ORGANIC LETTERS

2005
Vol. 7, No. 12
2401−2404

Mechanism of Reversible Fluorescent Staining of Protein with Epicocconone

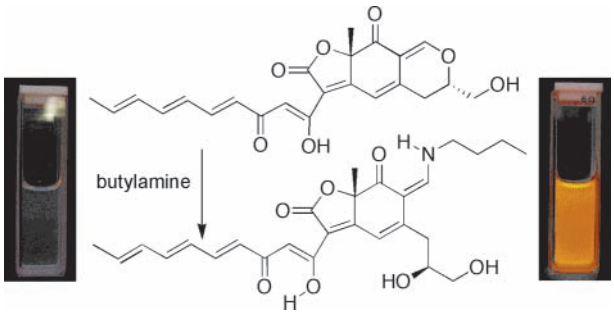
Daniel R. Coghlan,[†] James A. Mackintosh,[†] and Peter Karuso*[,†,‡]

FLUOROtechnics Pty Ltd., Sydney, NSW 2109, Australia, and Department of Chemistry & Biomolecular Sciences, Macquarie University, Sydney, NSW 2109, Australia

peter.karuso@mq.edu.au

Received March 29, 2005

ABSTRACT



Epicocconone is the active ingredient in Deep Purple Total Protein Stain and responsible for the apparent noncovalent staining of proteins in polyacrylamide gel and electroblots. Reaction of epicocconone with amines has shown that epicocconone reacts reversibly with primary amines to produce a highly fluorescent enamine that is readily hydrolyzed by base or strong acid such as in conditions used in post-electrophoretic analysis such as peptide mass fingerprinting or Edman degradation.

The use of fluorescent dyes to monitor biological systems has increased as the range and specificity of fluorescent probes has grown. Whether the probes are used in cell tracking, flow cytometry, microscopy, or protein detection, their usefulness is dependent upon the excitation sources available and the excitation and emission range of the dye.[1]

Epicocconone (**1**), a novel fluorescent natural product,[2] is the active ingredient in the new protein stain called "Deep Purple Total Protein Stain".[3] Deep Purple is popular for staining 1D and 2D protein gels[4] because of its unsurpassed sensitivity, mass-spec compatibility, ease of use, and environmental friendliness. However, the further application of epicocconone in labeling of specific proteins inside cells,[5] chemosensing,[6] fluorescence resonance energy transfer (FRET),[7] or as a molecular beacon[8] will rely on the elucidation of how this molecule interacts with proteins to achieve its unique fluorescent properties. Epicocconone is only weakly fluorescent in water (green) but becomes highly fluorescent (orange-red) in the presence of proteins and SDS.[2] The protein−epicocconone adducts can potentially be used in multiplexing as the red emission can be stimulated by the same frequencies used for fluorescein (UV or blue) that emits in the green. Herein, we report the isolation and structure elucidation of a highly fluorescent enamine that results from the reaction of epicocconone with butylamine and the MS analysis/kinetics of a lysine containing peptide derivatized with epicocconone.

[†] FLUOROtechnics Pty Ltd.
[‡] Macquarie University.

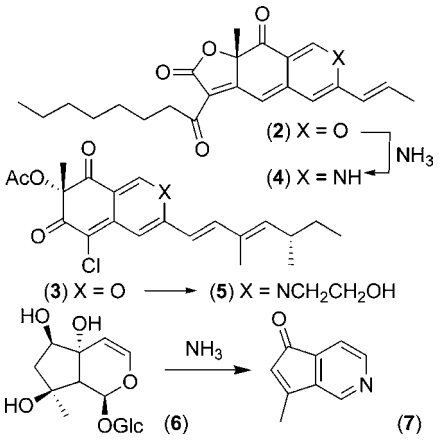
(1) Patton, W. F. *Electrophoresis* **2000**, *21*, 1123.
(2) Bell, P. J. L.; Karuso, P. *J. Am. Chem. Soc.* **2003**, *125*, 9304.
(3) Available from GE Healthcare − Biosciences.
(4) Mackintosh, J. A.; Choi, H.-Y.; Bae, S.-H.; Veal, D. A.; Bell, P. J.; Ferrari, B. C.; Van Dyk, D. D.; Verrills, N. M.; Paik, Y.-K.; Karuso, P. *Proteomics* **2003**, *3*, 2273.
(5) Griffin, B. A.; Adams, S. R.; Tsien, R. Y. *Science* **1998**, *281*, 269.
(6) (a) Simeonov, A.; Matsushita, M.; Juban, E. A.; Thompson, E. H.; Hoffman, T. Z.; Beuscher, A. E.; Taylor, M. J.; Wirsching, P.; Rettig, W.; McCusker, J. K.; Stevens, R. C.; Millar, D. P.; Schultz, P. G.; Lerner, R. A.; Janda, K. D. *Science* **2000**, *290*, 307. (b) Chen, C. T.; Wagner, H.; Still, W. C. *Science* **1998**, *279*, 851.
(7) Selvin, P. R. *Nature Struct. Biol.* **2000**, *7*, 730.
(8) Tyagi, S.; Marras, S. A.; Kramer, F. R. *Nature Biotech.* **2000**, *18*, 1191.

10.1021/ol050665b CCC: $30.25
Published on Web 05/17/2005

Epicocconone has a long Stokes' shifts (>100 nm) and does not to suffer from self-quenching, is less affected by Rayleigh scattering and can be multiplexed with more common fluorescent dyes. The understanding of epicocconone's mechanism is fundamental to its application in biology.

Epicocconone is an azaphilone that reacts with primary amines and NH_3 almost instantaneously to produce red fluorescent compounds.[4] Azaphilones, such as monascorubrin (**2**, X = O) and (+)-sclerotiorin (**3**, X = O), also react with primary amines (Scheme 1) to produce 1*H*-4-pyridylidenes

Scheme 1. Azaphilones and Their Amine Adducts



(e.g., **4**, X = NH; **5**, X = NCH_2CH_2OH, respectively).[9] Similarly, iridoids such as harpagide (**6**) when treated with ammonia and HCl produced aucubinine B (**7**).[10] Epicocconone could react similarly with primary amines to produce analogous structures (Scheme S1).[11]

To understand the action of epicocconone with proteins, epicocconone was reacted with butylamine to simulate the side chain of lysine. A yellow epicocconone solution reacted instantly with butylamine to produce a bright pink solution. ESI (+ve) mass spectrometry analysis of the reaction mixture indicated that the product had a molecular weight of 483.[12] This represented an addition product, not the expected 1*H*-4-pyridylidene derivative that would result from the elimination of two molecules of water (MW 447). Reaction of epicocconone with butylamine in an NMR tube ($CDCl_3$) indicated only one product, even after months of storage.

HPLC purification of the reaction mixture yielded a product whose UV and fluorescence spectra qualitatively matched the changes observed when epicocconone was added to protein[2] (Figure 1). The product's NMR spectrum was very similar to that of epicocconone but with the addition of several mutiplets attributable to butylamine. In the

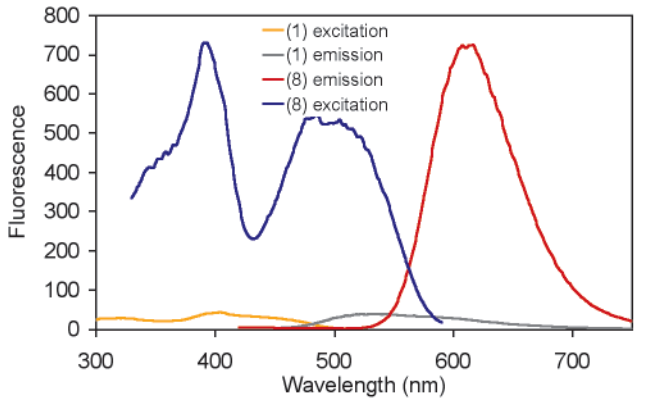
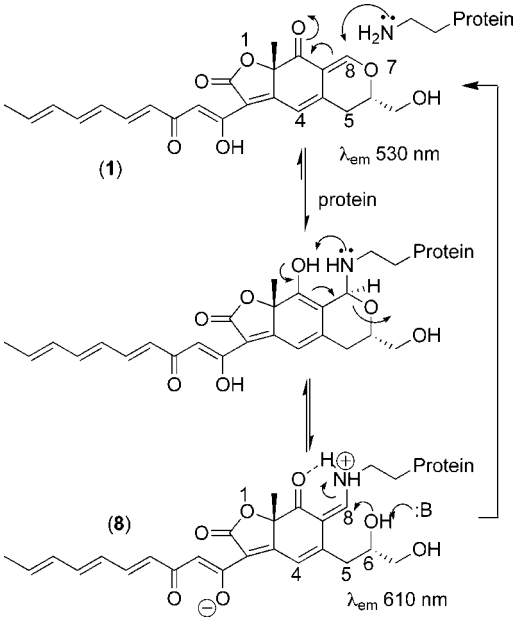


Figure 1. Fluorescence spectrum of epicocconone (green; λ_{em}, orange λ_{ex}) and the butylamine adduct (red; λ_{em}, blue λ_{ex}) in acetonitrile.

aromatic region, the signal for H8 in epicocconone (δ 7.85, s) was now split into a doublet (δ 7.65, J_{HH} = 13 Hz), coupled to an exchangeable proton at 10.4 ppm. The latter was further coupled to a methylene (δ 3.41, q) that was part of the butylamine spin system.

This is consistent with the expected regioselectivity for the nucleophilic addition of an amine to a dihydropyran[13] (Scheme 2). HMBC data indicated that H8 had correlations

Scheme 2. Proposed Mechanism for the Reversible Labeling of Protein with Epicocconone



to C9 and C4a as expected but also the NCH_2- of butylamine. However, there was no correlation to C6 as

(9) (a) Michael, A. P.; Grace, E. J.; Kotiw, M.; Barrow, R. A. *Aust. J. Chem.* **2003**, *56*, 13. (b) Wild, D.; Toth, G.; Humpf, H.-U. *J. Agric. Food Chem.* **2003**, *51*, 5493.

(10) Baghdikian, B.; Ollivier, E.; Faure, R.; Debrauwer, L.; Rathelot, P.; Balansard, G. *J. Nat. Prod.* **1999**, *62*, 211.

(11) Supporting Information.

(12) HRMS: found 506.2146 [$C_{27}H_{33}NO_7 + Na^+$], requires 506.2149.

(13) Zhu, S. Z.; Xu, G. L.; Qin, C. Y.; Chu, Q. L.; Xu, Y. *Monatsch. Chem.* **1999**, *130*, 671.

would be expected if a 1*H*-pyridylidene had formed. H6 did have the largest change in chemical shift from δ 4.39 in epicocconone to δ 3.96 and $(H5)_2$ went from an AB system to a 2H multiplet, indicative of free rotation about the C4a−C5 bond in the butylamine adduct. This evidence could only be interpreted as structure **8**, where the dihydropyranylidene had opened to an enamine.

Several tautomeric forms of **8** are possible, but the NMR data[11] indicated that only one was present in $CDCl_3$. The chemical shift of the amine hydrogen (δ 10.4) is indicative of strong intramolecular hydrogen bonding. A ROESY experiment showed H8 to be in proximity to $(H5)_2$ and H6. This evidence was used to suggest the configuration around the enamine double bond was *Z*. This was confirmed by 1D NOE difference spectroscopy where irradiation of the NH resulted in only a small NOE to H8 (<0.6%), whereas irradiation of the H8 resulted in a large NOE to $(H5)_2$ (5.3%).[11] In contrast, reactions of dihydropyrans with amines have previously been reported to give exclusively the *E*-isomers.[13] Whether the exchangeable proton was on the nitrogen (β-enaminone) or on the oxygen (β-iminenol) was inferred from ab initio calculations (B3LYP/6-31G(d,p)//B3LYP/6-31G(d,p))[11] of the epicocconone skeleton. The results indicated that the iminenol was 8.05 kcal/mol higher in energy than the corresponding enaminone. Interestingly, the *E*-enamine was only 6.69 kcal/mol higher in energy suggesting that this difference is due primarily to the H-bond. This is in agreement with previous calculations of model enaminones where a stabilization energy of 5.22 kcal/mol was found for the intramolecular β-enaminone H-bond.[14]

From the unexpected stability of the enamine, it was inferred that **1** should react similarly with lysine in proteins and peptides. This was confirmed using ESI mass spectrometry where an adduct was seen for lysine but not asparagine or methionine. Weaker adducts were seen for arginine and histidine.[11] The preference for lysine residues over arginine and histidine was illustrated by the mass spectrometric sequence analysis of epicocconone labeled peptides formaecin 1 (GRPNPVNNKPTPHPRL; *m*/*z* 1997) and formaecin 2 (GRPNPVNTKPTPYPRL; *m*/*z* 2010).[15] Each was labeled with only one epicocconone molecule (*m*/*z* 2407, 2420 respectively) and the automatic sequencing terminated at the lysine residue. It is known that epicocconone-stained 2D gels leave the proteins compatible to downstream processing such as MS and Edman degradation.[4] This could suggest that either epicocconone minimally labels proteins in gels or that the binding of epicocconone to proteins is reversible or both. The reversibility of epicocconone binding to peptides was observed by the change in color of aqueous or basic solutions of peptides treated with epicocconone that are initially red but reverted to the greenish yellow fluorescence of epicocconone over time. Additionally, NMR samples of the butylamine adduct also slowly reverted back to epicocconone after many months in $CDCl_3$ (singlets at δ 7.85; H8 and 7.13; H4).

The stability of the epicocconone adduct to proteins was examined under different pH conditions by MALDI-MS. Using formaecin 1, aglycon combined with an equimolar amount of epicocconone in 4 mM sodium carbonate. After 1.5 h, 10 μL aliquots were removed and added to 90 μL aliquots of water, sodium carbonate (5 mM), aqueous acetic acid (0.01, 0.05, 0.1, 0.5, 1.0, and 5.0%), and aqueous trifluoroacetic acid (0.01, 0.05, 0.1, 0.5, and 1.0%) for up to 144 h. The relative amount of formaecin−epicocconone adduct was analyzed by MALDI-MS. The results plotted against time (Figure 2) indicated that the enamine was stable

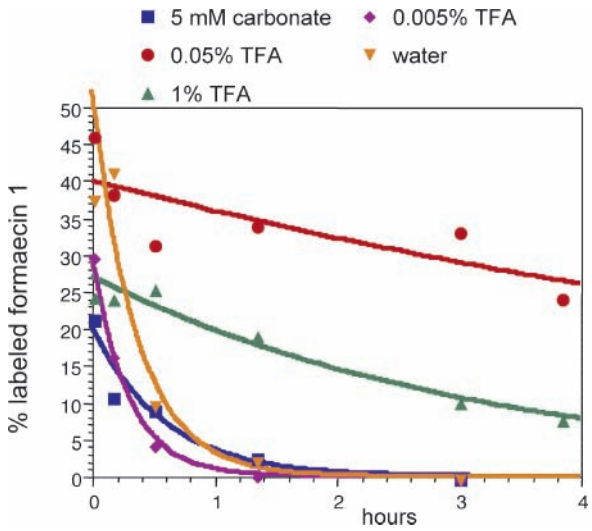


Figure 2. Least-squares fit of rates of hydrolysis of formaecin 1-epicocconone adduct over 4 h as measured by the relative integrals of molecular ion clusters of formaecin (1997 amu) and the epicocconone adduct (2407 amu) measured by MALDI-MS.

under acidic conditions. Fitting of each curve to a simple exponential decay (assuming pseudo-first-order kinetics; k_O') gave a maximum stability (Figure 3) at pH 2.4 ($k_O' = 0.015$ hr^{-1}). Fitting the combined k_O' to an equation that assumes hydrolysis can be catalyzed by water (k_W'), acid (k_A'), or base (k_B')[11] gave a value of k_B' of 1.69×10^9 h^{-1} and much smaller values for k_A' or k_W'.[11]

The impressive increase in quantum yield and shift in emission wavelength for the amine-conjugated epicocconone explains the compound's utility as a protein gel/blot stain. In particular, the shift in wavelength and covalent modification allow the almost complete removal of background signal, which is not possible with other dyes such as SYPRO Ruby that are inherently fluorescent. In addition, the conditions used for peptide mass fingerprinting and Edman degradation ensure that the epicocconone is hydrolyzed from the protein by the pH-controlled mechanism elucidated here. These unique features lend themselves to many other applications where the removal of background signal is important or where a simple pH controlled mechanism can be used to

(14) Gilli, P.; Bertolasi, V.; Ferretti, V.; Gilli, G. *J. Am. Chem. Soc.* **2000**, *122*, 10405.
(15) Mackintosh, J. A.; Veal, D. A.; Beattie, A. J.; Gooley, A. A. *J. Biol. Chem.* **1998**, *273*, 6139.

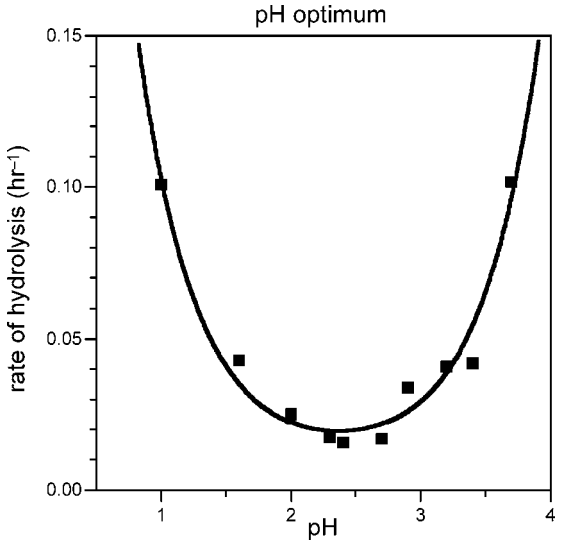


Figure 3. Hydrolysis rates vs pH using mixtures of acetic acid and TFA to achieve pH values near the stability maximum.

advantage. Some of these applications will be reported in due course.

$$k_O' = k_W' + k_A'[H_3O^+] + k_B'[OH^-]$$

$$k_O' = k_W' + k_A'[H_3O^+] + k_B'\left(\frac{K_W}{[H_3O^+]}\right)$$

In this paper, we provide a mechanism by which epicocconone reacts with primary amines in proteins by analysis of model systems using mass spectrometry, fluorescence, and NMR spectroscopy. The results identify a new mechanism by which epicocconone reacts that is different from the known mechanism of other azaphilones. This work also demonstrates the selective formation of only the *Z*-enamine, in contrast to previously reported examples of amine addition to dihydropyrans, which give exclusively *E*-enamines. Through 2D NMR spectroscopy in conjunction with ab initio calculations we have identified an intramolecular hydrogen bond responsible for stabilizing the β-enaminone. The reaction of epicocconone with amines is reversible and we obtained a pH profile for hydrolysis and identifies pH 2.4 as the point of maximum stability for the adduct, with both higher and lower pHs showing greater rates of hydrolysis. This work is important for understanding the conditions under which the dye can be used in proteomics applications. For example, in 2D gel electrophoresis, the fluorescence behavior of Deep Purple is qualitatively the same as that of epicocconone when reacted with butylamine (Figure 1) and with a model protein (BSA).[2] These results lead us to believe that proteins in 2D gel electrophoresis react in the same way as butylamine with epicocconone and explains why Deep Purple stains proteins in 2D gels uniformly, sensitively and with very little background.

Acknowledgment. We thank Lewis Adler for the acquisition of MS/MS data, Garry Willet (UNSW) for acquisition of high-resolution mass spectra, and Andrew Piggott for NMR support.

Supporting Information Available: Experimental procedures and full spectroscopic data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

OL050665B