Synthesis and Inhibition Activity of N-benzylbenzamide Derivatives against Tyrosinase

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Abstract

Tyrosinase is an enzyme that converts the amino acid tyrosine into L-DOPA, then converts L-DOPA into dopaquinone, which, through a chain of additional enzymes is converted into melanin, a group of pigment proteins that imparts a brown color to both plants, fungi and animals. The inhibition of tyrosinase directly inhibits the production of melanin. In this project, an optimized method for producing an inhibitor, *N*-benzylbenzamide and its derivatives, was developed, which is intended to be used in a classroom setting. The synthesized derivative compounds were then tested against tyrosinase to determine their effectiveness as inhibitors.

Introduction

Melanin is a pigment protein that is responsible for the dark pigments in plants, fungi and animals. In plants, melanin is responsible for the oxidation and browning of fruits. In fungi, it is responsible for the dark pigmentation. In animals and humans, melanin is the general name for three pigments. Neuromelanin is a black pigment present only in neurons in the brains of animals. Pheomelanin, a red pigment, creates red tones in skin and hair. Eumelanin, the most recognized melanin pigment, is a dark brown pigment, responsible for dark hair, eye and skin color. It also appears in high concentrations in skin discolorations such as moles, freckles and age spots. High concentrations of eumelanin have also been observed in malignant melanoma, a rare skin cancer that accounts for the majority of skin cancer deaths. It is believed that the high concentration of melanin plays a role in the progress of the disease, and so preventing its production may prove to be beneficial to patients.

Tyrosinase is an enzyme that catalyzes the first two steps of several in a reaction sequence that produces melanin, and is found in the same organisms as melanin. The synthesis of all melanin pigments initially begins with the enzyme tyrosinase. Tyrosinase oxidizes the amino acid tyrosine to L-DOPA, and then converts L-DOPA to dopaquinone, which then can be utilized by several additional enzymes, resulting in the production of melanin pigments. Preventing the action of tyrosinase halts the production of all types of melanin. This can be accomplished by the addition of an inhibitor that competes with the substrate, or compound the enzyme acts on, and prevents it from carrying out the reaction.

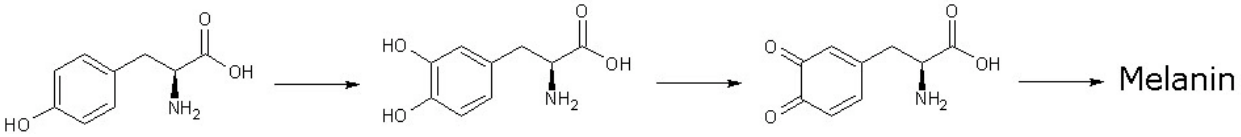


Fig X. Conversion of tyrosine to L-DOPA by tyrosinase.

Several natural inhibitors, such as kojic acid, are already in use in the pharmaceutical industry to lighten cosmetic skin discolorations. Three other classes of chemicals are being studied as tyrosinase inhibitors: the stilbenes, resorcinols and chalcones. Stilbenes and resorcinols are currently in use in over the counter products, but not as tyrosinase inhibitors. These compounds are being researched further to improve their effectiveness as inhibitors.

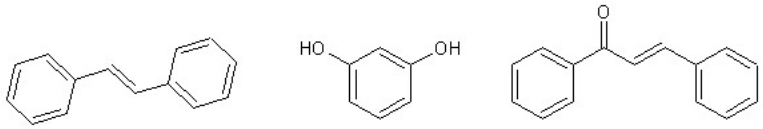


Fig X. The structures of *trans*-Stilbene, Resorcinol, Chalcone

Project Goals

In 2006, *Cho et al* published a paper detailing the inhibitory effects of a class of molecules structurally similar to the chalcones, the *N*-benzylbenzamides.[1] These molecules arestructurally similar to the chalcones, and are effective inhibitors.

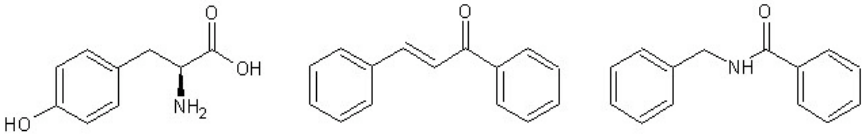


Fig X. Tyrosine, Chalcone and *N*-benzylbenzamide.

Cho et al focused on hydroxy substituted *N*-benzylbenzamides to determine how the number and positions of the group affected the compounds ability to inhibit tyrosine. They determined that the optimal positions for substituent groups were the 3 and 5 position on the A ring and 2 and 4 position on the B ring. *Cho et al* also indicated that the A ring and its substituents, which are structurally similar to resorcinols, seems to be more important to inhibiting tyrosinase than the B ring and its substituents.

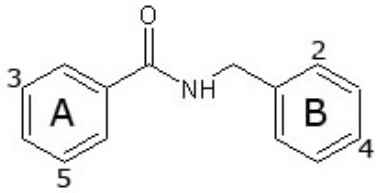


Fig X. Optimal substituent positions on *N*-benzylbenzamide derivatives

This project had two goals. The first was to prepare a method of *N*-benzylbenzamide synthesis that is appropriate for classroom use for organic chemistry students. An appropriate synthesis necessitated the elimination of any especially hazardous, toxic or flammable chemicals in the procedure, then to ensure that the method produced the desired compound even when the experimental procedure varied slightly. The second was to prepare several substituted *N*-benzylbenzamide compounds and test them against tyrosinase to determine their inhibitionary activity. The electronic effects of the substituents were studied to determine if the electronegativity and size had any effect on the inhibition activity. The chosen substuituents chosen were fluorine (F), chlorine (Cl) and the methoxy group. The first two groups, which are halogens, have high electronegativity, and are electron withdrawing groups, pulling electron density towards the halogen atoms making these groups more electron rich, similar to the hydroxy group in tyrosine. Conversely, the methoxy group has lower electronegativity and is considered an electron releasing group, and so is least similar to the hydroxy group. In terms of size, fluorine is the smallest of the studied substituent groups, followed by chlorine and then the methoxy group.

Results

N-benzylbenzamide Synthesis

The *N*-benzylbenzamide derivitives were synthesized using the Schotten-Baumann reaction.[2] The Schotten-Baumann reaction requires a large amount of base in solution to absorb the acid that is produced. In this case, excess HCl was produced as a gas, in addition to being produced in solution.

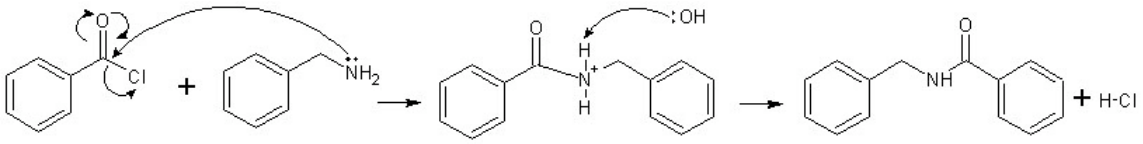


Fig X. Schotten-Baumann reaction

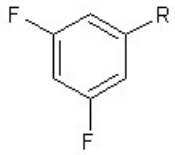
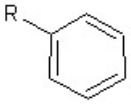
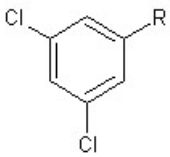
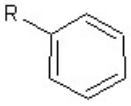
Three reaction conditions were considered:

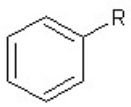
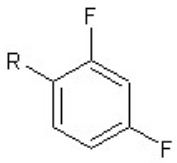
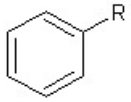
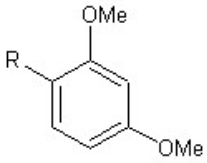
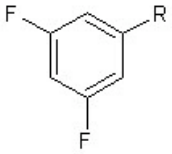
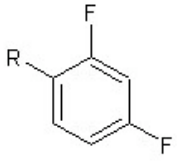
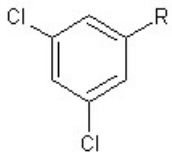
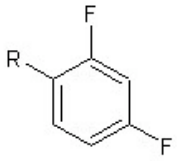
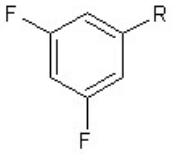
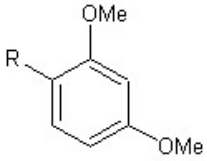
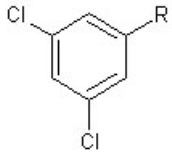
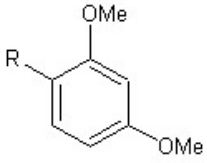
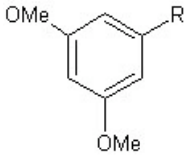
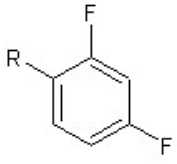
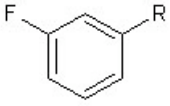
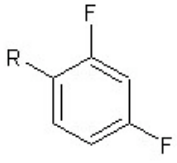
1. THF (solvent) / TEA (base): This procedure was outlined in *Cho et al*. It used tetrahydrofuran (THF) as the solvent and triethylamine (TEA) as the base. While this reaction was successful, the flammability of THF prevented this method from being useful in a classroom setting.

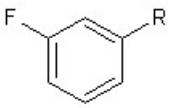
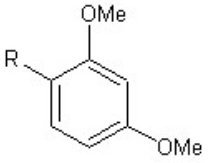
2. H2O (solvent) / NaOH (base): This procedure was adapted from the amine synthesis described by *Marvel et al.*[3] *It* used H2O as the solvent and NaOH as the base. Although a competing reaction occurred between benzoyl chloride and water, this ultimately proved most successful, even though it produced lower yields than the THF/TEA reaction.

3. H2O (solvent) / SDS: This procedure was adapted from *Naik et al* and used H2O as the solvent and sodium dodecyl sulfate (SDS) as the phase transfer catalyst.[4] This procedure was written as an alternative to the use of strong bases as an example of environmentally friendly chemistry. While these conditions were desirable for the classroom setting, the desired product proved difficult to separate from the SDS.

Table 1. Synthesized *N*-benzylbenzamide Derivatives

Compound #	A Ring	B Ring	Molecular Weight	Percent Yield
1			247.24	41.62
2			280.15	71.07

3			247.24	52.93
4			271.31	62.47
5			283.22	42.03
6			316.13	37.91
7			307.29	27.72
8			340.20	53.81
9			307.29	43.64
10			265.23	53.67

11			289.30	49.69

Enzyme Assay

The assay showed the effectiveness of the *N*-benzylbenzamide derivatives as inhibitors of mushroom tyrosinase. The derivative compounds were tested as competitive inhibitors against L-DOPA. If the reaction was not inhibited, then the dark brown dopaquinone appeared in the solution. If the reaction was inhibited, then colorless L-DOPA remained in the solution, producing no color. The assay was analyzed by the ultraviolet light absorption at 342 nm of the solution and was measured with a Bio-Rad Benchmark Plus Microplate Spectrophotometer.

Table 2. Enzyme Assay Results

Compound	Trial 1	Trial 2	Trial 1&2 Avg.	Trial 3	Trial 4	Trial 3&4 Avg.
1	61.0	56.0	58.5	-	-	-
2	46.0	63.0	54.5	-	-	-
3	49.0	38.3	43.7	22.0	14.0	18.0
4	54.0	29.5	41.8	11.0	19.0	15.0
5	40.0	38.0	39.0	24.0	23.0	23.5
6	59.0	49.0	54.0	-	-	-
7	40.0	60.0	50.0	43.0	16.0	29.5
8	60.0	75.0	67.5	38.0	15.0	26.5
9	33.0	6.0	19.5	-	-	-
10	37.0	44.0	40.5	66.0	29.0	47.5
11	41.0	53.0	47.0	10.0	22.4	16.2

Discussion

The synthesis of substituted *N*-benzylbenzamide derivatives was carried out with substituted benzoyl chloride and benzylamine using the H2O/NaOH system. The optimal ratios were determined to be 1:1:3 of benzoyl chloride to benzylamine to NaOH, although varied ratios

produced the desired product, though in smaller quantities. The desired product was initially recrystalized in methanol, but ethanol was later used as the compounds' solubility was lower in ethanol than methanol, making recrystalization easier.

The synthesized compounds were then tested in an visible/ultraviolet light enzyme assay. The assay velocity report indicated that the most effective compounds were compounds 3, 5, 9 and 11. These compounds had halogen groups on the A ring, while the B ring substituent seemed to have little impact as an inhibitor. The electron withdrawing nature of the halogens, which is similar to the hydroxyl group on tyrosine, is sufficient to act as an inhibitor for the enzyme.

Future work

Future work on *N*-benzylbenzamide derivative synthesis should include the use of additional functional groups, with more examples of electron releasing groups, as only one was used in this experiment. Also, the effective groups should be combined with hydroxy groups to determine their effects.

Experimental

Enzyme assay

The enzyme assay was taken from a procedure developed by Winder et al.[5] Each compound was dissolved in dimethylformamide (DMF). The compounds were then added to a 96-well plate containing 70 µL buffer, 100 µL L-DOPA and 30 µL tyrosinase and 2 µL of the synthesized compound. The data was recorded on the Benchmark Plus Microplate Spectrophotometer produced by Bio-Rad

Synthesis

The reaction is carried out in water with a three molar equivalent amount of NaOH added to it. A one molar equivalent of the benzylamine is added to the solution and is vigorously stirred with a magnetic stir bar. A one molar equivalent of benzoyl chloride is added slowly using a dropping funnel and allowed to stir for one hour. The solid is then isolated using vacuum filtration, then recrystalized using ethanol. The resulting product is then vacuum filtered to remove the solution and dry. Each compound was characterized by its melting point, IR Spectra, and 1H NMR in *d*-chloroform.

N-benzyl-3,5-difluorobenzylbenzamide

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.6796 g NaOH was added to 30 mL H2O. 1 mL benzylamine was added to the flask and was set to stir vigorously. 1 mL 3,5-difluorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in MeOH. Percent yield: 41.61%; Mp (°C): 115.2-117.0; IR (cm^{-1}): 3271.83, 3060.60, 2360.55, 2339.55, 1640.40; 1H NMR (ppm): 7.337-6.925 multiplet, 6.451 small bulge, 4.615-4.662 doublet.

N-benzyl-3,5-dichlorobenzylbenzamide

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.5644 g NaOH was added to 30 mL H2O. 1 mL benzylamine was added to the flask and was set to stir vigorously. 1 mL 3,5-dichlorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in MeOH. Percent yield: 71.07%; Mp (°C): 144.5-146.3; IR (cm^{-1}): 3266.70, 3063.73, 3028.56, 2359.94, 1636.20; 1H NMR (ppm): 7.348-7.631 multiplet, 4.665-4.557 doublet.

N-(2,4-difluorobenzyl)benzamide

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.6729 g NaOH was added to 30 mL H2O. 1 mL 2,4-difluorobenzylamine was added to the flask and was set to stir vigorously. 1.5 mL benzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in MeOH. Percent yield: 52.93%; Mp (°C): 90.0-91.4; IR (cm^{-1}): 3300.99, 3061.73, 2360.38, 2339.73, 1635.70; 1H NMR (ppm): 7.706-6.818 multiplet, 4.678-4.595 doublet.

N-(2,4-dimethoxybenzyl)benzamide

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.9525 g NaOH was added to 30 mL H2O. 1 mL 2,4-dimethoxybenzylamine was added to the flask and was set to stir vigorously. 1.5 mL benzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in MeOH. Percent yield: 62.47%; Mp (°C): 102.2-103.2; IR (cm^{-1}): 3309.57, 3054.15, 2004.72, 2930.02, 2832.00, 2359.94, 1623.43; 1H NMR (ppm): 7.813-7.270 multiplet, 4.624-4.536 doublet, 3.866-3.813 doublet.

N-(2,4-difluorobenzyl)-3,5-difluorobenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.5100 g NaOH was added to 30 mL H2O. 0.53 mL 2,4-difluorobenzylamine was added to the flask and was set to stir vigorously. 0.5 mL 3,5-difluorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 42.03%; Mp (°C): 138.4-140.4; IR (cm^{-1}): 3302.69, 3098.56, 1642.11; 1H NMR (ppm): 7.311-6.858 multiplet, 4.685-4.586 doublet.

N-(2,4-difluorobenzyl)-3,5-dichlorobenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.4233 g NaOH was added to 30 mL H2O. 0.24 mL 2,4-difluorobenzylamine was added to the flask and was set to stir vigorously.

0.5 mL 3,5-dichlorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 37.91%; Mp (°C): 142.9-145.9; IR (cm^{-1}): 3250.28, 2360.53, 2339.59, 1738.30; 1H NMR (ppm): 7.640-7.259 multiplet, 4.678-4.585 doublet.

N-(2,4-difluorobenzyl)-3,5-dimethoxybenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.3833 g NaOH was added to 30 mL H2O. 0.55 mL 2,4-difluorobenzylamine was added to the flask and was set to stir vigorously. 0.834 g 3,5-dimethoxybenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 43.64%; Mp (°C): 96.8-98.3; IR (cm^{-1}): 3247.57, 3079.97, 3007.14, 2943.31, 2838.60, 2359.59, 1641.82; 1H NMR (ppm): 7.268-6.565 multiplet, 4.679-4.576 doublet, 3.826-3.813 doublet.

N-(2,4-difluorobenzyl)-3-fluorobenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.505 g NaOH was added to 30 mL H2O. 0.5 mL 2,4-difluorobenzylamine was added to the flask and was set to stir vigorously. 0.55 mL 3-fluorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 53.67%; Mp (°C): 85.0-86.6; IR (cm^{-1}): 3304.72, 1635.55 1H NMR (ppm): 7.476-6.856 multiplet, 4.709-4.601 doublet.

N-(2,4-dimethoxybenzyl)-3,5-difluorobenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.3980 g NaOH was added to 30 mL H2O. 0.5 mL 2,4-dimethoxybenzylamine was added to the flask and was set to stir vigorously. 0.4 mL 3,5-difluorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 27.72%; Mp (°C): 103.2-104.3; IR (cm^{-1}): 3274.03, 3084.01, 2969.33, 2937.05, 2910.20, 2830.70, 1619.38; 1H NMR (ppm): 7.260-6.511 multiplet, 4.589-4.495 doublet, 3.863-3.807 doublet.

N-(2,4-dimethoxybenzyl)-3,5-dichlorobenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.5905 g NaOH was added to 30 mL H2O. 0.3 mL 2,4-dimethoxybenzylamine was added to the flask and was set to stir vigorously. 0.4 g 3,5-dichlodobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 53.81%; Mp (°C): 133.6-134.9; IR (cm^{-1}): 3285.45, 3005.00, 2931.52, 2834.19, 2360.60, 2339.63, 1739.58; 1H NMR (ppm): 7.611-6.509 multiplet, 4.584-4.400 doublet, 3.866-3.807 doublet.

N-(2,4-dimethoxybenzyl)-3-fluorobenzylamine

In a 50 mL Erlenmeyer flask with a magnetic stir bar 0.3980 g NaOH was added to 30 mL H2O. 0.5 mL 2,4-dimethoxybenzylamine was added to the flask and was set to stir vigorously. 0.45 mL 3-fluorobenzoyl chloride was added to a 75 mL dropping funnel and added dropwise. The reaction was allowed to stir for one hour. The product was vacuum filtered and recrystalized in EtOH. Percent yield: 49.69%; Mp (°C): 83.5-85.5; IR (cm^{-1}): 3301.81, 3072.60,

2994.04, 2931.23, 2832.70, 2359.78, 1626.15; 1H NMR (ppm): 7.253-6.503 multiplet, 4.608-4.512 doublet, 3.862-3.86 doublet.

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