Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytical Biochemistry 334 (2004) 193–195
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Notes & Tips

Nonenzymatic preparative-scale synthesis of dityrosine and 3-bromotyrosine☆

Michael Tilley[a,*], Rachel E. Benjamin[b], Phatthranith Srivarin[c], Katherine A. Tilley[b,c]

[a] *USDA-ARS Grain Marketing and Production Research Center, 1515 College Ave., Manhattan, KS 66502, USA*
[b] *Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA*
[c] *Food Science Institute, Kansas State University, Manhattan, KS 66506, USA*

Received 30 January 2004
Available online 25 August 2004

Dityrosine (3,3′-bityrosine) occurs in many biological systems and is formed by the action of peroxidases, reactive oxygen species/free radicals, or ionizing radiation. Dityrosine is found in extensible proteins such as elastin, resilin, and calmodulin and polymeric structures found in insect eggs, yeast spores, and sea urchin egg developmental stages [1]. Dityrosine has shown promise as a marker for identification of cellular aging and damage from protein cross-linking in several pathological conditions such as atherosclerosis [2] and amyloid fibril formation [3]. Eosinophil peroxidase has been postulated to promote oxidative tissue injury in conditions such as asthma, allergic inflammatory disorders, and helminthic infections [4]. Halogenated tyrosine residues in proteins are specific biomarkers of eosinophil-mediated tissue injury in vivo. Recent reports in the literature have identified 3-bromotyrosine as a specific indicator of eosinophil-peroxidase-mediated protein oxidation [5].

To adequately measure dityrosine and 3-bromotyrosine formation in biological samples, an efficient method for production of sufficient quantities of standard compounds is necessary. Several methods that employ an enzymatic synthesis of dityrosine using peroxidase have been reported [6], as have chemical methods for dityrosine synthesis [7]. Synthesis of dityrosine via these methods typically results in conversion of 25–50% of the theoretical maximum. Protocols for synthesizing 3-bromotyrosine are laborious and require formation of hypobromous acid from bromic acid and result in low recovery [4]. In this paper a simple, rapid, nonenzymatic method for the simultaneous preparation of both dityrosine and 3-bromotyrosine resulting in high (~72%) recovery of both tyrosine derivatives is presented.

L-Tyrosine (10 mg) was dissolved in 2 mL deionized water with the addition of 100 μL 1.6 M HCl (resulting solution = 0.08 N HCl) and divided into six glass 13 × 100-mm culture tubes. Then 2 mL aqueous potassium bromate ($KBrO_3$) [400 ppm (2.4 mM)] were added to each tube; the tubes were covered with aluminum foil, heated at 150 °C for 25 min in a convection oven, cooled, and lyophilized to dryness if needed. The material in each tube was dissolved in 0.5 mL deionized water, centrifuged through 0.22-μm nylon centrifuge tube filters (Corning, Midland, MI) at 2700*g*, and analyzed by high-pressure liquid chromatography (HPLC).

Semi-preparative-scale collections were performed using a Hewlett–Packard 1100 Series HPLC system (Wilmington, DE) fitted with a quaternary pump, a degasser, an autosampler, and a diode array detector (285 nm) with a standard flow cell (13 μL, 10-mm path). Aliquots of 100 μL were injected onto a C18 reversed-phase column (LUNA RP 10 μ, 250 × 10.0 mm, Phenomenex, Torrance, CA) using a stepwise gradient (9, 15, 85, and 9%) of acetonitrile containing 0.1% trifluoroacetic

☆ Cooperative investigation, USDA, Agricultural Research Service and the Department of Grain Science and Industry, Kansas State University. Contribution No. 05-20-J from the Kansas Agricultural Experiment Station, Manhattan, KS 66506. Names are necessary to report factually on available data; mention of firm names or trade products does not constitute endorsement by the U.S. Department of Agriculture over others that may also be suitable.

* Corresponding author. Fax: +1 785 537 5534.
E-mail address: mtilley@gmprc.ksu.edu (M. Tilley).

0003-2697/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.ab.2004.07.014

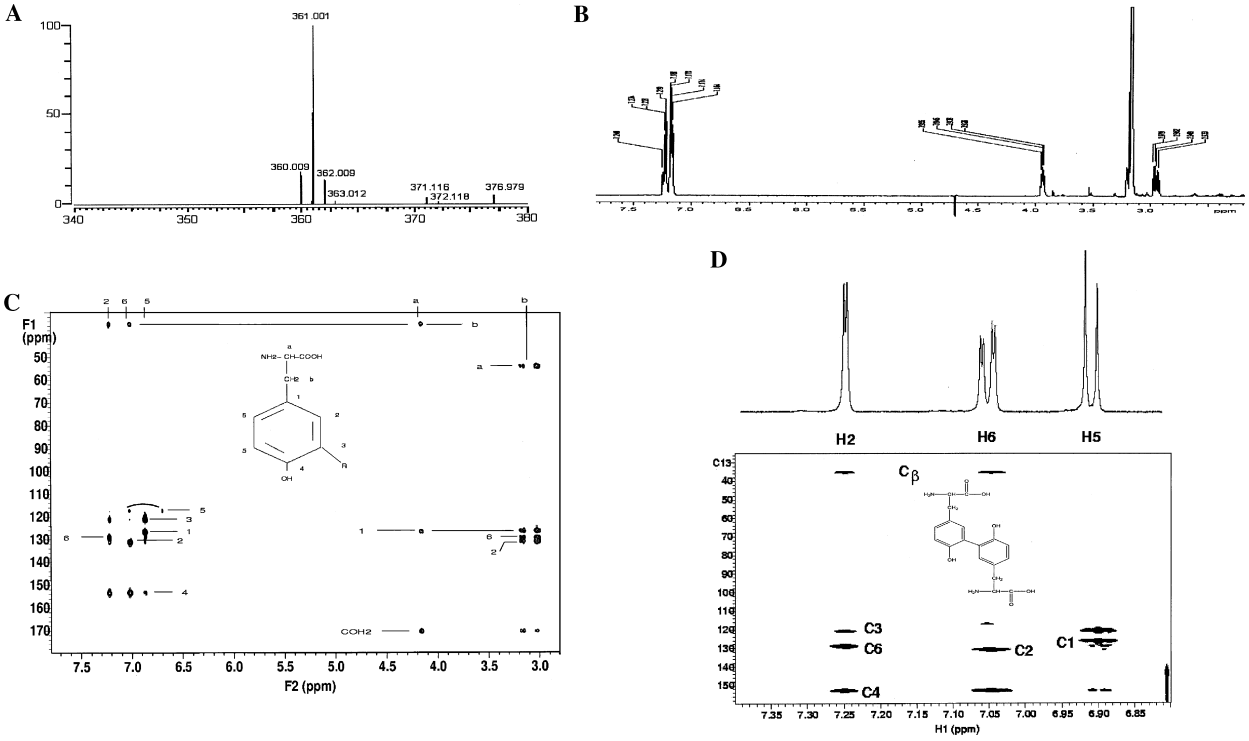

Fig. 1. (A) Mass spectral analysis, (B) proton (1D) NMR spectrum, (C) 2D NMR spectra, and (D) 2D NMR spectra of the expanded section between 6.80 and 7.30 ppm of the peak eluting at 24.9 min during HPLC analysis showing the aromatic portions of the molecule and confirming the biphenyl linkage. These spectral analyses are consistent with the known dityrosine structure.

acid (TFA)[1] at 0, 8, 18, and 23 min, respectively, with a flow rate of 2.5 mL/min and a column temperature of 30 °C. Tyrosine eluted at approximately 9.3 min, dityrosine eluted at approximately 13.6 min, 3-bromotyrosine (identified after purification) eluted at approximately 15.0 min (data not shown). Each peak was collected for further purification using a fraction collector. The collected fractions were lyophilized and individually purified using an analytical method on the same HPLC system described above by a reversed-phase column (LUNA RP 5 μC18(2); 250 × 4.6 mm, Phenomenex) and stepwise gradient (3, 10, 40, 85, and 3%) of acetonitrile containing 0.1% TFA at 0, 35, 50, 60, and 70 min, respectively, with a flow rate of 1.0 mL/min and a column temperature of 30 °C. Tyrosine eluted at approximately 12.6 min, dityrosine eluted at approximately 24.9 min, and 3-bromotyrosine (identified after purification) eluted at approximately 29.5 min. Each pure peak was collected and lyophilized to dryness.

Compounds representing each collected peak were confirmed by comparing them with either a known standard in the case of simple tyrosine or by mass spectral and nuclear magnetic resonance (NMR) analyses at the Complex Carbohydrate Research Center, University of Georgia as previously described [8] in the cases of dityrosine and 3-bromotyrosine, respectively (Figs. 1 and 2). The identity of dityrosine is difficult to distinguish from the related structure of isodityrosine due to identical mass and 1D NMR profiles. Both dityrosine and isodityrosine have masses of 361 and very similar proton (1D) NMR spectra. It is necessary to perform 2D NMR analyses to distinguish between the biphenyl cross-link of dityrosine and the ether linkage of isoditytrosine. The unequivocal determination was obtained through 2D NMR (Fig. 1C). The 2D NMR data show cross peaks between the H6 (7.05 ppm) and the β carbon (~35 ppm) and absence of the H3. Isodityrosine is ruled out in the 2D spectra as the OH group connectivity to C4 is present. The only possible alternative structure with the second tyrosine (R) at position 2 would allow only one cross peak between the H6 and the β carbon (b), whereas there is a cross peak from the H2 also. This alternative has never been described in the literature but needed to be included as a possibility. A strong cross peak between the H2 and the C4 is present, indicating dityrosine as the structure; the alternative structure would result in only a weak cross peak between the H3 and the C4. The identity of 3-bromotyrosine was determined by mass spectral analysis and by 1D and 2D NMR (Fig. 2) and is consistent with that reported in the literature [5].

Eight-point standard curves were prepared by serial dilution of tyrosine, dityrosine, and 3-bromotyrosine using dityrosine and 3-bromotyrosine, standards

[1] *Abbreviations used:* TFA, trifluoroacetic acid; 1D, one-dimensional; 2D, two-dimensional.

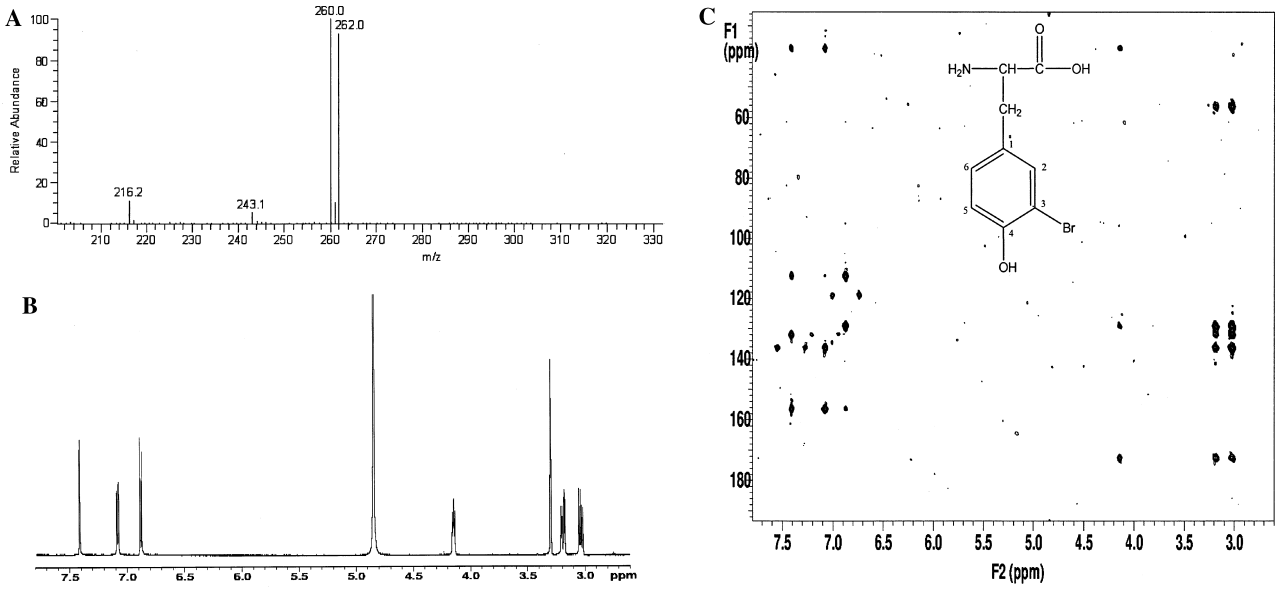

Fig. 2. (A) Mass spectral analysis, (B) complete proton (1D) NMR spectrum, and (C) 2D NMR spectra of the peak eluting at 29.5 min during HPLC analysis. These spectral analyses indicate a structure consistent with 3-bromotyrosine.

prepared in our laboratory. Under these experimental conditions, nearly 100% of tyrosine was converted to either dityrosine or 3-bromotyrosine, resulting in an average of 4.35 mg (SD = 0.31) of dityrosine and 7.55 mg (SD = 0.43) of 3-bromotyrosine from the original 10 mg of L-tyrosine. This represents 43–52% of the maximum theoretical yields for dityrosine and 3-bromotyrosine. Following purification, averages of 3.1 mg of dityrosine and 5.4 mg of 3-bromotyrosine were recovered. This results in recovery of approximately 72% for both dityrosine and 3-bromotyrosine. The protocol can be scaled up to suit the requirements and instrumentation of individual investigators. The procedure is simple, rapid, and relatively inexpensive. The tyrosine-derived compounds produced are nearly pure when initially separated on the semipreparative HPLC column, but it is advisable to further purify them using the analytical method described herein. This will allow laboratories to synthesize very pure stocks of these compounds.

Acknowledgment

We thank Sushma Prakash and Brian Ioerger (USDA-ARS GMPRC) for technical assistance. We also thank John Glushka of the Complex Carbohydrate Research Center, University of Georgia for mass spectral and NMR analyses. This work was supported by USDA-NRI Grant 2002-01752 and by Kansas wheat producers through a grant from the Kansas Wheat Commission.

References

[1] D.A. Malencik, S.R. Anderson, Dityrosine as a product of oxidative stress and fluorescent probe, Amino Acids 25 (2003) 233–247.
[2] J.W. Heinecke, Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress, Free Radicals Biol. Med. 32 (2002) 1090–1101.
[3] J.M. Souza, B.I. Giasson, Q. Chen, V.M.-Y. Lee, H. Ischiropoulos, Dityrosine cross-linking promotes formation of stable α-synuclein polymers, J. Biol. Chem. 275 (2000) 18344–18349.
[4] W. Wu, Y. Chen, A. d'Avignon, S.L. Hazen, 3-Bromotyrosine and 3,5-dibromotyrosine are major products of protein oxidation by eosinophil peroxidase: potential markers for eosinophil-dependent tissue injury in vivo, Biochemistry 38 (1999) 3538–3548.
[5] W. Wu, M.K. Samoszuk, S.A.A. Comhair, M.J. Thomassen, C.F. Farver, R.A. Dweik, M.S. Kavuru, S.C. Erzurum, S.L. Hazen, Eosinophils generate brominating oxidants in allergen-induced asthma, J. Clin. Invest. 105 (2000) 1455–1463.
[6] D.A. Malencik, J.F. Sprouse, C.A. Swanson, S.R. Anderson, Dityrosine: preparation, isolation and analysis, Anal. Biochem. 242 (1996) 202–213.
[7] C.A. Hutton, O. Skaff, A convenient preparation of dityrosine via Miyaura borylation-Suzuki coupling of iodotyrosine derivatives, Tetrahedron Lett. 44 (2003) 4895–4898.
[8] K.A. Tilley, R.E. Benjamin, K.E. Bagorogoza, B.M. Okot-Kotber, O. Prakash, H. Kwen, Tyrosine crosslinks: the molecular basis of gluten structure and function, J. Agric. Food Chem. 49 (2001) 2627–2632.