98 µg/mL would be that of 99 µg/mL + the incremental increase. The concentration that has an optical density closest to the calculated IC$_{50}$ OD will be your IC$_{50}$. Submit this in a 5 unit range (i.e IC$_{50}$ = 80-85).

When the IC$_{50}$ for all penicillins in every lab group have been calculated, these results will be distributed, and you should make a list ranking the penicillins from most effective to least effective. You should also discuss in your lab report if there are any relationship between acyl chloride structure and effectiveness of antibiotic.