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Aspects of the stability of isoindoles derived from the reaction of *o*-phthalaldehyde–ethanethiol with primary amino compounds

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Abstract: The stability of a series of fluorescent isoindoles formed under analytical conditions following the reaction of *o*-phthalaldehyde (OPA) and ethanethiol (ET) with a series of primary amines is reported. Increasing the bulk and degree of substitution of the isoindole *N*-substituent resulted in substantial increases in isoindole stability. The effects of excess reagents on isoindole stability is examined and OPA is observed to accelerate isoindole degradation whilst ET provides a stabilizing effect. Comparison with previously reported data involving the use of 2-mercaptoethanol revealed that ET clearly forms the more stable isoindole derivatives, i.e. a minimum of five-fold improved stability based on the time for 10% degradation to occur. Identification of the major degradation product together with kinetic data suggests that degradation proceeds via autoxidation.

Keywords: *Derivatization of primary amines; o-phthaldehyde–ethanethiol reagent; stability of isoindoles; autoxidation reactions; high-performance liquid chromatography.*

Introduction

Since the initial report by Roth [1], derivatization of primary alkylamines with the *o*-phthalaldehyde-thiol reagent to produce fluorescent 1-thiosubstituted-2-alkylisoindoles [2–4] has become the basis for a widely accepted analytical method. When used in conjunction with high-performance liquid chromatography (HPLC) the reaction provides one of the most sensitive methods available for the determination of amino acids and primary alkyl amines [5–8]. While both pre-column and post-column derivatizations have been applied analytically, in general the precolumn approach offers the advantages of improved detection limits, simplification of the chromatographic system and reduced analysis times [7, 8]. Unfortunately, 1-thiosubstituted-2-alkylisoindoles are not stable [5–15] and thus place some limitations on the pre-column method.

For the majority of applications the thiol component of the reagent has been the hydroxyalkylthiol, 2-mercaptoethanol (2-ME), despite evidence that the non-hydroxyl bearing alkanethiol, ethanethiol (ET), results in isoindole derivatives of improved

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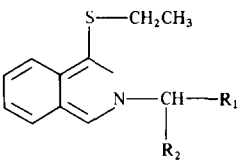
stability [13, 14]. Previously, it has been shown that both amine structure and excess OPA/2-ME reagent significantly affect derivative stability [8, 11, 15, 16]. The purpose of the present study was to evaluate the influence of excess OPA/ET reagent and primary amine structure upon the stability of isoindole derivatives under conditions which would allow a direct stability comparison with the derivatives previously studied [15] when 2-ME was the thiol component of the reagent (Table 1). Additionally, isolation and characterization of the major degradation product from one of the isoindoles (**I**) has been carried out. This information, in conjunction with the observed kinetics, enables an interpretation of the type of process responsible for the loss of isoindole.

Table 1
Isoindole derivatives investigated



Derivative	Precursor amine	R_1	R_2
I	*n*-Propylamine (PA)	$-CH_2CH_3$	$-H$
II	γ-Amino-*n*-butyric acid (GABA)	$-CH_2CH_2CO_2H$	$-H$
III	β-Alanine (BALA)	$-CH_2CO_2H$	$-H$
IV	Glycine (GLY)	$-CO_2H$	$-H$
V	β-Amino-*n*-butyric acid (BABA)	$-CH_2CO_2H$	$-CH_3$
VI	Alanine (ALA)	$-CO_2H$	$-CH_3$

Experimental

Apparatus

The HPLC system consisted of a Waters Associates Model 6000A pump, U6K injector, a Model 440 absorbance detector, and a Schoffel FS-970 fluorescence detector equipped with a Corning 7-51 primary filter and 418 nm cutoff secondary filter. 10 μm Hypersil-ODS columns 150 × 4.6 mm i.d. (Shandon) packed in our laboratories by an established method [17] and 10 μm μ Bondapak phenyl columns 300 × 3.9 mm i.d. (Waters Associates) were used. Chromatograms were recorded on a two-channel Houston Instruments Omniscribe recorder. Perkin–Elmer 727 and Varian FT-80A spectrometers were used to acquire IR and ^{1}H-NMR spectra, respectively. Preparative separations (MPLC) were carried out on a modular system consisting of a Fluid Metering, Inc., Model RPSYI-SSY pump, 1.0 m × 15 mm i.d. glass column (Altex), and a ISCO Instruments, Model 1840 variable wavelength detector. Low resolution mass spectra were obtained using a Varian model CH-5 spectrometer using electron impact (70 eV) or chemical ionization (CI) modes. Elemental analyses were performed by the Department of Medicinal Chemistry with a Hewlett–Packard Model 195-B CHN analyzer.

Materials

Solvents were freshly distilled or of HPLC grade. *n*-Propylamine (Aldrich) was redistilled and all other chemicals (Aldrich) were of reagent grade and used as received.

Preparative chromatography and TLC (0.2 mm layer, F254 indicator) were performed on silica gel 60 (E. Merck, Darmstadt).

Borate buffer. Sodium tetraborate decahydrate was dissolved in glass distilled water and the ionic strength adjusted with anhydrous sodium perchlorate ($Na_2B_4O_7.10H_2O$, 0.01 M; $NaClO_4$, 0.28 M; μ = 0.30 M; pH 9.05 at 25.0°C).

OPA/ET reagent preparation. OPA was weighed accurately into a 10.0 ml volumetric flask and dissolved in 5.0 ml of CH_3CN, then the required quantity of ET was added and the solution made up to volume with borate buffer.

Amine solutions. The desired amine was weighed into a 10.0 ml volumetric flask and dissolved in borate buffer. Further dilution of the stock solutions with borate buffer provided the concentrations of the amine required for kinetic studies.

Derivatization procedure

To 9.6 ml of borate buffer equilibrated at the required temperature was added 0.100 ml of the OPA/ET reagent solution followed by 0.100 ml of the required amine. The flask was quickly brought to volume with borate buffer, mixed and returned to the thermostated bath.

Kinetic studies

The degradation kinetic profiles for several isoindoles (**I–VI**) were established. In all cases, the required isoindole was generated *in situ* as described above. Isoindole loss was measured by HPLC (chromatographic parameters described in Table 2) utilizing fluorescent detection with excitation wavelengths of 230–260 nm and an 418 nm emission cut-off filter.

Table 2
HPLC parameters utilized for monitoring the degradation of specific isoindoles

Compound	Column	Mobile phase	
I	Phenyl*	65% CH_3OH/Acetate‡	(A)
II	ODS†	20% CH_3OH/20% THF/Acetate‡	(B)
III	ODS	20% CH_3OH/16% THF/Acetate‡	(C)
IV	ODS	20% CH_3OH/12% THF/Acetate‡	(D)
V	ODS	30% THF/Acetate‡	(E)
VI	ODS	25% THF/Acetate‡	(F)

*Phenyl: Water Associates; Bondapak Phenyl; flow rate = 1.5 ml min^{-1}.

†ODS: Column packed in these laboratories with Hypersil-ODS (Shandon); 5 μm particles; 150 × 4.6 mm i.d.; flow rate = 1.0 ml min^{-1}.

‡Acetate: Sodium acetate–acetate acid buffer, 0.05 M, pH 5.70; present mobile phase composition as follows: A = methanol–aqueous 0.05 M acetate buffer pH 5.70 (65:35, v/v). B = methanol–tetrahydrofuran–aqueous 0.05 M acetate buffer pH 5.70 (20:20:60, v/v). C = methanol–tetrahydrofuran–aqueous 0.05 acetate buffer pH 5.70 (20:16:64, v/v) or alternatively as B but (20:16:64, v/v). D = as B but (20:12:68, v/v). E = tetrahydrofuran–aqueous 0.05 acetate buffer pH 5.70 (30:70, v/v). F = as E but (25:75, v/v).

Synthesis

3-Ethylthio-2-n-propylphthalimidine (**VIII**). Methyl-2-formylbenzoate (3.28 g, 0.020 mol), ethanethiol (4.96 g, 0.10 mol) and *n*-propylamine (2.36 g, 0.04 mol) were dissolved in THF (25 ml) and refluxed for 24 h. Removal of the solvent and volatiles under reduced pressure left a liquid mixture which was resolved by MPLC with hexane–ethyl acetate, 75:25, v/v eluent and, after solvent removal from the various fractions, provided the product (2.71 g, 58%) as a light yellow liquid. IR (neat) 2980, 2940, 2880, 1690, 1620, 1605, 1470, 1400, 1190, 1070, 890, 775 and 705 cm^{-1}. ^{1}H-NMR ($CDCl_3$) 0.93 (t, 6H), 1.36–2.17 (m, 4H), 3.10–4.20 (m, 2H), 5.53 (s, 1H), 7.23–7.90 (m, 4H).

EIMS (70 eV) *m/e* (relative intensity) 174 (M-SCH_2CH_3, 100), 132 (M-SCH_2CH_3-propylene, 58), 104 (14), 90 (14), 77 (26), CIMS (ammonia) MH^+, *m/e* = 236.

Anal. Calc'd for $C_{13}H_{17}NOS$: C, 66.34; H, 7.28; N, 5.95. Found: C, 66.45; H, 7.33; N, 5.69.

Identification of degradation product

For HPLC observation, **I** was generated *in situ* (*ca* 10^{-5} M) as previously described and monitored with detection at 254 nm. For isolation and identification of larger quantities, **I** was prepared by reacting OPA (537 mg, 4 mmol), ET (297 μl, 4 mmol) and n-propylamine (329 μl, 4 mmol) in 200 ml of 50% acetonitrile–water solution. Decomposition of **I** was monitored by HPLC and found to be complete after 48 h of exposure to the atmosphere. The acetonitrile was removed and the aqueous phase extracted with chloroform (50 ml, 3x). The resulting mixture was fractionated by column chromatography (silica gel, hexane–ethyl acetate 75:25, v/v) and the major degradation product isolated. HPLC analysis revealed an identical retention time compared to the major decomposition peak observed for dilute solutions. Spectral characterization (IR, ^{1}H-NMR and MS) revealed the isolated degradation product to be identical in all respects to authentically prepared 3-ethylthio-2-*n*-propylphthalimidine (**VII**).

Results and Discussion

Structure–stability relationships

The influence of primary amine structure on isoindole derivative stability was investigated for a series of amino acids (Table 1) under conditions similar to those encountered analytically except that the temperature was increased to 40.5°C to facilitate kinetic study and enable direct stability comparison with the same OPA/2-ME derivatized amino acids previously studied. In contrast to the results previously reported [14] for some similar 1-ethylthio-2-alkylisoindoles, the kinetics of decomposition were not first order. In contrast to the results previously reported [14] for some similar 1-ethylthio-2-alkylisoindoles, the kinetics of decomposition was not first order as semilogarithmic plots of the data in Fig. 1 resulted in curved plots. Close examination of the kinetic profiles during the early stages of degradation (Fig. 2) revealed that derivatives **II** and **III** immediately declined whilst **IV–VI** exhibited an inhibition phase prior to degradation. Such kinetic results are characteristic of free-radical mediated processes [18].

The kinetic profiles for degradation of homologs **II–IV** (Fig. 1) suggest that the ω-carboxylate group of the *N*-substituent exerts a modest improvement in derivative stability as the moiety resides nearer the isoindole ring. In contrast, for the homologs **V** and **VI**, markedly improved, but indistinguishable stability profiles were observed despite the differing proximity of the ω-carboxylate group to the isoindole ring. Thus,

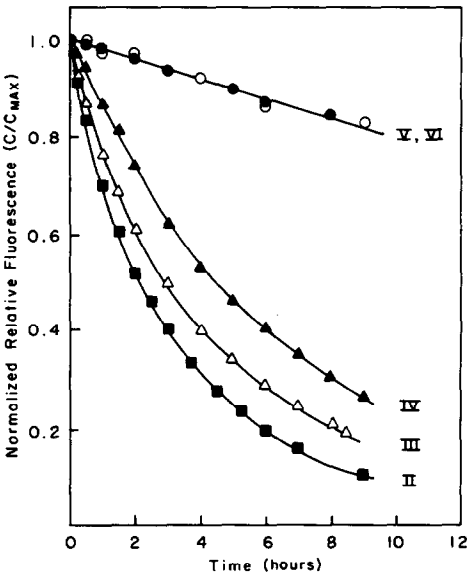


Figure 1
Structure–stability relationships: Extended degradation profiles for a series of amino acid derivatives. The derivatives were generated *in situ* utilizing reactant ratios of 1430:150:1 where 1 = 1.0×10^{-6} M (ET/OPA/amino acid). Temperature was maintained at 40.5°C, pO_2 = atmospheric and the solutions were protected from light of <500 nm. Derivative loss was monitored by HPLC. Each profile was normalized to aid visual comparison and the individual points represent the average of duplicate determinations which differed by <3% in all cases. The curves drawn do not represent a calculated result but were drawn to aid visual comparison.

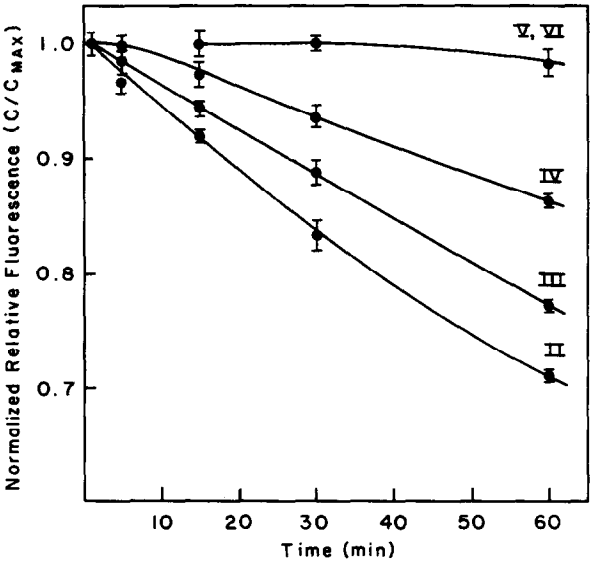


Figure 2
Structure–stability relationships: Initial degradation profiles for amino acid derivatives. Experimental conditions were as described in Fig. 1. Individual time points represent the mean of triplicate determinations.

from the present kinetic data it would appear that steric bulk (e.g. existence of a branched *N*-alkyl substituent) adjacent to the isoindole ring rather than electronic effects exerted by a carboxylate group is of significantly greater importance for isoindole derivative stability.

These stability trends are in accord in reports [5] that several amino acids (glycine, lysine and ornithine) form particularly unstable OPA/ET derivatives. From the present structure–stability relationships, it is readily seen that primary amines bearing a primary alkyl group would be expected *a priori* to form the least stable OPA/ET derivatives and would be the most problematic for pre-column application of this analytical derivatization reaction.

Reagent effects — qualitative aspects

The OPA/ET derivative of *n*-propylamine (**I**) was chosen as a model compound for elucidating the effect of excess reagent components (OPA, ET) on derivative stability. In all studies, **I** was generated *in situ* and decomposition monitored by the HPLC conditions described in Table 2. In experiments where the quantity of ET was increased relative to a fixed OPA concentration the stability of the resulting isoindole was enhanced, as shown in Fig. 3. However, in the experiments where the excess OPA present after derivatization was varied with the ET concentration invariant, the decomposition rate of **I** was accelerated with increased OPA concentration, as illustrated by Fig. 3B and D. It is interesting to note that when OPA was the limiting reactant and theoretically was completely consumed by the derivatization reaction, a significant inhibition period prior to degradation of **I** was observed, as illustrated by curve A in Fig. 3.

These kinetic results, the non-first order degradation profiles and the observation of induction periods, are consistent with loss of **I** occurring by a free radical autoxidation

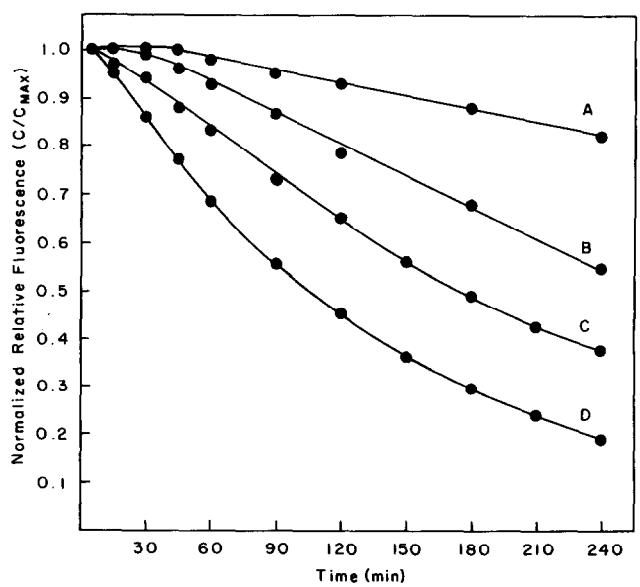


Figure 3
Reagent effects on derivative **I** stability: The isoindole was generated *in situ* utilizing the following ET:OPA:PA reagent ratios: A, 675:1:15; B, 675:15:1; C, 27:15:1; D, 675:75:1 where 1 = 1.0×10^{-6} M. Experimental conditions, except temperature = 25°C, and data treatment were as described in Fig. 1.

[18] rather than an ionic process. The present results also suggest that the excess thiol may, at least in part, be stabilizing the isoindole via a well-known antioxidant action [19]. The destabilizing effect of OPA may result from the fact that aldehydes autoxidize via radical intermediates [20], which may serve to catalyze any free-radical process responsible for decomposition of isoindole **I**.

From these data two analytically significant facts emerge concerning the use of this reaction in pre-column HPLC applications. First, while excess OPA is necessary for complete derivatization of an analyte, disregard for the quantity used, as appears to be the case in many applications, can dramatically compromise the stability of derivatives formed from primary alkyl amines, thus, potentially compromising the ease of use and sensitivity of the method. Secondly, from these data it may readily be seen that on-column degradation is not likely to be a serious problem because maximum stability is achieved once excess OPA has been separated from the isoindole derivative and thus allows for ample elution time for even the more unstable derivatives fomed. For instance, with **I** <10% degradation occurs in approximately 2 h as can be seen from curve A in Fig. 3.

Reagent effects — quantitative aspects

For many analytical applications, large excesses of OPA are employed. As seen previously, such excesses may accelerate isoindole decomposition (see Fig. 3), with no induction period being observed. Under such experimental conditions, whilst the decomposition profile still does not exhibit true first-order kinetics, it is possible to model the analytically important early phase of the degradation as such a process. Decomposition profiles for the isoindole formed from the unbranched primary amine GABA in the presence of excess OPA is shown in Fig. 4 and the rate constants are summarized in Table 3. These rate data were fitted to a linear expression to give a measure of OPA-independent degradation;

$$k_{obs} = k_o + k_l\,[\mathrm{OPA}]$$

of the isoindole, where $k_o = 1.17 \times 10^{-3}$ min^{-1}, the OPA-dependent term, $k_l = 27.8$ min^{-1} l mol^{-1} and the corresponding linear correlation coefficient $r > 0.9999$. Previously the effects of excess OPA on a similar unbranched primary amine derivative (BALA), prepared using OPA/2-ME, have been treated similarly to yield an OPA catalytic rate constant of $k_l = 100.5$ min^{-1} l mol^{-1}. Although not comparing the same unbranched primary amine, (the results in Fig. 1 suggest similar instability for all unbranched primary amines), the present data suggest that the use of ET results in derivatives with a reduced tendency to undergo degradation in the presence of OPA than in the case of the OPA/2-ME system.

The degradation process

Previously, it has been suggested [14] that 1-ethylthio-2-alkyl isoindoles undergo degradation by a hydrolytic process to form *N*-alkylphthalimidines (**VIII**). In general, the isoindole ring system is known to be susceptible to autoxidation [21, 22] and for 1-*tert*-butylthio-2-*n*-propyl isoindole we have previously shown that decomposition does occur via autoxidation to yield one major product, 3-*tert*-butylthio-2-*n*-propylphthalimidine [23]. Thus, in the present case the formation of **VII** would be indicative of decomposition occurring via autoxidation rather than hydrolysis.

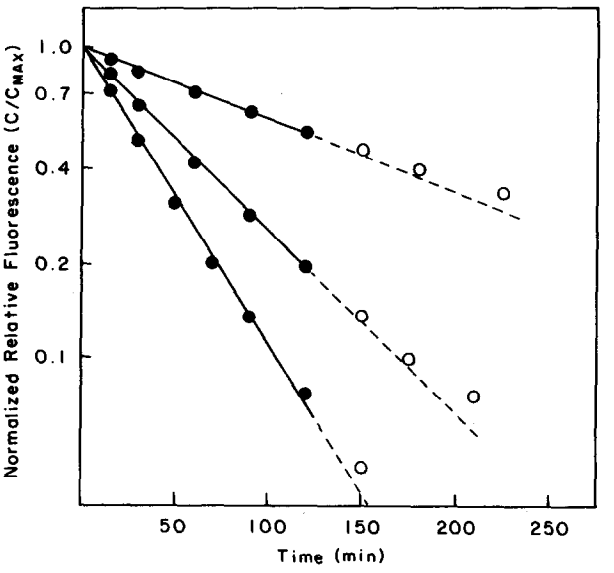


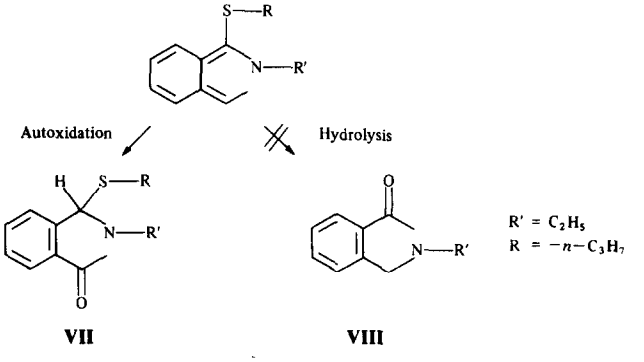
Figure 4
Apparent first-order loss of **II** in the presence of large OPA excesses: The derivative was generated *in situ* using ET = 1.43×10^{-3} M, GABA = 1.0×10^{-6} M and the following amounts of OPA; A = 1.50×10^{-4} M, B = 4.5×10^{-4} M and C = 7.5×10^{-4} M. Conditions were as described in Fig. 1.

Table 3
Influence of large OPA excesses on the stability of isoindole **II**

[OPA]·10^6 M*	$k_{obs} \cdot 10^3$ (min^{-1})	$t½$ (min)	Number of $t_{½}$ followed†
150	5.36	129.3	1
450	13.69	50.6	2
750	22.07	31.4	4

*II was generated *in situ* (see experimental) by reaction of GABA (1.0×10^{-6} M), ET (1.43×10^{-3} M) and the required OPA concentration. The conditions of the experiment are described in Fig. 1.

†These were the maximum times (120 min) for which significant curvature of the first-order plots did not occur (see Fig. 4) and form the basis for the rate constant calculation.



VII VIII

When dilute solutions, *ca* 10^{-5} M, of **I** were generated *in situ* and monitored by HPLC the appearance of a major degradation product (**VII**) of increased polarity (**I**, t_R = 9.9 min; **VII**, t_R = 5.4 min) was observed, as shown in Table 2. When preparative quantities of **I** were generated in 50% acetonitrile–water solution (necessary because of limited aqueous solubility) the same major degradation product was observed by HPLC. This product was isolated by column chromatography and upon spectral characterization (IR, NMR, MS) found to be identical to an authentic sample of 3-ethylthio-2-*n*-propyl-phthalimidine (**VII**) prepared by synthesis. Thus, based on the present kinetic and structural data it does appear that degradation of **I** proceeds by autoxidation.

Quantitative stability comparisons between OPA/2-ME and OPA/ET derivatized amino acids

Previously, we have reported kinetic data describing the decomposition of several isoindoles obtained by derivatizing amino acids with OPA/2-ME [15]. In the present work, these same amino acids were derivatized with OPA/ET under identical conditions, thus allowing for quantitative comparison of the isoindole stability (reported as t_{90} values in Table 4) of the two most widely used OPA reagent systems. The most unstable isoindole studied, derived from GABA, was five times more stable when ET rather than 2-ME was used as the thiol component of the reagent. In general, the use of ET results in isoindoles of enhanced stability (Table 3) and in cases where the amines being derivatized would be expected to form particularly unstable isoindoles (primary alkyl amines), the use of ET may thus prove valuable in enhancing sensitivity and precision.

Table 4
Stability comparison between OPA/ET and OPA/2-ME derivatized amino acids

Precursor amine	t_{90} (min)* ET	2-ME	Stability enhancement ET/2-ME
γ-Amino-*n*-butyric acid	18	3.2	5.6
β-Alanine	27	3.8	7.1
Glycine	44	4.7	9.4
β-Amino-*n*-butyric acid	290	8.8	33
Alanine	290	15.0	19.3

*Time for 10% degradation. Values for ET series taken from average curves shown in Fig. 2. Values for 2-ME series were calculated from the rate constants previously reported.

Recently, both 3-mercaptopropionic acid (3-MPA) and *tert*-butylthiol (TBT) have been used as the thiol to provide isoindole derivatives of improved stability with fluorescence [24] and electrochemistry [25] detection, respectively. Currently, extensive use has not been made of either alternative but 3-MPA would be likely to have the same attributes and limitations as ET while TBT results in derivatives of reduced fluorescence response (relative to 2-ME or ET), although not altering electrochemical detectability. The present results show that ET (and probably 3-MPA) provides a significant advantage compared to 2-ME without compromising fluorescence output or the kinetics of product formation.

Conclusions

The use of ET rather than 2-ME as the thiol component of the reagent results in enhanced derivative stability in all cases examined, offering greater than a five-fold increase in stability for amino acids previously reported to form particularly unstable isoindoles. Kinetic experiments revealed that, when excess OPA (>150-fold) was present, the acceleration of the isoindole degradation showed apparent first-order behaviour with respect to OPA. Furthermore, in the absence of excess OPA excellent derivative stability was realized, as would be the case once the derivatization mixture is fractionated on a HPLC column, thus resulting in minimal on-column degradation.

The results presented here support the contention that 1-alkylthio-2-alkylisoindoles undergo autoxidation in the presence of atmospheric oxygen. The presence of unreacted thiol in the reaction medium stabilizes the isoindole whilst excess OPA promotes isoindole degradation. From the degradation profiles for a series of isoindoles derived from amino acids, it was found that isoindole stability is predominantly influenced by the steric bulk about the *N*-substituent rather than electronic contributions provided by the carboxylate group.

As a consequence of this work it is now clear that isoindole stability is a function of the amine structure from which it is derived as well as the reagent composition. Thus, stability problems may be anticipated for derivatives of primary amines which do not provide steric bulk about the isoindole nitrogen and for maximum stability only the amount of excess OPA required to ensure complete derivatization in a convenient time should be utilized.

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