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

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Abstract

Feeding of [1-¹³C]glucose to *Prototheca wickerhamii* followed by ¹³C NMR analysis of the resulting ¹³C-labeled ergosterol demonstrated this yeast-like alga operates the mevalonate-independent pathway. Based on the ¹³C NMR signal assignment of [2,6,11,12,16,18,19, 21,23,27-¹³C₁₀] ergosterol synthesized from [1-¹³C]glucose indicated the pro-*Z* methyl group of cycloartenol is derived from C-5 of IPP and that protonation at C-25 of the Δ²⁵⁽²⁷⁾-sterol intermediate takes place from the *Si*-face of Δ²⁵ to form the isopropyl *pro-R* methyl group. © 2000 Elsevier Science Ltd. All rights reserved.

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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 cholest-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 ¹³C NMR of the ¹³C-labeled

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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 24 α -ethyl cholest-5-en-3 β -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 24 α -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

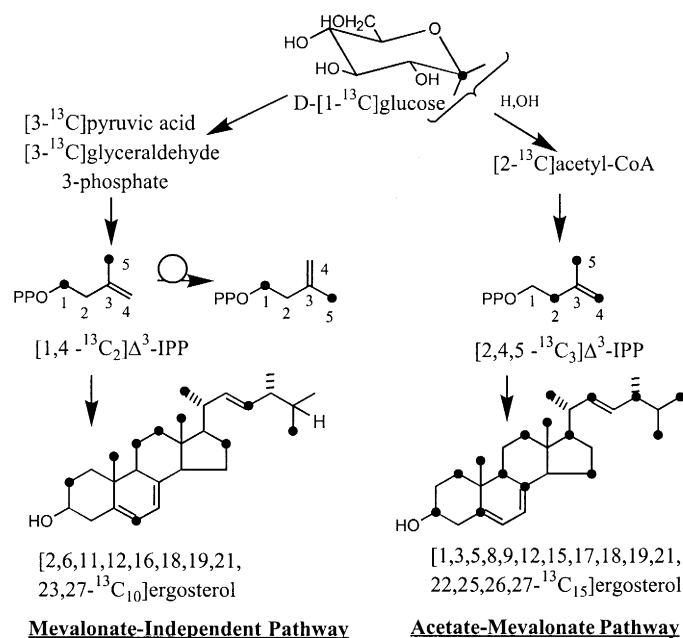


Fig. 1. Expected ^{13}C -labeling patterns of ergosterol via the mevalonate-independent and acetate-mevalonate pathways

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

^aThe 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 ¹³C 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 [¹³C₁₀]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 [¹³C₁₀]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 ¹³C in the specimen of [¹³C₁₀]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 protothecasterol (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 ¹³C NMR spectrum of cycloartenol (2 mg) derived from cells of *P. wickerhamii* cultured on [1-¹³C]acetate (no glucose added) and 25-azacycloartenol to impair carbon flux to ergosterol⁸ showed only six enhanced signals.

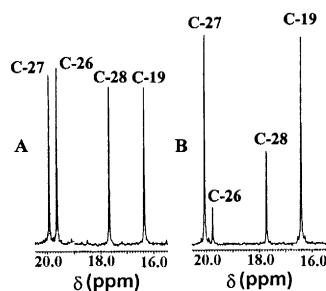
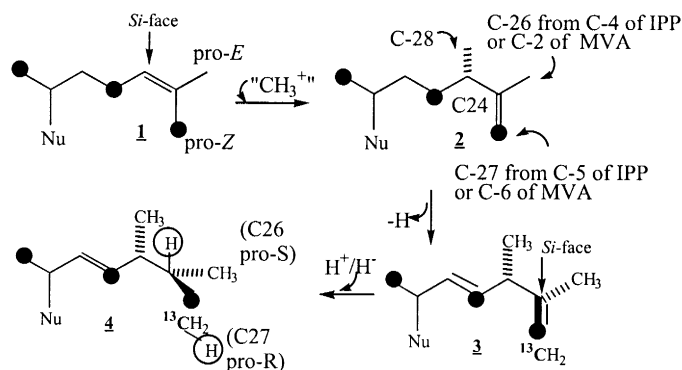


Fig. 2. Upfield portion of the ^{13}C NMR spectra of unlabeled ergosterol (A) and ergosterol derived from D-[1- ^{13}C]glucose (B)



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- $^{13}\text{C}_6$]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 24 β -ethyl cholesta-7,22-dien-3 β -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.

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