Comprehensive and definitive structural identities of *Pneumocystis carinii* sterols

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Abstract ***Pneumocystis*** **causes a type of pneumonia in immunodeficient mammals, such as AIDS patients. Mammals cannot alkylate the C-24 position of the sterol side chain, nor can they desaturate C-22. Thus, the reactions leading to these sterol modifications are particularly attractive targets for the development of drugs against fungal and protozoan pathogens that make them. In the present study, the definitive structures of 43 sterol molecular species in rat-derived *Pneumocystis carinii* were elucidated by nuclear magnetic resonance spectroscopy. Ergosterol, $\Delta^{5,7}$ sterols, trienes, and tetraenes were not among them. Most (32 of the 43) were 24-alkylsterols, products of *S*-adenosyl-L-methionine:C-24 sterol methyl transferase (SAM:SMT) enzyme activity. Their abundance is consistent with the suggestion that SAM:SMT is highly active in this organism and that the enzyme is an excellent anti-*Pneumocystis* drug target. In contrast, the comprehensive analysis strongly suggest that *P. carinii* does not form Δ^{22} sterols, thus C-22 desaturation does not appear to be a drug target in this pathogen. The lanosterol derivatives, 24-methylenelanost-8-en-3β-ol and (Z)-24-ethylidenelanost-8-en-3β-ol (pneumocysterol), previously identified in human-derived *Pneumocystis jiroveci*, were also detected among the sterols of the rat-derived *P. carinii* organisms.—** Giner, J-L., H. Zhao, D. H. Beach, E. J. Parish, K. Jayasimhulu, and E. S. Kaneshiro. **Comprehensive and definitive structural identities of *Pneumocystis carinii* sterols. *J. Lipid Res.* 2002.** 43: **1114–1124.**

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Pneumocystis pneumonia (PcP) remains among the most prevalent opportunistic infections in immunocompromised individuals such as AIDS patients. It has becoming evident that the high incidence of PcP in AIDS patients is global and that the organism can infect other immunodeficient people such as patients undergoing chemotherapy for cancer or solid organ transplant (1, 2), children prior to becoming fully immunocompetent, and the elderly with diminished immune systems. *Pneumocystis* can also transiently colonize normal, healthy people and animals without causing overt symptoms of respiratory disorder. The combination of trimethoprim and sulfamethoxazole and other agents (e.g., pentamidine, atovaquone) used for prophylaxis and for clearing PcP has successfully reduced the number of deaths directly attributed to PcP. However, AIDS and other patients with prolonged immunodeficiency experience recurrent *Pneumocystis jiroveci* (*Pneumocystis carinii* f. sp. *hominis*) (3, 4) infections. Also, some individuals cannot tolerate these drugs and suffer undesirable side effects. The development of drug-resistant pathogen populations is of serious concern, making it imperative to develop a larger armamentarium of diverse drugs to circumvent these problems (5–7). Although the organism can be maintained in long-term, small-volume axenic cultures (8), growth is very slow and insufficient numbers of organisms are obtained for most biochemical studies. Thus, *Pneumocystis* remains an organism considered difficult to manipulate for experimental work. Despite this difficulty, much is now known about the organism's lipids from analyses performed on preparations purified from infected rat lungs (9–11).

Our group earlier reported that *P. carinii* (*P. carinii* f. sp. *carinii*) organisms isolated and purified from infected lungs of corticosteroid-immunmosuppressed rats contained sterols distinct from those in the rat lung (12), and more than 20 sterol components were resolved by gas-liquid chromatography (GLC) using capillary columns (13).

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Abbreviations: GLC, gas-liquid chromatography; MS, mass spectrometry; PcP, *Pneumocystis* pneumonia; RRT, relative retention time; SAM:SMT, *S*-adenosyl-L-methionine:C-24 sterol methyl transferase.

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Several of these were identified as 24-alkysterols but ergosterol, the dominant sterol of most fungal pathogens, was not among them (9, 11, 14, 15). The structural assignments of several of these sterol components were made by analyses using TLC, GLC, and GLC-mass spectrometry (MS) in conjunction with chemically synthesized authentic standards. Subsequently, Furlong et al. (16) and Urbina et al. (17) confirmed the structural assignments for some of the major *P. carinii* sterol components using comparable analytical methods. However, these workers assigned structures for some sterols containing more than one double bond, which is not possible solely by GLC and GLC-MS analyses. Nuclear magnetic resonance (NMR) spectroscopy can provide definitive structural characterizations, including the elucidation of double bond positions and the detection of stereoisomers. This powerful analytical method was not applied in all previous studies of *P. carinii* sterols because the sensitivity of NMR is relatively low, and therefore requires more material than that needed for GLC and MS.

Purified *P. carinii* preparations isolated from an individual infected rat routinely provides <1 mg total lipids, containing only microgram amounts of total sterols. Recently, we pooled large numbers of TLC-purified *P. carinii* sterol samples that had been previously extracted, isolated, and stored. This pooled sample, containing 35 mg total sterols, made it possible to fractionate the components by nondestructive HPLC and to analyze the isolated fractions by NMR. Here we present a comprehensive NMR analysis providing unambiguous structural assignments for 43 *P. carinii* sterols and correlate their identities with those previously characterized by GLC and GLC-MS (9, 11, 13). Most of the sterol molecular species were 24-alkylsterols, consistent with the suggestion that the *Pneumocystis S*-adenosyl-L-methionine:C-24 sterol methyl transferase (SAM:SMT) activity and other sterol biosynthesis reactions in this pathogen are excellent targets for the development of new therapies against PcP (2, 9–22). 20-Piperidin-2-yl-5α-pregnan-3β-20(*R*)-diol and 24,25-(*R,S*)-epiminolanosterol were reported to inhibit both the proliferation of *P. carinii* in vitro (static activity) and the accumulation of 24-alkylsterols, presumably as a consequence of SAM:SMT inhibition (17). In another study, it was shown that 24,25-epiminolanosterol did not kill *P. carinii* organisms; however, several other inhibitors that target different reactions in sterol biosynthesis had cidal activity against *P. carinii* in vitro (21).

MATERIALS AND METHODS

Corticosteroid-immunosuppressed PcP rat model, organism isolation, and purification

P. carinii was isolated from corticosteroid-immunosuppressed, viral antibody-negative, female Lewis rats (Harlan Sprague Dawley, Indianapolis, IN, Room 202B) that had been twice intratracheally inoculated with cryopreserved organisms according to the animal model developed by Boylan and Current (23). The animals were fed balanced rat food (Techlad, type LM-485, sterilizable rat chow, Harlan, Madison, WI) and hyperchlorinated water (0.074% sodium hypochloride, Aldrich Chemical Co., Milwaukee, WI). *Pneumocystis* organisms were isolated from the infected rat lungs ~8 to 10 weeks post-inoculation when the animals were moribund with PcP. These animals routinely have high pathogen loads, as determined by enumerating organisms in lung impression smears stained with DiffQuik (Baxter Healthcare Corp., Miami, FL) and scored under bright field, oil immersion optics. Organisms were routinely isolated and purified from PcP animals having infection scores of +5 (>100 <1,000 organisms/oil immersion field) to +6 (>1,000 organisms/oil immersion field).

After euthanasia using halothane, the lungs were perfused, excised, and cut into small pieces. The organisms were isolated by homogenization (Stomacher, Tekmar, Cincinnati, OH) of lung tissue in a buffered salt solution containing the mucolytic sulfhydryl agent glutathione, which caused the detachment of *P. carinii* adhered to type I alveolar epithelial cells and to each other (13). Purification involved passage through a 50- to 60-mesh sieve followed by centrifugation steps using different speeds to remove host material, especially lung surfactant. A high percentage of the *P. carinii* population is lost during the centrifugation steps (only 15% of organisms in the sievate were recovered in the final preparation), but yield was sacrificed in favor of purity (13). The preparation was then subjected to microfiltration, first through membranes with 8 μm and then through 5 μm straight-channel pores. Routinely, the preparations from a single rat with heavy pathogen load contained 10^8 to 10^9 organisms. Most organisms in the final preparation were trophic forms and included 10% to 30% cystic forms (spore cases). The purity of the *P. carinii* preparations (>95% to 100%) was previously demonstrated by a variety of light and electron microscopic, biochemical, immunochemical, and microbiological criteria (13, 24).

Lipid extraction, fractionation, GLC, and MS analysis of sterols

Total lipids were extracted from individual *P. carinii* preparations (25), and purified by biphasic partitioning (26). Total lipids were fractionated by adsorption column chromatography (Unisil, Clarkson Co., Williamsport, PA) by elution with chloroform ($CHCl_3$) for the neutral lipid fraction, followed by elution with methanol (CH_3OH) for the polar lipid fraction. The neutral lipid fraction, containing free and esterified sterols, was isolated by TLC using petroleum ether-diethyl ether-acetic acid (80:20:1, v/v/v) as the solvent system. Free sterols or total sterols (free plus esterified) were isolated by TLC, dried, and stored under N_2 at −20°C in shell vials sealed with Teflon-lined screw caps. The GLC and GLC-MS procedures used in the present study were similar to those used for the initial characterization of *P. carinii* sterols previously described (13). The sterols were dissolved in hexane and analyzed in a Hewlett-Packard 5790 GLC equipped with a 30 m × 0.32 mm capillary column coated with 0.25 μm of SPB-5 (Supelco Inc., Bellefonte, PA). Isothermal analyses were performed using an oven temperature of 280°C; injection temperature was 290°C and the flame ionization detector was 305°C. The carrier gas was helium at a flow rate of 1 ml/min. Relative retention times (RRT) were calculated using the cholesterol retention time as reference.

GLC-MS analysis was performed on a Thermo-Finnigan Trace 2000 instrument using a split/splitless injector in the splitless mode. Injection temperature was 310°C with the splitless timing set to use a 15 psig surge pressure for 2 min after injection; total splitless time was 3 min, and then the split gas flow was run at 80 ml/min. The 30 m long, 0.32 mm ID GLC capillary column was coated with 0.25 μm of Restek XTI-5 (5% diphenyl, 95% dimethyl polysiloxane) and was equipped with a 10 m Integra-

TABLE 1. *Pneumocystis carinii* sterols identified by 600 MHz ^{1}H-NMR chemical shifts of olefinic, hydroxyl, and methyl signals[a,b]

Sterol	Total	Structure[c]	Olefin	OH Group (C-3)	Methyl Groups	Others
	%					
Lanosta-8,24-dien-3β-ol (lanosterol)	0.40	2A	5.098(t, 7.0)[d]	3.237(dd, 11.7, 4.4)	1.682(s), 1.601(s), 0.997(s),0.977(s), 0.909(d, 6.3), 0.870(s), 0.807(s), 0.684(s)	
24-Methylenelanost-8-en-3β-ol	0.29	2B	4.714(s), 4.661(s)	3.236(dd,11.7, 4.4)	1.030(d, 6.9), 1.024(d, 6.9), 1.001(s), 0.983(s), 0.924(d, 6.6), 0.883(s), 0.811(s), 0.694(s)	
(*Z*)-24-Ethylidenelanost-8-en-3β-ol (pneumocysterol)	0.31	2C	5.113(q, 6.7)	3.234(dd, 11.7, 4.4)	1.591(d, 6.7), 1.001(s), 0.983(s),0.977(6Hs, d, 6.6), 0.920(d, 6.4), 0.880(s), 0.811(s), 0.693(s)	2.832(C-25, sep, 7.0)
4,4-Dimethylcholesta-8,24-dien-3β-ol (14-norlanosterol)	0.15	2D	5.103(t, 7.0)	3.237(m)	1.680(s), 1.598(s), 0.994(s), 0.982(s), 0.936(d, 6.6), 0.808(s), 0.588(s)	
4,4-Dimethylergosta-8,24(28)-dien-3β-ol (24-methylene-14-norlanosterol)	0.21	2E	4.709(s), 4.654(s)	3.239(m)	1.025(d, 6.9), 1.019(d, 6.9), 0.994(s), 0.983(s), 0.950(d, 6.6), 0.808(s), 0.592(s)	
(*Z*)-4,4-Dimethylstigmasta-8,24(28)-dien-3β-ol (24*Z*)-ethylidene-norlanosterol)	0.10	2F	5.102(q, 6.9)	3.240(m)	1.587(d, 6.9), 0.994(s), 0.982(s), 0.972(6Hs, d, 7.0), 0.947(d, 6.6), 0.808(s), 0.591(s)	2.827(C-25, sep, 7.0)
4α-Methylcholesta-8,24-dien-3β-ol	0.10	2G	5.083(t, 7.0)	3.103(m)	1.681(s), 1.600(s), 0.991(d, 6.4), 0.970(s), 0.940(d, 6.4), 0.603(s)	
4α-Methylergosta-8,24(28)-dien-3β-ol	0.27	2H	4.711(s), 4.655(s)	3.103(m)	1.025(d, 6.9), 1.020(d, 6.9),0.991(d, 6.9), 0.971(s), 0.965(d, 6.6), 0.608(s)	
(*Z*)-4α-Methylstigmasta-8,24(28)-dien-3β-ol (4α-methyl-(24*Z*)-ethylidene-cholest-8-en-3β-ol	0.04	2I	5.105(q, 6.9)	3.102(m)	1.587(d, 6.9), 0.991(d, 6.4),0.974(d, 6.4), 0.971(s), 0.950(d, 6.9), 0.876(d, 7.0), 0.606(s)	2.828(C-25, sep, 7.0)
4α-Methylcholest-7-en-3β-ol	0.09	2J	5.176(m)	3.123(m)	0.984(d, 6.3), 0.914(d, 6.3), 0.864(d, 6.7), 0.860(d, 6.9), 0.822(s), 0.528(s)	
4α-Methylergosta-8(14),24(28)-dien-3β-ol	0.05	2K	4.719(s), 4.664(s)	3.105(m)	1.030(d, 6.9), 1.024(d, 6.9), 0.990(d, 6.4), 0.965(d, 6.9), 0.845(s), 0.717(s)	
Cholesta-7,24-dien-3β-ol	0.34	2L	5.161(m), 5.093(t, 7.3)	3.595(m)	1.683(s), 1.603(s), 0.944(d, 6.6),0.799(s), 0.538(s)	
Cholest-7-en-3β-ol (lathosterol)	0.29	2M	5.158(m)	3.591(m)	0.922(d, 6.6), 0.870(d, 6.6), 0.866(d, 6.6), 0.799(s), 0.537(s)	
Ergosta-7,24(28)-dien-3β-ol (24-methylenecholest-7-en-3β-ol)	1.16	2N	5.158(m), 4.712(s), 4.655(s)	3.597(m)	1.026(d, 6.9), 1.020(d, 6.9), 0.951(d, 6.6), 0.793(s), 0.536(s)	
(24*S*)-Ergost-7-en-3β-ol (24β-methyl-cholest-7-en-3β-ol, fungisterol)	2.51	2O	5.155(m)	3.596(m)	0.921(d, 6.6), 0.853(d, 6.9), 0.792(s), 0.779(d, 6.9), 0.772(d, 6.9), 0.528(s)	
(*Z*)-Stigmasta-7,24(28)-dien-3β-ol ([24*Z*]-ethylidenecholest-7-en-3β-ol)	2.60	2P	5.157(m), 5.105(q, 6.9)	3.597(m)	1.588(d, 6.9), 0.973(6Hs, d, 6.9), 0.948(d, 6.4), 0.793(s), 0.535(s)	2.829(C-25, sep, 7.0)
(24*S*)-Stigmast-7-en-3β-ol (24β-ethyl-cholest-7-en-3β-ol)	1.30	2Q	5.156(m)	3.596(m)	0.926(d, 6.4), 0.851(t, 7.4), 0.828(d, 6.9), 0.807(d, 6.9), 0.791(s), 0.531(s)	
(24*S*)-Stigmasta-7,25-dien-3β-ol (24β-ethylcholesta-7,25-dien-3β-ol)	0.06	2R	5.153(m), 4.731(s), 4.643(s)	3.595(m)	1.570(s), 0.913(d, 6.4), 0.805(t, 7.3), 0.796(s), 0.531(s)	
Cholest-5-en-3β-ol (cholesterol)	81.16	2S	5.350(m)	3.524(m)	1.004(s), 0.910(d, 6.6), 0.862(d, 6.6), 0.858(d, 6.6), 0.673(s)	
Cholesta-5,24-dien-3β-ol (desmosterol)	0.35	2T	5.350(m), 5.087(t, 7.1)	3.525(m)	1.680(s), 1.599(s), 1.005(s), 0.933(d, 6.6), 0.675(s)	
Cholesta-5,25-dien-3β-ol	0.03	2U	5.350(m), 4.681(s), 4.657(s)	3.524(m)	1.708(s), 1.004(s), 0.922(d, 6.2), 0.675(s)	
Ergosta-5,24(28)-dien-3β-ol (24-methylenecholest-5-en-3β-ol)	0.65	2V	5.351(m), 4.710(s), 4.653(s)	3.525(m)	1.024(d, 6.9), 1.020(d, 6.9), 1.006(s), 0.946(d, 6.6), 0.680(s)	
(24*S*)-Ergost-5-en-3β-ol (24β-methyl-cholest-5-en-3β-ol)	1.92	2W	5.351(m)	3.523(m)	1.007(s), 0.918(d, 6.6), 0.854(d, 6.9), 0.782(d, 6.7), 0.774(d, 6.7), 0.676(s)	

(Continued)

TABLE 1. (Continued)

Sterol	Total	Structure[c]	Olefin	OH Group (C-3)	Methyl Groups	Others
	%					
(24*R*)-Ergost-5-en-3β-ol (24α-methyl-cholest-5-en-3β-ol campesterol)	0.96	2X	5.351(m)	3.523(m)	1.008(s), 0.910(d, 6.5), 0.850(d, 6.9), 0.802(d, 6.9), 0.772(d, 6.6), 0.679(s)	
(24*R*)-Ergosta-5,22-dien-3β-ol (24β-methylcholesta-5,22-dien-3β-ol, brassicasterol)	0.04	2Y	5.348(m), 5.203(dd, 15.3, 7.1), 5.158(dd, 15.3, 7.6)	3.522(m)	1.010(d, 6.6), 1.009(s), 0.910(d, 6.9), 0.834(d, 6.9), 0.818(d, 6.9),0.692(s)	
(*Z*)-Stigmasta-5,24(28)-dien-3β-ol ([24*Z*]-ethylidenecholest-5-en-3β-ol, isofucosterol)	0.68	2Z	5.353(m), 5.108(q, 6.9)	3.524(m)	1.590(d, 6.7), 1.012(s), 0.978(6Hs, d, 7.0), 0.949(d, 6.6), 0.686(s)	2.828(C-25, sep, 7.0)
(24*S*)-Stigmast-5-en-3β-ol (24β-ethyl-cholest-5-en-3β-ol)	0.35	2AA	5.351(m)	3.523(m)	1.008(s), 0.914(d, 6.6), 0.853(t, 7.4), 0.830(d, 6.9), 0.810(d, 6.9), 0.678(s)	
(24*R*)-Stigmast-5-en-3β-ol (24α-ethyl-cholest-5-en-3β-ol,β-sitosterol)	1.38	2BB	5.351(m)	3.523(m)	1.008(s), 0.921(d, 6.4), 0.845(t, 7.4), 0.834(d, 6.9), 0.812(d, 6.9), 0.679(s)	
(24*S*)-Stigmasta-5,25-dien-3β-ol (24β-ethylcholesta-5,25-dien-3β-ol)	0.04	2CC	5.347(m), 4.723(s), 4.639(s)	3.522(m)	1.568(s), 1.002(s), 0.901(d, 6.6), 0.796(t, 7.4), 0.666(s)	
(24*S*)-Stigmasta-5,22-dien-3β-ol (24α-ethylcholesta-5,22-dien-3β-ol, stigmasterol)	0.05	2DD	5.349(m), 5.151(dd, 15.1, 8.6), 5.016(dd, 15.2, 8.7)	3.524(m)	1.021(d, 6.7), 1.010(s), 0.846(d, 6.3), 0.805(t, 7.4), 0.796(d, 6.3), 0.698(s)	
25-Methylergosta-5,24(28)-dien-3β-ol	0.02	2EE	5.352(m), 4.829(s), 4.657(s)	3.526(m)	1.054(9Hs, s), 1.008(s), 0.961(d, 6.6), 0.686(s)	
Cholest-8-en-3β-ol	0.01	2FF	—	3.617(m)	0.947(s), 0.907(d, 6.6), 0.868(d, 6.4), 0.864(d, 6.6), 0.595(s)	
Cholesta-8,24-dien-3β-ol (zymosterol)	0.03	2GG	5.094(t, 7.0)	3.619(m)	1.683(s), 1.603(s), 0.953(s), 0.946(d, 6.6), 0.604(s)	
Ergosta-8,24(28)-dien-3β-ol (24-methy-lenecholest-8-en-3β-ol)	0.11	2HH	4.715(s), 4.659(s)	3.616(m)	1.030(d, 6.9), 1.024(d, 6.9), 0.960(d, 6.9), 0.953(s), 0.618(s)	
(24*S*)-Ergost-8-en-3β-ol (24β-methyl-cholest-8-en-3β-ol)	0.17	2II	—	3.612(m)	0.946(s), 0.924(d, 6.6), 0.852(d, 6.9), 0.778(d, 6.9), 0.772(d, 6.9), 0.602(s)	
(*Z*)-Stigmasta-8,24(28)dien-3β-ol ([24*Z*]-ethylidenecholest-8-en-3β-ol)	0.15	2JJ	5.104(q, 7.0)	3.617(m)	1.587(d, 6.9), 0.972(6Hs, d, 6.9),0.953(d, 6.6), 0.946(s), 0.608(s)	2.828(C-25, sep, 7.1)
24-Methylenecholest-8(14)-en-3β-ol	0.05	2KK	4.714(s), 4.660(s)	3.622(m)	1.024(d, 6.9), 1.019(d, 6.9), 0.960(d, 6.70), 0.841(s), 0.687(s)	
(24*S*)-Ergost-8(14)-en-3β-ol (24β-methylcholest-8(14)-en-3β-ol)	0.48	2LL	—	3.619(m)	0.936(d, 6.7), 0.856(d, 6.9), 0.842(s), 0.786(d, 6.9), 0.784(d, 6.9), 0.691(s)	
(*Z*)-Stigmasta-8(14),24(28)-dien-3β-ol ([24*Z*]-ethylidenecholest-8(14)-en-3β-ol)	0.29	2MM	5.109(q, 6.9)	3.617(m)	1.589(d, 6.9), 0.973(d, 7.0), 0.970(d, 7.0), 0.956(d, 6.9),0.839(s), 0.686(s)	2.830(C-25, sep, 7.0)
(24*S*)-Stigmast-8(14)-en-3β-ol (24β-ethylcholest-8(14)-en-3β-ol)	0.24	2NN	—	3.621(m)	0.936(d, 6.7), 0.853(t, 7.5), 0.836(s), 0.824(d, 6.9), 0.806(d, 6.9), 0.686(s)	
5β-Cholestan-3β-ol (5β-cholestanol, coprostanol)	0.33	2OO	—	4.105(m)	0.957(s), 0.896(d, 6.6), 0.862(d, 6.6), 0.856(d, 6.6), 0.644(s)	
(24*S*)-5β-Ergostan-3β-ol (24β-methyl-5β-cholest-3β-ol, [24*S*]-methylcoprostanol)	0.15	2PP	—	4.102(m)	0.962(s), 0.906(d, 6.6), 0.856(d, 6.9), 0.785(d, 6.9), 0.768(d, 6.9),0.650(s)	
(24*R*)-5β-Stigmastan-3β-ol (24α-ethyl-5β-cholest-3β-ol, [24*R*]-ethylcoprostanol)	0.13	2QQ	—	4.105(m)	0.957(s), 0.902(d, 6.4), 0.840(t, 7.6), 0.828(d, 6.70), 0.806(d, 6.7), 0.645(s)	

[a] Chemical shift in ppm (δ).
[b] Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; sep, septet; m, multiplet.
[c] Figure showing structure in this report.
[d] [Multiplicity, coupling constant (*J*) in Hz.]

Guard pre-column (Restek Corp., Bellefonte, PA). The GLC oven initial temperature was 50°C for 3 min after injection, the first ramp was 4°C/min to 250°C, and the second ramp was 5°C/min to 350°C, which was held for 2 min. A 6 inch section of deactivated 0.10 mm ID fused silica tubing was used to restrict post-column flow in the GLC-MS transfer line to increase the back pressure on the column. The transfer line was held at 290°C throughout the analyses; helium gas flow through the column was 1 ml/min.

The single quadrupole Trace MS (Thermo Finnegan, San Jose, CA) was equipped with a electron impact (EI) ion source optimized for maximum sensitivity using perflouro tributyl amine (PFTBA) at an electron energy of 70 eV. The source temperature was held at 300°C throughout the runs. The MS was operated in full scan mode collecting the full mass spectrum from mass 39 to mass 518 amu at a rate of 2.2 scans/s.

HPLC fractionation of *P. carinii* pooled total sterol sample and NMR spectroscopy

To prepare a large pooled sample of sterols to perform NMR analysis, individual samples were redissolved in chloroform-methanol (2:1, v/v), pooled, and dried under a stream of N_2. The high concentration of cholesterol in the *P. carinii* sample relative to the other sterol components required a combination of TLC and HPLC procedures to isolate individual sterols or groups of sterols. Samples were applied on glass-backed plates coated with a 0.25 mm layer of Silica gel 60 F254, and then developed twice in hexane-ethyl acetate (4:1, v/v). Four TLC fractions were isolated, corresponding to triterpenols, 4α-methylsterols, Δ^7 sterols, and all other sterols. These TLC fractions were further fractionated by reversed-phase HPLC using a Waters 6000A pump, Waters 410 differential refractometer, and two 10 mm × 250 mm columns (packed with Altex Ultrasphere ODS-5 μm) in series. Sterols were eluted with methanol at a flow rate of 3 ml/min, and the relative proportions of sterols were determined by integrations in HPLC chromatograms. HPLC fractions containing mixtures were further fractionated by HPLC using acetonitrile-methanol-ethyl acetate (11:4:4, v/v/v) as the solvent. The HPLC-separated sterol fractions were evaporated under a stream of N_2, and then analyzed by ^{1}H-NMR spectroscopy. NMR spectra were acquired with a Bruker Avance-600 MHz instrument using $CDCl_3$ as the solvent and referenced to residual $CHCl_3$ signals (7.26 ppm).

RESULTS

Forty-three different sterol molecular species were identified in the NMR analysis of HPLC fractions obtained from the pooled *P. carinii* sterol sample (**Table 1**, **Figs. 1** and **2**). The definitive structures, which included stereoisomers, represented 10 different sterol nuclei and 15 different side chains. Most were known compounds for which NMR spectral data have previously been published, although on lower-field instruments. The 600 MHz NMR data for all sterols found in the present study are given in Table 1 and the spectra of representative *P. carinii* sterol molecular species are shown in Fig. 1. Of the 43 molecular species, 32 were 24-alkylsterols. Only two minor sterols, each comprising ≤0.05% of total sterols, had a double bond at C-22 (Figs. 2Y and 2DD).

These NMR analyses confirmed our previous GLC and GLC-MS analyses, indicating the absence in *P. carinii* of ergosterol (ergosta-5,7,22-trien-3β-ol, the major sterol of most fungi). Furthermore, no evidence of direct ergosterol precursors were detected; trienes, tetraenes and $\Delta^{5,7}$ sterol nuclei were not detected. The inability to detect ergosterol by all highly sensitive analytical methods and instrumentation used in this study further verifies that *P. carinii* prepared from our rat model and purified by our protocol (18) are pure and free from other fungi that synthesize this sterol. If ergosterol were present in this large

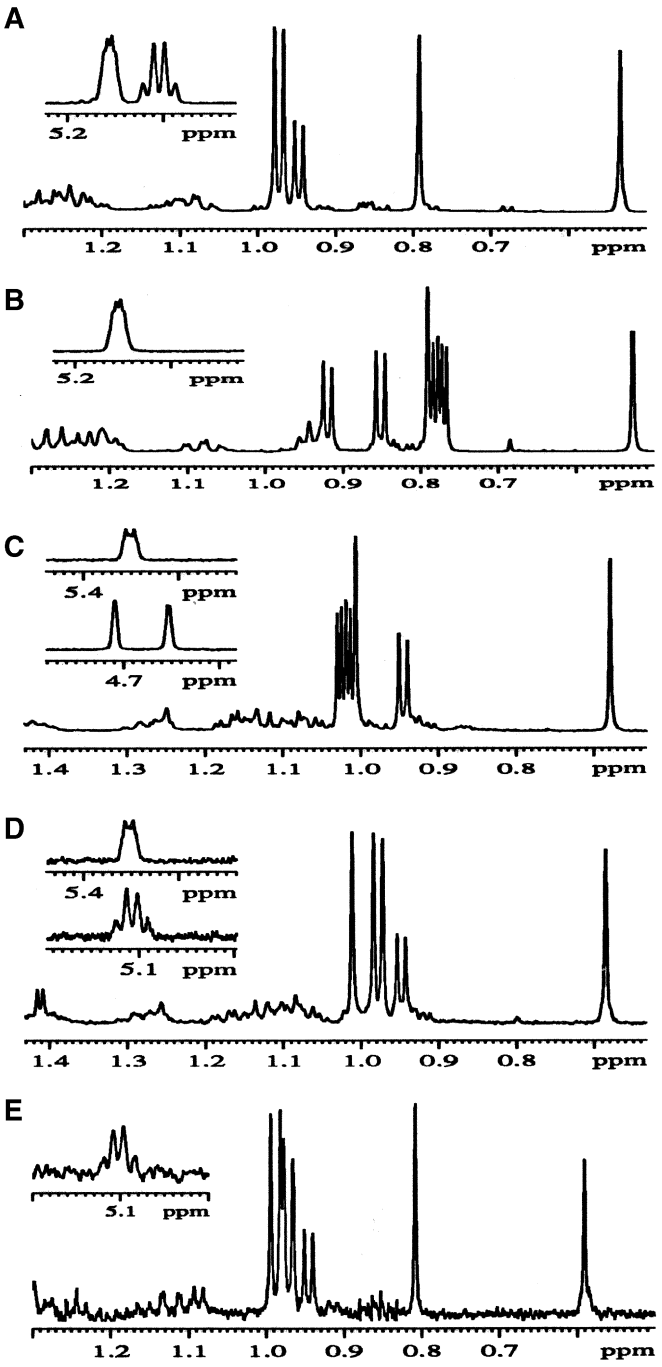


Fig. 1. NMR spectra of some *Pneumocystis carinii* sterols. A: (*Z*)-Stigmasta-7,24(28)-dien-3β-ol. B: (24*S*)-Ergost-7-en-3β-ol. C: Ergosta-5,24(28)-dien-3β-ol. D: (*Z*)-Stigmasta-5,24(28)-dien-3β-ol. E: (*Z*)-4,4-Dimethylstigmasta-8,24(28)-dien-3β-ol [(24*Z*)-ethylidene-14-norlanosterol], a novel sterol identified in this study.

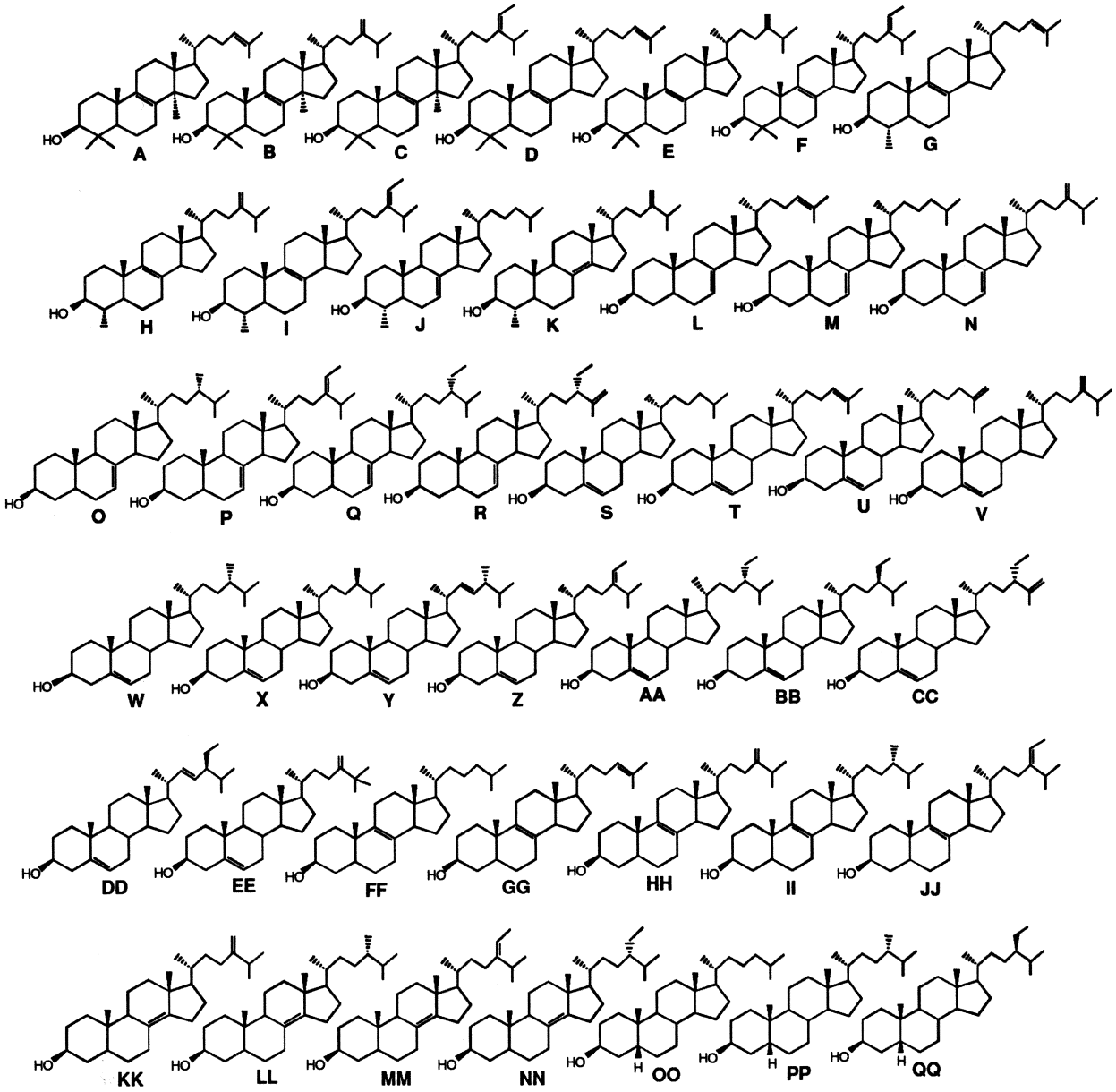


Fig. 2. *P. carinii* sterols: 4,4-dimethyl and 4α-methyl sterols (A−K); Δ^7 sterols (L−R); Δ^5 sterols (S−EE); and other sterols (FF−QQ). The $\Delta^{8(14)}$ sterols may be intermediates formed during the isomerization of Δ^8 sterols to Δ^7 sterols. A: Lanosta-8,24-dien-3β-ol (lanosterol). B: 24-Methylenelanost-8-en-3β-ol. C: (*Z*)-24-Ethylidenelanost-8-en-3β-ol (pneumocysterol). D: 4,4-Dimethylcholesta-8,24-dien-3β-ol (14-norlanosterol). E: 4,4-Dimethylergosta-8,24(28)-dien-3β-ol (24-methylene-14-norlanosterol). F: (*Z*)-4,4-Dimethylstigmasta-8,24(28)-dien-3β-ol [(24*Z*)-ethylidene-14-norlanosterol]. G: 4α-Methylcholesta-8,24-dien-3β-ol. H: 4α-Methylergosta-8,24(28)-dien-3β-ol. I: (*Z*)-4α-Methylstigmasta-8,24(28)-dien-3β-ol [4α-methyl-(24*Z*)-ethylidenecholest-8-en-3β-ol]. J: 4α-Methylcholest-7-en-3β-ol. K: 4α-Methylergosta-8(14),24(28)-dien-3β-ol. L: Cholesta-7,24-dien-3β-ol. M: Cholest-7-en-3β-ol (lathosterol). N: Ergosta-7,24(28)-dien-3β-ol (24-methylenecholest-7-en-3β-ol). O: (24*S*)-Ergost-7-en-3β-ol (24β-methylcholest-7-en-3β-ol, fungisterol). P: (*Z*)-Stigmasta-7,24(28)-dien-3β-ol [(24*Z*)-ethylidenecholest-7-en-3β-ol]. Q: (24*S*)-Stigmast-7-en-3β-ol (24β-ethylcholest-7-en-3β-ol). R: (24*S*)-Stigmasta-7,25-dien-3β-ol (24β-ethylcholesta-7,25-dien-3β-ol). S: Cholest-5-en-3β-ol (cholesterol). T: Cholesta-5,24-dien-3β-ol (desmosterol). U: Cholesta-5,25-dien-3β-ol. V: Ergosta-5,24(28)-dien-3β-ol (24-methylenecholest-5-en-3β-ol). W: (24*S*)-Ergost-5-en-3β-ol (24β-methylcholest-5-en-3β-ol). X: (24*R*)-Ergost-5-en-3β-ol (24α-methylcholest-5-en-3β-ol, campesterol). Y: (24*R*)-Ergosta-5,22-dien-3β-ol (24β-methylcholesta-5,22-dien-3β-ol, brassicasterol). Z: (*Z*)-Stigmasta-5,24(28)-dien-3β-ol [(24*Z*)-ethylidenecholest-5-en-3β-ol, isofucosterol]. AA: (24*S*)-Stigmast-5-en-3β-ol (24β-ethylcholest-5-en-3β-ol). BB: (24*R*)-Stigmast-5-en-3β-ol (24α-ethylcholest-5-en-3β-ol, β-sitosterol). CC: (24*S*)-Stigmasta-5,25-dien-3β-ol (24β-ethylcholesta-5,25-dien-3β-ol). DD: (24*S*)-Stigmasta-5,22-dien-3β-ol (24α-ethylcholesta-5,22-dien-3β-ol, stigmasterol). EE: 25-Methylergosta-5,24(28)-dien-3β-ol. FF: Cholest-8-en-3β-ol. GG: Cholesta-8,24-dien-3β-ol (zymosterol). HH: Ergosta-8,24(28)-dien-3β-ol (24-methylenecholest-8-en-3β-ol). II: (24*S*)-Ergost-8-en-3β-ol (24β-methylcholest-8-en-3β-ol). JJ: (*Z*)-Stigmasta-8,24(28)-dien-3β-ol [(24*Z*)-ethylidenecholest-8-en-3β-ol]. KK: 24-Methylenecholest-8(14)-en-3β-ol. LL: (24*S*)-Ergost-8(14)-en-3β-ol [24β-methylcholest-8(14)-en-3β-ol]. MM: (*Z*)-Stigmasta-8(14),24(28)-dien-3β-ol [(24*Z*)-ethylidenecholest-8(14)-en-3β-ol]. NN: (24*S*)-Stigmast-8(14)-en-3β-ol [24β-ethylcholest-8(14)-en-3β-ol]. OO: 5β-Cholestan-3β-ol (coprostanol). PP: (24*S*)-5β-Ergostan-3β-ol [(24*S*)-methylcoprostanol]. QQ: (24*R*)-5β-Stigmastan-3β-ol [(24*R*)-ethylcoprostanol].

TABLE 2. GLC and MS analyses of *P. carinii* total sterol samples and fractions isolated by HPLC

Sterol	Structure[b]	GLC Peak Assigned to Sterols in P. carinii[c]	GLC (RRT)[a] Total Sterols[d]	GLC (RRT)[a] HPLC Fractions[e]	Molecular Mass[f] (Mass spectrum)
Cholest-5-en-3β-ol (Cholesterol)	2S	1	1.000−1.000	1.000	386 (Fig. 2 in ref. 13)
Cholesta-7,24-dien-3β-ol	2L	2	1.028[g]	1.054	384 (Fig. 5A in ref. 13)
Cholesta-5,24-dien-3β-ol (Desmosterol)	2T	3	1.046−1.019	1.058	384 (Fig. 4A)
Cholesta-7-en-3β-ol (Lathosterol)	2M	5	1.076[g]	1.086	386 (Fig. 4B)
Ergosta-5,24(28)-dien-3β-ol	2V	7	1.144−1.085	1.177	398 (Fig. 4C)
(24*S*)-Ergost-5-en-3β-ol	2W	8	1.175−1.123	1.198	400 (Fig. 4A in ref. 13)
(24*R*)-Ergost-5-en-3β-ol (Campesterol)	2X	8	1.175−1.123	1.198	400 (Fig. 4A in ref. 13)
Cholest-5-en-3-one		10	1.221−1.157	NA[h]	384 (Fig. 1 in ref. 13)
(24*S*)-Ergost-8(14)-en-3β-ol	2HH	11	1.226[g]−1.179	1.189	400
Ergosta-7,24(28)-dien-3β-ol	2N	12	1.278−1.214	1.293	398 (Fig. 4C in ref. 13)
(24*S*)-Ergost-7-en-3β-ol (Fungisterol)	2O	13	1.294−1.234	1.323	400 (Fig. 4B in ref. 13)
(24*R*)-Stigmast-5-en-3β-ol (β-Sitosterol)	2BB	15	1.379−1.313	1.408	414 (Fig. 5A in ref. 13)
(24*S*)-Stigmast-5-en-3β-ol	2AA	15	1.379−1.313	1.408	414 (Fig. 5A in ref. 13)
(*Z*)-Stigmasta-5,24(28)-dien-3β-ol (Isofucosterol)	2Z	16	1.421−1.347	1.413	412 (Fig. 4D)
Lanosta-8,24-dien-3β-ol (Lanosterol)	2A	17	1.459−1.381	1.443	426 (Fig. 4E)
(24*S*)-Stigmast-7-en-3β-ol	2Q	19	1.520−1.444	1.532	414 (Fig. 5B in ref. 13)
(*Z*)-Stigmasta-7,24(28)-dien-3β-ol	2P	20	1.567−1.491	1.611	412 (Fig. 5C in ref. 13)
24-Methylenelanost-8-en-3β-ol (Eburicol)	2B	23	1.619−1.169[i]	NA[h]	440 (Fig. 3A in ref. 20)
(*Z*)-24-Ethylidenelanost-8-en-3β-ol (Pneumocysterol)	2C	24	2.166−2.026[j]	NA[h]	454 (Fig. 3C in ref. 20)

[a] RRT, GLC relative retention time.
[b] Figure showing structure in this report.
[c] GLC peak assignments from references 13 and 20.
[d] RRT values obtained in analyses of total sterol samples.
[e] RRT values obtained in analyses of sterol fractions obtained by HPLC.
[f] Obtained by mass spectrometry of GLC peak component.
[g] RRT from reference 13.
[h] NA, HPLC fraction not analyzed by GLC.
[i] RRT of authentic standard.
[j] RRT from reference 20.

pooled *P. carinii* total sterol sample, it should have been detected in this study or in previous studies using GLC-MS techniques.

The lanosterol derivatives (*Z*)-24-ethylidenelanost-8-en-3β-ol (pneumocysterol; Fig. 2C) and 24-methylenelanost-8-en-3β-ol (24-methylenelanosterol; Fig. 2B) were previously identified and characterized in human-derived *P. jiroveci* (15, 20, 27, 28). These two compounds were identified in the present study of rat-derived *P. carinii* sterols. We here correct the trivial name (eburicol) for 24-methylenelanost-8-en-3β-ol, which was incorrectly referred to as euphorbol in a previous report on *P. jiroveci* lanosterol derivatives (20). In the present study the definitive structure of 24-methylenelanosterol in *P. carinii* was confirmed. Also, GLC-MS was previously used to detect and identify eburicol in *P. jirovec*, and since it is difficult to obtain consistent RRT from system to system (due to the configuration of the instruments), in the present study, the RRT of an authentic standard of this sterol was determined using capillary column GLC alone. This sterol eluted earlier from the SPB-5 GLC column than it did under the GLC-MS conditions used previously, which was confirmed in the present study (**Table 2**). Other compounds with methyl groups at C-4 of the sterol nucleus and/or a double bond at C-8, apparently derived from lanosterol and/or 24-alkyllanosterol compounds, were also present (Fig. 2A−K). The majority of the molecular species in this group were 24-alkylsterols.

Among the sterols apparently synthesized de novo by *P. carinii* are its distinct Δ^7 24-alkylsterols (9–11, 13, 16, 17, 19) (Fig. 2L−R). Several were present in relatively high concentrations. We earlier identified (*Z*)-stigmasta-7,24(28)-dien-3β-ol (24-ethylidenecholest-7-en-3β-ol; Fig. 2P) by GLC and MS techniques as a C_{29} diene 24-alkylsterol (13). Based on those earlier data, double bond positions could not be definitively assigned then, but the NMR data now unambiguously identified their locations within the molecule.

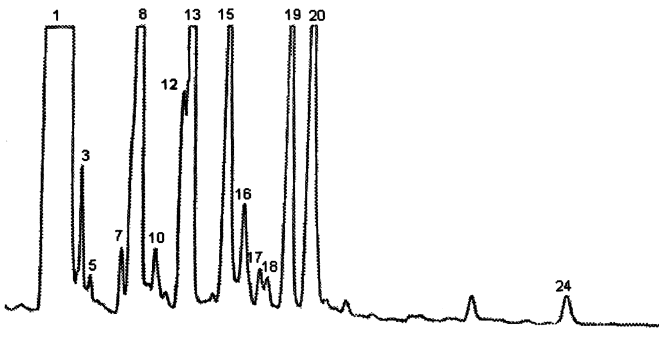
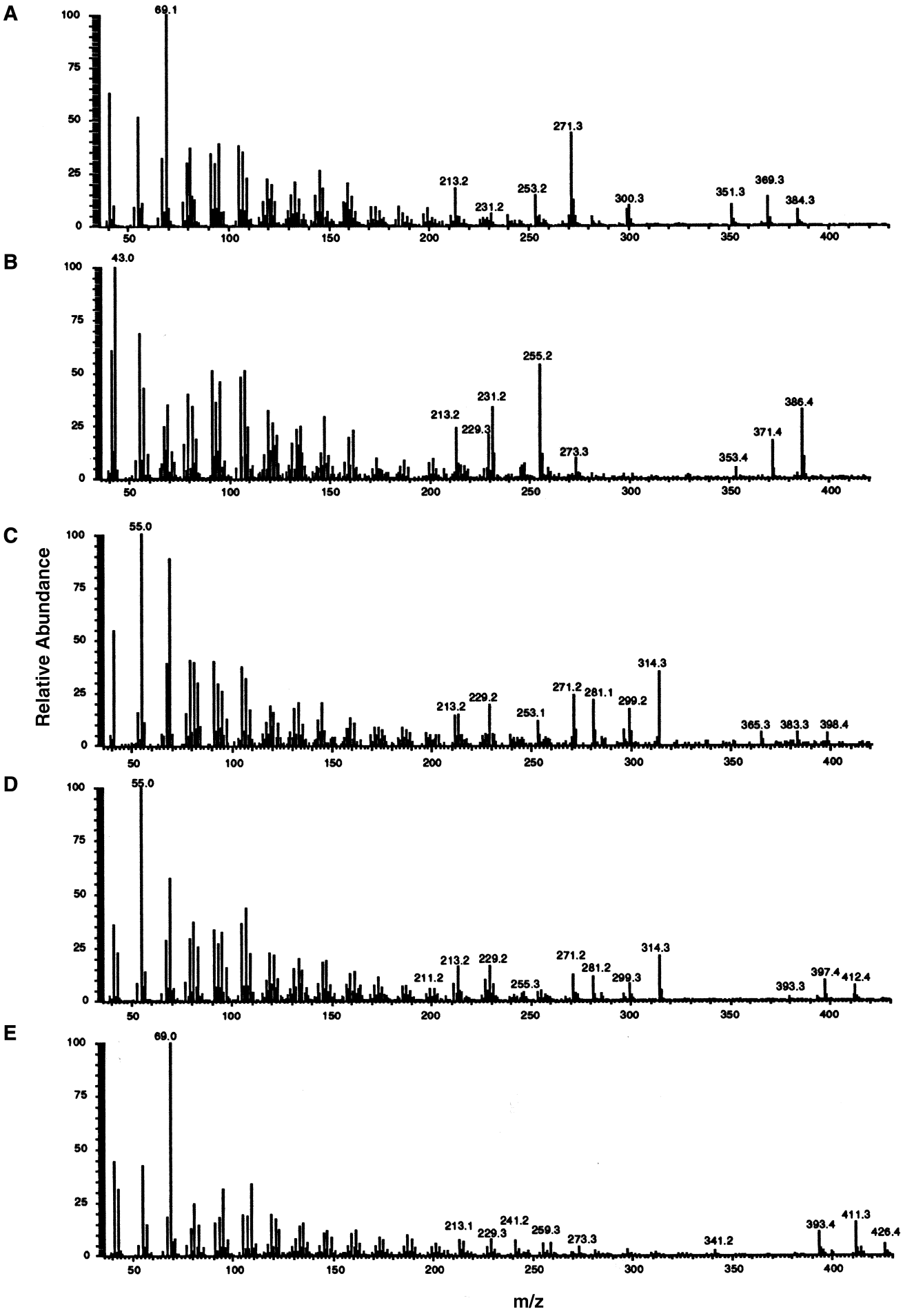


Fig. 3. Gas chromatogram of total *P. carinii* sterols analyzed by overloading the gas-liquid chromatography (GLC) column to detect and integrate peaks representing minor components in the sample. GLC peak numbers are designated according to the order of elution. The sterol GLC profile is equivalent to that reported previously (13) indicating stable sterol composition in the organisms and the purity of the isolated preparations.



Four 24-alkylsterols with an 8(14) double bond (Fig. 2KK–NN) may represent intermediates formed during Δ^8 to Δ^7 isomerization occurring within the sterol nucleus. (*Z*)-4,4-Dimethylstigmasta-8,24(28)-dien-3β-ol [(24*Z*)-ethylidene-14-norlanosterol; Fig. 2R] identified in the present study is novel; this sterol has not been previously reported.

The large numbers of Δ^5 sterol molecular species, including those with alkyl groups at C-24, suggest that host-derived sterols with Δ^5 nuclei have their side chains modified by *P. carinii*. We previously identified ergosta-5,24(28)-dien-3β-ol (24-methylenecholest-5-en-3β-ol; Fig. 2W) only as a C_{28} diene 24-alkylsterol using GLC-MS (13). In the present study, NMR spectroscopy enabled the definitive assignments of the two double bond positions. Urbina et al. (17) had reported identifying ergosta-5,24(25)-dien-3β-ol and 24-ethylcholesta-5,24(25)-dien-3β-ol as major sterols in *P. carinii* by employing argentation TLC, GLC, and GLC-MS methods. In contrast, no 24-alkylsterols with a 24(25)-double bond were detected in the present study. Descriptions of their chromatographic properties and relative concentrations suggest that the structures of these sterols were ergosta-5,24(28)-dien-3β-ol (24-methylenecholest-5-en-3β-ol; Fig. 2V) and (*Z*)-24-stigmasta-5,24(28)-dien-3β-ol (24-ethylidenecholest-5-en-3β-ol; Fig. 2Z), respectively.

GLC analysis of the sterols from *P. carinii* organisms isolated and purified from this corticosteroid-immunosuppressed rat model showed highly stable and reproducible compositional profiles (**Fig. 3**, Table 2). The relative proportions of GLC sterol components varied from sample to sample, but considering that we are not working with clonally-derived organisms grown in culture, the GLC profiles published in 1994 (13) were remarkably similar to those of organisms currently isolated; the major sterol components remained the same. Since cholesterol comprises about 75% of the *P. carinii* sterols, in order to detect and integrate the GLC peak areas of minor sterol components, high concentrations of total sterol samples were injected onto the column. Under over-load conditions, the RRT values for GLC peaks are altered; elution of the compounds from the column, including cholesterol (to which retention times of other sterols were referenced), is delayed. Thus, the broad ranges in RRT values presented in Table 2 reflect the necessity to perform some analyses under sub-optimal GLC conditions.

It is known that GLC peaks and chromatographic fractions can represent multiple components that are not resolved by the technique. However, NMR analysis is not feasible to perform for many individual experiments on *P. carinii*, as explained above. GLC is a highly sensitive analytical tool useful for studies of sterol metabolism. Thus, *P. carinii* sterol fractions separated by HPLC and total *P. carinii* sterol samples were analyzed by GLC to correlate the GLC peaks in chromatograms with the major components for which definitive structures were assigned by NMR analysis (Table 2).

Correlations were made between mass spectra of components represented by the GLC peaks (**Fig. 4**). The sterol molecular weights and structures (based on elemental compositions and fragmentation patterns) were all consistent with the assignments made by the NMR analyses.

DISCUSSION

The vast number of sterol molecular species in *P. carinii* is best explained by the existence of a variety of sterol metabolism enzymes and branched pathways that lead to their formation in the organism. The sterol biosynthetic pathways in this important pathogen have yet to be elucidated and mapped. Only a few experiments have been reported on the direct analysis of sterol metabolism reactions in the organism. However, much insight into sterol metabolism in these organisms proliferating in rat lungs can be gained from this comprehensive analysis of *P. carinii* sterol structures. The assemblage of sterol molecules suggests the following mechanisms by which the organism obtains and produces appropriate sterols for optimal functioning of its cellular membranes.

One mechanism is the scavenging of sterols present in the lung alveolus and incorporating them unchanged into the organism's own membranes. The major sterol available for scavenging in the rat lung is cholesterol, which is synthesized by mammals, including humans. 24-Alkylsterols of plant origins can be ingested and become incorporated in small amounts into mammalian tissues (29). Our organism preparations contain the plant-derived 24-alkylsterols, campesterol [(24*R*)-ergost-5-en-3β-ol, 24-methylcholest-5-en-3β-ol], β-sitosterol [(24*R*)-stigmast-5-en-3β-ol, 24-ethylcholest-5-en-3β-ol], brassicasterol [(24*R*)-ergosta-5,22-dien-3β-ol, 24β-methylcholesta-5,22-dien-3β-ol], and stigmasterol [(24*S*)-stigmasta-5,22-dien-3β-ol, 24α-ethylcholesta-5,22-dien-3β-ol]. We previously examined control lungs from normal untreated rats and corticosteroid-treated uninoculated rats and identified campesterol and β-sitosterol among the lung sterols (13). Furlong et al. (16) reported that brassicasterol [(24*R*)-ergosta-5,22-dien-3β-ol, 24-methylcholesta-5,22-dien-3β-ol] and stigmasterol (24-ethylcholesta-5,22-3β-ol) were among the eight major sterols in their *P. carinii* preparations. They did not report on the sterol composition of control rat lungs to determine whether these sterols could have been scavenged from the host by the organisms in their experimental system. In the present study, these sterols were identified only as minor components, suggesting that *P. carinii* does not form Δ^{22} sterols. As a working hypothesis, we tenta-

Fig. 4. *(Preceding page)*. Mass spectra of some *P. carinii* sterols. A: Cholesta-5,24-dien-3β-ol, MW 384 (desmosterol, GLC peak 3). B: Cholest-7-en-3β-ol, MW 386 (lathosterol, GLC peak 5). C: Ergosta-5,24(28)-dien-3β-ol, MW 398 (GLC peak 7). D: (*Z*)-Stigmasta-5,24(28)-dien-3β-ol, MW 412 (isofucosterol, GLC peak 16). E: Lanosterol, MW 426 (GLC peak 17).

tively conclude that the four plant sterols, campesterol, β-sitosterol, brassicasterol, and stigmasterol, were taken up by the animals from their diet of rat chow, incorporated into lung lipids, and subsequently scavenged by *P. carinii* along with rat-synthesized cholesterol. These sterols were then incorporated unchanged into *P. carinii* membranes.

A second mechanism for the formation of sterols found in *P. carinii* is by modifying sterols that have been scavenged from the host. It is likely that the various Δ^5 sterols identified in *P. carinii* were compounds initially scavenged and then metabolized by the organism. Since most of the Δ^5 sterol species are alkylated at the C-24 position of the sterol side chain and because mammals cannot form these structures, it is likely that the organism can use Δ^5 sterols (e.g., cholesterol or desmosterol found in the lung) as productive substrates for the organism's SAM:SMT. There is currently no evidence that *P. carinii* can form a double bond at C-5 of the sterol nucleus. Preliminary data indicate that the *P. carinii* recombinant SAM:SMT enzyme expressed in a bacterial system transfers methyl groups to the C-24 position of Δ^5 sterols (unpublished observations).

A third mechanism is de novo synthesis. It was previously shown that *P. carinii* is capable of synthesizing sterols de novo via the acetate/mevalonate pathway (9, 10, 19, 24, 30). Metabolic incorporations of HMG-CoA, mevalonate, and squalene into sterols were demonstrated (12, 19, 24, 30). Also, lovastatin-sensitive HMG-CoA reductase activity, which converts HMG-CoA to mevalonic acid, was reported in *P. carinii* (19).

The first identification and report of naturally occurring (*Z*)-24-ethylidenelanost-8-en-3β-ol was in the lauracean plant *Neolitsea sericea* (pencil cactus) (31). This was later given the trivial name pneumocysterol when it was found to comprise a major component of some human-derived *P. jiroveci* (*P. carinii* f. sp. *hominis*) isolated organism populations (20). Pneumocysterol and 24-methylenelanosterol have now been definitively identified in rat-derived *P. carinii* (*P. carinii* f. sp. *carinii*). The detection of 24-alkylated lanosterol molecules and their products is consistent with our finding that the recombinant *P. carinii* SAM:SMT expressed in *Escherichia coli* has a unique substrate specificity favoring the biomethylation of sterols with the lanosterol nucleus (22, 32). It has been reported that cycloartenol, a key sterol intermediate in some plants, is the preferred substrate for the SAM:SMT enzyme in *Neolitsea* (31), but cycloartenol was not detected in *P. carinii,* SAM:SMT activity on this substrate was not detected. Thus, although pneumocysterol is synthesized in both *Neolitsea* and *Pneumocystis*, the intermediates and metabolic pathways leading to its formation differ in the two organisms.

Mammals cannot desaturate C-22 or alkylate the C-24 position of the sterol side chain. From the data obtained in the present study, we conclude that it is highly unlikely that *P. carinii* desaturates sterol C-22 and hence the reactions leading to this sterol modification do not represent a potential drug target in this organism. In contrast, evidence mounts for high C-24 alkylation by SAM:SMT activity (22, 32). Also, the *P. carinii* SAM:SMT has unusual properties; its affinity for the major sterol intermediate C_{30} lanosterol appears to be greater than for its biosynthetic products (e.g., C_{27} zymosterol) that have undergone sterol nucleus demethylation. This enzyme property provides additional possibilities for the development of drugs specifically directed against *Pneumocystis*. In *P. carinii,* the substrate for sterol nucleus demethylation reactions (e.g., cytochrome P-450-dependent lanosterol 14α-demethylase homologs in other organisms) is apparently not lanosterol but appears to be 24-alkylsterols (e.g., pneumocysterol and 24-methylenelanosterol). In the present study, these putative substrate intermediates were unambiguously identified in *P. carinii* organisms, consistent with the suggestion that the major pathways for sterol biosynthesis in this pathogen are unusual.

Isomerization of the sterol nucleus double bond from the C-8 to the C-7 position is also a possible target worthy of further investigation. With the exception of some plant pathogenic rust fungi in the class Uredinales, production and accumulation of C_{28} and C_{29} Δ^7 24-alkylsterols are not typical of most fungi (33). The efficacy of the sterol Δ^8-to-sterol Δ^7 inhibitor AY 9944 in reducing *P. carinii* viability (21) indicates that this modification of the sterol nucleus may be another excellent target for the development of chemotherapeutic agents against this ubiquitous opportunistic pathogen.

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