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AN ENZYMATIC SYNTHESIS OF 2-AZIDO-L-TYROSINE

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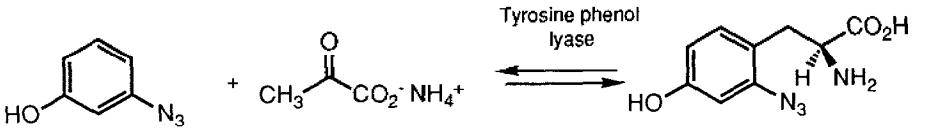
Abstract: Tyrosine-phenol lyase-catalyzed coupling of 3-azidophenol and pyruvic acid produced 2-azido-L-tyrosine in good yield. Preliminary results indicate that this new analogue may serve as a replacement for tyrosine in several enzymatic processes.

As an extension of our research with analogues of biogenic amines and their amino acid precursors,[1] we have initiated synthetic efforts to prepare azido-substituted analogues of such naturally occuring catechols as 3-(3,4-dihydroxyphenyl)alanine (DOPA), dopamine (DA) and norepinehrine (NE). These analogues would have the potential of serving as active analogues which, at the same time, could function as photoaffinity labels and irreversible inhibitors of key enzymes in the production and metabolism of these neurotransmitters. While the sensitive nature of the azido group has complicated the design of synthetic routes, we are currently pursuing chemical syntheses of such compounds. As a first step in studying the effects of the azido group on biological activity in this series, we have used an enzymatic procedure for the synthesis the 2-azido analogue (**1**) of tyrosine, the biological precursor of DOPA, DA and NE.

Tyrosine-phenol lyase has been used to prepare a series of tyrosine and DOPA analogues from phenols or catechols and pyruvic acid or serine.[2] A requirement for enzyme-catalyzed coupling is the presence of an unsubstituted position *para* to the phenolic OH in the substrate. *meta*-Azidophenol (**2**)[3] is a good substrate for tyrosine phenol lyase, and **1** can be isolated in 54% yield (55% conversion). Thus, a solution of 450 mg of **2** (3.33 mmol), 407 mg pyruvic acid, 1.8 mg of pyridoxal-5'-phosphate, 711 mg of ammonium acetate and 10 units of tyrosine phenol lyase[3] in 74 ml of water was adjusted to pH 8 with dilute NH_4OH and stored in the dark at room temperature for 4 d. The mixture was then acidified with dilute acetic acid and filtered through Celite. The filtrate was extracted twice with 50 ml of ethyl acetate to remove unreacted azidophenol [200 mg (1.48 mmol) azidophenol was recovered]. An Amberlite IRA-118H column (1.5 x 10 cm) was activated with 2 N HCl and washed with water. The reaction mixture was passed through the column and eluted with 4% NH_4OH. The fractions that gave a positive ninhydrin reaction after spotting on silica gel plates were combined and lyophilized to give **1** as

a white solid. Recrystallization from water gave 225 mg (1.01 mmol) of analytically pure **1**, mp 200-210 °C (decomp), $[\alpha]_D^{23}$ -8.62 (c = 4, 1 N HCl), IR (KBr) cm^{-1} 3700-2600, 2110, 1605, UV (H_2O) λ_{max} 219, 250, 290, MS (CI, NH_3): 223 (M + 1)$^+$, 179 (M - COOH + 1)$^+$, 149 [$HOPhN_3(CH_3)$]$^+$, ^1H NMR (D_2O) δ 2.93 (1H, dd, J_1 = 14.5 Hz, J_2 = 8.2 Hz), 3.20 (1H, dd, J_1 = 14.5 Hz, J_2 = 5.3 Hz), 3.94 (1H, dd, J_1 = 8.2 Hz, J_2 = 5.3 Hz), 6.64 (1H, dd, J_1 = 8.3 Hz, J_2 = 2 Hz), 6.75 (1H, d, J = 2 Hz), 7.12 (1H, d, J = 8.3 Hz).

Anal. calcd for $C_9H_{10}N_4O_3$ + 1/3 H_2O, C 47.37, H 4.68, N 24.56; found, C 47.44, H, 4.80, N 24.48.



2 **1**

The effect of 1 on amino acid incorporation and on DNA synthesis. The effect of **1** on the incorporation of [^{14}CU]tyrosine and [^{3}H]leucine into protein were examined in cell cultures of PC12 cells. Culture of PC12 cells was carried out as previously described.[4] The incorporation (2μCi/well) of [^{14}CU]tyrosine, [^{3}H]leucine, and [^{3}H]thymidine were measured in the presence and absence of 2-azidotyrosine (1.0 mM) over a period of 72 hours. As shown in figure 1, the incorporation of [^{14}CU]tyrosine was inhibited by approximately 58 to 38% (hatched bars) compared to untreated controls (solid bars) at 24 and 72 hrs. The incorporation of [^{3}H]leucine was not significantly different from control. The inhibition of [^{3}H]thymidine incorporation was 34 and 24% at 24 and 72 hours respectively. Thus, the apparent incorporation of tyrosine into PC12-cellular protein was significantly reduced in the presence of **1** over a 72 hr period of linear growth. The incorporation of leucine was less than that of tyrosine and was not significantly inhibited by the presence of **1**. These results suggest that 2-azidotyrosine can specifically inhibit the incorporation of tyrosine into protein in PC12 cells.

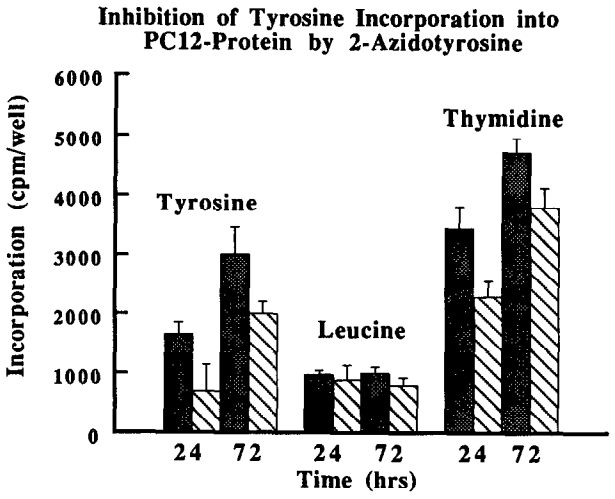


Figure 1. Incorporation of [^{14}CU]Tyrosine and [^{3}H]Leucine into PC12 Cell Protein: Inhibition by 2-Azidotyrosine. PC12 cells were grown as monolayers in 150 cm^2 tissue culture flasks at 37°C in 6% CO_2 in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum, 7% horse serum, and 100 µg streptomycin and 100 units of penicillin per ml. The cells were split after 3 days and aliquots (2 ml) transferred to 6 well culture plates. 2-Azidotyrosine [final concentration, 1 mM, (hatched bars)], and either [^{14}CU]tyrosine (2 µCi/well), or [^{3}H]leucine (2 µCi/well), or [^{3}H]thymidine (2 µCi/well) were added in 0.5 ml solutions in cell media. The final volume of each well was adjusted to 3.0 ml. The cultures were incubated in the dark at 37°C in 6% CO_2 for 12, 24, 48 and 72 hrs. Cells were removed and transferred to conical tubes (10 ml), sedimented by centrifugation, and washed 3X with phosphate buffered saline, pH 7.2. The cell sediment was dissolved in 0.5 ml of a solution of 0.5 N NaOH containing 1% SDS over a period of 30 min., neutralized with 0.5 ml 0.5 N HCl, and the isotope content measured by scintillation spectrometry. The results are expressed as the mean ± SEM for 3 wells for each set of conditions.

As expected, **1** is a good substrate for tyrosine-phenol lyase catalyzed hydrolytic cleavage to ammonium pyruvate and 3-azidophenol, with a K_m of 1 mM (L-tyrosine K_m = 0.28 mM)[5] and V_{max} 18% that of L-tyrosine. Suprisingly, **1** does not appear to be hydroxylated by mushroom tyrosinase (Sigma), since there were no detectable changes in its UV and visible absorption

spectra upon extensive incubation (data not shown). However, addition of **1** (0.1 mM) to tyrosinase solutions containing equimolar L-tyrosine caused the normal lag period for oxidation of tyrosine to increase by as much as 3-fold, as determined by the production of dopaquinone at 480 nm. Thus, **1** is apparently able to bind to mushroom tyrosinase.

From these preliminary results, it appears that **1** may mimic L-tyrosine in certain biochemical processes. We are expanding our biological evaluation of **1** to include other tyrosine-processing enzymes. The apparent binding of **1** to mushroom tyrosinase, with no apparent oxidation, suggests the potential for **1** as a photoaffinity label of this enzyme. These and other labelling possibilities are being explored.

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References and Notes

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2. Nagasawa, T.; Utagawa, T.; Goto, J.; Kim, C.-J.; Tani, U.; Kumagai, H.; Yamada, H.; *Eur. J. Biochem.*, **1981**, *117*, 33. The tyrosine phenol-lyase used in these studies was prepared from Citrobacter freundii cells (ATCC 29063) grown on a medium containing 0.1% L-tyrosine.

3. Ugi, I.; Perlinger, H.; Behringer, L.; *Chem Ber* , **1958**, *91*, 2330. The literature procedure included neutralization of a solution of 3-hydroxybenzenediazonium chloride in HCl prior to addition of sodium azide. This resulted in extensive azo coupling, chlorophenol formation, and gave very low and inconsistent yields of azidophenol. To avoid these complications, we carried out the diazotization of 3-aminophenol in cold fluoroboric acid. Sodium azide was added prior to neutralization of the cold solution. After adjustment of the pH to 5-7, the solution was extracted with ether, washed with water, and prepurified by passage through a suction funnel containing silica gel. Evaporation of the residue and chromatography over silica gel (petroleum ether-ethyl acetate; 4:1) gave pure **2** in 80 % yield.

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