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BENZOYLPHENOXY ANALOGS OF ISOPRENOID DIPHOSPHATES AS PHOTOACTIVATABLE SUBSTRATES FOR BACTERIAL PRENYLTRANSFERASES

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Abstract. Photoactivatable benzophenone-containing analogs of isoprenyl diphosphates have been synthesized and evaluated as substrates and inhibitors of three bacterial prenyltransferases: farnesyl diphosphate synthase (FPS), hexaprenyl diphosphate synthase (HexPS) and undecaprenyl diphosphate synthase (UPS). These analogs undergo chain extension and will allow identification of hydrophobic active site residues of these enzymes.

Prenyltransferases consist of two main families: (i) the isoprenoid diphosphate synthases, which catalyze 1'-4 condensation reactions of isopentenyl diphosphate to an allylic diphosphate substrate; and (ii) the protein prenyltransferases, which catalyze the addition of farnesyl or geranylgeranyl diphosphate to the sulfhydryl group of a cysteine residue in a C-terminal CAAX motif of a membrane-targeted protein.[1] Both proteins possess hydrophobic clefts that bind polyisoprenoid diphosphates as substrates, and the identification of specific substrate–protein interactions can be probed using photoaffinity labeling.[2-5] In addition, the prenylated proteins interact with membranes through hydrophobic interactions[6] and with other proteins through specific protein–protein and potentially protein–isoprenoid interactions.[7-10] Three-dimensional structures of two relevant proteins, protein farnesyl transferase (FPTase)[11] and rab GDP dissociation inhibitor (rab GDI)[12] do not explicitly reveal the locations of the isoprenoid groups.

Farnesyl diphosphate synthase (FPS) [EC 2.5.1.10] is the most widely occurring and the most extensively studied prenyltransferase, and the three-dimensional structure of the avian FPS has been solved.[13] FPS can be converted by random mutagenesis[14] or directed mutagenesis[15] to have transferase activity that produces geranylgeranyl (or higher polyisoprenyl) diphosphates. FPS occupies an important branch point of the pathway of isoprenoid biosynthesis; the product, farnesyl diphosphate (FPP), is the substrate for the dimerization to squalene in cholesterol biosynthesis,[16] and for important isoprenoids such as glycosyl carrier lipids, respiratory quinones, heme a, and prenylated proteins.[17] The FPS from the thermophilic bacterium *Bacillus stearothermophilus* has been cloned and overproduced in *Escherichia coli*.[18] This abundant, thermostable recombinant enzyme is readily purified, and significant residues have been identified in the substrate binding site by mutagenesis based on sequence comparisons.[19]

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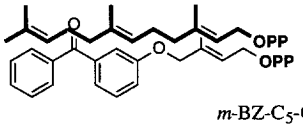
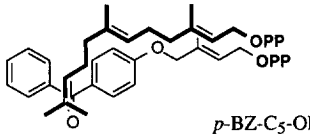
The C_{30} chain elongation is catalyzed by hexaprenyl diphosphate synthase (HexPS) [EC 2.5.1.33]. It catalyzes condensation of three molecules of isopentenyl diphosphate (IPP) with FPP to form (all-*E*)-hexaprenyl diphosphate (HexPP). The HexPS of *Micrococcus luteus* B-P 26 consists of two dissociable components, designated as component A and B, neither of which has catalytic activity until both are combined.[20] Undecaprenyl diphosphate (UPP) synthase (UPS) [EC 2.5.1.31] catalyzes the construction of a *Z*-prenyl chain onto FPP as a primer to yield a C_{55}-prenyl diphosphate with *E,Z*-mixed stereochemistry.[21] For these and other multicomponent prenyltransferases,[22] three-dimensional structural data are as yet unavailable, and approaches are needed for identification of the substrate-binding subunits and the binding sites for the isoprenyl diphosphate substrates.

Farnesyl diphosphate mimics

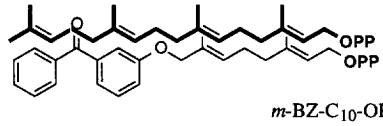


m-BZ-C_5-OPP (**1m**)

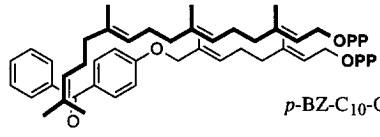


p-BZ-C_5-OPP (**1p**)

Geranylgeranyl diphosphate mimics



m-BZ-C_{10}-OPP (**2m**)



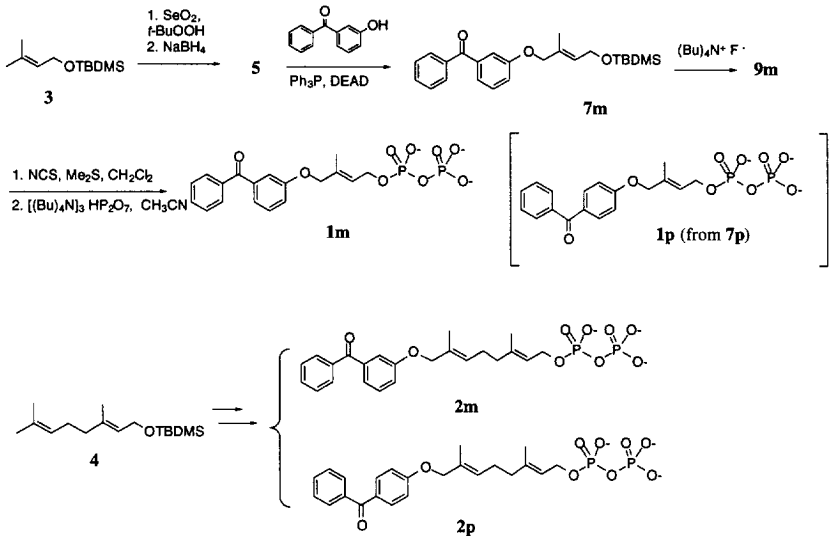
p-BZ-C_{10}-OPP (**2p**)

Scheme 1. Structures of farnesyl diphosphate (**1m, 1p**) and geranylgeranyl diphosphate (**2m, 2p**) incorporating the *meta*- or *para*-benzoylphenoxy ether moieties.

The design of photoaffinity labels for characterization of the prenyl chain binding site analogs initially employed diazoesters appended to the isopropylidene terminus.[23-25] More recent designs[3,4,26] incorporate the benzophenone (BZ) photophore,[27] which shows improved chemical and ambient light stability, non-photodissociative activation at longer wavelengths, and improved efficiency of specific covalent attachment to hydrophobic amino acids.[2] The reported synthesis and in vitro active site photoaffinity labeling of FPTase with the 4-benzoylbenzoate-containing analogs of FPP[3,4] and the 4-benzoylbenzyl ether analogs[26] coincided with our independent development of similar but chemically new analogs. We describe herein the synthesis of two 3-substituted (**1m, 2m**) and two 4-substituted benzoylphenoxy analogs (**1p, 2p**) corresponding to the FPP and GGPP structures, respectively (Scheme 1). In addition, we present data on the biochemical activity of these new analogs with three prenyltransferases: FPS, HexPS, and UPS. These analogs are competitive inhibitors, alternative substrates, and exhibit light-activated inhibition of enzyme activity.

Synthesis of the photoactivatable analogs is shown in Scheme 2. For the FPP analogs, selective oxidation of the TBDMS ether (**3**) of dimethallyl alcohol gave (*E*)-allylic alcohol **5**, and Mitsunobu coupling[28] with either *m*-benzoylphenol or *p*-benzoylphenol afforded the phenyl ethers **7m** and **7p** in high yields. Fluoride-induced deprotection furnished the prenol analogs **9m** and **9p**, which were then converted to the corresponding

m-BZ-C_5-OPP (**1m**) and *p*-BZ-C_5-OPP (**1p**) via displacement of the allylic chlorides with tris[tetra(*n*-butyl)-ammonium] hydrogen diphosphate.[29] The GGPP analogs *m*-BZ-C_{10}-OPP (**2m**) and *p*-BZ-C_{10}-OPP (**2p**) were prepared by an analogous route starting with geraniol.



Scheme 2. Synthesis of photoaffinity analogs of farnesyl diphosphate (**1m**, **1p**) and geranylgeranyl diphosphate (**2m**, **2p**).

The inhibitory effects of the four BZ-prenyl diphosphates, **1m**, **1p**, **2m**, and **2p**, were assayed with three prenyltransferases: FPS, HexPS, and UPS.[30-32] These three enzymes provided activity data for catalytic proteins with quite different product specificity and protein subunit composition. Table 1 shows their effectiveness on FPS from *B. stearothermophilus*, HexPS from *M. luteus* B-P 26, and UPS from *B. stearothermophilus*. Both of the BZ-C_{10}-OPPs (**2**) showed higher inhibition than the BZ-C_5-OPPs (**1**) toward the prenyltransferases examined. Furthermore, the BZ-prenyl diphosphates were readily accepted as substrates for each of the enzymes.

Table 1. Inhibition of Prenyltransferases by BZ-Prenyl Diphosphates (n.d., not determined). Values are percent inhibition relative to the standard assay with no inhibitor added.

	m-BZ-C_5-OPP (**1m**)			*p*-BZ-C_5-OPP (**1p**)			*m*-BZ-C_{10}-OPP (**2m**)			*p*-BZ-C_{10}-OPP (**2p**)		
[Inhibitor] (μM)	50	125	250	50	125	250	5 0	125	250	50	125	250
FPS (GPP)[b]	n.d.	14.6%	19.8%	n.d.	24.5%	27.6%	86.0%	94.9%	96.1%	84.4%	94.4%	95.5%
HexPS (FPP)	3.1%	3.5%	10.6%	8.8%	23.4%	37.0%	9.2%	37.9%	80.5%	14.4%	49.5%	86.4%
[Inhibitor] (μM)	16	32	125	16	32	125	16	32	63	16	32	63
UPS (FPP)	12.5%	22.5%	68%	12.5%	17.5%	57%	38%	58%	63%	29%	53%	63%

Substrate	FPS	HexPS	UPS
Control	100 (GPP)	100 (FPP)	100 (FPP)
1m	0.22	0	n.d.
1p	0.06	5.7	n.d.
2m	1.83	21.6	55.7
2p	0.78	46.4	49.1

Table 2. Relative activities of BZ-prenyl diphosphates as substrates for prenyltransferases.

As shown in Table 2, the BZ-C_{10}-OPPs (**2**) are better substrates than the C_5-OPPs (**1**) for FPS and HexPS. Interestingly, the *meta*-isomer (**2m**) showed higher reactivity than the *para*-form for FPS; in contrast, however, the *para*-isomer (**2p**) was the preferred substrate for HexPS. R_f Analyses of the reaction products derived from the BZ-C_{10}-OPPs on reversed-phase TLC clearly indicated that FPS synthesized the corresponding BZ-C_{15}-OPP product (R_f, system A, 0.38) by the condensation of one molecule of IPP, while the HexPS gave both of the corresponding BZ-C_{15}-OPP (R_f, system B, 0.87) and BZ-C_{20}-OPP (R_f, system B, 0.83) products in a ratio of ca. 3:1. Analyses of the product(s) produced by the UPS reactions have not been carried out.

Concentration	357 μM	714 μM	1428 μM
Control (GPP)	100[a]	100[a]	100[a]
2m	62.2[a] 66.6[b] 37.0[c]	61.2[a]	75.9[a]
2p	67.3[a] 48.1[b] 17.0[c]	64.4[a]	36.2[a]

Table 3. Relative FPS activity remaining after irradiation.
Key: a, 30 min; b, 60 min; c, 120 min.

Effects of UV-irradiation are shown in Tables 3 (on FPS) and 4 (on HexPS). Though the HexPS was more sensitive to UV-light than to FPS, each of the BZ-prenyl diphosphates was found to be effective as specific photoaffinity-labeling reagents for these two prenyltransferases. Experimental details for synthetic and biochemical experiments are summarized below.[33]

Concentration	125 μM	250 μM	125 μM	250 μM
Control (GPP)	101[a]	101[a]	14.3[b]	14.3[b]
2m	32.5[a]	5.5[a]	10.8[b]	1.9[b]
2p	30.7[a]	9.8[a]	9.2[b]	4.3[b]

Table 4. Relative HexPS activity remaining after irradiation.
Key: a, 5 min; b, 15 min.

Photoaffinity labeling with benzophenone-containing photophores has emerged as a powerful tool for isolation of target proteins for ligands, determination of the binding subunit in a multicomponent system, and localization of modified peptide fragments within a catalytic domain.[2,27] To apply this technique to determination of protein--ligand interactions involving isoprene groups, analogs of the prenyl chain that preserved the hydrophobicity, general steric size and shape, and pi-electron richness of the parent farnesyl and geranylgeranyl

substrates (Scheme 1) were synthesized. Importantly, bacterial FPS showed a reasonably broad substrate specificity compared to the vertebrate enzymes; a variety of aromatic and aliphatic replacements for the terminal isoprenyl unit of GPP were tolerated.[34] Moroever, the MAP kinase pathway was activated by H-ras that was C-terminally modified by saturated and unbranched analogs of the farnesyl moiety.[35] Investigations of the protein interactions with prenyl groups contacting proteins are currently in progress using these and other BZ analogs.

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References

1. Casey, P. J.; Seabra, M. C. *J. Biol. Chem.* **1996**, *271*, 5289.
2. Prestwich, G. D.; Dormán, G.; Elliott, J. T.; Marecak, D. M.; Chaudhary, A. *Photochem. Photobiol.* **1997**, *65*, 222.
3. Gaon, I.; Turek, T. C.; Weller, V. A.; Edelstein, R. L.; Singh, S. K.; Distefano, M. D. *J. Org. Chem.* **1996**, *61*, 7738.
4. Turek, T. C.; Gaon, I.; Distefano, M. D. *Tetrahedron Lett.* **1996**, *37*, 4845.
5. Liu, J. G.; Stipanovic, R. D.; Benedict, C. R. *J. Labelled Cmpnd. Radiopharm.* **1996**, *38*, 139.
6. Parish, C.; Rando, R. *Biochemistry* **1996**, *35*, 8473.
7. Maltese, W.; Wilson, A.; Erdman, R. *Biochem. Soc. Trans.* **1996**, *24*, 703.
8. Pfeffer, S.; Dirac-Svejstrup, B.; Soldati, T. *J. Biol. Chem.* **1995**, *270*, 17057.
9. Zhang, F. L.; Casey, P. J. *Annu. Rev. Biochem.* **1996**, *65*, 241.
10. Yasuda, H.; Lindorfer, M. A.; Woodfork, K. A.; Fletcher, J. E.; Garrison, J. C. *J. Biol. Chem.* **1996**, *271*, 18588.
11. Park, H. W.; Boduluri, S. R.; Moomaw, J. F.; Casey, P. J.; Beese, L. S. *Science* **1997**, *275*, 1800-1804.
12. Schalk, I.; Zeng, K.; Wu, S. K.; Stura, E. A.; Matteson, J.; Huang, M. D.; Tandon, A.; Wilson, I. A.; Balch, W. E. *Nature* **1996**, *381*, 42.
13. Tarshis, L.; Yan, M.; Poulter, C.; Sacchettini, J. *Biochemistry* **1994**, *33*, 10871.
14. Ohnuma, S. I.; Nakazawa, T.; Hemmi, H.; Hallberg, A. M.; Koyama, T.; Ogura, K.; Nishino, T. *J. Biol. Chem.* **1996**, *271*, 10087.
15. Tarshis, L. C.; Proteau, P. J.; Kellogg, B. A.; Sacchettini, J. C.; Poulter, C. D. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 15018.
16. Abe, I.; Tomesch, J. C.; Wattanasin, S.; Prestwich, G. D. *Natural Prod. Rept.* **1994**, 279-302.
17. Clarke, S. *Annu. Rev. Biochem.* **1992**, *61*, 355.
18. Koyama, T.; Obata, S.; Osabe, M.; Takeshita, A.; Yokoyama, K.; Uchida, M.; Nishino, T.; Ogura, K. *J. Biochem. (Tokyo)* **1993**, *113*, 355.
19. Koyama, T.; Tajima, M.; Sano, H.; Doi, T.; Koike-Takeshita, A.; Obata, S.; Nishino, T.; Ogura, K. *Biochemistry* **1996**, *35*, 9533.
20. Fujii, H.; Koyama, T.; Ogura, K. *J. Biol. Chem.* **1982**, *257*, 14610.
21. Keenan, M. V.; Allen, C. M., Jr. *Arch. Biochem. Biophys.* **1974**, *161*, 375.
22. Koike-Takeshita, A.; Koyama, T.; Obata, S.; Ogura, K. *J. Biol. Chem.* **1995**, *270*, 18396.
23. Yokoyama, K.; McGeady, P.; Gelb, M. *Biochemistry* **1995**, *34*, 1344.
24. Baba, T.; Muth, J.; Allen, C. M. *J. Biol. Chem.* **1985**, *260*, 10467.
25, Bukhtiyarov, Y. E.; Omer, C. A.; Allen, C. M. *J. Biol. Chem.* **1995**, *270*, 19035.
26. Gaon, I.; Turek, T. C.; Distefano, M. D. *Tetrahedron Lett.* **1996**, *49*, 8833.
27. Dormán, G.; Prestwich, G. D. *Biochemistry* **1994**, *33*, 5661.
28. (a) McCarthy, J. R.; Wiedeman, P. E.; Schuster, A. J.; Whitten, J. P.; Barbuch, R. J.; Huffman, J. C. *J. Org. Chem.* **1985**, *50*, 3095; (b) Bittner, S.; Assaf, Y. *Chem. & Ind.* **1975**, 281.
29. Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768.
30. Yoshida, I.; Koyama, T.; Ogura, K. *Biochemistry* **1987**, *26*, 6840.
31. Koyama, T.; Yoshida, I.; Ogura, K. *J. Biochem. (Tokyo)* **1988**, *103*, 867.
32. Fujii, H.; Koyama, T.; Ogura, K. *Biochim. Biophys. Acta* **1982**, *712*, 716.
33. The oxidation[3,26] of **3** was modified to give 63% yield of **5** (based on consumed **3**); HR-EI MS calcd. for $C_{11}H_{24}O_2Si$ 216.1546; found 216.1473. Starting with **4** to give 32% of homogeneous **6**; HR-EI MS calcd. for $C_{16}H_{32}O_2Si$ 284.2172; found 284.2115.

The Mitsunobu reaction of alcohol **5** (400 mg, 1.85 mmol) with 3-hydroxybenzophenone (385 mg, 1.94 mmol), and Ph_3P (605 mg, 2.31 mmol) in 1 mL dry THF at -4 °C, with dropwise addition of diethyldiazodicarboxylate (DEAD) (0.40 mL, 2.4 mmol) gave, after SiO_2 purification (EtOAc:Hex, 1:20), **7m** as a pale yellow oil (610 mg, 83% yield). ^1H NMR ($CDCl_3$) δ 0.05 (6H, s), 0.85 (9H, s), 1.6 (3H, s), 4.25 (2H, d), 4.45 (2H, s), 5.65 (1H, t), 7.0–7.9 (9H, m); ^{13}C NMR ($CDCl_3$) δ -5.2, 25.9, 59.8, 73.4, 119.5, 122.8, 128.2, 128.4, 129.2, 130.0. Crystalline **7p** was obtained in 99% yield, while oil **8m** was obtained in 84% yield and solid **8p** in 90% yield. Cleavage of the TBDMS ether in THF at 0 to 25 °C gave **9m** (75%): ^1H NMR ($CDCl_3$) δ 1.65 (3H, s), 4.15 (2H, d), 4.35 (2H, s), 5.75 (1H, t), 7.0-7.8 (9H, m); ^{13}C NMR ($CDCl_3$) δ 13.9, 58.8, 73.1, 115.4, 119.6, 123.0, 127.3, 128.2, 129.2, 130.0, 132.4, 133.5, 138.8, 158.6. Anal. calcd. for $C_{18}H_{18}O_3$: C, 76.57; H, 6.43; found: C, 76.35; H, 6.64. Similar reaction afforded alcohol **9p** (42%), mp 68–69 °C; anal. calcd. for $C_{18}H_{18}O_3$: C, 76.57; H, 6.43; found: C, 76.78; H, 6.48. Alcohol **10 m** (97%): ^1H NMR ($CDCl_3$) δ 1.65 (6H, 2s), 2.1 (4H, m), 4.1 (2H, d), 4.4 (2H, s), 5.35 (1H, t), 5.5 (1H, t), 7.0–7.9 (9H, m). Anal. calcd. for $C_{23}H_{26}O_3$: C, 78.83; H, 7.48; found: C, 78.67; H, 7.54. Alcohol **10p** (95%): mp 51–52 °C; anal. calcd. for $C_{23}H_{26}O_3$: C, 78.83; H, 7.48; found: C, 79.33; H, 7.79.

*Synthesis of Diphosphates (**1m**, **1p**, **2m**, **2p**).* The diphosphate analogs were synthesized from the corresponding alcohols **9m**, **9p**, **10m**, and **10p** and were purified by ion exchange.[29]

Measurement of Prenyltransferase Activities. Prenyltransferase activities were measured by determination of the amount of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. FPS from *B. stearothermophilus* was purified from the *E. coli* cells harboring the expression plasmid pEX11 as previously described.[18] HexPS from *M. luteus* B-P 26 was partially purified.[30] UPS from *B. stearothermophilus* was partially purified essentially according to the procedure for the purification of UPS from *M. luteus* B-P 26.[31]

(a) For *B. stearothermophilus* FPS, the incubation mixture (1.0 mL) contained 50 mM Tris-HCl buffer, pH 8.5, 50 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM 2-mercaptoethanol, 25 μM geranyl diphosphate (GPP), 25 μM [1-^{14}C]IPP (spec. act. 37 MBq/mol), and a suitable amount of enzyme. After incubation at 55 °C for 15 min, 0.3 mL of 1 M HCl was added, incubated at 37 °C for 15 min to hydrolyze the FPP produced. Then 2 mL of saturated NaCl solution was added and the reaction product was extracted with hexane. Duplicates were performed and averaged.

(b) Assay of HexPS of M. luteus B-P 26. The incubation mixture (200 μL) contained 50 mM Tris-HCl buffer, pH 8.5, 20 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM 2-mercaptoethanol, 25 μM FPP, 50 μM [1-^{14}C]IPP (1.95 GBq/mol), and an appropriate amount of a partially-purified HexPS solution. The mixture was incubated at 37 °C for 5 min and products extracted with 1-butanol. Duplicates were performed and averaged.

(c) Assay of UPS from B. stearothermophilus. The incubation mixture contained, in a final volume of 200 μL, 50 mM Tris-HCl buffer, pH 7.7, 5 mM $MgCl_2$, 50 μM 2-mercaptoethanol, 50 μM NH_4Cl, 1.3 μM [1-^{14}C]IPP (2.22 GBq/mol), 25 μM of FPP, 0.25% Triton X-100, and an appropriate amount of partially-purified UPS from *B. stearothermophilus*. The mixture was incubated at 55 °C for 30 min and products extracted with 1-butanol. Triplicate assays were performed and the average value is reported.

Product Analysis of Prenyltransferases. The allylic diphosphate was replaced with the BZ-prenyl diphosphate and incubated as above. Radioactive prenyl diphosphates in the reaction mixture were hydrolyzed to the corresponding alcohols with potato acid phosphatase,[32] and extracted with pentane and analyzed by TLC (LKC-18 plate, Whatman) with acetone/water solvent systems (A, 4:1; or B, 19:1). The positions of authentic standards (D. M. Marecak, unpublished results) were visualized with iodine vapor, and the distribution of radioactivity was determined by autoradiography

Photoinactivation. After incubation with a BZ-prenyl diphosphate at 37 °C for 5 min, each mixture was irradiated by UV lamp. Irradiation of FPS solutions was carried out at 360 nm with a UV lamp (500 W) equipped with a UV-D35 colored glass filter for indicated periods. A Bio-Rad GS Gene Linker UV chamber was used for the irradiation of HexPS solutions using an 8 W UV lamp.

34. Maki, Y.; Masukawa, A.; Ono, H.; Endo, T. ; Koyama, T. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1605.
35. McGeady, P.; Kuroda, S.; Shimizu, K. Takai, Y.; Gelb, M.H. *J. Biol. Chem.* **1995**, *270*, 26347.

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