ORGANIC LETTERS

2003
Vol. 5, No. 5
609−612

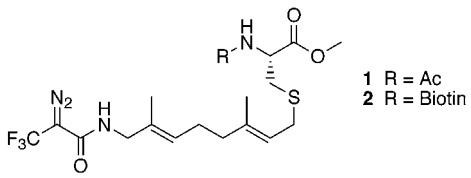
Diazotrifluoropropionamido-Containing Prenylcysteines: Syntheses and Applications for Studying Isoprenoid–Protein Interactions

Tamara A. Kale and Mark D. Distefano*

Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455
distefan@chem.umn.edu

Received August 19, 2002

ABSTRACT



Photoaffinity-labeled prenylcysteines (1 and 2) incorporating a diazotrifluoropropionamide-based photophore have been prepared. Photolyses of 2 in the presence of RhoGDI, a protein that interacts with prenylated proteins, and prenylcysteine-containing competitors demonstrate the effectiveness of this photoaffinity-labeled analogue as a tool for studying isoprenoid binding sites.

Protein prenylation, whereby certain cysteine residues are appended with one or more isoprenoid units, has been extensively studied in the past 15 years due to the role prenylated proteins play in the development of cancer. Considerable progress has been made in developing prenylation inhibitors for chemotherapeutic purposes.[1] However, an alternative to this mode of attack could lie in the disruption of prenylated protein−protein interactions. Isoprenoid binding sites in several proteins that interact with prenylated proteins have been either detected or suggested. Interruption of this type of isoprenoid−protein interaction may provide an alternative, and possibly more favorable, target of clinical interest.[2,3] Prenylated proteins have numerous opportunities to interact with other proteins, both cytosolic and membrane bound.[4,5] Various methodologies have been employed to study the way in which prenylated proteins interact with other proteins at the molecular level, including NMR, X-ray crystallography, and photoaffinity labeling. Photoaffinity labeled probes can lead to the identification of specific amino acid residues involved in recognition and/or binding events. Along with others,[6−8] our laboratory has exploited this technique, incorporating photoactivatable benzophenone and diazotrifluoropropanoyl moieties into either prenyl diphosphates[9−13] or prenylcysteines[14−16] and then photochemically cross-linking these compounds to proteins of interest (see Figure 1).

The benzophenone unit has proved to be a desirable photoaffinity labeling reagent in the field of protein prenylation, in part due to the structural similarities between the photo-

* To whom correspondence should be addressed. Phone: (612) 624-0544. Fax: (612) 626-7541.

(1) Sebti, S. M.; Hamilton, A. D. *Oncogene* **2000**, *19*, 6584−6593.
(2) Cox, A. D.; Der, C. J. *Curr. Opin. Cell. Biol.* **1992**, *4*, 1008−1016.
(3) Marshall, C. J. *Science* **1993**, *259*, 1865−1866.
(4) Zhang, F. L.; Casey, P. J. *Annu. Rev. Biochem.* **1996**, *65*, 241−269.
(5) Geyer, M.; Wittinghofer, A. *Curr. Opin. Struct. Biol.* **1997**, *7*, 786−792.
(6) Marecak, D. M.; Horiuchi, Y.; Arai, H.; Shimonaga, M.; Maki, Y.; Koyama, T.; Ogura, K.; Prestwich, G. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1973−1978.
(7) Yokoyama, K.; McGeady, P.; Gelb, M. H. *Biochemistry* **1995**, *34*, 1344−1354.
(8) Omer, C. A.; Kral, A. M.; Diehl, R. E.; Prendergast, G. C.; Powers, S.; Allen, C. M.; Gibbs, J. B.; Kohl, N. E. *Biochemistry* **1993**, *32*, 5167−5176.
(9) Turek, T. C.; Gaon, I.; Distefano, M. D.; Strickland, C. L. *J. Org. Chem.* **2001**, *66*, 3253−3264.
(10) Turek, T. C.; Gaon, I.; Gamache, D.; Distefano, M. D. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 2125−2130.
(11) Gaon, I.; Turek, T. C.; Distefano, M. D. *Tetrahedron Lett.* **1996**, *37*, 8833−8836.
(12) Edelstein, R. L. Ph.D. Thesis, University of Minnesota, Minneapolis, Minnesota, 1997.
(13) Edelstein, R. L.; Distefano, M. D. *Biochem. Biophys. Res. Commun.* **1997**, *235*, 377−382.
(14) Kale, T. A.; Raab, C.; Yu, N.; Dean, D. C.; Distefano, M. D. *J. Am. Chem. Soc.* **2001**, *123*, 4373−4381.

10.1021/ol026752a CCC: $25.00
Published on Web 02/14/2003

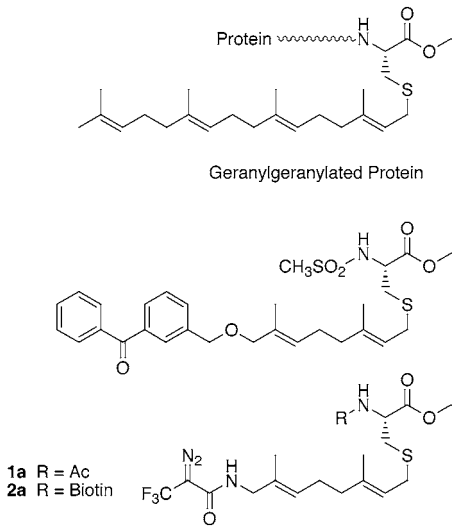
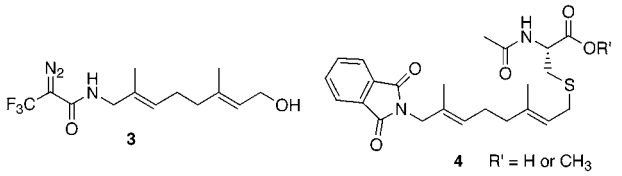


Figure 1. Examples of benzophenone- and diazotrifluoropropanoyl-containing mimics of prenylcysteines.

phore and an isoprene unit. Despite its successes, there are some potential drawbacks to using this reactive group: the lack of precise steric complementarity and the relatively long irradiation times that are often needed for optimal cross-linking emphasize the benefits of having a different photoactive structure.[17] For these reasons, the diazotrifluoropropanoyl (DATFP) group may prove to be a useful alternative. Recent work by Quellhorst et al., who showed that a DATFP-containing isoprenoid could be enzymatically incorporated into Rab5 and used to photolabel GDI-1, validates the use of this photophore as a probe for isoprenoid binding sites.[18] Prenylcysteine analogues such as *N*-acetyl farnesylcysteine (AFC) and *N*-acetyl geranylgeranylcysteine (AGGC) have been used to examine prenylated protein−protein behavior because they mimic the prenylated C-terminal cysteine residue of prenylated proteins.[19] Here, we describe the synthesis and cross-linking properties of a new class of small molecule probes that incorporate a DATFP photophore into prenylated cysteine methyl esters **1a** and **2a**.[20] To confer greater stability on the probe, we elected to incorporate an amide linkage between the DATFP group and the prenyl moiety instead of the ester linkage that has been used in most DATFP-containing isoprenoid analogues; this change significantly complicated the syntheses of the target compounds.

To commence with the synthesis of **1a**, we initially pursued the use of a phthalimide for introduction of the DATFP amide nitrogen due to the success of this protecting group in the preparation of amide-linked benzophenone- and DATFP-containing prenyl diphosphates.[10,12] Compound **3** was prepared on the basis of previous work[12] in hopes that halogenation of the C-1 alcohol would lead to a species primed to alkylate a cysteinyl thiol. Unfortunately, preparation of a brominated or chlorinated intermediate was unsuccessful. The presence of the secondary amide linkage (although not related tertiary amides) appears to cause several side reactions to occur.[10] Thus, a new route was conceived that would proceed via intermediate **4**.[21] Unfortunately, either cleavage of the phthalimide protecting group or removal of the cysteinyl methyl ester resulted in the formation of several degradation products.



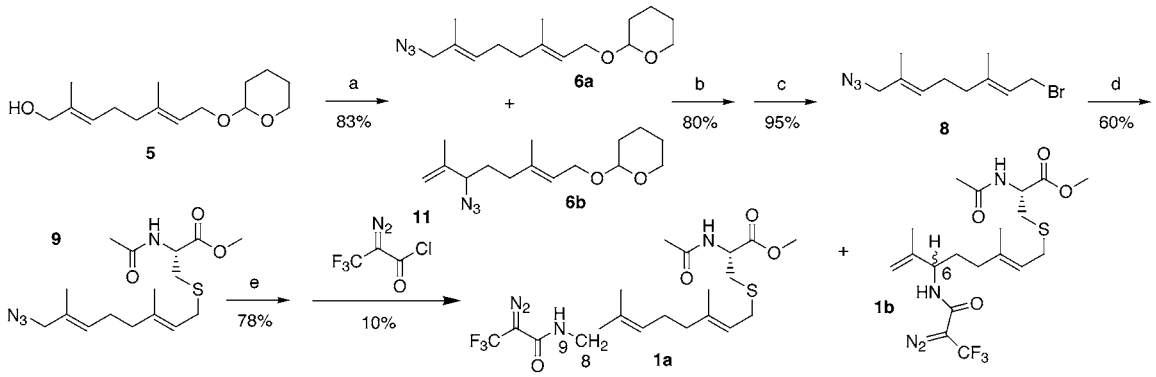
As an alternative source for the amine nitrogen, the azidation method employed by Thompson et al.[22] was found to be effective (83% yield) whereby the hydroxyl group of **5** (Scheme 1), a previously reported compound,[10] was directly converted to the azide (**6**). However, regioisomers resulted in each attempt to introduce an azide at the C-8 position (**6a** and **6b**).[23,24]

Azidations of geraniol and farnesol, using a variety of conditions, including palladium-catalyzed chemistry and Mitsunobu-type substitution employing zinc azide,[22,23,25−27] also resulted in two sigmatropically rearranged products in an invariably equimolar ratio. Furthermore, isomers persisted during the syntheses of our prenylcysteine analogues, with each new compound being formed as an inseparable mixture (see Supporting Information). This is not altogether surprising since allyl azides are known to undergo 1,3 thermal rearrangements at room temperature.[28] However, this complication was not seen as an impediment: in principle, both isomers of **1** and **2** could be photoaffinity labeling reagents for isoprenoid binding sites, although the targeted isomers, derived from the primary azides, would most likely be the more effective cross-linkers since the other isomers (obtained from the secondary azides) are less structurally similar to isoprenoid units.

Thus, the THP protecting group of compounds **6a** and **6b**, as a mixture, was removed, and the resulting C-1 position was brominated (**7**). Using acidic conditions,[29] the thiol of

(15) Kale, T. A.; Raab, C.; Yu, N.; Aquino, E.; Dean, D. C. *J. Labelled Compds. Radiopharm.* **2003**, *46*, 29−54.
(16) Kale, T. A.; Turek, T. C.; Chang, V.; Gautam, N.; Distefano, M. D. *Methods Enzymol.* **2002**, *344*, 245−258.
(17) Dormán, G.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661−5673.
(18) Quellhorst, G. J., Jr.; Allen, C. M.; Wessling-Resnick, M. *J. Biol. Chem.* **2001**, *276*, 40727−40733.
(19) Mondal, M. S.; Wang, Z.; Seeds, A. M.; Rando, R. R. *Biochemistry* **2000**, *39*, 406−412.
(20) Syntheses of **1a** and **2a** produced inseparable mixtures containing regioisomers **1b** and **2b**. References to **1** and **2** (and intermediates in the syntheses) in the text refer to mixtures of these compounds. The presence and ratio of these isomers was relatively easy to determine via ^{1}H NMR.
(21) Both cysteine methyl esters and their free acid counterparts are desirable prenylcysteine analogues, as not all proteins are methylesterified.
(22) Thompson, A. S.; Humphrey, G. R.; DeMarco, A. M.; Mathre, D. J.; Grabowski, E. J. J. *J. Org. Chem.* **1993**, *58*, 5886−5888.
(23) Murahashi, S.-I.; Taniguchi, Y.; Imada, Y.; Tanigawa, Y. *J. Org. Chem.* **1989**, *54*, 3292−3303.
(24) Compound **6b** and subsequent products therefrom are, in reality, each mixtures of two diastereomers due to the chirality at C-6.
(25) Viaud, M. C.; Rollin, P. *Synthesis* **1990**, 130−133.
(26) Safi, M.; Fahrang, R.; Sinou, D. *Tetrahedron Lett.* **1990**, *31*, 527−530.
(27) Trost, B. M.; Pulley, S. R. *Tetrahedron Lett.* **1995**, *36*, 8737−8740.
(28) Gagneux, A.; Winstein, S.; Young, W. G. *J. Am. Chem. Soc.* **1969**, *82*, 5956−5957.

Scheme 1 [a]



[a] Reagents and conditions: (a) $(PhO)_2PON_3$, DBU, toluene; (b) PPTS, EtOH; (c) PPh_3 (polymer supported), CBr_4, CH_2Cl_2; (d) *N*-acetyl cysteine methyl ester, $Zn(OAc)_2$, 2:1:1 DMF/butanol/0.025% TFA; (e) PPh_3 (polymer supported), THF/H_2O. Isomers are present for **7** and **8** as well as intermediates leading up to **1**; the undesired isomers were omitted for clarity.

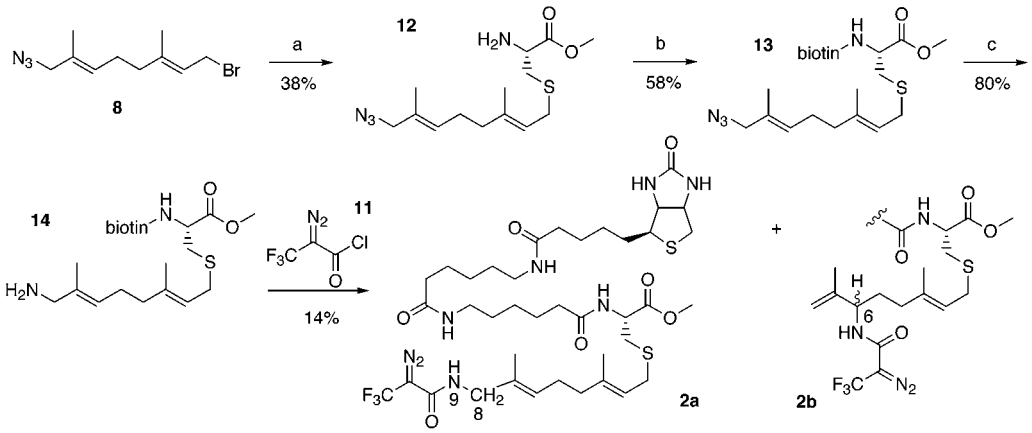
N-acetyl cysteine methyl ester was alkylated by **7**, followed by reduction of the azide using polymer-supported PPh_3.[30] This resin-bound reagent was easily removed by filtration, and the free amine was then acylated using diazotrifluoropropionyl chloride (**11**) to produce compound **1a** and its isomer **1b**. Fortuitously, the ratio of isomers ranged from 2.5 to 4:1 in favor of **1a** as PPh_3 appeared to preferentially reduce the primary azide in **8**.

Triazolone formation is sometimes a competing problem with the preparation of DATFP-containing compounds.[31,32] Thus, ^{1}H NMR, IR, and UV techniques were used to verify the production of **1a** and its isomer **1b**. ^{1}H COSY NMR of **1** showed coupling between key protons of **1a** (at C-8 and N-9, Scheme 1) as well as its isomer **1b** (at C-6 and its neighboring amide proton), suggesting the presence of the intact DATFP moiety. This was corroborated by IR and UV analyses that further indicated the presence of the DATFP group.

Once a route had been established for the synthesis of DATFP-containing, *N*-acetylated prenylcysteine **1**, our next focus was to incorporate a tag to trace any protein products cross-linked by the DATFP group following irradiation, as well as provide the means to eventually identify specific cross-linked amino acids. Biotin was chosen because subsequent blotting to a nitrocellulose membrane and treatment with HRP-conjugated streptavidin would allow the visualization of a chemiluminescent signal signifying cross-linked protein.[33,34] Scheme 2 shows the synthesis of biotinylated prenylcysteine **2**, starting from **8**.

Alkylation of cysteine methyl ester using bromide **8** proceeded efficiently using DIEA in DMF to produce **12**,[35] which was then biotinylated. Reduction of the azide with PPh_3 and incorporation of the DATFP moiety proceeded in a fashion similar to the generation of **1** to give **2**.[36] ^{1}H NMR analysis indicated that the primary DATFP isomer (**2a**) was formed in a 7:1 ratio over the secondary isomer (**2b**). Triazolone formation was again avoided in the synthesis of **2** as evidenced by ^{1}H and COSY NMR, IR, and UV analyses, all of which support the assigned structure of **2**.

Scheme 2 [a]



[a] Reagents and conditions: (a) cysteine methyl ester hydrochloride, DIEA, DMF; (b) biotin−XX, DIEA, DMF; (c) PPh_3 (polymer supported), THF/H_2O. Isomers are present at each stage of this synthesis; undesired isomers were omitted for clarity, excluding **2**.

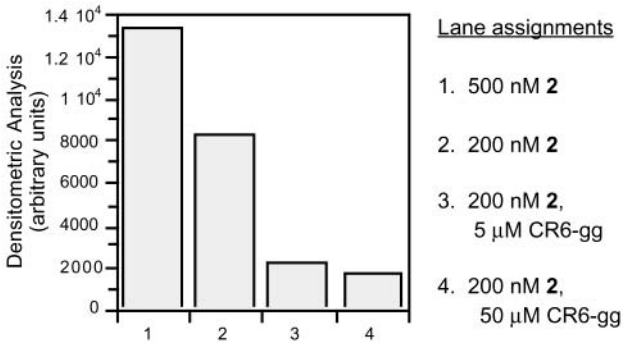


Figure 2. Autoradiographic analysis of reaction products detected by SDS-PAGE, blotting, and treatment with HRP-conjugated streptavidin following photolabeling of RhoGDI by **2** alone and in the presence of various potential competitors. Reactions, irradiated for 2 min, were performed using 4.0 μM RhoGDI. Lane assignments: (1) 500 nM **2**; (2) 200 nM **2**; (3) 200 nM **2**, 5 μM CR6-gg; (4) 200 nM **2**, 50 μM CR6-gg.

To examine the utility of photoprobe **2**, we chose RhoGDI for photolysis experiments because this cytosolic protein is known to interact with geranylgeranylated Rho proteins, and has been shown to possess an isoprenoid binding site.[37] Irradiating RhoGDI under various conditions established that 2 min of irradiation in our system resulted in no detectable protein degradation, as judged by SDS-PAGE. RhoGDI (4.0 μM) was then irradiated in the presence of **2** at multiple concentrations, and the reactions were first subjected to SDS-PAGE, blotted to nitrocellulose and treated with HRP-conjugated streptavidin. Cross-linking by **2** was detected in concentrations ranging from 200 nM to 2.0 μM, and a histogram representing a portion of the resultant chemiluminescent signals associated with the blots, as detected by autoradiography, is presented in Figure 2. Competition experiments were performed using a prenylated peptide that not only mimics the C-terminal residues of Rho proteins but whose farnesylated counterpart is known to affect residues within the isoprenoid binding site of RhoGDI: Ac-KKSRRC (*S*-geranylgeranyl) (termed CR6-gg). The cross-linking signal is decreased by roughly 75% in the presence of 5 μM CR6-gg, and nearly 80% in the presence of 50 μM prenylated peptide; notable competition was also observed using AFC as a competitor. These results imply that photoprobe **2** and CR6-gg are competing for the same isoprenoid binding site, thus suggesting that **2** is an effective prenylcysteine mimic. Direct comparison between DATFP-based **2** reported here and earlier benzophenone-based probes is difficult because the methods used for detection were different (biotin/streptavidin versus radioactivity, respectively). However, using a compound analogous to **2** that retained the biotin reporter but replaced the DATFP moiety with a benzophenone group made a comparison possible. Irradiation of **2** and its benzophenone-containing counterpart with RhoGDI at comparable concentrations suggests that **2** may be a somewhat more efficient cross-linking agent. Moreover, **2** appears to be the more specific reagent since its photolabeling of RhoGDI can be significantly attenuated by the inclusion of competitors such as CR6-gg and AFC. In contrast, cross-linking by the benzophenone homologue of **2** is relatively unaffected by the addition of these competitors at comparable concentrations.

In addition to protein prenylation processing enzymes, there are numerous effector, activator, and inhibitory proteins that contribute to the regulation of prenylated Ras and Ras-like proteins, virtually all of which have the potential of possessing isoprenoid binding sites. Discovering the molecular basis for prenylated protein recognition is a necessary step in understanding how these proteins, and their possible oncoprotein isoforms, operate. Small molecules such as **1** and **2**, acting as prenylated protein mimics, offer a means to access these details. Since many putative prenyl-binding proteins may be membrane bound, photoaffinity labeling reagents may prove to be superior to other methods such as X-ray crystallography and NMR for elucidating such structural information. Greater specificity of photoprobe−protein interactions may likely occur by using photoactivatable peptides, and efforts are underway to develop such agents. The simple compounds presented here, however, appear to be well-suited for providing initial guidance in studying proteins that interact with prenylated proteins.

Acknowledgment. The authors thank Dr. George O'Doherty for invaluable advice during the preparations of these compounds. This work was supported by a grant from the National Institutes of Health (GM58442).

Supporting Information Available: Product syntheses and characterization as well as a description of the preparation of acyl chloride **11**, experimental procedures for photolysis experiments, and details of the comparison between **2** and its benzophenone-containing homologue. This material is available free of charge via the Internet at http://pubs.acs.org.

OL026752A

(29) Xue, C.-B.; Becker, J. M.; Naider, F. *Tetrahedron Lett.* **1992**, *33*, 1435−1438.
(30) Bernard, M.; Ford, W. T. *J. Org. Chem.* **1983**, *48*, 326−332.
(31) Hahn, K. M.; Hastie, S. B.; Sundberg, R. J. *Photochem. Photobiol.* **1992**, *55*, 17−27.
(32) Theodore, L. J.; Nelson, W. L.; Dave, B.; Giacomini, J. C. *J. Med. Chem.* **1990**, *33*, 873−877.
(33) Willchek, M., Bayer, E. A., Eds. *Avidin−Biotin Technology*; Academic Press: San Diego, 1990; Vol. 184.
(34) Biotinylated prenylated peptides have been prepared via solid-phase synthesis, where biotin−avidin chemistry was utilized in subsequent enzymatic assays (Dolence, E. K.; Dolence, J. M.; Poulter, C. D. *Bioconjugate Chem.* **2001**, *12*, 35−43. Liu, L.; Jang, G.-F.; Farnsworth, C.; Yokoyama, K.; Glomset, J. A.; Gelb, M. H. *Methods Enzymol.* **1995**, *250*, 189−206).
(35) Yang, C.-C.; Marlowe, C. K.; Kania, R. *J. Am. Chem. Soc.* **1991**, *113*, 3177−3178.
(36) It should be noted that instead of biotinylation of the amine-containing prenylcysteine compound **12** in Scheme 2, attempts were made to synthesize *N*-biotinylated cysteine methyl ester and then alkylate its free thiol with bromide **8**. This method did not give reproducible results. The potential for oxidation of the cysteinyl sulfhydryl group as well as disulfide formation complicated this reaction. The method shown in Scheme 2 is considerably more reliable.
(37) Hoffman, G. R.; Nassar, N.; Cerione, R. A. *Cell* **2000**, *100*, 345−356.