Synthesis of Fluorogenic Phosphodiesterase Substrates. Preparation of Di(coumarin-7-yl)phosphate and Di(4-methylcoumarin-7-yl)phosphate

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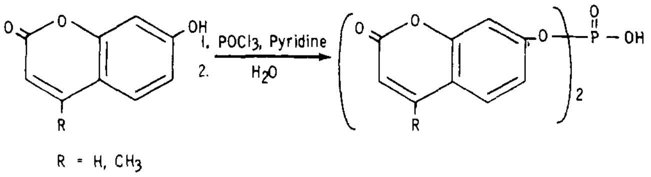
Di(coumarin-7-yl)phosphate and di(4-methylcoumarin-7-yl)phosphate were synthesized by conventional methods. These compounds served as fluorogenic phosphodiesterase substrates.

In the course of our studies, it became necessary to assay for low concentrations of phosphodiesterases selectively released from *Escherichia coli* (*2, 4*). We needed a more sensitive assay system than that offered by available colorimetric methods (*3, 4*); thus, we found it necessary to synthesize some fluorogenic phosphodiesterase substrates. Hopefully, these materials, themselves nonfluorescent, would be hydrolyzed by the enzymes to yield fluorescent products and provide the basis of an assay with increased sensitivity.

We prepared phosphoric acid diesters of the fluorescent compounds 7-hydroxycoumarin and 4-methyl-7-hydroxycoumarin essentially by the method of Friedman and Seligman (*1*). The synthetic scheme pursued (as depicted in the structure) involved the reaction of appropriate amounts of either compound with phosphorus oxychloride and pyridine followed by hydrolysis.



Infrared spectra of the esters revealed the expected carbonyl and conjugated olefinic absorption bands in addition to the phosphorus oxygen absorption bands at 7.9 and 8.7 μ and the disappearance of the broad phenolic oxygen-hydrogen absorption band at 3.1 μ.

The di(coumarin-7-yl)phosphate isolated from the reaction mixture resisted all attempts at recrystallization and was purified by digestion, in turn, in refluxing acetone and benzene followed by precipitation from distilled water through the dropwise addition of concentrated hydrochloric acid. Both esters served as phosphodiesterase substrates.

Experimental

The infrared spectra were measured in potassium bromide discs with a Perkin-Elmer spectrophotometer, Model 521. All melting points are uncorrected. All solvents were dried and redistilled before use. The phosphorus oxychloride was also distilled before use, and the starting materials were recrystallized.

Di(coumarin-7-yl)phosphate. To a slurry of 3.24 grams (0.02 mole) of 7-hydroxycoumarin in 50 ml of benzene heated at reflux was added with vigorous stirring 1.53 grams (0.01 mole) of phosphorus oxychloride. Thereafter, 1.6 grams (0.02 mole) of pyridine in 5 ml of benzene was added dropwise over a 15-min period. The reaction mixture was heated for 2 hr and allowed to stand overnight. The solid that formed was separated from the benzene solution and refluxed for 4 hr in a mixture of 100 ml of water and 150 ml of acetone. The resulting solution was evaporated, and the oily residue dissolved in 10% sodium bicarbonate before being extracted three times with ethyl acetate. The aqueous layer was treated with dilute hydrochloric acid to form a white solid.

The benzene solution obtained above was evaporated to yield a yellow, gummy residue. This material was treated with a solution of 10 ml of water in 25 ml of acetone. This mixture was refluxed for 4 hr and then evaporated to give an oil which was dissolved in 10% sodium bicarbonate; the bicarbonate solution was extracted with ethyl acetate and treated with dilute HCl. The white precipitate obtained here was combined with the solid previously obtained. This material was then, in turn, digested in refluxing acetone and then benzene before being dissolved in water and titurated with concentrated HCl to yield 1.2 grams (0.003 mole, 15% yield) of the phosphoric acid diester, mp 235–8°C.

Anal. Calcd $C_{18}H_{11}PO_8$: C, 55.97; H, 2.87. Found: C, 55.61; H, 2.87.

Di(4-methylcoumarin-7-yl)phosphate. A mixture of 7.08 grams (0.04 mole) of 4-methyl-7-hydroxycoumarin and 3.06 grams (0.02 mole) of $POCl_3$ was heated at reflux with stirring, and over a period of 30 min, a solution of 3.16 grams (0.04 mole) of dry pyridine in 5 ml of benzene was added. Heating was continued for 2 hr, and the mixture was allowed to stand at room temperature for two days.

The oil and benzene layers which formed were separated and subjected to thin-layer chromatography (solvent methanol–benzene 1:9). Tlc indicated that the oil was mainly starting material, and the product could be found in the benzene solution. The benzene solution was evaporated and the residue was treated with acetone and filtered. An equal volume of water was added to the acetone filtrate, and the mixture was heated until the desired diester formed as a white solid. This material was recrystallized from water to give 2.43 grams (0.006 mole, 15% yield) of a white solid, mp 233–5°C.

Anal. Calcd $C_{20}H_{15}PO_8$: C, 57.97; H, 3.65. Found: C, 57.82; H, 3.68.

Literature Cited

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