Pergamon

Tetrahedron Letters 41 (2000) 2791–2795

TETRAHEDRON LETTERS

Stereochemistry of hydrogen introduction at C-25 in ergosterol synthesized by the mevalonate-independent pathway

Wen-xu Zhou and W. David Nes *

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409, USA

Received 12 January 2000; accepted 1 February 2000

Abstract

Feeding of [1-^{13}C]glucose to *Prototheca wickerhamii* followed by ^{13}C NMR analysis of the resulting ^{13}C-labeled ergosterol demonstrated this yeast-like alga operates the mevalonate-independent pathway. Based on the ^{13}C NMR signal assignment of [2,6,11,12,16,18,19, 21,23,27-$^{13}C_{10}$] ergosterol synthesized from [1-^{13}C]glucose indicated the pro-*Z* methyl group of cycloartenol is derived from C-5 of IPP and that protonation at C-25 of the $\Delta^{25(27)}$-sterol intermediate takes place from the *Si*-face of Δ^{25} to form the isopropyl *pro-R* methyl group.

Keywords: biosynthesis; non-mevalonate pathway; *Prototheca wickerhamii*; ergosterol.

The biosynthesis of ergosterol has been investigated by several groups and found to involve the same intermediary steps from acetate to mevalonate (AC/MVA) to isopentenyl diphosphate (IPP) as in the genesis of cholesterol synthesized by animals.[1] In accord with the 'biogenetic isoprene rule', organisms operating the AC/MVA pathway incorporate C-2 of MVA into C-4 of IPP, and C-4 of IPP subsequently becomes C-26 of the sterol side chain, whereas C-6 (C-3′) of MVA becomes C-5 of IPP, and C-5 of IPP subsequently becomes C-27 of the sterol side chain. However, the recent discovery of a mevalonate-independent pathway to phytosterols in vascular plants and algae,[2] cast doubt on the operation of the AC/MVA pathway generally and of the origin of carbons associated with sterol molecules from pathogenic microbes with a photosynthetic lineage, such as *Plasmodium falciparum*[3] or *Prototheca wickerhamii*.[4]

It has been claimed that the sterol ring system and side chain of ergosterol (24β-methyl cholesta-5,7,22*E*-trien-3β-ol) and 24β-ethyl cholest-7-en-3β-ol are formed in some green algae by the mevalonate-independent pathway.[2] The two diastereotopic methyl groups at C-25 of the 24-ethyl sterol, the pro-*R* methyl group (C-26) and pro-*S* methyl group (C-27), are proposed to be derived from C-5 and C-4 of IPP, respectively, and by implication, so is the ergosterol isopropyl carbon atoms — C-26 and C-27.[2b] The origin of the IPP units is based on the signal assignments in the ^{13}C NMR of the ^{13}C-labeled

* Corresponding author.

0040-4039/00/$ - see front matter
PII: S0040-4039(00)00265-3

phytosterol derived from [1-^{13}C]glucose, and these assignments are diagnostic for a *C*-methylation pathway of the Δ^{24}-bond that proceeds stereoselectivity from the *Re*-face.[2b,5]

The labeling pattern of ergosterol from the algal non-mevalonate pathway is unexpected in view of the results of Arigoni and coworkers, studying the non-mevalonate pathway in vascular plants where C-26 and C-27 of 24α-ethyl cholest-5-en-3β-ol (sitosterol) synthesized by cell cultures of *Cantharanthus roseus* were found to originate with C-4 and C-5 of IPP, respectively.[6] The assembly of C-4 and C-5 IPP units into C-26 and C-27 of sitosterol from the non-mevalonate pathway is consistent with *C*-methylation of a sterol acceptor molecule from the *Si*-face of the Δ^{24}-bond followed by a *syn*-S_E2-type reduction of the 24,25-double bond to generate the 24α-ethyl group and 25*S*-26,27-isopropyl group characteristic of sitosterol, as we and others have demonstrated recently.[1c,7] In as much as the origin of the carbon of fungal ergosterol synthesized from the methyl and carboxyl carbon atoms of acetic acid have been established using ^{13}C NMR spectroscopy,[1d] and there is some confusion as to the biosynthesis of algal sterols, a reexamination of the distribution pattern of isotopic carbon in algal ergosterol synthesized from [1-^{13}C]glucose would be of considerable interest. In this way one could ascertain whether the biosynthetic pathway to ergosterol in fungi and plants is similar. Furthermore, by employing ^{13}C NMR spectroscopy to determine the magnetic non-equivalency of C-26 and C-27, the biochemical non-equivalency of these methyl groups can be assigned and the stereochemistry of hydrogen introduction at C-25 determined.

Glucose was isotopically diluted with [1-^{13}C]glucose (Aldrich: 99 atom% ^{13}C) (3:1 w/w) and 3 g of the labeled carbon source added to 300 ml medium of *P. wickerhamii* and grown in the dark for 48 h. The stationary phase cultures were harvested and the resulting ^{13}C-labeled ergosterol (ca. 1 mg) isolated from the non-saponifiable lipid fraction by reverse-phase HPLC, as described.[8] The ^{13}C NMR results are shown in Table 1. The routes of incorporation involving the AC/MVA pathway or the mevalonate-independent pathway from proffered [1-^{13}C]glucose to ergosterol are shown in Fig. 1. The ergosterol obtained from the mevalonate-independent pathway should be labeled at 10 positions whereas

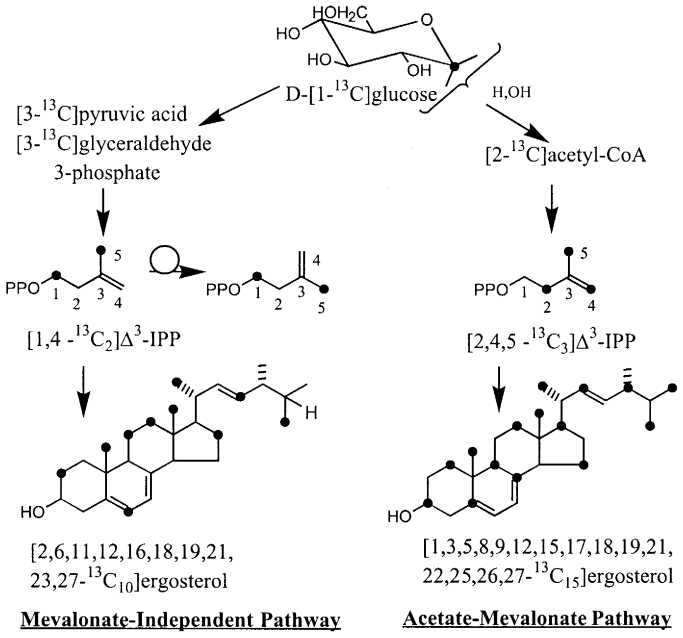


Fig. 1. Expected ^{13}C-labeling patterns of ergosterol via the mevalonate-independent and acetate-mevalonate pathways

Table 1
^{13}C-Chemical shifts and normalized peak height of ergosterol derived from D-[1-^{13}C]glucose

Carbon	Chemical shift δ (ppm)	Normalized peak height [a]
1	38.39	1.07
2	32.00	**5.87**
3	70.45	1.29
4	40.81	3.68
5	139.79	1.00
6	119.59	**5.35**
7	116.31	1.04
8	141.33	3.25
9	46.27	1.00
10	37.04	3.08
11,21	21.12	**7.53**
12	39.10	**5.69**
13,24	42.84	1.76
14	54.57	3.05
15	23.00	1.26
16	28.29	**5.04**
17	55.75	1.09
18	12.06	**6.80**
19	16.29	**6.97**
20	40.42	3.45
22	135.57	1.25
23	131.99	**5.63**
25	33.10	3.03
26	19.66	1.18
27	19.96	**6.70**
28	17.62	3.14

[a]The signal intensities were corrected by those of unlabeled ergosterol and normalized to C-9. Bold signals indicate the carbons labeled from the mevalonate-independent pathway

the ergosterol synthesized from the AC/MVA pathway should be labeled at 15 positions. The ergosterol isolated from *P. wickerhamii* contained 10 significantly enhanced signals in the ^{13}C NMR spectrum corresponding to the positions at 2, 6, 11, 12, 16, 18, 19, 21, 23, and 27. This compound will be referred to as [$^{13}C_{10}$]ergosterol. The intensity of C-26 signal is minimally above background (reference to C-9 signal), thereby excluding an active AC/MVA pathway.[9]

Comparison of the spectra of the [$^{13}C_{10}$]ergosterol with a control sample shows that of the two terminal carbon atoms marked 26 and 27 (Fig. 2), the latter carbon was enriched with ^{13}C in the specimen of [$^{13}C_{10}$]ergosterol. We know from an earlier set of reports that *C*-methylation by *P. wickerhamii* proceeds from the *Si*-face of the Δ^{24}-bond of cycloartenol (native substrate) and that the C-27 (pro-*Z*) becomes the carbon bearing the olefinic bond ($\Delta^{25(27)}$) in the *C*-methylated product-cyclolaudenol.[8] *P. wickerhamii* was also found to synthesize ergosterol from a pathway involving cycloartenol, cyclolaudenol and prototheca sterol (Scheme 1; side chains 4, 1, 2, and 3, respectively).[4,8]

In further support of the absence of the AC/MVA pathway is our finding that the ^{13}C NMR spectrum of cycloartenol (2 mg) derived from cells of *P. wickerhamii* cultured on [1-^{13}C]acetate (no glucose added) and 25-azacycloartenol to impair carbon flux to ergosterol[8] showed only six enhanced signals.

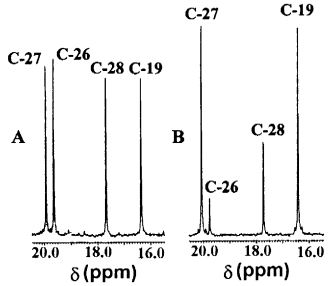
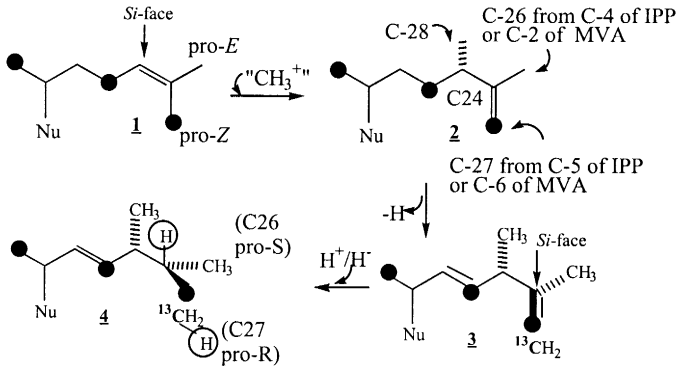


Fig. 2. Upfield portion of the ^{13}C NMR spectra of unlabeled ergosterol (A) and ergosterol derived from D-[1-^{13}C]glucose (B)



Scheme 1. Hypothetical Δ^{24}-C-methylation-Δ^{25}-reduction pathway to the ergosterol side chain of *P. wickerhamii*. The new hydrogens introduced are marked with circles. Solid dots represent the predicted position of ^{13}C labeled carbons of the sterol side chain synthesized from [1-^{13}C]glucose by the mevalonate-independent pathway

These signals corresponded to the species [1,7,15,22,26,30-$^{13}C_6$]cycloartenol (data not shown).[10] [1-^{13}C]Acetate is considered to be incorporated into carbon 4 of IPP in the mevalonate-independent pathway.[2b] The observation that C-26 (pro-*E* methyl group) of cycloartenol is labeled from a [1-^{13}C]acetate treatment is consistent with a mevalonate-independent pathway to cycloartenol. Hence, C-27 is correctly identified as the pro-*Z* methyl group of the Δ^{24}-sterol that gives rise to the $\Delta^{25(27)}$-sterol intermediate on the path to ergosterol synthesized by *P. wickerhamii* (Scheme 1).

Two research groups have studied the reduction of the Δ^{25}-bond in higher plants with conflicting observations. Nagano et al. report the conversion of $\Delta^{25(26)}$-cholesterol to cholesterol proceeds by the 25-*Re*-addition of hydrogen on $\Delta^{25(26)}$-cholesterol during its transformation into cholesterol (same steric course as proceeds in the conversion of desmosterol into cholesterol) whereas Seo et al. report an opposite 25-*Si*-face attack of hydrogen occurring in the biosynthesis of 24β-ethyl cholesta-7,22-dien-3β-ol from the Δ^{25}-olefinic precursor.[11] We reported the ^{13}C NMR spectrum of ergosterol formed by the yeast mutant GL7 fed [27-^{13}C]-lanosterol or [27-^{13}C]24(28)-methylene-24,25-dihydrolanosterol generated from a cell preparation of corn seedlings.[12] The resonance for the enhanced signal corresponding to C-27 in each of the ergosterol samples from yeast and *P. wickerhamii* was the same, indicating that in the reduction of the 25(27)-double bond both hydrogens are added to the *Si*-face of the double bond, equivalent to the *cis* addition of the hydride ion to C-25 from pyridine nucleotide and of a proton to C-25. It follows, since C-25 in ergosterol is a prochiral center, that C-26 and C-27 of cycloartenol become, respectively, the pro-*S* (C-26) and pro-*R* (C-27) methyl groups in the side chain of ergosterol.

We conclude that the assignments made by Rohmer and coworkers[2b] for C-26 and C-27 of phytosterols derived from the mevalonate-independent pathway are not correct and should be reversed. Interestingly,

the chirality associated with C-26 and C-27 of ergosterol and sitosterol are stereochemically opposite to that of animal cholesterol. Nonetheless, in all cases the sterol side chain carbons are derived from IPP in a similar manner as indicated in Scheme 1. The ability of a Δ^{25}-reductase to generate stereochemically opposite end products may be from proximity effects related to the recognition of increased steric bulk at C-24 at binding. Further study is warranted.

References

1. (a) Nes, W. R.; McKean, M. L. *Biochemistry of Steroids and Other Isopentenoids*; University Park Press: Baltimore, 1977; pp. 325–533; (b) Popják, G.; Edmond, J.; Anet, F. A. L.; Easton Jr., N. R. *J. Amer. Chem. Soc*. **1977**, *99*, 931–935; (c) Nes, W. D.; Norton, R. A.; Benson, M. *Phytochemistry* **1992**, *31*, 805–811; (d) Seo, S.; Uomori, A.; Yoshimura, Y.; Ken'chi, T.; Seto, H.; Yutaka, E.; Nguchi, H.; Sankawa, U. *J. Chem. Soc., Perkin Trans 1* **1988**, 2407–2413.
2. (a) Guo, D.; Venkatramesh, M.; Nes, W. D. *Lipids* **1995**, *30*, 203–212; (b) Schwender, J.; Seeman, M.; Lichtenthaler, H. K.; Rohmer, M. *Biochem. J*. **1996**, *316*, 73–80; (c) Disch, A.; Schwender, J.; Muller, C.; Lichtenthaler, H. K.; Rohmer, M. *Biochem. J*. **1998**, *333*, 381–388.
3. Jomaa, H.; Wiesner, J.; Sanderbrand, S.; Altincicek, B.; Weidemyer, C.; Hintz, M.; Tubachova, I.; Eberl, M.; Zeidler, J.; Lichtenthaler, H. K.; Soldati, D.; Beck, E. *Science* **1999**, *285*, 1573–1576.
4. Nes, W. D.; Norton, R. A.; Crumley, F. G.; Madigan, S. J.; Katz, E. R. *Proc. Natl. Acad. Sci.* **1990**, *87*, 7565–7569.
5. (a) Colombo, D.; Ronchetti, F.; Russo, G.; Toma, L. *J. Chem. Soc., Chem. Commun*. **1990**, 263–264; (b) Seo, A.; Uomori, Y.; Takeda, K. *J. Am. Chem. Soc*. **1983**, *105*, 6343–6344; (c) Wright, J. L. C. *Phytochemistry* **1981**, *20*, 2403–2405.
6. Arigoni, D.; Sagner, S.; Latzel, C.; Eisenreich, W.; Bacher, A.; Zenk, M. H. *Proc. Natl. Acad. Sci*. **1997**, *94*, 10600–10605.
7. Nes, W. D.; McCourt, B. S.; Marshall, J. A.; Ma, J.; Dennis, A. L.; Lopez, M.; Li, H.; He, L. *J. Org. Chem*. **1999**, *64*, 1535–1542 (and references cited therein).
8. Mangla, A. T.; Nes, W. D. *Bioorg. Med. Chem*. **2000**, in press.
9. Ergosterol structures were determined by ^{1}H and ^{13}C NMR, (JEOL Delta 500 MHz NMR). The mass spectrum of ^{13}C-ergosterol gave a cluster of ions in the high mass region of *m/z* (M^{+} 397 and other diagnostic ions at 364, 337 and 324).
10. We have described the complete ^{13}C carbon assignments for cycloartenol in: Nes, W. D.; Koike, K.; Jia, Z.; Sakamoto, Y.; Satou, T.; Nikaido, T.; Griffin, J. F. *J. Am. Chem. Soc*. **1998**, 5970–5980.
11. (a) Nagono, M.; Yamada, J.; Yazaki-Yagi, T.; Morisaki, M; Nakagawa, T.; Fujimoto, Y. *Chem. Pharm. Bull.* **1997**, *45*, 944–946; (b) Seo, S.; Uomori, A.; Yoshimura, Y.; Takeda, K.; Seto, H.; Ebizuka, Y.; Noguchi, H.; Sankawa, U. *J. Chem. Soc., Perkin Trans. 1* **1989**, 1969–1974.
12. (a) Zhou, W.; Guo, D.; Nes, W. D. *Tetrahedron Lett*. **1996**, *37*, 1339–1442; (b) Guo, D.; Jia, Z; Nes,W. D. *J. Am. Chem Soc*. **1996**, *118*, 8507–8508; (c). The support of the Welch Foundation (D-1276) is acknowledged.