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Pneumocysterol [(24*Z*)-ethylidenelanost-8-en-3β-ol], a rare sterol detected in the opportunistic pathogen *Pneumocystis carinii hominis*: Structural identity and chemical synthesis

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ABSTRACT ***Pneumocystis carinii* pneumonia (PcP) remains among the most prevalent opportunistic infections among AIDS patients. Currently, drugs used clinically for deep mycosis act by binding ergosterol or disrupting its biosynthesis. Although classified as a fungus, *P. carinii* lacks ergosterol. Instead, the pathogen synthesizes a number of distinct Δ^7, 24-alkylsterols, despite the abundance of cholesterol, which it can scavenge from the lung alveolus. Thus, the pathogen-specific sterols appear vital for organism survival and proliferation. In the present study, high concentrations of a C_{32} sterol were found in human-derived *P. carinii hominis*. The definitive structural identities of two C-24 alkylated lanosterol compounds, previously not reported for rat-derived *P. carinii carinii*, were determined by using GLC, MS, and NMR spectroscopy together with the chemical syntheses of authentic standards. The C_{31} and C_{32} sterols were identified as euphorbol (24-methylenelanost-8-en-3β-ol) and pneumocysterol [(24*Z*)-ethylidenelanost-8-en-3β-ol], respectively. The identification of these and other 24-alkylsterols in *P. carinii hominis* suggests that (*i*) sterol C-24 methyltransferase activities are extraordinarily high in this organism, (*ii*) 24-alkylsterols are important components of the pathogen's membranes, because the addition of these side groups onto the sterol side chain requires substantial ATP equivalents, and (*iii*) the inefficacy of azole drugs against *P. carinii* can be explained by the ability of this organism to form 24-alkysterols before demethylation of the lanosterol nucleus. Because mammals cannot form 24-alkylsterols, their biosyntheses in *P. carinii* are attractive targets for the development of chemotherapeutic strategies against this opportunistic infection.**

Sterols and their biosyntheses are excellent targets for chemotherapeutic attack against infectious microbes, especially the fungi. Polyene antibiotics such as amphotericin B bind avidly to ergosterol in fungal cell membranes. After the sterol–drug complexes aggregate, large pores in the membranes are formed, dissipating ion gradients. Fluconazole and some other compounds routinely used clinically for systemic mycosis target ergosterol biosynthesis at nuclear demethylation steps. Ergosterol was not detected in *Pneumocystis carinii carinii* that was isolated and purified from the lungs of corticosteroid-immunosuppressed rats. In this respect, the pathogen appears to be unlike higher fungi. However, the organism synthesizes its own distinct sterols, e.g., fungisterol (24-methylcholest-7-en-3β-ol and 24-ethylcholest-7-en-3β-ol; refs. 1–4). Parasites generally scavenge sterols (e.g., cholesterol) from the host and utilize them for membrane formation and other cell functions. If host sterols do not fulfill the precise stereochemical requirements of the parasite sterol, the pathogen synthesizes at least low levels of its own sterol for these vital functions. The parasite-specific sterols have been described as "metabolic" sterols, and represent attractive targets for drug development (5). Beside representing putative metabolic sterols, the rare occurrence of these molecules make these good markers—or signature lipids—of microorganisms. Improved diagnostic procedures for *P. carinii* pneumonia (PcP) could be developed based on the detection of *P. carinii*-specific sterols.

In the present study, two sterols that have not been reported for *P. carinii carinii* were detected in a *P. carinii hominis*-infected lungs, in human bronchoalveolar lavage fluid (BALF), and in organisms isolated from human lungs with PcP. The structural identities of C_{31} euphorbol and a rare C_{32} sterol, for which the trivial name pneumocysterol was proposed (6), are herein described.

MATERIALS AND METHODS

Biological Specimens. A whole formalin-fixed human lung from an AIDS patient who did not receive treatment for, and died of, PcP was generously provided by Miercio Perreira, (Tufts New England Medical Center, Boston, MA). Pieces ($\approx$100 g) were removed and homogenized with distilled water in a 125-ml stainless steel blender cup (Baxter Scientific Products, McGaw Park, IL). Alternatively, samples were homogenized with organic solvents for simultaneous extraction of lipids. Most structural analyses were performed on pneumocysterol purified from this large specimen. Formalin-fixed autopsied lung specimens from individuals with no histological evidence of *P. carinii* infection served as controls.

To examine whether formalin fixation destroyed or altered the *P. carinii*-specific sterols, PcP-containing rat lungs were fixed in 2% formalin and left at room temperature for 1–18 weeks. The sterols of these infected rat lungs were compared with those of fresh unfixed PcP-containing rat lungs.

Organisms were isolated from cryopreserved human PcP lungs by using a protocol similar to that described previously for rat PcP lungs (7). By using this protocol, the purity of organism preparations isolated from fresh rat PcP lungs had been rigorously quantified by using microscopic, biochemical, and immunochemical criteria, and found to be >95–100% pure (7). Because human lung samples were not liquified by the homogenization solution, glutathione was replaced with 10% DTT as the mucolytic sulfhydryl agent; the buffer solution contained 25 mM Hepes buffer, 5 mM EDTA, 0.85% NaCl, and 75 mM DTT, pH 7.4.

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Abbreviations: BALF, bronchoalveolar lavage fluid; PcP, *Pneumocystis carinii* pneumonia; NOESY, nuclear Overhauser effect spectroscopy; THF, tetrahydrofuran.

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Cryopreserved BALF recovered from PcP patients and non-PcP controls were analyzed. Approximately 1.0 ml of the BALF material was suspended in 4× vol of the DTT-containing buffer solution before extraction with organic solvents.

Lipid Extraction and Fractionation. Lung homogenates, isolated organisms, and BALF were extracted (8) for at least 2 hr at room temperature. Large particles were removed by using centrifugation or filtration. The lipids were purified by using biphasic partitioning (9), and the lower organic phase was recovered, concentrated under reduced pressure, and dried under N_2.

Total neutral lipid fractions were isolated as described (2). In some studies, the neutral lipid fraction was subjected to preparative TLC to obtain the free sterols and steryl esters (10). The steryl esters were saponified (2), and the resultant free sterols were isolated by using TLC.

Purification of Pneumocysterol. Pneumocysterol was isolated and purified by preparative GLC using a glass column packed with 15% OV-101 on 60–80-mesh Gas Chrom Q (Supelco) and a thermal conductivity detector (2). Pneumocysterol was collected at the detector as it condensed onto the inner surfaces of 100-μl glass capillary tubes. The samples were recovered by rinsing the capillary tubes with hexane; the purity and elution time of pneumocysterol were verified by analytical GLC with a flame ionization detector.

GLC and MS Analyses. Samples dissolved in hexane were analyzed by GLC using a DB-5 or SPB-5 capillary column and a flame ionization detector (2). Relative retention times were calculated using cholesterol as reference. Some samples were too small to enable the detection of *P. carinii* sterols by using these procedures. Hence, for this report, we included only data from samples in which both pneumocysterol and fungisterol were present in substantial amounts as indicated by $>10^5$ ions detected by the flame ionization detector.

Total sterols from human PcP lungs, isolated organisms, GLC-purified pneumocysterol, and chemically synthesized pneumocysterol (see below) were analyzed by GLC–high resolution electron-impact MS as described (2). Electron-impact mass spectra were continuously collected and processed on a Data General NOVA/4C computer with a DS-55 data system.

NMR Spectroscopy. The ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker (Billerica, MA) AMX-400 WB broad band spectrometer with spectrometer frequencies of 400.13 MHz and 100.614 MHz, respectively, by using an inverse 5-mm probe. All samples were dissolved in deuterated chloroform ($CDCl_3$). The sample temperature was maintained at 30°C by using a Bruker VT-1000 variable-temperature unit. Proton and carbon chemical shifts were reported relative to $CDCl_3$ at 7.20 ppm and 77.0 ppm, respectively. For the one-dimensional ^{1}H spectra, the spectral width was set at 5,600 Hz with a relaxation delay time of 4.0 s. The same parameters were used for the two-dimensional H–H homonuclear shift correlation spectroscopy and two-dimensional nuclear Overhauser effect spectroscopy (NOESY) acquisitions (512 experiments and 1,024 data points were run for each). The data were zero-filled to 1K × 1K, processed, phased, and symmetrized. A mixing time of 500 ms was used for the NOESY spectrum. The two-dimensional H–C heteronuclear shift correlation spectroscopy spectra were obtained in the inverse mode (128 experiments of 1,024 data points were collected). Two mixing times, 3.25 ms and 50 ms, were used to obtain one- and three-bond connectivities, respectively, between the H and C nuclei.

Chemical Synthesis of (24*Z*)- and (24*E*)-Ethylidenelanost-8-en-3β-ol. Procedures and conditions for obtaining melting points, IR spectra, ^{1}H-NMR spectra, MS, TLC, GLC, and column chromatography were as described (11). Commercial lanosterol (Sigma) was purified by using multiple (4) recrystallization from acetone/water, and after recrystallization was found to be a mixture of lanosterol (Fig. 1, compound **I**) and 24,25-dihydrolanosterol. Authentic **I** and 24,25-dihydrolanosterol were obtained from commercial lanosterol by using standard methods (12, 13). Acetylation of each purified sample, using pyridine and acetic anhydride, yielded authentic lanosteryl acetate, (Fig. 1, compound **II**) and 24,25-dihydrolanosteryl acetate. The C-24 double bond of lanosterol acetate (Fig. 1, compound **II**) was selectively reacted with borane/tetrahydrofuran (THF) to yield the C-24 organoborane (14), which was then oxidized by pyridinium fluorochromate in refluxing methylene chloride to yield 3β-acetoxylanost-8-en-24-one (Fig. 1, compound **III**). Cleavage of the acetate in **III**, using potassium carbonate in CH_3OH/THF (2:1), resulted in sterol **IV**, which was subjected to Wittig reaction by using ethyl triphenylphosphonium iodide in THF with potassium *tert*-butoxide. Purification with column chromatography resulted in the 24-ethylidene lanosterol derivatives **V** and **VI**. The assignments of the ^{1}H-NMR methyl resonance in **III** and **IV** were based on previously reported values (15, 16). The final products (24*E*)- and (24*Z*)-ethylidenelanost-8-en-3β-ol (**V** and **VI**) were isolated as a white solid (85%): ^{1}H-NMR, 0.699 (s, ^{3}H, C-18-CH_3), 1.008 (s, 3H, C-19-CH_3), 1.521 (d, 3H, ≈80%, C-29-*Z*-CH_3), 1.613 (d, 3H, ≈20%, C-29-*E*-CH_3), 2.818 (heptet, 1H, ≈80%, C-25-H), 2.200 (heptet, 1H, ≈20%, C-25-H), 3.240 (m, 1H, C-3α-H).

The C-24 configurations of the final authentic standard products could be determined by the ^{1}H-NMR spectra and were compared with those of authentic fucosterol (Sigma). The signal for the C-25 proton appears as a seven-line multiplet coupled to six protons at 2.20 ppm for the *E* configuration (e.g., in fucosterol, (24*E*)-ethylidenecholest-5-en-3β-ol), and the signal at C-25 is

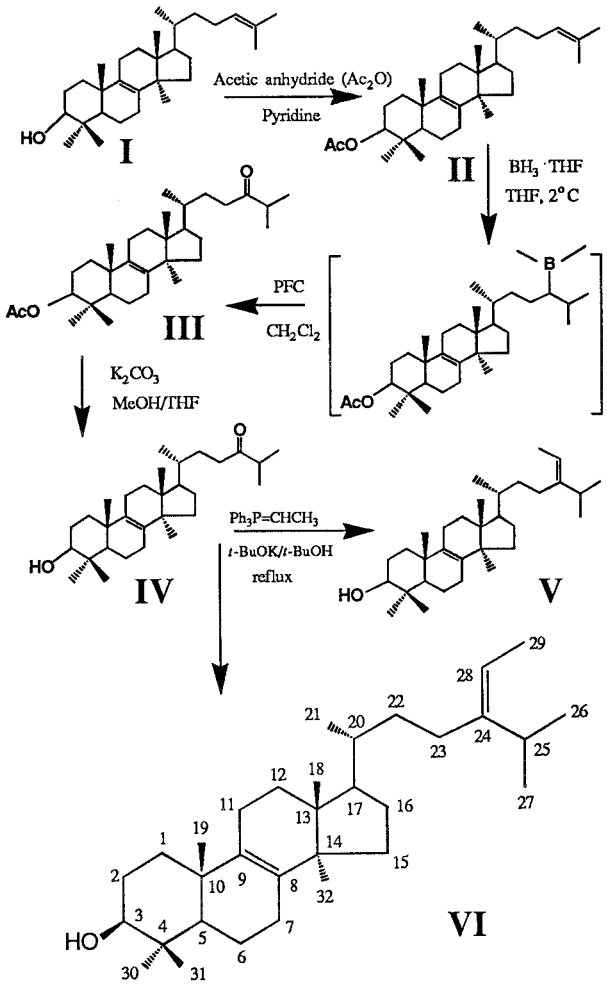

FIG. 1. Chemical synthesis of authentic 24*Z*- and 24*E*-ethylidenelanost-8-en-3β-ol. **I**, lanosterol; **II**, lanosteryl acetate; **III**, 3β-acetoxylanost-8-en-24-one (24-ketolanosteryl acetate); **IV**, lanost-8-en-3β-ol-24-one; **V**, 24*E*-ethylidenelanost-8-en-3β-ol; **VI**, 24*Z*-ethylidenelanost-8-en-3β-ol (numbering is according to ref. 31).

found downfield at 2.82 ppm for the *Z* configuration (e.g., in isofucosterol).

RESULTS

Chemically Synthesized Authentic Standards of (24*Z*)- and (24*E*)-Ethylidenelanost-8-en-3β-ol and Quantitation by Using GLC. In this study, hydroboration (17) was used to form products in essentially quantitative yields. We found that the C-24 double bond of lanosteryl acetate selectively reacts with BH_3/THF to yield the C-24 organoboranes. Direct oxidation of this intermediate with pyridinium fluorochromate in refluxing methylene chloride gave the corresponding ketone **III** in 88% yield. The isolation of 24,25-dihydrolanosteryl acetate from the latter reaction mixture, which remained after the consumption of **II** from commercial lanosteryl acetate, makes this procedure a useful method of preparing this compound.

Base-catalyzed cleavage of the acetate at C-3 in **III** was accomplished by using potassium carbonate in CH_3OH/THF. This mild hydrolysis condition selectively cleaved the ester function without significant enolization (and condensation) of the C-24 keto group to give a high yield of lanost-8-en-3β-ol-24-one (**IV**). The procedure for the preparation of **III** and **IV** represents a unique approach to the synthesis of these sterols in high yield, from which the final products **V** and **VI** were prepared by using a modified Wittig reaction (18–21).

The relative amounts of the two isomers in the chemically synthesized authentic sample of (24*Z*)- and (24*E*)-ethylidenelanost-8-en-3β-ol mixture were first estimated by the intensity of ^{1}H-NMR signals. By using this method, the *Z* and *E* isomers in the final product were estimated at 80% and 20%, respectively (22). Resolution and quantitation by using analytical SPB-5 capillary column GLC (Fig. 2*Aa*) showed that the 24*Z* isomer actually constituted 85% and the 24*E* isomer accounted for 15% of the mixture. The major 24*Z* isomer eluted later than the 24*E* isomer. The authentic standard mix was cochromatographed with preparative GLC-purified *P. carinii* pneumocysterol (Fig. 2*Ab*). The peak of the late-eluting larger component (24*Z* isomer) was enhanced. Thus, *P. carinii* pneumocysterol was identified with GLC as (24*Z*)-ethylidenelanost-8-en-3β-ol.

Lung Controls. Although formalin has no groups that would react with sterols, because pneumocysterol used for structural analyses was isolated from a formalin-fixed human PcP lung, fresh and formalin-fixed rat PcP lungs were compared to verify the stability of sterols (Fig. 2*B*). After fixation with formalin and storage for up to 18 weeks at room temperature, the free sterols of rat PcP lungs were examined. The major 24-alkylsterols (2) present in the free sterol fraction of isolated organisms (Fig. 2*Bc*) also were found in the free sterol fraction of both fresh (Fig. 2*Bb*) and formalin-fixed, stored (Fig. 2*Ba*) rat PcP lungs. Excluding cholesterol, *P. carinii*-specific sterols in peaks 13, 16, 19, and 20 composed 65.6% of the free sterols in formalin-fixed rat PcP lungs ($n = 4$). These sterols composed 47.5% of the free sterol fraction of the fresh rat PcP lungs ($n = 7$) and 63.6% of the free sterol fraction of purified organism preparations (2). Thus, formalin fixation and storage did not affect the integrity of the sterols.

Formalin-fixed autopsied human lungs that had been found *P. carinii*-free by using microscopic analysis also served as controls. Of 11 specimens obtained from different PcP-negative control lungs, 10 did not contain detectable pneumocysterol or other *P. carinii* 24-alkylsterols.

GLC and GLC–MS of *P. carinii hominis* Sterols. In most samples containing sufficient numbers of organisms, *P. carinii*-specific sterols were detected by GLC of the total nonsaponifiable lipid fraction. The major signature 24-alkylsterols, identified in rat-derived *P. carinii carinii*, also were present in human-derived *P. carinii hominis* organisms isolated from

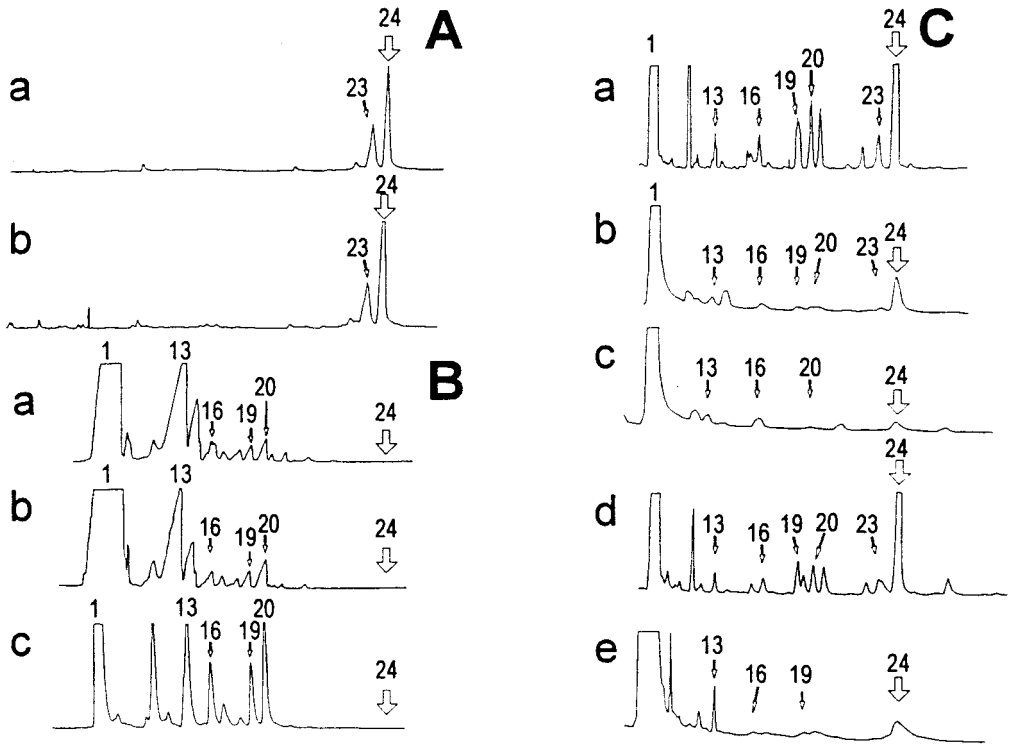

FIG. 2. GLC analyses. (*A*) Authentic 24*Z*- and 24*E*-ethylidenelanost-8-en-3β-ol mixture analyzed by using GLC with flame ionization detection. The major component (24*Z* isomer) comprised 85% and the minor component (24*E* isomer) comprised 15% of the chemically synthesized product (*a*). Co-chromatography of the authentic standard mix and GLC-purified *P. carinii hominis* pneumocysterol. The minor 24*E*-isomer peak was unchanged, whereas the major 24*Z*-isomer peak was enhanced (*b*). (*B*) Effects of formalin fixation on rat PcP-lung free sterols. Formalin-fixed rat PcP lungs stored for 12 weeks at room temperature (*a*); freshly isolated rat PcP lungs (*b*); and purified *P. carinii carinii* organisms (*c*). (*C*) *Pneumocystis carinii hominis* sterols. Total sterols from a formalin-fixed PcP lung (*a*); total sterols from organisms isolated from a frozen PcP lung (*b*); total sterols of BALF from a PcP patient (*c*); free sterol fraction from a formalin-fixed PcP lung (*d*); and steryl ester sterols from a formalin-fixed PcP lung (*e*). The ratios of peak 24 (pneumocysterol) to peak 13 (fungisterol) were: *a*, 21.9; *b*, 2.5; *c*, 2.2; *d*, 21.8; and *e*, 0.3.

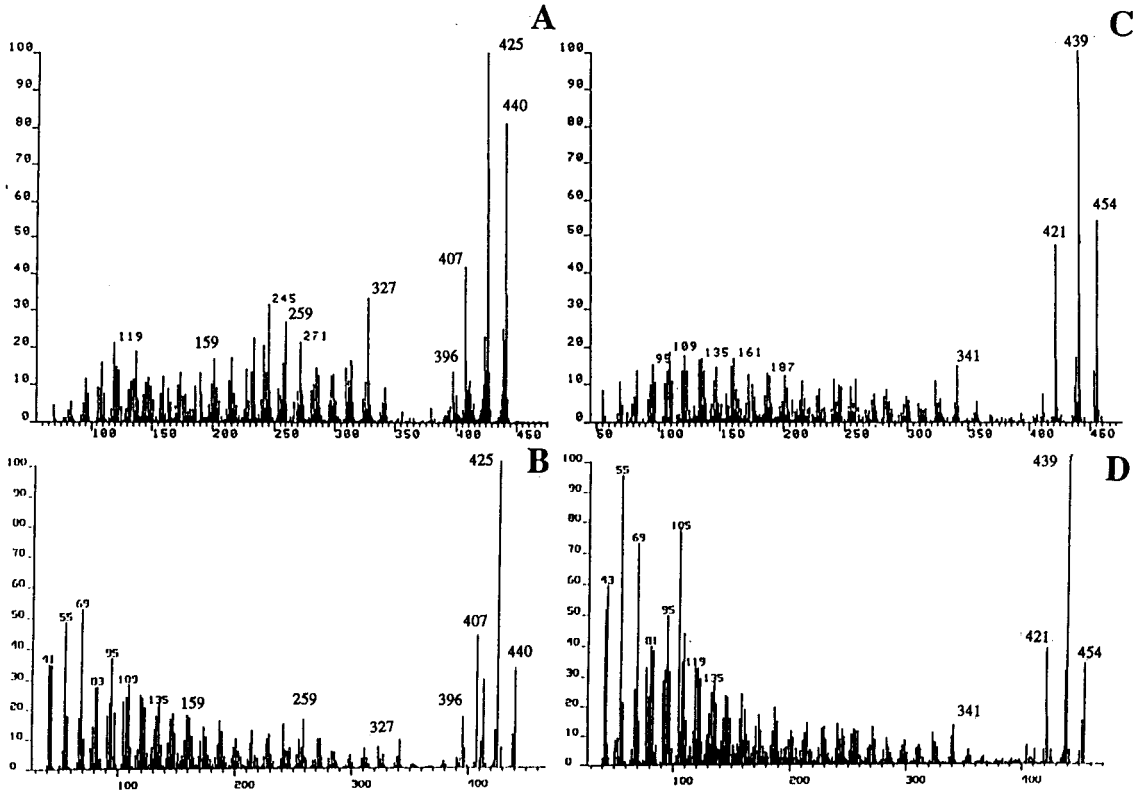

FIG. 3. GLC–MS analyses of *P. carinii hominis* C_{31} and C_{32} sterols. (*A*) Mass spectrum of the *P. carinii hominis* sterol in peak 23. (*B*) The mass spectrum of authentic euphorbol (24-methylenelanost-8-en-3β-ol) identified peak 23 as 24-methylenelanost-8-en-3β-ol. (*C*) Mass spectrum of the *P. carinii hominis* sterol in peak 24. (*D*) Mass spectrum of authentic pneumocysterol (24*Z*-ethylidenelanost-8-en-3β-ol) identified peak 24 as 24*Z*-ethylidenelanost-8-en-3β-ol.

cryopreserved autopsied lungs, BALF from PcP patients, and formalin-fixed human PcP lungs. The sterols detected in these human samples included 24-methylcholest-7-en-3β-ol (fungisterol, peak 13), 24-ethylcholestatriene-3β-ol (peak 16), 24-ethylcholest-7-en-3β-ol (peak 19) and 24-ethylcholestadiene-3β-ol (peak 20).

In addition to these *P. carinii* 24-alkylsterols previously identified in rat-derived *P. carinii carinii* (2, 4, 5), two late-eluting components with relative retention time values (cholesterol as reference) of 2.15 $\pm$ 0.02 SEM (n = 6) and 2.23 $\pm$ 0.02 SEM (n = 19) were seen in GLC traces of the total sterol fraction in formalin-fixed human PcP lungs (Fig. 2*Ca*). The smaller earlier, and larger later eluting components were designated as peaks 23 and 24, respectively. Peak 24 also was detected in the total sterol fraction of *P. carinii hominis* organisms isolated from cryopreserved lungs (Fig. 2*Cb*) and in BALF obtained from human PcP patients (Fig. 2*Cc*).

Analyses of the free and esterified sterols of formalin-fixed human PcP lungs indicated that peak 24 was present in both fractions. Excluding cholesterol, it comprised up to 50% of the free sterols (Fig. 2*Cd*). In contrast, peak 24 was only a minor component of the sterols in the steryl ester fraction (Fig. 2*Ce*). These observations are consistent with the report that *P. carinii*-specific sterols were difficult to detect in the steryl ester fraction of rat-derived organisms (2). Cholesterol comprised 75% of the free sterols of *P. carinii carinii*, whereas it accounted for 92% of the steryl ester sterols, suggesting that the free sterols were in cellular membranes and the steryl esters were within storage compartments (2).

High-resolution electron-impact GLC–MS indicated that the material in both peaks were sterols with molecular weights of 440 and 454 (C_{31} and C_{32} sterols, respectively; Fig. 3). The component in peak 23 was identified by GLC–MS as euphorbol, 24-methylenelanost-8-en-3β-ol (Table 1). Peak 24, pneu-

Table 1. Elemental compositions of pneumocysterol and euphorbol major ions obtained by high-resolution GLC–MS analyses

Molecular ion ($M^{+\cdot}$) and characteristic ion fragments (m/z)	Elemental composition	Calculated exact mass	Experimental exact mass	Deviation, molecular mass units
Pneumocysterol				
454 ($M^{+\cdot}$)	$C_{32}H_{54}O$	454.4174	454.4199	2.5
439 ($M^{+\cdot}$–CH_3)	$C_{31}H_{51}O$	439.3940	439.3921	−1.9
436 ($M^{+\cdot}$–H_2O)	$C_{32}H_{52}$	436.4069	436.4046	−2.3
421 ($M^{+\cdot}$–CH_3, –H_2O)	$C_{31}H_{49}$	421.3834	421.3824	−1.0
Euphorbol				
440 ($M^{+\cdot}$)	$C_{31}H_{52}O$	440.4018	440.4055	3.7
425 ($M^{+\cdot}$–CH_3)	$C_{30}H_{49}O$	425.3783	425.3774	−0.9
422 (–H_2O)	$C_{31}H_{50}$	422.3912	422.3878	−3.4
407 ($M^{+\cdot}$–CH_3, –H_2O)	$C_{30}H_{47}$	407.3677	407.3617	−6.0

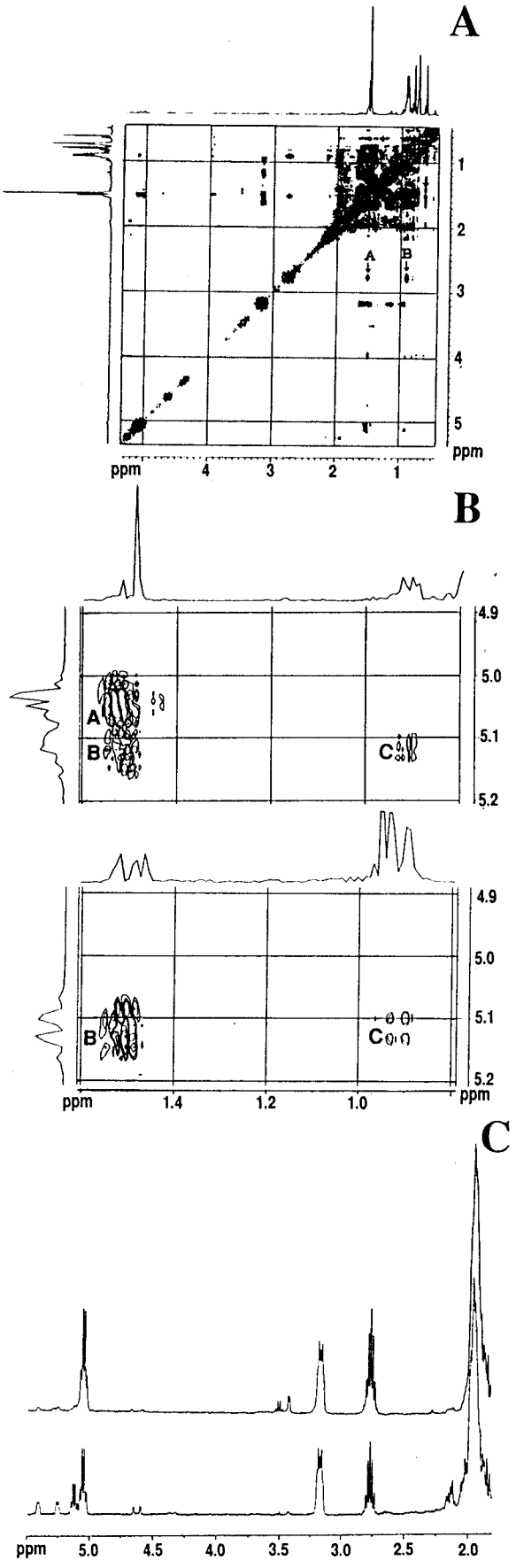

FIG. 4. NMR analyses. (*A*) The NOESY spectrum of the synthetic mixture at 400 MHz. The "normal" one-dimensional spectrum lies along the diagonal, and cross peaks are observed off the diagonal at the chemical shifts of two protons that are in close proximity with each other. The expected cross peaks between the methine proton at 2.818 ppm and the C-29 methyl group at 1.521 ppm (*A*) and the C-26 and C-27 methyl groups at 0.916 ppm (*B*) of the 24*Z*-isomer are indicated. (*B*) The expanded region of the NOESY spectrum of the synthetic mixture (*Upper*) and of authentic fucosterol (*Lower*). Only one cross peak is observed between the vinyl proton at 5.097 ppm and the C-29 methyl groups of the major synthetic product at 1.521 ppm (labeled A), whereas two cross peaks are observed between the vinyl proton at 5.122 ppm and the C-29 methyl group at 1.521 ppm (labeled B) and the C-26 and C-27 methyl groups at 0.916 ppm (labeled C) for both the minor product and fucosterol. (*C*) The 400-MHz ^{1}H spectra of pneumocysterol isolated from PcP lung (*Upper* trace) and the synthetic mixture (*Lower* trace). The chemical shifts of the vinyl proton on the C-28 carbon and the methine proton on the C-25 carbon are at 5.097 ppm and 2.818 ppm, respectively, for the 24*Z* isomer, similar to that of pneumocysterol isolated from PcP lungs.

mocysterol, required further characterization with NMR to determine its structure.

^{1}H-NMR of the Chemically Synthesized Standards and of Pneumocysterol Purified from *P. carinii hominis*-Infected Lung. The 24*E* and 24*Z* isomers of ethylidenelanost-8-en-3β-ol were distinguished by using the NOESY spectrum of the chemically synthesized mixture (Fig. 4*A*) and comparing it to that of authentic fucosterol, a 24*E* sterol isomer. For the 24*Z* isomer, the expected cross peaks were observed between the methine proton of C-25 at 2.818 ppm and the methyl group at C-26 and C-27 at 0.916 ppm and the methyl group at C-29 at 1.521 ppm. Only one cross peak was observed between the C-29 methyl group at 1.521 ppm and its vinyl proton at 5.097 ppm for the major synthetic product (Fig. 4*B Upper*). In contrast, the fucosterol NOESY spectrum had cross peaks between the vinyl proton at 5.122 ppm and the methyl group at C-29 at 1.521 ppm and the methyl groups at C-26 and C-27 at 0.916 ppm (Fig. 4*B Lower*). The minor product showed two cross peaks between the C-29 methyl group at 1.521 ppm and the C-26 and C-27 methyl groups at 0.916 ppm and its vinyl proton at 5.122 ppm (similar to fucosterol). These data showed that the major product was the 24*Z* isomer.

The ^{1}H spectrum of the chemically synthesized isomer mixture showed the characteristic signals from C-25 at 2.818 ppm (*Z*) and 2.201 ppm (*E*), and C-28 at 5.097 ppm (*Z*) and 5.122 ppm (*E*) (Fig. 4*C*). The 24*E*-isomer resonances were similar to those reported for fucosterol (23). The spectrum for the *P. carinii hominis* pneumocysterol side-chain moiety was identical to the spectrum of the major product in the synthetic mixture.

The assignments of the ^{1}H and ^{13}C chemical shifts were obtained from the one-bond and three-bond heteronuclear shift correlation spectroscopy spectra. Resonances were assigned in a stepwise manner starting with the previously assigned ^{1}H resonances (Table 2). Although all nine methyl groups were resolved in both the synthetic and *P. carinii hominis* pneumocysterol, the observed one-bond correlations were not in agreement with previously published assignments (18). The predicted correlations (18) of the proton and carbons at C-31 of 0.69 ppm and 28.6 ppm, at C-32 of 0.88 ppm and 15.4 ppm, and at C-19 of 1.00 ppm and 18.2 ppm were not observed. The protons of the C-30 and C-31 methyl groups exhibited a strong NOESY correlation at 0.742 and 0.933 ppm. The downfield resonance was assigned to the methyl group on the same face as the hydroxyl group. The doublets for the C-21, C-26, C-27, and C-29 methyl groups were assigned. Of the remaining methyl groups, the C-19 and C-32 were assigned to lower field because of their proximity to the double bond. In all NMR analyses performed, the spectra of the isolated biological compound were found identical to those of the authentic chemically synthesized *Z* isomer.

DISCUSSION

Occurrence of Pneumocysterol. This study provides definitive structural identity of pneumocysterol by using GLC, GLC-high

Table 2. ^{1}H and ^{13}C chemical shifts of pneumocysterol

Carbon number	^{1}H δ	^{13}C δ
3	3.182	78.7
8	NA	134.2
9	NA	134.4
18 or 19	0.920	21.0
19 or 18	0.631	15.5
21	0.850	19.0
24	NA	146.1
25	2.818	28.4
26	0.916	21.0
27	0.916	21.0
28	5.097	116.2
29	1.521	12.6
30	0.742	15.3
31	0.933	28.2
32	0.813	24.2

NA, not applicable.

resolution electron-impact MS, and TLC and ^{1}H-NMR, and IR spectroscopy. Thus far, the bark of the plant *Neolitsea sericea* (18) is the only other known source of pneumocysterol, indicating that it is not a common sterol. The fungus *Gibberella fujikuroi* may be able to synthesize it, because cells treated with the inhibitor 24,25-(*R,S*)-epiiminolanosterol (24) was reported to form 24-ethylidenelanost-8-en-3β-ol (25). However it was only a minor sterol component, and data for the C-29 isomeric assignment was not reported. The C_{31} sterol euphorbol was previously identified in *Euphorbia lathyris* latex (26), in the lauracean plant *Neolitsea sericea* (18), and in the fungi *Phycomyces blakesleeanus* (27) and *Mucor rouxii* (19).

Given the difficulty in obtaining human materials, it is not feasible to quantitatively and qualitatively define the purity of the human-derived preparations used in this study. It cannot be ruled out that pneumocysterol originates from other microbes, but it is most likely that *P. carinii hominis* is its source, because light microscopic analyses indicated that *P. carinii* was the only identifiable (or dominant) microbe in the isolated organism and BALF samples. Furthermore, because *P. carinii carinii* is known to contain fungisterol (peak 13) and other *P. carinii*-specific C-24-alkyl sterols (e.g., peaks 16, 19, and 20), the presence of these with pneumocysterol further suggests that the sterols originate from *Pneumocystis* organisms. Because mammals, including humans, cannot form 24-alkylsterols, these would not be expected to be synthesized by the host. Because bacteria do not synthesize sterols, these could not orginate from bacteria that also may be in these human lung specimens. Most higher fungi that occur in human lungs (e.g., *Aspergillus*) contain ergosterol as their major sterol component.

Pneumocysterol and the Δ^7 24-alkylsterols may be useful signature lipids of *P. carinii hominis* and could serve as markers in the diagnosis and therapy of PcP. Thus far, pneumocysterol has been detected with other distinct *P. carinii* sterols in 18 human BALF samples. Furthermore, *P. carinii* 24-alkylsterols have been detected in seven samples of organisms isolated from autopsied lungs, three of which had high proportions of pneumocysterol. Several *P. carinii*-specific sterols, but not pneumocysterol, have been detected in organisms of rabbit, SIV-positive monkey, rat, ferret, and mouse origins (Z.A. and E.S.K., unpublished data). In some *P. carinii hominis* preparations, pneumocysterol was not detected, and because it is known that sterol profiles can be changed by therapy, samples obtained from patients with known medical histories are being examined. However, there is evidence that great genetic diversity exists in *P. carinii* organisms from a single mammalian host species, including humans (28, 29), thus, pneumocysterol may accumulate in some, but not all, genetically distinct populations of *P. carinii hominis*.

Sterol Metabolism and Drug Targets. Sterols that commonly accumulate in cells and tissues are formed from intermediates such as lanosterol (cycloartenol in some plants). The C_{30} intermediate, lanosterol, usually undergoes modifications including demethylation of the sterol nucleus and isomerization of the C-8 double bond. In general, yeasts require nuclear demethylation before alkylation of C-24. In contrast, lanosterol serves as the substrate for C-24 methyltransferase activity in plants and the filamentous fungi (30). The elucidation of pneumocysterol and euphorbol structures indicates that the sterol biosynthetic mechanisms in *P. carinii* resemble those in plants and filamentous fungi. Azole antimycotics are active against pathogens that require lanosterol demethylation to form the final sterol molecules required for their membranes. Thus, the inefficacy of fluconazole and other azoles against PcP can be explained by the ability of the *P. carinii* sterol C-24 methyltransferase activity to use lanosterol as a substrate.

Mammals are incapable of forming 24-alkylsterols; hence, drugs that interfere with these reactions could be effective anti-*P. carinii* agents with little toxicity to the host. The azasterol, 20-piperidin-2-yl-5α-pregnan-3β-20(*R*)-diol, which inhibits *Trypanosoma cruzi* $\Delta^{24(25)}$ and $\Delta^{24(28)}$ sterol methyltransferase activities, was tested on rat-derived *P. carinii* (4). The drug inhibited cell proliferation in a dose-dependent manner, and the sum of C_{28} plus C_{29} sterols relative to C_{27} sterols decreased. Transmethylation of sterols using *S*-adenosylmethionine as the methyl donor is one of the most energy-utilizing biochemical reactions in cells. Each methyl transfer requires 12–14 ATP equivalents (30). The accumulation of pneumocysterol, euphorbol, and other 24-alkylsterols in *P. carinii hominis* indicates that sterol C-24 methyltransferase activities can be extraordinarily high in this opportunistic pathogen.

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