THESIS

BIOLOGICALLY ACTIVE AROMATIC ACIDS IN PHOSPHATIDYLCHOLINE LIPOSOMES: BENZOIC AND SALICYLIC ACIDS

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ABSTRACT

BIOLOGICALLY ACTIVE AROMATIC ACIDS IN PHOSPHATIDYLCHOLINE LIPOSOMES: BENZOIC AND SALICYLIC ACIDS

The interactions of benzoic acid and salicylic acid with phosphatidylcholine liposomes were characterized to understand interfacial interactions of the two weak aromatic acids with the membrane. The liposomal system was comprised of soy l-α-phosphatidylcholine (SPC) bilayers, which allowed the determination of interfacial interactions and position within the membrane using 1D ^{1}H NMR. Benzoic acid was considered due to its effects as a food stabilizer, where salicylic acid was considered as a derivative due to its effects as an anti-acne agent. Both were found to penetrate the membrane interface deeper when in their protonated forms. The presence of the weak acids on the membrane surface allowed stabilization through hydrogen bonding with liposomal headgroups, which allowed deprotonation to occur. Broadening of aromatic peaks demonstrated a pH dependence for both benzoic acid and salicylic acid, showing a deeper penetration around the pKa values of the weak acids. This study offers justification for the antimicrobial activity of benzoic and salicylic acids in lower pH environments. Thus, this study provides the next piece in understanding the uptake of benzoic acid and salicylic acid in bacteria for microbial inhibition.

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Chapter 1: Introduction

Food and beverage spoilage is a major concern for industrial production and human consumption worldwide.[1,2,3] Benzoic Acid (HB) is commonly used to prevent the spoilage of food caused by bacteria, such as yeast,[4,5] and eukaryotic organisms.[6,7] HB is one of many weak aromatic acids, which are powerful antimicrobial agents for food preservation in an acidic environment.[6, 7] Additionally, HB becomes less effective if the pH of the food is far from the pK_a of HB,[6] which is why HB is primarily used in foods around a 4.0 pH.

Other weak aromatic acids, such as salicylic acid (H_2SAL), have shown similar inhibitory effects on bacteria.[8] H_2SAL is a derivative of HB that has established antimicrobial properties.[9,10,11] Commonly, H_2SAL is used in conjunction with other weak acids for topical creams as the pK_a of H_2SAL is acidic enough to cause stomach ulcers.[7,12] For example, H_2SAL has been paired with HB for use in antifungal creams.[7] H_2SAL compared to HB has strong inhibitory effects with gram-negative membranes as observed with E. coli.[10,13,14] Both HB and H_2SAL are more active against Gram-positive than Gram-negative membranes due to the prior having a single lipid bilayer and the latter having two.[7,15] This is the result of the inability of the anionic forms to cross membranes, where Gram-negative membranes can more easily prevent HB and H_2SAL from crossing into the interior cytosol. H_2SAL is better able to prevent growth on Gram-negative membranes than HB due to its structural differences. H_2SAL was thus considered for its ortho positioned phenol group that acts as an electron withdrawing group.

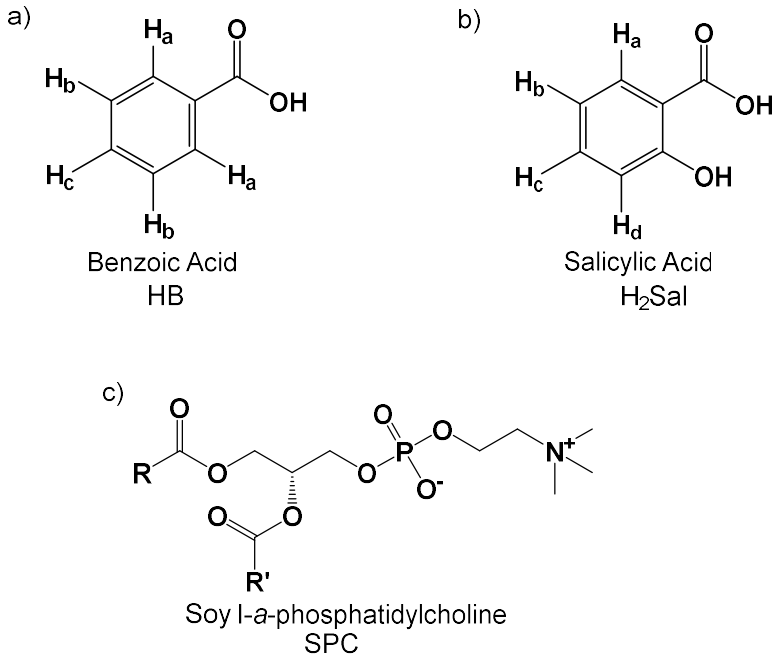

Figure 1: Structure of (a) benzoic acid (HB), (b) salicylic acid (H_2SAL), and (c) l-α-phosphatidylcholine from soy (SPC) with labeled aromatic protons on HB and H_2SAL. R and R′ represent 16:0, 18:0, and 18:1 carbon chains.

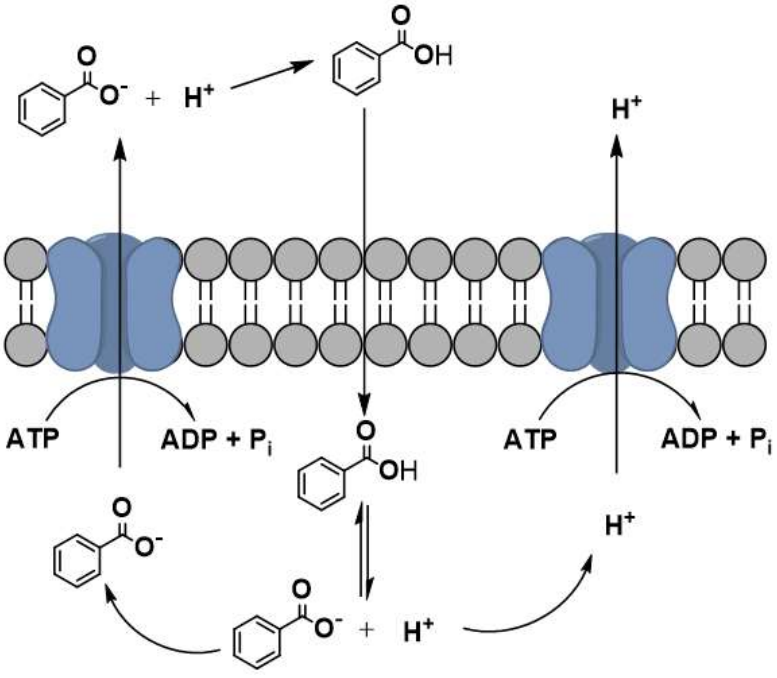

Figure 2: The "Futile Cell" as described by Piper et al. for yeast (S. cerevisiae) with the weak acid HB. Passive intake trumping facilitated export of H+ and B- anions. H+, ATP, and ADP + Pi respectively represent a free proton, adenosine triphosphate, and adenosine diphosphate.[16]

Benzoic acid is thought to be more active in acidic conditions and more permeable than its anionic counterpart.[6,7] Inside the cell HB dissociates into its anionic form (benzoate) and a free proton that effectively lowers the cytosol pH.[17,13] Decreased pH inside the cell and an accumulation of benzoate result in the need for the cell to pump protons and benzoate molecules back across the membrane. Transporter Pdr12 regulates the release of carboxylic anions out of the cell while ATPase Pma1 also pumps excess protons out of the cell.[18,19] Extracellular anions and protons reform HB, and the process is repeated until it reaches equilibrium. An equilibrium is established to the point when HB enters the cell faster than the cell can export anions and protons, being described as a futile cell as seen in Figure 2.[20,21] This proposed mechanism effectively describes the ability of HB as an uncoupler to inhibit bacterial growth within an acidic environment and can be applied to other uncoupling compounds.[22,23,24]

In order to expand our understanding of the inhibition process of HB, mimetic studies were proposed to study liposome systems *in* vitro. While there are numerous types of lipids in both eukaryotic and prokaryotic cell environments that can be used in mimetic studies, the selection of appropriate lipids becomes important in accurate modeling of the desired biological system. Many lipids found in the endothelial cells lining the primary contact of food comprise phosphatidylcholine, a two-tail lipid bilayer.[25] It is desirable to mimic the natural environment of these cells to best identify noticeable trends between small molecules and their active environments. Previous studies have looked at the interactions of benzoic acid in reverse micellar and Langmuir monolayer environments, which provided the basis for this study.[6] These environments are comprised solely of a lipid monolayer, and while these are useful in

characterizing lipid interactions, they lack the length and organization of a true bilayer.[26,27] For this reason, a liposome is the next step for understanding fundamental interactions of HB in vivo.

This study is focused on the characterization of benzoic and salicylic acids with SPC unilamellar vesicles in the hope of establishing: (i) the mechanism by which HB and H_2SAL interact with the membrane (ii) the effect of HB and H_2SAL at different pH values and (iii) the inhibitory effects of HB and H_2SAL in model membrane systems.

Chapter 2: Materials and Methods

General Materials and Methods: The reagents were purchased and used without further purification unless otherwise specified. Benzoic acid (abbreviated as HB, Sigma-Aldrich ≥ 99.5%), salicylic acid (abbreviated as H_2SAL, Sigma-Aldrich ≥ 99.0%), deuterium oxide (abbreviated as D_2O, Sigma-Aldrich 99.0%), Soybean L-a-phosphatidylcholine (abbreviated as SPC, Sigma-Aldrich), 3-(trimethylsilyl)-propane-1-sulfonic acid (abbreviate as DSS, Wolmad), and chloroform (Cambridge Isotope) were used as received.

The pH measurements were all completed using a sympHony pH meter with a VWR semimicro pH probe. The pH measurements were adjusted in the presence of deuterium (pH + 0.4 = pD).[6, 28] Commonly pD is referred to as pH and so this paper will refer to pD measurements as pH.[6, 28] The NMR measurements were conducted using a 400 MHz Bruker NMR spectrometer with deuterium oxide solvent and the spectra were referenced using an internal DSS standard. Dynamic light scattering (DLS) experiments were performed using a Zetasizer nano-ZS instrument using default parameters at 25 ˚C. Liposomes were prepared using thin-film hydration techniques followed by hand extrusion using an Avanti mini-extruder with a 13 mm polycarbonate filter with 0.1 µm pore size.

Preparation of SPC Liposomes with D_2O Solutions for NMR Experiments: To prepare the lipid bilayer, SPC lipids (10.0 mg, 0.013 mmoles) were dissolved in a minimal amount of chloroform. Excess chloroform was removed *in vacuo*, leaving a thin film of lipids on the round bottom flask. The lipids were then hydrated by adding HB or H_2SAL (10.0 mmol, 1.00 mL) in deuterium oxide (D_2O) by heating the solution to 40 ˚C form multilamellar vesicles.[29] The transition temperature

of the lipid is 35 ˚C.[30] Homogeneity of vesicles was achieved in the suspension using sonication and extrusion to form unilamellar vesicles of a specific size using a hand extruder with a pore size of 0.1 µm.[30,31] The reported pH of each sample was determined from the D_2O solutions of HB or H_2SAL prior to the addition to the round bottom with the lipid film.

Preparation of Stock Solutions for NMR: The 10 mM stock solutions were prepared by adding HB (6.11 mg) and H_2SAL (6.91 mg) solid compound in 5.00 mL of D_2O. The suspensions were sonicated until clear and no visible particles remained. The pH was adjusted using 1.0 M solutions of either DCl or NaOD.

^{1}H NMR Experiments on Liposomes and Standard D_2O Samples: The ^{1}H NMR studies were done at 400 MHz with N = 32 and routine parameters. All samples are referenced with regard to DSS, which were added as an internal standard after the ^{1}H NMR spectrum was recorded of the sample. The data was processed using Mestre Nova NMR processing software (version 14.1.2). The pK_a was determined from the titration curve by plotting chemical shifts at varying pH values.

^{1}H – ^{1}H 2D NOESY NMR Experiments on Liposomes: 2D NMR studies were carried out at 400 MHz at 25 ˚C. A standard NOESY pulse sequence was used of 16 scans in the f1 domain using a 45˚ pulse angle and a 2.0 s relaxation decay. The data was also processed using Mestre Nova NMR processing software (version 14.1.2).

DLS Experiments: DLS analysis was used to confirm the formation of LUVs of the desired size. Experiments were performed using a Zetasizer nano-ZS instrument to confirm liposome formation. Vesicle sizes were analyzed and confirmed using Zetasizer software.

Chapter 3: Results

NMR Spectra of HB in Aqueous Environment: The spectra of HB in D_2O were found for the pH range of 2.35 – 9.48, generating the titration curve seen in Figure 3. There is an upfield shift between the pH values of 3.27 – 6.06 for the aromatic protons (-0.15 ppm (H_c), -0.08 (H_b), and -0.19 (H_a)). Outside of this range the aromatic protons do not shift for the acidic/basic forms of HB. Above 6.06 pH all of HB has been deprotonated. Using the chemical shifting for H_a, the pK_a was found to be 4.57. This is in agreement with a reported pK_a 4.65 of HB in D_2O.[32]

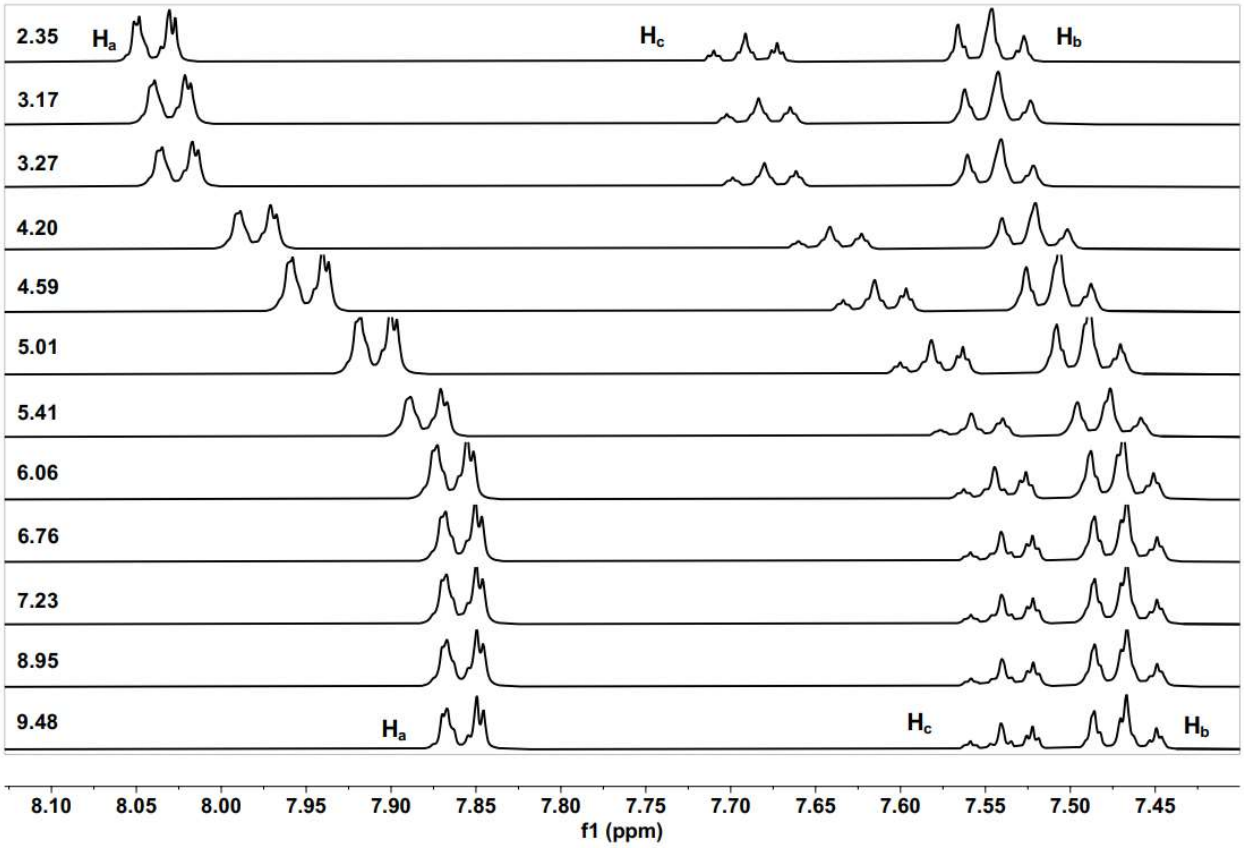

Figure 3: 1D ^1H NMR of 10 mM HB in D_2O. The generated titration curve is consistent with acid deprotonation. Aromatic protons are labeled according to Figure 1.

1D ^{1}H NMR Spectroscopy of HB in SPC Liposomes: Benzoic and salicylic acids were characterized in both aqueous and liposomal environments with 1D ^{1}H NMR spectroscopy. Aqueous environments were used the basis to assign aromatic proton peaks for each compound in their liposomal environments. Theoretical peak shifts were determined using $CDCl_3$ as a solvent and were considered when assigning peak labels. No peak overlap was observed for the acids and the liposome.

HB has been previously studied in micellar, reverse micellar and monolayer environments.[6,33] It has been previously seen for hydroxybenzoic acid derivatives that upfield shifts, when comparing aqueous to reverse micelle environments, are consistent with proton location in the palisade layer of a micelle, (the hydrophobic tail region).[6,33] Downfield shifts place the proton in the Stern layer, (the area between polar headgroups and D_2O pools).[6,33] Consistent data was shown for HB in reverse micelle environments by placing the carboxylic acid in the Stern layer and the para proton to the carboxylic acid deepest in the palisade layer (in the acyl chains).[6]

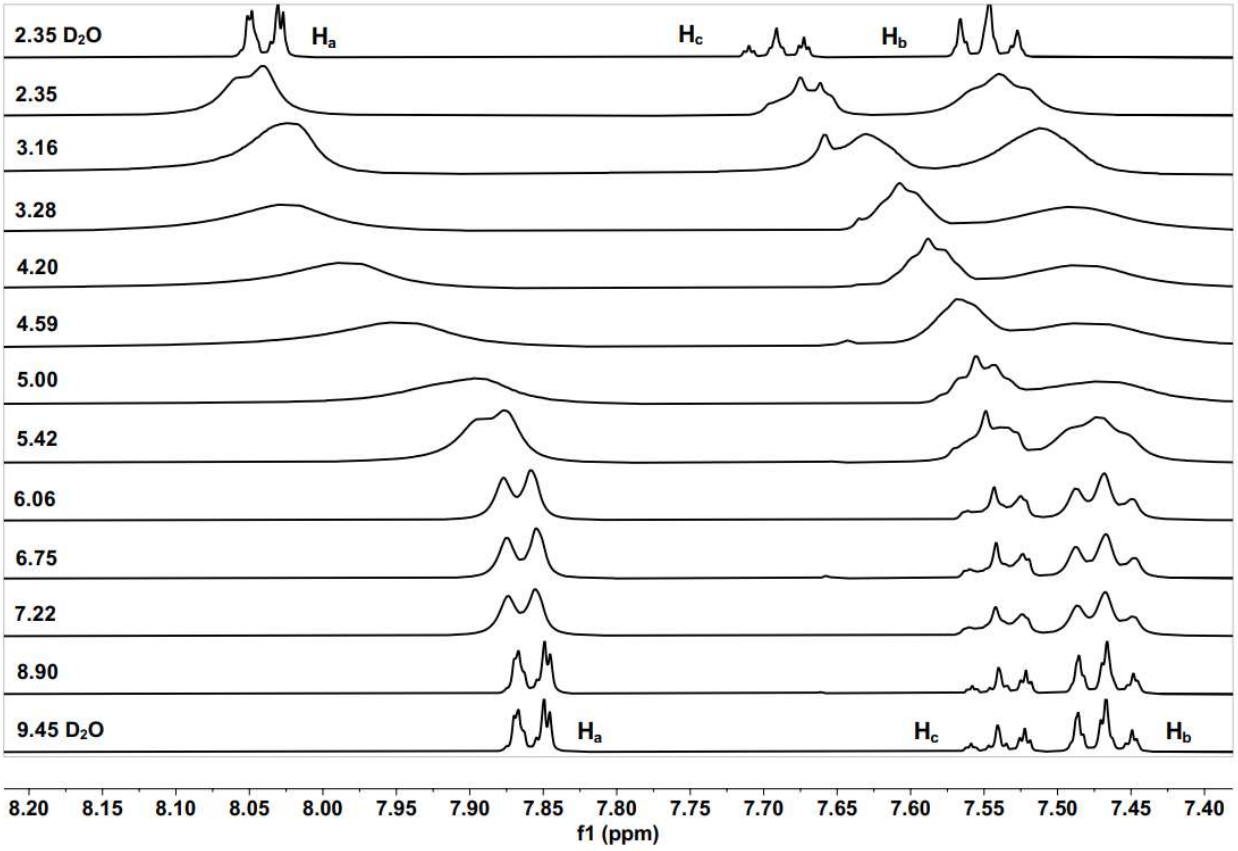

Figure 4: 1D ^{1}H NMR stacked spectra of HB in 0.1 µm SPC liposomes. The top and bottom are D_2O samples for reference.

After peak assignments of HB were done, comparisons were made between the spectra. Significant peak broadening was seen in Figure 4 for the pH region of 2.35 to 5.42. The broadening made it difficult to see major shifts, but they were visible the most in the 5.42 spectrum. An upfield shift was more pronounced for this part of the titration curve where broadening also occurred. This gave a preliminary analysis that HB was more incorporated into the membrane in this pH range (before most of the HB formed B^- anions). The protons followed a similar titration curve to what was seen in the aqueous environment. Figure S1 shows the different shifts when comparing aqueous and liposomal environments, where protons H_a and H_c are shown to have the greatest difference. Using chemical shifting for H_a, the pK_a of HB was found to be 4.37 for the

liposomal environment. The difference in pK_a, when compared to D_2O (-0.27), is the result of liposomal interactions which will be discussed further. Table 1 below shows pK_a values for aromatic protons in different environments.

Table 1. Determined pK_a values for aromatic protons of benzoic acid in aqueous and liposomal environments as compared to literature.

Solvent / Environment	H_a	H_c	H_b	Average pK_a	Source
H_2O		4.21		4.21	34
D_2O		4.65		4.65	32
MeOH		9.41		9.41	34
EtOH		10.25		10.25	34
AOT Reverse Micelle w_o 8	3.7	-	1.4	2.6	6
AOT Reverse Micelle w_o 12	4.1	3.7	3.2	3.7	6
AOT Reverse Micelle w_o 16	4.1	4.0	4.0	4.0	6
AOT Reverse Micelle w_o 20	4.0	4.0	4.0	4.0	6
D_2O	4.57 ±0.04	4.47 ±0.04	4.60 ±0.04	4.55 ±0.02	Experimental
SPC Liposomes	4.37 ±0.04	4.41 ±0.04	4.51 ±0.04	4.43 ±0.02	Experimental

2D ^{1}H-^{1}H NMR Spectra of HB in SPC Liposomes: 2D NMR was run on HB in SPC liposomes at three pH points where the protonation state of HB was expected to be majorly protonated, deprotonated, or between the two.

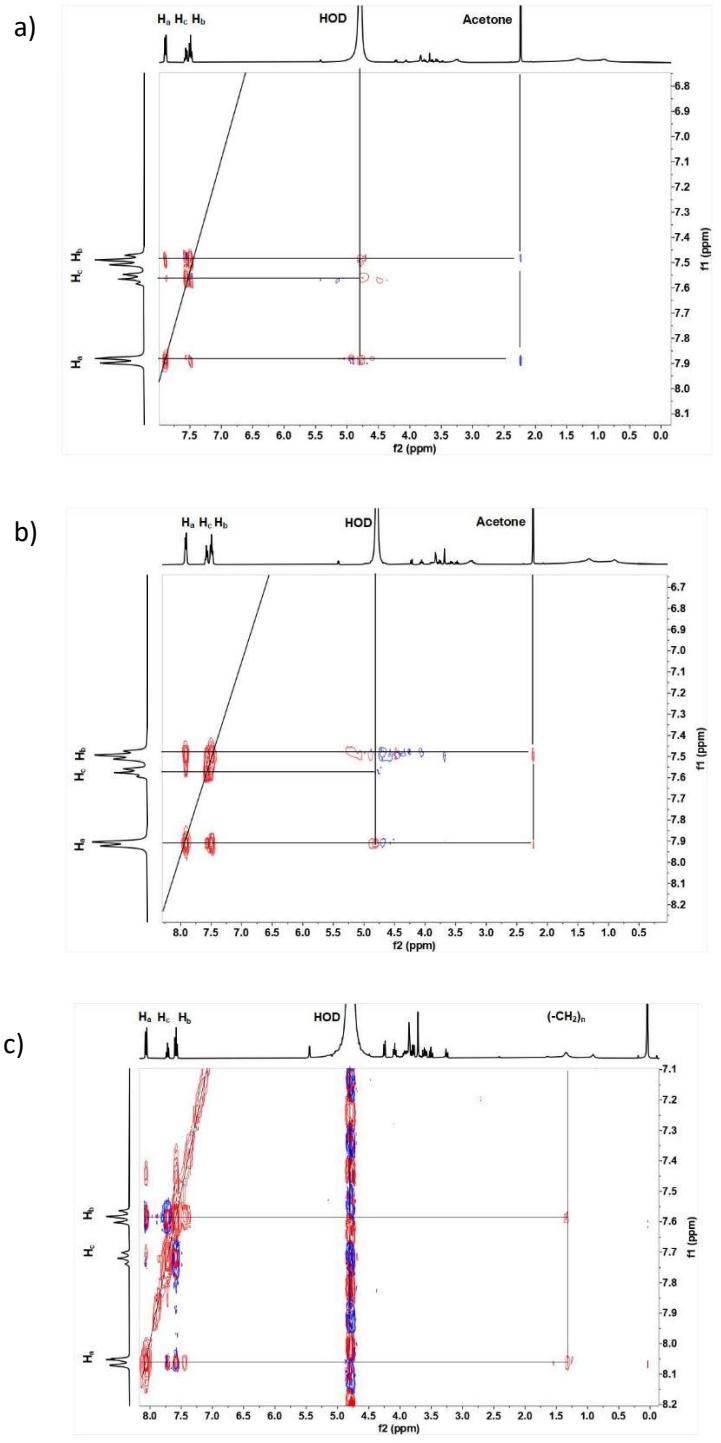

Figure 5: 2D ^{1}H-^{1}H NMR spectra of 10 mM HB in SPC liposomes at pH values of a) 7.18 b) 5.40 and c) 2.54. All spectra were run using a standard ^{1}H-^{1}H NOSEY NMR pulse sequence and spectra were processed using a sine weighting function where cross peaks and the diagonal are shown with lines on the spectra. Impotant peaks are labeled according to Figure 1 for the structure of HB.

NMR Spectra of H_2SAL in Aqueous Environment: The spectra of H_2SAL in aqueous were found for the pH range of 1.65 – 8.44 as seen in Figure 6. The generated curve is consistent for an acid titration curve. Aromatic protons H_b and H_d are more indistinguishable from each other as the solution approaches a basic pH. A noticeable downfield shift is seen for all the aromatic protons with the largest shifts for H_a and H_c (-0.12 ppm for each). These results can be interpreted that there was deprotonation on the carboxylic acid above pH 3.53. Once the pH went above 4.27, it is expected that all of the H_2SAL was deprotonated. Using chemical shifting for H_a, the pK_a of H_2SAL was found to be 3.39, which is in agreement with a reported pK_a of 3.46 SH in D_2O.[35]

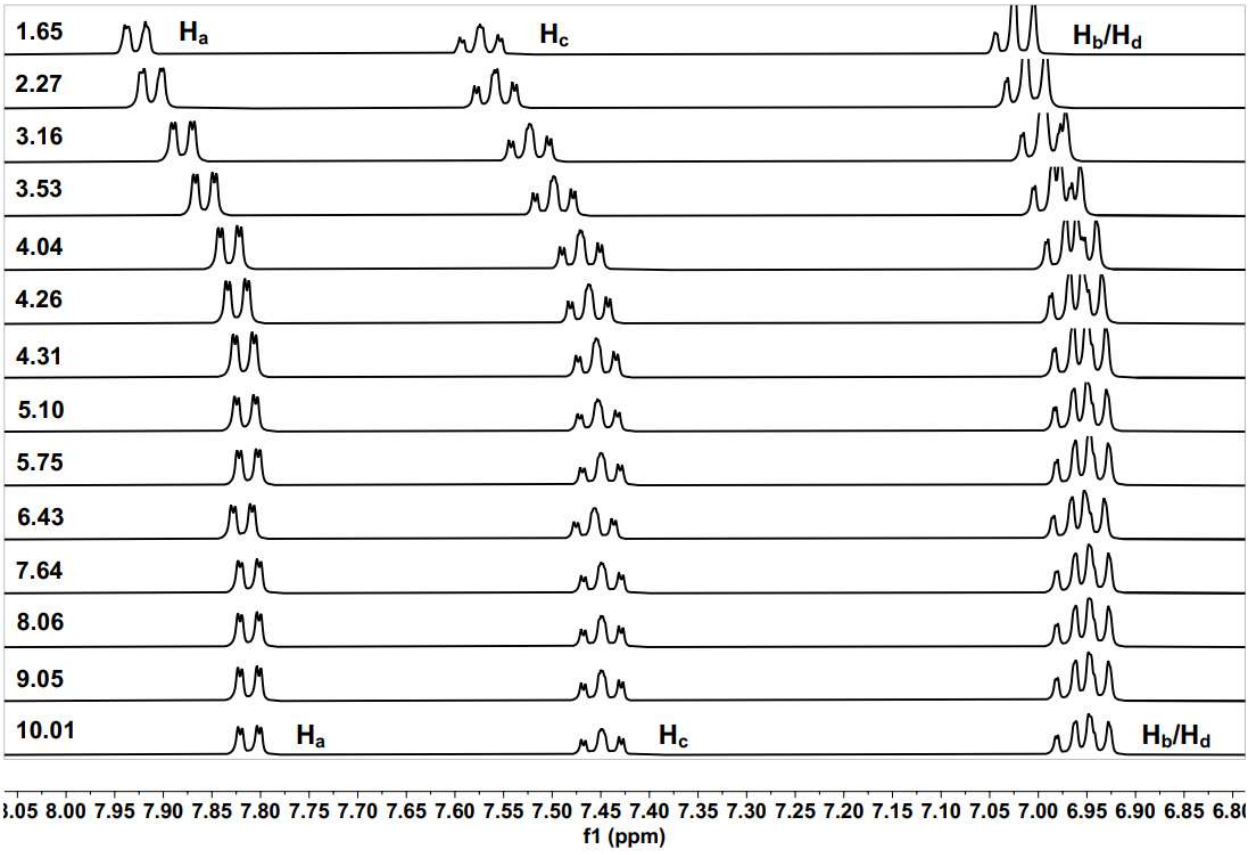

Figure 6: Titration curve of H_2SAL in D_2O. Aromatic protons are labeled according to Figure 1.

NMR Spectra of H_2SAL in SPC Liposomes: The spectra of H_2SAL in liposomal environments yield the titration curve shown in Figure 7. A similar broadening pattern as seen for HB was observed between the pH range from 2.27 to 4.69 with proton H_a broadening the most. There was a consistent upfield shift in the broadened range seen below in Figure 6.

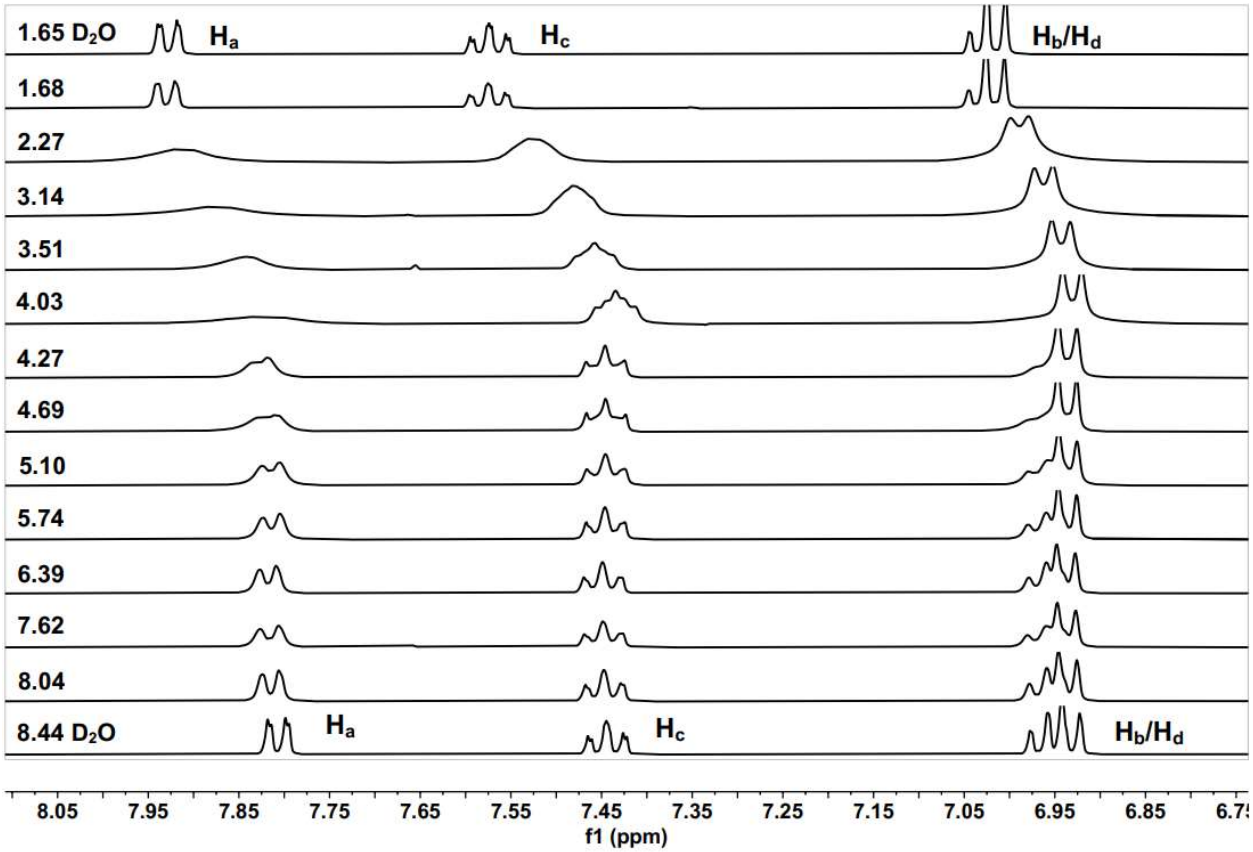

Figure 7: Stacked spectra of H_2SAL in 0.1 µm SPC liposomes. Aromatic protons are labeled according to Figure 1.

It has been previously established that the presence of H_2SAL on phosphatidylcholine lipids in an aqueous environment resulted in significant aromatic peak broadening.[36] Using the chemical shifts of H_a, the pK_a of H_2SAL was found to be 2.87 for the liposomal environment. The lowered pK_a for the liposomal environment (-0.52) is due to interactions of H_2SAL with the liposome. The low pK_a of H_2SAL is what makes it most suitable as a preservative in topical creams such as acne

treatment.[37] Table 2 below shows calculated pK_a values for all aromatic protons comparing to literature.

Table 2: Determined pK_a values for aromatic protons of salicylic acid in aqueous and liposomal environments as compared to literature.

Solvent / Environment	H_a	H_c	H_b/H_d	Average pK_a	Source
H_2O	2.97			2.97	38
D_2O	3.46			3.46	35
D_2O	3.39 ±0.01	3.36 ±0.01	3.39 ±0.01	3.38 ±0.01	Experimental
SPC Liposomes in D_2O	2.87 ±0.01	2.84 ±0.01	2.87 ±0.01	2.86 ±0.01	Experimental

DLS Measurements of HB in SPC Liposomes: Further characterization of the interactions of HB with liposomes was done using dynamic light scattering (DLS) measurements to confirm the formation of liposomes. Measurements confirmed that liposomes were formed with an average size of 0.1 µm. It has previously been mentioned that HB with DPPC did not affect the size of reverse micelles.[6] Our data shows consistent results with an average liposome size of 0.17 µm.

Chapter 4: Discussion:

HB Interactions with Liposomes: Reverse micelles are monolayers formed around water pools in organic solvents, where hydrophilic heads face the water pool and the hydrophobic tails face outward towards organic solvent. These systems provide useful information for understanding membrane interactions but are lacking in their comparison to *in vivo* studies. The monolayer nature of reverse micelles is roughly half the bilayer diameter of a typical liposome, causing interactions to be different than in liposomes. It has been previously noted with HB and some other systems in reverse micelles that upfield shifts when comparing to aqueous NMR data were consistent with molecular placement in the aliphatic chains of AOT.[6,33,39] The organic solvent also plays a role in the interface interactions and can effect peak shifting depending on the location and interactions with the solute. In contrast, it is possible for a hydrophobic molecule to be trapped at the midplane of a bilayer in liposomes, where the water exists both inside and outside. Therefore, the monolayer reverse micelle system is fundamentally different than the bilayer liposome system.

In a liposome preparation, when solute is added it ends up both inside the liposome and outside in the bulk aqueous solution. The liposomes prepared can be represented as spherical particles suspended in an aqueous solution. When an NMR spectrum is recorded of a solute within a liposome, the solute inside the liposome has an upfield shift when comparing to bulk solution.[40,41] This has been attributed to the difference in susceptibility between the solute inside the liposome versus outside. The magnetic susceptibility is related to the sample's geometry, when the magnetic field applied is applied to the sample it is affected by the molecule's shape and size.[40,41] These effects imply, for our system, that upfield shifts when comparing the aqueous and

liposomal environments correspond to molecular placement inside the membrane.[41] Each aromatic proton experienced an upfield shift (-0.05 ppm average), suggesting HB resides primarily inside the liposomal membrane. Its anionic form likely cannot cross the liposome due to the charge formed by deprotonation of the carboxylic acid. Thus, the deprotonated form of HB is limited to the aqueous environment inside/outside of the liposome. Major peak shifting (-0.05 ppm) from acidic to basic pH is in agreement with the aqueous titration curve.[6]

Peak shifting can potentially be caused by dimerization. Dimerization of HB, due to intermolecular hydrogen bonding of carboxylic acids, has previously been reported in organic solvents.[6,42] While it is possible for HB to form dimers, it is unlikely that liposomes contain HB with a high affinity to bind with itself. The comparison of HB in isooctane, where it would be likely to form dimers, showed a different shift of protons H_a and H_b, suggesting that HB is a monomer in the liposomal state.[6] Thus, it is reasonable to conclude dimerization is not relevant to our system.

The pK_a of HB in liposomes is closer to the pK_a in an aqueous environment than in reverse micelles. This can be attributed to the size of the two systems and the nature of NMR. The average size of the prepared liposomes was 150 nm (0.15 µm), which is ~35 times larger than the w_o 16 reverse micelle. Since NMR is an average of signals within a system, and the liposomes are prepared in an aqueous solvent, it is likely that HB in liposomes would show a pK_a similar to when it is in an aqueous environment. This is consistent with reverse micelles having only a few HB molecules in the water pools, where the average NMR signal would be less affected by water.[6]

In D_2O, the pK_a of HB is 4.65, which is 0.44 above the pK_a of HB in H_2O.[7,34] Using the conversion from pH to pD, this difference is to be expected. The determined pK_a of HB was 4.57 ±0.04 using H_a, 4.47 ±0.04 using H_c, and 4.60 ±0.04 for H_b, all being within reasonable error to be comparable to literature. As well, MeOH and EtOH have pK_a values significantly larger than other solvents. This is consistent with the change in environment and is to be expected for organic solvents.

The pK_a in the liposomal environment is significantly different when compared to the aqueous environment, where H_b is significantly different than both H_a and H_c. Both H_a and H_c are more sensitive to the deprotonation of the carboxylic acid as a result of ortho and para resonance effects. These results suggest that overall the liposomal system favors the deprotonation of HB more, causing the lower pK_a, where the difference in pK_a values for aromatic protons can be attributed to relative position to the carboxylic acid. HB is a member of uncouplers, (compounds that can alter the pH gradient across cell membranes that leads to a changed cytosol pH).[6,22,23] The data presented agree with HB's ability to alter cytosol pH, where a reduced pK_a could be attributed to the ability of HB to lower cytosol pH.

While the pK_a gives good insight on the location of HB, it is not enough to state for certain where HB is located in the liposome. Because of this, peak broadening was also evaluated to further understand the membrane interactions with HB. Peak broadening in the pH range 2.35 to 5.42 suggests that HB is able to penetrate further into the liposome, which is consistent with the active pH range for HB.[7] Since liposomes are sensitive to their environments, it is likely that the presence of HB on the liposome allows hydrogen bonding, stabilizing its ionization states.[38] Peak broadening in this region suggest that HB is interacting with the phospholipid headgroups, where it is more favorable to exist in its anionic form. This is supported by the lowered pK_a values in

liposomal environments,[43] which show HB is readily able to donate a proton. The significant broadening on protons H_a and H_b suggest that HB enters the lipid with the aromatic portion first, meaning that they reside deeper in the membrane. This is consistent with hydrogen bonding occurring on the carboxylic acid, which suggests that the aromatic portion is located deeper in the liposome.

Since 1D NMR has limited chemical shifting, 2D ^{1}H-^{1}H NMR was used as a conformational method to further evaluate the location of HB within the liposome. Figure 5 shows portions of the 2D NMR spectra for HB at three pH values where HB can be expected to be mostly deprotonated (a), both protonated and deprotonated (b), and mostly protonated (c). The full spectra can be seen in Figures S3-S5 in the supplemental information. Off-diagonal cross peaks in Figure 5 between protons H_a and H_b/H_c show that these protons are close in proximity and serves as an internal control for the system. For the more basic pH, it is expected that HB would be deprotonated and reside primarily in the aqueous layer. The off-diagonal cross peak at 4.79 ppm in the f2 dimension in both Figure 5a and 5b relates to the direct interaction of the deprotonated acid and the aqueous environment around the liposomal membrane. A small off-diagonal cross peak at 2.23 ppm in the f2 dimension for both 5a and 5b correlates with an acetone impurity. The signal is These signals are consistent with deprotonated HB interactions with acetone in the solution.

When the acid is protonated as seen in Figure 5c, it is expected that HB would be able to transverse the membrane and show interactions with the tails of the liposomal bilayer. Large off-diagonal cross peaks of H_a and H_b/H_c protons are expected for the internal control. Smaller off-diagonal cross peaks relating to the carbon chain of the hydrophobic tails of SPC at about 1.30 ppm in the f2 dimension indicate an interaction of aromatic protons H_a and H_b on HB with the

hydrophobic tails of the liposomal membrane. The intensity of the two signals is weak and can be attributed to both a low concentration of the sample as well as a low theta angle, indicating low NOE intensity. This implies that the interaction between these compounds is weak. The lack of a signal for H_c is likely a result of low concentration when prepping the sample and is low intensity. It is likely that H_c has an interaction but that it is indistinguishable from spectral noise.

This data shows a direct dependence of HB on the pH of the aqueous environment with the SPC liposomal interface. The HB species penetrating deeper than its deprotonated form shows that the pH of the environment heavily influences the protonation state of HB, thus influences the ability of HB to transverse the membrane. Combined, this effectively describes the usefulness of HB as a food preservative. By the placement in the membrane, as suggested by peak broadening and off-diagonal cross peaks, HB is able to donate a proton to bacterial cytosol better in acidic environments. This suggests that HB resides with its aromatic ring deeper in the membrane to lower the bacterial pH. HB functions best if the pH of the food is within the active range.[7] Outside of this active range, HB will either be deprotonated outside the cell and unable to pass through the membrane, or the pH of the food environment will be acidic enough to prevent bacterial growth. In order to maintain an ideal pH range in which food will be acidic enough not to have bacterial growth, while being basic enough to not cause consumer harm, HB is desirable because it promotes inhibition in a wider range of pH that is more suitable for consumption. Inhibition is most effective in aqueous media, where it the overall pH of the sample is better maintained for optimal inhibition of bacteria. Other weak acids such as sorbic and citric acid can be used to maintain the pH of food products, ensuring shelf stability for longer periods of time. Preservatives

are less effective in an air interface due to the lack of water pools with dissolved acids in them. These products are likely to see a larger amount of growth over time.

H_2SAL Interactions with Liposomes: There are consistent upfield shifts for all aromatic protons when comparing the liposomal and aqueous environment. As seen with HB, upfield shifting places H_2SAL inside the liposomal membrane. Figure S2 shows the different shifts, when comparing aqueous and liposomal environments, where protons H_b/H_d and H_c are shown to have the greatest difference in shifts. Peak shifting was used to determine pK_a of the sample to further characterize membrane interactions.

Dimerization of H_2SAL is expected for our system due to the ability of H_2SAL to form intermolecular hydrogen bond with its phenol group.[44] Thus, H_2SAL is expected to dimerize in solution and is expected for this system. This dimerization provides an explanation as to the lower pK_a of H_2SAL when compared to HB, where H_2SAL is more available for deprotonation as a result of stability from forming intermolecular hydrogen bonds.

The pK_a of H_2SAL in D_2O is 3.46, 0.41 above the pK_a in H_2O. This is consistent with the pH to pD conversion and is an expected change. The average determined pK_a of H_2Sal was 3.38, which is lower than literature but within a reasonable range, implying that the experimental data is comparable to literature. When H_2SAL was prepared in liposomes, the pK_a was reduced by 0.52. This pK_a reduction is significantly different than the aqueous environment and is consistent with H_2SAL interacting in the membrane. It is notable that the aromatic proton pKa values are statistically the same, implying that the deprotonation of the carboxylic acid has less of an effect than what was previously seen for HB. This can be attributed to the presence of the phenol group

that stays protonated at higher pH values, enacting a sort of stabilization as the carboxylic acid deprotonates. The data can be interpreted that H_2SAL deprotonation has a uniform effect on the aromatic portion of the weak acid.

A lowered pK_a suggests that H_2SAL has stabilization in its deprotonation, where the phenol group and carboxylic acid are placed closer to the lipid head groups the liposome. However, the pKa alone is not enough to fully understand the placement of H_2SAL in liposomes, so peak broadening was considered to further characterize these interactions. Broadening on all aromatic protons suggests that they are deeper in the membrane. This follows with similar ideas of membrane stabilization, where H_2SAL can deprotonate at acidic pH values, stabilized by hydrogen bonding to liposomal headgroups.[36,38,45] Hydrogen bonding between liposomal headgroups were likely reduced in the presence of H_2SAL, which allowed deeper penetration into the liposome.[36,43] H_2SAL favors deprotonation at lower pH ranges due to its ability to stabilize in the membrane with both phenol and carboxylic acid groups, suggesting that it is more suitable for deprotonation in acidic environments.

The largest peak broadening can be seen on H_a and H_c. This suggest that they are interacting the most with the membrane. It is noticeable that the H_b/H_d peak is reduced to a doublet for the active range of H_2SAL, suggesting that it is also interacting with the membrane. Outside of the active range of H_2SAL, it is likely that the charge resulting from the deprotonation of the carboxylic acid prevents it from crossing the membrane. This resulted in less interactions with the acyl tail region, leading to sharper peaks at a basic pH. It has been established that the presence of H_2SAL prepared in D_2O is consistent with a lower lipid transition temperature and a lower enthalpy of the system, where changes in pH had no effect on the transition temperature.[36]

Our results are consistent with previous findings, where significant broadening can be seen in the active range of the weak acid. This data suggests that H_2SAL is more readily available to donate a proton to microbials, likely a direct result of the stability the phenol group provides in proximity to the membrane.

Comparison Overview: HB has been used as a food preservative in foods with acidity around its pK_a. Its ability to inhibit bacterial growth can be justified by the data presented here. Both HB and H_2SAL have upfield shifts from the aqueous to liposomal environment, indicating that the majority of interactions were inside the liposome. Significant peak broadening, presented in Figures 4 and 6 shows that both HB and H_2SAL are both inside the liposomal membrane and show interaction with head groups. H_2SAL has an extra phenol group available that results in a larger disruption of the lipid headgroup-headgroup interactions/more stability in the membrane than HB. This can be seen by the change induced on pK_a values, where the pK_a of H_2SAL is lowered more than HB.

HB is able to penetrate deeper into the membrane, which was justified by the larger peak broadening seen. All protons have significant peak broadening, suggesting HB is deeper in the membrane than H_2SAL. While the pK_a of HB is not affected as much as H_2SAL, it is a result of the stability of H_2SAL in the presence of the polar headgroups. Both HB and H_2SAL are able to stabilize when deprotonating through hydrogen bonding to polar head groups, but the phenol group on H_2SAL allows for stronger hydrogen bonding to polar head groups, increasing its stability at a more acidic pH. Figure 8 shows the diffusion of HB and H_2SAL across a lipid bilayer with theorized stabilization orientation inside the membrane via hydrogen bonding and a potential charge attraction with zwitterionic headgroups.

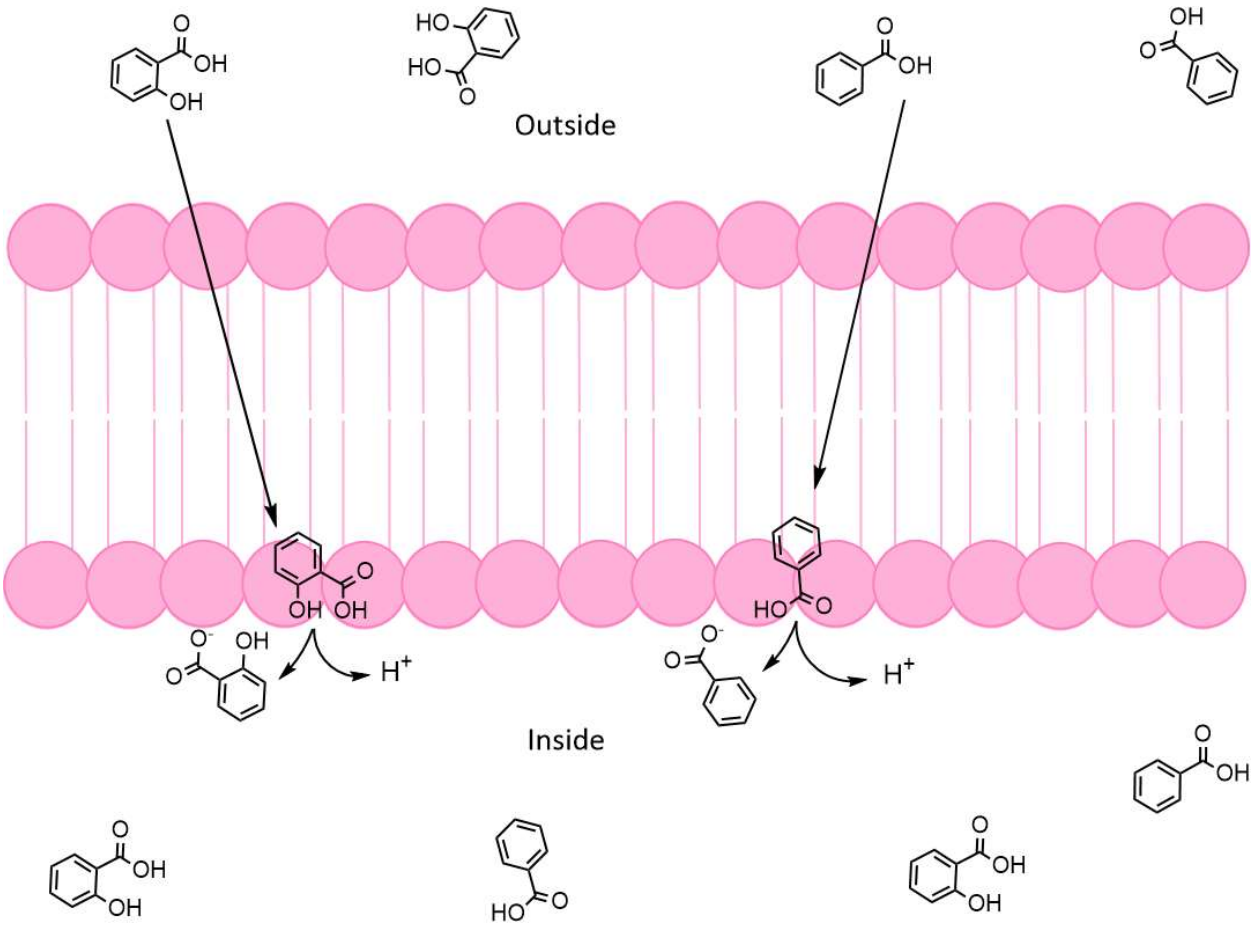

Figure 8: Image of HB and H_2SAL diffusing across a lipid bilayer. Hydrogen bonding and charge attraction stabilizes anionic forms inside the membrane.

Both acids are used as preservatives, but HB is a more desirable food preservative since it is active at higher pH values. H_2SAL can be used in topical creams where the pH is able to be more acidic.[37] However, H_2SAL is not as applied in food science due to its reduced inhibition at a higher pH. The lower pK_a of H_2SAL can result in stomach ulcer formation and is thus not desirable for consumption.[38] With its phenol group, H_2SAL is likely more active against Gram-negative membranes due to the higher pK_a of the phenol group, allowing H_2SAL to donate a proton across the inner membrane. The data presented in the study is consistent with the inhibitory properties of HB and H_2SAL, where HB is more active on Gram-positive membranes at higher pH values. This

can be seen with the higher pK_a of HB and the peak broadening in Figure 4, which suggests its ability to lower cytosol pH.

Chapter 5: Conclusion:

We found that benzoic acid (HB) and salicylic acid (H_2SAL) were both able to penetrate the liposome membrane. Both weak acids penetrated the membrane deeper at acidic pH values close to the pK_a of the weak acid. This placement by internal liposome headgroups is supported by aromatic peak broadening around the pK_a of the weak acid as well as off-diagonal cross peaks on 2D ^1H-^1H NOSEY NMR with HB. These interactions are primarily attributed to stabilization of anionic forms to liposomal headgroups, allowing a greater cytosol pH change. To summarize, we showed that the presence of the weak acid on the membrane allows for penetration into the headgroups due to altering the lipid interface. These observations can explain the antimicrobial properties of HB and H_2SAL.

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Appendices

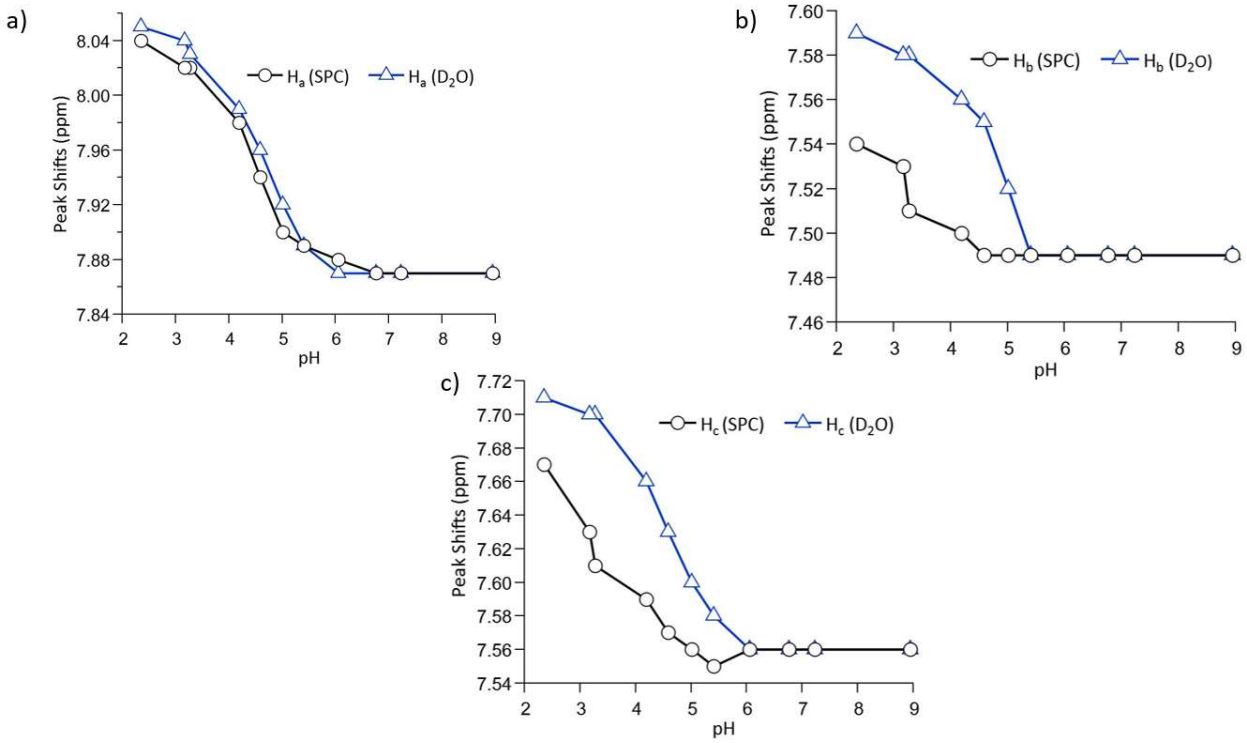

Figure S1: Peak shifts for aromatic protons, (as labeled in Figure 1), in aqueous and liposomal environments. Error bars calculated were smaller than symbols selected.

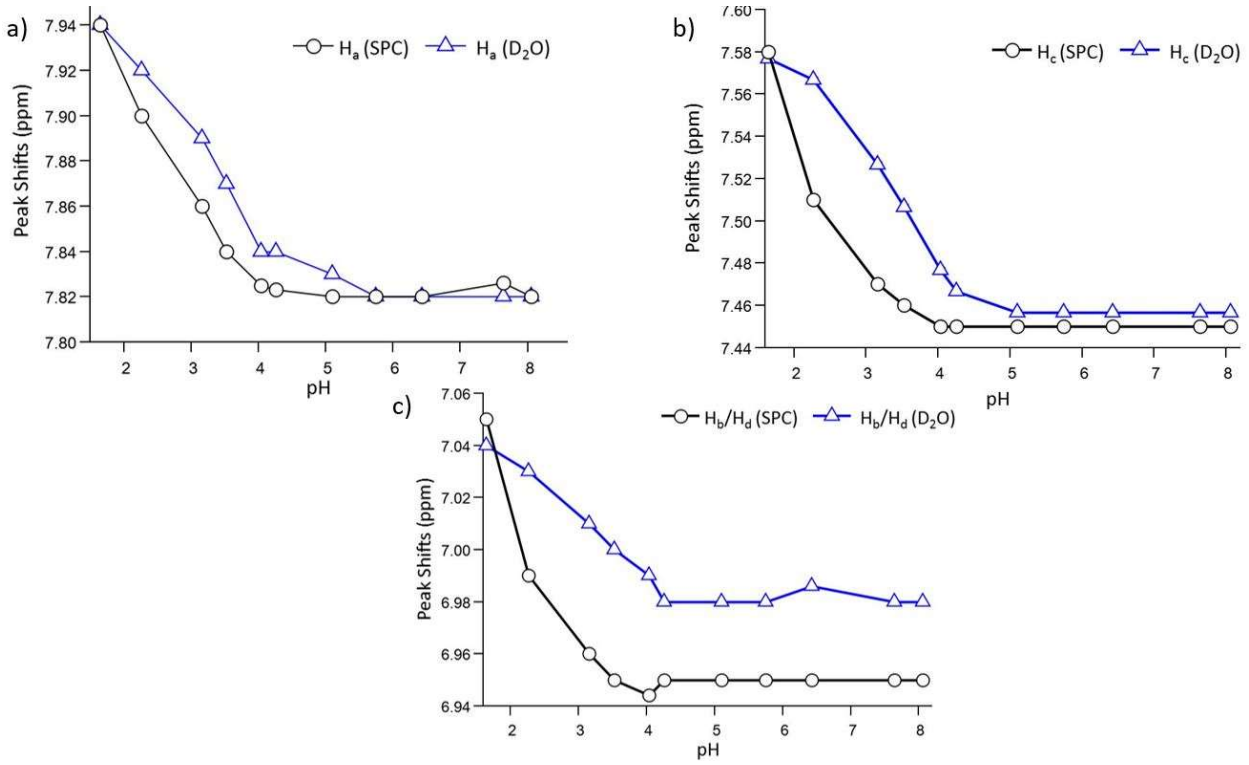

Figure S2: Peak shifts for aromatic protons (as labeled in Figure 1), in aqueous and liposomal environments. Error bars calculated were smaller than symbols selected.

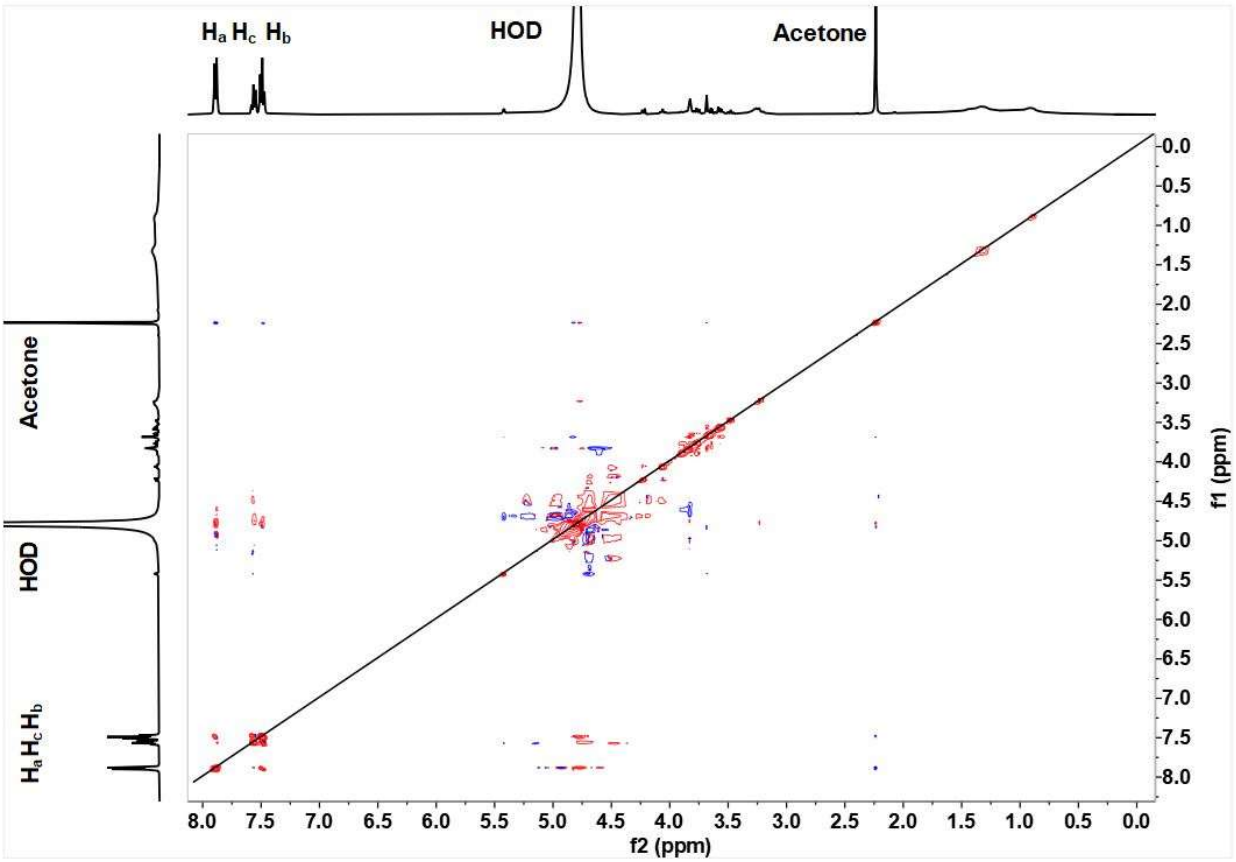

Figure S3: ^{1}H-^{1}H NOSEY NMR Spectra of 10 mM HB in SPC Liposomes at 7.18 pH with aromatic protons of HB labeled according to Figure 1.

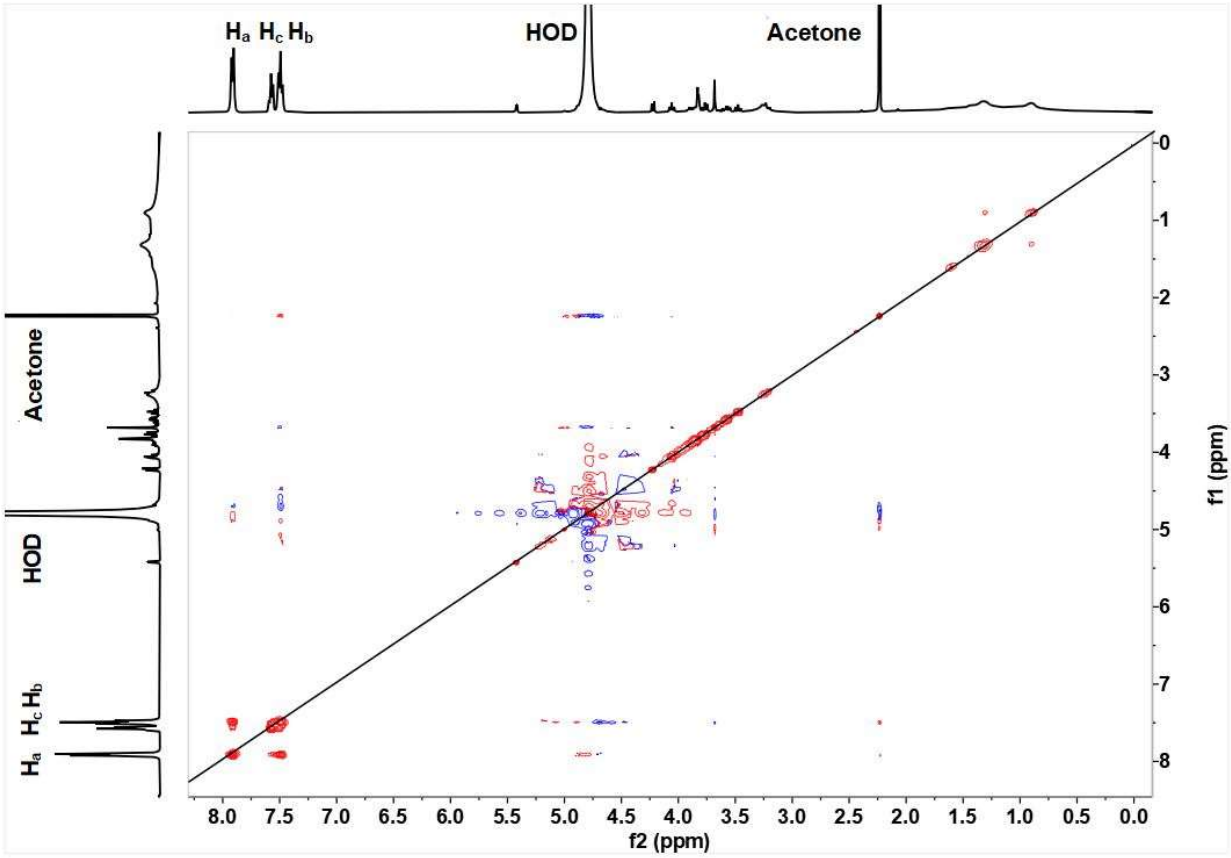

Figure S4: ^{1}H-^{1}H NOSEY NMR Spectra of 10 mM HB in SPC Liposomes at 5.40 pH with aromatic protons of HB labeled according to Figure 1.

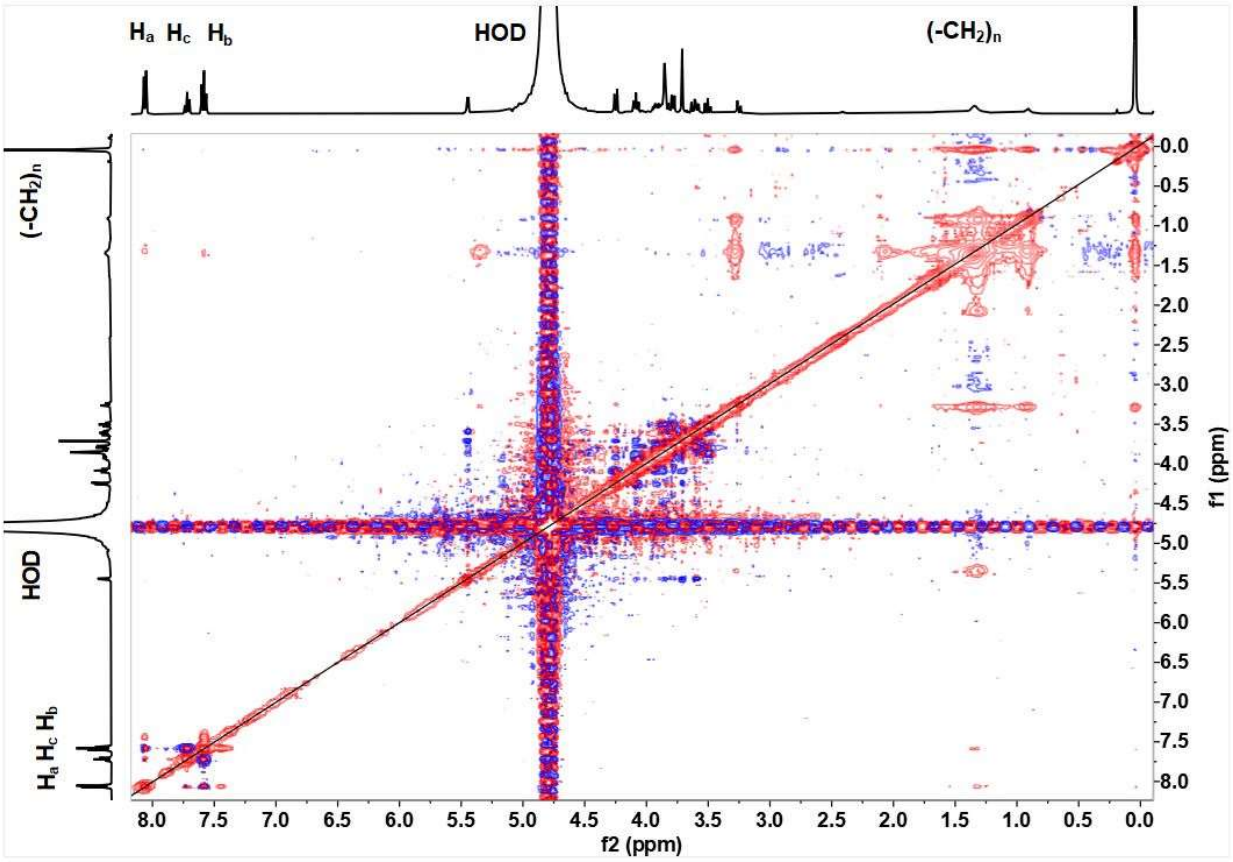

Figure S5: ^{1}H-^{1}H NOSEY NMR Spectra of 10 mM HB in SPC Liposomes at 2.53 pH with aromatic protons of HB labeled according to Figure 1.

Table S1: Dynamic light scattering of HB in SPC Liposomes.

Sample	Size (d. nm)	Z-Average (d. nm)	Standard Deviation (d. nm)	pH
Control (blank SPC liposomes)	139.58	126.64	1.51	x
10 mM BH in SPC	183.57	159.13	3.32	2.35
10 mM BH in SPC	160.90	140.85	3.20	5.40
10 mM BH in SPC	166.12	214.52	11.44	7.18