Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 16, pp. 2125-2130, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00373-9

SYNTHESIS AND EVALUATION OF BENZOPHENONE-BASED PHOTOAFFINITY LABELING ANALOGS OF PRENYL PYROPHOSPHATES CONTAINING STABLE AMIDE LINKAGES

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Abstract: The syntheses of two photoactive prenyl pyrophosphate analogs (**1a** and **1b**) that incorporate stable amide-linked benzophenones are described. Compound **1a** contains a single isoprene (C_5) unit between the pyrophosphate and benzophenone functionalities while **1b** contains a geranyl (C_{10}) moiety. Compounds **1a** and **1b** are competitive inhibitors of yeast farnesyl protein transferase with respect to farnesyl pyrophosphate and have K_I values of 6000 nM and 700 nM. Upon irradiation, [^{32}P]-**1b** preferentially labels the β-subunits of yeast farnesyl protein transferase and human geranylgeranyl protein transferase. © 1997 Elsevier Science Ltd.

Protein prenylation is a common post-translational protein modification that involves the attachment of a farnesyl (C_{15}) or geranygeranyl (C_{20}) moiety to a specific protein derived cysteine residue; prenylated proteins have been isolated from animal, plant and fungal sources.[1] The discovery that Ras protein is farnesylated and that the isoprenoid is essential for the transforming activity of mutant Ras proteins has generated considerable interest in this area. A number of groups are designing inhibitors of farnesyl protein transferase (FPTase) as potential anticancer agents.[2] Photoaffinity labeling is a useful technique for studying the interactions between small molecules and proteins.[3] To gain information about the interactions between proteins and prenyl pyrophosphates, we have synthesized a number of analogs of farnesyl pyrophosphate and geranylgeranyl pyrophosphate that incorporate photoactive benzophenone crosslinking groups. In earlier work, we demonstrated the utility of benzophenone-containing analogs by showing that they are potent competitive inhibitors of yeast FPTase (yFPTase), they irreversibly inactivate yFPTase upon photolysis, and they preferentially label the β-subunit of yFPTase.[4, 5] This paper describes the preparation and use of new analogs shown in Figure 1 (**1a** and **1b**) that replace the ester linkages that coupled the benzophenone units to the isoprenoids in earlier compounds with more stable amide linkages.

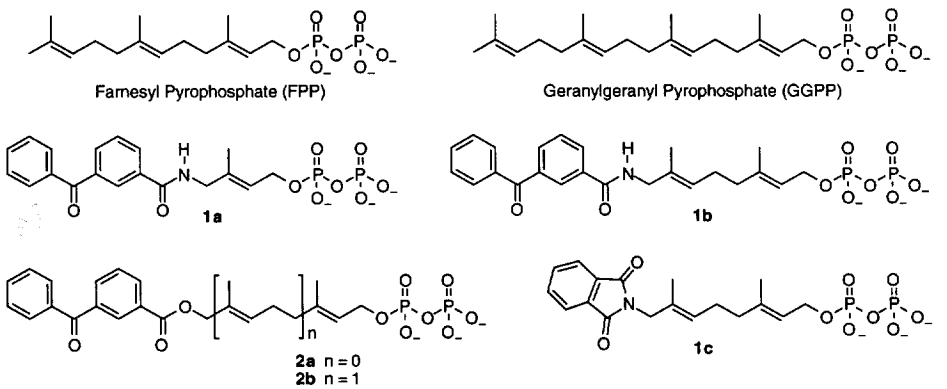

Figure 1

Synthesis. Compounds **1a** and **1b** were each synthesized in seven steps as illustrated in Figure 2. The allylic hydroxyl groups of dimethyl allyl alcohol (**3a**) and geraniol (**3b**) were first protected as tetrahydropyranyl ethers followed by oxidation with *t*-butyl hydroperoxide and H_2SeO_3 to produce **5a** and **5b** in 56% and 33% yield after purification by flash chromatography; it should be noted that the oxidation of **4a**, which contains an allyl ether adjacent to the alkene undergoing reaction requires more vigorous conditions than are necessary for **4b**. Compounds **5a** and **5b** were then reacted under Mitsunobu[6] conditions with potassium phthalamide to produce **6a** and **6b** in 81% and 79% yields, respectively.[7] The *E*-stereoselectivity[8] for the hydroxylation reactions of **4a** and **4b** was confirmed by difference NOE spectroscopy of **6a** and **6b**; the structure of **6a** was also confirmed by x-ray crystallography as shown in Figure 3. Selective deprotection of the phthalamides (**6a** and **6b**) was achieved by treating them with one equivalent of $NH_2NH_2{\bullet}H_2O$ in EtOH at rt for 24 hours; using these conditions **7a** and **7b** were obtained in 68% and 69% yields. The free amines were then acylated with *m*-benzoylbenzoyl chloride (*m*-BBCl) to give **8a** and **8b** which were subsequently deprotected to yield **9a** and **9b**.[9] Substantial difficulty was experienced in converting **9a** and **9b** to the corresponding pyrophosphates via the allylic chlorides using *N*-chlorosuccinamide and dimethyl sulfide or through the allylic bromides by employing triphenylphosphine and carbon tetrabromide; this appears to be due to the presence of the amide linkage since similar problems were not experienced with earlier ester-linked compounds (**2a** and **2b**). To circumvent these problems, alcohols **9a** and **9b** were converted directly to the corresponding pyrophosphates using the procedure developed by Cramer.[10] Diphosphates **1a** and **1b** were purified by reversed-phase chromatography and characterized by ^1H NMR, UV and MALDI mass spectrometry.[11] Compound **1c** was prepared from **6b** by deprotection with PPTS and clean conversion to the bromide using Ph_3P and CBr_4 followed by pyrophosphorylation with tris (tetra-*n*-butylammonium) hydrogen pyrophosphate.[12, 13] The ability to prepare the above bromide in the presence of the tertiary phthalamide in contrast to the results described with the secondary amides suggests that it is the amide N-H that is problematic in these latter reactions.

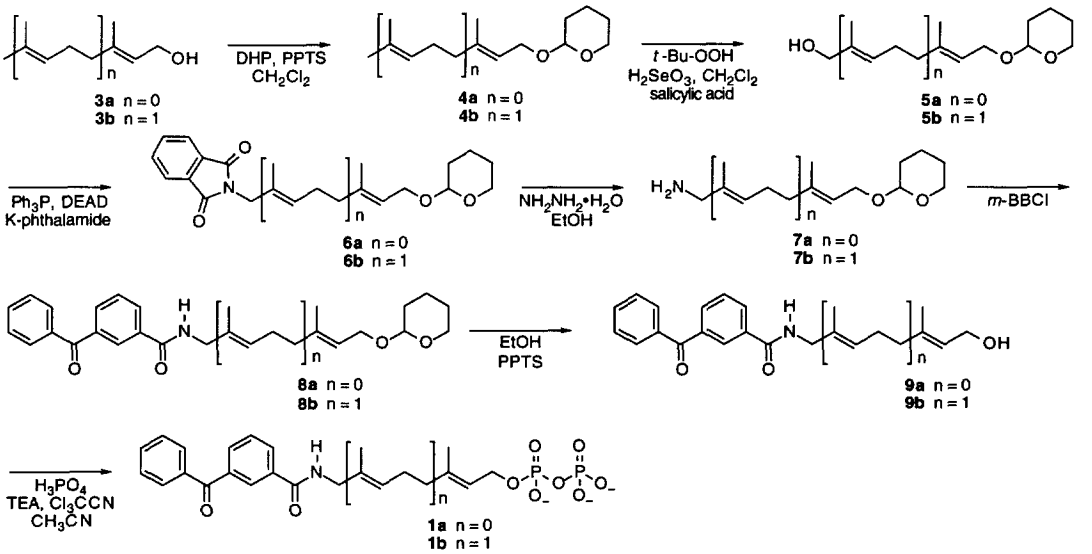

Figure 2

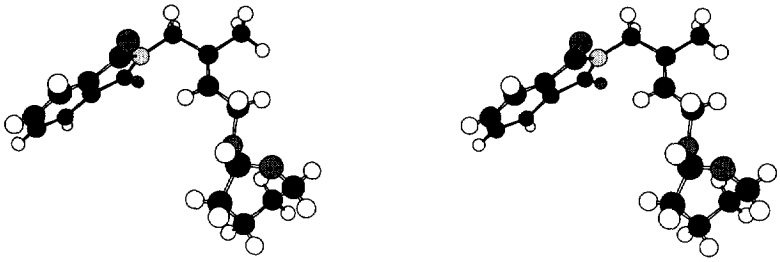

Figure 3. Structure of **6a** determined by x-ray diffraction showing the trisubstituted alkene stereochemistry.

Enzyme Inhibition Experiments. To evaluate the utility of the above compounds as potential prenyl pyrophosphate analogs, the rate of yFPTase catalyzed prenylation of a peptide substrate (*N*-Dansyl-GCVIA) was measured in the presence of **1a**, **1b** and **1c**. This was accomplished using an assay that measures the time dependent increase in the fluorescence of the dansyl group as the neighboring cysteine is farnesylated.[14] No change in the fluorescence of the peptide was observed in reactions containing **1a**, **1b** or **1c** without FPP; these results are similar to those obtained with the ester-linked benzophenone-containing FPP analogs (**2a** and **2b**) and suggests that these compounds are not efficient substrates for yFPTase. In the presence of FPP, **1a**, **1b** and **1c** caused a decrease in the rate of enzyme catalyzed peptide farnesylation indicating that these compounds are enzyme inhibitors. Plots of 1/v versus [I] gave IC_{50} values of 37 μM for **1a**, 3.7 μM for **1b**, and 10 μM for **1c**. Comparison of these values with data obtained in previous work with the ester-linked analogs suggests that the amide compounds are weaker in their inhibitory potency (Table 1). Additional kinetic experiments in which the reaction rate was measured at various concentrations of FPP and **1a** or **1b** gave competitive inhibition patterns in Lineweaver-Burke plots as shown in Figure 4; further analysis of these data using the method of Eadie-Hoftsee gave K_I values of 6000 nM (**1a**) and 700 nM (**1b**). These results indicate that **1a** and **1b** are competitive inhibitors with respect to FPP and that substitution of the ester linkages present in earlier analogs with more stable amide linkages does have an effect on the affinity of these compounds for yFPTase (see Table 1 for a summary of the K_I values). Comparison of the K_I values for the ester-linked C_5 analog (**2a**) and the corresponding amide analog (**1a**) shows that the amide linkage decreases the affinity for yFPTase 6.3 fold; this may reflect the more rigid nature of **1a** due to the presence of the amide and suggests that the conformation of this region of FPP may not be planar when bound to yFPTase. A similar comparison of K_I values for **1b** and **2b** indicates a 1.8 fold decrease in yFPTase affinity upon introduction of the amide linkage. This smaller effect suggests greater tolerance for the planar amide at this position within FPP. Thus, although amide **1b** is somewhat less potent than ester **2b**, **1b** is still a good inhibitor (K_I = 700 nM) for yFPTase and hence a useful analog of FPP.

Compound	IC_{50} (nM)	K_I (nM)	Compound	IC_{50} (nM)	K_I (nM)
1a	37,000	6,000	**2a**[4a]	2000	960
1b	3,700	700	**2b**[4b]	2300	380
1c	10,000	-	FPP[15]	-	75 (K_D)

Table 1

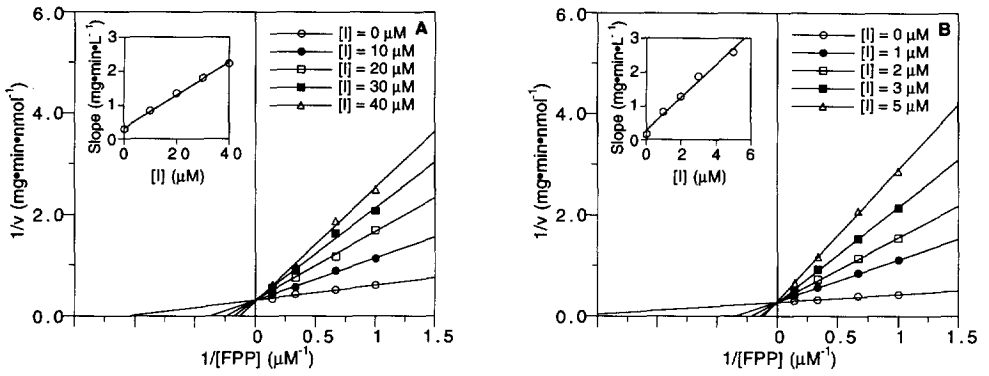

Figure 4. Kinetic analysis of the inhibition of yFPTase by **1a** (A) and **1b** (B).

Photoaffinity Labeling of yFPTase and hGGPTase. To examine the utility of **1b** as a photoaffinity labeling reagent, [^{32}P]-**1b** was prepared[16] and photolyzed[17] in the presence of yFPTase; the results of these experiments are shown in Figure 5. Irradiation of yFPTase in the presence of [^{32}P]-**1b** resulted in preferential labeling of the β-subunit 4.2 fold greater than the α-subunit. The β-subunit labeling decreased 5.5 fold when irradiation was performed in the presence of [^{32}P]-**1b** and the natural substrate, FPP, suggesting that the labeling is occurring specifically at the active site.

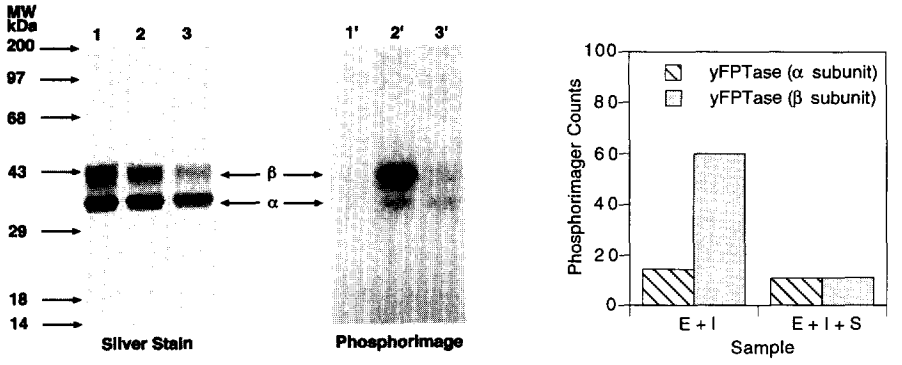

Figure 5. Photoaffinity labeling of yeast farnesyl protein transferase using [^{32}P]-**1b**. Left: Electrophoretic analysis (SDS-PAGE) of reactions containing yFPTase and [^{32}P]-**1b**. Lanes 1 and 1': yFPTase (2.9 μg) and [^{32}P]-**1b** (1.0 μM) without irradiation. Lanes 2 and 2': yFPTase and [^{32}P]-**1b** after irradiation. Lanes 3 and 3': yFPTase and [^{32}P]-**1b** after irradiation in the presence of FPP (50 μM). Silver staining of the gel shown in lanes 1-3 illustrates the total protein present. Phosphorimaging of the gel shown in lanes 1'-3' shows the associated radioactivity after irradiation. Right: Quantitation of crosslinking of [^{32}P]-**1b** with the α- and β-subunits of yFPTase by phosphorimager analysis. (E+I): yFPTase and [^{32}P]-**1b** after irradiation. (E+I+S): yFPTase and [^{32}P]-**1b** after irradiation in the presence of FPP.

Benzophenone-containing compounds have not been previously employed in studies with geranylgeranyl protein transferases. Thus, analog **1b** was evaluated in photoaffinity labeling experiments with the human enzyme (hGGPTase).[18] Irradiation of hGGPTase in the presence of [^{32}P]-**1b** also resulted in preferential labeling of the β-subunit as shown in Figure 6. However, in this case the β-subunit was labeled 14.8 fold greater than the α-subunit. A decrease in β-subunit labeling (8.5 fold) was again observed in the presence of the physiologically relevant substrate, GGPP.

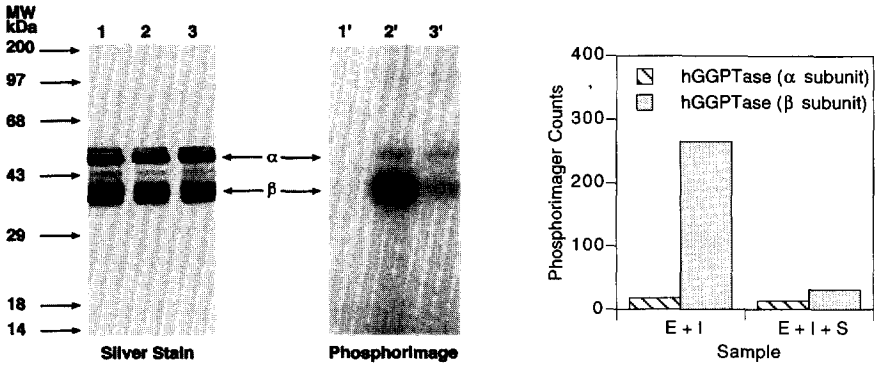

Figure 6. Photoaffinity labeling of human geranylgeranyl protein transferase using [^{32}P]-**1b**. Left: Electrophoretic analysis (SDS-PAGE) of reactions containing hGGPTase and [^{32}P]-**1b**. Lanes 1 and 1': hGGPTase (3.5 μg) and [^{32}P]-**1b** (1.0 μM) without irradiation. Lanes 2 and 2': hGGPTase and [^{32}P]-**1b** after irradiation. Lanes 3 and 3': hGGPTase and [^{32}P]-**1b** after irradiation in the presence of GGPP (50 μM). Silver staining of the gel shown in lanes 1-3 illustrates the total protein present. Phosphorimaging of the gel shown in lanes 1'-3' shows the associated radioactivity after irradiation. Right: Quantitation of crosslinking of [^{32}P]-**1b** with the α- and β-subunits of hGGPTase by phosphorimager analysis. (E+I): hGGPTase and [^{32}P]-**1b** after irradiation. (E+I+S): hGGPTase and [^{32}P]-**1b** after irradiation in the presence of GGPP.

The results reported in this paper broaden the scope of enzymes that can be studied by photoaffinity labeling using benzophenone-containing analogs to include GGPTases. The subunit labeling results described here for yFPTase and hGGPTase are similar to those previously obtained with the ester-linked benzophenone, **2b**, and other diazoester-based compounds.[4,5] Thus, these observations strengthen the current model for prenyltransferase action in which isoprenoid binding occurs predominantly on the β-subunit.[19] Additionally, the greater stability of the amide-linked analog (**1b**) makes this molecule particularly attractive for peptide mapping and site identification applications. Compound **1b** should be useful for photoaffinity labeling experiments with prenyltransferases as well as other FPP and GGPP utilizing enzymes involved in sesquiterpenoid and diterpenoid biosynthesis.

Acknowledgements. This research was supported by funds from the American Cancer Society (BE-222, IN-13-33-47, and JFRA-584). We thank C. D. Poulter for providing *E. coli* DH5α/pGP114 and C. A. Omer for providing *E. coli* BL21(DE3)/pRD578. The structure of **6a** was determined by Dr. V. G. Young, Jr. in the x-ray crystallographic laboratory in the Department of Chemistry at the University of Minnesota.

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7. Compound **6a**: ^{1}H NMR (300 MHz, $CDCl_3$): 1.54-1.86 (m, 6H), 1.74 (s, 3H), 3.49-3.52 (m, 1H), 3.83-3.86 (m, 1H), 4.04-4.11 (m, 1H), 4.23-4.33 (m, 1H), 4.25 (s, 2H), 4.62 (t, 1H, *J*=6.0), 5.55 (t, 1H, *J*= 6.0), 7.73-7.81 (m, 2H), 7.90-7.99 (m, 2H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 15.0 (primary), 19.4, 25.5, 30.6, 44.5, 62.2, 63.2 (secondary), 97.8, 123.4, 133.4, 134.0 (tertiary), 132.0, 133.9, 168.1 (quaternary). HRFAB-MS: $[M+H]^+$, calcd. 316.1542, found 316.1569.
Compound **6b**: ^{1}H NMR (300 MHz, $CDCl_3$): 1.51-1.70 (m, 6H), 1.63 (s, 6H), 2.02-2.13 (m, 4H), 3.48-3.50 (m, 1H), 3.80-3.86 (m, 1H), 3.93-4.00 (m, 1H), 4.15-4.21 (m, 1H), 4.17 (s, 2H), 4.59 (t, 1H, *J*=6.0), 5.31-5.34 (m, 2H), 7.67-7.80 (m, 2H), 7.81-7.83 (m, 2H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 14.5, 16.4 (primary), 19.6, 25.4, 30.7, 39.0, 44.9, 62.2, 63.5, 65.4 (secondary), 97.7, 120.0, 123.2, 127.2, 133.8 (tertiary), 129.0, 131.8, 139.7, 168.2 (quaternary). LRFAB-MS: $[M+H]^+$, calcd. 384.2, found 384.3.
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9. Compound **9a**: ^{1}H NMR (300 MHz, $CDCl_3$): 1.77 (s, 3H), 4.07 (d, 2H, *J*=6.0), 4.24 (d, 2H, *J*=6.0), 5.64 (t, 1H, *J*=6.0), 6.42 (s, 1H), 7.51-7.56 (m, 2H), 7.58-7.67 (m, 2H), 7.83 (d, 2H, *J*=9.0), 7.94 (d, 1H, *J*=9.0), 8.11 (d, 1H, *J*=9.0), 8.22 (s, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 18.3 (primary), 46.5, 58.4 (secondary), 126.9, 128.7, 128.9, 130.1, 131.5, 132.9, 133.7, 134.5 (tertiary), 128.5, 137.0, 137.9, 142.5, 169.2, 194.3 (quaternary). HRFAB-MS: $[M+H]^+$, calcd. 310.1437, found 310.1425.
Compound **9b**: ^{1}H NMR (300 MHz, $CDCl_3$): 1.68 (s, 6H) 2.07-2.22 (m, 4H), 4.02 (d, 2H, *J*=6.0), 4.18 (d, 2H, *J*=6.0), 5.33-5.41 (m, 2H), 6.51 (s, 1H), 7.38-7.57 (m, 2H), 7.60-7.67 (m, 2H), 7.82-7.87 (m, 2H), 8.06 (d, 1H, *J*=9.0), 8.34 (d, 1H, *J*=9.0), 8.45 (s, 1H). ^{13}C NMR (75.5 MHz, $CDCl_3$): 14.0, 16.0 (primary), 25.4, 26.8, 39.1, 47.1 (secondary), 123.7, 126.0, 127.5, 128.6, 128.9, 129.1, 130.2, 130.3, 131.7 (tertiary), 133.0, 133.8, 134.5, 137.1, 142.1, 176.4, 196.0 (quaternary). LRFAB-MS: $[M+H]^+$, calcd. 378.2, found 378.2.
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11. Compound **1a**: ^{1}H NMR (300 MHz D_2O, adjusted to pH 8 with ND_4OD): 1.58 (s, 3H), 3.82 (s, 2H), 4.18 (t, 2H, *J*=6.0), 5.44 (t, 1H, *J*=6.0), 7.42-7.47 (m, 2H), 7.51-7.62 (m, 2H), 7.68 (d, 2H, *J*=9.0), 7.83 (d, 1H, *J*=9.0), 7.93 (d, 1H, *J*=9.0), 8.00 (s, 1H). UV (H_2O), λ_{max} = 258 nm, ε = 17,800 $M^{-1}\cdot cm^{-1}$. MALDI-MS: $[M-H]^-$, calcd. 468.0614, found 468.0615.
Compound **1b**: ^{1}H NMR (300 MHz D_2O, adjusted to pH 8 with ND_4OD): 1.51 (s, 6H), 1.95-2.07 (m 4H), 3.78 (s, 2H), 4.12 (t, 2H, *J*=6.0), 5.25 (m, 2H), 7.23-7.50 (m, 2H), 7.52-7.62 (m, 2H), 7.68 (d, 2H, *J*=9.0), 7.77 (d, 1H, *J*=9.0), 8.01 (d, 1H, *J*=9.0), 8.07 (s, 1H). UV (H_2O), λ_{max} = 258 nm, ε = 32,300 $M^{-1}\cdot cm^{-1}$. MALDI-MS: $[M-H]^-$, calcd. 536.1240, found 536.1265.
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13. Compound **1c**: ^{1}H NMR (300 MHz D_2O, adjusted to pH 8 with ND_4OD): 1.49 (s, 3H), 1.51 (s, 3H), 1.90-2.05 (m, 4H), 4.01 (s, 2H), 4.25 (m, 2H), 5.09 (t, 1H, *J*=6.0), 5.29 (m, 1H), 7.43-7.66 (m, 2H), 7.69-7.75 (m, 2H). ESI-MS: $[M-H]^-$, calcd. 458.1, found 458.0.
14. Yeast FPTase was purified from *E. coli* DH5α/pGP114 as described by Mayer, M. P.; Prestwich, G. D.; Dolence, J. M.; Bond, P. D.; Wu, H.-Y.; Poulter, C. D. *Gene* **1993**, *132*, 41-47. Enzyme assays were performed spectrofluorometrically as described by Bond, P. D.; Dolence, J. M.; Poulter, C. D. *Methods Enzymol.* **1995**, *250*, 30-43. Solutions of **1a**, **1b** and **1c** were prepared by dissolving the solid in 25 mM NH_4HCO_3 and their concentrations determined by phosphate analysis as described by Reed, B. C.; Rilling, H. C. *Biochemistry* **1976**, *15*, 3739-3745.
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17. Photolysis reactions were conducted at 4 °C in a UV Rayonet Mini-Reactor equipped with 8 RPR-3500° lamps. All reactions (100 μL) were performed in silinized quartz test tubes (10 x 45 mm) and were irradiated for 2 hours.
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(*Received in USA* 25 *June* 1997; *accepted* 11 *July* 1997)