# *N*-(2-Naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol, a Fluorescent Cholesterol Analogue†

Yin J. Kao,‡ Anne K. Soutar,§ Kong-Yi Hong, Henry J. Pownall, and Louis C. Smith*

ABSTRACT: Naturally occurring structural analogues of cholesterol were tested as substrates for lecithin:cholesterol acyltransferase and cholesterol oxidase. Minor differences in reactivity were associated with the nature of the 17$\beta$ substituent of the sterol nucleus. A cholesterol analogue, *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol, was synthesized by reaction of 3$\beta$-acetoxy-23,24-dinor-5-cholen-22-oyl chloride with 2-aminonaphthalene followed by reduction of the intermediate amide with $LiAlH_4$. The analogue showed high structural and functional similarity to cholesterol by the following criteria: comparable limiting areas in surface monolayers, precipitability by digitonin, and reactivity as a substrate in the enzymic reaction catalyzed by lecithin:cholesterol acyltransferase. The position of $\lambda_{max}$ of the fluorescent emission of *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol in a series of solvents and in phosphatidylcholine vesicles indicated that the hydrocarbon region of the phospholipid bilayer is quite polar, comparable to acetonitrile. Cholesterol, 30 mol % in the vesicles, reduced the apparent polarity of the bilayer interior to that of 2,2,4-trimethylpentane. The solvent-sensitive fluorescence properties of *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol reveal a previously unrecognized role of cholesterol, i.e., the regulation of the polarity of the hydrocarbon region of membranes.

The importance of cholesterol as a regulator of lipoprotein and membrane function is well recognized (Demel & de Kruyff, 1976; Jain, 1975). Molecular details of the role of cholesterol have been difficult to elucidate because there are few spectral features which are sensitive to the microenvironment of cholesterol in membranes. Several compounds similar in structure to cholesterol have been used in studies of cholesterol function in membranes. For example, quenching of the fluorescence of a cholesterol analogue, 1-methyl-19-norcholesta-1,3,5(10)-trien-3-ol, was used to determine the rate of movement from one side of bilayer vesicle to the other (Smith & Green, 1974a). This sterol contains a planar aromatic nucleus with a phenolic hydroxyl group in lieu of the A ring and the secondary 3$\beta$-alcohol of cholesterol. The stereochemistry of the ring systems and the ionization of the hydroxyl groups are not comparable and may account for the difference in the rate of transbilayer movement of this analogue, $t_{1/2}$ = 72 min, and that for [$^3$H]cholesterol, $t_{1/2} > 3$ day (Pozanansky & Lange, 1976). By contrast, measurement of 3-thiocholesterol indicated that about 70% of the sterol was in the exterior leaflet of a single bilayer vesicle of phosphatidylcholine (Huang et al., 1970). This distribution has been confirmed by other techniques (Pozanansky & Lange, 1976; Huang et al., 1974). A conjugated sterol, choles-5,7,9(11)-trien-3$\beta$-ol, was used as a probe of cholesterol:apoprotein interaction in plasma lipoproteins (Smith & Green, 1974b). Generally, there has been little comparison of the physical and biological properties of these analogues with cholesterol. The acceptance of these compounds as probes of cholesterol function depends substantially on such studies.

In this report we have determined which features of the 17$\beta$ substituent of cholesterol are necessary for action of lecithin; cholesterol acyltransferase,[1] LCAT, and cholesterol oxidase. The results of our experiments indicated that substitutions in the alkyl side chain at the 17$\beta$ position did not affect enzymic activity appreciably. Consequently, we undertook the synthesis of several cholesterol analogues in which a spectroscopic reporter group replaced the 17$\beta$-alkyl substituent. Physical and enzymic studies showed that one of these analogues, *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol, NCA, was essentially identical with cholesterol.[2] In addition, the fluorescence properties of this analogue were dependent on solvent polarity. With this new membrane probe, we were able to identify a previously unknown role of cholesterol, modulation of the solvent-like polarity of the hydrocarbon region of phosphatidylcholine bilayers. A preliminary report of this work has appeared (Smith et al., 1974).

## Experimental Procedures

### *A. Synthesis*

The general scheme for synthesis of cholesterol analogs (Figure 1) involved reaction of an amine with 3$\beta$-acetoxy-23,24-dinor-5-cholen-22-oyl chloride. After purification the intermediate amide was reduced with $LiAlH_4$ to give the desired product.

†From the Departments of Medicine and Biochemistry, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030. *Received October 4, 1977; revised manuscript received March 14, 1978.* This work was supported by the Robert A. Welch Foundation, Grant Q-343; The American Heart Association 73-778, U.S. Public Health Service HL-15648 and 17679, and NHLBI Lipid Research Clinic Contract 71-2156. H.J.P. and L.C.S. are Established Investigators of The American Heart Association.

‡ Robert A. Welch Predoctoral Fellow.

§ Current address: MRC Lipid Metabolism Unit, Hammersmith Hospital, London W12 OHS, England.

[1] Abbreviations are: LCAT, lecithin:cholesterol acyltransferase; NCA, *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; Ans, 1-anilinonaphthalene-8-sulfonic acid; $T_m$, thermotropic melting temperature of the phosphatidylcholines; ESR election spin resonance; cmc, critical micelle concentration.

[2] *N*-(2-Naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol suppressed 3-hydroxy-3-methylglutaryl-CoA reductase in cultured normal human fibroblasts as effectively as did cholesterol (S. H. Gianturco, unpublished experiments).

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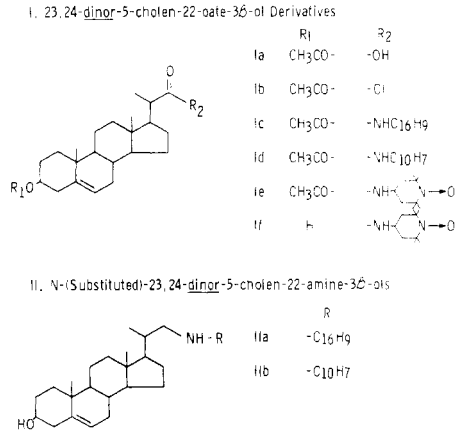


FIGURE 1: Derivatives of 23,24-dinor-5-cholen-22-amid-3β-ol and 23,24-dinor-5-cholen-22-amin-3β-ol. The 3-pyrenyl and 2-naphthyl groups are $C_{16}H_9$ and $C_{10}H_7$, respectively.

*3β-Acetoxy-23,24-dinor-5-cholen-22-oic Acid (Ia).* This compound, purchased from Steraloids, Inc., was purified by precipitation of the commercial material from benzene with 2 N NaOH. The free acid was regenerated in benzene with 2 N HCl after the soluble impurities were removed by filtration. Following removal of solvent, the residue of 3β-acetoxy-23,24-dinor-5-cholen-22-oic acid was treated with activated charcoal in acetone, recrystallized from the same solvent, and stored in a vacuum desiccator, mp 232–234 °C [lit. mp 232–236 °C (Fernholz, 1933)]. All melting points are uncorrected.

*3β-Acetoxy-23,24-dinor-5-cholen-22-oyl Chloride (Ib).* Four grams of purified Ia was dissolved with stirring in 100 mL of dry benzene containing 5 mL of oxalyl chloride and 0.1 mL of pyridine. After 2 h, the solvent and unreacted oxalyl chloride were removed under vacuum. Rotary evaporation of additional dry benzene removed traces of oxalyl chloride. For analysis, the product was recrystallized from benzene as needle-like crystals. Recrystallization usually was omitted: yield, 4.1 g; mp 130 °C [lit. mp 130–131 °C (Cole & Julian, 1945)].

*3-Acetoxy-N-(3′-pyrenyl)-23,24-dinor-5-cholen-22-amide (Ic).* To 4 g of Ib dissolved in 100 mL of benzene, 5 g of 3-aminopyrene (Aldrich) was added in small portions over 10 min. The amide, which precipitated during reflux, was collected after 4 h and washed successively with benzene, 1 N HCl, and $H_2O$: yield 6 g; mp >300 °C. After recrystallization from THF, only one fluorescent spot was found on an Eastman silica gel chromagram developed in chloroform:hexane (70:30 v/v). Mass spectra were obtained using an LKB Model 9000 single focusing instrument with a direct inlet probe; ionizing energy was 70 eV. Mass spectrum: m/e (relative abundance) 587 ($M^+$, 42%); 52 (7%); 283 (7%); 272 (4%); 244 (7%); 217 (100%); 201 (4%). For this and other new compounds, the relative abundances of other ions were less than 2%, or the *m/e* was less than 200.

*N-(3′-Pyrenyl)-23,24-dinor-5-cholen-22-amin-3β-ol (IIa).* Ic was refluxed with 2 g of $LiAlH_4$ in 200 mL of tetrahydrofuran for 24 h. After addition of 5 mL of ethyl acetate and 200 mL of 2 N NaOH, the product was obtained by extraction with 200-mL portion of $CH_2Cl_2$. The dried residue from the organic layer was triturated with benzene. Light yellow plates from benzene were stored protected from light in a vacuum desiccator. During preparation and handling, light was excluded whenever possible to minimize photodecomposition: yield 1.4 g; mp 280 °C (dec). Mass spectrum: *m/e* (relative abundance) 531 ($M^+$, 60%); 513 (1%); 230 (100%); 217 (11%); 202 (19%). With excitation at 350 nm, fluorescence maxima typical of pyrene appeared at 380 nm, 405 nm, and 425 nm with relative intensities of 1:0.7:0.23, respectively, in ethanol.

*3β-Acetoxy-N-(2-naphthyl)-23,24-dinor-5-cholen-22-amide (Id).* Three grams of 2-naphthylamine (Aldrich) was added in small portions during 10 min to 4 g of Ib dissolved in 100 mL of benzene. After refluxing overnight, 2 N NaOH was added and the solution was dried by rotary evaporation. The amide was recrystallized from aqueous acetone as colorless rhombic crystals: yield 4.2 g; mp 214–216 °C. Mass spectrum: *m/e* (relative abundance) 513 ($M^+$, 9%); 453 (26%); 283 (5%); 199 (7%); 173 (9%); 143 (100%). With excitation at 355 nm, fluorescent emission maxima occurred at 388, 410, and 430 nm with relative intensities of 1:0.7:0.24, respectively, in ethanol:$CH_2Cl_2$ (2:1).

*N-(2-Naphthyl)-23,24-dinor-5-cholen-22-amin-3β-ol (IIb).* A solution of 1 g of Id and 2 g of $LiAlH_4$ in 100 mL of tetrahydrofuran was refluxed for 16–24 h. Product formation was monitored by analytical reversed phase chromatography on Waters C-18 Bond-A-Pak, 37–74 μm (Waters Associates) in 80% ethanol. When product formation ceased, 5 mL of ethyl acetate and 500 mL of 2 N NaOH was added and the product was extracted with three aliquots of 200 mL of $CH_2Cl_2$. The amine in the combined organic layers was further purified by preparative reverse phase chromatography and was finally crystallized from $CH_2Cl_2$ to yield pale yellow needles. Only one component was detected after recycling ten times in this solvent system: yield 0.6 g; mp 180–182 °C. Mass spectrum: *m/e* (relative abundance) 457 ($M^+$, 100%); 439 (5%); 271 (18%); 212 (7%); 196 (7%); 183 (14%); 170 (7%); 155 (25%); 142 (59%); 127 (39%). Spectra in various solvents are recorded in Figure 4.

*3β-Acetoxy-N-[4′-(1′-oxyl-2′,2′,6′,6′-tetramethylpiperidinyl)]-23,24-dinor-5-cholen-22-amide (Ie).* To 3.9 g of purified Ia and 4.2 g of dicyclohexylcarbodiimide (Aldrich) dissolved in methylene chloride were added 2 g of 1-oxyl-4-amino-2,2,6,6-tetramethylpiperidine (Eastman) in methylene chloride over 20 min. After refluxing overnight, the reaction mixture was treated with 2 N NaOH, filtered, and chromatographed on silica gel (Baker, 60–200 mesh) in chloroform:hexane (85:15). The products were located by gel comparison of aliquots of the reaction mixture, starting materials and column fractions by TLC. Brick-red crystals formed as the solvent evaporated: yield 3.5 g; mp 258 °C (dec). Mass spectrum: *m/e* (relative abundance) 541 ($M^+$, 2%); 526 (1%); 511 (5.5%); 481 (2.8%); 456 (3%); 395 (9%); 388 (3%); 328 (6%); 299 (1.3%); 283 (6%); 121 (100%). The absorption maximum occurred at 457 nm, with a molar extinction coefficient of 1700.

*N-[4′,4′-(1′-Oxyl-2′,2′,6′,6′-tetramethylpiperidinyl)]-23,24-dinor-5-cholen-22-amid-3β-ol (If).* The acetyl group was removed from 130 mg of If by refluxing in 30 mL of ethanol containing 30 mg of KOH for 30 min. After the solvent was removed under vacuum, the product was dissolved in $CH_2Cl_2$ and filtered to remove all insoluble material. Light orange crystals were recovered by evaporating the solvent: yield 113 mg; mp 198–201 °C. Mass spectrum: *m/e* (relative abundance) 499 ($M^+$, 16%); 484 (14%); 469 (13%); 413 (40%); 346 (15%); 301 (9%); 283 (9%); 172 (8%); 154 (14%); 140 (34%); 124 (100%).

*Methyl 2-Bromododecanoate (Rogozinski, 1964) and Methyl 12-Bromododecanoate (Woolford, et al., 1964).* Reaction of the appropriate bromoacids (Pfaltz and Bauer) with oxalyl chloride followed directly by treatment with methanol after removal of excess oxalyl chloride gave the desired esters, which were chromatographed on silica gel (Baker,

60–200 mesh) in benzene. The esters contained no contaminants as visualized by $I_2$ vapors after thin-layer chromatography on silicic acid in ether:hexane:acetic acid (90:10:1 v/v/v).

*1-N-[4-(3′-Pyrenyl)butanoyl]amino-2-(11-bromoundecanoyl)propanol and 1-N-[4-(3′-Pyrenyl)butanoyl]amino-2-dodecanoylpropanol.* 4-(3′-Pyrene)butanoic acid, 2.3 g in 50 mL of benzene containing 2 drops of pyridine as catalyst, was added dropwise to 5 mL of oxalyl chloride. The mixture was irradiated in a bath sonicator for 30 min. Benzene and excess oxalyl chloride were then removed under reduced pressure. 4-(3-Pyrenyl)butanoyl chloride was dissolved in 10 mL of benzene and added dropwise to 50 mL of redistilled 1-amino-2-propanol. After reflux for 2 h, the reaction mixture was taken to dryness in vacuo. The residue was dissolved in methylene chloride and washed twice with 1 N HCl and 1 N NaOH. The product was recovered as yellow crystals from methylene chloride and used without further characterization.

11-Bromoundecanoyl chloride and dodecanoyl chloride were synthesized by reaction of the corresponding fatty acids with oxalyl chloride in benzene. The acyl chlorides, isolated and used without characterization, were reacted with 1-*N*-[4-(3′-pyrenyl)butanoyl]amino-2-propanol in methylene chloride for 30 min. The final purification was accomplished by chromatography on silicic acid in $CH_2Cl_2$/MeOH (99:1). Both products were white crystals. 1-*N*-[4-(3′-pyrenyl)butanoyl]-amino-2-(11-bromoundecanoyl)propanol, mp 64–66 °C. Mass spectrum: *m/e* (relative abundance) 593 ($M^+$, 21%); 591 (18%); 379 (9%); 377 (7.3%); 365 (19%); 363 (19%); 322 (54%); 320 (36%); 207 (73%); 305 (59%); 284 (66%); 279 (52%); 277 (47%); 265 (38%); 263 (36%); 249 (42%); 247 (40%); 243 (49%); 229 (68%); 225 (100%); 213 (34%). 1-*N*-[4-(3-pyrenyl)butanoyl]amino-2-dodecanoylpropanol, mp 67–69 °C. Mass spectrum: *m/e* (relative abundance) 527 ($M^+$, 100%); 344 (5%); 328 (9.1%); 287 (2.2%); 271 (6.1%); 243 (3.4%); 229 (17%); 215 (14%); 201 (614%).

### *B. Spectroscopic Studies*

The UV absorption spectra were obtained with a Beckman Acta V spectrophotometer and fluorescent spectra were recorded on an Aminco-Bowman spectrophotofluorometer. Fluorescence lifetimes were obtained at room temperature, 23 °C, with a SLM Model 480 subnanosecond spectrofluorimeter. Single bilayer phospholipid vesicles containing cholesterol or NCA were prepared by the method of Batzri & Korn (1973). An ethanolic solution of phosphatidylcholine and sterol, 10 μL, was injected with a Hamilton syringe into 1.0 mL of 50 mM Tris-Cl, pH 7.4, containing 1 mM EDTA and 100 mM NaCl, at 37 °C. To obtain quenching constants, the spectra of the fluorophore in 2 mL of ethanol were obtained before and after addition of 10-μL aliquots of either 1 M ethanolic solutions of 2-bromododecanoic acid, 12-bromododecanoic acid, methyl 2-bromododecanoate, and methyl 12-bromododecanoate or 5 M aqueous KI solution containing $10^{-4}$ M $Na_2S_2O_3$ as a reducing agent. No correction for the dilution was made since the dilution was less than 5% in all cases. All spectra are uncorrected.

The temperature dependence of the fluorescence of NCA was measured with a fluorimeter constructed in this laboratory. This instrument gives a plot of intensity vs. temperature automatically as the temperature of the sample changes. A 200-W high pressure mercury lamp with a stable power supply (less than 1% RMS; Opti-Quiet Co.), coupled with a GCA/McPherson monochrometer Model EU-700, was used as the light source. A light tight box with a thermostatically controlled cuvette holder in the center was attached to the monochrometer. The temperature of the sample was controlled by a Laude R-2/K thermoregulator and monitored by a thermocouple, directly immersed in the sample. The thermocouple was connected to the X axis of the X-Y recorder. The fluorescent emission was monitored with a photomultiplier tube (IP22) at a 90° angle after passage through a 390-nm interference filter. Photocurrent from the phototube was amplified with an operational amplifier. The signal was connected to the Y axis of the X-Y recorder. The rate of temperature change was controlled by a linear programmer at 0.5° $min^{-1}$. Slower rates of temperature change (0.1° $min^{-1}$) gave essentially identical results.

### *C. Characterization in Monomolecular Films*

Changes in surface pressure were measured in a polyfluorethylene trough, 10 × 50 × 0.5 cm with a Cahn recording electrobalance with a lightly sanded Pt plate, 1.0-cm perimeter. Changes in the surface potential were measured with an apparatus similar to that described by Shah & Schulman (1965) with a $^{208}Po$ (3M Co.) as the α source. The reference electrode was Ag coated with AgCl. The aqueous subphase contained 20 mM Tris-Cl, pH 7.4, and 150 mM NaCl. The surface film was compressed by a motorized barrier which moved at a rate of 0.3 cm $min^{-1}$. The limiting surface areas were reproducible within 5%.

### *D. Characterization as Enzyme Substrate*

LCAT activity was measured as described by Soutar et al. (1974) with single bilayer vesicles of phosphatidylcholine containing 10 mol % sterol as the substrate. The steryl ester formation was quantified by liquid scintillation counting after isolation of the product by thin-layer chromatography. The LCAT preparation had been purified about 1000-fold (Soutar et al., 1975) and was about 25% pure compared with the apparently homogeneous preparation of Albers et al. (1976). The reaction mixture at 37 °C contained 40 μg of partially purified LCAT, 1 μmol of egg PC containing 0.05 μCi of [U-$^{14}C$]-labeled algae PC and 10 mol % of either cholesterol or NCA. Cholesterol oxidase activity was measured as previously described (Allain et al., 1974). The initial rates of reaction were obtained from the first 10% of the reaction monitored with a Gilford recording spectrophotometer.

### *E. Precipitation with Digitonin*

NCA was characterized by coprecipitation with digitonin (Dittmer & Wells, 1969). The sterol, 0.5 μmol of [$^3H$]cholesterol or NCA, dissolved in 2 mL of acetone–ethanol (1:1), was added to 1 mL of 0.5% digitonin in 50% ethanol. The precipitates were collected after 24 h at 23 °C with a G-51 glass fiber filter (Whatman). The NCA in the filtrate was quantified by absorption at 288 nm.

### *F. Materials*

All solvents (Burdick and Jackson; Mallinckrodt Nanograde) were used without further purification. Egg phosphatidylcholine was isolated by the method of Singleton et al. (1965) and purified on alumina (Wells & Hanahan, 1969). The purities of cholesterol, demosterol, and β-sitosterol (Applied Science), DMPC and DPPC (sigma), and DLPC (Supelco) were established to be greater than 98% by thin-layer chromatography before use. DMPC containing [1-$^{14}C$]-myristic acid in the 2-position (0.36 mCi/mg) was supplied by Applied Science; [U-$^{14}C$]phosphatidylcholine was from algae (1.846 Ci/mmol), [26-$^{14}C$]demosterol (13.33 Ci/mol), [22,23-$^3H$]-β-sitosterol (41.6 Ci/mol), and [7-$^3H$]dehydro-

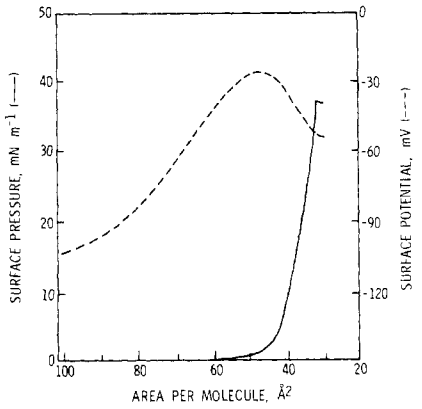


FIGURE 2: Surface properties of *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol. NCA, 18 $\mu$L of a 2 mM solution in ether, was added to the monolayer tray. After 2 min, the initial surface area of 260 cm$^2$ was reduced by a motorized barrier at 2 cm min$^{-1}$ as described in Experimental Procedures while the surface pressure and surface potential were recorded continuously.

epianodrosterone (41.6 Ci/mol) were by New England Nuclear, and [G-$^3$H]cholesterol (3 Ci/mol) was by Amersham/Searle.

## Results

*Enzyme Specificity.* Naturally occurring cholesterol analogues were tested as substrates in the reaction catalyzed by LCAT to determine if the enzyme was specific for the 17$\beta$ substituent of the cholesterol. In single bilayer vesicles of 0.4 mM DLPC containing 0.04 mM sterol, 125, 180, and 67 pmol of ester $\mu$g$^{-1}$ protein were formed h$^{-1}$, when cholesterol, desmosterol, and $\beta$-sitosterol, respectively, were acyl acceptors. Dehydroepiandrosterone was not effective as a substrate; only 6 pmol of steryl ester $\mu$g$^{-1}$ protein was formed h$^{-1}$. The lack of ester formation was attributed to the low amount of acyl acceptor in the vesicles, since about 75% of this polar sterol partitioned into the aqueous phase during concentration of the substrate vesicles.

To determine if various sterols would compete with cholesterol, LCAT activity was determined with DLPC vesicles containing 10 mol % [$^3$H]cholesterol and 10 mol % of unlabeled sterol, either cholesterol, $\beta$-sitosterol, desmosterol, or dehydroepiandrosterone. The relative activity of the respective sterols was 45, 49, 26, and 94% of the control vesicles in which there were no unlabeled sterols. These experiments indicated LCAT did not have high specificity for the 17$\beta$-side chain. On the basis of this information, the series of compounds derived from 23,24-dinor-5-cholen-22-oic acid-3$\beta$-ol was synthesized (Figure 1) and tested as substrates for LCAT.

*N*-(2-Naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol was a satisfactory substrate for LCAT. With DMPC as the acyl donor, NCA was about 50% as active as cholesterol as an acyl acceptor. When the reaction was measured by transacylation with $^{14}$C-labeled algae PC in egg PC, NCA was 70% as efficient as cholesterol. In 30 min, 1.63 nmol of cholesteryl ester were formed. Since NCA functioned as a reasonable good substrate for LCAT, this analogue of cholesterol was characterized further.

It has been reported cholesterol oxidase does not have high specificity for cholesterol; a steroid nucleus with a 3$\beta$-hydroxyl group and a bulky substituent are necessary for activity (Brooks & Smith, 1975; Munster et al., 1976). Under our experimental conditions, cholesterol was oxidized much faster than all the other sterols tested. The relative velocity for desmosterol, $\beta$-sitosterol, dehydroepiandrosterone, and NCA was 50, 40, 6, and 20%, respectively, of the reaction rate observed with cholesterol.

Steryl ester formation was shown qualitatively with the 3-pyrenylamine analogue (IIIb) with egg phosphatidylcholine as the acyl donor; however, no further experiments were done since the pyrene-containing analogues, IId and IIIb, were extremely sensitive to light and decomposed during purification. The spin-labeled cholesterol analogue (IIg) showed no activity in the LCAT assay system, although it was shown by the absorption spectra to be present in the substrate vesicles. The lack of activity of the spin-labeled cholesterol analogue was ascribed to improper orientation at the interface. The nitroxide appeared to be sufficiently more polar than the 3$\beta$-hydroxyl group of the sterol, that the molecule was oriented with the nitroxide moiety in the interface. The hyperfine splitting of the ESR spectrum (Knauer & Napier, 1976) of IIb was identical with that of 16-doxylstearate below the cmc, confirming the presence of the nitroxide group in the aqueous phase.

*Comparison of NCA and Cholesterol in Monomolecular Films.* The relationship of the surface pressure and surface potential of NCA with molecular area is shown in Figure 2. The limiting areas of NCA and cholesterol were 43 and 39 Å$^2$ (Demel et al., 1972), respectively, indicating that both molecules occupied approximately the same area in the monolayer. The force area curve of NCA was similar to cholesterol with a collapse pressure of about 37 dynes. The surface potential curve decreased after the limiting area per molecule was reached. This transition is ascribed to a change in the orientation of naphthyl group from parallel to perpendicular to the plane of the interface.

Phosphatidylcholine containing saturated fatty acids are known to form mixed films with cholesterol in which the area per molecule is less than the sum of the areas occupied by the two lipids in pure component films (Demel et al., 1967). For 33 mol % NCA and cholesterol, respectively, in mixed films with DPPC, the reduction in area per molecule was 10.5% and 11%.

*Precipitation of NCA and Cholesterol by Digitonin.* Digitonin forms an insoluble precipitate with cholesterol (Sperry & Webb, 1950) and is highly specific for the $\beta$-hydroxyl group. The ability of NCA and cholesterol to precipitate digitonin was comparable. Under the experimental conditions, 92% of cholesterol was precipitated by digitonin as compared with 89% of NCA.

*Orientation of NCA in Single-Walled Phosphatidylcholine Vesicles.* To establish that orientation of a fluorescent compound in a phospholipid bilayer could be determined by quenching, two derivatives of 1-*N*-[4-(3-pyrenyl)]butanoylamino-2-propanol were synthesized. 11-Bromoundecanoyl group was used as the heavy atom quencher; an unsubstituted fatty acid, dodecanoic acid, was used for reference. The results with these compounds allow us to evaluate the quenching of NCA in a phospholipid matrix.

A. Enhancement of Intramolecular Quenching by Orientation in a Phospholipid Vesicle. The fluorescent emission spectra of the 11-bromoundecanoyl and dodecanoyl esters of 1-*N*-[4-(3-pyrenyl)]butanoylamino-2-propanol had the usual pyrene spectral shape. At the same concentration, 10 $\mu$M, the fluorescence spectra of the two compounds are essentially identical, except for an expected small reduction (3%) in relative intensity in the brominated compound. The fluorescence spectra of the two compounds in egg yolk PC vesicles were quite different. The relative intensity of the compound containing the 11-bromo fatty acid was half that of the unsubstituted reference compound. Quenching is a function of molecular distance (Stryer & Haugland, 1967). The virtual ab-

TABLE I: *N*-(2-Naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol Fluorescence.

| Solvent | Emission max (nm) | Rel intensity | *Z* value | $E_T$ (30) |
|---|---|---|---|---|
| Benzene | 387.4 | 60 | 54 | 34.5 |
| 2,2,4-Trimethylpentane | 389.4 | 61 | 60.1 | |
| Dioxane | 394.3 | 71 | | 36.7 |
| Dimethoxyethane | 395.6 | 75 | 62.1 | 38.2 |
| Acetonitrile | 401.4 | 57 | 71.3 | 46.0 |
| 2-Propanol | 400.5 | 78 | 76.3 | 48.6 |
| 1-Butanol | 401.8 | 76 | 77.7 | 50.2 |
| 1-Propanol | 403.5 | 69 | 78.3 | 50.7 |
| Ethanol | 403.7 | 69 | 78.3 | 50.7 |
| Methanol | 405.2 | 51 | 83.6 | 55.5 |
| 80% ethanol | 407.5 | 89 | 84.8 | 53.6 |
| Ethylene glycol | 411.6 | 100 | 85.1 | 56.3 |
| 60% ethanol | 408.5 | 105 | 87.9 | |

sence of quenching in an isotropic solvent is consistent with a random orientation of the fluorophore and quencher. The reasonably efficient quenching in the vesicle indicates that confinement and orientation of these compounds in the phospholipid bilayer alter the configuration so that the heavy atom quencher and the fluorophore are next to each other in the interior of the bilayer.

B. Orientation of NCA in Single-Walled Vesicles. The Stern–Volmer (1919) plots of the quenching of NCA in ethanol by 2- and 12-bromododecanoic acid gave $K_{sv}$ of 41.5 and 6 $M^{-1}$, respectively. NCA fluorescence was not quenched by dodecanoic acid. The eightfold difference in the $K_{sv}$ showed that 2-bromododecanoic acid was significantly more potent as a quencher than was 12-bromododecanoic acid. The reason for the difference in quenching efficiency is not known. It is not due to the greater ionization of the 2-bromododecanoate ion, since the $K_{sv}$ values for both methyl 2-bromododecanoate and methyl 12-bromododecanoate, 55.9 and 18.2 $M^{-1}$, respectively, were both higher than the corresponding free acids.

The quenching of NCA fluorescence in phosphatidylcholine vesicles with 2-bromododecanoic acid was less than twice the quenching observed with 12-bromododecanoic acid. However, since the 2-bromo fatty acid is an eightfold better quencher when there are no restrictions on orientation or diffusion, we suggest that the relatively inefficient quenching indicates the fluorescent moiety of NCA in the bilayer is much closer to the heavy atom in 12-bromo isomer than in the 2 isomer. This conclusion that the orientation of NCA with the naphthylamino group inside the bilayer agrees with the efficient activity of this compound as an LCAT substrate.

*Fluorescence Properties of NCA.* We anticipated that the fluorescence properties of the arylamino group would be influenced by the nature of the solvent and thereby provide a measure of the polarity of the hydrocarbon region of a lipid bilayer. The wavelength of the fluorescence emission maximum, $\lambda_{max}$, was solvent dependent (Table I; Figure 3B). For example, in benzene, $\lambda_{max}$ was 387.4 nm, while in ethylene glycol, $\lambda_{max}$ was 414.6 nm. The relative fluorescence intensities of equivalent concentrations of NCA in ethylene glycol and 60% ethanol were about twofold greater than those found with methanol and acetonitrile as solvents. The relative fluorescence intensities of NCA in other solvents were found between these limits and were not correlated with solvent polarity. The fluorescence lifetimes of NCA in ethanol (8.6 ns), in acetonitrile (7.5 ns), in benzene (7.9 ns), and in ethylene glycol (15.5 ns) were not correlated with solvent polarity.

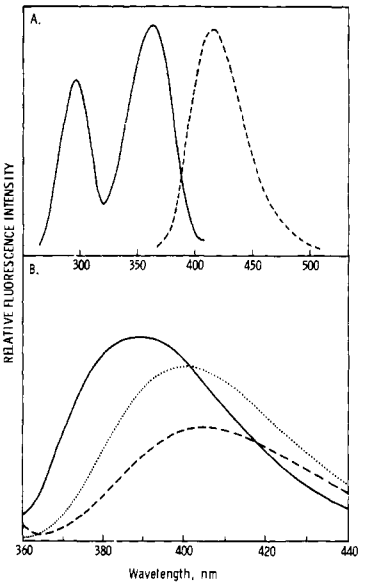


FIGURE 3: Fluorescence properties of *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol. (A). The concentration of NCA in ethylene glycol was 1.6 $\mu$M. For the emission spectrum (- - -), the excitation wavelength was 365 nm. The emission was monitored at 412 nm for the excitation spectrum (—). (B) The solvents were ethylene glycol (- - -), acetonitrile (· · ·), and benzene (—). NCA concentration was 1.6 $\mu$M, with the excitation wavelength set at 365 nm.

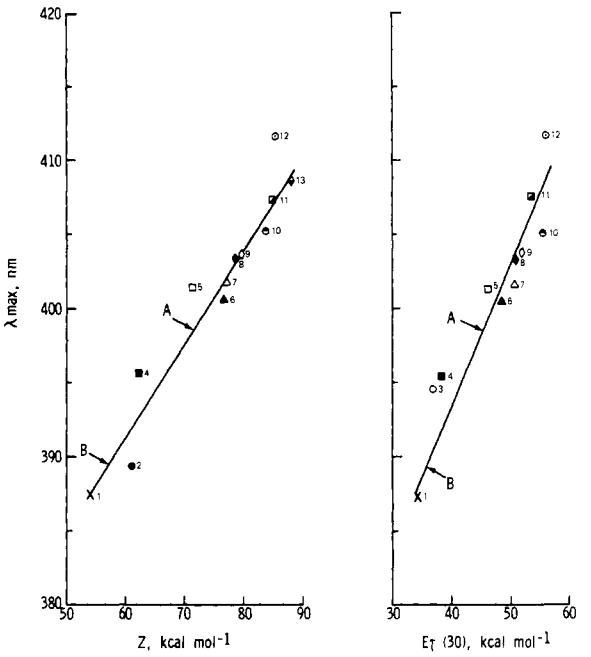


FIGURE 4: Effect of solvent polarity on *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol fluorescence. The solvents were benzene, 1 (X); isooctane, 2 (●); dioxane, 3 (○); dimethylethylene glycol, 4 (■); acetonitrile, 5 (□); 2-propanol, 6 (▲); 1-butanol, 7 (△); 1-propanol, 8 (♦); ethanol, 9 (◇); methanol, 10 (◓); 80% ethanol, 11 (◪); ethylene glycol, 12 (⊙); 60% ethanol, 13 (◈). The concentration of NCA was 10 $\mu$M with the excitation wavelength set at 365 nm. Point A indicates the $\lambda_{max}$ for 1 mol % NCA in 1 mM egg PC vesicles; point B denotes $\lambda_{max}$ for 1 mol % NCA in 1 mM egg PC vesicles which also contain 50 mol % cholesterol.

The $\lambda_{max}$ of NCA shifted to longer wavelengths with linearly increasing solvent polarity (Figure 4). When plotted on the basis of two different empirical measures of solvent polarity, the Kosower *Z* values (Figure 4A) (Kowsower, 1958) and the

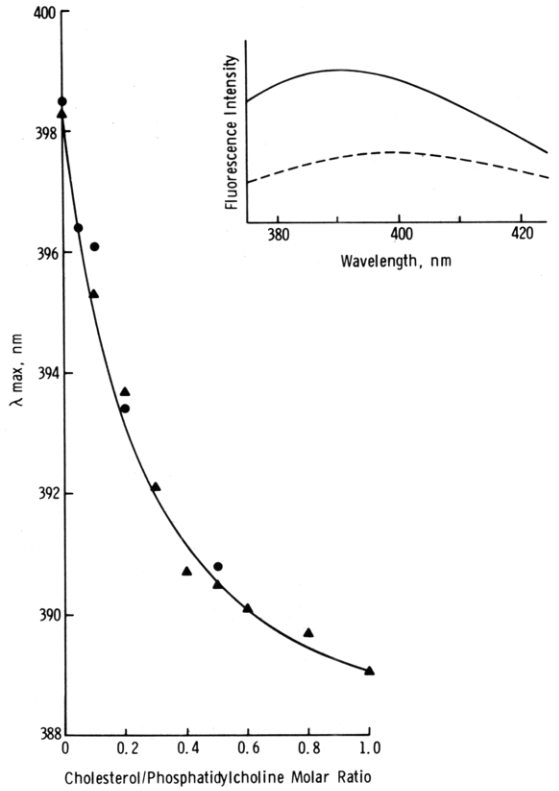


FIGURE 5: Effect of cholesterol on the $\lambda_{max}$ of *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3$\beta$-ol fluorescence in egg phosphatidylcholine vesicles. Egg PC, 1 mM, containing 1 mol % NCA and varying amounts of cholesterol were prepared in a solution consisting of 150 mM NaCl, 50 mM Tris-Cl, pH 7.4, and 1 mM EDTA. The spectra were recorded at 37 °C. The inset shows the spectra obtained for 0 (- - -) and 50 (—) mol % cholesterol.

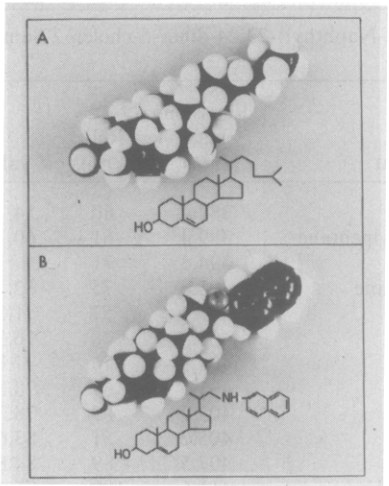


FIGURE 6: Space-filling models of cholesterol and *N*-(2-naphthyl)-23,24-dinor-cholen-22-amin-3$\beta$-ol.

Dimroth-Reichardt $E_T(30)$ values (Figure 4B) (Reichardt, 1965), there is an approximate linear correspondence of the $\lambda_{max}$ and solvent polarity for NCA. The correlation coefficient for $Z$ values and $\lambda_{max}$ was $r = 0.96$; for $E_T(30)$, $r = 0.95$.

The $\lambda_{max}$ of NCA in single bilayer vesicles of egg PC and DMPC were 395.5 and 396.5 nm, respectively, at 20 °C. The fluorescent cholesterol probe indicates that the polarity of the hydrocarbon region is surprisingly high, comparable to that of acetonitrile. The apparent $Z$ value is 71.5 (point A from the left panel of Figure 4A); for $E_T(30)$, the value is 45.5 (point A in the right panel of Figure 4B). The $\lambda_{max}$ of NCA in egg PC vesicles and in DMPC vesicles appeared at longer wavelengths at 37 °C, 398.5 and 399.2 nm, respectively. The apparent polarity increased with temperature above $T_m$, but was constant below $T_m$ between 21.8 and 5 °C. The fluorescence of NCA in DPPC multilayers is temperature dependent. The relative fluorescence intensity decreased about 10% between 41 and 40 °C. This change was reversed when the sample temperature was increased again. The change in the fluorescence intensity reflects the reorganization in the structure of the PC vesicles at the $T_m$, 41 °C (Ladbrooke & Chapman, 1969). In egg and 1-palmitoyl-2-oleyl-PC vesicles, there were no inflection points in the temperature profile; with DMPC multilayers, the inflection point was observed at 24 °C.

The effect of cholesterol on the polarity of the hydrocarbon region of egg PC vesicles is presented in Figure 5. With increasing amounts of cholesterol, the $\lambda_{max}$ decreased significantly and continuously. The apparent $Z$ value for egg PC vesicles containing an equimolar amount of cholesterol is 57, taken as point B in the left panel of Figure 4; the value of $E_T(30)$ is 36 (point B in the right panel of Figure 4). The polarity is comparable to that of 2,2,4-trimethylpentane. In these experiments, the phospholipid vesicles contained 1 mol % NCA. The $\lambda_{max}$ of 5 mol % NCA in NMPC vesicles was about 5 nm less than that found in egg PC vesicles at the same sterol content. The less polar hydrocarbon region of DMPC can be attributed to the uniformity of the polymethylene acyl moieties of the phospholipid.

## Discussion

The features that are necessary for a biologically active cholesterol analogue have been described in the literature. The structurally important regions of cholesterol are (1) intact alicyclic rings, (2) the 3$\beta$-hydroxyl group, (3) the planar double bond between C-5 and C-6, (4) the angular methyl groups, and (5) a branched seven-carbon alkyl side chain at 17$\beta$ position (Butler et al., 1970; Demel et al., 1972; de Kruyff et al., 1973). These determinants are present in both NCA and cholesterol, shown by the space filling models (Figure 6). The difference between NCA and cholesterol in actual limiting area values in surface monolayers is less than 10%. The slight differences in the compression curves of the surface films of cholesterol and NCA are attributed to the interactions of the planar aromatic nucleus of NCA. The condensing effect caused by NCA in DPPC monolayers is only slightly less than that of cholesterol and indicates the interaction between NCA and phosphatidylcholine is comparable to that of cholesterol. The results of the quenching studies show that NCA is oriented in the phosphatidylcholine matrix so that the 3$\beta$-hydroxyl group is at the interface and the naphthylamino group is in the apolar interior. There are no detectable differences between NCA and cholesterol in the digitonin complex formation, again indicating high structural similarity between the two molecules.

As a part of the evaluation of cholesterol analogues, two enzymatic assays were performed. The reactivity of NCA in the acyl transfer catalyzed by LCAT paralleled that of cholesterol (Soutar et al., 1974) and depended on the nature of the acyl donor, with egg PC being the most effective. The lower reactivity of NCA in the reaction catalyzed by cholesterol oxidase may be related to the polarity of the side chain which could alter the orientation with respect to the interface or possibly binding of the NCA to the enzyme. The physical structure of the usual substrate dispersion containing Triton X-100 is not known (Smith & Brooks, 1976). Cholesterol oxidase did not oxidize cholesterol, demosterol, $\beta$-sitosterol, or NCA in egg PC single bilayer vesicles. Only dehydroepian-

derosterone, which partitioned into the aqueous phase, was a substrate under these conditions.

The advantage of probe molecules is that they can be designed with unique reporter groups which will give spectroscopic information about their microenvironment. Fluorescent compounds have been used extensively as probles of both the structure and function of biological membranes (Brocklehurst et al., 1970; Waggoner & Stryer, 1970; Brand & Gohlke, 1972; Radda & Vanderkooi, 1972; Azzi, 1975). Generally, the relationship of the structural and functional features of the probe with those of the components of the membrane has not been considered. For example, Ans has no structural similarity with any membrane component. Consequently, the changes in fluorescence intensity of this probe are difficult to evaluate (Azzi, 1975). By a variety of criteria, NCA is a reasonable analogue of cholesterol.

The hydrocarbon region of biological membranes acts as a solvent for a variety of lipophilic molecules. The amounts of cholesterol, the degree of acyl chain unsaturation, and the nature of the phospholipid polar head group influence the solvent properties of the membrane and consequently the course of chemical and enzymic reactions that occur in this hydrophobic matrix. It is well established in organic chemistry that solvents influence the course of chemical reactions and, in many instances, determine which of several competing reactions give the principal products (Reichardt, 1965). Solvent polarity is basically an empirical quality and is the time and distance weighted sum of all intermolecular interactions in which the solvent may participate. These interactions create a cybotactic region, the volume around a solute molecule in which the order of the solvent molecules has been affected by the solute (Knauer & Napier, 1976). The $Z$ and $E_T(30)$ values are based on effects which the cybotactic region, as a microenvironment, has on solute molecules. For example, the lifetime of the chemically reactive species generated by metabolic activation (Heidelberger, 1975; Suwa et al., 1977; Hamman & Seliger, 1976), could conceivably be quite long in the membrane and modulated by the amount of cholesterol present.

Solvent polarity is an important factor in determining solubility (Karger et al., 1973). The rate of permeation of small molecules through a membrane depends on the solubility of these substances in the hydrocarbon region of the bilayer, or to be more precise, the partition coefficient between the aqueous phase and the hydrophobic bilayer (Ohki, 1976). Consequently, a change in the solvent polarity parameter of the bilayer will influence the rate of permeation through the membrane. It is well established that cholesterol decreases the rate of diffusion of small hydrophilic molecules through phospholipid bilayers (Inoue, 1974; Blok et al., 1977).

The fluorescent properties of NCA are ideally suited as a probe for this important membrane parameter, which has not been studied previously. The $\lambda_{max}$ of NCA shows the microenvironment of the fluorescent moiety in the phospholipid bilayer is relatively polar. With higher temperatures, the apparent polarity increases. By contrast, the addition of cholesterol shifts the $\lambda_{max}$ to shorter wavelength. Thus the apparent polarity at high cholesterol content corresponds to that of hydrocarbon solvents. We conclude that NCA is reasonably similar to cholesterol and mimics cholesterol in both monomolecular systems and in lipid aggregates. The fluorescence behavior of NCA should be highly useful in studying the effect of cholesterol on the solvent polarity properties of the hydrocarbon regions of various biological membranes, on the chemical events which occur in this microenvironment, and on the rate of permeation through these membranes.

Acknowledgment

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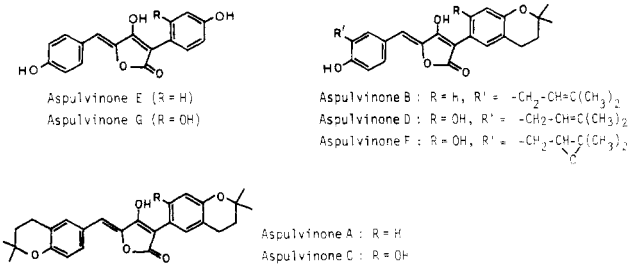
# Purification and Characterization of Dimethylallyl Pyrophosphate: Aspulvinone Dimethylallyltransferase from *Aspergillus terreus*†

Ikuko Takahashi, Nobutoshi Ojima,‡ Kyozo Ogura,* and Shuichi Seto

ABSTRACT: Dimethylallyl pyrophosphate:aspulvinone dimethylallyltransferase, the prenylation enzyme for the biosynthesis of aspulvinone pigments, has been purified from mycelia of *Aspergillus terreus*. The transferase catalyzed the transfer of the dimethylallyl moiety from dimethylallyl pyrophosphate to either of the two aromatic rings of aspulvinone E to give the mono- and diprenylated derivatives which were identified with the metabolites aspulvinone I and aspulvinone H, respectively. Aspulvinone G, another fundamental metabolite of this series, also acted as substrate to afford the corresponding diprenylated derivative, which is assumed to be a precursor for aspulvinone C, D, and F. The molecular weight of the enzyme was estimated to be 240 000–270 000 by gel filtration. Since the subunit molecular weight determined by NaDodSO$_4$–polyacrylamide disc gel electrophoresis was 45 000, the native enzyme appears to be a hexomeric protein composed of identical molecular weight subunits. The apparent $K_m$ values for aspulvinone E, aspulvinone G, and dimethylallyl pyrophosphate were 13.7, 7.7, and 40.0 $\mu$M, respectively. The enzyme shows the maximum activity at pH 7.0, and no metal ion is necessary for the activation. Sulfhydryl blocking agents or mercaptoethanol has no effect. Bromophenol blue binds specifically to the transferase and strongly inhibits the enzyme activity.

A large number of phenolic compounds with prenyl groups attached to the aromatic ring occur widely in nature. Although it is generally conceived that enzymatic prenylation of an aromatic nucleus is involved as the juncture step in the biosynthesis of such mixed terpenoid molecules, only a few papers have appeared describing the prenylation enzyme. Recent works from this laboratory have shown that *Aspergillus terreus* produces several yellow pigments called aspulvinones (Ojima et al., 1973, 1975a, 1976). These compounds have provided us a chance to study a prenylation enzyme, because they are of a series of prenylated phenol derivatives which seem to be biogenetically related with one another. We communicated preliminarily that a crude extract of *A. terreus* catalyzed the transfer of the isoprene unit from dimethylallyl pyrophosphate to the aromatic rings of aspulvinone E, the fundamental




compound of this series of pigments (Ojima et al., 1975b). The present paper reports the purification and characterization of this new prenylating enzyme, dimethylallyl pyrophosphate: aspulvinone dimethylallyltransferase.

## Materials and Methods

*Cell Culture. Aspergillus terreus* IAM 2054 (origin NRRL 1960 Raper ATCC 10020) were grown at 27 °C on Czapek Dox medium in stationary culture. Three-liter stationary culture flasks containing 1 L of medium were inoculated with 20 mL of a mycelial suspension from a 3-day-old shaking culture. After the culture was grown stationarily for 1 week,

† From the Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, Sendai 980, Japan. *Received February 7, 1978.* This work was supported by a Grant-in-Aid for scientific research (254168) from the Ministry of Education, Science, and Culture of Japan and by the Asahi Glass Foundation for the Contribution to Industrial Technology.
‡ Present address: Nippon Shinyaku Co., Ltd., Kyoto, 601, Japan.

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