AN ULTRAMICRO FLUORESCENT SPRAY REAGENT FOR DETECTION AND QUANTIFICATION OF CARDIOTONIC STEROIDS ON THIN-LAYER CHROMATOGRAMS*

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INTRODUCTION

JENSEN[1], and later WELLS, KATZUNG AND MEYERS[2], have described a useful reagent for the fluorometric analysis of cardiotonic steroids. This reagent has been reliably used by MURPHY for assay of digoxin content in *Digitalis lanata*[3]. Basically, the reagent is a mixture of concentrated hydrochloric acid, methanol, ascorbic acid, and dilute aqueous hydrogen peroxide.

This report describes a modification of the above reagent into a useful spray for the detection of 0.01 μg amounts of cardiotonic steroids on thin-layer chromatograms. This is accomplished by the simple expedient of substituting microliter amounts of 30 % aqueous hydrogen peroxide for the very dilute solutions used by the above authors. This modification permits more rapid drying of the sprayed plate so that areas of interest can be easily scraped off for subsequent elution and quantification, using the original fluorometric reagents[1,2]. This spray reagent also appears well suited for direct photofluorometric quantitative analysis upon the thin-layer plate.

METHODS

General reagents and glassware

ACS reagent grade formamide, concentrated hydrochloric acid (specific gravity = 1.19), concentrated nitric acid, L-ascorbic acid (Eastman Organic Chemicals) and 30 % aqueous hydrogen peroxide (Matheson, Coleman and Bell) were all used as received. The hydrogen peroxide was stored in a refrigerator.

ACS reagent grade methylene chloride and chloroform were redistilled from anhydrous K_2CO_3 before use and stored in the dark. ACS reagent grade methanol was redistilled before use, using glass beads and a few (about three) micro-porous boiling chips (Van Waters and Rogers).

Deionized water (Sparkletts) was used throughout as received, both as a reagent and for all glasswashing procedures.

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All glassware was first well washed in dilute detergent (Royal Drene Shampoo, Proctor and Gamble), 1 ml per gallon of water. It was then rinsed 10 times with water, soaked overnight in concentrated HNO_3–H_2O, 1:1, rinsed 10 times with water, and either dried at room temperature or in an oven at 60°.

Preparation of spray reagent

The spray reagent was prepared as follows: 20 mg ascorbic acid were dissolved in a mixture of 19 ml methanol and 30 ml concentrated HCl. Fresh 30 % aqueous hydrogen peroxide (2.1 μl) was then added. The contents were mixed and used immediately. The chromatographic plate was sprayed thoroughly with this reagent until it was uniformly well wetted and slightly translucent. It was placed in the dark for 50 min and then examined in a chromatographic view box (Ultra-Violet Products, Inc., San Gabriel, Calif.) containing a 30 watt long-wave ultraviolet lamp (main wavelength, 360 mμ) which could shine down on the plate, and/or a similar 90 watt long-wave lamp upon which the plate could be placed for transillumination.

Elution and fluorometric quantification of digoxin

Digoxin was then subjected to a one-step elution and quantification procedure as follows: The fluorescent spots were scraped from the plate into 50 ml centrifuge tubes, and 3 ml of the original reagent of either JENSEN[1] or WELLS, KATZUNG AND MEYERS[2] was added. In our laboratory this reagent was prepared as follows: 20 mg ascorbic acid were dissolved in a mixture of 19 ml methanol and 30 ml concentrated HCl. 1.25 ml of a solution containing 85 μl of 30 % H_2O_2 dissolved in 50 ml H_2O were then added. The centrifuge tubes were then vigorously agitated on a vortex mixer for exactly 5 min, centrifuged for 10 min at 2000 r.p.m. and the supernatants were then carefully aspirated and read in a Turner Model 110 Fluorometer 50 min after the reagent was originally added. The primary filter was Turner's No. 7-60 (360 mμ). The secondary was an interference filter (No. 339), with peak transmission at 470 mμ, made by Optics Technology, Inc., Palo Alto, Calif. Maximal sensitivity of the fluorometer was used.

Although the fluorescence maximum of this product of digoxin was 490 mμ when larger amounts were examined on an Aminco-Bowman Model 4-8202 spectrophotofluorometer, the analysis of 0.01 to 0.03 μg amounts per 3 ml in the Turner fluorometer at 490 mμ was difficult because of apparent fluorescence of the quartz cuvettes at 500 mμ[4]. The situation was much improved when the 470 mμ filter was used, which effectively excluded the interfering fluorescence.

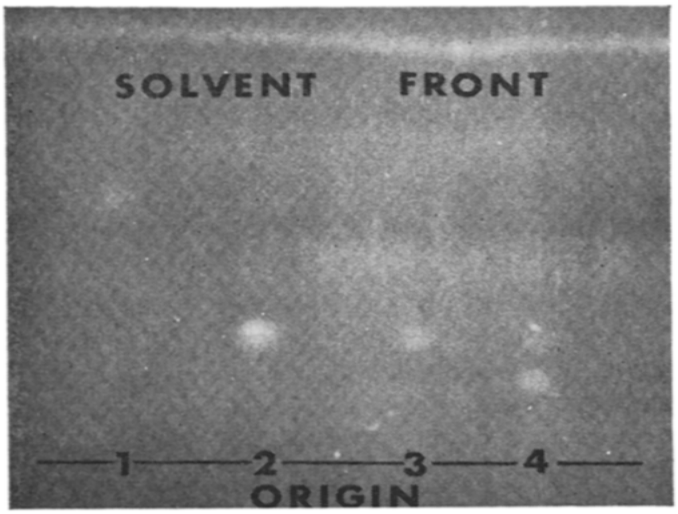
RESULTS

Detection of cardiotonic steroids on thin-layer chromatograms

When sprayed as described and examined with either or both of the ultraviolet lights described, 0.01 μg amounts of cardiotonic steroids were reliably detected.

Fig. 1 is a polaroid photograph of a thin-layer chromatogram showing fluorescent spots produced by 0.02 μg amounts of digitoxigenin, digitoxin, digoxigenin, and digoxin after treatment with the spray reagent. Close inspection shows that the digoxin sample (spot 4) also contained small amounts of material resembling digoxigenin. These compounds were chromatographed as follows: 30 g Silica Gel G (Research

Specialities Company) was shaken with 60 ml of 0.05 *N* NaOH and spread in a 0.25 mm thick layer with a Desaga spreader. The plates were activated at 110° for 30 min, cooled in a desiccator, and then spotted with the above four cardiotonic steroids. Ascending development was performed for 10 cm in methylene chloride–methanol–formamide (90:9:1; v/v/v). The plate was removed, allowed to dry for 5 min, and then treated with the spray reagent as described above. Fluorescence development was maximal after 50 min. Digitoxin gave a slightly reddish-blue fluorescence which after several hours gradually changed to a brick-red fluorescence. Digoxin, on the other hand, gave a brilliant sky-blue fluorescence which persisted overnight without significant fading. These findings are consistent with excitation and fluorescence spectra previously published[2]. Similar amounts of these fluorescent compounds were detected with the spray reagent following chromatography on Eastman Silica Gel thin-layer sheets, using only a small, four-watt, hand-held, long-wave ultraviolet light.



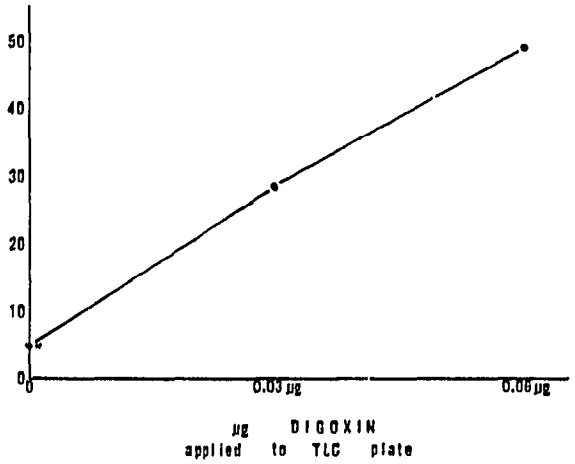


Fig. 1. Polaroid photograph of Silica Gel G plate containing 0.02 µg amounts of cardiotonic steroids after treatment with the spray reagent. 1 = Digitoxigenin; 2 = digitoxin; 3 = digoxigenin; 4 = digoxin.

Fig. 2. Graph of fluorescence developed by varying amounts of digoxin when sprayed, eluted, and quantified as described. * = TLC blank set to 5 units.

Subsequent elution and quantification of digoxin

Fig. 2 compares the fluorescence developed by the reagent blank and by 0.03 and 0.06 µg of digoxin after elution and quantification as described. Reference standards of digoxin were also prepared. The slope of fluorescence *versus* amount of digoxin eluted from the thin-layer plate is similar to that obtained from test tube reference standards. It is felt that digoxin can probably be eluted and quantified in this manner with an analytic error of less than $\pm$ 0.003 µg.

In addition, preliminary studies with digitoxin show that it, too, can be similarly eluted and quantified, although with less sensitivity due to its less brilliant fluorescence.

Examination of the fluorescent products

It was of interest to compare the chromatographic behavior of the fluorescent products formed by the spray reagent with that of the original compounds. A marked loss of polarity occurs due to treatment with the spray reagent, suggesting not only

a loss of the sugars, but also a loss of most of the hydroxyl groups present on the genins.

One microgram amounts of digitoxigenin, digoxigenin, digitoxin, and digoxin were spotted on a Silica Gel G plate prepared as described above. The plate was then treated with the spray reagent and allowed to stand in the dark for 50 min. The fluorescent products thus produced by the spray reagent were then subjected to ascending chromatography in chloroform–methanol (180:5, v/v) for 10 cm. The plate was removed, allowed to dry, and then examined in the view box. Fig. 3 shows a polaroid photograph of this chromatogram. One major fluorescent spot was seen when digitoxin or digitoxigenin were treated in this manner, while two major spots were seen when digoxin or digoxigenin were thus treated. Gitoxigenin, in similar studies, yielded a single sky-blue fluorescent product with chromatographic mobility quite similar to that of the fluorescent product of digitoxin and the faster moving fluorescent product of digoxin.

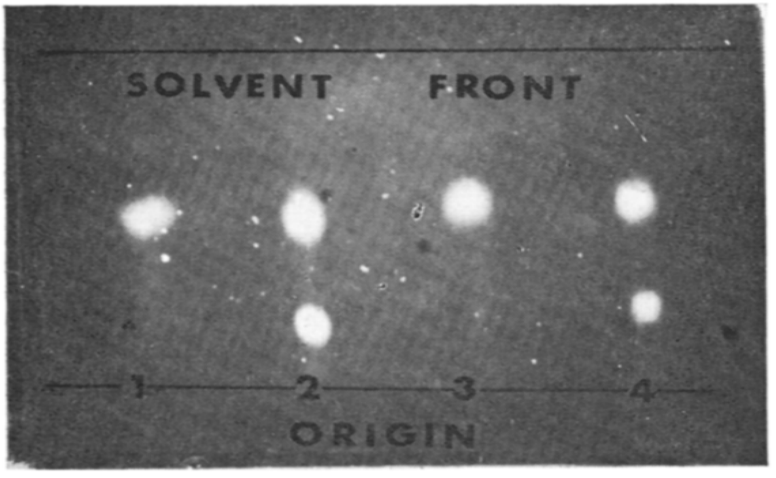


Fig. 3. Chromatogram of the fluorescent products formed by treatment of cardiotonic steroids with the spray reagent. Polaroid photograph. Steroids were spotted on Silica Gel G at the origin, treated with the spray reagent, dried, and subsequently subjected to thin-layer chromatography in chloroform–methanol, 180:5. 1 = Digitoxin; 2 = digoxin; 3 = digitoxigenin; 4 = digoxigenin. One fluorescent product is formed from digitoxin or digitoxigenin. Two fluorescent products are formed from digoxin or digoxigenin.

Excitation and emission spectra of the fluorescent products of digitoxin, of gitoxigenin, and of both fast and slow moving products of digoxigenin are shown in Fig. 4, along with appropriate control spectra from blank portions of the thin-layer plate. Digitoxin (Fig. 4a) yields a product with an excitation maximum at 410 mμ and an emission maximum at 570 mμ. The spectrum of the product of digitoxigenin is identical. A small excitation peak at 300 mμ was also present in the thin-layer chromatogram blank but was absent in unchromatographed samples. It is therefore probably due to material present in the Silica Gel G.

The fluorescent product of gitoxigenin in Fig. 4b and both fast and slow moving products of digoxigenin (Figs. 4c and 4d) all gave identical spectra with an excitation maximum at 365 mμ and an emission maximum at 490 mμ. The fluorescent spectrum of digoxin was identical.

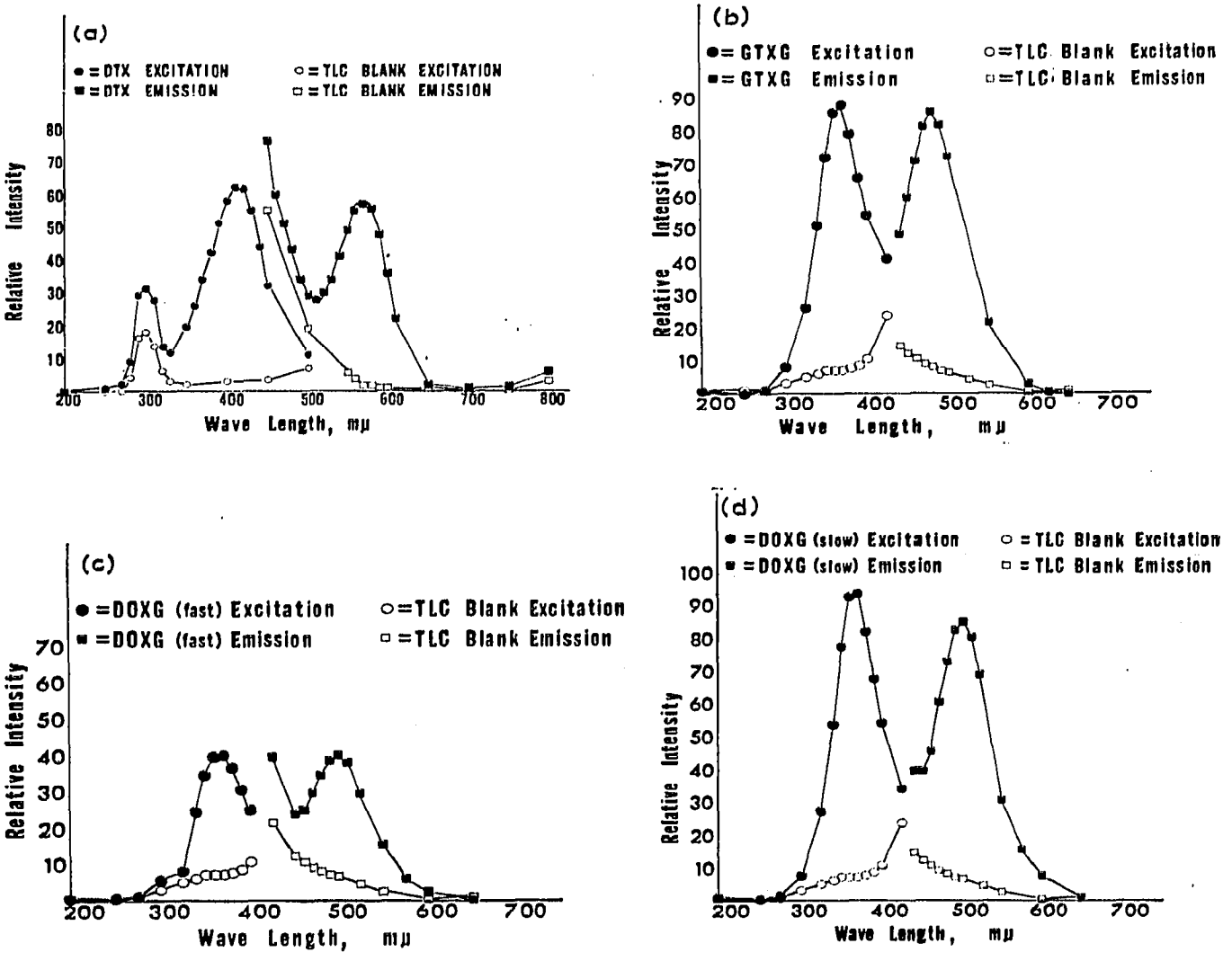


Fig. 4. Excitation and emission spectra of fluorescent products of cardiotonic steroids following thin-layer chromatography. Control spectra from appropriate blank areas of the thin-layer plate are also shown (TLC blank). (a) Digitoxin (DTX). (b) Gitoxigenin (GTXG). (c) Digoxigenin, fast moving component by thin-layer chromatography (DOXG, fast). (d) Digoxigenin, slower moving component by thin-layer chromatography (DOXG, slow).

DISCUSSION

The spray reagent reported here is well suited for detection of ultramicro amounts of cardiotonic steroids, followed by their subsequent elution and quantification by a well-proven fluorometric procedure[1,2]. The fluorescent reaction products are stable. They can be subjected to further chromatographic manipulations. Furthermore, the spray reagent also appears well suited for direct photofluorometric quantitative analysis upon the chromatographic plate itself.

Several other spray reagents appear to be at least as sensitive as that reported here, but they appear to have been used primarily for identification purposes. Ethanolic trichloracetic acid[5,6] is sensitive, but the A series compounds do not fluoresce. Trichloracetic acid–chloramine reagent[5,7] is also approximately as sensitive

(0.01 to 0.5 μg) and the fluorescent products are reported to be stable. It does not appear to have been used for quantitative analysis. Phosphoric acid and bromine vapor[3] is a very sensitive spray reagent (0.002 μg of digoxin, 0.008 μg of digitoxin), but the procedure is complicated and may well be less reproducible than the spray described here. Concentrated acid and sodium hypochlorite reagent[6,8] is also sensitive and may well be reproducible, but does not appear to have been used for quantitative analytic purposes. *p*-Anisaldehyde–perchloric acid reagent[9] is also sensitive. Other fluorescent spray reagents appear to be not nearly so sensitive[10–15].

Much of the variation in sensitivity reported in the literature with various fluorescent spray reagents may in fact be due to variation in intensity and wave length of ultraviolet illumination, and to variation in thickness and opacity of the chromatographic medium in which the fluorescent product is formed. Several authors[6,16–18,22] marshall impressive evidence that several different treatments of gitoxigenin with strong acid all result in the production of $\Delta^{14,16}$-dianhydrogitoxigenin. This compound has an ultraviolet absorption maximum at 337 mμ[18] and yields a stable blue fluorescence[6]. Similar treatments of digitoxigenin yield only the non-fluorescent Δ^{8-14}- or Δ^{14-15}-anhydrodigitoxigenin[1,6].

However, when digitoxin, digoxin, or their genins are exposed to an oxidizing agent such as hydrogen peroxide, as well as to strong methanolic hydrochloric acid, as in the spray reagent reported here, it is conceivable that the following four-step reaction may occur:

(1) Hydrolysis of glycoside to genin.

(2) Production of Δ^{8-14}- and Δ^{14-15}-anhydrogenins, probably in equilibrium with each other.

(3) Conversion of the Δ^{14-15}-anhydrogenin to its corresponding 16-hydroxy or 16-chloro compound.

(4) Conversion of this intermediate 16-substituted compound to a Δ^{14-16}-dianhydrogenin, with ultimate conversion of the majority of steroid to such a fluorescent product by the spray reagent.

Other spray reagents[5–8] also produce fluorescent products of the A series compounds, possibly by formation of similar 16-substituted derivatives of Δ^{14}-anhydrodigitoxigenin.

Further, it is possible that compounds of series C may also be similarly converted to fluorescent 16-substituted derivatives of Δ^{14}-anhydrodigitoxigenin[2,6,8]. Digoxin, for example, is converted by the spray reagent reported here to two products which both fluoresce a brilliant blue. One has almost the same chromatographic mobility as the fluorescent product of digitoxin. The other is slightly more polar. It is likely that similar $\Delta^{14,16}$-dianhydrogenins of digoxin may be produced by treatment with the spray reagent as are produced from digitoxin, and for the same reasons. This hypothesis is supported by the great similarity between the fluorescent products of digoxin and of gitoxigenin, both in chromatographic mobility and in their fluorescence spectra.

The lactone ring of digitalis glycosides has an ultraviolet absorption maximum at 220 mμ[19]. When one adds two more conjugated double bonds to this system, as with $\Delta^{14,16}$-dianhydrogitoxigenin, one finds an ultraviolet absorption peak at approximately 340 mμ[18]. Thus, each new conjugated double bond appears to advance the absorption maximum by about 60 mμ. The fact that the fluorescent products of

digoxin have an excitation maximum fairly close to 340 mμ suggests that a total of at least four conjugated double bonds may well have been produced by the spray reagent. In addition, one or both of the hydroxyl groups present on digoxigenin may have been oxidized to keto groups, or possibly substituted and dehydrated to form a C=C double bond. One might suggest, then, that the less polar fluorescent product of digoxin produced by the spray reagent might possibly be 3,12-diketo-$\Delta^{14-15,\ 16-17}$-dianhydrogitoxigenin or Δ^{3}, $\Delta^{11-12,\ 14-15,\ 16-17}$-tetraanhydrogitoxigenin, and that the more polar product might possibly be 3-keto-12-hydroxy-$\Delta^{14-15,\ 16-17}$-dianhydrogitoxigenin or Δ^{3}, -12-keto-$\Delta^{14-15,\ 16-17}$-trianhydrogitoxigenin.

On the other hand, since the fluorescent product of digitoxin produced here has an excitation maximum at 410 mμ, it is likely that one more, or at least five conjugated double bonds may be present in this molecule. Possible structures for this fluorescent product might then be 3-keto (or Δ^{3-4}), $\Delta^{8-9,\ 14-15,\ 16-17}$-anhydrogitoxigenin, or 3-keto (or Δ^{3-4}), $\Delta^{7-8,\ 14-15,\ 16-17}$-anhydrogitoxigenin.

These hypothetical structures are somewhat supported by FAUCONNET AND FAZAN[6]. However, it is not clear why only digitoxigenin seems to yield a product with at least five probable conjugated double bonds, while gitoxigenin and digoxigenin do not.

That the proposed less polar derivative of digoxin may in fact have a chromatographic mobility quite similar to the fluorescent product of digitoxin and gitoxigenin is rendered possible by the following:

(A) 3,12-Digoxigenone yields only a monoxime and a monosemicarbazone[20]. A molecular model of this compound reveals that the C_{12} carbonyl group is quite hindered by the C_{18} methyl group, by the lactone ring, and also by the D ring of the steroid nucleus, which is in a position *cis* to the A ring.

(B) Δ^{14}-Anhydrodigoxigenone, on the other hand, yields a dioxime and a mixture of mono- and di-semicarbazones[21]. Such enchancement of activity at C_{12} may well occur because both the D ring and the lactone ring have been swung well away from the C_{12} carbonyl group by the formation of the double bond at C_{14-15}.

(C) If one then goes on to add still another double bond at C_{16} to the above molecule, the lactone ring is once again swung back into close proximity to the C_{12} carbonyl group, causing what appears to be at least as much steric hindrance of the C_{12} carbonyl as exists in the case of 3,12-digoxigenone.

It is therefore likely that the C_{12} position is so hindered that a Δ^{11-12} or a C_{12} carbonyl group may not exert much influence upon chromatographic behavior.

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SUMMARY

A spray reagent is described which permits detection of 0.01 μg amounts of digitoxin, digoxin, and their genins on thin-layer chromatograms. The fluorescent products formed by the reagent are reproducible and stable. They lend themselves

well to subsequent elution and quantification by well proven, previously described methods[1,2]. Furthermore, the spray reagent appears well suited for direct photofluorometric quantitative analysis. The excitation and emission spectra and chromatographic behavior of the fluorescent products produced by this reagent have been examined, and hypothetical structures of these products have been suggested.

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