University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
May 2017

Preparation and Analysis of Novel Urinary Phthalate Conjugates

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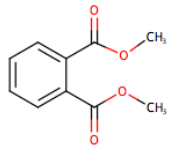
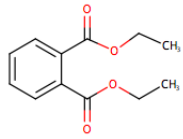
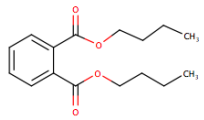
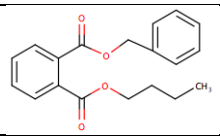
Abstract

Phthalate diesters are toxic compounds found in personal care products and soaps that consist of a benzene ring with two ortho-substituted ester substituents. Phthalates are excreted after glucuronidation. However other possible metabolic pathways have not been examined. One possible route, amino acid conjugation, has been observed in compounds that are structurally similar to phthalate monoesters. An amino acid conjugate of monoethyl phthalate (MEP) was synthesized in the lab with a relatively low yield (<10%). LC/MS, LC/MS/MS, IR, and NMR instrumentation were all utilized in the analysis of the synthesis product. A product ion scan was performed on the compound using 252 and 209 m/z as precursor ions in positive ion mode. LC peaks and corresponding mass spectrum data collected for the compound demonstrated a fragmentation pattern comparable to MEP, with abundant masses of 149 and 177 m/z. The product was used as a standard for the analysis of human urine using the same instrumentation methods. A series of full scans and SIM scans were completed for urine extracts to identify candidate chromatogram peaks for subsequent product ion scans to elucidate the presence or absence of the desired compound. A pepsin digestion was performed on both the standard and one of the urine samples to attempt to identify amino acids present within the analytes of interest. Digestion of both the synthesis product and extract resulted in near or total disappearance of chromatogram peaks from precursor ions of the proposed conjugates, which was most likely a result of pepsin cleavage. The synthesis and characterization coupled with pepsin digestion of the samples provided evidence that suggests amino acid conjugation may be a feasible route of metabolism for phthalate monoesters. Proving phthalate monoesters follow this metabolic pathway would have lasting ramifications by increasing exposure estimates stemming from previously unknown metabolites.

1. Introduction

Washing hands or using personal care products exposes people to endocrine disrupting chemicals known as phthalate diesters. Phthalates are manmade chemicals used to make plastics and personal care products.[1] These chemicals are widely used across several industrialized countries and humans have ubiquitous exposure. Dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP) and benzylbutyl phthalate (BzBP) are among the most common phthalates utilized in industry and have been observed in significant concentrations in people.[2] A structure for each of these phthalates along with their estimated mean concentration in urine in 2003-2004 is given in Table 1.

Table 1. Structures of common phthalates and their respective concentrations in urine are listed. Levels are based on US CDC data from 2001-2002.[2]

Name	Structure	Metabolite name	Mean Concentration in Urine (US; creatinine corrected in μg/g creatinine)
DMP		Mono-methyl phthalate	1.08
DEP		Mono-ethyl phthalate	167
DBP		Mono-isobutyl phthalate, Mono-n-butyl phthalate	2.54; 17.8
BzBP		Mono-benzyl phthalate	14.1

These concentrations are viewed as estimates because the metabolites of phthalates are the only substances being measured. Parent compounds of phthalate metabolites are not quantified due to their abundance in the environment.[2] These chemicals are not permanently bound to the products they form, enabling them to enter the air or fragment from the site to which they are attached.[3] Disrupting this weak interaction allows them to enter humans through inhalation, ingestion, or through the skin.[2] However, dermal absorption from personal care products is the most common means of entry into humans.

Studies using rats suggest that in high enough doses, phthalates could pose risk to humans in the form of developmental issues, including decreased testicular weight and lowered testosterone levels.[2,4,5] Phthalates have exhibited low acute toxicity in animals, but chronic exposure in lab rats has resulted in incidences of testicular injury, liver injury, and teratogenicity. In addition, phthalates have shown anti-androgenic effects in lab animals by inhibiting testosterone production and even estrogen production at higher exposure levels.[2] These findings have also been associated with infertility in some groups of men with high levels of urinary phthalate metabolites.[2,6]

Upon entering the body, phthalate diesters are rapidly converted to a phthalate monoester in Phase I metabolism, utilizing an esterase enzyme that hydrolyzes one of the ester substituents of the phthalate into a carboxylic acid functional group.[3] The formation of a phthalate monoester slightly increases hydrophilicity but substantially increases reactivity. A reaction scheme for Phase I is included in Figure 1.

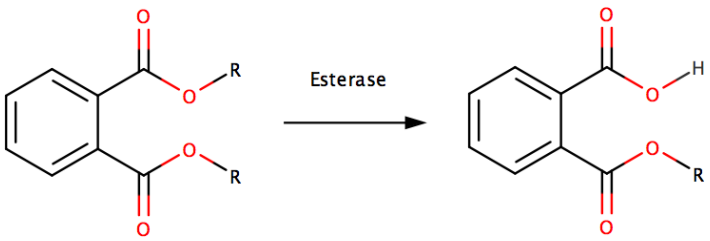


Figure 1. Phase I metabolism showing the conversion of a phthalate diester to a phthalate monoester.

The reaction effectively reduces the stability of the compound, which can causes toxic effects associated with phthalates or prepares the phthalate for Phase II metabolism.[5]

After Phase I metabolism, phthalate monoesters then enter a Phase II metabolic pathway called glucuronidation, which promotes urinary excretion as a glucuronidated conjugate.[3] Phase II pathways are characterized by a decrease in reactivity and a sharp increase in hydrophilicity.[5] This pathway increases hydrophilicity of the phthalate by oxidizing the monoester created from the esterase.[7] A reaction scheme depicting Phase II glucuronidation is given in Figure 2.

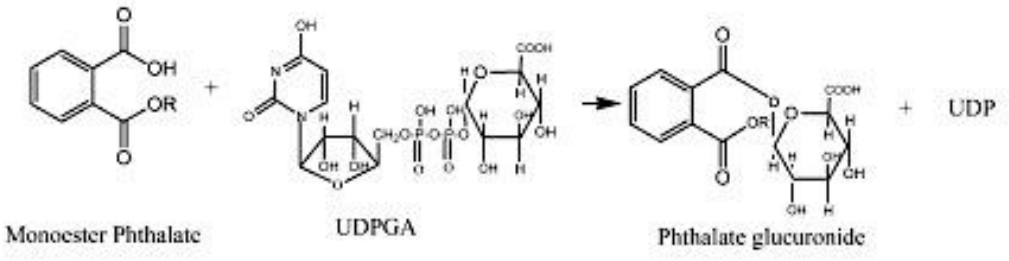


Figure 2**.** Shows the formation of a phthalate glucuronide by glucuronidation.

The UDPGA equivalent forms a bond at the carboxylic acid of the monoester through nucleophilic attack at its carbonyl carbon, greatly increasing the phthalate's ability to interact with water, thus allowing renal excretion.[5]

Amino acid conjugation is another common metabolic pathway for elimination of toxins. It is an ATP dependent process involving the conjugation of xenobiotics using short-chain amino acids such as glycine and glutamine. These residues form amide bonds with carboxylic acid-containing compounds to facilitate excretion through the kidney.[5,8] An example of how this process proceeds with benzoate is given in Figure 3.

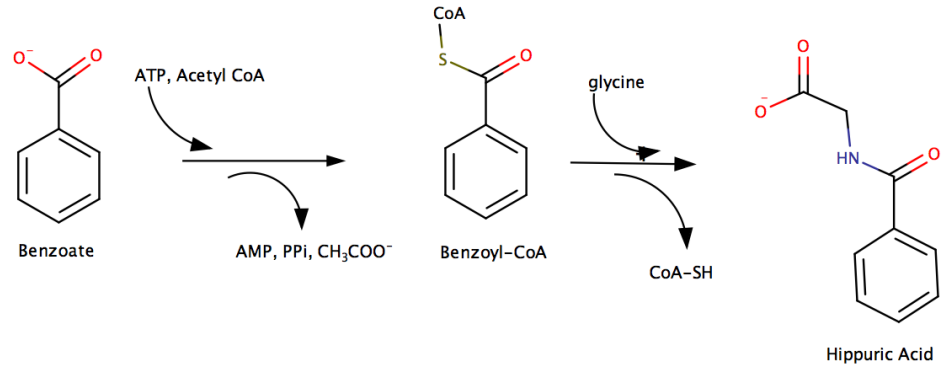


Figure 3**.** Reaction scheme of benzoic acid being converted to hippuric acid in its deprotonated form, benzoate. PPi is a diphosphate ion, ATP is adenosine triphosphate, and AMP is adenosine monophosphate.[5]

Amino acid conjugation has been observed in the metabolism of compounds found in food preservatives like benzoic acid, but it has not been previously studied as a means of phthalate metabolism.[9]

The goal of the project is to search urine samples in order to quantify how much, if any, amino acid conjugated phthalates are present utilizing established instrumentation methods. The findings derived from this work will provide new insight into the metabolism of phthalate monoesters by identifying additional routes of their excretion from the body. Characterization of the mechanism of amino acid conjugation in relation to phthalate metabolism will help determine the viability of this pathway. It will also potentially identify a unique pathway not previously found, which may alter the current known exposure estimates of phthalates. Increasing these estimates to account for amino acid conjugation would have lasting ramifications on the importance of studying phthalates due to their high toxicity at higher concentrations.

The results produced by research groups in the field have provided new understanding towards the function and effect of exposure to these molecules in both animals and humans. Studies have focused on evaluating the toxicity of phthalates and displaying the importance of quantifying humans' exposure to them by measuring changes in enzymatic activity in rats. Crucial metabolic enzymes like glucuronyl transferase remove xenobiotic molecules which are harmful to the body but have shown marked reduction in activity in the presence di-ethylhexyl phthalate (DEHP).[4] Phthalates have prominent stability due to their ring structure combined with two polyalkylated esters.[10] Decreased activity of

the glucuronyl transferases that catalyze the hydrolysis of these compounds results in the retention of these compounds in the body, with potentially detrimental effects on metabolic processes in the body. This inhibited activity is evident in data, which recorded nearly a 20% decrease in enzyme activity at a concentration of 0.2 mg/kg DEHP.[4] Other phthalates that are structurally similar to DEHP produce the same effects. However, DEHP is one of the most common phthalate diesters used in production and has also been one of the better studied.

Research aimed at characterizing the metabolic pathway of phthalates has also led to the discovery of numerous metabolites derived from these molecules. Many phthalates utilize the glucuronidation pathway previously mentioned as a Phase 2 pathway after being converted into a phthalate monoester in Phase 1 metabolism.[7] The main reaction pathway for DEHP with the glucuronidation step is shown in Figure 4.

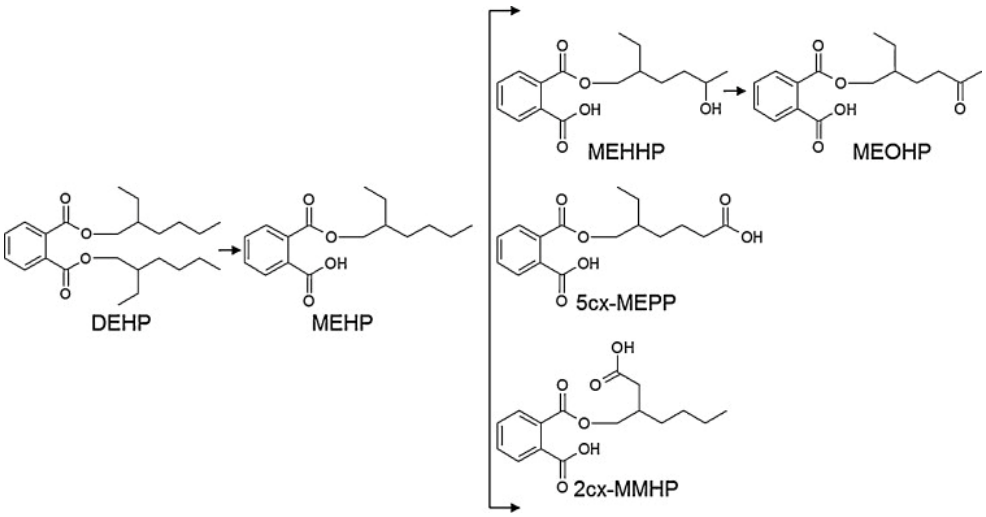


Figure 4**.** Reaction scheme of DEHP with esterase (DEHP to MEHP) and the glucuronidation step (MEHP to various hydrolyzed Phase 2 metabolites).[11]

This pathway improves solubility of the molecule and allows urinary excretion, which prevents the build up of phthalates in the body. Glucuronidation has been the main focus of the metabolism of phthalates, but similar routes of excretion may exist. Amino acid conjugation is carried out by a group of enzymes known as N-acyltransferases, which results in similar hydrophilic end products conjugating much the same way as glucuronidation. Amino acid conjugation may be a potential pathway for the elimination of these phthalates *in vivo*.[12]

Amino acid conjugation has been observed as a significant means of metabolism in living organisms, including humans and plants.[9,13] The process by which compounds are metabolized through amino acid conjugation has been divided into two steps. The first involves the activation of the parent compound through the addition of acetyl-CoA, an ATP consuming process improving the compound's nucleophilicity. The second and most important step in this process involves the attack with an amino acid, usually glycine and other small side chain residues. The CoA intermediate to increases hydrophilicity, thus enabling the metabolite to be excreted via the urine.[5,8] Amino acid conjugation of salicylic acid showing these steps is given in Figure 5.

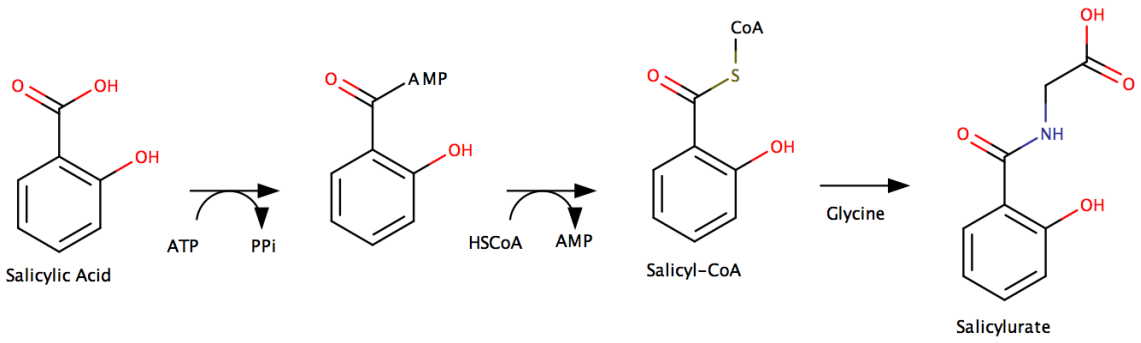


Figure 5. Conjugation of salicylic acid including the activation step with ATP, the formation of salicyl-CoA, and the addition of glycine.[9]

Steric hindrance at the ortho position has shown to reduce amino acid conjugation compared to the para and meta positions. However, recent findings have shown that substrates in the ortho confirmation can still undergo conjugation at significant rates. Small side chains at this position as found in compounds such as salicylic acid above enable the amino acid residue to still form the amide bond with the carboxylic acid.[9,13]

Several factors have been determined to influence the rate of amino acid conjugation. Aside from the configuration of substituents on aromatic rings previously mentioned, concentrations of free amino acids as well as inhibition of either step in the pathway have been linked to decreased rates of amino acid conjugation in the body.[8] These findings were outlined in the work of Badenhorst et al. and Knights et al., which dealt primarily with aromatic acids structurally similar to phthalates as substrates for amino acid conjugation. It was determined that low dietary intake of amino acids, especially glycine, have led to decreased rates of conjugation and are compounded if not enough ATP is present to form the CoA intermediate prior to the addition of the amino acid, as two ATP are required to form the precursor to the final step in conjugation.[14] Alcohol may serve as an inhibitor to this process, as it puts additional stress on the liver where these reactions occur.[9] Applying these findings to variations in human lifestyles may prove invaluable to the quantification of metabolites from amino acid conjugation, and it could also establish a set of guidelines for the determination of the viability of phthalates to undergo this process in addition to those compounds listed in this research.

Various methods have been used in these studies to correctly identify phthalate metabolites and to quantify their concentration in urine. The type of instrumentation commonly used to perform this analysis is liquid chromatography tandem mass spectrometry (LC/MS/MS), which can accurately quantify metabolites in the urine despite the large number of other non-target substances present in this biological fluid.[15] Prior to sample injection, the concentration of urinary phthalates must be known. The standards are intentionally spiked with a known quantity of C_{13}-labeled phthalate, and they are corrected by monitoring concentrations of chemicals constantly released in urine that determine the dilution of the urine.[11,16] Recognizing creatinine concentration in urine samples in these studies provide a baseline of diluted and concentrated urine since creatinine is constantly released from the body.[16] In order to correctly determine the amount of phthalate that is metabolized, the standards are compared to other urine samples to measure an individual's exposure.[11]

A high performance liquid chromatography atmospheric pressure chemical ionization tandem mass spectrometry (HPLC-APCI-MS/MS) method was developed by Silva et al. to selectively quantify phthalate metabolites in the urine. Carbon-13 labeled standards were added to urine, and the samples were measured for total and free phthalate concentrations. The method utilized an automated solid-phase extraction system, followed by chromatographic separation with a nonlinear solvent gradient prior to mass spectrometer analysis. Instrument settings were tuned for each analyte, and each run consisted of a blank with multiple unknown samples and two quality control samples.[17] The validity of the method was tested through multiple runs of samples, and alterations to extraction settings on multiple instruments determined its ruggedness.

It may be possible to synthesize labeled phthalate amino acid conjugates following a previously described procedure.[18] The scheme was developed to synthesize glycine conjugates from substituted benzene compounds such as salicylic acid and benzoic acid. Two steps were utilized to produce the final glycine conjugated product. These consisted of addition of a N-succinimide with a coupling reagent to provide a good leaving group for subsequent glycine addition.[18] The products formed during the reaction corresponded to the desired glycine conjugates and were

present in high yields (>75%). Most notably however was the successful synthesis of an ortho-substituted salicylic acid glycine conjugate, as ortho substituted conjugates are more difficult to synthesize than para and meta substituted products. Furthermore, it was important to consider the small ortho substituent on the final product. Addition of glycine would likely not be a viable option for compounds such as phthalates that contain long side-chains due to steric hindrance, thus making this process selective towards small side-chains only.

One method used to confirm the presence of amino acid conjugates is by introducing an enzyme in solution with the desired analyte. Depending on the enzyme used, it is possible to preferentially cleave the amide bonds present in amino acid conjugates. Pepsin has the capability of cleaving the amide bond at the end of glycine residues, and it has been extensively researched in its applications for enzymatic digestion. One protocol has been used by the Promega Corporation to perform a pepsin based enzyme digestion.[19] By manipulating factors such as the pH and the ratio of enzyme to substrate, this procedure was successful in cleaving the amide bond of a variety of amino acid residues. The analysis of the products through LC/MS/MS is one method of confirming successful digestion.

3. Experimental

3.1. Overview

The present study examines the glycine conjugation of phthalate diesters. Salicylic acid was first used as a template for the synthesis of an amino acid conjugated standard. The proposed synthesis scheme based on the research of Van Brussel et al. was tested to optimize yields and isolation of products. The scheme was then applied to monoethyl phthalate to determine its candidacy for amino acid conjugation and subsequent use as a standard in LC/MS/MS. NMR, IR, liquid chromatography mass spectrometry (LC/MS), and LC/MS/MS were used in the characterization of the synthesized products. The data were analyzed for characteristic markers unique to each analytical technique to properly identify each product. The LC-MS/MS data of the synthesized standard provided a guide for the analysis of amino acid conjugates in urine. The urine samples were prepared using a solid-phase extraction method to analyze using LC/MS/MS. The objective of the urine analysis was to search for an amino acid conjugated phthalate within the urine based on the LC/MS/MS data of the synthesized standard. Fragmentation patterns were screened in order to delineate between the two analytes to verify their similarities. Finally, a preliminary pepsin digestion was performed on both the synthesized standard and the urine sample to further solidify the analytes as being glycine conjugates. Based on current knowledge, the study is the first to focus on phthalate metabolism by amino acid conjugation.

3.2. Safety

dicyclohexylcarbodimide (DCC)- Harmful if swallowed, causes severe eye damage
N-hydroxysuccinimide- Non-toxic for all absorption routes
dioxane- Highly flammable carcinogen, causes severe eye irritation
salicylic acid- Harmful if swallowed, causes eye irritation
sodium bicarbonate- Non-toxic for all absorption routes
glycine- Non-toxic for all absorption routes
monoethyl phthalate- Endocrine disruptor, irritating vapors when heated
acetonitrile-Reproductive toxin, skin and eye irritant
methanol- Flammable, skin and eye irritant
concentrated HCl- Causes severe burns, irritant, corrosive
phosphoric acid- Causes severe burns, corrosive, causes severe irritation
sodium phosphate- mild irritant to skin, eyes, or digestive tract
pepsin- hazardous if inhaled, mild skin and eye irritant

3.3. Materials

DCC, dioxane, salicylic acid, sodium bicarbonate, and concentrated HCl were obtained from Sigma Aldrich or Fisher Scientific. Dioxane was distilled over sodium metal prior to use. All other reagents, including monoethyl phthalate and N-hydroxysuccinimide, were purchased from Fisher Scientific. Pooled Urine was obtained through anonymous donors and given to the Brock Analytical Toxicology Research Lab. Solid pepsin was purchased from the Promega

Corporation. Solvents used in the synthesis, solid phase extraction, and pepsin digestion were all readily available within the lab.

3.4. Instrumental Analysis

A Thermoscientific Nicolet IS10 infrared spectrophotometer (IR), an Oxford 200 nuclear magnetic resonance spectrometer (NMR), and a Shimadzu LCMS-2020 Spectrometer were used in the analysis of salicylic acid and phthalate conjugates. Solid phase extraction using a Zymark Turbovap LV Evaporator was used to prepare urine samples for LC/MS/MS analysis. A Shimadzu LC/MS/MS-8040 spectrometer was used for the characterization of the conjugated phthalate standard and the urine samples as well as their respective pepsin digestion products. Parameters for the IR and NMR were kept on the default settings for all analytes. Parameters such as the collision energy, injection volume, and precursor ions were manipulated for both the LC/MS and the LC/MS/MS analyses and were recorded for each sample run. The "Novel conjugated phth monoesters ESI product scan" method was used by the LC/MS/MS until clogging issues with the injection tubes and introduction of a new LC column prompted the development of a new instrument method. The new method was titled "Novel conjugated phth monoesters ESI" and retained most prior settings but altered the LC program to run 60 minutes with a linear solvent gradient of acetonitrile from 0%-50%. The LC column used during Spring 2017 was a Kintetex 5µm C18 100Å LC column 150x2.1mm.

3.5. Salicylic Acid Trial Synthesis

The procedure used by Van Brussel et al. was adapted to synthesize salicylic glycine. Dioxane was dried by distilling 200 ml of dioxane and 2 small blocks of sodium metal under inert conditions. The mixture was magnetically stirred and heated for approximately 45 minutes with the sodium metal. The vapor recondensed and was collected in a 50 ml round bottom flask. A total of 100 ml of dry dioxane was collected and stored at room temperature in two separate 100 ml round bottom flasks.

The synthesis of salicylic glycine followed the reaction scheme given in Figure 6.

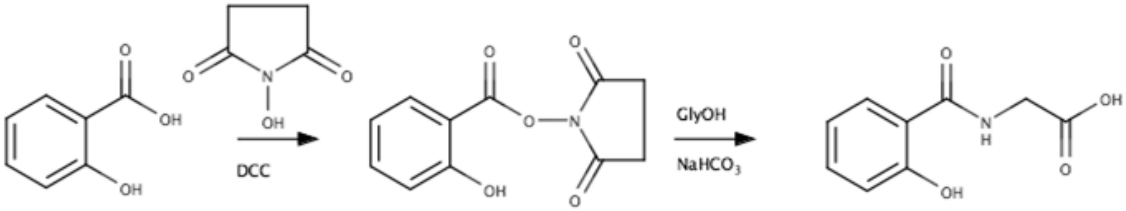


Figure 6. Reaction pathway for salicylic acid to salicylic glycine. The pathway was comprised of two major steps: the addition of N-hydroxysuccinimide in the presence of DCC and the addition of glycine with sodium bicarbonate.

A 100 ml round bottom flask was heat-dried and 30 ml of dry dioxane, 12 mmol of DCC, 12 mmol of N-hydroxysuccinimide, and 10 mmol of salicylic acid were added and magnetically stirred for 5 hours at room temperature. The formed dicyclohexylurea were filtered off using gravity filtration and were rinsed with 5ml of dioxane. The mixture was rotovaped to dryness [using a Buchi Rotovaporator R-124].

The formed intermediate, 15 mmol of glycine, 15 mmol of sodium bicarbonate, and 10ml of water were added to a 100 ml round bottom flask and stirred for 20 hours at room temperature. The reaction mixture was extracted using 5 ml of ethyl acetate three consecutive times, each time filtering the organic layer from the water layer. The organic layer was stored while the aqueous layer was acidified to pH 2 with concentrated HCl. The water layer was cooled and gravity filtered to isolate the product, and the sample was analyzed via IR, NMR, and LC/MS.

3.6. Phthalate Standard Synthesis

The same procedure as mentioned for the synthesis of salicylic glycine directed the synthesis of the phthalate conjugate. The amounts of reagents were 25% of the number of millimoles and volume used for the salicylic glycine synthesis. The reaction scheme for the preparation of the phthalate conjugate is given in Figure 7.

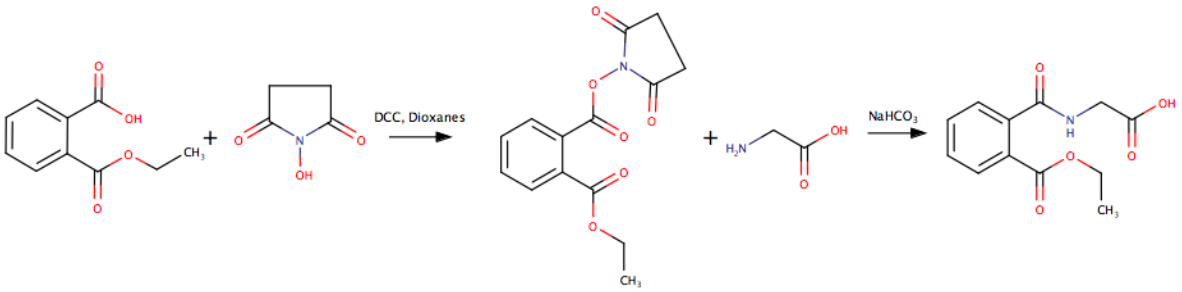


Figure 7. The reaction scheme for the synthesis of the phthalate glycine conjugate. The two steps from the previous synthesis were applied in the same way except monoethyl phthalate (MEP) was used as the starting material.

The product formed in the reaction was analyzed using IR, NMR, and LC/MS. The sample was prepared for LC/MS analysis by dissolving in deionized water (DI) and filtration using a microfilter.

3.7. Phthalate Standard Revised Synthesis

The synthesis of the phthalate glycine conjugate was also attempted using a "one-pot" approach with only MEP, glycine, and DCCD following the scheme given in Figure 8.

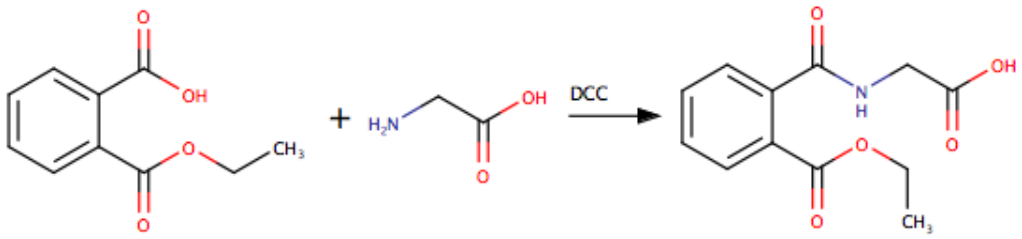


Figure 8. One step reaction involving the coupling of MEP and glycine through DCC.

To a solution of 7.5 ml of dry dioxane, 0.0025 mol of monoethyl phthalate, 0.0025 mol glycine, and 0.0025 mol DCC were added. The reaction mixture was stirred for 4.5 hours at room temperature under a nitrogen balloon. The DCC urea was filtered off using gravity filtration and rotovaped to dryness. The crude product was recrystallized in 100% water, and the solid was filtered by gravity filtration to isolate the final product, giving approximately a 30% yield. IR, NMR, LC/MS, and LC/MS/MS data were collected for the compound.

3.8. Solid Phase Extraction

Solid phase extraction (SPE) turbovap tubes were labeled A-D, and tube A was used as a blank in the extraction process. 2 ml of water was added to tube A, and 1 ml of water and pooled urine were added to tubes B-D. Oasis HLB 60 mg cartridges were placed on the turbovap manifold at. 1 ml of acetonitrile was added to each cartridge at -400 kPa and rinsed through until still wet. 1 ml of acid buffer consisting of sodium phosphate and phosphoric acid in water were rinsed through the cartridges until almost all of the liquid had evaporated. The samples were loaded by pulling them through the cartridges until only a small amount of liquid remained at the top. Each cartridge was rinsed with 1 ml water until dry, and the labeled test tubes were placed underneath the cartridges for collection. 1 ml of acetonitrile and 1 ml of ethyl acetate were pulled through all cartridges until dry for 30 seconds to elute the desired analytes.

The analytes in each test tube were evaporated to dryness with nitrogen in approximately 1 hour. Lastly, the samples were resuspended in 200 µl of water and transferred to autosampler vials containing 200 µl inserts for LC/MS/MS analysis.

3.9. Pepsin Digestion

HCl (1M) was added to both the synthesis product and the urine extract until the solutions were at pH 2. 6 mg of Pepsin was resuspended in acidified water at pH 2, and the pepsin solution was mixed with the urine and synthesis samples. The solutions were incubated at 37° C for 24 hours and stopped by heating at 95° C for 10 minutes. Each sample was refrigerated after heating to store them.

4. Results and Discussion

4.1. Salicylic Glycine Analysis

Thin-layer chromatography of both the intermediate and product for the salicylic reaction implemented a 15%:85% ethyl acetate:hexane mobile phase on a silica coated glass TLC plate. Two products had small R_f values (<20%) while the third product had an R_f of >50%. Despite the lack in purification, the goal of this synthesis was to simply achieve the target fragment masses corresponding to the product to determine its identity. Therefore, the isolation of the salicylic glycine product was not as essential to the study so long as the crude product was present.

IR data collected for the final product from the reaction showed the addition of a secondary amine N-H stretch (sharp, 3316 cm^{-1}) and an amide stretch (sharp, 1622 cm^{-1}). The full IR data are available as supplemental material. These two stretches were closely associated with the predicted final product spectrum and suggested the addition of glycine to the salicylic acid.

NMR data from the final product demonstrated clear shifts in the aromatic and amide regions, which were observed at 6-8 ppm and ~4.6 ppm respectively. However, issues with the purification of the final product led to several unknown chemical shifts between 1-4 ppm, thus the full structure of the desired product was unable to be determined from NMR. The full data are available as supplemental material.

LC/MS data further showed the presence of impurities in the final product. Despite peaks from side products, the most intense peak was characterized to have the exact mass expected. The LC/MS print-out is included in Figure 9. In addition to finding the product mass of 196 g/mol, the ratio of 196 g/mol to 197 g/mol was compared to find the ratio of C_{12}-C_{13} detected by the instrument. The concept was based on the theory that each carbon in the salicylic glycine has about a 1% chance of being found in the C_{13} isotope, therefore a compound with 9 carbons would have roughly 9% of its carbons as C_{13}. The product identity was verified as salicylic glycine after calculating the mass intensity of 197 g/mol to be roughly 9% of the mass intensity for 196 g/mol.

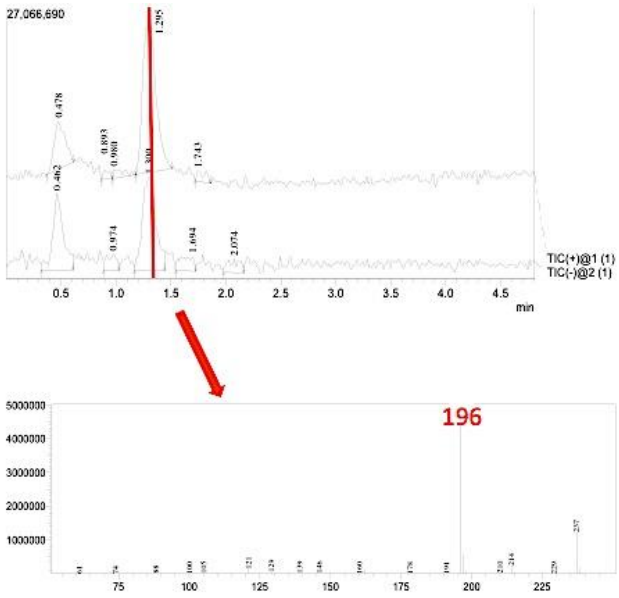


Figure 9. (20160616001) Salicylic glycine LC-MS chromatogram. Method performed a product scan of sample on positive ion mode with a non-linear gradient from 10-50% acetonitrile in water for 5 minutes. Top image shows chromatogram and bottom is corresponding mass spectrum. Y-axis of top is instrument response, x-axis retention time. Y-axis of bottom is intensity, x-axis is m/z ratio.

4.2. Phthalate Standard Synthesis

A similar approach was taken to characterize the product of the MEP reaction as the salicylic glycine reaction, but a greater emphasis was placed on the LC/MS data as the fragmentation of the product was vital to determining its structure.

Two major findings were derived from the LC/MS chromatograms for the standard synthesis, and the chromatograms from the instrument are available as supplemental material. The intermediate phthalate succinimide ester mass (292 g/mol on positive ion mode) was clearly distinguishable among the data, and the collision energy (-15 eV) was sufficient enough to fragment the product to generate the most characteristic phthalate fragment (149 g/mol). IR data also supported this result, containing no carboxylic acid stretch while having a potential N-O stretch at 1744 cm^{-1}.

The chromatogram of the final product did not provide the anticipated results based on the data for the intermediate product. When a product scan was completed for the sample, netiher the 195 g/mol mass appeared, nor were any phthalate fragments (149, 177, and 163 g/mol) detected. Collision energies ranged from -15 eV to -35 eV, each time with the same result. The IR data as well as the NMR data concurred with the LC-MS chromatograms, both of which did not contain the desired data to verify the presence of the amide bond of the proposed product.

4.3. Viability of Alternate Method for Standard Synthesis

The “one pot” synthesis of the phthalate standard demonstrated more reliable results than the procedure based on the synthesis of the salicylic glycine compound. The final product from the reaction was characterized by an oily consistency, which was an indicator of impurities found within the compound. This result was to be expected however since few measures were taken to protect the ester substituents of the MEP from side reactions. These data prevented identification of the product using solely LC/MS, IR, and NMR, but these methods combined with LC/MS/MS proved effective in interpreting the data as a whole.

Full IR and NMR data collected for the product is available are available as supplemental material. IR spectroscopy showed vibrational frequencies that supplemented other analytical data that suggested the conjugated product was formed. The primary frequencies are 3332 cm^{-1} (amine N-H stretch) and 1652 cm^{-1} (amide C-O stretch) while two additional vibrations at 1724 cm^{-1} and 1807 were believed to stem from the other two carbonyl stretches evident in

the synthesis product of Figure 7. NMR shifts for the proposed product were observed at 7.04ppm (singlet, aromatic), 6.95ppm (triplet, amide N-H), 4.4ppm (quartet, ester), and 2.3ppm (doublet, CH_2 near carbonyl). All hydrogens were accounted for in the analysis except for those of the CH_3 of the ethyl ester, which could have been found within the shifts between 1-2ppm in a pure sample of product.

LC/MS/MS data provided perhaps the clearest results among all analytical methods. Originally, the fragment masses displayed on the mass spectrum for the LC/MS chromatogram did not correlate to the characteristic fragmentation of MEP. The solvent used in preliminary runs consisted of 10-90% acetonitrile. The NMR and IR data conflicted with that of the LC/MS in addition to the sample being partially insoluble in the solvent. The solution to the issue was to use a mixture of 50/50 methanol/water. The solvent was used in the LC/MS/MS analysis, and the chromatogram for one of the runs is shown in Figure 10. Completing a product scan for the precursors 252 and 209 both resulted in detection of the masses 149 g/mol and 177 g/mol, while 163 g/mol was also detected for the 209 product scan (Figure 10). These data support the claim for successful synthesis for the amino acid conjugated MEP.

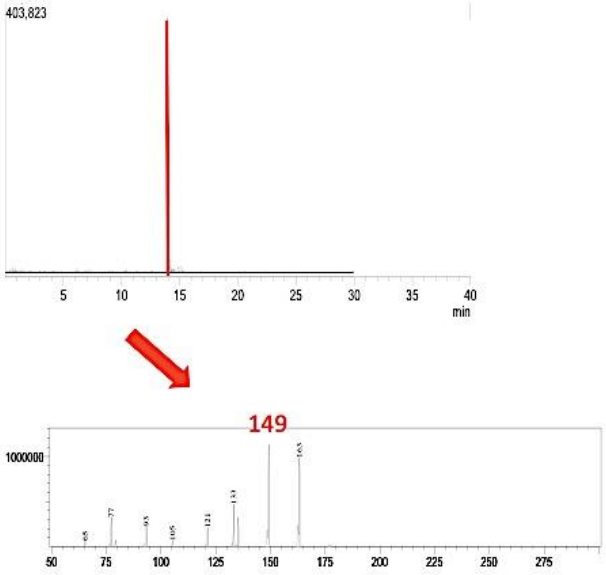


Figure 10. Chromatogram of precursor 209 g/mol, representing the loss of CO_2 from the desired product in positive ion mode.

Parameters for analysis used the method "Novel conjugated phth monoesters ESI product scan" at -25 eV, 10-90% acetonitrile. Top image is chromatogram, bottom is mass spectrum for corresponding chromatogram. Axes for chromatogram are instrument response vs. retention time. Axes for mass spectrum are intensity vs. m/z ratio.

4.4. Urine Analysis

Urine samples were analyzed using LC/MS/MS according to the method used for the standard synthesis. The LC/MS/MS chromatogram showing data for the 252 g/mol product ion scan is provided in Figure 11.

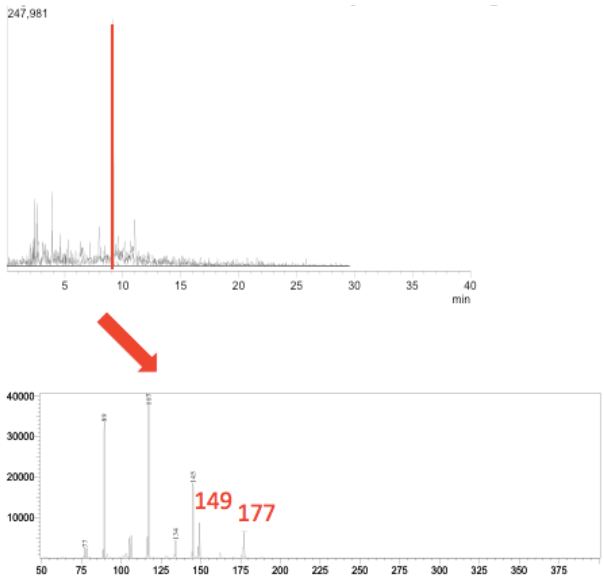


Figure 11. (20160802003) LC-MS/MS chromatogram for urine sample at -35 CE in positive ion using same method as standard synthesis.

Chromatogram peak corresponding to mass spectrum is highlighted in red. Top image is chromatogram, bottom is corresponding spectrum. Axes for top are instrument response vs. retention time. Axes for bottom are intensity vs. m/z ratio.

The masses of 149 g/mol as well as 177 g/mol were observed from the data, thus validating the presence of the phthalate fragmentation pattern for this selected precursor. Unlike the data for the standard product, an additional 209 g/mol precursor product scan did not reveal the fragmentation pattern for MEP. Thus, a complete match could not be found between the urine sample and the synthesized standard.

Full scans of additional urine extracts aimed to increase reproducibility of results from preliminary urine analyses. The three different extracts that were run on the LC/MS/MS with the new LC program and column exhibited a multitude of chromatogram peaks and marked separation each one. Manipulation of data view parameters was successful in identifying chromatogram peaks corresponding to previous target masses. Elution times of compounds in these extracts differed significantly from prior results due to alterations in the LC program but remained nearly identical for all extracts run after the changes that were made.

SIM scans of the three extracts further supported consistency of data. All target masses were found in both positive and negative ion mode, and retention times only differed slightly from run to run. These data were used to isolate candidate chromatogram peaks for further analysis by selectively fragmenting compounds present in accord with the previous methodology of the first urine analysis.

4.5. Pepsin Digestion Results

Pepsin digestions for both the synthesized standard and the urine sample were analyzed using identical parameters on the LC/MS/MS. The data for the standard did not change significantly after the digestion, except the retention time for the chromatogram peak increased by about 9 minutes. Additionally, the MEP peak increased from the original LC/MS/MS data. However, the urine sample chromatogram peaks for all chosen precursors were absent from the data, much different from the original LC/MS/MS analysis.

5. Conclusion

The data presented in the study led to a number of different findings concerning amino acid conjugation of MEP and phthalate monoesters. The successful addition of the N-hydroxysuccinimide to MEP refuted the notion that ortho

substituted compounds such as phthalates are not likely to form products. The use of a coupling reagent in these reactions was important however, as the desired product was likely formed by the one step approach that added glycine through DCC rather than the prior two step method that did not use the reagent in the second step. It may be possible to improve the selectivity of the reaction to isolate the final product by using a protecting group on the glycine at its C-terminus. Protecting groups along with a refined multiple step approach instead of a "one pot" synthesis would decrease the number of side products, thus allowing for improved analytical data interpretation.

The procedures for analysis show promise in identifying amino acid conjugates in the urine. The synthesis of the standard material, albeit impure, combined with the presence of the same mass fragmentations being found in urine may pave the way for future analyses utilizing the same method. The disappearance of the chromatogram peaks for the urine sample following pepsin digestion provides strong evidence for the process of amino acid conjugation being extended to phthalate monoesters. However, additional runs using different product ion scans corresponding to phthalates other than MEP must be completed in order to justly conclude amino acid conjugation of phthalates in the urine. Pepsin digestion for these other precursors could potentially verify the hypothesis made in this study, proving amino acid conjugation actually occurs in humans.

6. Acknowledgements

The author wishes to express his appreciation to Dr. John Brock for his assistance throughout the project, Dr. Herman Holt for his guidance in completing the synthesis portion of the project, the UNC Asheville Department of Chemistry for the resources it has provided, and the UNC Asheville Undergraduate Research Program for the grant money used during the course of the study.

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