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*FLUOROGENIC SUBSTRATES FOR β-D-GALACTOSIDASES AND PHOSPHATASES DERIVED FROM FLUORESCEIN (3, 6-DIHYDROXYFLUORAN) AND ITS MONOMETHYL ETHER**

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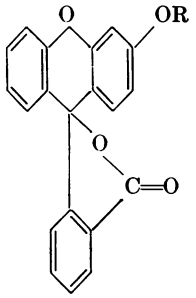
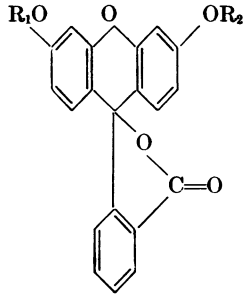
The sensitivity and usefulness of fluorogenic assays has been demonstrated by measuring the β-D-galactosidase activity of single molecules of this enzyme,[1] individual bacterial cells,[2] and ribosomes.[3]

Syntheses of several new biochemically useful fluorogenic substrates for β-D-galactosidases and phosphatases are described. In addition, evidence is presented that the fluorogenic substrate for β-D-galactosidase, previously believed to be 6-hydroxyfluoran-3-D-galactopyranoside (I*b*),[1,5] is not a derivative of 6-hydroxyfluoran (I*a*) but rather fluorescein-di-(β-D-galactopyranoside) (II*d*).





I*a* R = H
b R = β-D-galactopyranosyl

II*a* $R_1 = R_2 = H$
b $R_1 = R_2$ = Acetyl
c $R_1 = R_2$ = 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl
d $R_1 = R_2$ = β-D-galactopyranosyl
e $R_1 = R_2 = PO_3^-$
f R_1 = H, R_2 = Methyl
g R_1 = 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl, R_2 = Methyl
h $R_1 = PO_3^-$, R_2 = Methyl

In 1929, Ghatak and Dutt[4] claimed to have prepared 6-hydroxyfluoran (I*a*), mp 181°, by treatment of 2,4-dihydroxybenzoyl benzoic acid with phenol and concentrated sulfuric acid at 140° for 17 hr. Upon repeating this procedure,[1,5] it has been possible to isolate an amorphous solid with melting point *ca.* 181°; how-

ever, the lack of reproducibility and low yields of this synthesis prompted a more thorough examination of the reaction. We found that 3,6-dihydroxyfluoran (fluorescein) (II*a*) is formed in this reaction as a major product. At least six other fluorescent compounds were detected.

Fluorescein itself has many polymorphic forms[6, 7] and presents difficulties in terms of positive identification. Its presence in the reaction products was confirmed by direct comparison with authentic fluorescein via paper chromatography, infrared spectroscopy, and by conversion to a pure crystalline diacetate, mp 203–205°. Comparison of this diacetate with authentic fluorescein diacetate[6, 7] (II*b*), by the melting point of a mixture of both compounds, elemental analyses, and spectroscopic analyses, proved the samples to be identical. Moreover, when the sample previously described[1, 5] as 6-hydroxyfluoran-β-D-galactopyranoside (I*b*) was compared by paper chromatography with authentic fluorescein-di-(β-D-galactopyranoside) (II*d*) (see below), both samples were found to have identical mobilities. Likewise, the octa-O-acetyl derivatives of these samples have the same chromatographic behavior, melting point, and infrared spectrum.

Presumably, in the presence of sulfuric acid at 140°, 2,4-dihydroxybenzoyl benzoic acid undergoes decomposition to yield resorcinol which then reacts with the remaining parent molecules to form fluorescein. Direct evidence for this hypothesis rests on the fact that fluorescein production occurs even in the absence of phenol.[7]

The preparation of authentic fluorescein-di-(β-D-galactopyranoside) (II*d*) was carried out by treatment of fluorescein (II*a*) with 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide[8] and silver oxide in benzene solution. The resulting octaacetate (II*c*) was then isolated by column chromatography and hydrolyzed to yield pure II*d*. In addition to the isolation of the octaacetate (II*c*), large quantities of a second substance were always obtained. This highly colored, fluorescent material has been tentatively identified as mono-β-D-galactoside derivative of fluorescein.

It was also of interest to attempt the preparation of the diphosphate (II*e*) of fluorescein to be used as a substrate for phosphatases. When fluorescein was treated with either phosphorous oxychloride in pyridine[16] or cyanoethylphosphate,[9] gross mixtures were obtained, and pure compounds could not be isolated in large amounts. Assuming that these phosphorylations were being complicated by the presence of two hydroxyl functions in fluorescein, we investigated the utility of fluorescein monomethyl ether[10] (3-hydroxy-6-methoxyfluoran) (II*f*), where one of the hydroxyl groups is firmly protected. In this case phosphorylation with phosphorous oxychloride readily yielded the desired derivative (II*h*) which was eventually isolated as the monocyclohexylamine salt. It is of interest to note that, whereas this salt is a colorless solid with good stability at room temperature, the corresponding sodium salt undergoes rapid decomposition in the dry state.

Experimental.—General methods:[17] Thin layer chromatography was carried out on silica gel according to Stahl[11] using benzene:ethyl acetate = 1:3 as the solvent. Descending paper chromatography on Whatman No. 1 or S & S orange was employed with the following solvent systems: solvent I, 1-butanol:pyridine:water = 315:175:240; solvent II, 1-pentanol:1-propanol:water = 40:11:15; solvent III, 2-propanol:ammonium hydroxide (sp. gr. 0.9):water = 7:1:2; solvent IV, 1-propanol:ammonium hydroxide (sp. gr. 0.9):water = 6:3:1; solvent V, isobutyric

acid:1 *M* ammonium hydroxide:0.1 *M* sodium ethylene diaminetetraacetate= 100:60:1.6; solvent VI, ethanol:0.5 *M* ammonium acetate, pH 3.8 = 5:2; solvent VII, ethanol:1 *M* ammonium acetate, pH 7.5 = 5:2.

The chromatograms were examined under long-wave ultraviolet light for fluorescence and then were irradiated with short-wave ultraviolet for a few minutes. Fluorogenic substances were found to give blue fluorescence after this treatment. Phosphates and carbohydrates were detected with enzymes and by spraying with molybdate[12, 13] or ammoniacal silver nitrate,[14] respectively. Rapid examination of column eluants was accomplished by boiling with *1 M* alcoholic potassium hydroxide and visually observing the appearance of fluorescence.

Purification of fluorescein: Commercial fluorescein (Eastman No. 780) was converted to its diacetate[6] and recrystallized three times from ethyl acetate. Hydrolysis was then effected by heating the sample (7.5 gm) for 30 min in 1 *M* alcoholic potassium hydroxide (110 ml). After evaporation to dryness, the residue was precipitated from dilute aqueous potassium hydroxide with 1 *M* hydrochloric acid. Filtration followed by washing with water, and drying gave material of sufficient purity for use in the next step.

Fluorescein-di-(2',3',4',6'-tetra-O-acetyl-β-D-galactopyranoside) (IIc): Purified fluorescein (5 gm, 15 mmole), 2,3,4,6-tetra-O-α-D-galactopyranosyl bromide[8] (12.4 gm, 30 mmole), freshly prepared silver oxide[15] (3.5 gm, 15 mmole), dried benzene (75 ml), and four drops of freshly distilled quinoline were stirred at room temperature in the dark for 60 hr. After removal of the solids by filtration (2.7 gm fluorescein was recovered), the filtrate was washed with 0.5 *M* aqueous sodium hydroxide (3 × 100 ml), and then with water (5 × 100 ml). The solution was then dried over sodium sulfate and, after evaporation to dryness, the residue was taken up in 200 ml of benzene and chromatographed on 100 gm of silicic acid (Mallinckrodt No. 2847) in a 3 × 26 cm column. After washing the column with 200 ml benzene, elution was commenced using a linear gradient of ethyl acetate. The mixing vessel initially contained 700 ml benzene and the reservoir 700 ml benzene–ethyl acetate (4:1). The (4:1) mixture was used for further elution. After eluting with 1100 ml, a peak containing the desired compound was obtained. The solvent was removed under reduced pressure yielding 2.2 gm (2.2 mmole) of material. Two recrystallizations from methanol provided the analytical sample, mp 153–155°, which was colorless and nonfluorescent. λ_{max} (CH_3OH) 223 mμ (log ε 4.72), 273 mμ (3.74); λ_{max} (KBr) 5.70, 6.18, 6.65, 7.28, 8.0–8.3μ; R_f (silica plate) 0.70.

Analysis calculated for C_{48} $H_{48}O_{23}$: C, 58.1; H, 4.9; O, 37.1; CH_3CO, 34.7. Found: C, 58.0; H, 5.2; O, 37.1; CH_3CO, 33.9.

After elution of the column with pure ethyl acetate, 2.5 gm of a crystalline, orange red, strongly fluorescent substance was obtained. This compound is probably a derivative of fluorescein monogalactoside. On the basis of fluorescein, the over-all yield of the reaction was 65%.

Fluorescein-di-(β-D-galactopyranoside) (IId): A solution of 700 mg (0.7 mmole) of IIc in 35 ml of absolute methanol was mixed at 0° with 2 ml of 0.85 *M* sodium methoxide in the same solvent. No starting material could be detected by thin layer chromatography after 15 min. After 2 hr at 0°, the reaction mixture was diluted with an equal volume of water and was passed through Dowex 50W-X8 in the pyridine form. The solution was evaporated to dryness under reduced pressure,

and the residue, dissolved in dioxane and lyophilized, afforded 460 mg (0.66 mmole) of a nonfluorescent yellowish powder (95 per cent yield). The analytical sample, recrystallized twice from ethanol and once from water, was crystalline, colorless, and nonfluorescent. The compound becomes colored when stored in dry form. It is stable in solutions at neutral pH which are kept frozen, mp 202°-dec., λ_{max} (CH_3OH) 224 mμ (log ϵ 4.82), 273 mμ (3.83); λ_{max} (KBr) 5.75, 6.25, 6.70, 7.05 μ; R_f 0.68 in solvent I, 0.17 in solvent II.

Analysis calculated for $C_{32}H_{32}O_{15}.2H_2O$: C, 55.5; H, 5.2; fluorescein, 48.0. Found: C, 55.4; H, 5.3; fluorescein, 47.8.

Fluorescein was determined spectrophotometrically before and after complete hydrolysis of II*d* with β-D-galactosidase. In this way, it was shown that the analytical sample of II*d* contained fluorescence and color corresponding to 0.13% fluorescein. Partial hydrolysis with the enzyme yielded three compounds separated on paper chromatography with solvent II. The R_f of two of these compounds corresponded to those of fluorescein and fluorescein digalactoside. The third compound was probably the monogalactoside of fluorescein.

Fluorescein-3-O-methyl-6-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside) (IIg): Similar treatment of 1.8 gm (5.2 mmole) fluorescein monomethyl ether (II*f*)[10] with 2 gm (4.9 mmole) of 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide[8] and 5.8 gm (25 mmole) of Ag_2O,[15] followed by recrystallization from ethyl acetate-hexane, provided 2.4 gm (0.36 mmole) of the title product. In this case, column chromatography was not necessary. The analytical sample was colorless, nonfluorescent, mp 241-242°; λ_{max} (CH_3OH) 224 mμ (log ϵ 4.83), 273 mμ (3.91); λ_{max} (KBr) 5.75, 6.20, 6.32 m, 6.66, 6.78 m, 7.00, 8.0–8.3 μ, R_f (silica plate) 0.75.

The free galactoside, obtained by deacetylation and purification by paper chromatography, was characterized only by enzymatic degradation. This galactoside was found to be less sensitive than II*d* for the fluorogenic assay of β-D-galactosidase.[1]

Monocyclohexylammonium 3-O-methyl-fluorescein phosphate (IIh): A sample of II*f*[10] (5.2 gm, 15 mmole) was dissolved in 50 ml of pyridine, cooled in an ice bath, and then added all at once to two equivalents (2.75 ml) of phosphorous oxychloride in 50 ml of pyridine. After 30 min at 0°, the mixture was poured onto 100 gm of ice, and the solvent evaporated under vacuum at 30°. The residue was dissolved in 200 ml of 2-butanol or 1-butanol (some insoluble material always remains), and extracted five times with 100 ml portions of saturated aqueous sodium sulfate. The organic phase was dried over sodium sulfate and then evaporated to dryness under vacuum at 30°. The residue was dissolved in a minimum of water (*ca.* 50 ml) and the pH carefully adjusted to 7 with 1 *M* sodium hydroxide. Ethanol (*ca.* 100 ml) was then added until precipitation of the sodium salt began. After standing at 0° overnight, the precipitate was collected by centrifugation and washed with a small amount of cold ethanol. The resulting solid could then be stored in the freezer as an ethanolic suspension and under these conditions the salt appears not to undergo any decomposition. If one attempts to store this salt in a dry form, rapid decomposition ensues which is easily detectable by the resulting coloration.

The precipitate obtained from about 5 ml of suspension was dissolved in a minimum volume of water (*ca.* 5 ml) and added to a suspension of Dowex 50W-X8 in the cyclohexylamine salt form (10 ml) in water (10 ml). This mixture was then

stirred for 10 min whereupon a yellow solid precipitated. Methanol (*ca.* 50 ml) was added until the yellow solid had completely dissolved. After filtration, the Dowex resin was washed with 50 ml of methanol and the combined filtrates were evaporated to a small volume (*ca.* 10 ml). The solid, which had then precipitated, was filtered and dissolved in a minimum volume of warm methanol. Ether was then added until precipitation began. After storing overnight at 0°, the precipitate was collected and recrystallized four or five times to obtain a white amorphous solid, nonfluorescent, mp 218–223° dec. Precipitation takes place with difficulty after the first recrystallization and it may be necessary either to evaporate part of the methanol and add more ether, or to leave the solution in the freezer 4–5 days. While this sample appeared homogeneous on Whatman No. 1 paper in solvent systems III–VI, it was noted that on S & S orange paper in solvent system IV a faint bluish streak could be seen following the phosphate spot. In solvent systems III, IV, and VI, a barely perceptible yellow streak may be observed just ahead of the phosphate spot. The intensities of these streaks indicate that they are present only in trace amounts. For analysis, the samples were dried for 3 days at room temperature over phosphorous pentoxide under 0.1 mm pressure.

Analysis calculated for $C_{27}H_{28}O_8NP$: C, 61.7; H, 5.4; N, 2.7; P, 5.9. Found: C, 61.3; H, 5.9; N, 2.8; P, 5.7; λ_{max} (CH_3OH) 224 mμ (log ϵ 4.81), 273 mμ (3.84); λ_{max} (KBr) 5.70, 6.10, 6.20, 6.45 m, 6.65, 6.78 m, 7.02 μ; R_f values 0.49, 0.71, 0.81, 0.74, 0.83 in solvents III, IV, V, VI, and VII, respectively.

The over-all yields for the cyclohexyamine salt were in excess of 10%.

Summary.—Detailed and reproducible procedures are reported for the synthesis of fluorescein-di-(2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside), fluorescein-di-(β-D-galactopyranoside), 3-O-methyl-fluorescein-2′,3′,4′,6′-tetra-O-acetyl-β-D-galactopyranoside, and monocyclohexylammonium 3-O-methyl-fluorescein phosphate. These compounds are important as fluorogenic substrates for determination of β-D-galactosidases and phosphatases because the sensitivity of the assay extends to single molecules of enzyme. The material employed previously, assumed to be 6-hydroxyfluoran-β-D-galactopyranoside, is shown to be fluorescein-di-(β-D-galactopyranoside).

We wish to thank Dr. Koert Gerzon for suggesting that fluorescein could be the product in the condensation of 2,4-dihydroxybenzoyl benzoic acid and phenol. We are also grateful to Dr. John G. Moffatt for his constant interest in this work and invaluable advice, and to Dr. P. G. Holton (Syntex, S.A., Mexico City) for a supply of fluorescein monomethyl ether (II*f*).

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[17] All melting points are uncorrected. Microanalyses were performed by Midwest Microlab, Inc. Infrared spectra were determined with a Perkin-Elmer Model 237 spectrophotometer.

THE IONIC CENTRIFUGE CAN GIVE FUSION NUCLEAR POWER

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Different equations for the behavior of plasmas have been given by different authors. Since the results of experiments on the Ionic Centrifuge seem to accord better with one of these than with another, it seems desirable to compare them, particularly as the Ionic Centrifuge seems to offer the possibility of successful nuclear fusion.

1. *The Equations Offered by Spitzer.*—The equations offered by Spitzer in his book[1] (p. 18) are for the positive ions

$$n_i m_i\left(\frac{\partial \mathbf{v}_i}{\partial t} + \mathbf{v}_i \cdot \nabla \mathbf{v}_i\right) = \frac{n_i Ze}{c}(\mathbf{E} + \mathbf{v}_i \times \mathbf{B}) - \nabla \cdot \psi_i - n_i m_i \nabla \Phi + \mathbf{P}_{ie}, \quad \text{Sp(2-4)}$$

$$\mathbf{v}_i = \frac{1}{n_i \Delta V}\sum \mathbf{w}_i, \quad \text{Sp(2-5)}$$

$$\psi_i = \frac{m_i}{\Delta V}\sum (\mathbf{w}_i - \mathbf{v}_i)(\mathbf{w}_i - \mathbf{v}_i), \quad \text{Sp(2-6)}$$

where Φ is the gravitational potential, $\mathbf{v}_i$ the mean velocity of the positive ions in an element of volume ΔV, $\mathbf{w}_i$ the actual velocity of the individual ion, and ψ_i is the stress tensor or dyadic of the ions. $\mathbf{P}_{ie}$ is the total momentum transferred to the ions per unit volume per unit time by "collisions" with the electrons.

The corresponding equations for the electrons are obtained by replacing the quantities $\mathbf{v}_i, n_i, m_i, \psi_i, \mathbf{P}_{ie}$ with $\mathbf{v}_e, n_e, m_e, \psi_e, \mathbf{P}_{ei}$. Z is replaced by -1.

Of course, by Newton's laws, $\mathbf{P}_{ei} + \mathbf{P}_{ie} = 0$, but it cannot be concluded that $\mathbf{P}_{ei} = 0$ and $\mathbf{P}_{ie} = 0$, no matter what the density of the ions and electrons may be, so long as the electric field and magnetic field are determined by the over-all current, $\mathbf{j} = eZn_i\mathbf{v}_i - en_e\mathbf{v}_e$.

Spitzer then gives as an approximate equation (ref. 1, p. 21)

$$\rho\frac{\partial \mathbf{v}}{\partial t} = \mathbf{j} \times \mathbf{B} - \nabla p - \rho \nabla \phi, \quad \text{Sp(2-11)}$$

where

$$\mathbf{v} = \frac{1}{\rho}(n_i m_i \mathbf{v}_i + n_e m_e \mathbf{v}_e), \quad \text{Sp(2-8)}$$