

Synthesis of 5-Iodo- and 5-Nitro-3-indolyl Phosphates as Cytochemical Substrates for Acid Phosphatase (1,2)

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The synthesis of 5-iodo-3-indolyl and 5-nitro-3-indolyl phosphate for cytochemical demonstration of acid phosphatase is reported. Enzymatic hydrolysis of these compounds liberates the corresponding indoxyl which is oxidized to the substituted indigo at an acid pH. In contrast, the 5-bromo and the unsubstituted indoxyl are not oxidized readily at an acid pH. Comparison of the UV and visible spectra of the disubstituted indigo has offered a partial explanation for this difference. E.S.R. measurements showed that 5,5'-diiodoindigo, unlike 5,5'-dibromoindigo, gives a detectable radical ion at an acid pH.

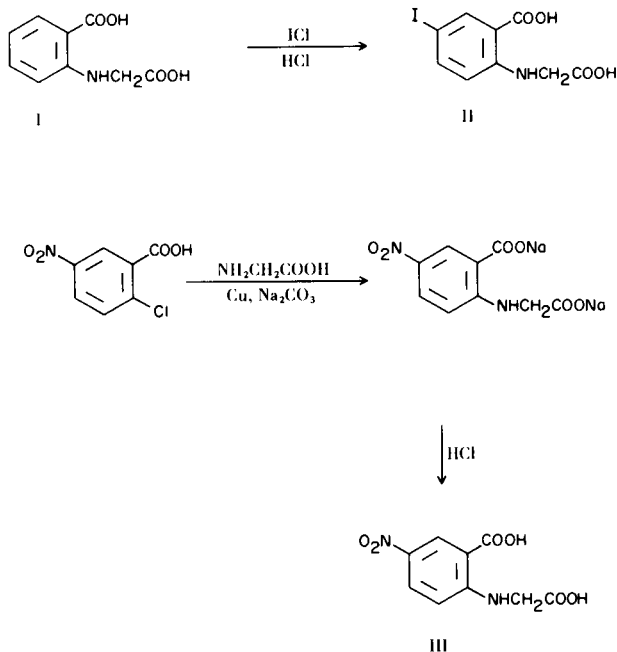
Previously we reported the synthesis of 3-indolyl phosphate and 5-bromo-3-indolyl phosphate and their use for the histochemical demonstration of alkaline phosphatase (3). Because of the relatively slow rate of oxidation of indoxyl at an acid pH, these substrates are not suitable for acid phosphatase. In searching for other derivatives, we now wish to report the synthesis of 5-iodo and 5-nitro-3-indolyl phosphate which do lead to the formation of 5,5'-diiodo- and 5,5'-dinitroindigo when hydrolyzed at an acid pH. These substrates are therefore suitable for the histochemical demonstration of acid phosphatase (4). The

purpose of this paper is to report the synthesis and structure study of these substrates. These data are essential for their histochemical application at both light and electron-microscope levels (5).

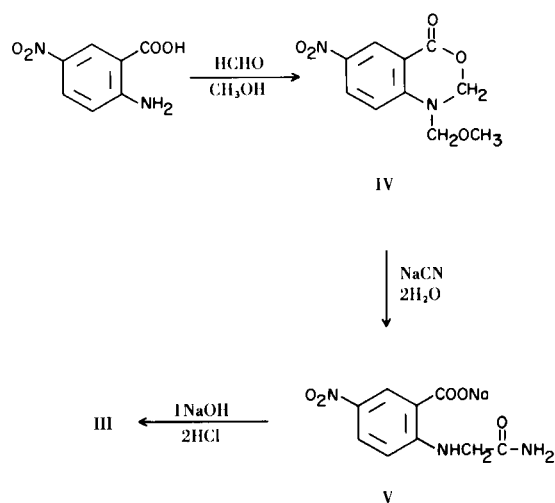
Anthranilic acid is reacted first with chloroacetic acid to give 2-carboxyphenylglycine (I). Iodination of I with iodine monochloride then yielded *N*-(4-iodo-2-carboxyphenyl)glycine (II). The corresponding nitro-substituted acid (III), however, was prepared only when 2-chloro-5-nitrobenzoic acid was condensed with glycine, in the presence of both copper powder and sodium carbonate (Scheme 1).

Alternately, 5-nitroanthranilic acid was reacted with formaldehyde in methanol to give 1-methoxymethyl-6-nitro-1,3-benzoxazine-4-one which was treated with sodium cyanide and hydrolyzed to yield the sodium salt of *N*-(4-nitro-2-carboxyphenyl)glycine amide. Further alka-

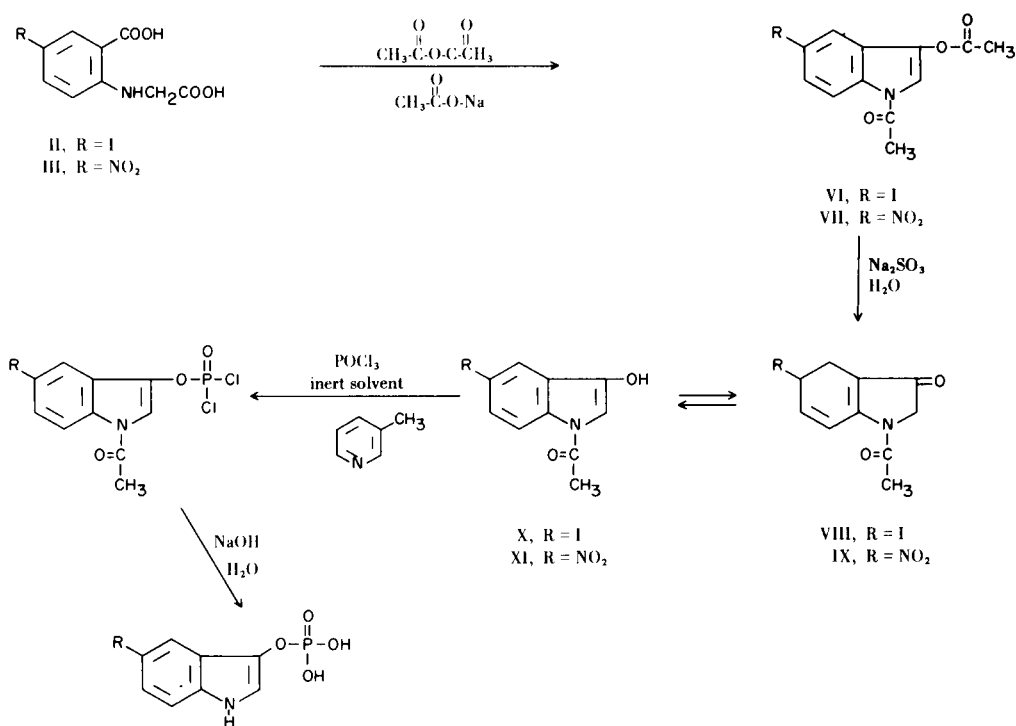
SCHEME 1



SCHEME 2



SCHEME 3

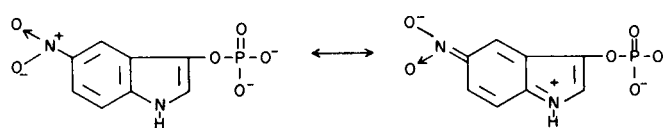


line hydrolysis of the latter compound and careful treatment with hydrochloric acid then gave the acid III (Scheme 2).

Once II and III are made, the preparation of the substituted indolyl phosphate can be summarized (Scheme 3) and is essentially similar to that given previously (3). The phosphorylation step was found to proceed better however in an inert solvent such as benzene or *p*-dioxane and in the presence of β -picoline. Isolation of the free 3-indolyl phosphate was best done *via* its calcium salt and then converted to the sodium salt by treatment with sodium carbonate.

The rate of hydrolysis and subsequent oxidation of the unsubstituted 5-bromo- and 5-iodo-3-indolyl phosphates with intestinal alkaline phosphatase were compared at pH 9.2 (6) and with acid phosphatase at pH 5.4. While it was found that the 5-iodoindolyl substrate can also be used for alkaline phosphatase, at acid pH only the 5-iodo and the 5-nitro substrates lead to indigo formation.

The UV and visible spectra of the sodium salts of substituted indolyl phosphates in water solution were compared (Table I). Halogen substitution in 3-indolyl phosphates resulted in a bathochromic shift of the absorption maxima. The longer displacement for the nitro group can be due to the following resonance contribution.



While IR spectra of all the substituted indigo were similar, UV and visible spectra obtained in polyvinyl alcohol suspension were different (Table I).

In PVA suspension, the visible spectra of the substituted indigo stand in the hypsochromic order as to wavelength position: H > Br > I > NO₂. The unsubstituted compound absorbs at the longest wavelength and the I-substituted at the shortest. However, the iodine substituted indigo does have another low intensity band at 690 nm ($\epsilon \sim 700$). This spectroscopic data accounts for the mixed blue and purple appearance of the dye deposits that one sees with this substrate upon hydrolysis and subsequent oxidation, and is the basis for the *in vivo* use of 5-iodo-3-indolyl phosphate for ruby laser damage in cells (8), since ruby laser emits at 694 nm.

E.S.R. study of the 5-iodo- and 5-bromo compounds were also compared. Only in the 5-iodo case was there a signal present at an acid pH (Fig. 1). It is thus our opinion that the usefulness of the 5-iodo derivative must be related to the stability of the intermediate free radical at the acid

pH. Attempts to detect this reaction *in vivo* with E.S.R. technique have not yet been successful in our hands.

TABLE I

UV and Visible Spectra of Substituted Indolyl Phosphates (a) and the Corresponding Indigo (b).

Compound	λ max nm ($\times 10^{-3}$)	Compound	λ max nm ($\times 10^{-3}$)
H	221.3 (42.3) 278.8 (5.58)	indigo	215.0 (20.4) 250.0 (20.0) 287.5 (22.8) 662.5 (12.6)
Br	230.0 (34.9) 288.8 (4.77)	5,5'-dibromoindigo	213.8 (14.0) 251.3 (19.1) 290.0 (16.2) 577.5 (4.70)
I	232.5 (32.7) 288.8 (4.10)	5,5'-diiodoindigo	217.5 (19.6) 282.5 (13.4) 550.0 (3.46) 690.0 (0.70)
NO ₂	211.3 (7.80) 277.5 (4.65) 340.0 (2.66)	5,5'-dinitroindigo	317.5 (12.4) 550.0 (8.9)

(a) Concentration $0.465 \times 10^{-4} M$ in water. (b) Concentration $0.167 \times 10^{-4} M$ in 0.5% PVA aqueous solution.

E.S.R. Spectra of Radical Ion of 5,5'-diiodoindigo



Figure 1

EXPERIMENTAL

Melting points were determined on a Mel-Temp laboratory device. Elementary analyses were performed by Dr. S. M. Nagy. UV Spectra.

UV and visible spectra were obtained with a Beckman DB-G Grating Spectrophotometer equipped with a Deuterium lamp.

The indigo compounds were determined as colloidal suspensions in 0.5% (wt/wt) polyvinyl alcohol (PVA) in water, and they were generated *in situ* by the hydrolysis of the corresponding 3-indolyl acetate.

N-(4-iodo-2-carboxyphenyl)glycine II (10).

2-Carboxyphenyl glycine (9) (19.5 g., 0.1 mole) was suspended in 145 ml. of 0.8 *N* hydrochloric acid. While the suspension was cooled to 20°, iodine monochloride (20 g., 0.125 mole) dissolved at 5° in 100 ml. of 1*N* hydrochloric acid, was added with stirring. The reaction mixture was stirred at room temperature for 5 hours, and then allowed to stand overnight. The crude product was collected by filtration and recrystallized from ethanol-water to give 26.5 g. (82.6%) of II, m.p. 170-172°.

Anal. Calcd. for C₉H₈INO₄: C, 33.67; H, 2.51; N, 4.36. Found: C, 33.59; H, 2.70; N, 4.29.

5-iodo-1-acetyl-3-indoxyl Acetate (11).

The acid II (28.2 g., 0.088 mole) was added slowly with stirring to a hot stirred mixture of anhydrous sodium acetate (28.2 g., 0.344 mole) and 264 ml. (285 g., 2.80 moles) of acetic anhydride. Stirring and heating were continued until carbon dioxide evolution was complete. The reaction mixture was then cooled and treated with ice and cold water. The crude product was recrystallized from methanol-water to give 5-iodo-1-acetyl-3-indolyl acetate, m.p. 108-110°, yield 16.2 g. (53.6%).

Anal. Calcd. for C₁₂H₁₀INO₃: C, 42.00; H, 2.94; N, 4.08. Found: C, 41.78; H, 2.90; N, 4.14.

5-Iodo-1-acetyllindol-3-ol (12).

An aqueous solution (125 ml.) of 8 g. (0.0318 mole) of sodium sulfite heptahydrate was added to 5-iodo-1-acetyl-3-indolyl acetate (10.0 g., 0.0293 mole) in 75 ml. of hot ethanol. The reaction mixture was refluxed for 20 minutes and then cooled. The crude product was collected by filtration and recrystallized from methanol to give 5-iodo-1-acetyllindol-3-ol, m.p. 189-191°, yield 4.8 g. (54.9%).

Anal. Calcd. for C₁₀H₈INO₂: C, 39.89; H, 2.68; N, 4.65. Found: C, 70.10; H, 2.75; N, 4.78.

Calcium Salt of 5-Iodo-3-indolyl Phosphate.

5-Iodo-1-acetyllindol-3-ol (4.44 g., 0.0148 mole) was dissolved in 160 ml. of boiling dry benzene. Pure β -picoline (1.6 ml., 0.0165 mole) was then added to the benzene solution, followed by the addition of phosphorus oxychloride (1.4 ml., 0.014 mole). The resulting reaction mixture was heated for 22 hours at 60°. The β -picoline hydrochloride was removed by filtration under nitrogen. The filtrate was added slowly with stirring to 100 ml. of 0.1 *N* sodium hydroxide with cooling, and the resulting solution was stirred for 1½ hours. The sodium salt of di(5-iodo-3-indolyl)-pyrophosphate which had formed was removed by filtration. The aqueous layer of the filtrate was separated from the benzene layer and adjusted to pH 9 with glacial acetic acid. A solution of 0.75 g. (0.0029 mole) of magnesium nitrate hexahydrate and 0.75 g. (0.014 mole) of ammonium chloride in 6 ml. of water was then added to the aqueous layer, and the resulting reaction mixture was stirred for 1 hour. A small amount of charcoal was then added, and the precipitate containing magnesium di(5-iodo-3-indolyl)-pyrophosphate was removed by filtration. The filtrate was heated rapidly to 70-80° and then treated with a solution of 5.2 g. (0.030 mole) of calcium acetate monohydrate in 26 ml. of water. The calcium salt of 5-iodo-3-indolyl phosphate was collected by filtration, washed with water, ethanol and acetone, dried briefly in air, and then stored in a desiccator over calcium sulfate, yield 3.0 g. (54.0%).

Disodium Salt of 5-Iodo-3-indolyl Phosphate.

The calcium salt of 5-iodo-3-indolyl phosphate (3.0 g., 0.008 mole) was finely powdered and mixed with 1.31 g. (0.0123 mole)

of sodium carbonate in 450 ml. of water and heated to boiling. The mixture was then stirred for 1 hour without further heating, and decolorized with a small amount of charcoal. The charcoal and the calcium carbonate which had been formed in the reaction were then removed by filtration. The filtrate was freeze-dried to give the disodium salt of 5-iodo-3-indolyl phosphate (3.07 g., 100% yield). It was recrystallized from water and ethanol to yield 1.25 g. of pure product, and it was analyzed as a dihydrate.

Anal. Calcd. for $C_8H_5INO_4PNa_2 \cdot 2H_2O$: C, 22.93; H, 2.16; N, 3.34. Found: C, 22.53; H, 2.25; N, 3.12.

5-Nitroanthranilic Acid.

This preparation was a modified procedure of Dunn and Prysiazniuk (13). Carboxy-4-nitroacetanilide (7.0 g., 0.031 mole) was hydrolyzed with 84 ml. of concentrated hydrochloric acid in 21 ml. of ethanol by heating the mixture under reflux for 2 hours. The resulting 5-nitroanthranilic acid was removed by filtration, washed with water, and allowed to dry in air, m.p. 273-280°, yield 4.5 g. (79.1%). The pure product was obtained by recrystallization from boiling water. After drying under vacuum over phosphorus pentoxide at 80° for 5 hours, it had a m.p. of 280-282°. The previous authors reported a m.p. of 268-269°.

1-Methoxymethyl-5-nitro-1,3-benzoxazine-4-one (IV).

This compound was prepared by a method similar to that of Holt and Sadler (15). 5-Nitroanthranilic acid (4.5 g., 0.025 mole) in 225 ml. of boiling methanol was added an excess of 40% formalin (9 ml., 0.12 mole) while refluxing. After 5 hours, the reaction mixture was added. The yellow crude product was collected by filtration and washed with 5% sodium bicarbonate solution. The insoluble residue was recrystallized from methanol twice to yield 3.73 g. (62.6%) of pure IV, m.p. 182-184°.

Anal. Calcd. for $C_{10}H_{10}N_2O_5$: C, 50.47; H, 4.24. Found: C, 50.70; H, 4.51.

2-Carboxy-4-nitrophenylglycineamide (V).

When the exact conditions based on Holt and Sadler (15) for the preparation of *N*-cyanomethyl anthranilic acids were followed, we obtained instead the amide V. A suspension of IV (0.166 g., 0.0007 mole) in an aqueous solution (75 ml.) of sodium cyanide (0.094 g., 0.0019 mole) was stirred and the temperature was gradually raised to 60° over a period of 45 minutes and maintained at 60-65° for an additional 1½ hours. The mixture was then cooled and acidified with 4 *N* hydrochloric acid to precipitate V which was collected by filtration, m.p. 205-212°, yield 0.075 g. (31.6%). An analytical sample was prepared by dissolving it in aqueous sodium bicarbonate, filtering, and reprecipitating it from the filtrate with 4 *N* hydrochloric acid. This was followed by recrystallization twice from aqueous ethanol, dried under vacuum over phosphorus pentoxide at 110° for 2 hours, m.p. 265-267°.

Anal. Calcd. for $C_9H_9N_3O_5$: C, 45.19; H, 3.79; Found: C, 43.38; H, 3.52

N-(4-Nitro-2-carboxyphenyl)glycine (III).

This compound was prepared by a minor modified procedure of Holt and Sadler (15). When V (0.06 g., 0.003 mole) was refluxed with 25 ml. (0.05 mole) of 2 *N* sodium hydroxide for 2½ hours, the reaction mixture was cooled and acidified with 4 *N* hydrochloric acid to obtain III, m.p. 210-215°, yield 0.041 g. (57.0%).

Another procedure for the preparation of III was based on that of Holt and Petrow (16). A mixture of 2-chloro-5-nitrobenzoic acid (10.0 g., 0.050 mole), glycine (5.0 g., 0.67 mole), sodium carbonate (5.5 g., 0.052 mole), copper powder (1.0 g., 0.0158 mole) and 100 ml. of water was stirred and refluxed for

2 hours. It was then filtered while hot and the filtrate was cooled. Concentrated hydrochloric acid was added until the solution was acid to Congo-red paper. The crude III was collected, washed with water, and allowed to air dry. The crude product weighed 11.7 g., m.p. 142-175°. Recrystallization from boiling water yielded 4.5 g. (35.9%) of pure material, m.p. 225-227° dec., (Lit. [16], m.p. 226-227°).

Attempts to prepare III from 5-nitroanthranilic acid and chloroacetic acid under a variety of reaction conditions were unsuccessful. 5-Nitro-1-acetyl-3-indolyl Acetate (VII).

The nitro acid III (2.0 g., 0.008 mole) was added slowly with stirring to a hot mixture of sodium acetate (2.0 g., 0.025 mole) and 34 ml. of acetic anhydride (36.6 g., 0.35 mole) over a period of 15 minutes. The product VII was isolated in the same manner, and was recrystallized from aqueous methanol to yield pure VII, 1.09 g. (49.5%), m.p. 215-217° (Lit. [16], m.p. 219-220° on recrystallization from glacial acetic acid).

5-Nitro-1-acetylindol-3-ol (IX).

To 5-nitro-1-acetyl-3-indolyl acetate (11.93 g., 0.46 mole) in 300 ml. of hot ethanol was added a hot solution (300 ml.) of 11.4 g. (0.046 mole) of sodium sulfite heptahydrate. The reaction mixture was refluxed for 20 minutes and then cooled. The crude IX was collected by filtration, m.p. 234-237°, yield 8.7 g. (86.6%). Pure IX was obtained by recrystallization from methanol, 2.6 g. (26.5%), m.p. 235-244°.

Anal. Calcd. for $C_{10}H_8N_2O_4$: C, 54.58; H, 3.66; N, 12.74. Found: C, 54.55; H, 3.89; N, 12.91.

Calcium Salt of 5-Nitro-3-indolyl Phosphate.

A dioxane solution (3.25 ml.) of IX (3.26 g., 0.015 mole) was added, 1.6 ml. (0.017 mole) of β -picoline first, then 1.4 ml. (0.014 mole) of phosphorus oxychloride, under a nitrogen atmosphere. It was stirred and heated at 60° for 20 hours. The reaction mixture was then cooled in an ice bath and transferred to a glove bag filled with nitrogen. The precipitate of β -picoline hydrochloride which had formed in the reaction mixture was removed by filtration. The filtrate was added slowly with stirring to a solution of 3.05 g. (0.077 mole) of sodium hydroxide in 86 ml. of water, the temperature being maintained below 30°. Benzene (150 ml.) and water (100 ml.) were added and the mixture was stirred for 1½ hours. A precipitate which had formed at this point was removed by filtration. The aqueous layer was separated from the organic layer of the filtrate. The aqueous layer had a pH of approximately 7. A solution of 0.75 g. (0.0029 mole) of magnesium nitrate hexahydrate and 0.75 g. (0.014 mole) of ammonium chloride in 6 ml. of water was added to the aqueous layer and the resulting mixture was stirred for 1 hour. A small amount of charcoal was then added and the mixture was stirred for 5 minutes. The charcoal and any solid material were removed by filtration. The filtrate was heated to 70-80° and a solution of 5.2 g. (0.0295 mole) of calcium acetate monohydrate in 26 ml. of water was added with stirring. A precipitate of the calcium salt of 5-nitro-3-indolyl phosphate formed. The mixture was allowed to come to room temperature. The precipitate was collected by filtration, washed with water, ethanol and acetone, allowed to dry briefly in air, and then stored in a dessicator over calcium sulfate, yield 3.2 g. (73.2%).

Disodium Salt of 5-Nitro-3-indolyl Phosphate.

The calcium salt of XI (2.37 g., 8 mmoles) was finely powdered and mixed with 0.85 g. (8 mmoles) of sodium carbonate. The mixture was stirred into 450 ml. of water and heated to boiling.

Stirring was continued for 1 hour without further heating. After decolorizing with charcoal, and removal of the charcoal and the calcium carbonate which had formed in the reaction, the filtrate was freeze-dried to give the disodium salt of XI, yield 1.34 g. (55.6%). This sample was purified further by dissolving in water and adding ethanol slowly to give a yellow purified sample suitable for histochemical purposes. For analytical purposes, a derivative of bis(cyclohexylamine) salt of XI was prepared which had m.p. 180° dec., and analyzed to contain 5H₂O·1C₂H₅OH. Repeated drying caused decomposition.

Anal. Calcd. for C₂₀H₃₃N₄O₆P·(5H₂O: 1 H₂O): C, 44.57; H, 8.34. Found: C, 44.27; H, 8.44. The similarity between X and XI leaves no doubt that the solvent of crystallization must be situated in a similar lattice.

E.S.R. Determination

E.S.R. was carried out with a Varian V-4500 ESR spectrometer and the spectra was recorded with 0.03 gauss modulation field of 100 kc/sec. upon the operating microwave frequency of 9521.12 MC/sec. The expanded second derivative spectrum of X, determined from the hydrolysis of X at pH 5.4, could be seen in Figure 1, H₁=3.917 gauss and H₂=0.741 gauss, g=2.0045 ± 0.0010.

Acknowledgement.

The authors acknowledge the help of Drs. G. Kollman and Helen C. F. Su in the early phase of the synthetic work, and Mr. W. Fineman and Mrs. Cecelia Whinney for their assistance in preparing samples for histochemical experiments. Dr. C. J. Lin of the Franklin Institute also assisted in the E.S.R. study.

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Received November 11, 1969

Philadelphia, Pa. 19104