

Substrates for Cytochemical Demonstration of Enzyme Activity. II. Some Dihalo-3-indolyl Phosphates and Sulfates¹

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A recent communication² from this laboratory described the syntheses of several dihalo-3-indolyl- β -D-glycopyranosides for the histochemical demonstration of corresponding glycosidases in mammalian tissue. The utility³ of these substrates derives from the rapid deposition of an insoluble, microcrystalline tetrahaloindigo at the sites of activity through oxidation of an enzymically released intermediate dihaloindoxyl. This same chromogenic reaction sequence has been applied to the localization of alkaline phosphatase through the use of both sodium 3-indolyl phosphate⁴ and calcium 5-bromo-3-indolyl phosphate.⁵ The fact that improved staining results^{3,6} are obtained with indoxyl derivatives that carry 5,6- and in particular 5,4-bromo-chloro substituents prompted the preparation of the corresponding dihalo-3-indolyl phosphates. Moreover, indigogenic staining has been extended to the localization of arylsulfatase(s) through syntheses of 5,6- and 5,4-bromochloro-3-indolyl sulfates.

Experimental Section⁷

***p*-Toluidinium 5-Bromo-6-chloro-3-indolyl Phosphate.**—To a suspension of 0.5 g (1.69 mmoles) of 1-acetyl-5-bromo-6-chloroindol-3-ol² in 5 ml of dry pyridine, cooled to -20° , was added 0.2 ml (2.18 mmoles) of POCl₃ and the reaction mixture was stirred magnetically at 0° overnight (18 hr) with care to exclude moisture. The dark suspension was then stirred at room temperature for an additional 5 hr to achieve complete homogeneity. The amber solution was evaporated to dryness and the -POCl₂ intermediate was hydrolyzed by the addition of ice (*ca.* 5 g). The aqueous mixture was adjusted to pH 9 with 10% KOH and the solution was evaporated to dryness. Traces of water were removed from the residue after three evaporations from 5-ml portions of absolute ethanol. The solid was dissolved in 5 ml of methanol containing 1 equiv of KOCH₃ and the solution was held at room temperature overnight. The reaction mixture was then neutralized with acetic acid and evaporated to dryness. The residue was dissolved in 5 ml of water, decolorized with Norit, and the filtrate was treated with a solution of 0.250 g (1.72 mmoles) of *p*-toluidine hydrochloride in 5 ml of water. The off-white solid that was deposited was collected, air dried, and then crystallized (Norit) from absolute ethanol; 0.38 g (51% yield), mp 198–200° dec.

Anal. Calcd for C₁₅H₁₅BrClN₂O₄P: C, 41.54; H, 3.49; N, 6.46; P, 7.14. Found: C, 41.34; H, 3.67; N, 6.80; P, 7.55.

***p*-Toluidinium 5-Bromo-4-chloro-3-indolyl Phosphate.**—The extension of the above method to 1-acetyl-5-bromo-4-chloroindol-3-ol² required only that the period of stirring at room temperature

be increased to 24 hr. From 0.5 g (1.69 mmoles) of the indoxyl derivative there was obtained 0.3 g (45% yield) of product which crystallized from absolute ethanol in the form of colorless short needles, mp 194–195° with immediate resolidification of the melt and ultimate decomposition at 230°.

Anal. Found: C, 41.49; H, 3.73; N, 6.66.

Potassium 5-Bromo-4-chloro-3-indolyl Sulfate.—To a solution of 0.25 ml (4.3 mmoles) of chlorosulfonic acid in 5 ml of pyridine cooled to 0° , was added, all at once, 1.0 g (3.3 mmoles) of 1-acetyl-5-bromo-6-chloroindol-3-ol. The mixture was stirred magnetically at 0° for 40 hr followed by an additional 4 hr at room temperature. The deep red solution was evaporated to dryness and the residue was treated with *ca.* 1 ml of ice water. The solution was again evaporated and the last traces of moisture were removed by several evaporations from ethanol. The oil was dissolved in a small volume of methanol, and the pH of the solution was adjusted to 9–10 with a saturated solution of methanolic KOH. After *ca.* 10 min, the reaction mixture was neutralized with glacial acetic acid and evaporated to dryness. The product crystallized from a small volume (*ca.* 2 ml) of water in the form of colorless plates, after prior treatment with Norit; 0.715 g (60% yield), mp 200–202° dec after drying at 100° (10⁻³ mm).

Anal. Calcd for C₈H₄BrClKNO₄S: C, 26.35; H, 1.11; N, 3.84. Found: C, 25.94; H, 1.42; N, 3.91.

The product was characterized further as a *p*-toluidine salt which was obtained from the dissolution of equimolar quantities of the potassium salt and *p*-toluidine hydrochloride in a minimum of water at 60°. The product crystallized from water as colorless plates, mp 109–113° dec.

Anal. Calcd for C₁₅H₁₄BrClN₂O₄S: C, 41.54; H, 3.25; N, 6.46; S, 7.39. Found: C, 41.68; H, 3.21; N, 6.66; S, 7.62.

Potassium 5-Bromo-6-chloro-3-indolyl Sulfate.—The application of the procedure described above to 1-acetyl-5-bromo-4-chloroindol-3-ol provided the product in 26% yield, mp 170–175° dec.

Anal. Found: C, 26.16; H, 1.24; N, 4.04.

The conversion of the potassium salt to the corresponding toluidine derivative was accomplished as described above, mp 170–175° dec.

Anal. Found: C, 41.33; H, 3.40; N, 6.45.

N-Substituted DL-Aspartic Acids and β -Methyl Esters

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In continuation of our investigation of compounds which are effective in lowering blood cholesterol levels,² a number of additional N-substituted aspartic acid derivatives were prepared.

Experimental Section³

N-Substituted DL-Aspartic Acids β -Methyl Esters.—A modification of the method of Zilkha and Bachi⁴ was employed. Maleic anhydride (0.22 mole) was refluxed in 60 ml of methanol for 0.5 hr. After cooling, 40 ml of pyridine, followed by 0.2 mole of the amine was added, and the solution was heated at 100–110° (oil bath) for 0.5 hr while 30 ml of methanol was removed. After cooling, 50 ml of ether was added to the yellow reaction mixture, and the resulting solid was filtered, washed with several 50-ml portions of ether, and recrystallized. The new esters, thus prepared, are listed in Table I.

(1) Author to whom inquiries should be addressed.

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(7) All melting points were taken with a Thomas-Hoover apparatus and are corrected. Elementary analyses were performed by Micro-Tech Laboratories, Skokie, Ill. All evaporations were carried out *in vacuo* at $<50^{\circ}$.

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