FLUOROMETRIC ASSAY OF SECONDARY AMINO ACIDS

Manfred Weigele, Silvano DeBernardo, and Willy Leimgruber

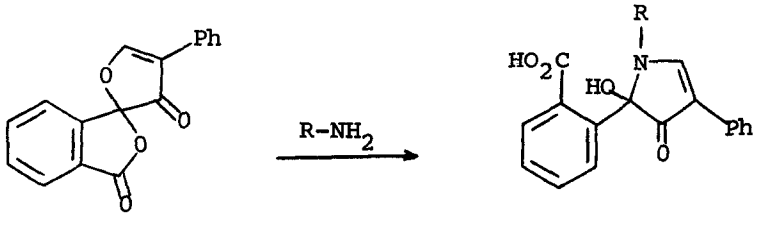
Chemical Research Department, Hoffmann-La Roche Inc.

Nutley, New Jersey 07110

Received November 21,1972

Summary: A method is described for the conversion of secondary amino acids to primary amines which can be assayed with fluorescamine (I). Secondary amino acids undergo oxidative decarboxylation when reacted with halogenating agents. The resulting imines are hydrolyzed to primary amines, which are subsequently allowed to react with fluorescamine (I) to yield fluorescent pyrrolinones (II). This reaction sequence provides an efficient fluorometric assay for secondary amino acids. Thus, the fluorescamine procedure is now applicable to the full array of natural amino acids.

Fluorescamine, 4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione (I), has recently been introduced as a novel reagent for the fluorometric quantitation of primary amines (1). Fluorescamine reacts with primary amines to form highly fluorescent pyrrolinones (II). This reaction proceeds efficiently and very rapidly at room temperature and allows the assay of sub-micromolar concentrations of amines, particularly those of biological importance,



I II

in aqueous solutions. A most important application of fluorescamine is the fluorometric assay of amino acids (2). Picomole

quantities of amino acids can be separated and analyzed in a recently described automated chromatography apparatus in which the eluate is monitored with fluorescamine (3,4). However, a shortcoming of the reagent has been its inability to produce fluorescence with secondary amino acids. This problem has now been resolved. We report here a method for the efficient transformation of secondary amino acids to primary amines and their subsequent estimation by the fluorescamine procedure.

Of particular interest is the analytical determination of proline. It was found that strong fluorescence is produced when acidic solutions of proline are subjected to oxidation by N-chlorosuccinimide (or other sources of active halogen), and then allowed to react with fluorescamine at pH 9. The observed fluorescence (390nm excitation, 475nm emission) is of the same type as the one generated from fluorescamine with primary amines (1). The fluorescence intensity is linearly dependent upon the concentration of proline.

In detail, the data contained in Fig. 1 were obtained by successively combining at room temperature at 10-second intervals 1 ml aliquots of 4×10^{-6} to 4×10^{-5} M solutions of proline, or hydroxyproline, resp., at pH 2 with 1 ml of 4×10^{-4} M aqueous N-chlorosuccinimide, 1 ml of 2% sodium bicarbonate and 1 ml of 2×10^{-3} M fluorescamine solution in acetone. Fluorescence was measured two minutes after addition of the latter reagent. Optimal fluorescence from sarcosine (Fig. 2) was generated by the use of bromine water (2×10^{-3} M) in place of N-chlorosuccinimide.

In analogy with documented transformations of amino acids (5), it could reasonably be assumed that N-chlorosuccinimide causes the oxidative decarboxylation of proline, by way of

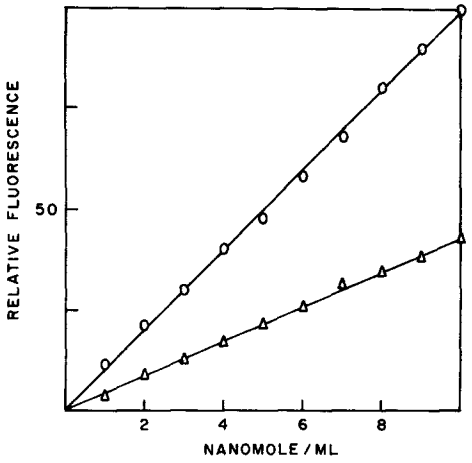


Fig. 1.

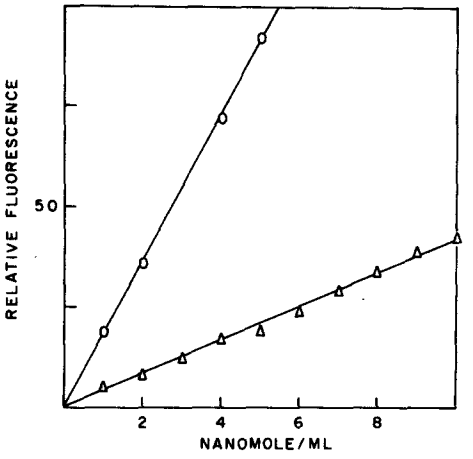
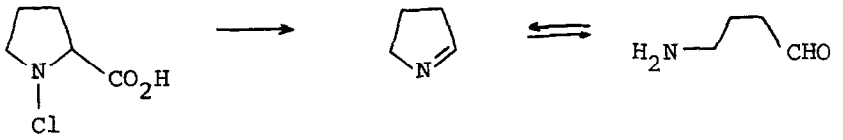


Fig. 2.

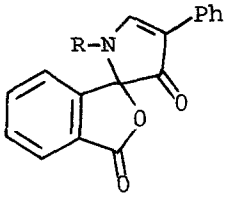
Fig. 1 Fluorescence generated from proline (- o -) and from hydroxyproline (-Δ-) upon oxidation with N-chlorosuccinimide followed by reaction with I. In both figures, fluorescence is expressed in arbitrary units (100 for the fluorescence obtained with 10 nmol/ml of proline) and corrected for blank.

N-chloroproline (III), resulting in the formation of Δ^1-pyrroline (IV), which in aqueous solution is in equilibrium with 4-amino-n-butyraldehyde (V). In order to prove the involvement of V in the production of fluorescence from proline, the fluorogenic reaction sequence was carried out on a preparative scale. After acidification of the reaction mixture, the resulting fluorophor II [R = $-(CH_2)_3CHO$] was extracted with benzene. Brief heating of the extract converted the fluorescent product to the thermally more stable nonfluorescent lactone VIa.

The structure of VIa was confirmed by an independent synthesis. Reaction of I with equimolar amounts of 4,4-diethoxy-1-butylamine in acetonitrile gave the fluorophor II [R = $-(CH_2)_3CH(OC_2H_5)_2$; mp 104-108° dec., uv max (EtOH) 275 (ε 18,200) and 390 nm (6,450); ir (KBr) 1720 (sh), 1680, 1545 cm^{-1}; nmr (DMSO-d_6) δ 9.08 (s,



III IV V



VI

a: R = $-(CH_2)_3-CHO$

b: R = $-(CH_2)_3-CH(OC_2H_5)_2$

N-CH=)]. Brief heating of II [R = $-(CH_2)_3CH(OC_2H_5)_2$] in benzene solution afforded the lactone VIb [yellow oil; uv max (EtOH) 269 (ε 19,000) and 288 nm (5,800); ir ($CHCl_3$) 1785, 1700, 1605 and 1580 cm^{-1}; nmr ($CDCl_3$) δ 8.47 (s, N-CH=)]. Hydrolysis of its acetal function with dilute hydrochloric acid in acetone yielded VIa [mp 137-138°, dec.; uv max 286 (ε 19,000), and 385 nm (6,000); ir (KBr) 1785, 1725, 1680, 1600, 1575 cm^{-1}; nmr ($CDCl_3$) δ 8.50 (s, N-CH=), 9.70 (s, CHO)]. The substance obtained by this route was identical in all respects (mp, mixture mp, spectra and tlc) with VIa prepared from proline under the conditions of the assay.

By analogy, it is inferred that sarcosine produces the methylamine-derived fluorophor II (R = $-CH_3$). This substance [mp 116°; uv max (EtOH) 274 (ε 18,000) and 390 nm (5,500); ir (KBr) 1680, 1620, 1545 cm^{-1}; nmr (DMSO-d_6) δ 9.02 (s, N-CH=); 3.01 (s, CH_3N)] was synthesized from I and methylamine

in acetonitrile. Its fluorescence was measured at varying concentrations in aqueous solution (pH 8) and compared with the fluorescence generated *in situ* from sarcosine. The results (Fig. 2) demonstrate that the conversion of sarcosine to the observed fluorophor (II, R = $-CH_3$) proceeds with an overall yield of 23% independent of concentration.

Preliminary results indicate that the outlined procedure will be readily adaptable to automation (6). Hence, fluorescamine can now be utilized for the assay of the full array of natural amino acids.

Acknowledgment. We thank Mrs. Karin Manhart for fluorescence measurements.

REFERENCES

(1) M. Weigele, S.L. DeBernardo, J.P. Tengi, and W. Leimgruber, J.Amer.Chem.Soc., 94, 5927 (1972).

(2) S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber, and M. Weigele, Science, 177 (1972), in press.

(3) S. Udenfriend, S. Stein, P. Böhlen, and W. Dairman, Third American Peptide Symposium, Boston, Mass., June 19-23, 1972, in press.

(4) S. Stein, P. Böhlen, J. Stone, W. Dairman, and S. Udenfriend, manuscript in preparation.

(5) A. Schönberg, P. Moubasher, and M.Z. Barakat, J.Chem.Soc., 2504 (1951).

(6) A.M. Felix, private communication.