proof of structure as their spectra should be nearly the same, since the electronic configurations are similar under the existing conditions.[15]

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BETHESDA 14, MD.

[CONTRIBUTION FROM THE RADIOISOTOPE SERVICE, VETERANS ADMINISTRATION HOSPITAL, MINNEAPOLIS, AND THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY, UNIVERSITY OF MINNESOTA]

Protein Binding of Model Quinone Imides. III. Preparation of N^{ϵ}-(1-Hydroxy-2-acetamido-4-fluorenyl)-DL-lysine[1]

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The synthesis of an amino acid derived from a metabolite [*N*-(1-hydroxy-2-fluorenyl)acetamide] of the carcinogen, *N*-2-fluorenylacetamide, is described. The amino acid, N^{ϵ}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine, was obtained by reaction of 1,2-fluorenoquinone-2-acetimide with N^{α}-tosyl-DL-lysine benzyl ester, with subsequent removal of the protecting groups of the lysine moiety.

It has been suggested that the proximate metabolite of the carcinogen *N*-2-fluorenylacetamide which is bound to cellular proteins is the *o*-quinoneimine, 1,2-fluorenoquinone-2-imine.[4] More recently, in studies on the reaction of 1,2-fluorenoquinone-2-acetimide with crystalline bovine serum albumin,[5] the ϵ-amino group of the lysine residues of the protein was shown to be involved in the binding reaction by way of 1,4-addition to the *o*-quinone imide. Since the unequivocal identification of the adduct, *N*-(1-hydroxy-2-amino-4-fluorenyl)-L-lysine or its hydrochloride salt (V), in hydrolysates of tissue proteins obtained from rats after feeding *N*-2-fluorenylacetamide would require the availability of an authentic sample of V, the synthesis of this adduct was undertaken.

Reaction of N^{α}-tosyl-DL-lysine benzyl ester (I) with 1,2-fluorenoquinone-2-acetimide (II) gave a product which was homogeneous upon paper chromatography. Since primary amines react with quinone imides either by 1,2-addition or by 1,4-addition,[6,7] the isolated product should have either structure VI or III. The elemental analysis eliminated structure VI and, by inference, structure III has been assigned to the product. III was further characterized as its hydrochloride salt.

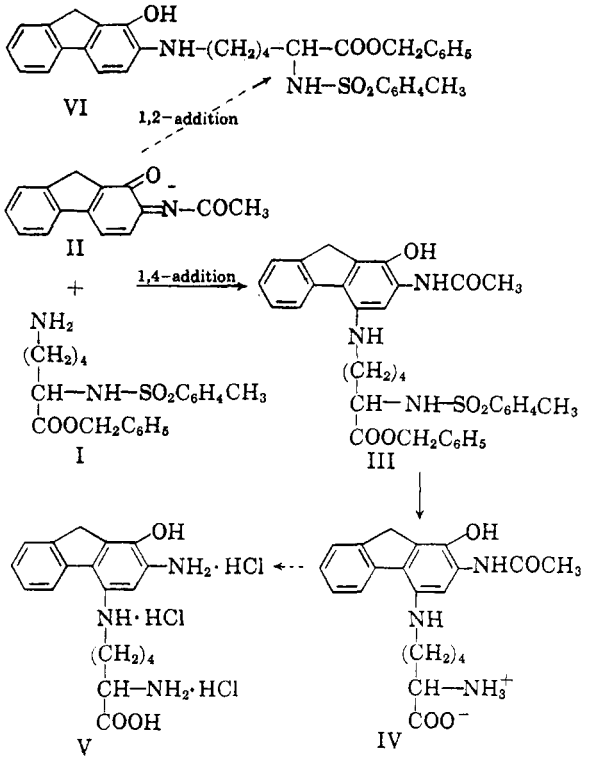

Fig. 1. Preparation of N^{ϵ}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine (IV)

Removal of the protecting groups on the lysine moiety of III with 37% hydrogen bromide in glacial acetic acid[8] and subsequent removal of hydrogen bromide with an anion exchange resin yielded

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N^{ϵ}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine (IV). Traces of contaminating lysine in samples of IV proved difficult to remove.

Both III and IV were readily oxidized by air. Solutions of III or IV in methanol became red in color upon standing overnight and exhibited an absorption maximum at 475 mμ. It is of interest that the protein conjugate of 1,2-fluorenoquinone-2-acetimide and of bovine serum albumin also showed an absorption maximum at 475 mμ.[5] Addition of lead tetraacetate to a solution of III in dry chloroform likewise yielded an intense red solution. No attempts were made to isolate these oxidation products.

Neither III nor IV had sharp melting points, but turned red at the temperatures indicated and became very viscous over a range of several degrees. Morrison[9] has made a similar observation with an amino acid derived from fluorene, DL-β-(2-fluorenyl)alanine. He reported that this compound discolored at 210–215° and decomposed to a brown liquid at 225–233°.

Attempts to remove the acetyl group of IV by refluxing in ethanol-hydrochloric acid (1:1) showed that the acetyl group is very resistant to hydrolysis. This is in marked contrast to the ease with which the acetyl group of *N*-(1-hydroxy-2-fluorenyl)-acetamide is removed by acid hydrolysis. The removal of the acetyl group of IV and the characterization of V are subjects of continued experimentation.

EXPERIMENTAL

1,2-Fluorenoquinone-2-acetamide[10] and N^{α}-tosyl-DL-lysine benzyl ester[11] were prepared by previously published methods. Melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. Chloroform was purified according to Vogel.[12] The ultraviolet spectra were recorded on a Beckman Model DR Spectrophotometer. The Folin-Ciocalteau phenol reagent was either prepared[13] or obtained commercially (Fisher Scientific Co.). All paper chromatography was carried out with Whatman No. 1 paper.

N^{α}-Tosyl-N^{ϵ}-(1-hydroxy-2-acetamido-4-fluorenyl)-DL-lysine benzyl ester (III). N^{α}-Tosyl-DL-lysine benzyl ester, 0.162 g. (0.42 mmole), was partially dissolved in 250 ml. of dry ether with stirring. A solution of 0.099 g. (0.42 mmole) of 1,2-fluorenoquinone-2-acetimide in 10 ml. of purified chloroform was added dropwise during 20 min. The bright red color of the quinone imide disappeared during the addition and a clear, pale pink colored solution resulted. Stirring was continued for 15 min. after the addition had been completed. The solvent was removed under reduced pressure at 35°, yielding a pink-red, semisolid. The product was dissolved in 5 ml. of benzene and the solution was added slowly with stirring to 100 ml. of petroleum ether (b.p. 38–52°). The precipitate was collected at once, washed with petroleum ether, and then dried in air. There was obtained 0.238 g. (0.39 mmole, 91% yield) of pink-red material. The product did not melt sharply, but became very viscous and turned dark red at 67–74°. In numerous other similar preparations, the yield was always about 90%, the product exhibiting a similar behavior upon heating.

Because of the melting behavior of this material the possibility that it was a mixture of products III and VI was considered and the homogeneity of the material was investigated by the use of paper chromatography. Owing to the tendency for the compound to decompose, no equilibration of the paper with the solvent was allowed. The material (10–30 μg.) was detected by spraying the chromatograms first with the Folin-Ciocalteau reagent and subsequently with 20% sodium carbonate. The most satisfactory solvent was cyclohexane: *tert*-butyl alcohol:pyridine:water (16:2:2:1)[14] using the ascending technique and 7–8 hr. development. The R_f value in this solvent system was 0.82 and a faint spot remained at the origin. In other solvents [70% methanol (R_f, 0.87) and 1-butanol:acetic acid:water, 4:1:5 (R_f, 0.96)], a single spot was obtained.

Attempts to crystallize this material were unsuccessful. The compound was purified as follows: 133 mg. was suspended in 20 ml. of water and dissolved by the dropwise addition of 2*N* sodium hydroxide. The solution was filtered and the filtrate adjusted to *p*H 6 with dilute hydrochloric acid. The product which precipitated was collected and washed with water. After drying *in vacuo* over phosphorus pentoxide at room temperature, there was obtained 90 mg. of III, which melted indefinitely above 83°, turning red and becoming quite viscous during heating.

Anal. Calcd. for $C_{35}H_{37}N_3O_6S$: C, 67.0; H, 5.94; N, 6.69; S, 5.11. Found: C, 66.6; H, 5.78; N, 6.34; S, 5.16, 5.39.

The ultraviolet spectrum of III in 95% ethanol showed λ_{max} = 293 mμ (ε, 22,300) and λ_{min} = 255 mμ (ε, 8000) with a shoulder at 282 mμ.

Attempts to purify III by partition chromatography on a silicic acid column according to the method of Weisburger *et al.*[14] with cyclohexane:*tert*-butyl alcohol:pyridine:water (16:2:2:1) as the solvent were not successful. However, III could be chromatographed on a column of cellulose. Whatman cellulose powder (standard grade) (10 g., 35 ml.) was wetted with 5 ml. of the aqueous phase of the solvent system cyclohexane:*tert*-butyl alcohol:pyridine:water (16:2:2:1) and thoroughly mixed. Small portions of the cellulose were transferred as a slurry in the upper phase to a 1 × 30 cm. column with the intermittent application of a nitrogen pressure of 5 lbs./sq. in.[2] until a column 20 cm. high had been obtained. The column was allowed to stand under the upper phase for 4 hr. A sample of III (17.9 mg.), melting at 65–73°, was dissolved in 10 drops of 1-butanol, applied to the column and washed onto the column with 10 drops of 1-butanol. The column was then developed with the upper phase of the solvent, 2-ml. fractions being collected by gravity flow. A red band moved down the column leaving a yellow and an orange band behind. Aliquots of the fractions were examined by paper chromatography, and the fractions containing III (fractions 9–14) were combined and taken to dryness under reduced pressure at 40°. The residue was taken up in 2 ml. of benzene and the solution was added rapidly to 75 ml. of petroleum ether. The product was collected and washed with petroleum ether. After drying *in vacuo* over phosphorus pentoxide at room temperature, there was obtained 10.1 mg. of III, which melted indefinitely above 70°, turning red and becoming quite viscous during heating.

Anal. Calcd. for $C_{35}H_{37}N_3O_6S$: C, 67.0; H, 5.94; N, 6.69. Found: C, 66.6; H, 5.68; N, 6.31.

Hydrochloride of III. The preparation of III was carried out as described above except that ether instead of chloroform was used as solvent. The 1,2-fluorenoquinone-2-

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acetimide (0.108 g.) was added as a slurry in 100 ml. of dry ether in small portions to the suspension of the N^{α}-tosyl-DL-lysine benzyl ester (0.178 g.) in 300 ml. of dry ether during the course of 15 min. After the mixture had been stirred an additional 10 min., the clear, pale pink solution was cooled in an ice bath. Dry hydrogen chloride was passed into the solution, which resulted in the immediate appearance of a white, flocculent precipitate. After standing for 10 min., the product was collected by centrifugation and washed five times with 30 ml. of ether. After drying *in vacuo* over potassium hydroxide, there was obtained 0.251 g. of III hydrochloride (83%), which melted indefinitely above 100–105°, turning red and becoming viscous during heating. Attempts to crystallize the hydrochloride were not successful.

Anal. Calcd. for $C_{35}H_{38}N_3O_6ClS$: C, 63.3; H, 5.77. Found: C, 62.6; H, 5.83.

N^{ϵ}-(1-Hydroxy-2-acetamido-4-fluorenyl)-DL-lysine (IV). A mixture of 0.238 g. of III (0.38 mmole) and 0.110 g. of phenol (1.16 mmoles) was suspended in 2 ml. of 37% hydrogen bromide in glacial acetic acid in a glass-stoppered 10 ml. Erlenmeyer flask and heated at 70° for 2 hr.[8] The clear brown solution was then cooled to room temperature and 6 ml. of ether was added. The precipitate was collected by centrifugation and washed five times with 10 ml. of ether. The tan colored product, which was very hygroscopic, was dissolved in 5 ml. of 50% ethanol and the red-brown solution was passed through a column (1 × 10 cm.) of IRA-400 (acetate). The column was washed with 50% ethanol and the fractions containing ninhydrin-positive material were collected, combined and concentrated under reduced pressure at 35° to approximately one-half volume. The mixture was centrifuged to remove a small amount of precipitate and the slightly turbid supernatant solution was lyophilized. There was obtained 0.104 g. of crude IV (68% yield). The compound became red at about 155° and turned brown, becoming viscous, at 165–168°. The crude IV was purified by dissolving 58.6 mg. in 2.5 ml. of warm methanol. A small amount of insoluble material (4.7 mg.), which was gray-black and did not melt below 300°, was removed by centrifugation. The clear red supernatant solution was filtered through charcoal and the charcoal was washed with methanol. The filtrate and washings were combined, diluted with ether to incipient turbidity and cooled. The brown-red product was collected by centrifugation and washed with ether. After drying *in vacuo*, there was obtained 10.5 mg. of IV. The product softened at 160–162° and melted at 171–174°.

Anal. Calcd. for $C_{21}H_{25}N_3O_4 \cdot H_2O$: C, 62.8; H, 6.78; N, 10.47. Found: C, 63.0; H, 6.40; N, 10.44.

The ultraviolet spectrum of IV in 95% ethanol showed λ_{max} = 292 mμ (ε, 15,000) and λ_{min} = 253 mμ (ε, 7760).

IV was soluble in Methyl Cellosolve, ethanol, methanol, and very soluble in 50% aqueous ethanol or methanol, but insoluble in chloroform, ether, water, dioxane, toluene, or acetone. The compound gave a strongly positive ninhydrin reaction as well as a positive reaction with the Folin-Ciocalteau reagent.

On paper chromatograms, IV (10–30 μg.) was detected by spraying separate strips with ninhydrin solution (0.3% in ethanol) or with the Folin-Ciocalteau reagent as described above. The most suitable solvent was 1-butanol:acetic acid: water (4:1:5). Using this solvent and the descending technique with 15–18 hr. development time, a single spot (R_f = 0.53), giving *both* a positive ninhydrin reaction and a positive Folin reaction, was obtained. In addition, there was a spot which gave only a positive ninhydrin reaction (R_f, 0.20); this spot was shown to be due to lysine. All samples of IV were contaminated with traces of lysine. Using the solvent systems listed below and the ascending technique with shorter development times, a single spot was obtained for IV except that there were traces of lysine as indicated: *tert*-butyl alcohol:formic acid:water 70:15:15 (R_f of IV, 0.55), methyl ethyl ketone:propionic acid:water 75:25:30 (R_f of IV, 0.51, R_f of lysine, 0.10) and phenol:water 88:12 (R_f of IV, 0.98). The compound did not migrate in the solvent system cyclohexane: *tert*-butyl alcohol:pyridine:water (16:2:2:1).

In subsequent experiments, it was found to be more expeditious to detect IV by the use of paper electrophoresis. Using the Beckman Spinco Model R paper electrophoresis cell and Duostat power supply and Whatman 3MM paper with 0.25*N* acetic acid as the electrolyte, IV was readily separated from the contaminating lysine with a constant voltage of 200 volts (about 2.2 milliamps) in 2.5 hr. The mobilities (cm./sec. per volt/cm.) of IV and lysine were 6.7 and 15, respectively, under these conditions. The compounds were detected after electrophoresis on the air-dried strips as described above for the paper chromatography of IV.

MINNEAPOLIS, MINN.

[CONTRIBUTION FROM THE COLLEGE OF PHARMACY, UNIVERSITY OF MICHIGAN]

Derivatives of 7-Methyl-6-thia-1,6-dihydro- and 7-Methyl-6-thia-1,2,3,6-tetrahydropurine 6,6-Dioxide

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Derivatives of 7-methyl-6-thia-1,6-dihydro- (A) and 7-methyl-6-thia-1,2,3,6-tetrahydropurine 6,6-dioxide (B), as well as the parent compound A, have been prepared by interaction of 1-methyl-4-amino-5-sulfamylimidazole with ortho esters, phosgene or thiophosgene.

A number of derivatives of 1-methyl-4-nitro- and 1-methyl-4-amino-5-substituted imidazoles were synthesized.

This paper describes the preparation of derivatives of 7-methyl-6-thia-1,6-dihydro- (A) and 7-methyl-6-thia-1,2,3,6-tetrahydropurine-6,6-dioxide (B). These products were of interest as potential antimetabolites. In addition, the parent compound A has been synthesized.

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