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APPLICATION OF 3-(2-FUROYL)QUINOLINE-2-CARBALDEHYDE AS A FLUOROGENIC REAGENT FOR THE ANALYSIS OF PRIMARY AMINES BY LIQUID CHROMATOGRAPHY WITH LASER-INDUCED FLUORESCENCE DETECTION

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SUMMARY

3-(2-Furoyl)quinoline-2-carbaldehyde (FQCA) has been synthesized and characterized as a fluorogenic derivatizing reagent for liquid chromatography. The reagent forms highly fluorescent isoindoles upon reaction with primary amines. The spectral properties and optimum reaction conditions for the formation of isoindoles have been investigated. The utility of FQCA for the high-sensitivity analysis of amino acids by laser-induced fluorescence with an argon-ion laser is demonstrated.

INTRODUCTION

Increasing the sensitivity of the determination of primary amines (*e.g.*, amino acids and peptides) resulting from the controlled degradation of biologically important macromolecules is currently a focus of intense research in modern biochemistry. Over the years, a number of chromatographic procedures have been developed for use in this capacity. A suitable derivatizing reagent which adds a chromophore or fluorophore to the molecule, thereby converting the target compounds into a form amenable to high-performance liquid chromatography (HPLC), is often the key to such determinations. Suitable reagents have included ninhydrin[1], fluorescamine[2,3], 7-chloro-4-nitrobenzene-2-oxa-1,3-diazole (NBD chloride)[4], and *ortho*-phthalaldehyde (OPA)[5–8]. Recently, naphthalene-2,3-dicarboxaldehyde (NDA)[9] has been added to the list of effective fluorogenic reagents.

OPA has been perhaps the most popular reagent for use in both pre- and post-column derivatization schemes. This is due to the fact that its reaction with a primary amine results in very rapid formation of an intensely fluorescent isoindole from non-fluorescent starting materials. However, sensitivity of the OPA analysis has typically been limited to the picomole range. Moreover, OPA derivatives have been plagued by their sensitivity to light, air oxidation, and attack by acids[10]. Thus, the isoindoles formed by this reaction tend to be unstable.

A newly developed reagent, NDA[9] is structurally very similar to OPA in that it features the *ortho*-dialdehyde moiety and forms an isoindole in the presence of a primary amine and suitable nucleophile. Considerable work has been done to opti-

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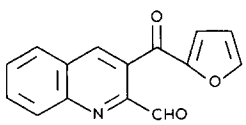
mize the nucleophile for the derivatization reaction[9,11]. The cyanide ion was found to be superior to the more conventional thiol, yielding more stable derivatives with a higher quantum yield[11].

Most fluorogenic reagents have been developed from the perspective of conventional HPLC and commercially available detection systems. As a consequence, the sensitivity exhibited by such methods is adequate for most applications, but not for state-of-the-art protein "nanoscale" isolations[12,13]. Isolation of low picogram quantities of protein has recently been demonstrated with short, packed capillary LC columns[12]. The degradation of a protein on this scale will yield femtogram or lesser amounts of each amino acid. Determinations at such levels require ultra-sensitive detection combined with a separation technique suited to low-volume trace analysis. The combination of capillary LC with laser-induced fluorescence detection provides a unique means for meeting such a challenge.

Since virtually all fluorogenic reagents for primary amines do not yield derivatives with excitation maxima near the lines of convenient high-intensity light sources, such as the helium-cadmium or argon-ion lasers, we have undertaken the task to synthesize and test novel reagents[14]. As the result of our systematic studies in this area, 3-benzoyl-2-quinolinecarboxaldehyde (BQCA)[15] and 3-benzoyl-2-naphthaldehyde (BNA)[16] were prepared and characterized as superior reagents for chromatographic analysis and detection with a helium-cadmium laser at the 442 nm blue line. In this communication we wish to report on yet another promising reagent, 3-(2-furoyl)-quinoline-2-carbaldehyde (FQCA), the structure of which is shown below. FQCA yields fluorescent isoindoles with an excitation maximum near the 488 nm (main line) of the argon-ion laser.



FQCA

EXPERIMENTAL

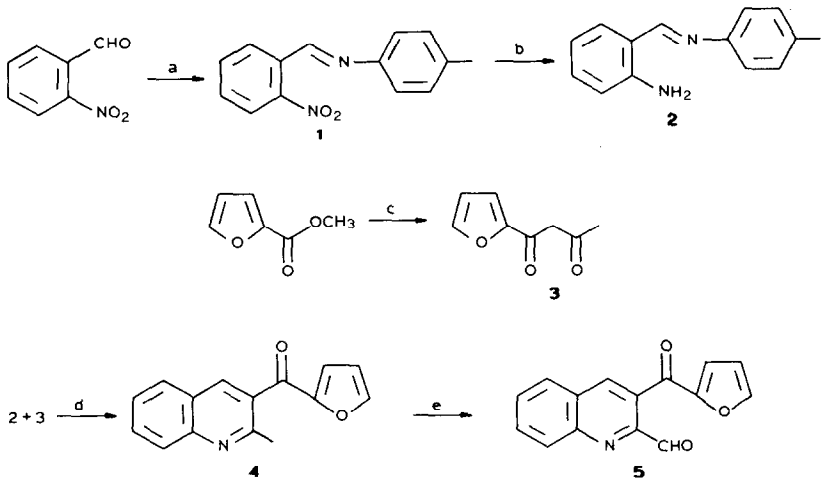
Chemicals

The starting materials for the FQCA synthesis were obtained from Aldrich (Milwaukee, WI, U.S.A.). A set of L-amino acids (Kit No. LAA-21; Sigma, St. Louis, MO, U.S.A.) was used as the amino acid standard. The solvents for the synthesis, chromatography, and fluorescence measurements were from Mallinckrodt (St. Louis, MO, U.S.A.). Water was doubly distilled and deionized in-house.

Synthesis

The synthetic pathway leading to FQCA is shown below. The identity of all intermediates was established by NMR and/or mass spectral data.

*N-(p-Tolyl)-o-nitrobenzaldimine (**1**).* A mixture of 9.68 g (90.5 mmol) *p*-toluidine and 10.89 g (72.1 mmol) *o*-nitrobenzaldehyde in 25 ml ethanol was kept at 25°C. The precipitate, which began to form after 5 min, was collected on a filter, washed



a: *p*-toluidine; b: $Na_2S \cdot 9H_2O$; c: NaH, $(CH_3)_2CO$; d: piperidine; e: SeO_2

with cold ethanol, and air-dried. Additional precipitate was obtained by dilution of the filtrate with water and refrigeration. A yield of 16.0 g, 66.7 mmol (93%) was obtained.

*N-(p-Tolyl)-o-aminobenzaldehyde (**2**).* The procedure for the preparation of **2** was carried out as described by Borsche *et al.*[17]. To a solution of 16.0 g (66.7 mmol) nitroamine **1** in 33 ml 95% ethanol at reflux temperature was added a solution of 31 g (129 mmol) $Na_2S \cdot 9H_2O$ in 30 ml 50% aq. ethanol over 15 min, so as to maintain reflux. The mixture was heated under reflux an additional 15 min and cooled to effect crystallization. Solid was collected on a filter, washed with water, and air-dried, yielding 4.09 g (19.5 mmol, 29%) **2**. No attempt was made to secure additional product from the filtrate.

*(2-Furoyl)acetone (**3**).* Intermediate **3** was prepared according to a modification of the procedure of Swamer and Hauser[18]; 4.27 ml (5.04 g, 40 mmol) methyl 2-furoate was added to a suspension of NaH (made from 3.83 g commercial NaH slurry by removal of the mineral oil with pentane) in 30 ml anhydrous diethyl ether. To this was added, so as to maintain the temperature below 30°C, 5.87 ml (4.64 g, 80 mmol) acetone (dried over $CaCl_2$). This mixture was heated under reflux for 2 h, cooled, and divided between diethyl ether and 3 *M* HCl. The ether layer was washed with brine, dried ($MgSO_4$), and concentrated to yield 6.06 g of a red oil (raw yield 99%) which was purified by vacuum distillation (b.p. 85–90°C/1 torr).

*3-(2-Furoyl)-2-methylquinoline (**4**).* A solution of 410 mg (1.95 mmol) aminoimine **2**, 326 mg (2.15 mmol) diketone **3**, and 50 mg piperidine in 4 ml 95% ethanol was heated overnight under reflux. Removal of the volatile materials left a black, gummy oil which was taken up in dichloromethane and, after being washed with 3 *M* NaOH, brine, and dried ($MgSO_4$), purified by flash chromatography [eluent, 3:2 pentane–diethyl ether (3:2)] yielding 355 mg (1.50 mmol, 77%) product.

*3-(2-Furoyl)quinoline-2-carbaldehyde (**5**).* A mixture of 355 mg (1.50 mmol) **4** and 183 mg (1.65 mmol) SeO_2 in 6 ml acetic acid was heated for 17 h in an 80°C bath. The solvent was removed by rotary evaporation. The dichloromethane extract of the

residue was washed with 1 *M* NaOH and brine and dried ($MgSO_4$). Concentration yielded 284 mg oil, 1.13 mmol, 75% raw. The ultimate purification was done by LC as discussed below.

Liquid chromatography

A Perkin-Elmer (Norwalk, CT, U.S.A.) Series 3B liquid chromatograph with detection (Perkin-Elmer LC-55 absorbance detector) at 330 nm was employed for purification of the FQCA reagent. The separation was performed on a Phenomenex (Rancho Palos Verdes, CA, U.S.A.) 5-μm, C_{18} column. A linear gradient from 60% aq. methanol to 100% methanol, in 30 min, was sufficient to isolate FQCA from contaminants introduced during the synthetic procedure.

The micro-LC system consisted of an ISCO (Lincoln, NE, U.S.A.) Model 314 syringe pump equipped with an ISCO HPLC pressure controller and a Model 314 metering pump. Separations were accomplished on a 0.5 m × 0.25 mm fused-silica (Polymicro Technologies, Phoenix, AZ, U.S.A.) capillary, packed in-house with 5-μm C_{18} particles (Shiseido Corp., Kyoto, Japan). Detection was accomplished with an argon-ion laser-based detection system similar in design to that described in previous studies[15,16]. Injections were made in the stopped-flow mode. The derivatized amino acids were eluted with a stepwise gradient, beginning with 20% acetonitrile in water containing 0.2% triethylamine and 0.2% acetic acid, in steps of 5% to 50% acetonitrile in water containing 0.2% triethylamine and 0.2% acetic acid.

Fluorescence measurements

Static fluorescence measurements were made with a Perkin-Elmer 650 spectrofluorometer. For quantum yield studies, the emission spectrum was corrected according to the method of Parker[19], and compared to that of quinine bisulfate.

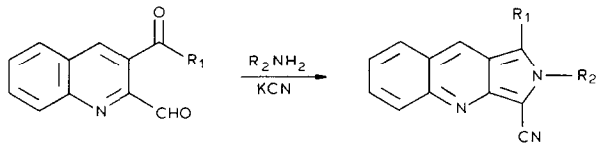
RESULTS AND DISCUSSION

The development, synthesis, and characterization of fluorogenic derivatizing reagents for primary amines has been of continuing interest to our laboratory[14–16]. We are mainly concerned with the design of reagents that combine the advantages of OPA and NDA with the added feature of an absorbance maximum closely matching the principal output radiation wavelength of an inexpensive and easily maintained laser system. Use of a laser-based detection scheme for HPLC has been shown to enhance the detection limits in a variety of experiments[20–24]. Our efforts have centered on the development of reagents having absorption bands in the visible range. Exploitation of a band in the visible range eliminates many interferences present in biological samples, which typically feature absorption maxima in the UV region. Moreover, two lasers (He-Cd and Ar ion), which are both relatively inexpensive and easy to operate, feature output radiation in the visible range. We have previously described reagents compatible with the 442 nm He-Cd line [14–16], and in this paper we present a similar reagent for use with the 488 nm line of the Ar-ion laser.

Our design considerations for a fluorogenic reagent include the formation of an isoindole upon reaction with a primary amine and the capability of building a variety of functional moieties into the reagent. The ability to modify the reagent facilitates adaptation to almost any type of detection scheme. In addition, the absorbance of the

derivative should be sufficiently different from that of the reagent itself, thus eliminating interferences from unreacted starting materials. The final criterion of our developmental strategy is the stabilization of the resulting isoindoles so that they may be reliably used in the pre-column derivatization mode, since post-column derivatization is often impractical for capillary LC.

We postulated that the stability of isoindoles can be enhanced through substitution of the proton adjacent to the nitrogen atom of the indole ring. This led us to the *ortho*-aroylaraldehyde backbone instead of the more conventional *ortho*-dialdehydes[14]. In the scheme shown below, both positions adjacent to the indolic nitrogen are substituted. Also, the use of a keto-aldehyde:



facilitates the incorporation of various moieties into the parent molecule, resulting in the ability to "fine-tune" the absorption maximum of the derivatized molecule.

In a previous communication[14], we outlined a number of criteria for comparison of the various fluorogenic reagents developed. These are: (1) high quantum yield; (2) good reaction rate; (3) stability of the derivative with time; and (4) proximity of the absorption maximum to the target laser line. Here, we examine FQCA with respect to these criteria and optimization of the derivatization conditions.

FQCA was originally synthesized as an analogue of BQCA[15], which has an absorption maximum at 460 nm. In order to effect a hypsochromic wavelength shift,

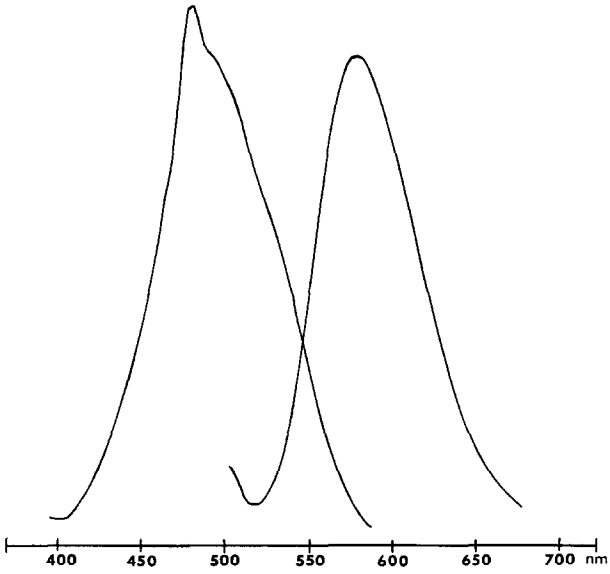


Fig. 1. Absorption and emission spectra of the methionine product.

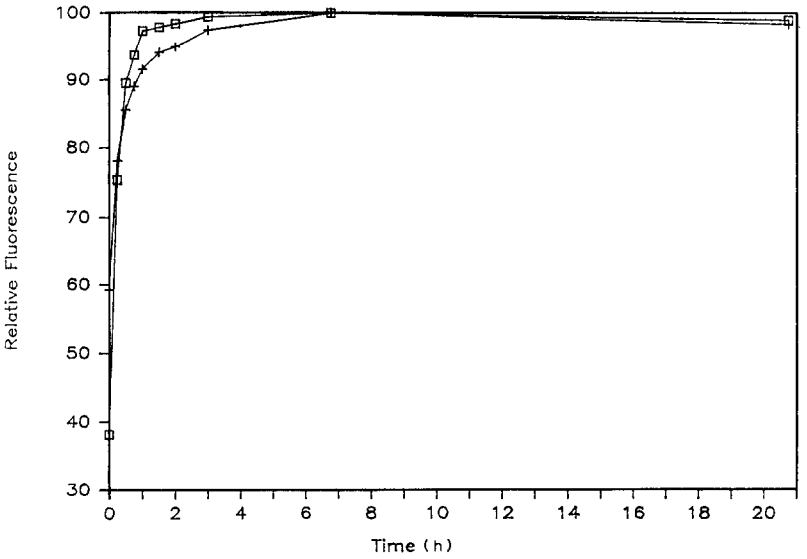


Fig. 2. Stability–time curves for (□) alanine and (+) valine.

we replaced the R_1 (scheme above) phenyl group with a furan moiety. In actuality, the absorption was bathochromically shifted to 480 nm (emission maximum 590 nm), which happens to coincide with the main line (488 nm) of the argon-ion laser. Fig. 1 depicts the absorption and emission spectra for a typical FQCA derivative. Further investigation indicated that the quantum yield of FQCA, derivatized with glycine, is 0.3.

When OPA reacts with primary amines, the fluorescent product forms within 5 min. While FQCA does not react that quickly, the isoindole product reaches its fluorescence maximum in about 45 min. Reaction time on this scale does not present major problems. Primarily, we are concerned with the formation of a stable derivative within a reasonable time and under mild conditions (37°C without stirring or agitation). Fig. 2 depicts the time–stability curve for FQCA-derivatized glycine and lysine. Once the fluorescence maximum has been reached, the derivatives are stable in solution for more than 2 h. In fact, when the dry derivatives are stored in the freezer, they maintain their fluorescence intensity for about 2 weeks.

The optimum concentrations of cyanide ion and reagent for maximum reaction yield have been investigated. The dependence of the reaction yield upon the concentration of cyanide ion and reagent is depicted in Figs. 3 and 4, respectively. These experiments were carried out by allowing FQCA to react with glycine or lysine under the appropriate conditions for 45 min. The reaction mixture was then diluted with methanol to 1.5 ml, and triplicate peak height readings were acquired from the spectrofluorometer, with excitation at 490 nm and emission at 590 nm. Each point on the curve represents an average of three readings. Examination of these data reveals that the fluorescence maximum is reached with *ca.* four-fold molar excess of FQCA and a two-fold excess of cyanide ion. In a previous study[15], we found that the optimum pH for the derivatization of amino acids is between 7 and 9. This finding also applies to

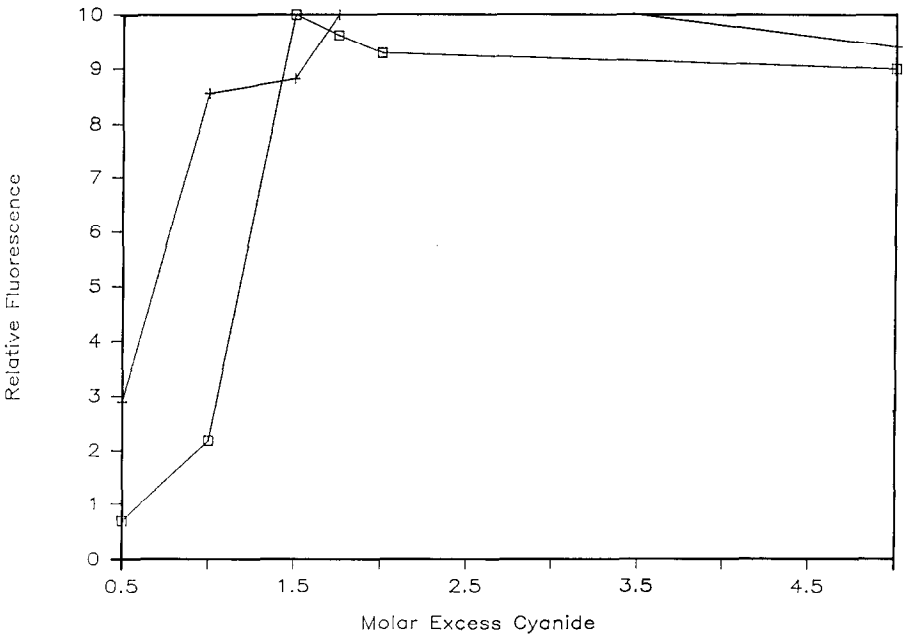


Fig. 3. Dependence of reaction yield on cyanide concentration. □ = Glycine; + = lysine.

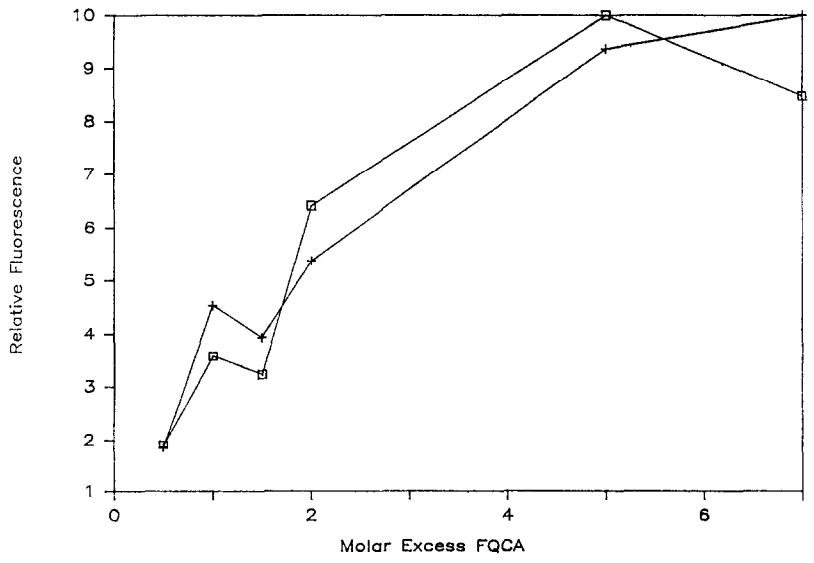


Fig. 4. Reaction yield as a function of reagent concentration. □ = glycine; + = lysine.

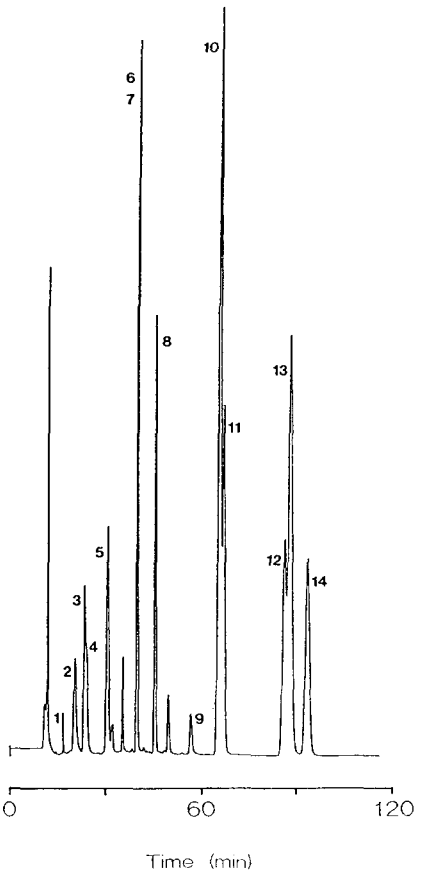


Fig. 5. Standard amino acid chromatogram (conditions given in the text). Peaks 1 = Glu; 2 = Asp; 3 = His; 4 = Ser; 5 = Arg; 6 = Gly; 7 = Tyr; 8 = Ala; 9 = Thr; 10 = Met; 11 = Val; 12 = Phe; 13 = Ile; 14 = Leu.

FQCA, so that the reaction may be carried out in methanol, as opposed to the traditional borate buffer.

Fig. 5 represents a microcolumn LC chromatogram of amino acid standards with high-sensitivity laser-induced fluorescence detection. The chromatographic conditions are as described in the Experimental section. Peak identities are given in the figure legend. The practical detection limits obtained with FQCA-derivatized amino acids were found to be in the femtomole range.

In summary, we have presented the design considerations and synthesis of a fluorogenic reagent for primary amines, rendering them amenable to high-sensitivity detection following microcolumn LC separation. FQCA is shown to be compatible with argon-ion laser-induced fluorescence detection. In addition, flexibility in the design of our reagents through a generalized synthesis scheme[14] is demonstrated.

FQCA in the presence of an amino acid and a suitable nucleophile (cyanide ion, chosen here) forms an intensely fluorescent isoindole the absorption maximum of which closely matches the 488 nm output radiation of the main line of the argon-ion

laser. The reaction proceeds reproducibly under mild conditions (pH 7–9, 37°C) to yield stable products which can be satisfactorily separated chromatographically. Future studies will aim at the optimization of retention.

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