Pneumocysteryl [(24Z)-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 sterols. Even though the abundance of cholesterol, which it can scavenge from the host and utilize them for membrane functions 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 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 C31 euphorbol and a rare C32 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 (~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 pneumocysteryl purified from this large specimen. Formalin-fixed autopsy lung specimens from individuals with no histological evidence of P. carinii infection served as controls.

To examine whether formalin fixation 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 immunochromatographic 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.

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 N2.

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 1H- and 13C-NMR spectra were obtained on a Bruker (Billericia, 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 (CDCl3). 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 CDCl3 at 7.27 ppm and 77.0 ppm, respectively. For the one-dimensional 1H 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 24Z- and 24E-Ethylidenelanolast-8-en-3β-ol.** Procedures and conditions for obtaining melting points, IR spectra, 1H-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 lanosterol acetate, (Fig. 1, compound II) and 24,25-dihydrolanosterol 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 (24-ketolanosterol acetate); III, lanosterol acetate; IV, lanost-8-en-3β-ol-24-one; V, 24E-ethylenelanolast-8-en-3β-ol; VI, 24Z-ethylenelanolast-8-en-3β-ol (numbering is according to ref. 31).

**Fig. 1.** Chemical synthesis of authentic 24Z- and 24E-ethylidenelanolast-8-en-3β-ol. I, lanosterol; II, lanosterol acetate; III, 3β-acetoxylanost-8-en-24-one (24-ketolanosterol acetate); IV, lanost-8-en-3β-ol-24-one; V, 24E-ethylenelanolast-8-en-3β-ol; VI, 24Z-ethylenelanolast-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 (24Z)- and (24E)-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 BH₃ in 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 CH₃OH/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-ene-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 (24Z)- and (24E)-ethylidenelanost-8-en-3β-ol mixture were first estimated by the intensity of ¹H-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 SBP-5 capillary column GLC (Fig. 2A) showed that the 24Z isomer actually constituted 85% and the 24E isomer accounted for 15% of the mixture. The major 24Z isomer eluted later than the 24E isomer. The authentic standard mix was cochromatographed with preparative GLC-purified P. carinii pneumocystosterol (Fig. 2Ab).

Lung Controls. Although formalin has no groups that would react with sterols, because pneumocystosterol 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. 2B). 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. 2Bc) also were found in the free sterol fraction of both fresh (Fig. 2Bb) and formalin-fixed, stored (Fig. 2Ba) 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 pneumocystosterol 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 24Z- and 24E-ethylidenelanost-8-en-3β-ol mixture analyzed by using GLC with flame ionization detection. The major component (24Z isomer) comprised 85% and the minor component (24E isomer) comprised 15% of the chemically synthesized product (a). Co-chromatography of the authentic standard mix and GLC-purified P. carinii hominis pneumocystosterol. The minor 24E-isomer peak was unchanged, whereas the major 24Z-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 (pneumocystosterol) to peak 13 (fungisterol) were: a, 21.9; b, 2.5; c, 2.2; d, 21.8; and e, 0.3.](image-url)
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 (fungi-sterol, 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 (2, 4, 5), two late-eluting components with relative retention time values (cholesterol as reference) of 2.15 ± 0.02 SEM (n = 6) and 2.23 ± 0.02 SEM (n = 19) were seen in GLC traces of the total sterol fraction in formalin-fixed human PCP lungs (Fig. 2Ca). 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. 2Cb) and in BALF obtained from human PCP patients (Fig. 2Cc).

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. 2Gi). In contrast, peak 24 was only a minor component of the sterols in the steryl ester fraction (Fig. 2Cc). 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, 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 (C31 and C32 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, pneumocysterol (24Z-ethylidenelanost-8-en-3β-ol) identified peak 24 as 24Z-ethylidenelanost-8-en-3β-ol.

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

<table>
<thead>
<tr>
<th>Molecular ion (M+) and characteristic ion fragments (m/z)</th>
<th>Elemental composition</th>
<th>Calculated exact mass</th>
<th>Experimental exact mass</th>
<th>Deviation, molecular mass units</th>
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<tr>
<td>Pneumocysterol</td>
<td>C32H52O</td>
<td>454.4174</td>
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<tr>
<td>439 (M+-CH3)</td>
<td>C31H51O</td>
<td>439.3940</td>
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<tr>
<td>436 (M+-H2O)</td>
<td>C32H52</td>
<td>436.4069</td>
<td>436.4046</td>
<td>2.3</td>
</tr>
<tr>
<td>421 (M+-CH3, -H2O)</td>
<td>C31H49</td>
<td>421.3834</td>
<td>421.3824</td>
<td>1.0</td>
</tr>
<tr>
<td>Euphorbol</td>
<td>C31H52O</td>
<td>440.4018</td>
<td>440.4055</td>
<td>3.7</td>
</tr>
<tr>
<td>425 (M+-CH3)</td>
<td>C30H49O</td>
<td>425.3783</td>
<td>425.3774</td>
<td>0.9</td>
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<tr>
<td>422 (-H2O)</td>
<td>C31H49O</td>
<td>422.3912</td>
<td>422.3878</td>
<td>3.4</td>
</tr>
<tr>
<td>407 (M+-CH3, -H2O)</td>
<td>C30H47</td>
<td>407.3677</td>
<td>407.3617</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Fig. 3. GLC–MS analyses of P. carinii hominis C31 and C32 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 (24Z-ethylidenelanost-8-en-3β-ol) identified peak 24 as 24Z-ethylidenelanost-8-en-3β-ol.
mocysterol, required further characterization with NMR to
determine its structure.

1H-NMR of the Chemically Synthesized Standards and of
Pneumocysterol Purified from P. carinii hominis-Infected Lung.
The 24E and 24Z isomers of ethylidenelanost-8-en-3β-ol were
distinguished by using the NOESY spectrum of the chemically
synthesized mixture (Fig. 4A) and comparing it to that of authen-
tic fucosterol, a 24E sterol isomer. For the 24Z 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. 4B 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. 4B 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 24Z isomer.

The 1H 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.
4C). The 24E-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 1H and 13C 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 1H reso-
nances (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 correla-
tions (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

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 24Z-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 1H 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 24Z isomer, similar to that of pneumocysterol isolated from PcP
lungs.
resolution electron-impact MS, and TLC and 1H-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)-epimedinolanosterol (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 C30 sterol euphorbol was previously identified in Euphorbia lathyris latex (26), in the lauracean plant Neolitsea sericea (18), and in the fungi Phymcymes 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 is known to contain fungosterol (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 originate 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 Δ2 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 stereoisomers 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, and other 24-alkylsterols in P. carinii species indicate that sterol C-24 methyltransferase activities can be extraordinarily high in this opportunistic pathogen.

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