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INOSITOL 1,4,5-TRISPHOSPHATE AFFINITY CHROMATOGRAPHY

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Summary : Inositol 1,4,5-trisphosphate (IP_3) affinity columns were made by coupling IP_3 analogs to a supporting matrix, Sepharose 4B. IP_3 5-phosphatase activity, IP_3 3-kinase activity and IP_3 binding activity from rat brain were adsorbed on the IP_3 columns, and were eluted by increasing KCl concentration. This purification procedure increased the specific activities of these parameters 5-200-fold. Thus Sepharose 4B immobilized IP_3 analogs can specifically interact with IP_3-binding proteins, demonstrating that IP_3 affinity columns are a good method for purifying such proteins. Furthermore, our results suggest that IP_3 analogs can be linked to other molecules to make useful derivatives without loss of their biological activities.

Inositol 1,4,5-trisphosphate (IP_3), a product from the receptor-activated hydrolysis of phosphatidylinositol 4,5-bisphosphate, plays an important role as an intracellular second messenger by mobilizing Ca^{2+} from non-mitochondrial store sites (1-3). IP_3 is metabolized by two known routes. One route, dephosphorylation, catalyzed by IP_3 5-phosphatase, results in the formation of 1,4-IP_2, which is subsequently degraded to free inositol by other phosphatase activities (4). Alternatively, phosphorylation of the 3-hydroxyl group of IP_3 by an ATP-dependent kinase produces 1,3,4,5-IP_4 (5), whose potential role is now being investigated (6,7). Thus, three proteins are listed as IP_3-interacting macromolecules: putative IP_3 receptors involved in the Ca^{2+} release and two enzymes related to IP_3 metabolism. To characterize the IP_3-recognizing domain of these proteins in relation to their functions, it is necessary to determine what structural features in IP_3 are essential for activity, and by chemical modification of the molecule to build up

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structure-activity profiles for the interaction of IP_3 with receptor sites and with the metabolic enzymes (8-11).

Recently, we synthesized a series of IP_3 analogs, in which bulky substituents such as 4-azidobenzoyl (designated as analog #195), 4-(5-benzamidoethyl-2-hydroxyphenylazo)benzoyl (#204), 4-aminocyclohexanecarbonyl (#206) or 4-aminobenzoyl group (#209) are coupled with the 2nd hydroxyl group of IP_3 (12). Using these analogs, we found that such modifications reduced little the ability of the analogs to interact with IP_3-recognizable proteins, but the potencies did vary among the proteins and analogs (12).

In addition to designing these analogs to allow us to build up structure activity profiles as described above, the analogs are capable of attaching to other molecules through the added substituents. We have now coupled these analogs with Sepharose 4B to obtain IP_3 affinity columns and the applicability was examined using cytosol fractions and detergent-extracts of rat brain membrane fractions.

MATERIALS AND METHODS

Materials. [^{3}H]IP_3 (specific radioactivity: 37 GBq/mmol or 1.67 TBq/mmol) was obtained from Amersham. IP_3 was prepared by alkaline hydrolysis of the phosphoinositide fraction (Sigma), according to Grado and Ballou (13), and purified by a SAX column on an HPLC system. Activated CH-Sepharose 4B was purchased from Pharmacia. Chemical synthesis of the IP_3 analogs will be described elsewhere (14). All other reagents were of the highest grade available.

Preparation of the cytosol fraction and detergent-extract from the particulate fraction of the rat brain. Whole brains, excised from Wistar rats of either sex, weighing 200-300 g, were homogenized with a Dounce homogenizer in 5 vol. of Buffer A containing 50 mM KCl, 10 mM Hepes buffer (pH 7.2), 2 mM NaN_3, 2 mM EDTA and 10 mM β-mercaptoethanol. The homogenate was centrifuged at 100,000 g for 60 min, and the resultant supernatant was designated as the cytosol fraction. The pellet was resuspended in the same buffer and recentrifuged. This washed precipitate was solubilized with 1% detergent in Buffer A at a density of 50 mg/ml wet weight at 4℃ for 6 hrs, as described by Supattapone et al. (15), in which they used Triton X-100 as the detergent. Mainly, we used the detergent polyoxyethylene 10 tridecyl ether (P10TE) because it has negligible absorbance at 280 nm and thus allows us to monitor protein elution from the IP_3 column at this wavelength. The extent of extraction by P10TE was similar to that with the same concentration of Triton X-100, in terms of the specific activities of IP_3 5-phosphatase and [^{3}H]IP_3 binding. The solubilized materials (detergent-extract) were obtained by centrifuging the mixture at 100,000 g for 2 hrs. Protein concentration was determined by the method of Lowry et al (16) using bovine serum albumin as a standard or by the method of Bradford (17), using a Bio-Rad kit.

Assay of IP_3 metabolic enzyme activities. IP_3 5-phosphatase activity was assayed in a mixture (0.5 ml) containing 20 mM Hepes buffer (pH 7.0), 5 mM $MgCl_2$ and 50 μM IP_3 (containing 370 Bq [^{3}H]IP_3 of lower specific radioactivity) at 37℃ for 5 min. The reaction was terminated by adding the

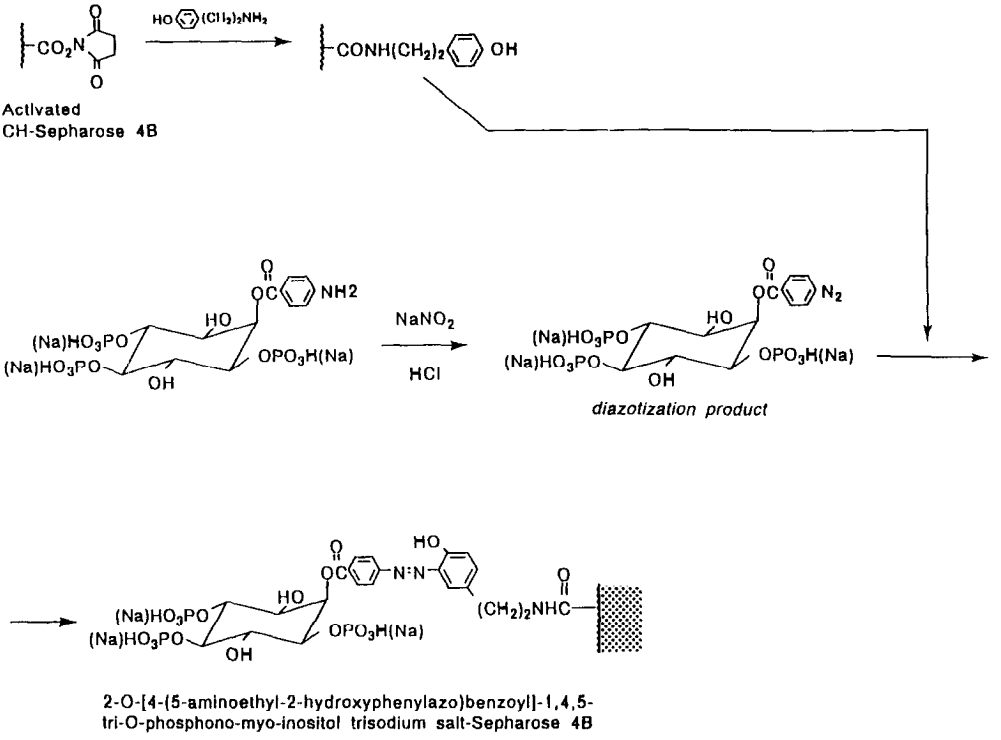

Fig. 1. Procedure used to prepare 2-O-[4-(5-aminoethyl-2-hydroxyphenylazo) benzoyl]-1.4.5-tri-O-phosphono-myo-inositol-Sepharose 4B (#204 resin). See text for details.

same volume of 20% trichloroacetic acid. After centrifugation at 3000 rpm for 10 min and extraction with diethylether (4 ml x 3), the reduction of [^{3}H]IP_3 was analyzed by applying the sample to a SAX column in an HPLC system (18). IP_3 3-kinase activity was assayed as previously described (12,18).

Assay for [^{3}H]IP_3 binding activity of the detergent-extract and its subfraction on an IP_3 affinity column. Binding assays with soluble fractions were performed as follows: the mixture (0.45 ml) contained 50 mM Tris-HCl buffer (pH 8.3), 1 mM EDTA, 0.98 nM [^{3}H]IP_3 of a higher specific radioactivity and 10-200 μg soluble fraction (final detergent concentration was adjusted at 0.2%). Following incubation on ice for 10 min, the mixture was added to 50 μl of bovine γ-globulin (10 mg/ml) as a carrier protein, followed by the addition of 500 μl of polyethyleneglycol 6000 (30%,w/v). Each tube was left on ice for 30 min. After centrifuging the mixture at 16,000 g for 10 min, the precipitate was dissolved in 1 ml of 0.1 N NaOH, and then counted for ^{3}H-radioactivity. The ^{3}H-radioactivity in the precipitate gradually decreased, with an IC_{50} of 52 nM (mean of three determinations), by adding increasing concentrations of unlabeled IP_3 (1-1000 nM). Thus, non-specific binding was determined in the presence of 1 μM unlabeled IP_3 and was subtracted from the total binding in its absence to determine the specific binding. In a typical assay with 200 μg detergent-extract, the ^{3}H-radioactivity of total or non-specific binding was about 900-1100 dpm or 100-300 dpm, respectively. The specific binding was linearly increased by increasing the amount of extract up to 800 μg.

Preparation of Sepharose 4B coupled with IP_3 analogs (i)2-O-[4-(5-aminoethyl-2-hydroxyphenylazo)benzoyl]-1,4,5-tri-O- phosphono-myo-inositol trisodium salt-Sepharose 4B (Fig. 1) : Step 1: Following washing activated CH-Sepharose 4B (1 g) with 1 mM HCl and subsequently with 0.1 M $NaHCO_3$ (pH 8.0), the resin was mixed with 2 μmol tyramine at room temperature for 1 hr, collected by

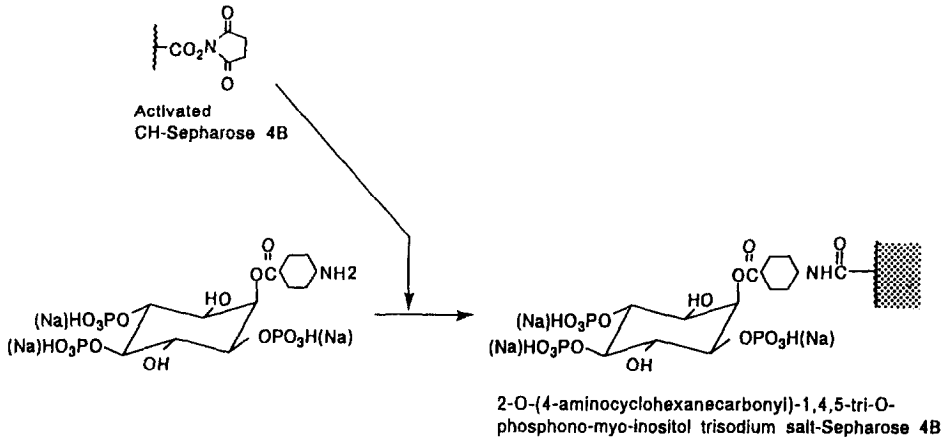

Fig. 2. Procedure used to prepare 2-O-(4-aminocyclohexanecarbonyl)-1,4,5-tri-O-phosphono-myo-inositol-Sepharose 4B (#206 resin). See text for details.

suction filtration, washed with 0.5 M NaCl plus 50 mM Tris-HCl buffer (pH 8.0) and 0.5 M NaCl plus 50 mM formic acid (pH 4.0), alternatively and finally suspended in 0.1 M $NaHCO_3$ (pH 8.0). Step 2: Twenty microliters of concentrated HCl and 5 μmol $NaNO_2$ were mixed with 2 μmol 2-O-(4-aminobenzoyl)-1,4,5-tri-O-phosphono-myo-inositol in water cooled to 0 ℃ by an ice-water bath and the mixture was left at the same temperature for 30 min for diazotization. Step 3: The diazotization product at Step 2 was mixed with the product at Step 1, and the mixture was incubated at room temperature for 1 hr. The resin coupled with the IP_3 analog was collected by passing the mixture through a sintered glass funnel, followed by washing as described above. Because the IP_3 analog used here was previously designated as compound #204 (12), we will call this the #204 resin or a #204-column when refering to it in this paper. (ii) 2-O-(4-aminocyclohexanecarbonyl)-1,4,5-tri-O-phosphono-inositol trisodium salt-Sepharose 4B (Fig. 2) : After first washing the activated CH-Sepharose 4B (1 g) with 1 mM HCl and subsequently with 0.1 M $NaHCO_3$ (pH 8.0), the resin was suspended in 0.1 M $NaHCO_3$ (pH 8.0) and mixed with 3 mg 2-O-(4-aminocyclohexanecarbonyl)-1,4,5-tri-O-phosphono- inositol, previously designated as compound #206 (12). The mixture was incubated at room temperature for 1 hr. The resin coupled with the IP_3 analog #206 was collected by passing the mixture through a filter, followed by alternately washing with 0.5 M NaCl plus 50 mM Tris-HCl (pH 8.0) or 0.5 M NaCl plus 50 mM formic acid (pH 4.0).

RESULTS AND DISCUSSION

The cytosol fraction of the rat brain was applied to either a #204- or #206-column, and the adsorbed proteins were eluted by a step-wise increase in potassium concentrations (Fig.3). All the media used contained 2 mM EDTA to protect the columns from attack by IP_3 5-phosphatase activity present in the sample: activation of this phosphatase, which can utilize IP_3 analogs as a substrate (12), requires the presence of Mg^{2+} (19) and inclusion of EDTA to chelate the Mg^{2+} present in the solution would prevent the activation of this enzyme. The cytosolic IP_3 5-phosphatase (a specific activity of 37

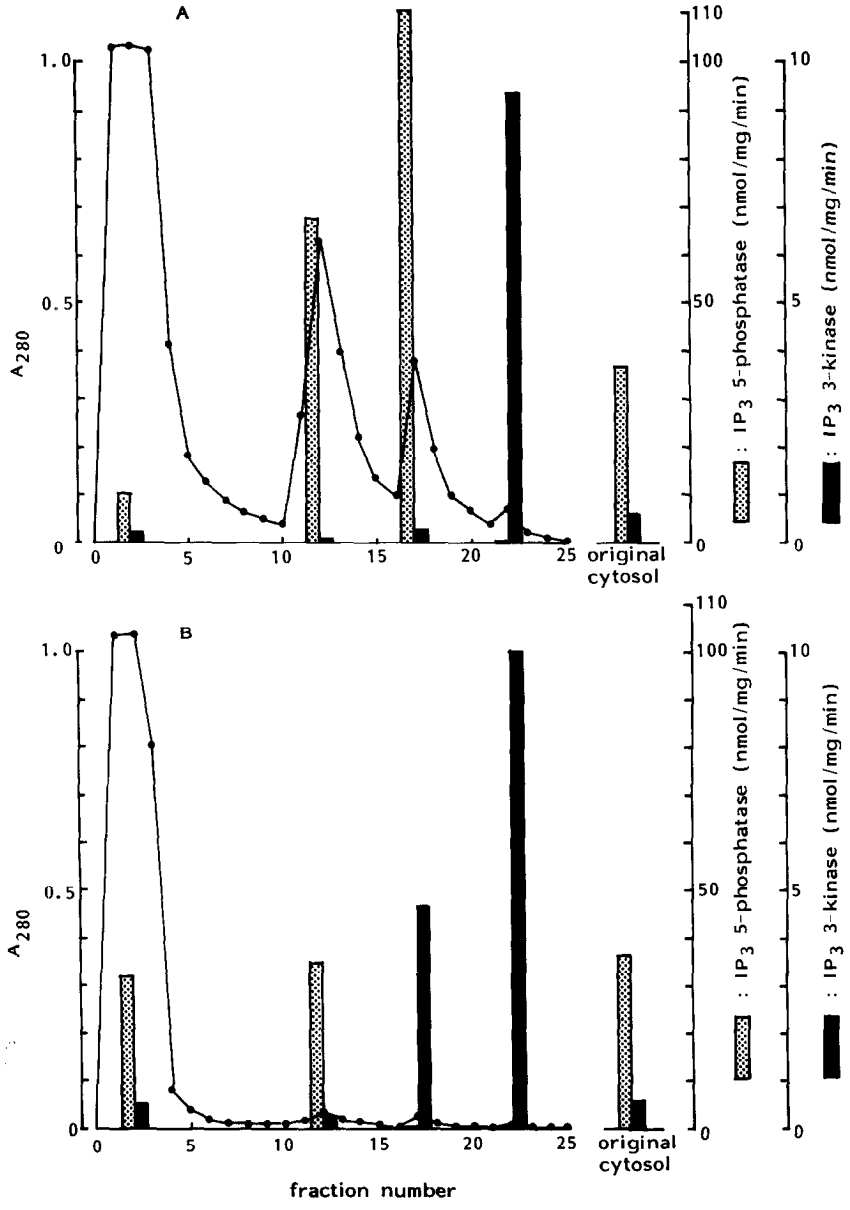

Fig. 3. IP_3 affinity column chromatography of the cytosol fraction from the rat brain. The cytosol fraction (5.6 mg/ml x 5 ml) was applied to a 1-ml #204- (A) or #206-column (B), equilibrated with buffer A. Ten 3-ml fractions were collected (up to tube #10). Then 0.2 M KCl elution was started at fraction 11, followed by 0.5 M KCl at fraction 16 and 2 M KCl at fraction 21. The KCl eluates were collected in 1-ml fractions. Fractions 2, 12, 17 and 22 were assayed for IP_3 5-phosphatase (▨) and IP_3 3-kinase (■). Each value is mean of duplicate determinations. Three other experiments gave essentially similar results.

nmol/mg/min) that was retained on the #204-column was eluted with 0.2 M and 0.5 M KCl, with a 3- to 5-fold increase in this specific activity (Fig. 3A). On the other hand, a #206-column adsorbed the phosphatase activity to the

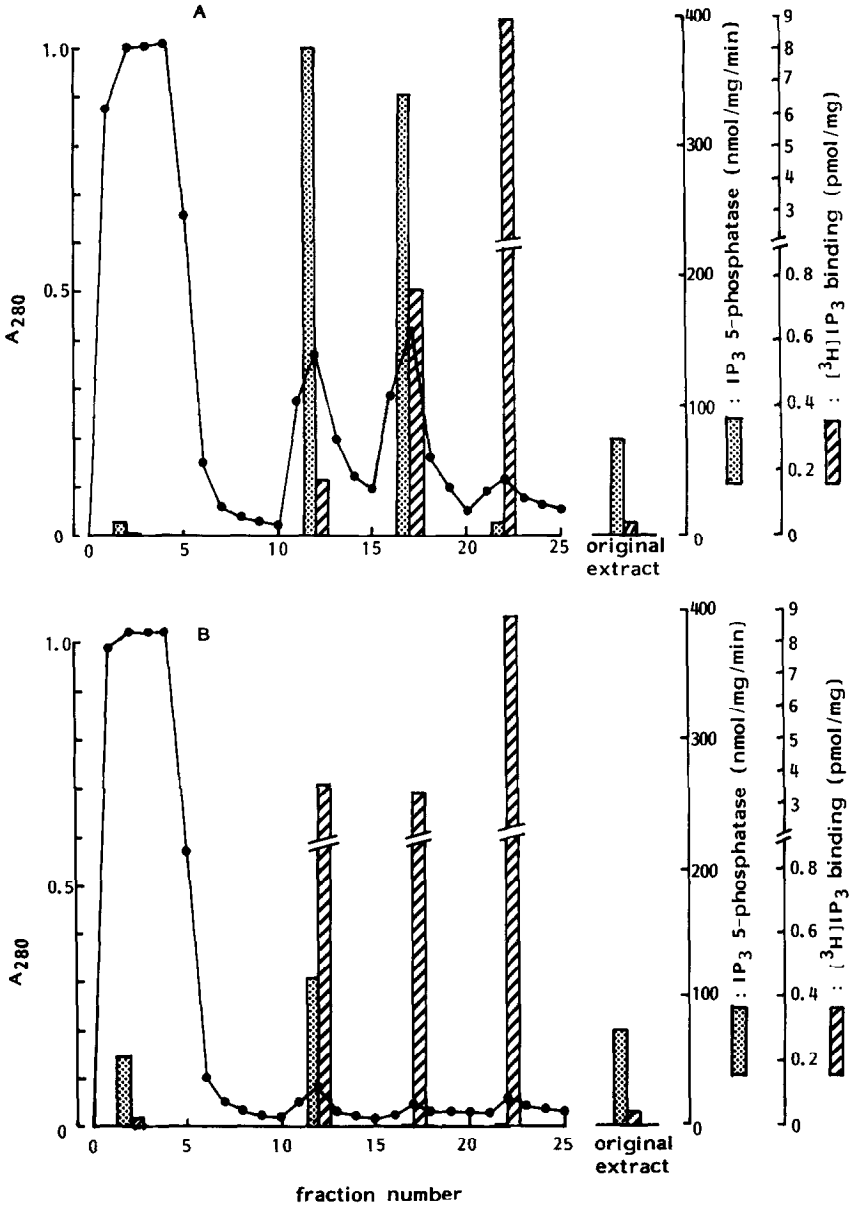

Fig. 4. IP_3 affinity column chromatography of detergent-extract of rat brain membrane fraction. The 1%-P1OTE extract of the membrane fraction (2.3 mg/ml x 20 ml) was applied to a #204- (A) or #206-column (B) equilibrated with Buffer A containing 1% P1OTE, and ten 5-ml fractions were collected. The KCl elution was performed as described in Fig. 3. Four fractions were assayed for IP_3 5-phosphatase (▨) and IP_3 binding (▨). Each determination was done in duplicate. Four other experiments gave essentially the same results. The extract with 1% Triton X-100 behaved in the same manner as the P1OTE extract.

least extent, i.e., most of the activity passed through at 50 mM KCl and little activity was eluted with 0.2 M KCl (Fig. 3B). The IP_3 3-kinase activity which is mainly present in the cytosol fraction (5, 20), was also

determined in each fraction. The enzyme activities were eluted from a #204-column by 2 M KCl (Fig. 3A). Both 0.5 M and 2 M KCl eluted enzyme activities from the #206-column (Fig. 3B). Purification over the #204- or #206-column increased the specific enzyme activity by 10- to 20-fold. The #204-column appeared to non-specifically adsorb other proteins in addition to the IP_3-recognizing proteins, probably due to the long spacer between the resin and IP_3.

We also used both columns to fractionate the detergent-extract from rat brain membrane fraction (Fig. 4). In this case, all the media used contained 1% detergent (P10TE or Triton X-100), in addition to 2 mM EDTA; and both of IP_3 5-phosphatase activity and [^{3}H]IP_3 binding in the sample were determined. The results on the IP_3 5-phosphatase activity in the membrane fraction were essentially the same as those for the cytosolic enzyme: more than 70% of the total enzyme activity was retained on the #204-column; thus, the specific enzyme activity in the 0.2 M or 0.5 M KCl eluate was about 5-fold greater than that in the extract. In contrast, 80% of the IP_3-phosphatase activity did not bind to the #206-column, appearing in the flow-through fraction. The [^{3}H]IP_3 binding activity, was retained on both columns, and could be eluted by raising the salt-concentration: the material eluted by the high salt concentration had a specific activity that was 100-200-fold greater than that of the extract (42 fmol/mg, mean of 4 determinations).

The IP_3 affinity columns could be used again after they were regenerated by washing them with a solution of 6M urea and 2M KCl, followed by re-equilibration with the initial buffer.

The characteristics of the examined IP_3 analogs (12) appeared to be related to the results of the column chromatography: (i) IP_3 5-phosphatase activities from both cytosolic and membrane fractions were retained by the #204-column, but not by the #206-column, probably due to differences in the potencies of the interaction of the analog with the phosphatase (the Ki value of analog #204 in the inhibition of [^{32}P]IP_3 hydrolysis by erythrocyte ghosts was 5.6 μM, while that of analog #206 was 16 μM (12)). (ii) The activities of both IP_3 3-kinase in the cytosol fraction and [^{3}H]IP_3 binding in the detergent-extract were highly concentrated by both these two columns because

analogs #204 and 206 interacted with the proteins with higher affinities (12). Thus, these Sepharose 4B-coupled analogs were capable of interacting with IP_3 recognizable proteins; therefore these columns are useful for concentrating these activities. These results further suggest that the IP_3 analogs may be linked to other molecules without loss of their biological activities; thus useful IP_3 derivatives such as spin-labeled or fluorescent-IP_3 may be synthesized, as proposed by Nahorski and Potter (21).

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