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SYNTHESIS OF A N-PHTHILIMIDO & ACETYLATED TYROSINE FOR TYROSINASE INHIBITORY ACTIVITY

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ABSTRACT

The present study deals with the application of α N-Phthilimido and acetylated tyrosine and determination of tyrosinase inhibitory activity which serves as an useful target in the treatment of hyper pigmentation skin disorder. The tyrosinase inhibitory activity of α N-Phthilimido tyrosine found to be highly significant ($p< 0.0001$) when compared with that of acetyltyrosine

KEYWORDS

α N-Phthilimido & Acetylated tyrosine, tyrosinase inhibition and hyper pigmentation.

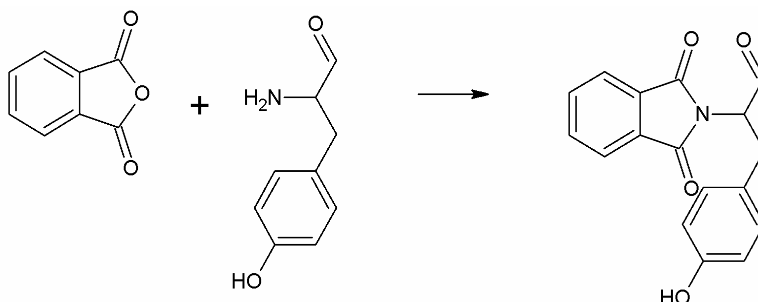
INTRODUCTION

As tyrosinase enzyme (copper containing polyphenol oxidase) is responsible for pigmentation of skin, eyes and hair through enhancement of melanin production, the determination of tyrosinase inhibitory activity would be useful in the treatment of skin pigmentation disorders. Phthalimido and acetamido analogues was found to exhibit antibacterial, antifungal, anticancer and antiviral activities. Hence in the current study an attempt was made to study the tyrosinase inhibitory. As tyrosinase enzyme (copper containing polyphenol oxidase) is responsible for pigmentation of skin, eyes and hair through enhancement of melanin production, the determination of tyrosinase inhibitory activity would be useful in the treatment of skin pigmentation disorders.

Melanin biosynthesis can be initiated from either the hydroxylation of L-Phenyl alanine to L-tyrosine or directly from L-tyrosine which is then hydroxylated to L-dihydroxy phenyl alanine (L-DOPA). L-DOPA acts as a precursor to both melanins, Catecholamines which act along separate pathways

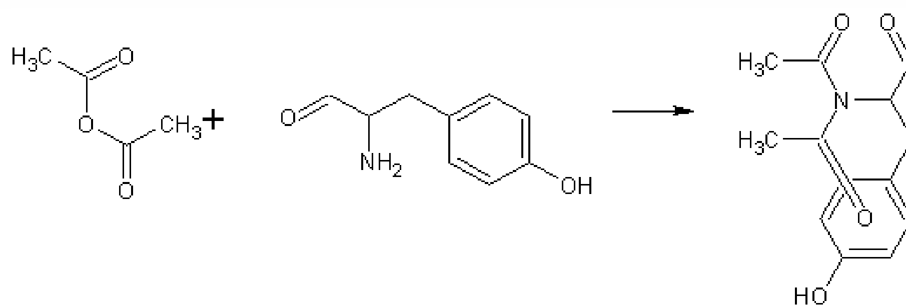
MATERIAL & METHOD

1. ***i)Synthesis of α N-Phthilimido Tyrosine(PT)***



Equimolar quantities of phthalic anhydride acid, tyrosine and distilled water. The above was mixed well and heated on a heating mantle and refluxed for two & half-hours. After reflux, the hot mixture was transferred and kept in a beaker at room temperature, over night. The crystalline product, which was found to be insoluble in benzene and chloroform but soluble in hot water, was collected by filtered & dried to remove moisture. The unreacted raw materials were removed by repeatedly shaking with 50ml portions of benzene A.R. and then with 50ml portions of chloroform A. R. filtered, dried and recrystallised from hot water. The pure product was collected by filtration and dried to remove moisture.

ii)*Synthesis of α N-Acetyl Tyrosine(AT):*



Equimolar quantities acetic anhydride, thyrosine and distilled water. The above was mixed well and heated on a heating mantle and refluxed for two & half-hours. After reflux, the hot mixture was transferred and kept in a beaker at room temperature, over night. The crystalline product, which was found to be insoluble in benzene and chloroform but soluble in hot water, was collected by filtered & dried to remove moisture. The unreacted raw materials were removed by repeatedly shaking with 50ml portions of benzene A.R. and then with 50ml portions of chloroform A. R. filtered, dried and recrystallised from hot water. The pure product was collected by filtration and dried to remove moisture.

Melting point of all synthesized compound were determined by using open capillary method and are uncorrected. The precoated alumina plates with silica gel GF254 (E.Merck) were used for purity determination and pet: ethyl acetate (1:2) was employed as irrigate. IR spectra were recorded in (cm^{-1}) ABB BOMEM FT-IR Spectrometer using KBr pellet technique. ^{1}H-NMR[11] and ^{13}C-NMR were recorded (in δppm) on BRUKER AV 400 using TMS as internal standard[11].

2. *Determination of total phenolics:*

Folin-ciocalteau method was used to determine total phenolics. About 10mg of analogues was made upto 10ml using methanol: water in the ratio of 20: 30. 1ml of the above solution was treated with 5ml of folin's ciocalteau phenol reagent (1:2) and 4ml of 7.5% sodium carbonate solution were added and mixed well. Standard solution of gallic acid in methanol (10 to 100µg/ml) were prepared and treated in identical manner. After 1hour 30 minutes, the absorbance of test and standard solutions at 750nm was measured on Lambda25 (Perkin Elmer UV Spectrophotometer). The total phenolic content was calculated graphically using Gallic acid standard curve and the results were expressed as gallic acid equivalent [12].

3. *Determination of reducing power*

About 2.5ml of analogues was treated with 2.5ml of phosphate buffer (0.2m, pH 6.6), 2.5ml of 1% potassium ferricyanide (10mg/ml) and was then incubated at 50°C for 20 minutes. It was then rapidly cooled and treated with 2.5ml of 10% trichloroacetic acid and then centrifuged at 6500 rpm for 10minutes. The supernatant was then treated with distilled water and 0.5ml of ferric chloride solution. It was then allowed to stand for 10minutes. The absorbance was measured at 700nm using L-ascorbic acid as standard [13].

4. Determination of tyrosinase inhibitory activity

About 20µL of Mushroom Tyrosinase solution (1000U/ml) was treated with 0.1M phosphate buffer (pH 6.8) and 20µL of test analogues was taken. Then sample solution without enzyme was also prepared in the same manner. Blank solution with and without enzyme was also prepared. About 20µL of

0.55mM L-DOPA solutions as substrate was added into every sample and blank. These assay mixtures were incubated at 37 °C for 10minutes, the amount of dopachrome produced in the reaction mixture was measured at 475nm (Perkin Elmer UV Spectrophotometer). The percentage inhibition of Tyrosinase inhibitory activity was calculated using following formula:

$$\text{Percentage Tyrosinase inhibition} = \frac{(A - B) - (C - D) \times 100}{(A - B)}$$

Where A = Absorbance of blank solution with enzyme; B = Absorbance of blank solution without enzyme; C = Absorbance of sample solution with enzyme; D = Absorbance of sample solution without enzyme [14, 15].

STATISTICAL ANALYSIS

The percentage inhibitory activity of the samples obtained was analyzed statistically by one way ANOVA followed by Dunnets test at $P < 0.0001$.

(1) Percentage yield of Analogues

The results of percentage yield of analogues obtained have been shown in table 1. The maximum percentage yield was obtained by PT analogue which was high when compared with that AT analogue.

Table 1

Percentage yield of Analogues

Analogues	Percentage yield
PT	92%
AT	87%

2) *Total phenolic content*

The extract obtained by MAE was found to contain maximum amount of phenolic content when compared with that obtained by SE method as shown in table 2

Table 2

Total phenolic content

Sample	Total phenolics (µg/mg)
PT	109
AT	50

(3)*Reducing power*

Similarly the reducing power indicated by an increase in absorbance value was evaluated for both the Analogues (PT & AT) The Product was found to possess more reducing ability as shown in table 3.

Table 3

Reducing power of Analogues obtained by different extraction methods

Concentration	Standard Ascorbic acid	PA	AT
30	0.1215±0.0091	0.0028±0.0004	0.0027±0.0003
45	0.1777±0.0030	0.0741±0.0034	0.0242±0.0468
60	0.2451±0.0030	0.1662±0.086	0.0933±0.047

The reducing power of Analogues was found to be highly significant at $p < 0.0001$

(4) Tyrosinase inhibitory activity

The Tyrosinase inhibitory activity of PT analogue was found to be more than that of AT analogue. The results had been shown in table 4.

Table 4

Tyrosinase inhibitory activity

Analogues	Percentage inhibition
PT	75.49± 0.32%
AT	65.85±0.13%

The percentage tyrosinase inhibitory activity of analogue was found to be highly significant at $p < 0.0001$ when compared with that obtained by PT- Phthalimido Tyrosine Inhibitory; AT – Acetyl Tyrosine Inhibitory

DISCUSSION

PT was considered to be the best Analogues due to significant reduction in extraction time which varied between few seconds to few minutes, improved extraction yield and suitability for thermo labile constituents. Also the results obtained showed the PT possesses increased reducing power which forms the basis for exploring the Tyrosinase inhibitory potential of Analogues. Tyrosinase is a copper containing enzyme hence any substance which reduces this metal ion was considered as an effective Tyrosinase inhibitor. The reducing power reported might be due to presences of tyrosine in analogues form. The possible mechanism underlying behind the Tyrosinase inhibitory ability might be chelation of copper ion present in Tyrosinase enzyme by it analogues and thereby suppression of tautomerisation to dopochrome by the plant extract, thereby the PT and AT extract acts as reducing agents on melanin intermediates by blocking oxidation chain reaction at various points from Tyrosinase/ DOPA to melanin and hence causing reduction of skin pigmentation.

CONCLUSION

Phthalimido and acetamido tyrosine was found to be rich in phenolic compounds consisting of hydrophobic part which would have acted as competitive inhibitor on the enzyme tyrosinase and thereby on melanin synthesis. Hence the determination of tyrosinase inhibitory potential of PT & AT paves a way for development of skin whitening agents. The investigation of Tyrosine analogues on human melanocytes has to be performed to confirm the anti hyper pigmentation effect

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