Isolation and Structural Determination of Enfumafungin, a Triterpene Glycoside Antifungal Agent That Is a Specific Inhibitor of Glucan Synthesis

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Received December 20, 1999

Abstract: Enfumafungin (**1**) is a hemiacetal, triterpene glycoside that was isolated from a fermentation of *Hormonema* sp. as a mixture of two interconverting forms. The primary structure of the major component of the mixture was determined as part of the mixture, mainly via NMR and comparison with hyalodendroside A (**2**), a related hemiacetal, triterpene glycoside that exists in a single form. The primary structure was confirmed, and the relative stereochemistry determined, based on a pair of methylacetal derivatives (**3** and **4**). Enfumafungin is an antifungal agent that acts as a specific inhibitor of glucan synthesis in cells and in vitro, and leads to morphological changes in yeasts and molds.

The echinocandin group of antifungal antibiotics variously known as echinocandins,[1] pneumocandins,[2] aculeacins,[3] and the mulundocandins,[4] in addition to the papulacandins,[5] represents the only naturally occurring compounds that show potent and specific inhibition of fungal glucan synthesis. In particular, the compound known as caspofungin acetate (MK-0991; L-743,872), derived from chemical modification of pneumocandin B_0, has demonstrated potent in vitro[6] and in vivo[7] antifungal activity in a variety of models and is presently in clinical studies.[8,9] The echinocandins and papulacandins belong to the lipopeptide and liposaccharide structural classes, respectively. In this paper the isolation and structural determination of enfumafungin (**1**) (Figure 1), a novel antifungal agent, will be presented. This compound is an inhibitor of glucan synthesis belonging to a new structural class, the triterpene glycosides. The fermentation and taxonomy of the producing organism will be published elsewhere,[10] as will the antifungal mode-of-action[11] of enfumafungin.

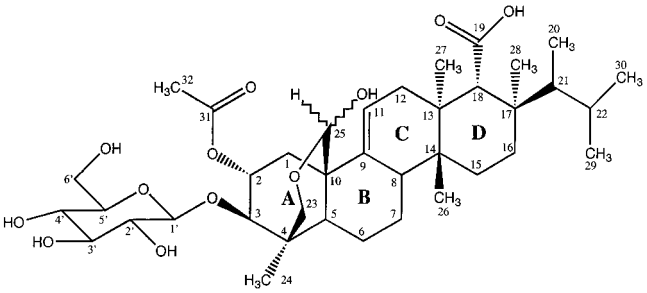

Figure 1. Structure of enfumafungin (**1**).

The structure determination of this compound was complex because it consisted of a mixture of two interconverting forms. To determine the structure, a multifaceted approach was used. The correct structure was proposed by comparison to a noninterconverting, related compound, as previously described[12] and confirmed using noninterconverting derivatives of enfumafungin. In addition, the relative stereochemistry was determined via NMR on one of the noninterconverting derivatives.

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10.1021/ja9944296 CCC: $19.00 © 2000 American Chemical Society
Published on Web 04/29/2000

Results and Discussion

Structure Determination of Enfumafungin. A methyl ethyl ketone extract (35 mL) of the original screening fermentation of *Hormonema* sp., as described by Pelaez et al.,[10] was partitioned, HPLC bioautographed, and assigned a high priority based upon the biological profile of the semipurified, active antifungal region. Enfumafungin was isolated and identified as the active antifungal principal from a 3.5 L fermentation of this organism.

The UV spectrum of **1** consisted of end absorption only; the IR spectrum indicated the presence of carbonyl and hydroxyl functionalities. High-resolution FAB-MS suggested a molecular formula of $C_{38}H_{60}O_{12}$ for enfumafungin, indicating nine degrees of unsaturation. Mass spectral fragmentation implied the presence of a hexose sugar ($C_6H_{11}O_6$, tetra-TMS modified) and an acetate moiety. Silylation of the parent molecule indicated the presence of six exchangeable protons.

Preliminary ^{1}H NMR spectroscopy showed doubling of all signals in an approximate 2:1 ratio; as expected, this doubling of signals was also observed in the ^{13}C NMR spectrum, complicating its interpretation and strongly suggesting that enfumafungin exists in two forms. Analytical reversed-phase HPLC indicated a single, broad peak under a variety of conditions, while a NOESY NMR experiment suggested that the two forms are not in fast chemical exchange on the NMR time scale. Silica gel TLC, EtOAc/MeOH/H_2O (90:10:4), clearly separated the active composite into two spots. These data suggested that enfumafungin consists of (1) a mixture of two very closely related, noninterconverting isomers or (2) a mixture of two slowly interconverting forms of a compound.

Fractions from a preparative silica gel HPLC of purified enfumafungin (EtOAc/MeOH/H_2O 95:5:4), as monitored via analytical TLC, indicated the separation of two nonoverlapping peaks. However, TLC of the separated components showed that they begin to reequilibrate into the original mixture within a few hours. NMR spectra of the early-eluting component obtained 5 h after separation, were enriched in the minor component, while the NMR spectra obtained after 3 days were back to the 2:1 major-to-minor ratio observed in the original sample. A similar phenomenon was observed for the late-eluting component, which had a greater than 2-fold enrichment in the major component at 5 h, but which also eventually reverted to the 2:1 equilibrium mixture. These data indicated that enfumafungin was a mixture of two slowly interconverting isomers.

Working from the premise that there are 38 carbon atoms (as determined from MS) and taking into account data obtained from DEPT and HMQC spectra, 38 pairs of carbon signals (8 CH_3, 8 CH_2, 14 CH, and 8 C) could be identified. This led to the determination that 54 of the 60 hydrogen atoms in **1** were attached to carbon atoms; thus, six of the hydrogens were deemed exchangeable, consistent with the six exchangeable protons noted in the MS data. In the following discussion, all spectroscopic data refer to the subset of spectra belonging to the major diastereomer.

^{1}H and ^{13}C NMR data (Table 1) indicated the presence of (1) an acetate group, (2) a hexose sugar, (3) a trisubstituted double bond, (4) an ester/carboxylic acid group, and (5) a doubly oxygenated methine carbon, likely belonging to an acetal/hemiacetal functionality. These functionalities accounted for four of the nine degrees of unsaturation (assuming a cyclized sugar) and all twelve of the oxygen atoms present in the molecule (Table 2). The remaining unsaturations were attributed to the presence of five rings in the aglycon portion of **1**. Assuming that the sugar moiety possessed four of the exchangeable protons, then the other two are associated with the carboxylic acid and the hemi-acetal group.

A deuterium shift experiment[13] was performed to substantiate this assumption. As indicated in Table 1, both carbons at δ^{13}C 97.2 d (C25) and 177.6 s (C19) experienced shifts consistent with their bearing hydroxyl groups. In addition, the adjacent carbons, C10 (δ^{13}C 45.3) and C18 (δ^{13}C 53.4), respectively, showed smaller, but significant shifts in this experiment. Confirmation of the presence of a carboxylic acid in **1** was achieved via the successful formation of a methyl ester derivative with diazomethane.

^{1}H−^{1}H NMR connectivity experiments (COSY, LRCOSY, TOCSY) and ^{1}H−^{13}C NMR connectivity experiments (HMQC, HMBC) clearly established the substructures **1a**−**1e** depicted in Figure 2. ^{1}H NMR coupling constant and ^{13}C NMR chemical shift data indicated that the sugar was glucose. The β-linkage was determined based on the ^{1}H coupling constant of the anomeric proton (7.7 Hz) and the ^{13}C chemical shift of the anomeric carbon (the anomeric carbon in a β-linked sugar has a much more downfield shift than that in the corresponding α-linked sugar).[14] At this point, the structure determination slowed since all of the spectral data had been recorded on the mixture of diastereomers and interpretation was made difficult due to signal overlap.

However, the structure of another compound, hyalodendroside A (**2**)[12] (Figure 3), had just been determined and a number of structural similarities were observed between it and **1**. Like **1, 2** had 38 carbons, a β-linked glucose moiety, an acetate at C2, and a hemiacetal functionality. Additionally, **2** existed in only one form, unlike **1**, although it also contained a hemiacetal. On the basis of these similarities, the assumption was made that enfumafungin was a triterpene glycoside rather than a steroidal glycoside.

With this assumption in mind, the spectral data for enfumafungin were reanalyzed. Substructure **1a** (Figure 2) was assumed to be a part of the triterpene A ring. Substructure **1b** (Figure 2) appeared to be the terminus of the triterpene skeleton, where the E ring found in **2** had been oxidatively cleaved between C19 and C20. However, neither C28 nor C29 were oxidized in **1** and, therefore, the hemiacetal moiety was not in the same location in enfumafungin as it was in **2**. Careful analysis of LRCOSY and HMBC data placed the hemiacetal moiety adjacent to substructure **1a** (Figure 2). Substructure **1f** accounted for two of the five rings present in the aglycon portion of **1**. Assuming that the other three rings were the six-membered B, C, and D rings of a triterpene skeleton, as in **2**, the NMR data for **1** were reexamined and a structure for this compound was proposed.

Some of the key ^{1}H−^{1}H and HMBC correlations that tied the structure together are shown in Figure 4. High-resolution electron impact MS fragmentation data fully supported the proposed structure for **1** (Figure 4).

Derivatization and the Relative Stereochemistry of Enfumafungin. Initial NMR studies suggested that the mixture might contain a hemiacetal moiety. Assuming that equilibration of the hemiacetal between its two anomers was responsible for the interconversion of this molecule, an attempt was made to methylate the hemiacetal and separate the two diastereomers. Analytically, either *p*-toluene sulfonic acid or dry HCl in MeOH successfully effected the conversion. Scale-up of the HCl procedure, followed by separation on HPLC, led to four major

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Table 1. ^{1}H and ^{13}C NMR Data for the Major Diastereomer of Enfumafungin (**1**) in CD_3OD and for **3** and **4** in CD_3OD[a]

		1				**3**				**4**			
	C type	δ ^{13}C	deut. shift?	δ ^{1}H (mult)		δ ^{13}C	δ ^{1}H	HMBC	ROESY	δ ^{13}C	δ ^{1}H	HMBC	ROESY[b]
1	CH_2	39.8	no	2.69 (dd, 13.7, 7.1)	e	40.5	2.65	4.30	H-1a, H-2	45.3	2.24	4.41	H-1a, H-2, H-11, H-25
				1.13 (dd, 13.1, 11.2)	a		1.12		H-3, H-5, H-2(s)		1.29		H-1e, H-3, H-5, H-2(s)
2	CH	74.4	no	5.75 (m)	a	74.5	5.74	2.65, 3.53	H-1a(s), H-1e, H-23e	74.6	5.67	1.29, 2.24, 3.52, 3.26	H-a(s), H-1e, H-3, H-23e, H-25
3	CH	87.8	no	3.54 (d, 8.9)	a	87.8	3.53	2.65, 5.74, 3.56, 4.02, 4.37	H-5, H-24,H-2, H-1a(s)	87.9	3.52	2.24, 3.66, 3.55, 0.91	H-1a, H-2, H-5, H-24, H-1′
4	C	41.3	no			40.0		3.53, 0.91		40.8		3.52, 3.55, 0.91	
5	CH	41.2	no	1.74 (m)	a	41.3	1.73	2.65, 3.53, 1.74, 3.56, 0.91	H-3, H-1a	38.8	1.58	2.24, 3.52, 3.55, 0.91	H-1a, H-3, H-24, H-8
6	CH_2	20.9	no	1.86 (m)	a	20.8	1.86		H-25, H-23a(s)	21.4	1.80		H-23a, H-24
				1.74 (m)	e		1.74				1.60		
7	CH_2	19.4	no	1.76 (m)	e	19.4	1.82		H-15a	18.7	1.68		H-8
				1.18 (m)	a		1.22		H-25(s), H-26		1.60		H-26
8	CH	41.5	no	2.05 (m)	a	41.4	2.06	1.86, 1.82, 5.62	H-12a, H-27, H-6e, H-5	40.1	2.10	0.80	H-7e, H-15a, H-27, H-5
9	C=	140.3	no	-		140.0		1.57		143.6		1.29, 1.98	
10	C	45.3	yes (+0.030)			44.8		2.65, 1.73, 5.62, 4.30		43.3		1.29, 2.24, 5.37	
11	CH=	125.1	no	5.63 (brd, 6.1)		125.0	5.62	1.57	H-12a/e, H-33	120.0	5.37	1.98	H-1e, H-12a/e, H-25
12	CH_2	38.6	no	2.02 (m)	e	39.0	1.95		H-27	38.5	1.98	1.16	H-12e, H-18, H-26
				1.60 (m)	a		1.57		H-18		1.54		H-11, H-12a
13	C	39.0	no			38.7		5.62, 1.57, 2.85, 1.17		38.9		5.37, 1.98, 0.80	
14	C	38.5	no			39.0		2.85, 1.26, 2.21, 1.21		38.8		2.85, 0.80, 1.16	
15	CH_2	29.0	no	1.48 (m)	a	29.0	1.50		H-8	29.3	1.48		H-16a
				1.37 (m)	e		1.42		H-7e, H-8(w) H-26		1.42	2.85, 0.78, 1.26, 2.21, 1.21	H-8, H-26
16	CH_2	30.0	no	1.60 (m)	e	30.0	1.63		H-18, H-22	30.0	1.60	1.21	H-16a, H-20
				1.20 (m)	a		1.24		H-18, H-22		1.24		H-16e
17	C	41.7	no			41.7		0.78, 1.26, 2.21, 1.21		41.7		0.80	
18	CH	53.4	yes (+0.046)	2.85 (s)	a	53.5	2.85	1.17, 1.21, 0.86	H-26, H-12a, H-22, H-16a	53.7	2.87	1.16, 1.21	H-12a, H-16a, H-21, H-22, H-26
19	C=O	177.6	yes (+0.099)			177.9		2.85		177.8		2.85	
20	CH_3	8.4	no	0.75 (d, 7.5)		8.4	0.78	2.21, 1.26, 0.91		8.4	0.78	1.26, 2.21	H-16e, H-21, H-28
21	CH	49.1	no	1.35 (m)		49.2	1.26	2.85, 0.78, 2.21, 0.91, 0.86	H-18	49.2	1.26	2.85, 0.78, 1.21, 0.91, 0.86	H-18, H-22, H-29
22	CH	27.7	no	2.27 (m)		27.7	2.21	0.78, 1.26, 0.91, 0.86	H-18	27.6	2.21	0.78, 1.26, 0.91, 0.86	H-21, H-18, H-29, H-30
23	CH_2	66.1	no	4.00 (d, 12.3)	e	65.9	4.02		H-2(w)	60.0	3.66	3.52, 0.91, 4.41	H-24, H-33, H-2(w)
				3.59 (d, 12.3)	a		3.56		H-25, H-23e, H-6a		3.55		H-6a, H-24, H-33
24	CH_3	17.5	no	0.91 (s)		17.5	0.91	3.53	H-23a/e, H-3	18.1	0.91	3.52	H-3, H-6a/e, H-7a, H-5, H-23a/e
25	CH	97.2	yes (+0.100)	4.69 (br s)		105.1	4.30	1.12, 2.65, 3.65, 3.41	H-7a(s), H-6a(w), H-23a(s)/e(w), H-33, H-26	106.3	4.41	2.24, 3.55, 3.26	H-1e, H-2, H-11, H-33
26	CH_3	16.1	no	0.78 (s)		16.0	0.78		H-18, H-11, H-33, H-12a, H-11, H-25	16.4	0.80		H-7, H-33, H-18, H-12a
27	CH_3	17.2	no	1.19 (s)		17.1	1.17	2.85	H-8, H-12e, H-15a	17.0	1.16	1.98, 2.85	H-8, H-12a, H-15a, H-28
28	CH_3	21.2	no	1.22 (s)		21.1	1.21	2.85		21.2	1.21	2.85, 1.60	
29	CH_3	25.3	no	0.90 (d, 6.7)		25.3	0.91	0.78, 1.26, 2.21		25.3	0.91	1.26, 2.21, 0.86	H-20, H-21, H-22
30	CH_3	18.9	no	0.85 (d 6.8)		18.8	0.86	1.26, 2.21, 0.91		18.8	0.86	1.26, 2.21, 0.91	H-20; H-22
31	C=O	173.3	no			173.6		2.04, 5.74		173.3		5.67, 2.05	
32	CH_3	21.7	no	2.05 (s)		21.7	2.04		H-6′a/b	21.6	2.05		H-6′a/b
33	CH_3	- -				57.8	3.41	4.30	H-25, H-1′	59.8	3.26	4.41	H-25, H-26
1′	CH_3	105.3	no	4.33 (d, 7.8)		105.4	4.37	3.53, 3.15	H-3(s)	105.4	4.34	3.16, 3.31	H-3, H-3′, H-5′, H-24
2′	CH	75.7	yes (+0.129)	3.16 (dd, 9.2, 7.8)		75.9	3.15	4.37		75.8	3.16	3.31	
3′	CH	78.1	yes (+0.183)	3.31 (m)		78.2	3.31			78.2	3.31	3.16, 3.28	H-1′
4′	CH	71.8	yes (+0.122)	3.26 (dd, 9.2, 9.1)		71.9	3.26	3.31, 3.21		71.7	3.28	3.31, 3.21, 3.66, 3.84	H-6′a, H-32
5′	CH	77.8	yes (+0.045)	3.22 (ddd, 9.2, 9.1, 2.5)		77.9	3.21	4.37, 3.15, 3.26, 3.66		77.8	3.21	3.28, 3.66, 3.84	H-1′, H-6′a, H-32
6′	CH_2	62.9	yes (+0.107)	3.85 (dd, 11.7, 2.5)		63.0	3.84	3.26		62.9	3.84	3.28	H-6′a, H-32
				3.66 (dd, 11.7, 9.1)			3.66				3.66		H-6′b, H-32

[a] ^{1}H NMR spectra were run at 500 MHz and were referenced to the solvent signal at 3.30 ppm; ^{13}C NMR spectra were run at 125 MHz and were referenced to the solvent signal at 49.0 ppm. HMQC was used to correlate carbon signals with their attached protons. The deuterium shift data were obtained by running a sample of enfumafungin in both CD_3OD and CD_3OH and comparing chemical shift values. [b] MeOD/Pyridine-d_6(30%).

Table 2. Tabulation of Functional Groups in **1**

functional group	δ ^{1}H and δ ^{13}C chemical shifts	no. of unsaturations	no. of O's
acetate	δ ^{1}H 2.05 s δ ^{13}C 21.7 q, 173.3 s	1	2
hexose sugar	δ ^{13}C 105.3 d, 78.1 d, 77.8 d, 75.7 d, 71.8 d, 62.9 t	1	6
double bond	δ ^{1}H 5.63 brd δ ^{13}C 140.3 s, 125.1 d	1	0
carboxylic acid	δ ^{13}C 177.6 s	1	2
dioxygenated methine	δ ^{1}H 4.69 brs δ ^{13}C 97.2 d	0	2
total		**4**	**12**

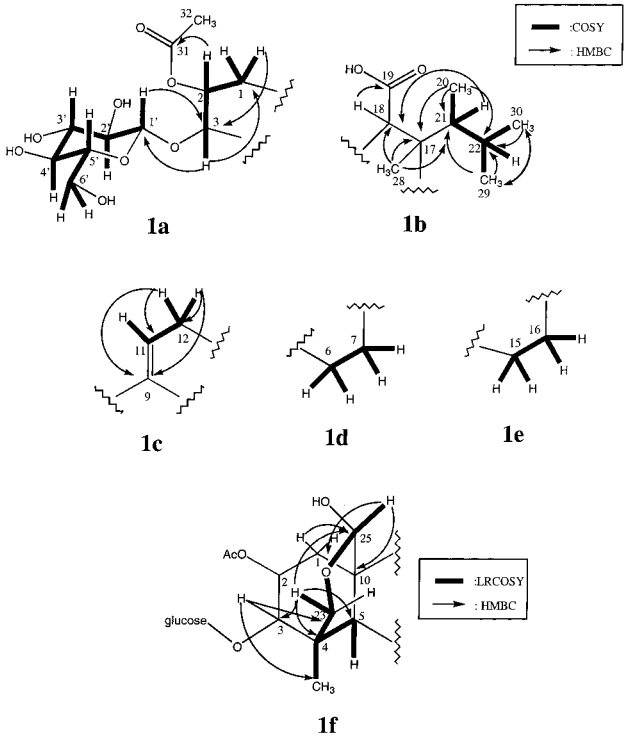

Figure 2. Substructures of enfumafungin (**1**).

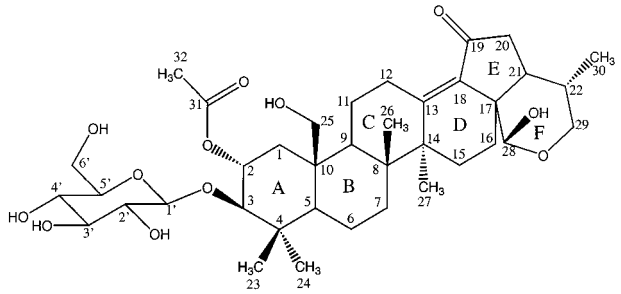

Figure 3. Structure of hyalodendroside (**2**).

noninterconverting components. ^{1}H NMR and MS suggested that components **3** and **4** were diastereomeric isomers of the methyl acetal derivative (Figure 5), while the other two components were diastereomeric isomers of the des-acetyl/methyl acetals. No doubling of the NMR peaks was observed for these derivatives.

Complete analysis of HMQC, HMBC, and COSY 2D NMR data for the methyl derivatives, **3** and **4** (Table 1), provided sufficient intra- and inter-ring connectivities to confirm the molecular structure initially proposed for **1**. The NMR data for these compounds suggest a system of four six-membered rings, fused in the manner of a pentacyclic triterpenoid. However, the

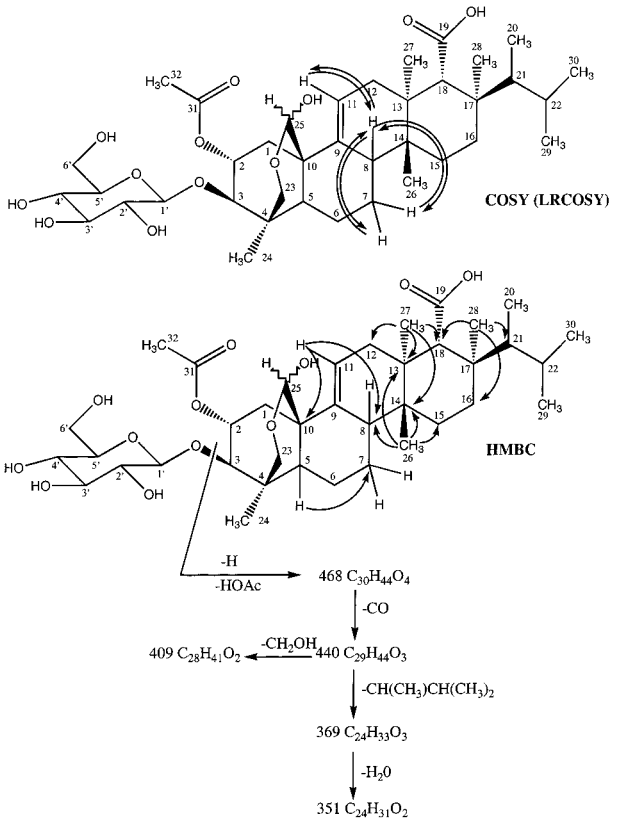

Figure 4. Selected ^{1}H−^{1}H and HMBC correlations in enfumafungin (**1**).

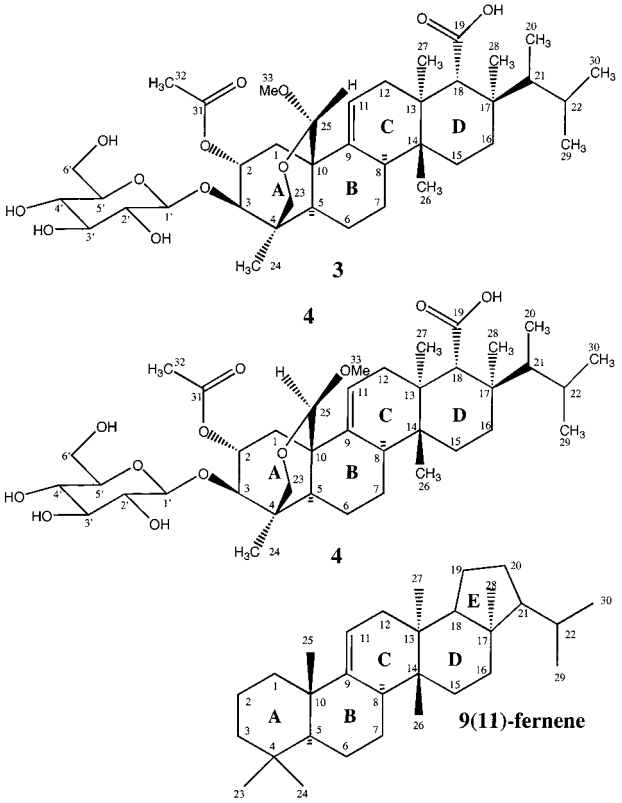

Figure 5. Structures of **3**, **4**, and 9(11)-fernene.

E ring, common to this structural family, appears to have opened up to a 1-methylisobutyl side chain attached at C17 on ring D. Another striking feature of the triterpenoid unit is the hemiacetal cyclization of C25 and C23 across ring A, which yields the

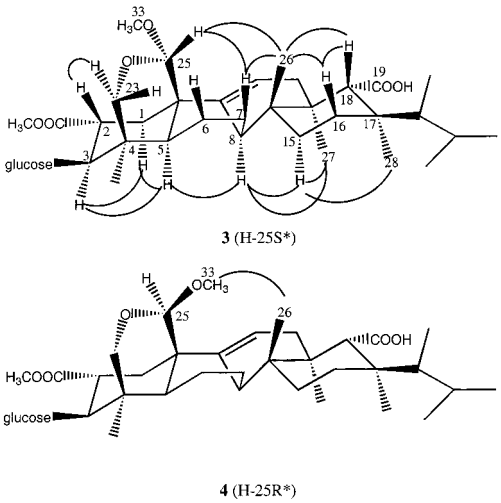

Figure 6. Selected ROE's **3** (H-25S*) and **4** (H-25R*).

interconverting diastereomers of the title compound and the noninterconverting methyl derivatives. Ring A is also substituted with an acetate and glucose at C2 and C3, respectively. Ring C is unsaturated at C9−C11, and the purported ring opening of ring E left a carboxylic acid attached to C18 on ring D. Given the high degree of substitution to the core ring system, it is likely that the structure assumes a relatively stable conformation in solution. Thus, careful studies of coupling constants and ROE measurements were undertaken to determine the relative stereochemistry of the hemiacetal anomers as well as the stereospecific assignment of the whole aglycon moiety.

The coupling constants between H1a, H2, and H3 ($J_{12} = 11.6$ Hz, $J_{23} = 8.9$ Hz) observed in the 1D proton spectra suggest that the dihedral angles between H2 and H1a, as well as those between H2 and H3, are near 180°. 2D ROESY correlations observed between H1a, H3, and H5 are consistent with a transdiaxial orientation of H1a, H2, and H3, and unambiguously place H1a, H3, and H5 on one face of ring A (Figure 6). Furthermore, ROE's between H5 and H8, and between H8 and the methyl protons of C27, locate H8 and the C27 on the same face of the four rings as H1a, H3, and H5. On the opposite face, ROE correlations between the methyl protons of C26 and H18, H7a, and H16 clearly differentiate the two sides of the aglycon unit. Large coupling constants between H8a and H7a, as well as those between H5a and H6a, along with ROE's consistent with their stereospecific assignment, further distinguish between the α and β faces of its ring system. These observations are consistent with the aglycon unit being a triterpenoid derived from 9(11) fernene, the stereochemistry of which is thoroughly documented.[15] It follows that the hemiacetal ring closure between C23 and C25 is on the β face of the triterpene ring system, proximal to, and on the same side as, H2 and H7a.

(15) (a) Oh, Y.; Maslen, E. *Acta Crystallogr.* **1966**, *20*, 852−864.(b) Ageta, H.; Iwata K.; Natori, S. *Tetrahedron Lett.* **1963**, *22*, 1447−1450.

The relative stereochemistry of the two anomers of enfumafungin and their methyl derivatives was deduced from chemical shift differences and ROE's observed in the NMR spectra of these compounds. The chemical shift changes of protons H1e and H7, observed between diastereomers, were relatively large ($|\Delta\delta| > 0.4$ ppm) and inversely related, implying the change in proximity of the anomeric oxygen to these two protons located on opposite sides of the hemiacetal bridge. Interestingly, H2, which is found at low field even for a proton α to an acetylated oxygen, shows little change in chemical shift although it also was close to the hemiacetal. This observation is probably due to a simultaneous change in the proximity of the bridging hemiacetal oxygen to H2. Finally, ROE's from the methyl protons of C26 to H25 in **3** and the methyl protons of C26 to the methoxy protons of C33 in **4** unambiguously confirmed the assignment of the *S** and *R** anomers, respectively (Figure 6). Thus the relative stereochemistry of enfumafungin was carried over from these observations.

Inhibition of Glucan Synthesis by Enfumafungin. In whole cells of both *Candida* and *Saccharomyces*, enfumafungin inhibits the incorporation of [^{14}C]-glucose into the alkaline insoluble cell wall extract without affecting mannan or chitin synthesis, indicating that it is a specific inhibitor of glucan synthesis. In addition, cellular changes observed upon treatment with enfumafungin are morphologically similar to those observed with other classes of glucan synthesis inhibitors. The IC_{50} of the glucan synthesis inhibition in *Candida albicans* is 0.05 μg/mL, comparable to the MIC of 0.2 μg/mL indicating that the mode-of-action of the antifungal activity is inhibition of glucan synthesis.[11]

Conclusion

Enfumafungin is the first inhibitor of glucan synthesis belonging to a new chemical class of antifungal compounds, the triterpene glycosides, since 1977, when the papulacandins were discovered. One reason for the renewed interest in screening for novel glucan synthesis inhibitors is the new antifungal clinical candidate caspofungin acetate (MK-0991; L-743,872) whose mode-of-action is inhibition of fungal glucan synthesis.[9] Thus, since inhibitors of fungal cell wall glucan synthesis are effective antifungal agents, it is desirable to identify additional compounds that act via this proven mode-of-action, but which have improved biological and chemical characteristics compared to the known classes of glucan synthesis inhibitors. The discovery of enfumafungin provides evidence that it is possible to find compounds that act via this proven mode-of-action and that natural products continue to be one of the best places to look for new classes of compounds.

Supporting Information Available: Experimental details and copies of the ^{1}H, ^{13}C, COSY, and HMBC NMR spectra for compounds **1**, **3**, and **4** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9944296