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Synthesis of [α-^{32}P]-8-N_3-NAD, a Photoaffinity Labeling Reagent for Pyridine Dinucleotide Binding Sites

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SUMMARY

The photoactive pyridine dinucleotide analogue [α-^{32}P]-nicotinamide 8-N_3-adenine dinucleotide ([α-^{32}P]-8-N_3-NAD) was synthesized in 30-50% radiochemical yield at a specific activity of 0.5 to 1.2 mCi/μmol by first obtaining [^{32}P]-8-N_3-5'-AMP and then coupling it to β-NMN. A chemo-enzymatic synthesis of [^{32}P]-8-N_3-5'-AMP began with conversion of 3'-AMP to 8-N_3-3'-AMP. 8-N_3-3'-AMP was converted to [^{32}P]-N_3-5'-AMP enzymatically by treatment with [γ-^{32}P]ATP and T4 polynucleotide kinase to obtain [5'-^{32}P]-8-N_3-adenosine-3',5'-bisphosphate. The labeled 3',5'-diphosphate was then transformed into [^{32}P]-N_3-5'-AMP using the 3'-specific phosphatase, nuclease P1. The enzymatic reactions were closely monitored by TLC during the course of which it was observed that the composition of the enzyme buffer as well as the incubation time and temperature were critical to the success of the T4 polynucleotide kinase catalyzed reaction.

Key words: phosphorus-32, [α-^{32}P]-8-N_3-NAD, photoaffinity label, NAD^+ analogue.

INTRODUCTION

Eight- and 2-azide analogues of nucleotides ATP (1) and NAD (2,3) have wide application as photoaffinity labels for labeling nucleotide binding proteins and in the identification of binding site peptides and amino acid residues of enzymes which utilize these nucleotides as substrates, cosubstrates or allosteric effectors (4). Although 8-N_3-NAD (**1**) was reported in 1976 (5), a radiochemical synthesis of 8-N_3-NAD has not yet appeared despite its obvious utility as a photoprobe. Its availability would complement applications of the 2-isomer (2,3) in cases where 2-substitution is

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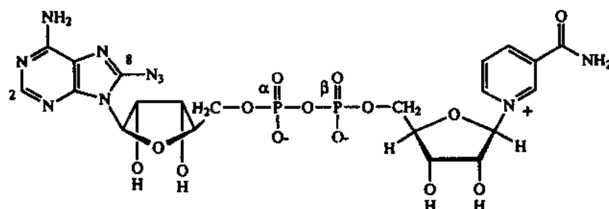

$8\text{-}N_3$-adenine nicotinamide adenine dinucleotide ($8\text{-}N_3$-NAD, **1**)

poorly tolerated (6) and because the isomeric compounds might photocrosslink different amino acid residues within the same binding site due to the different positioning of the photoactive groups (7).

Pyridine dinucleotides are synthesized by coupling nicotinamide mononucleotide (NMN) with AMP or an AMP analogue using a variety of well established techniques (8,9). Since the photoinduced crosslink between the photoprobe and the receptor will be formed from the purine, it is desirous that the adenosine nucleotide moiety bear the radioactive label. This prescribes a strategy in which a labeled $8\text{-}N_3$-AMP is produced and coupled to NMN forming **1**. The intended application of the photoprobe, to identify binding site peptides and amino acid residues, further requires incorporation of a high specific activity to permit facile isolation, purification, and sequencing of the photolabeled peptides.

Although it is possible to use $[^3H]$-$8\text{-}N_3$-adenosine (Moravek Biochemicals, Brea, CA) or $[^{14}C]$ or $[2\text{-}^3H]$AMP (10) for synthesis of labeled $8\text{-}N_3$-AMP, the high cost of the ^{14}C and ^3H isotopes verses ^{32}P argues strongly for the use of a ^{32}P label. High activity ^{32}P is commercially available at low cost both as $^{32}P\text{-}H_3PO_4$ and as $[\gamma\text{-}^{32}P]$ATP. Chemical methods for production of ^{32}P-labeled ribonucleoside 5'-monophosphate from the nucleoside and $^{32}P\text{-}H_3PO_4$ are available (11) but utilize harsh conditions that require protection of the nucleoside, and often difficult removal of side products. Enzymatic methods for the transfer of the γ-phosphoryl of ATP to the 5'-hydroxyl of a nucleoside or a 3'-nucleotide have been applied to radiochemical synthesis (12,13). In this work we report our adaptation of the two-step enzymatic method (13) to produce $[^{32}P]$-$8\text{-}N_3$-5'-AMP starting from $8\text{-}N_3$-3'-AMP and $[\gamma\text{-}^{32}P]$ATP which utilizes well characterized, pure, and commercially available enzyme catalysts (T4 nucleotide kinase and P1 nuclease). $[^{32}P]$-$8\text{-}N_3$-5'-AMP was then coupled with β-NMN using carbodiimide to produce $[\alpha\text{-}^{32}P]$-1.

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EXPERIMENTAL

Materials. 5'-AMP, 3'-AMP and β-NMN were purchased from Sigma (St. Louis, MO). Anhydrous DMF and other organic chemicals were purchased from Aldrich (Milwaukee, WI) and were used without further purification unless otherwise indicated. HPLC grade solvents were purchased from Baxter. [γ-^{32}P]ATP was from ICN (Irvine, CA). T4 polynucleotide kinase was purchased from Pharmacia Biotech (Piscataway, NJ). Nuclease P1 was obtained from United States Biochemicals (Cleveland, OH). Alkaline phosphatase and NAD glycohydrolase were purchased from Sigma (St. Louis, MO). Dowex AG 1-X2 anion exchange resin was purchased from Bio-Rad Laboratories (Richmond, CA). DE-52 cellulose was manufactured by Whatman Ltd. (Maidstone, England). Benzyl DEAE cellulose (product #B-2654, lot #74H5055) was purchased from Sigma.

Methods. Anion exchange thin-layer chromatography was performed on 5 x 20 cm polyethyleneimine (PEI-F) cellulose plates (Macherey-Nagel, Düren, Germany). Electronic absorption spectra were obtained using a Beckman DU-50 spectrophotometer. UV absorbance of fractions collected from low pressure chromatographic columns was monitored using a ISCO UA-6 UV detector (254 nm). The system for anion exchange HPLC consisted of two constaMetric metering pumps (Thermo Separation Products, Schaumburg, IL) and an SM 4000 programmable wavelength UV detector operated at 280 nm. Analysis was performed using a RSIL AN anion-exchange column (4.6 x 250 mm, 10 μ, Alltech, Deerfield, IL) developed isocratically with 250 mM KH_2PO_4 containing 20% v/v methanol, pH 3.5, at a flow rate of 1 mL/min (System A).

8-Bromoadenosine-3'-monophosphate (**2**). Compound **2** was synthesized according to the procedure described by Czarnecki et al. (1) for the synthesis of 8-bromo-5'-AMP. To a suspension of adenosine-3'-monophosphate (52.5 mg., 0.15 mmol) in 1 M sodium acetate (pH 3.8, 2 mL), 1 M NaOH was added dropwise with stirring until a solution was obtained. A solution of bromine (10 μL) in water (2 mL) was prepared by vigorous stirring on a vortex mixer. The diluted bromine solution was added to the reaction vial, the vial tightly sealed with a teflon-lined cap, wrapped with aluminium foil and allowed to stir at ambient temperature for 18 h. The reaction was monitored by anion exchange HPLC (system A). A shift in λ_{max} from 261 nm to 266 nm in the electronic absorption spectrum indicated formation of the brominated compound. After 20 h, excess bromine was removed by bubbling a stream of air through the mixture for a few minutes. The reaction mixture was diluted into 5 mM NH_4HCO_3 and the pH was adjusted to 7.5 with 1 M NH_4OH. The sample was applied to a DE-52 cellulose column (75 mL, 1.8 x 30 cm). A linear gradient formed between 5-250 mM NH_4HCO_3 (350 mL each) was applied to develop the column and 8 mL fractions were collected. Fractions

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containing 8-Br-5'-AMP were pooled and lyophilized resulting in the isolation of 40 mg (63% yield) of a white amorphous solid: HPLC (System A) retention time 9.3 min; UV λ_{max}(H_2O, pH 7) 266 nm, (literature (14): λ_{max} = 265.5 nm).

8-Azidoadenosine-3'-monophosphate (3). A solution of sodium azide in water (2 g in 10 mL) was passed through a column of Dowex AG 50W-X8 cation exchange resin (triethylammonium form) and the water removed by lyophilization to obtain triethylammonium azide. Triethylammonium azide (18.5 mg, 140 μmol) was dissolved in 0.5 mL of dry DMF. The azide was dried by adding 0.5 mL of dry DMF evaporating the solvent *in vacuo*, and repeating the procedure one time. The amorphous residue was resuspended in DMF (4 mL). Alternately, to avoid isolation and drying of the slightly volatile triethylammonium azide, solutions of triethylammonium azide in DMF can be prepared directly by ion-exchange (15). 8-Br-3'-AMP (12 mg, 28 μmol) was added and the reaction mixture was incubated at 75 °C with stirring. The reaction was monitored by anion exchange HPLC (System A) where the product appeared as a new peak with retention time 7.1 min, and by UV spectroscopy where product formation was indicated by a shift in the λ_{max} of the UV spectrum from 266 nm to 274 nm. The product was purified on a DE-52 cellulose column as described for 8-Br-3'-AMP (2). Fractions containing 8-N_3-3'-AMP were pooled and lyophilized yielding the product as white amorphous solid in 50% yield: TLC (PEI-F cellulose, 0.75 M KH_2PO_4, pH 3.5, R_f = 0.48).

[^{32}P]-8-N_3-5'-Adenosine monophosphate (5). 1-5 mCi of [γ-^{32}P] ATP (7000 Ci/mmol-supplied as 6-30 μL of solution in 1 mM HCl) was incubated in a total volume of 400 μL containing 40 μL One-phor ALL plus (®Pharmacia) buffer (100 mM Tris acetate, 100 mM magnesium acetate, 500 mM potassium acetate), 4 μL of T4 polynucleotide kinase (38 units) and 8-N_3-3'-AMP (10 μg, 26 nmol). One unit of T4 polynucleotide kinase catalyzes the transfer of 1 nmol of phosphate from ATP to nucleotide in 30 min at 37 °C. After incubation for 30 min at 37 °C, unlabeled ATP (10 μg) and an additional 1 μL (9.5 units) of polynucleotide kinase were added. Incubation was continued at ambient temperature for 30 min. At this stage the conversion of 8-N_3-3'-AMP into 8-N_3-adenosine-3',5'-bisphosphate (4) was complete as verified by TLC followed by autoradiography. Once the conversion to the bis-phosphate was judged complete, nuclease P1 was added to remove the 3'-phosphate. To the above reaction mixture containing (4), nuclease P1 (50 μL of 1 mg/mL stock) and 50 μL of 500 mM Tris HCl (pH 8.2) were added. After incubation at ambient temperature for one hour, the solution was diluted to 40 mL with 5 mM NH_4HCO_3. Product was purified by anion exchange chromatography on a column of benzyl DEAE cellulose (1 X 20 cm, column volume = 10 mL). Non-radioactive 8-N_3-5'-AMP (20 μg) was added before sample application. The

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chromatography was developed by applying a linear gradient formed between 200 mL of 5 mM NH_4HCO_3 and 200 mL of 200 mM NH_4HCO_3. Fractions (8 mL) were collected and radioactivity determined. Aliquots from the enzymatic reactions and pooled fractions from the chromatography were analyzed by TLC on 5 x 20 cm PEI-F cellulose plates (0.75 M KH_2PO_4, pH 3.5) R_f: ATP = 0.21; bis-phosphate (4) = 0.44; 8-N_3-5'-AMP (5) = 0.55). Fractions containing [^{32}P]-8-N_3-5'-AMP were lyophilized, redissolved in a small volume of water and concentrated under reduced pressure on a centrifugal evaporator.

[α-^{32}P]-8-N_3-Adenine nicotinamide adenine dinucleotide (1). To the concentrate containing 8-N_3-5'-AMP (5) was added β-NMN (0.3 mg, 0.84 μmol) in 10 mL H_2O followed by 50 mL pyridine and N, N'-1,3-diisopropylcarbodiimide (2 μL, 12.8 μmol). The reaction was incubated at 4 °C for four days, with intermittent addition of carbodiimide (2 μL) at intervals of 2-4 h during the day followed by 18 h overnight incubation. The dinucleotide was purified in two chromatographic steps. The crude reaction mixture was diluted to 40 mL with 5 mM NH_4HCO_3, non-radioactive 8-N_3-NAD (0.3 mg, 0.43 μmol) was added and the mixture applied to a 20 mL benzyl DEAE cellulose column (1 x 30 cm). The dinucleotide eluted almost at the start of a linear gradient formed between 5 and 150 mM NH_4HCO_3 (200 mL each). Fractions containing the dinucleotide were pooled and lyophilized. The lyophilizate was redissolved in water (40 mL) and applied to an AG1-X2 column (20 mL; 1 x 30 cm), acetate form. Here, [α-^{32}P] 8-N_3-NAD eluted in the latter half of the linear gradient formed between 200 mL of water and 200 mL of 2 M acetic acid. Appropriate fractions were pooled and lyophilized. The photoprobe was further concentrated on a centrifugal evaporator. Aliquots from the coupling reaction and the purified fractions were analyzed by TLC on 5 x 20 cm PEI-F cellulose plates (0.25 M KH_2PO_4, pH 3.5) R_f: 8-N_3-5'-AMP (5) = 0.34; 8-N_3-NAD (1) = 0.5. A molar extinction coefficient of 1.6 x 10^{+4} (λ_{max} = 274 nm) was determined by total phosphate analysis of chromatographically pure 8-N_3-NAD (literature (5) λ_{max} = 274; ε = 1.5 X 10^{+4}). Total phosphate was determined according to the method of Ames (16).

RESULTS AND DISCUSSION

The starting material 8-N_3-3'-AMP (3) was prepared from adenosine-3'-phosphate as shown in Scheme I. 8-Br-3'-AMP (2) was first obtained by treating 3'-AMP with bromine water in buffered aqueous solution at pH 4 (1,14). The bromo- compound was converted to azide 3 and purified by anion-exchange chromatography on DE-52 cellulose. Purity of 8-N_3-3'-AMP was established by TLC (PEI-F cellulose; 0.75 M KH_2PO_4) and anion exchange HPLC.

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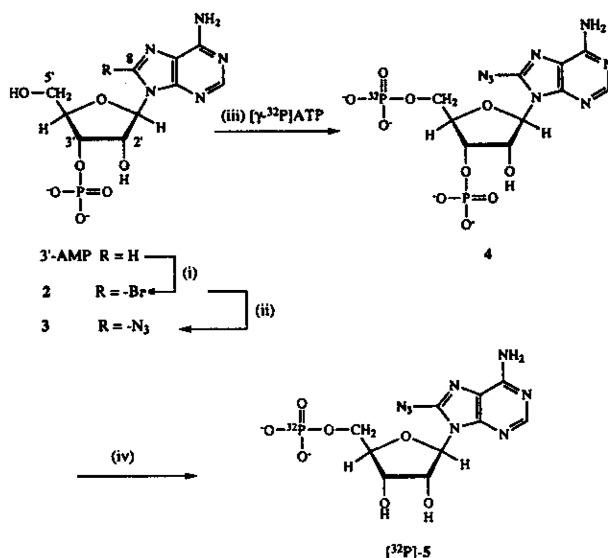
Scheme I. Syntheis of [^{32}P]-8-N_3-5'-AMP



Reagents and Conditions: (i) Br_2, NH_4OAc pH 3.8; (ii) triethylammonium azide, DMF; (iii) T4 polynucleotide kinase; (iv) nuclease P1

[^{32}P]-8-N_3-5'-AMP was prepared enzymatically from 8-N_3-3'-AMP (3) using the enzymes T4 polynucleotide kinase followed by nuclease P1. T4 polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to the 5'-hydroxy terminus of polynucleotides, oligonucleotides, and mononucleotides which contain a 3'-phosphate (17) whereas nuclease P1 is a 3'-specific phosphatase (18). 8-N_3-3'-AMP (3) was incubated with [γ-^{32}P]ATP and T4 polynucleotide kinase to obtain [5'-^{32}P]-8-N_3-3',5'-bisphosphate (4). Bis-phosphate 4 was then converted into [α-^{32}P]-8-N_3-5'-AMP (5) using nuclease P1. This general approach has been reported in the literature for the synthesis of related 5'-labeled nucleotides (13, 18).

In our first experiments on the enzymatic phosphorylation of 8-N_3-3'-AMP we observed the formation of large amounts of an extra unidentified radioactive component in addition to ATP, 8-N_3-3',5'-bisphosphate (4) and inorganic phosphate. This material typically had an R_f between that of ATP and 4 on our PEI-F cellulose TLC system. This impurity arose regardless of the source or purity of the starting 8-N_3-3'-AMP. The impurity was absent in experiments conducted under identical conditions using 3'-AMP as the substrate, where [5'-^{32}P]pAp was produced cleanly. The known sensitivity of azides to reduction by dithiothreitol (DTT) (19) and the presence of DTT in some commercial preparations of T4 polynucleotide kinase suggested that the azido group was being reduced to the amine under the conditions of phosphorylation. We confirmed this hypothesis by treating the mixture with nuclease P1 and obtaining [^{32}P]-8-amino-5'-AMP along with [^{32}P]-8-azido-5'-AMP and

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demonstrating that it was isographic with unlabeled 8-amino-5'-AMP. Authentic 8-amino-5'-AMP was obtained by reducing 8-azido-5'-AMP with DTT according to the procedure described by Cartright (19). In all the above experiments where the impurity was observed the enzyme was supplied in a buffer which contains 1 mM dithiothreitol, a reducing agent which is capable of reducing the azido group to the amino group. However, the rate of reduction is about two orders of magnitude slower with monothiols like β-mercaptoethanol (19). Therefore, solutions of polynucleotide kinase containing 10 mM β-mercaptoethanol (Pharmacia) were used for this reaction and the formation of 8-N_3-3',5'-bis-phosphate and the concomitant consumption of [γ-^{32}P]ATP was closely monitored by TLC. Upon prolonged incubation at ambient temperature, a small amount of the 8-amino-bisphosphate was still formed. Subsequent treatment of this reaction mixture with nuclease P1 yielded a mixture of [^{32}P]-8-azido and [^{32}P]-8-amino-5'-monophosphates. The formation of the amine can be suppressed or prevented entirely by decreasing the incubation time, increasing the temperature and stopping the reaction as soon as the formation of 4 is complete, but before appreciable 8-amine is formed. Thus, the composition of the enzyme buffer as well as the incubation time and temperature were found to be critical to the success of the T4 polynucleotide kinase catalyzed reaction. However, the inorganic ^{32}P, [^{32}P]-8-N_3-5'-AMP and [^{32}P]-8-amino-5'-AMP in the final reaction mixture were separable by anion exchange chromatography (Figure 1), enabling us to remove small quantities of [^{32}P]-8-amino-5'-AMP which were formed in the synthesis.

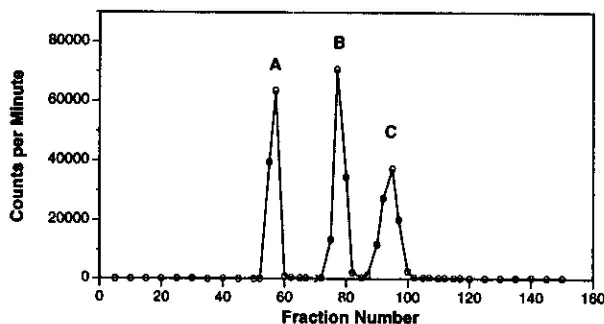

Figure 1. Purification of [^{32}P]-8-azido-5'-AMP by anion exchange chromatography on DEAE cellulose. Peak A: inorganic ^{32}P-phosphate; Peak B: [^{32}P]-8-azido-5'-AMP (5); Peak C: [^{32}P]-8-amino-5'-AMP. The sample was applied to a column of benzyl DEAE cellulose (1 X 20 cm) and the chromatography developed by the application of a linear gradient formed between 200 mL of 5 mM NH_4HCO_3 and 200 mL of 200 mM NH_4HCO_3.

Purified [^{32}P]-8-azido-5'-AMP was coupled with β-nicotinamide mononucleotide (β-NMN) using 1,3-diisopropylcarbodiimide as a coupling reagent (Scheme II). For this reaction, adding the

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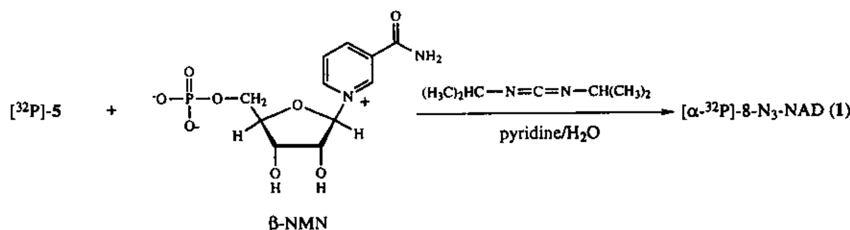
coupling reagent in small portions periodically led to a significant increase in the yield of the dinucleotide. Purification of [α-^{32}P]-8-N_3-NAD was performed in two chromatographic steps: anion exchange chromatography on benzyl DEAE cellulose (Figure 2A) followed by chromatography on Dowex 1-X2 (Figure 2B). [α-^{32}P]-8-N_3-NAD eluted from the benzyl DEAE column in the first half of the gradient and was contaminated with an unidentified component of the reaction mixture. This ^{32}P-labeled component was removed in the subsequent chromatography, where the impurity formed a leading shoulder of the major peak. Dowex 1-X2 chromatography by itself failed to separate [α-^{32}P]-8-N_3-NAD from all the components of the reaction mixture. Thus, both chromatographic steps were necessary to obtain pure material. The identity of [^{32}P]-8-N_3-5'-AMP and [α-^{32}P]-8-N_3-NAD were established by comigration of the radiolabeled compounds with authentic unlabeled markers on TLC plates and confirmed by treatment with alkaline phosphatase and NAD glycohydrolase respectively. Alkaline phosphatase converted [^{32}P]-8-N_3-5'-AMP to 8-N_3-adenosine and inorganic phosphate (^{32}P) which was identified by comparison to authentic ^{32}P. NAD glycohydrolase converted [α-^{32}P]-8-N_3-NAD to [α-^{32}P]-8-N_3-ADP-ribose which had an R_f almost identical to ADP-ribose.

Scheme 2: Synthesis of [α-^{32}P]-8-N_3-NAD (1)



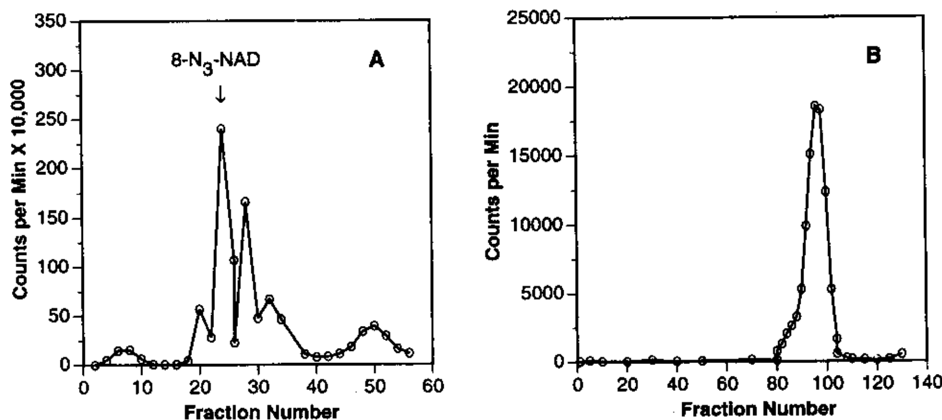


Figure 2. Purification of [α-^{32}P]-8-N_3-NAD (1) by anion exchange chromatography. A: chromatography on benzyl DEAE cellulose; B: re-chromatography on AG 1-X2 anion exchange resin. A description of the details of the conditions of chromatography are given in the Experimental section.

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