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ortho-HYDROXY DERIVATIVES OF THE CARCINOGEN 2-ACETYLAMINOFLUORENE

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Wiley (1) and Manson and Young (2) reported that one of the metabolites of the carcinogenic 2-naphthylamine was 2-amino-1-naphthol, which was excreted as the ethereal sulfate. Bonser, *et al.* (3) reported that this metabolite was a local carcinogen to the mouse bladder.

In view of these results we undertook the synthesis of the *ortho*-hydroxy derivatives of the carcinogen 2-acetylaminofluorene. By isotope dilution techniques it would then be possible to determine whether these compounds were actually metabolites of 2-acetylaminofluorene and to what extent this carcinogen was converted to these hydroxy derivatives.

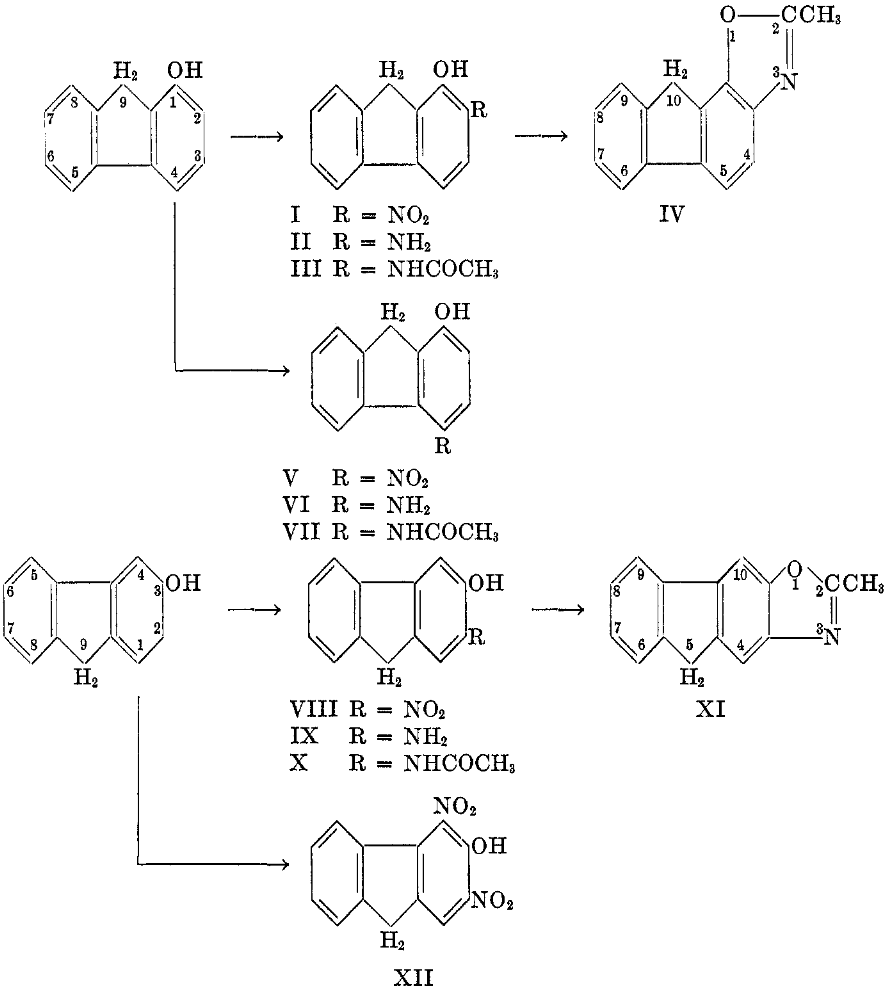
Nitration of 1-hydroxyfluorene (4) yielded a mixture which was separated by chromatographing in benzene on alumina. It was assumed that because of hydrogen bonding 1-hydroxy-2-nitrofluorene (I) would be less acidic and therefore would be the faster moving band. The more acidic and slower moving band would then be 1-hydroxy-4-nitrofluorene (V).

This conclusion was borne out when an oxazole (IV) was prepared from the amine derived from the 1-hydroxy-2-nitrofluorene, thus proving that the nitro group was *ortho* to the hydroxy group.

Further evidence for the structure of the nitration products of 1-hydroxyfluorene is adduced from a consideration of the ultraviolet spectra of the 2-nitro- and 4-nitro-1-hydroxyfluorene (Fig. 1), and of the corresponding 2- and 4-acetylamino derivatives (Fig. 4). The curves of the 2-nitro- and 2-acetylamino-1-hydroxyfluorenes resemble strongly those of the corresponding 2-nitro- and 2-acetylamino-fluorenes (Fig. 3) and (5). Similarly there is a close relationship of the 4-substituted derivatives, leaving no doubt that the structures of the nitro derivatives are correctly assigned.

The 1-hydroxy-2-amino- and -4-aminofluorenes were rather unstable in the presence of ammonium hydroxide, which probably acted as an oxidation catalyst, since it produced deep purple precipitates with these hydroxy amines. A similar reaction has been used by Bonser, Clayson, and Julle (6) for estimating the amount of 1-hydroxy-2-aminonaphthalene present in the excreta of animals fed 2-naphthylamine.

By nitration of 3-hydroxyfluorene (7) a mixture was also obtained which could be separated by chromatography into 2-nitro-3-hydroxyfluorene (VIII) and a dinitro derivative which was thought to be 2,4-dinitro-3-hydroxyfluorene (XII). An oxazole, 2-methyl-5*H*-fluoreno[2,3]oxazole (XI) was prepared from the 2-amino-3-hydroxyfluorene (IX), proving that nitration had taken place in a position *ortho* to the hydroxy group. Fluorene itself nitrates readily in the 2-position. It would be expected, therefore, that 3-hydroxyfluorene would nitrate in the



I $R = NO_2$
II $R = NH_2$
III $R = NHCOCH_3$

IV

V $R = NO_2$
VI $R = NH_2$
VII $R = NHCOCH_3$

VIII $R = NO_2$
IX $R = NH_2$
X $R = NHCOCH_3$

XI

XII

2-position. A consideration of the ultraviolet spectra of the nitration product and of 2-nitro- and 4-nitro-fluorene (Fig. 3) indeed shows a close similarity between the 2-substituted derivatives while the 4-nitrofluorene has an entirely different curve. This proves that the compound obtained is 2-nitro-3-hydroxyfluorene.

The spectrum of the dinitrated product (Fig. 2) exhibits one low peak at 326 mμ, suggesting that the second nitro group entered the 4-position. Indeed, if the second nitro group had substituted at the other possible point, the 7-carbon, the spectrum would be expected to have a considerably higher absorption at the maximum. This is exemplified by a comparison of the curves corresponding to the 2,7-dinitro and 2,5-dinitrofluorene (Fig. 3). It is also noteworthy in this series of fluorene derivatives that two peaks result from a *para*-hydroxynitro relationship. One peak only appears when the hydroxy group is *ortho* to a nitro substituent (Figs. 1 and 2).

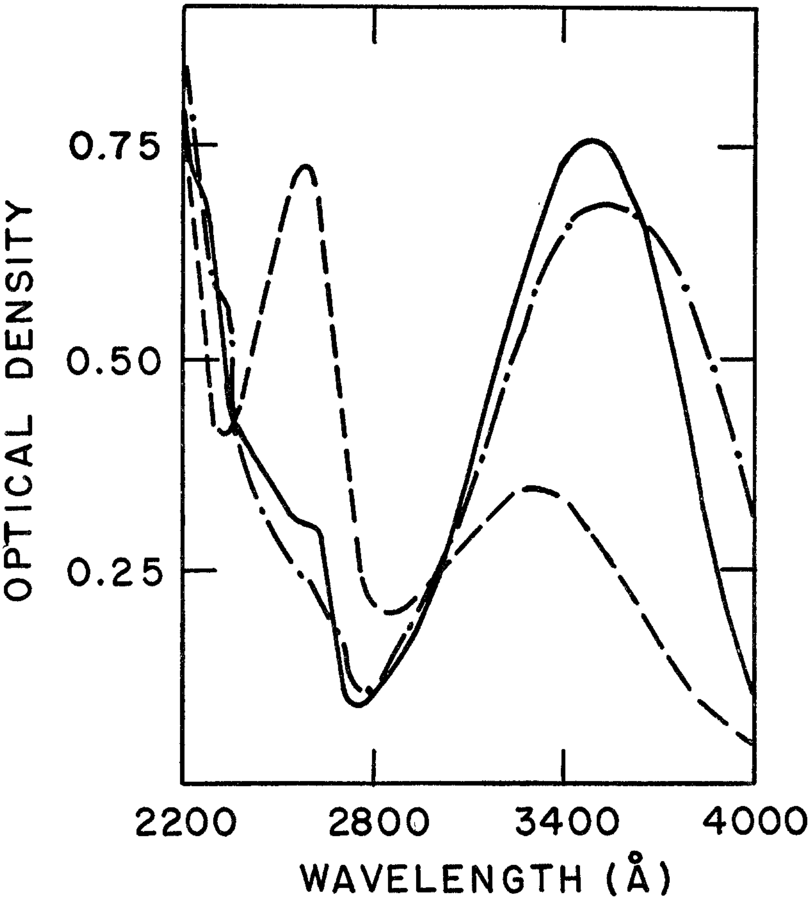


FIGURE 1. ULTRAVIOLET ABSORPTION SPECTRA IN ETHANOL:
——— 1-Hydroxy-2-nitrofluorene (max. 2610Å, ε 6,930; 3500Å, 17,200; min. 2560Å, 6,820; 2750Å, 20,400); — — 1-hydroxy-4-nitrofluorene (max. 2570Å, 16,400; 3290, 7,840; min. 2310Å, 9,320; 2840Å, 4,540); —·— 2-nitro-3-hydroxyfluorene (max. 3500Å, 15,600; min. 2775Å, 2,500). Each compound 10 mg./l. (4.4 × 10^{-5} moles/l.).

It was found that neither 1-hydroxy-2-aminofluorene nor 3-hydroxy-2-aminofluorene gave a diazonium test with nitrous acid and an alkaline solution of β-naphthol. These compounds did not give a true reading in the Westfall-Morris method (8) for determination of 2-aminofluorene but gave some interference in a method developed by Damron and Dyer (9) for the estimation of 2-amino-7-hydroxyfluorene. It had been reported by Ruiz (10) that an isomeric compound, 2-hydroxy-3-aminofluorene, did not form a diazo compound or couple with β-naphthol.

Upon coupling with diazotized *p*-nitroaniline or 2-amino-7-nitrofluorene only 2-acetylamino-1-hydroxy- and -7-hydroxyfluorene gave a dye. 2-Acetylamino-3-hydroxyfluorene, 4-acetylamino-1-hydroxyfluorene, and 3-acetylamino-2-hydroxyfluorene did not react. On the other hand, 1-hydroxy-, 2-hydroxy-, and 3-hydroxy-fluorene produced red dyes. In this series of compounds it would appear

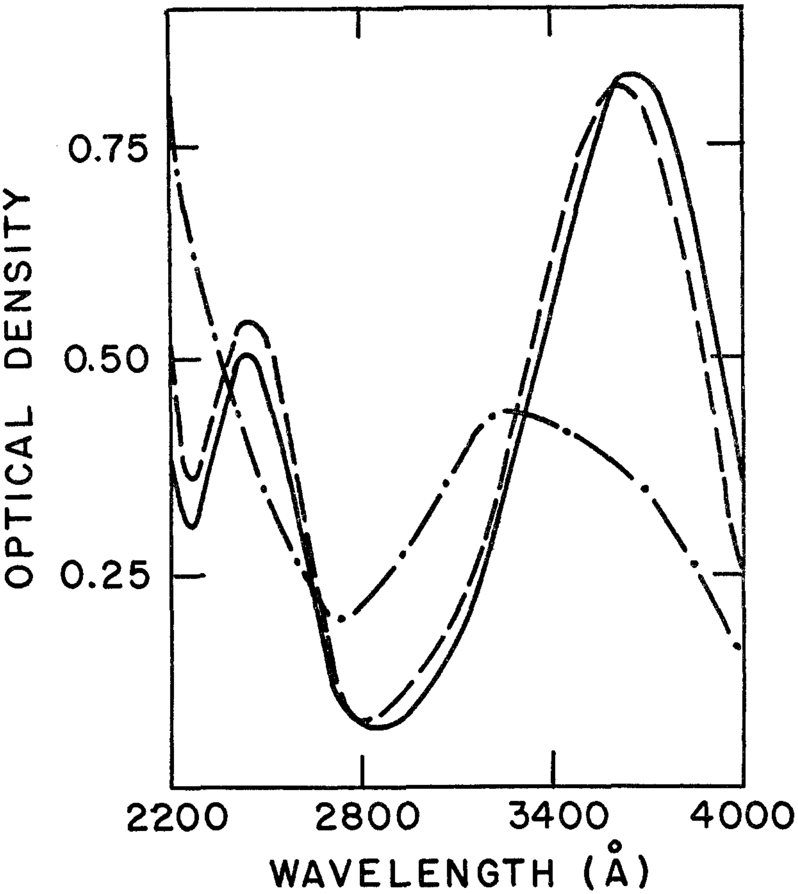


FIGURE 2. ULTRAVIOLET ABSORPTION SPECTRA IN ETHANOL:

——— 2-Nitro-7-hydroxyfluorene (4.4 × 10^{-5} moles/l.) (max. 2460Å, ε 11,700; 3660Å, 19,000; min. 2260Å, 6,820; 2840Å, 1,590); — — 2-nitro-7-methoxyfluorene (4.1 × 10^{-5} moles/l.) (max. 2430Å, 13,300; 3600Å, 20,000; min. 2240Å, 8,440; 2780Å, 19,300); —·— 2,4-dinitro-3-hydroxyfluorene (3.7 × 10^{-5} moles/l.) (max. 3260Å, 12,000; min. 2720Å, 5,310).

that coupling occurs only when the *para* position in the same ring is free, or when the hydroxy group is in a different ring from the acetylamino substituent. However, all the acetylaminohydroxyfluorenes noted above, except 1-hydroxy-4-acetylaminofluorene, gave a yellow color with nitrous acid indicating that nitrosation did occur.

By the use of carrier techniques on urine from rats fed 2-acetylaminofluorene-9-C^{14}, it was found that both 1-hydroxy- and 3-hydroxy-2-acetylaminofluorene were metabolites of 2-acetylaminofluorene. The details of these biochemical studies will be reported elsewhere.

EXPERIMENTAL

Nitration of 1-hydroxyfluorene. A solution of 4.2 g. of 1-hydroxyfluorene in 90 ml. of glacial acetic acid was cooled in an ice-bath with mechanical stirring. A mixture of 1.5 ml.

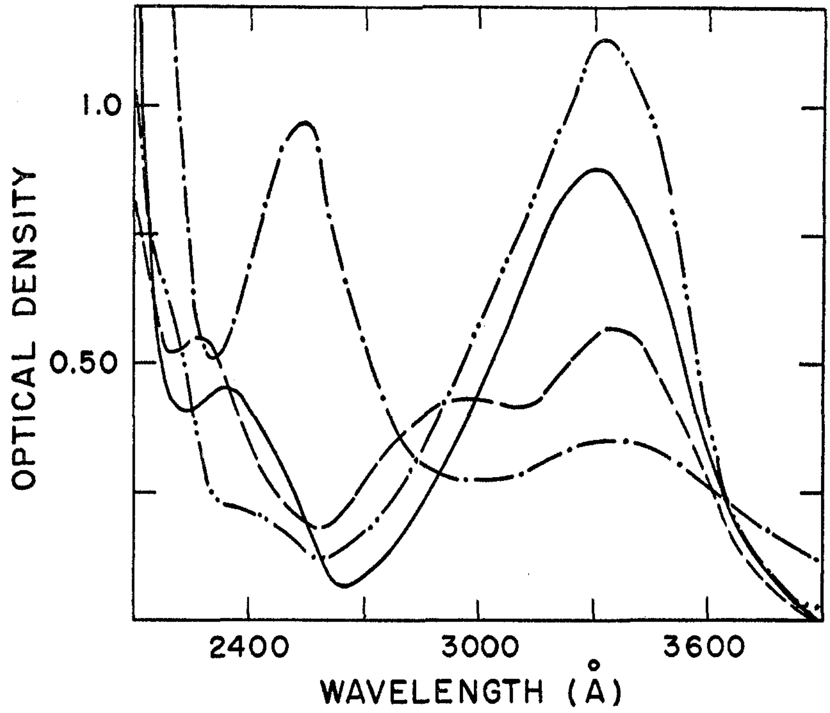


FIGURE 3. ULTRAVIOLET ABSORPTION SPECTRA IN ETHANOL:

———— 2-Nitrofluorene (4.8×10^{-5} moles/l.) (max. 2330Å, ε 9,460; 3300Å, 18,300; min. 2230Å, 8,230; 2660Å, 1,230); — — 2,5-dinitrofluorene (3.6×10^{-5} moles/l.) (max. 2270Å, 15,000; 3000Å, 12,000; 3350Å, 15,400; min. 2195Å, 14,000; 2580Å, 4,810; 3120Å, 11,300); —·— 4-nitrofluorene (5.0×10^{-5} moles/l.) (max. 2550Å, 21,600; 3350Å, 6,370; min. 2300Å, 9,990; 2930Å, 4,370); —·· — 2,7-dinitrofluorene (4.1×10^{-5} moles/l.) (max. 3320Å, 28,100; min. 2330Å, 5,540; 2450Å, 4,560; 2570Å, 3,080).

of concentrated nitric acid and 1.5 ml. of water was added dropwise. Stirring was then continued at room temperature for 1 hour longer. Dilution with water and filtering yielded 5 g. of a brownish-yellow precipitate, m.p. 135–160°. This material was dissolved in benzene and the solution was poured on an alumina column (2.5 × 15 cm.). Three bands formed in descending order, a narrow brown band, a broad orange-yellow band, and a broad orange band. The lower orange band was eluted with benzene and a 1:99 ethanol-benzene mixture. Evaporation of the eluates yielded 2.2 g. of bright yellow needles, m.p. 145–150°. Rechromatography of this material followed by crystallization from acetone or benzene gave 1.7 g. of *1-hydroxy-2-nitrofluorene* (I), bright yellow needles, m.p. 160–161°.

Anal. Calc'd for $C_{13}H_9NO_3$: C, 68.72; H, 3.99.

Found: C, 68.45; H, 3.92.

The orange-yellow band on the column was eluted with 1:1 ethanol-benzene giving 1.4 g. of darker yellow material, m.p. 190–210°. Rechromatographing followed by crystallization from benzene or dilute ethanol afforded 0.7 g. of long bright yellow needles of *1-hydroxy-4-nitrofluorene* (V), m.p. 215–216°.

Anal. Calc'd for $C_{13}H_9NO_3$: C, 68.72; H, 3.99.

Found: C, 68.46; H, 3.94.

1-Hydroxy-2-aminofluorene (II). To a solution of 1 g. of I in 125 ml. of warm ethanol there were added 1 g. of calcium chloride dissolved in 20 ml. of water and 7 g. of zinc dust. This mixture was refluxed for 2 hours and filtered into 10 ml. of concentrated hydrochloric

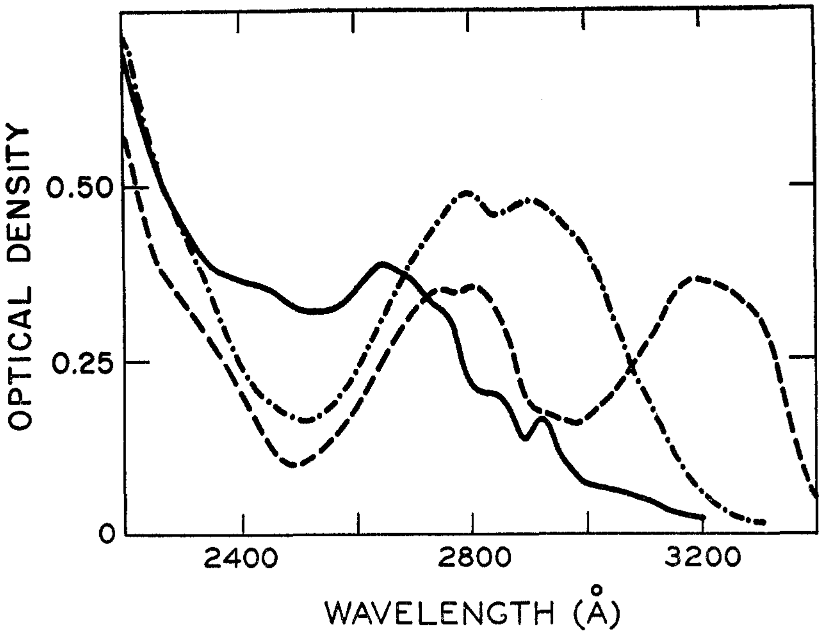


FIGURE 4. ULTRAVIOLET ABSORPTION SPECTRA IN ETHANOL:

———— 1-Hydroxy-4-acetylaminofluorene (max. 2650Å, ε 18,600; 2925Å, 8,040; min. 2520Å, 15,100; 2890Å, 6,600); — — 2-acetylamino-3-hydroxyfluorene (max. 2750Å, 16,700; 2800Å, 16,700; 3190Å, 17,400; min. 2500Å, 4,830; 2775Å, 16,500; 2980Å, 7,700); —·— 1-hydroxy-2-acetylaminofluorene (max. 2790Å, 23,500; 2910Å, 22,900; min. 2520Å, 7,750; 2840Å, 21,900). Each compound 5 mg./l. (2.1×10^{-5} moles/l.).

acid. The ethanol was removed on the steam-bath under a stream of nitrogen. The light pink crystalline precipitate weighed 1.1 g., m.p. indefinite > 265°. Addition of ammonium hydroxide to a solution of the amine hydrochloride produced a very dark purple precipitate which was mostly insoluble in dilute hydrochloric acid. The amine did not appear to diazotize and give a test with β-naphthol or R-salt. The free amine was prepared by addition of sodium bicarbonate or sodium acetate to a solution of the amine hydrochloride (0.4 g.) yielding light gray material which, after two crystallizations from dilute ethanol, gave 0.2 g. of shiny gray plates, m.p. 195–196°.

Anal. Calc'd for $C_{13}H_{11}NO$: C, 79.16; H, 5.62; N, 7.10.
Found: C, 78.95; H, 5.44; N, 6.82.

1-Hydroxy-2-acetylaminofluorene (III). A solution of 0.54 g. of the hydrochloride of II in 50 ml. of warm water and 0.5 ml. of concentrated hydrochloric acid was filtered to remove a small amount of dark material. The solution then was adjusted to *p*H 5 by addition of 0.5 g. of crystalline sodium acetate, upon which a light gray precipitate formed. The mixture was stirred mechanically in an ice-bath, 3 ml. of acetic anhydride was added and stirring was continued (in the cold) for 6 or 7 hours. The product, m.p. 206° (dec.)., weighed 0.53 g. Two crystallizations from dilute ethanol (1 vol. ethanol: 2 vols. water), using Norit, gave 0.297 g. of small white needles, m.p. 208° (dec.).

Anal. Calc'd for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48; N, 5.85.
Found: C, 75.50; H, 5.63; N, 5.58.

1-Hydroxy-4-aminofluorene (VI). This compound was prepared by the method used for the 2-isomer. From 1 g. of nitro compound 1.1 g. of amine hydrochloride was obtained. The free amine had m.p. 209° after crystallization from dilute ethanol.

Anal. Calc'd for $C_{13}H_{11}NO$: C, 79.16; H, 5.62; N, 7.10.
Found: C, 78.89; H, 5.81; N, 7.39.

The amine gave a yellow color with nitrous acid. However, when an alkaline β-naphthol solution was added to the nitrous acid solution a brightly colored precipitate was not obtained but only a yellow-brown solution. The same color was produced on addition of ammonium hydroxide to the diazonium solution.

1-Hydroxy-4-acetylaminofluorene (VII). *A*. This compound was prepared from the amine hydrochloride and it was crystallized in the same manner as the 2-isomer. It formed very shiny small white needles. The compound began decomposing at 274° and melted to a black paste at 278°.

Anal. Calc'd for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48; N, 5.85.
Found: C, 75.48; H, 5.57; N, 5.78.

B. A solution of 0.209 g. of sulfanilic acid dihydrate in 5 ml. of water and 0.53 g. of sodium carbonate was diazotized and coupled with 0.18 g. of 1-hydroxyfluorene according to the procedures given in Organic Syntheses (11) for preparation of 1,2- and 1,4-aminonaphthol hydrochloride. The free amine obtained (0.16 g.) had a m.p. over a range 145–155°. It was crystallized as the hydrochloride which was then acetylated in acetate buffer. The acetyl derivative melted at 248–250°. Further crystallizations gave a very small amount of VII, melting at 268°. A mixture m.p. with the material from Procedure A melted at 265–272°.

2-Methyl-10H-fluoreno[2,1]oxazole (IV). A mixture of 0.25 g. of the hydrochloride of II and 1 g. of fused sodium acetate was ground and then heated with 0.5 ml. of acetic anhydride at 150–160° for 8 hours. Water was added, the mixture was neutralized with sodium carbonate, and the precipitate was filtered off. The product, m.p. 150°, weighed 0.35 g. It was crystallized from benzene-petroleum ether, chromatographed on an alumina column (using benzene), and again crystallized from benzene-petroleum ether to yield 29 mg. of white crystals, m.p. 167–168°.

Anal. Calc'd for $C_{15}H_{11}NO$: C, 81.42; H, 5.01; N, 6.33.
Found: C, 81.04; H, 5.27; N, 6.44.

2-Nitro-3-hydroxyfluorene (VIII). A solution of 3 g. of 3-hydroxyfluorene in 80 ml. of glacial acetic acid was cooled to 16° with mechanical stirring. A mixture of 1.25 ml. of concentrated nitric acid and 1.25 ml. of water was added dropwise. A yellow precipitate formed. Stirring at room temperature was continued for 1 hour longer. Then 100 ml. of water was added with stirring and the tan-yellow precipitate, m.p. 146–149°, wt. 3.1 g., was filtered off. This was dissolved in benzene and chromatographed on an alumina column (17 × 2.5 cm.). In descending order a brown band, a broad bright-orange band, a thin yellow band, and a broad orange band formed. The lowest, fastest moving band was eluted with benzene yielding 1.65 g. of bright yellow crystals which, after crystallization from benzene or 80% ethanol, afforded 1.5 g. of material, m.p. 160–161°. Rechromatographing did not raise the melting point. A mixture with I sintered over a range 120–140° and melted at 140–144°.

Anal. Calc'd for $C_{13}H_9NO_3$: C, 68.72; H, 3.99.
Found: C, 68.68: H, 4.19.

The thin yellow band was eluted with ethanol but yielded only 8 mg. of an orange colored material which sintered and charred over a long range 260–285°. The slowest moving orange band was eluted with ethanol-acetic acid mixtures. This orange-colored material, wt. 1.3 g., apparently contained inorganic matter (probably aluminum acetate) since most of it was unmelted at 300°. By crystallization from ethanol 0.4 g. of brownish material, m.p. 226°, was obtained. After rechromatographing on alumina and crystallizing from dilute acetone fine bright yellow needles, m.p. 234–235°, were obtained. The analysis corresponded to that of a dinitro derivative, probably *2,4-dinitro-3-hydroxyfluorene* (XII).

Anal. Calc'd for $C_{13}H_8N_2O_5$: C, 57.36; H, 2.96.
Found: C, 57.44; H, 3.25.

2-Amino-3-hydroxyfluorene (IX). A mixture of 1.25 g. of VIII, 100 ml. of ethanol, 20 ml. of water, 1 g. of calcium chloride, and 7 g. of zinc dust was refluxed. After 1 hour 25 ml. of glacial acetic acid and 3 g. more of zinc dust were added and refluxing was continued 1 hour more. The mixture was filtered into 10 ml. of concentrated hydrochloric acid. After evaporation of the ethanol on the steam-bath, addition of ammonium hydroxide caused the amine, weighing 0.85 g., to precipitate. Two crystallizations from 30% ethanol gave 0.46 g. of amine, m.p. 209–210° (with decomposition).

Anal. Calc'd for $C_{13}H_{11}NO$: C, 79.16; H, 5.62; N, 7.10.
Found: C, 78.84; H, 5.63; N, 7.07.

2-Acetylamino-3-hydroxyfluorene (X). The amine was acetylated in almost quantitative yields in the same manner as the isomeric 1-hydroxy-2-aminofluorene. The acetyl derivative melted at 238° (decomposition) after recrystallization from dilute ethanol or dilute acetic acid.

Anal. Calc'd for $C_{15}H_{13}NO_2$: C, 75.29; H, 5.48; N, 5.85.
Found: C, 75.16; H, 5.71; N, 5.52.

2-Methyl-5H-fluoreno[2,3]oxazole (XI). A mixture of 0.26 g. of IX, 1 g. of fused sodium acetate, 1.5 ml. of acetic anhydride, and 0.5 ml. of acetic acid was heated at 150° for 7 hours. The crude product, m.p. 108–113°, was dissolved in benzene and was percolated through an alumina column (2.5 × 10 cm.). The benzene eluate gave 0.27 g. of tan material. Six crystallizations from benzene-petroleum ether gave cream-colored crystals, m.p. 144–145°, weighing 28 mg.

Anal. Calc'd for $C_{15}H_{11}NO$: C, 81.42; H, 5.01; N, 6.33.
Found: C, 80.92; H, 5.33; N, 6.07.

Color tests. A. Coupling reactions. Diazonium solutions were prepared from 50 mg. of 2-amino-7-nitrofluorene and 20 mg. of sodium nitrite in 20 ml. of 0.5 *N* hydrochloric acid or from 50 mg. of *p*-nitroaniline and 30 mg. of sodium nitrite in 20 ml. of 0.5 *N* hydrochloric acid. Urea was then added to destroy excess nitrite. Two drops of the diazonium solution was added to a small amount of the acetylaminohydroxyfluorene dissolved in glacial acetic acid on a porcelain spot test plate. Dropwise addition of 10% sodium hydroxide solution developed the color in those cases where coupling occurred.

B. Nitrosation. A cold nitrous acid solution (70 mg. of sodium nitrite in 25 ml. of water and 5 ml. of 1 *N* hydrochloric acid) was added dropwise to a cold solution of the acetylaminohydroxyfluorene in acetic acid.

Spectra. The ultraviolet spectra were recorded on a Cary instrument at a concentration of 5–10 mg./liter of ethanol.

SUMMARY

Nitration of 1-hydroxyfluorene gave a mixture which was separated by chromatography into 1-hydroxy-2-nitro- and -4-nitro-fluorene.

Upon nitration 3-hydroxyfluorene afforded 2-nitro- and 2,4-dinitro-3-hydrofluorene, which were separated by chromatography. Reduction of the nitro compounds, followed by acetylation, gave the corresponding hydroxy-2-acetylaminofluorenes.

The structures of the nitration products were proved by the preparation of derivatives and by a comparison of the ultraviolet absorption spectra of related fluorene compounds.

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