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NEW CHEMILUMINESCENT DERIVATIZING AGENT FOR THE ANALYSIS OF ALDEHYDES AND KETONES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PEROXYOXALATE CHEMILUMINESCENCE

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SUMMARY

Three methods of reductive amination have been investigated for the derivatization of aldehydes and ketones with a new chemiluminescent agent, 3-aminofluoranthene. Use of a borane–pyridine complex was found to give the highest product yield. Analytes were separated by high-performance liquid chromatography (HPLC) and detected by peroxyoxalate chemiluminescence. Detection limits were in the fmol range, chemiluminescence detectability being thirty times better than fluorescence.

INTRODUCTION

Peroxyoxalate chemiluminescence has been shown to be an effective post-column reaction for the detection of a number of compounds. Certain analytes can be sensitively detected if they have native fluorescence[1,2] or if they can be derivatized with a fluorescent agent[3]. However, not all fluorescent compounds can be sensitively detected with peroxyoxalate chemiluminescence. Development of new derivatizing agents which can be sensitively detected might extend the number of applications which would benefit from the sensitivity and wide linear dynamic range of peroxyoxalate chemiluminescence.

The nature of fluorophores efficiently detected by peroxyoxalate chemiluminescence has been investigated previously[4]. Amino-substituted polycyclic aromatic hydrocarbons (PAHs) are among the most sensitively detected fluorophores[2]. Here, we report an investigation of derivatization reaction conditions, effect on chromatographic efficiency, and detectability of 3-aminofluoranthene as a possible derivatizing reagent for aldehydes and ketones.

EXPERIMENTAL

Apparatus

A Gilson Model 302 HPLC pump (Gilson, Middleton, WI, U.S.A.), equipped with a 20-μl Rheodyne 7120 injection valve (Rheodyne, Cotati, CA, U.S.A.) and an Econosphere C_{18} column (5 μm, 25 cm × 4.6 mm I.D.) (Alltech, Deerfield, IL,

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U.S.A.) was used in the separation of analytes. A Schoeffel FS 970 fluorometer (Schoeffel, Westwood, NJ, U.S.A.) with excitation at 305 nm and a long pass 389 nm emission filter was employed for fluorescent detection. The mobile phase was acetonitrile–water (85:15) at a flow-rate of 1.2 ml/min.

For chemiluminescent detection, two pumps, a Gilson Model 302 pump, delivering 1 *M* hydrogen peroxide in acetonitrile at 1.2 ml/min and a Spectroflow 400 pump (Kratos, Ramsey, NJ, U.S.A.), delivering 4 m*M* bis(2,4,6-trichlorophenyl) oxalate in ethyl acetate at 2.1 ml/min, were connected so that the reagents were mixed before introduction into the column effluent. The fluorometer was operated with the lamp off and without emission filter. The mobile phase was acetonitrile–4 m*M* tris(hydroxymethyl)aminomethane in water at pH 7.5 (85:15, v/v) at a flow-rate of 1.2 ml/min.

Chemicals

The aldehydes *n*-hexanal, *n*-heptanal, *n*-octanal, and *n*-decanal (purity > 95%) and the ketones 2-hexanone, 2-heptanone, 2-octanone and 2-decanone (purity > 95%), borane–pyridine complex (BAP), sodium cyanoborohydride (95% pure), 3-aminofluoranthene (97% pure), tris(hydroxymethyl)aminomethane (99.9% pure) and toluene (99.9% pure) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC grade methanol and acetonitrile were purchased from Baker (Philipsburg, NJ, U.S.A.). Bis(2,4,6 trichlorophenyl) oxalate (TCPO) was donated by A. Mohan (NJ Department of Health) and hydrogen peroxide was obtained at 70% purity from DuPont (Wilmington, DE, U.S.A.).

Sample preparation

The aldehydes and the ketones were all subjected to the reaction conditions described below.

Acid-catalyzed reductive amination with sodium cyanoborohydride. This approach has been previously used to derivatize carbonyl compounds[5]. The carbonyl compound (0.3 mg) was dissolved in 2.0 ml methanol containing 1.6 mg of 3-aminofluoranthene. Trifluoroacetic acid (5 μl) and 0.5 ml of 4 m*M* sodium cyanoborohydride solution in methanol were added, and the mixture was heated in a capped tube at 60°C for 2 and 12 h for aldehydes and ketones, respectively. Samples of 50 μl were diluted with 5.0 ml methanol.

Aqueous reductive amination with sodium cyanoborohydride. Conditions similar to those described by Abraham and Low[6] were used, except sodium cyanoborohydride was added to reduce the imine intermediate directly instead of the two-step synthesis with sodium borohydride as the reductant.

The carbonyl compound (0.06 mg) was diluted to 2.0 ml with 2 m*M* 3-aminofluoranthene in 0.1 *M* phosphate buffer at pH 7.00. Sodium cyanoborohydride (1.6 mg) was added, and the solution was heated in a capped test tube at 60°C for 40 min. A 200-μl sample was diluted to 5.0 ml with methanol.

Reductive amination with BAP. Petter *et al.*[7] recently reported BAP to be an effective reductant in the synthesis of secondary amines from aldehydes and ketones. The carbonyl compound (0.3 mg) was mixed with 2.0 ml of 2 m*M* 3-aminofluoranthene in acetic acid–toluene (2:7, v/v) and 22 μl of BAP was added. Time and temperature effects are discussed below.

RESULTS AND DISCUSSION

Derivatization reaction conditions

Reductive amination is the reaction of an aldehyde or ketone with an amine in the presence of a reducing agent (Fig. 1). Several different sets of reaction conditions have been reported for this reaction including acid-catalyzed reduction with sodium cyanoborohydride, neutral reduction in aqueous medium with sodium cyanoborohydride, and acid-catalyzed reduction with BAP. These conditions were investigated using readily available aldehydes and ketones as model compounds. For each of the variables investigated, the relative reaction yield was determined by measuring relative peak heights of the chromatographically isolated derivatives.

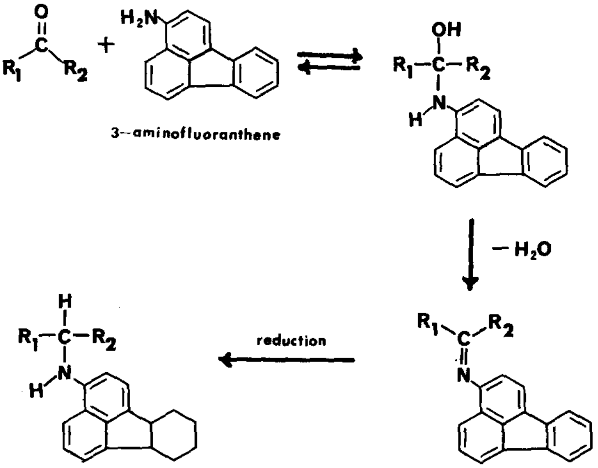


Fig. 1. Reductive amination reaction.

Acid-catalyzed reductive amination with sodium cyanoborohydride

Time. The derivatizing conditions for the acid-catalyzed reductive amination of aldehydes and ketones with sodium cyanoborohydride were first investigated at the temperature and molar ratio of reactants reported by Prakash and Vijay[5]. The maximum yield under these conditions for the derivatization of 2-octanal was achieved in *ca.* 2 h. Since no detectable derivatization product of 2-octanone was observed then, the reaction was allowed to continue overnight (12 h). The peak height of the product, measured at that time, was about two-thirds of that observed for the aldehyde product. To improve the yield of the ketone, the variables below were investigated, using 2-octanone as a model.

Solvents. Various solvents were investigated to determine the effect on reaction yield and to determine whether a higher-boiling solvent, used at a higher temperature, would increase the reaction rate. Of the solvents studied (Table I), only the hydroxylated solvents, methanol, 2-propanol and ethylene glycol led to a measurable product. Although ethylene glycol yielded slightly more product than methanol, more side-reaction products were also formed, resulting in the appearance of a large, skewed peak, eluted after the 2-octanone derivative. Therefore, methanol was used in subsequent experiments.

TABLE I

INFLUENCE OF SOLVENTS ON ACID-CATALYZED REDUCTIVE AMINATION WITH SODIUM CYANOBOROHYDRIDE

Solvent	*Peak height (mm)**
Methanol	30
Acetonitrile	0
2-Propanol	18
Ethylene glycol	43
1,4-Dioxane	0

* Derivative of 3-aminofluoranthene and 2-octanone.

Acid. Various amounts of acetic or trifluoroacetic acid were added to the reaction medium (Table II). Larger amounts of acetic acid resulted in increased yields of 2-octanone derivative but also larger amounts of unknown, poorly resolved, interfering, fluorescent materials. Small quantities of trifluoroacetic acid produced the highest yield of ketone derivative and the lowest amount of side products. The side products were produced in identical quantities when the reaction was carried out under argon, suggesting that the contribution of atmospheric oxygen to the production of fluorescent side products was negligible.

The acid dependence of the reaction may be understood in terms of the reaction mechanism. In an acid-dependent equilibrium reaction, a tetrahedral intermediate is formed by nucleophilic attack on the carbonyl carbon by the free amine. Dehydration occurs to produce the imine, which is then reduced to a secondary amine. Since dehydration is an acid-catalyzed process, the rate of imine formation increases with addition of acid. In competition with that process, a greater fraction of amine reactant exists in the unreactive, protonated form, as acid is added. Thus, optimal rates are obtained with intermediate amounts of acid. Competing side reactions, including reduction of the carbonyl group, limit the overall yield of secondary amine product.

TABLE II

EFFECT OF TYPE AND CONCENTRATION OF ACIDS IN CYANOBOROHYDRIDE REDUCTIVE AMINATION

Acid	*Volume (μl)*	*Peak height (mm)**
Acetic	2.5	10
	5.0	17
	25.0	36
	250.0	41
Trifluoroacetic	2.5	40
	5.0	40
	25.0	20
	50.0	5
	100.0	5

* Derivative of 3-aminofluoranthene and 2-octanone.

TABLE III

EFFECT OF CONCENTRATION OF 3-AMINOFLUORANTHENE ON ACID CATALYZED CYANOBOROHYDRIDE REDUCTIVE AMINATION

3-Aminofluoranthene added (mg)	*Peak height (mm)**
0.8	22
1.6	64
2.5	94
5.0	246
10.0	444

* Derivative of 3-aminofluoranthene and 2-octanone.

Concentration of 3-aminofluoranthene. Higher product yields are obtained with higher concentration of the derivatizing reagent (Table III) which accelerates the formation of the intermediate leading to the imine. Two factors determine the practical upper limit of derivatizing reagent concentration: (1) high reagent cost and (2) the increase in fluorescent products of side reactions with increased reagent concentration. A concentration of 1.6 mg of 3-aminofluoranthene per 2 ml was used for further work.

Concentration of reductant. The lowest concentration of reductant gave the highest product yields (Table IV) because of the role of the reductant in the major side reaction in the reduction of the carbonyl functional group. To ensure adequate concentrations of reductant for the range of concentrations of the other reagents, 4.0 m*M* sodium cyanoborohydride was used in subsequent experiments.

Reductive amination in aqueous medium

Under the conditions reported in the Experimental section only aldehydes could be derivatized to any measurable degree. The solubility of aminofluoranthene in water limited its concentration to 2.0 m*M*.

Time. The maximum yield of octanal derivative was obtained in 40 min (Fig. 2).

pH. Optimal yields of the derivative formed from 3-aminofluoranthene and octanal were obtained at pH 7 (Fig. 3). At this pH the dehydration and/or reduction

TABLE IV

EFFECT OF CONCENTRATION OF REDUCTANT ON ACID-CATALYZED CYANOBOROHYDRIDE REDUCTIVE AMINATION

Sodium cyanoborohydride concentration (mM)	*Peak height (mm)**
0.8	183
4.0	137
8.0	81
20.0	63
40.0	20

* Derivative of 3-aminofluoranthene and 2-octanone.

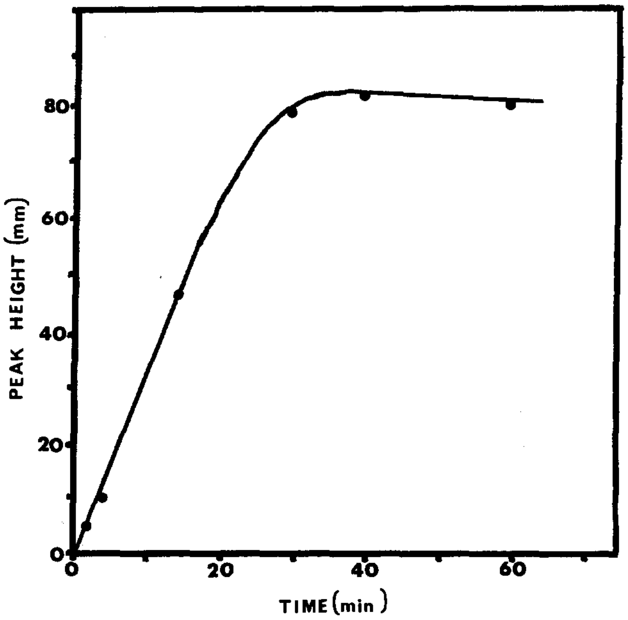


Fig. 2. Time dependence of formation of 3-aminofluoranthene derivative of *n*-octanal under reaction conditions for aqueous cyanoborohydride reaction (see Experimental).

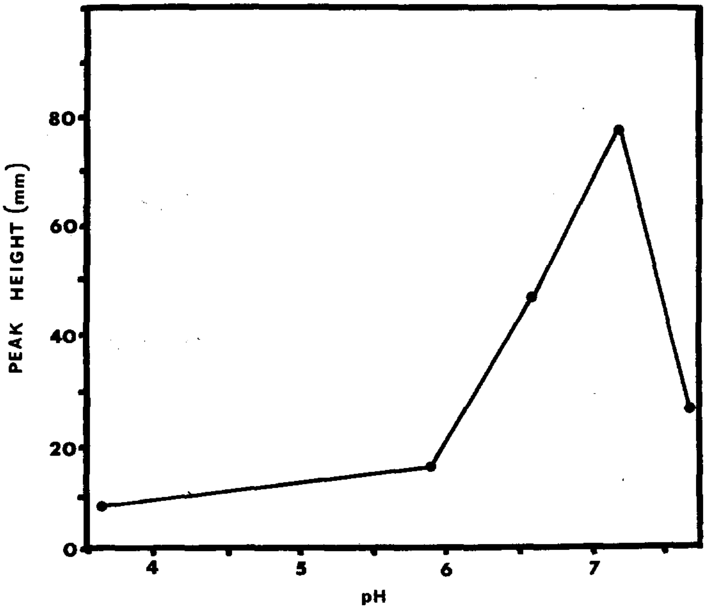


Fig. 3. Effect of pH on formation of 3-aminofluoranthene derivative of *n*-octanal under reaction conditions for aqueous cyanoborohydride reaction (see Experimental).

is rate-determining. The derivatization of as little as $2 \cdot 10^{-10}$ mol of aldehyde could be detected.

Reductive amination with BAP

Time and temperature. Previous reports indicate that yields of 80–90% of the reductive amination product for some carbonyl containing compounds can be obtained by using BAP at room temperature in *ca.* 4 h. With non-polar solvents, increasing temperature was shown to increase yields[7]. An investigation of this effect (Fig. 4) with 2-octanone showed that the highest yields were obtained at 40°C in about 14 h. Higher temperatures increase side reactions of the ketone. Aldehydes react immediately with 3-aminofluoranthene in the presence of BAP, even at room temperature. No increase in product is observed after several hours.

Chromatography

Aldehydes and ketones were used as model analytes to determine the effect of the derivatizing agent on chromatographic efficiency. Using the acid-catalyzed cyanoborohydride reaction, the 3-aminofluoranthene derivatives of *n*-hexanal, *n*-heptanal, *n*-octanal and *n*-decanal aldehyde (Fig. 5a), and 2-hexanone, 2-heptanone, 2-octanone and 2-decanone (Fig. 5b) were baseline-resolved in less than 30 min, demonstrating that the derivatizing reagent does not interfere with the separation. Since the products of the reductive amination are the same for each of the three reaction conditions above, the separation should be similar for each, as shown by the isolation of the same aldehyde products reduced in neutral aqueous medium (Fig. 6). One

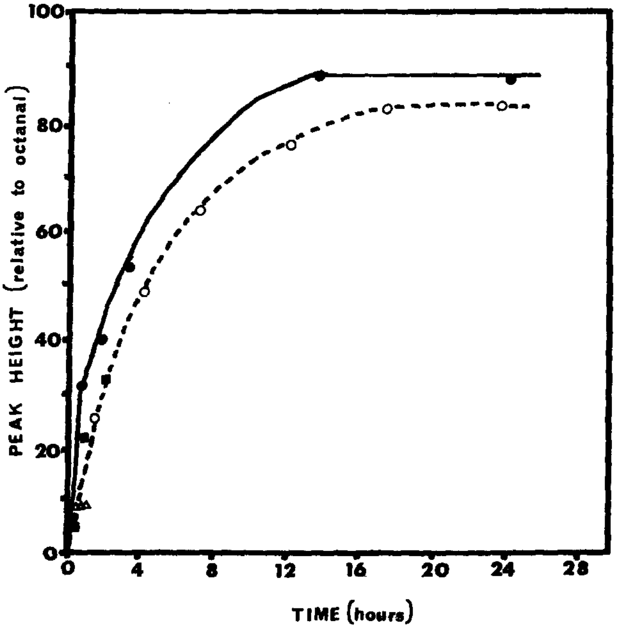
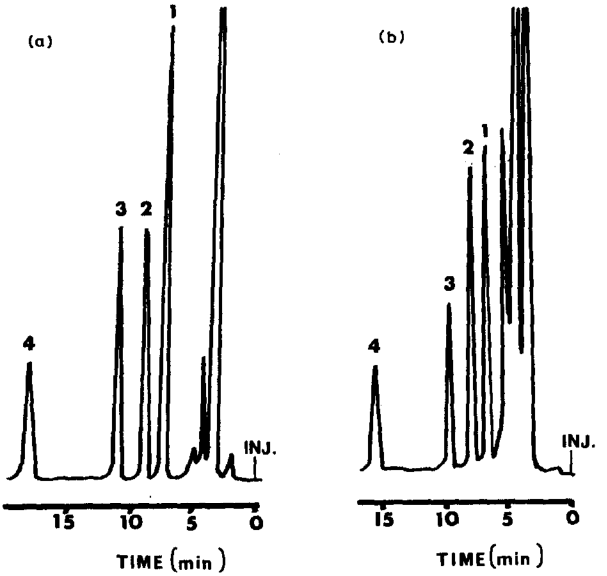


Fig. 4. Effect of time and temperature on formation of 3-aminofluoranthene derivative of 2-octanone under reaction conditions for acidic BAP (see Experimental). (○) 22°C, (●) 40°C, (■) 60°C and (△) 80°C.



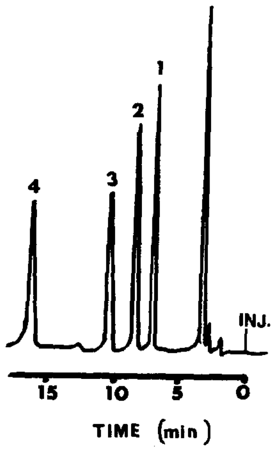


Fig. 5. (a) Chromatogram of 3-aminofluoranthene derivatives of aldehydes from acid-catalyzed cyanoborohydride reaction, detected with peroxyoxalate chemiluminescence (see Experimental). Peaks: 1 = *n*-hexanal, 2 = *n*-heptanal, 3 = *n*-octanal and 4 = *n*-decanal. (b) Chromatogram of 3-aminofluoranthene derivatives of ketones from acid-catalyzed cyanoborohydride reaction, detected with peroxyoxalate chemiluminescence (see Experimental). Peaks: 1 = 2-hexanone, 2 = 2-heptanone, 3 = 2-octanone and 4 = 2-decanone.

Fig. 6. Chromatogram of 3-aminofluoranthene derivatives of aldehydes from aqueous neutral cyanoborohydride reaction, detected with peroxyoxalate chemiluminescence (see Experimental). Peaks: 1 = *n*-hexanal, 2 = *n*-heptanal, 3 = *n*-octanal and 4 = *n*-decanal.

significant difference is that less of the unreacted amine and other fluorescent side products are present in this chromatogram because of low solubility of 3-aminofluoranthene in water. This becomes significant at low levels of analyte, because the presence of the unreacted amine affects the detectability of early eluted peaks, as discussed below.

Comparison of the derivatization reactions

Reaction yield

The greatest yield, measured as relative peak heights of products, was obtained for aldehydes (octanal) derivatized by the acid-catalyzed BAP procedure. This was used to estimate the relative yields for aldehydes and ketones for the three sets of reaction conditions by comparison of peak heights (Table V). The reaction conditions with the use of BAP also produced the highest yield of ketone derivative, although the peak height of the octanone derivative was only about 80% of that for the octanal derivative. Under both acid-catalyzed conditions, with cyanoborohydride or BAP, the reaction of ketones was much slower than that of aldehydes.

TABLE V

RELATIVE REACTION YIELDS AND CONDITIONS FOR THREE DERIVATIZATION PROCEDURES

	Time	*Temperature (°C)*	*Yield*[*]
Acidic cyanoborohydride			
Aldehydes	2 h	60	0.30
Ketones	12 h	60	0.25
Neutral cyanoborohydride			
Aldehydes	40 min	60	0.70
Ketones	No reaction		
Acidic BAP			
Aldehydes	< 2 min	25	1.00[**]
Ketones	14 h	40	0.80

[*] Relative to peak height of *n*-octanal derivative from acidic BAP derivatization.

[**] *n*-Octanal derivative.

Detectability

Because the highest yield was obtained under the acid-catalyzed BAP conditions, detectability was evaluated with derivatives produced under these conditions in two ways: (1) detector linearity for fluorescence and chemiluminescence was determined by dilution of an octanal sample, and (2) since the reaction cannot be assumed to produce an equal fraction of derivative at all concentrations, derivatization linearity and detectability were evaluated over a range of concentrations.

Detector linearity. A calibration curve was prepared by diluting the derivatized octanal sample ($1.2 \cdot 10^{-9}$ mol in 2 ml) and determining the limit of detection (LOD) for this sample at a signal-to-noise ratio of three. The LOD was $0.3 \cdot 10^{-12}$ mol with fluorescence detection and $9.0 \cdot 10^{-15}$ mol with chemiluminescence detection. The chemiluminescent detection was limited by instrumental conditions, primarily low frequency pulsing from the damped Gilson post-column pump. Other work done in this laboratory has shown that detection limits can be improved by at least one order of magnitude by improving detector electronics and lessening pulsations from pumps.

Derivatization linearity. Since the reactant concentrations affect the rate of the derivatization reaction, octanal was derivatized and analyzed by HPLC with fluorescence and chemiluminescence detection at several concentration levels. The calibration curves obtained were $y = (6.80 \cdot 10^{9})x + 0.12$ for fluorescent detection and $y = (1.15 \cdot 10^{10})x + 0.14$ for chemiluminescent detection. Using fluorescence detection, samples of $1.2 \cdot 10^{-9}$–$2.4 \cdot 10^{-10}$ mol in 2 ml could be derivatized and detected. With chemiluminescence detection, the range of derivatization and detection was extended to $1.2 \cdot 10^{-11}$ mol in 2 ml. The lowest level of derivatized octanal that can be determined by fluorescence is $3.0 \cdot 10^{-12}$ mol injected and for chemiluminescence it is $1.2 \cdot 10^{-13}$ mol injected. In both cases, the detection limits are higher than those obtained by diluting the sample because of the long tailing peak of the unreacted aminofluoranthene which is minimized by dilution. Statistical analysis of the data indicates that the chemiluminescence results are linear down to the lowest level, but that the lowest level measured by fluorescence does not fall in the linear range. This may be due to the relatively large background caused by the tailing peaks of the unreacted fluorophor and other fluorescent side products which are observable by fluorescence but not by chemiluminescence. This large fluorescent background not detected when observed by chemiluminescence may be due to some oxidized products of the aminofluoranthene since amino-substituted fluorophores can be detected at lower levels by chemiluminescence than by fluorescence[2]. Attempts to remove the unreacted fluorophore proved unsuccessful. When lower concentrations of the aminofluoranthene were used to reduce the amount of unreacted material, insufficient product for detection was produced. A possible advantage of the aqueous cyanoborohydride reaction is that less unreacted derivatizing agent is present because of solubility limitations.

CONCLUSION

Reductive amination with 3-aminofluoranthene is a suitable derivatization reaction for sensitive chemiluminescence detection of fmol amounts of aldehydes and ketones. Of the three sets of reaction conditions [(1) acid-catalyzed with cyanoborohydride, (2) neutral aqueous medium with cyanoborohydride and (3) acid-catalyzed with BAP], highest yields were obtained with the third set, although separation from unreacted derivative may be easier with the second set of conditions. The chemiluminescence detection limit of 3-aminofluoranthene derivatives is thirty times lower than that obtained with conventional fluorescence measurements.

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