[CONTRIBUTION FROM THE DAJAC LABORATORY OF THE BORDEN CHEMICAL CO.]

Synthesis of Bromo-substituted Indoxyl Esters for Cytochemical Demonstration of Enzyme Activity[1]

BY HELEN C. F. SU AND K. C. TSOU

RECEIVED JULY 6, 1959

5-Bromo-(II) and 5,7-dibromoindoxyl acetate (III) and their N-acetyl derivatives were prepared as chromogenic substrates for esterases. Modification of the acid moiety of such esters was made in the hope of providing further improvement in preventing diffusion of the indoxyl intermediate, and a higher degree of substantivity of the final pigment. In particular, a number of unsaturated esters of 5-bromoindoxyl were prepared for this purpose. 2-Acetylindoxyl (VII) was prepared from cyclization of ethyl 2-(acetonylamino)-benzoate. Acetylation of VII under various conditions gave 2-acetylindoxyl acetate (VIII) and 1,2-diacetylindoxyl acetate (IX). The infrared spectra of these indoxyl compounds are studied and correlation of infrared spectra with the structural assignment of the various compounds was made, and the mechanism of the formation of indoxyl esters has been suggested.

Indoxyl acetate (I) has been used for the histochemical localization of esterases by Barrnett and Seligman[2] and by Holt.[3] Enzymatic hydrolysis of I results in the liberation of indoxyl which is then readily oxidized to form an insoluble blue indigo at the site of enzymatic activity. In other synthetic studies designed to arrive at better substrates for histochemistry,[4] it was shown that the substantivity of the naphthol dye to tissues can be improved by introducing a bromine atom at the 6-position. It was therefore of interest to us to synthesize a number of bromo-substituted indoxyl esters as a possible means of improving the usefulness of this versatile method. While the histochemical work was still in progress and our synthetic work had been completed, such an improvement had, in fact, been achieved by Holt and his associates,[5] and 5-bromoindoxyl acetate (II) was reported as the best substrate. Although reports of their work are complete and admirable, we would like to report some independent observations that are related to the synthesis of these bromo-substituted indoxyl esters that have not been covered in their publications, and to report some hitherto unknown ester analogs as well.

Because the sodium salt of 5-bromoindoxyl is not accessible, the synthesis of 5-bromoindoxyl acetate (II) was accomplished *via* Heumann's method.[6] Ring closure of 4-bromophenylglycine-2-carboxylic acid in acetic anhydride and sodium acetate yielded 5-bromo-O,N-diacetylindoxyl (IV). The relative ease of CO_2 liberation led us to suggest that the reaction probably took place *via* an initial mixed anhydride formation followed by decarboxylation mechanism a or b. This plausible mechanism would then be facilitated by the continuous removal of acetic acid and retarded by the addition of acetic acid. It was indeed found that the addition of 10% acetic acid lowered the yield of IV from 42 to 17%, and the removal of acetic acid from the reaction mixture prior to decomposition of the acetic anhydride raised the yield to 80%. Selective deacetylation of IV gave 5-bromo-N-acetylindoxyl (V).

Reacetylation of V in the presence of sulfuric acid yielded IV, thus confirming the O,N-diacetyl structure IV and not the C,N-diacetyl structure VI.

In our earlier synthetic work, the possibility of VI had been considered because of the rather unexpected three carbonyl bands in the infrared spectrum of IV[7]: a shoulder at 5.60 μ in addition to a 5.65 and 5.80 μ pair. A negative iodoform test, its readiness to oxidize to indigo, and the reacetylation of V made this possibility unlikely. A more likely explanation would seem to be that such indoxyl esters may exist in hydrogen bonded form as illustrated in IVa where the quasi H can bond partially to both the ester carbonyl and the amide carbonyl. That such H bonding is of the internal type is verified by an identical spectrum determined in chloroform solution. Removal of either O- or N-acetyl groups increased the bonding capacity of the other carbonyl, and thereby further decreases the double bond character and results in a shift to longer wave lengths in each case (see Table I).

In the case of 5,7-dibromo-O,N-diacetylindoxyl, the effect is so accentuated that the 5.60 μ band disappears completely. The 5.80 μ band in the 5-bromo-N-acetyl compound V has been assigned to the keto form of the indoxyl (Va).[7]

To confirm the above assumption, we then undertook the synthesis of 2-acetylindoxyl (VII), 2-acetylindoxyl acetate (VIII) and 1,2-diacetylindoxyl acetate (IX). The ring closure of ethyl 2-(acetonylamino)-benzoate, in dry benzene with metallic sodium and a small amount of ethanol yielded the sodium salt of VII. Acidification of its aqueous solution with dilute acetic acid yielded the product VII.[8] By acetylation of its alkaline solution with acetic anhydride at room temperature, VIII was obtained. If VII was refluxed in acetic anhydride with sodium acetate, IX was formed.

A study of their spectra clearly supports our explanation to confirm the structure of IV instead of VI. The infrared spectrum of VII showed absorption at 2.98, 3.10 and 6.15 μ. The two lower bands indicate the existence of an –OH group and a –NH group. The 6.15 μ band confirmed the

(1) This work was supported by a Research Grant [C-2530] from the National Cancer Institute, National Institutes of Health, Department of Health, Education and Welfare, Bethesda 14, Md.

(2) R. J. Barnett and A. M. Seligman, *Science*, **114**, 579 (1951).

(3) S. J. Holt, *Nature*, **169**, 271 (1952).

(4) K. C. Tsou and A. M. Seligman, THIS JOURNAL, **74**, 3066 (1952); **74**, 5605 (1952).

(5) S. J. Holt, *et al.*, *Proc. Roy. Soc.* (*London*), **148**, 465, 481, 495, 506, 520 (1958).

(6) K. Heumann, *Ber.*, **23**, 3043 (1890).

(7) A study of infrared spectra of many halogenated indoxyl esters has also been reported recently by S. J. Holt, *et al.*, *J. Chem. Soc.*, 1217 (1958). This anomaly, however, was not recorded in their data; authors gave 1755 and 1706 cm.$^{-1}$.

(8) Hochster Farbw., German Patent 111,890; *Chem. Zentr.*, **71**, II, 614 (1900).

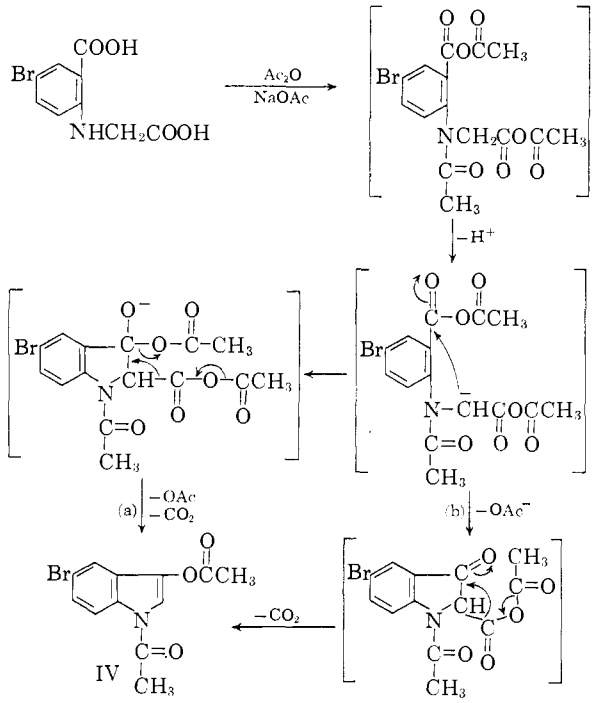

Fig. 1.

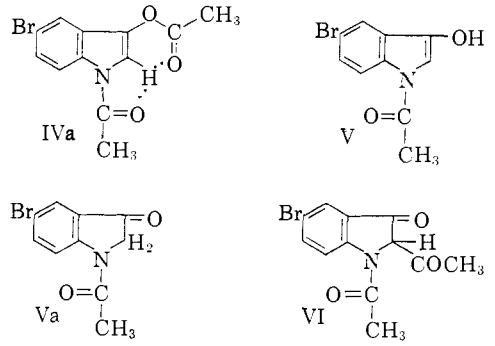

Fig. 2.

structure of 2-acetylindoxyl as existing in its enol form rather than the keto form shown in 5-bromo-N-acetylindoxyl (Va), since it is due to 1-keto-2-hydroxy-aryl ketone absorption. The shift from the normal carbonyl frequency can be explained by the "conjugate chelation" between VIIa and VIIb, rather than the ordinary hydrogen bonding VIIa. In the spectra of VIII and IX, the normal absorption at 5.68 and 6.00–6.07 for the former, and 5.65, 5.83, and 6.04 μ for the latter was observed.

The preparation of II was only accomplished by direct acetylation of 5-bromo-indoxyl which is formed *in situ* by the alkaline hydrolysis of IV.

We have synthesized a number of unsaturated esters of 5-bromoindoxyl with the hope of providing an even greater degree of substantivity and binding of the final indigo product in the tissue sections in which esterase is being demonstrated. It is anticipated that the unsaturated acid, liberated by enzymatic hydrolysis, will be polymerized during the oxidation process which converts indoxyl to indigo. If enough polymeric acid is formed, it may impede the diffusion of the indoxyl intermediate and provide sharper localization of the indigo to the actual sites of enzymatic activity. The synthesis of these compounds are recorded here, and histochemical evaluation will be published elsewhere.[9]

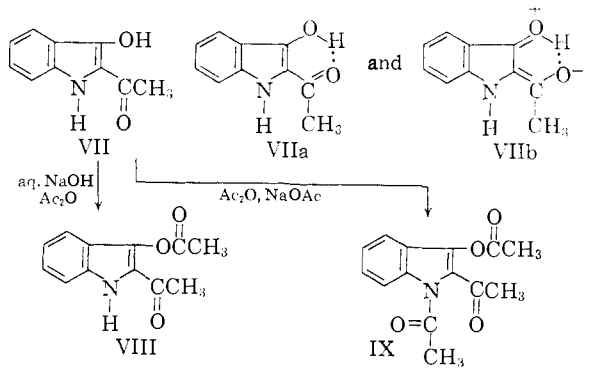

Fig. 3.

Experimental[10]

5-Bromo-O,N-diacetylindoxyl (IV). A. From Ring Closure.—4-Bromophenylglycine-2-carboxylic acid (19.2 g.) was refluxed with acetic anhydride (160 ml.) and freshly fused sodium acetate (10 g.) for four hours. The excess acetic anhydride was decomposed by the addition of 60 ml. of water. The acetic acid was removed *in vacuo*. The residue solidified after it was cooled to room temperature. It was suspended in ice-water and filtered. The crude product was recrystallized from methanol–water to give 9.9 g. (42.8%) of white, fluffy crystals, m.p. 123–123.8°.[5]

Anal.[11] Calcd. for $C_{12}H_{10}BrNO_3$ (296.14): N, 4.73. Found: N, 4.82.

B. From **Acetylation of 5-Bromoindoxyl Acetate (II).**—5-Bromoindoxyl acetate (0.25 g.) was added to a boiling mixture of acetic anhydride (6 ml.) and freshly fused sodium acetate (0.5 g.). The mixture was again refluxed for 30 minutes. After the solution was cooled to room temperature, water (15 ml.) was added. The suspension was heated to boiling and filtered while hot. On cooling, IV crystallized and was collected. Recrystallization from ethanol–water gave pure IV, m.p. 123.4–124.4°; no depression of melting point and identical infrared spectrum to that prepared by method A.

C. From **Acetylation of 5-Bromo-N-acetylindoxyl (V).**—To a mixture of acetic anhydride (3 ml.), glacial acetic acid (5 ml.) and 5-bromo-N-acetylindoxyl (0.2 g.) was added four drops of concentrated H_2SO_4 dissolved in 2 ml. of glacial acetic acid a 0°. After 20 minutes, the solution was warmed slowly to 40–50° for 5 minutes, and then cooled to room temperature. Water was added to precipitate the product. After recrystallization from ethanol–water, IV was obtained in white, fine crystals, m.p. 123.5–124°; no depression of melting point and identical infrared spectrum to that prepared by method A.

5-Bromo-N-acetylindoxyl (V).—To 1 g. of 5-bromo-O,N-diacetylindoxyl suspended in methanol (50 ml.) at room temperature, was introduced a small piece of sodium. The brown mixture turned amber-red with green fluorescence. After 30 minutes, it was acidified with acetic acid. Brown, glistening crystals were obtained after cooling. It was recrystallized from ethanol–water to obtain 0.48 g. (56%) of white, stout needles, m.p. 184–185°.

Anal. Calcd. for $C_{10}H_8BrNO_2$ (254.10): N, 5.51. Found: N, 5.55.

5-Bromoindoxyl Acetate (II).—Two grams of crude 5-bromo-O,N-diacetylindoxyl was added to a boiling 5% aqueous sodium hydroxide solution (100 ml.) with stirring.

(9) In collaboration with Dr. A. M. Seligman at Sinai Hospital and Johns Hopkins University Medical School in Baltimore, Md.

(10) All melting points are corrected. All starting materials prepared by methods published by Holt, *et al.*, in ref. 5 have been omitted.

(11) All analyses were done by Dr. Stephen Nagy, Microchemical Laboratory, Massachusetts Institute of Technology.

TABLE I

INFRARED SPECTRA OF BROMO-INDOXYL COMPOUNDS[a,b]

Indoxyl compounds	Wave lengths in microns											
5-Br-O,N-diAc[c]	..	..	5.60sh 5.65	..	5.80	..	6.25w	8.25	...	12.35	13.90	>15.50
5-Br-N-Ac	..	..	..	5.80	5.95	..	6.25	..	...	12.10	13.40	15.10
5-Br-O-Ac	..	3.02	5.70	..	..	..	6.45	8.25	...	12.50	13.40	15.50
5,7-diBr-O,N-diAc	..	..	5.65	..	5.80	..	6.25 6.45	8.25	...	12.60	13.80	15.40
5,7-diBr-O-Ac	..	3.00	5.72	..	..	..	6.35w 6.45	8.25	...	12.10	...	>15.50
5-Br-O-acrylate	..	2.93	5.70 5.77	..	..	6.12	6.35 6.45	8.22	10.20 10.40	12.48	13.42	>15.50
5-Br-O-methacrylate	..	3.04	5.82	..	..	6.10	6.35 6.45	8.13	10.60 10.65sh	12.50	12.65	15.40
5-Br-O-crotonate	..	2.98	5.72 5.78	..	..	6.13	6.47	8.25	10.25	12.50	13.43	>15.50
2-Ac	2.98	3.10	..	..	..	6.15	6.40 6.48	..	...	...	13.35	...
2-Ac-O-Ac	..	3.05	5.68	..	..	6.00 6.07	6.32 6.50	8.35	...	...	13.30	...
2-Ac-O,N-diAc	..	..	5.65	..	5.82	6.03	6.22 6.48	8.40	...	...	13.08 13.25	...

[a] The infrared absorption spectra were determined with a Perkin–Elmer spectrophotomer model 21. Acknowledgment is due to Mr. Mario Cardone of the Analytical Laboratory, The Borden Chemical Co., for the determination of these spectra. [b] All spectra are done in Nujol mull. [c] Infrared spectrum in chloroform solution was also determined, and was found to be identical.

The solution was refluxed for 20 minutes, and then cooled to room temperature. Without isolating the free indoxyl, acetic anhydride was added dropwise at −5 to 0° until the yellow solution turned colorless and the product precipitated as light, brown crystals. The product was recrystallized from ethanol–water to give 1.35 g. [78.4%] of white, shiny flakes, m.p. 133–133.5°.

Anal. Calcd. for $C_{10}H_8BrNO_2$ [254.10]: C, 47.27; H, 3.18; N, 5.51. Found: C, 47.33; H, 3.26; N, 5.79.

5,7-Dibromo-O,N-diacetylindoxyl.—4,6-Dibromophenylglycine-2-carboxylic acid (6 g.) was added with stirring into a boiling mixture of acetic anhydride (80 ml.) and anhydrous sodium acetate (3 g.). The brown solution was refluxed for 3 hours, and allowed to cool to 90°. Water (40 ml.) was added dropwise through the reflux condenser. The acetic acid was removed *in vacuo*. The brown solid obtained was suspended in ice-water and filtered. The crude product was recrystallized from methanol–water to give 0.9 g (14%) of white needles, m.p. 148.5–149.5°.

Anal. Calcd. for $C_{12}H_9Br_2NO_3$ (375.04): C, 38.43; H, 2.42; N, 3.74: Found: C, 38.55; H, 2.43; N, 3.77.

5,7-Dibromoindoxyl Acetate (III).—5,7-Dibromo-O,N-diacetylindoxyl (0.5 g.) in 5% aqueous sodium hydroxide solution (40 ml.) was refluxed for an hour, and then cooled to room temperature. Acetic anhydride was added dropwise −5 to 0° until the brown solution turned colorless and 5,7-dibromoindoxyl acetate separated as light blue crystals. The product was recrystallized from ethanol to give 0.3 g. (67.8%) of white needles, m.p. 127–128°.

Anal. Calcd. for $C_{10}H_6Br_2NO_2$ (333.01): C, 36.07; H, 2.12; N, 4.21. Found: C, 36.14; H, 2.05; N, 4.22.

5-Bromoindoxyl Acrylate.—One gram of crude 5-bromo-O,N-diacetylindoxyl was added to a boiling 5% aqueous sodium hydroxide solution (20 ml.) with stirring under nitrogen atmosphere. The solution was refluxed for 15–20 minutes and then cooled to room temperature. Acrylic anhydride was added dropwise at −5 to 0° until the solution turned colorless and the product precipitated as a soft green mass. It was extracted with ether. The product from the ether extract was recrystallized three times from methanol–water to obtain 92 mg. (10%) of white, shiny, small flakes, m.p. 78–79°.

Anal. Calcd. for $C_{11}H_8BrNO_2$ (266.11): C, 49.65; H, 3.03; N, 5.26. Found: C, 49.63; H, 3.16; N, 5.14.

5-Bromoindoxyl Methacrylate.—The method described in the preparation of 5-bromoindoxyl acrylate was used with methacrylic anhydride or methacrylyl chloride in place of acrylic anhydride. The methacrylate (0.4 g., 20%) was obtained in white, fine crystals, m.p. 130.5–132.4°.

Anal. Calcd. for $C_{12}H_{10}BrNO_2$ (280.14): C, 51.45; H, 3.60; N, 5.00. Found: C, 50.67; H, 3.68; N, 4.83.

5-Bromoindoxyl crotonate was prepared by the use of crotonyl chloride using the same method described for 5-bromoindoxyl acrylate. The crotonate (0.23 g., 24%) was obtained in white, glistening flakes, m.p. 87–88°.

Anal. Calcd. for $C_{12}H_{10}BrNO_2$ (280.14): C, 51.45; H, 3.60; N, 5.00. Found: C, 51.08; H, 3.73; N, 4.78.

2-Acetylindoxyl (VII).—Ethyl 2-(acetonylamino)-benzoate (10 g.) was dissolved in 200 ml. of dry benzene. To this was added small pieces of sodium (1.5 g.) with stirring. Ten ml. of ethanol was then added immediately. After refluxing for an hour, the yellow precipitate was collected. This was redissolved in 200 ml. of 0.5% aqueous sodium hydroxide and then acidified with dilute acetic acid. The brown solid collected was recrystallized from methanol–water to give 5.8 g. (73.5%) of tan solids, m.p. 158–159°. It was again recrystallized from benzene and then ethanol-water to give pale greenish-yellow needles, m.p. 161–161.5° [Hochster[8] reported m.p. 133° which we believe to be impure].

Anal. Calcd. for $C_{10}H_9NO_2$ (175.19): N, 8.00. Found: N, 8.00.

2-Acetylindoxyl Acetate (VIII).—2-Acetylindoxyl (0.8 g.) in 40 ml. of 5% aqueous sodium hydroxide was acidified with acetic anhydride at room temperature. The solid precipitated, was recrystallized from ethanol–water to obtain 0.8 g. (86%) of white, fluffy needles, m.p. 165–166°.

Anal. Calcd. for $C_{12}H_{11}NO_3$ (217.23): N, 6.45. Found: N, 6.36.

1,2-Diacetylindoxyl Acetate (IX).—2-Acetylindoxyl (0.8 g.), sodium acetate (0.8 g.) and acetic anhydride (12 ml.) were heated to reflux for three hours. After cooling, water (40 ml.) was added. The brown solid obtained was first recrystallized from ethanol–water and then from ethanol to obtain 0.13 g. (11%) of white needles, m.p. 104–105°.

Anal. Calcd. for $C_{14}H_{13}NO_4$ (259.27): N, 5.40. Found: N, 5.40.

PHILADELPHIA 24, PENNA.