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SYNTHESIS AND CHARACTERIZATION OF A PHOTOAFFINITY PROBE POSSESSING BIOTINYL AND AZIDOBENZOYL MOIETIES FOR IP_3-AFFINIATED PROTEIN

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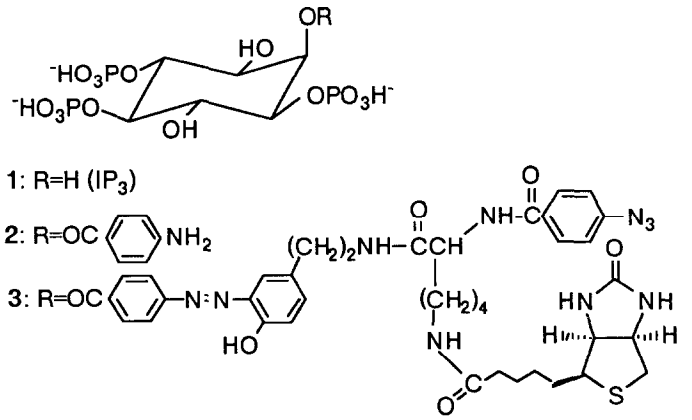
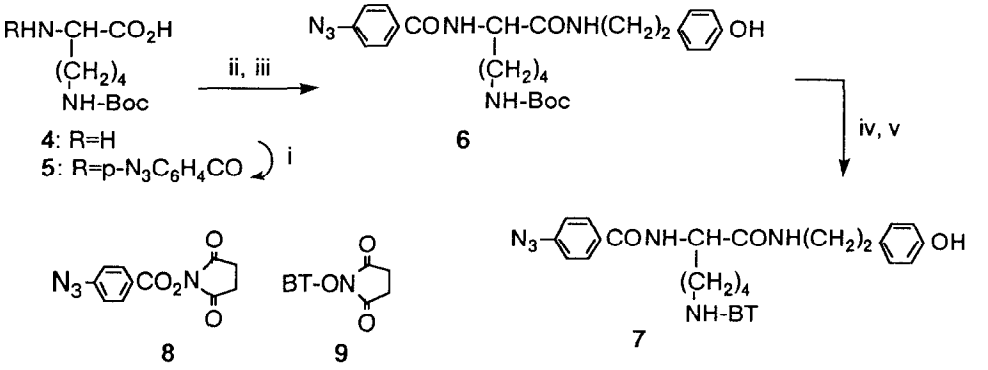
Abstract: A *myo*-inositol 1,4,5-tris(phosphate) analogue bearing *p*-azidobenzoyl and biotinyl moieties has been prepared and shown to act as a photoaffinity probe for *myo*-inositol 1,4,5-tris(phosphate) 5-phosphatase.

A new intracellular second messenger, D-*myo*-inositol 1,4,5-tris(phosphate) (**1**, IP_3) mobilizes Ca^{2+} from non-mitochondrial store sites.[1] The detection of IP_3-affiniated proteins such as its receptor on the store, IP_3 3-kinase, and 5-phosphatase and understanding of their functions at molecular level are quite important for knowledge of complex metabolic pathways and pharmacological invention. For these purposes, structurally modified IP_3 analogues have been synthesized.[2,3] However, until now there is little known an analogue having an additional functionality as a probe for studying active sites of IP_3-affiniated proteins and molecular mechanism of interaction between IP_3 and its receptor.[4] Hirata's group who is one of the present authors devised analogues bearing photosensitive azido and radioactive iodo (^{125}I) groups at the 2 position of IP_3 and utilized for detection of an IP_3 binding protein.[5] For further investigation along this line, we designed a non-radioactive IP_3 analogue possessing photo-sensitive and biotin-avidin complexing properties.[6] Very recently, Prestwich et al. have reported photoaffinity probes having azido and ^{125}I groups for IP_3.[4b] In this communication, we describe the synthesis and biological characterization of such a novel analogue **3**.



For a photoaffinity labelling, the *p*-azidobenzoyl group was employed which can be converted to the corresponding nitrene species on photolysis. The nitrene induces the insertion reaction at an active site in a receptor protein resulting in the formation of a covalently linked derivative. In addition, biotin was chosen as a non-radioactive marker. Function of biotin as a detecting tool is based on the strong interaction with avidin and visualization of the resulting complex. On the other hand, we have showed that modifications at the 2-position in IP_3 do not affect significantly its biological potency.[3] Among these analogues, 2-*p*-aminobenzoyl derivative **2** which showed to have especially similar characteristics to those of the native IP_3 was chosen for the present object.

Biotin and azidobenzoic acid was assembled by amido linkages on α- and ε-amino groups of lysine and its *N*-*p*-hydroxyphenylethylamide **7** was connected with **2** by azocoupling reaction leading to the final product **3**. Thus, N^{ε}-*t*-butyloxycarbonyl-*L*-lysine **4**[9] was treated with *N*-succinimidyl *p*-azidobenzoate **8** to give amide **5** in 93% yield which was then converted to lysine *N*-*p*-hydroxyphenylethylamide **6**[10a] in 60% yield by way of succinimidyl ester (Scheme 1). After a usual removal of the Boc group from **6**, the generated amine was allowed to react with the active ester **9**[11] of biotin (BT-OH) to afford the fully functionally-modified lysine derivative **7**[10b] in 70% yield. Finally, **7** was treated with diazonium salt *in situ* prepared conventionally from racemic **2** at pH about 8.5 at room temperature[12] to afford **3**. The structure of **3** was supported by ^1H-, ^{13}C- and ^{31}P-NMR and MS(FAB).



Scheme 1. *Reagents and conditions:* i) **8**/DMF/r.t./6h, ii) HOSu/DCC/DMF/r.t./4h, iii) *p*-HO-$C_6H_4(CH_2)_2NH_2$·HCl/Et_3N/CH_2Cl_2/r.t./3h, iv) TFA/CH_2Cl_2/r.t./1h, v) **9**/Et_3N/DMF/r.t./12h, *Abbreviations:* Boc, *t*-butoxycarbonyl; BT, biotinyl; DMF, *N,N*-dimethylformamide; HOSu, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; TFA, trifluoroacetic acid.

As several IP_3 analogues possessing acyl groups at C-2 exhibited,[3] the bifunctional analogue **3** thus synthesized was found to have also the same biological function and potency as the natural IP_3 by the experiment of inhibition in [^{3}H]IP_3 5-phosphatase with IP_3 or analogue **3** (Figure 1). This result prompted us to test its role for photoaffinity labelling. Thus, **3** was incubated on ice for 5 min with the 5-phosphatase-rich fraction which was obtained by IP_3 affinity column chromatography[13] of red-blood ghost treated with a detergent. The mixture was photolyzed (Toshiba FL-20E lamp) for 10 min at the same temperature followed by electrophoresis on an SDS-polyacrylamide gel.[14] The resulting gel was electro-blotted onto a nitrocellulose sheet[15] and the sheet was incubated in a solution containing the streptavidin-alkaline phosphatase conjugate and then stained with substrates

for alkaline phosphatase. The band corresponding to the molecular weight of 66 kDa was specifically stained. The ghost did not show IP_3 3-kinase and IP_3 binding activities. Consequently, it was concluded that the stained band was attributed to IP_3 5-phosphatase. Addition of excess IP_3 to the incubation medium reduced the labelling

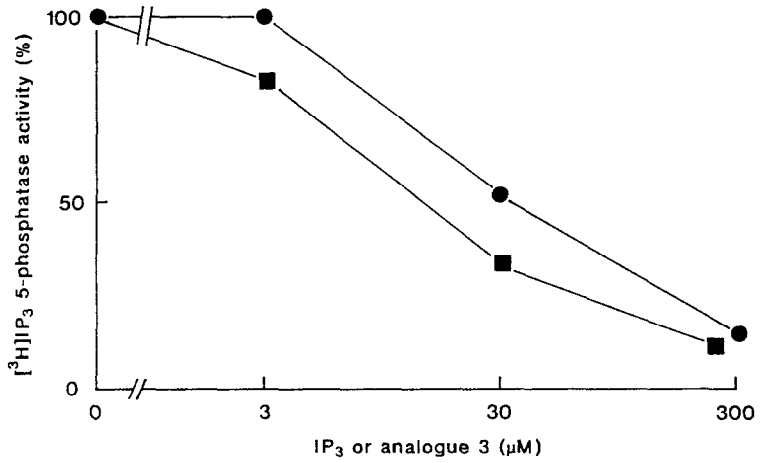


Figure 1. Inhibition in $[^3H]IP_3$ 5-phosphatase with IP_3 (●) or analogue 3 (■).

markedly. This result clearly indicates that 3 was photolyzed at the IP_3-recognizing domain of the protein resulting in the formation of the covalent linkage around the active site.

The IP_3 analogue with functionalities for photoaffinity labelling and biotin-avidin complexing at C-2 played a role well as a detector of IP_3 5-phosphatase. In analogy, 3 may provide a promising tool for detection and characterization of other IP_3-affiniated proteins. Consequently, 3 can be also expected to be utilyzed for discovering a new IP_3-recognizing protein.

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10. a) The compound was characterized by ^{1}H- and ^{13}C-NMR and MS(EI) as well as by combustion analysis. b) The compound was characterized by ^{1}H- and ^{13}C-NMR and MS(FAB) as well as by combustion analysis.
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12. *Experimental procedure:* To an ice-cold solution of **2** sodium salt (20 mg, 0.033 mmol) in H_2O (2 ml) was added *c*-HCl (28 μl, 0.33 mmol) and $NaNO_2$ (9 mg, 0.134 mmol), and the mixture was stirred for 30 min. The solution was made alkaline with $NaHCO_3$ (28 mg, 0.334 mmol) and **7** (26 mg, 0.04 mmol) in DMF (3 ml) was added at 0 °C. The *homogeneous solution* was stirred for 6 h at room temperature, concentrated in vacuo (about 0.1 mmHg) at below 20 °C, and neutralized by passing through a cation exchange column (H^+ form). After addition of pyridine to prepare a pyridinium salt, the eluate was concentrated and applied to a 30 x 1.5 cm column of the resin (Dowex 50W-X, Na^+ form). Elution with water followed by evaporation afforded the sodium salt of **3** (31.4 mg, 75% yield, calculated for $C_{44}H_{54}N_{10}O_{21}P_3SNa_3$) which was then precipitated from H_2O-MeOH: δ_P (109 MHz, D_2O, H_3PO_4 for reference: δ 0.00 ppm, 13.1 mg of **3** was dissolved in 2.9 ml of D_2O, pH 6.4) 1.06, 2.36, 3.22; δ_H (270 MHz, D_2O, HOD for reference: δ 4.64 ppm) 0.95-1.40 (m, 10H, CH_2x5), 1.55 (m, 2H, CH_2), 1.90 (t, *J* 7.3, 2H, $COCH_2$), 2.50 (d, *J* 13.5, 1H, H in SCH_2), 2.70 (m, 3H, benzylic CH_2 and H in SCH_2), 2.90 (m, 3H, $^{\varepsilon}NCH_2$ and SCH), 3.40 (m, 2H, NCH_2 in the *p*-hydroxyphenetyl moiety), 3.91 (dd, *J* 11.0 and 2.8, 1H, H-3 in the inositol ring), 3.98-4.20 (m, 3H, H-1, 5, 6 in the inositol), 4.33 (q-like, 1H, H-4 in the inositol), 5.77 (br t, 1H, H-2 in the inositol), 6.69 (d, *J* ca.8.4, 2H, H-*m* in the azidobenzoyl moiety), 6.78 (d, *J* ca.8.85, 1H, H-*m* in the *p*-hydroxyphenetyl moiety), 7.19 (d, *J* ca.8.4, 1H, H-*o* in the azidobenzoyl), 7.32 (m, 2H, H-*o* in the *p*-hydroxyphenetyl), 7.36 (m, 1H, H-*o* between azo and aminoethyl groups), 7.66 (d, *J* ca.8.24, 2H, H-*m* in the azobenzoyl group), 8.08 (d, *J* 8.24, 2H, H-*o* in the azobenzoyl); δ_C (67.8 MHz, dioxane for reference: δ 67.4 ppm. Most of carbon atoms showed doublet or multiplet mainly because of racemic 2-(2-aminobenzoyl)-*myo*-inositol 1,4,5-tris(phosphate). Chemical shifts of such carbons were shown by one of them.) 20.96, 23.37, 26.06, 28.36, 28.70, 28.75, 31.34, 34.13, 36.31, 39.49, 40.58, 55.44, 56.13, 61.01, 62.78, 71.07, 72.90, 73.54, 74.89, 77.47, 79.09, 119.06, 119.71 (2C), 123.03 (2C), 129.68 (2C), 130.57, 131.87 (2C), 131.89, 132.04, 132.06, 135.93, 137.77, 144.38, 151.86, 153.97, 165.96, 167.78, 169.34, 174.79, 177.13.
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