行政院國家科學委員會專題研究計畫　成果報告

氧化鯊烯環化酵素之產物多樣性與開發寡核酸先導藥物之研究

計畫類別：個別型計畫
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氧化鯊烯環化酵素之產物多樣性作用機制探討與開發寡核酸先導藥物之研究
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計畫類別：■個別型計畫　　□整合型計畫
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計畫編號：NSC 91－2113－M－009－023－

氧化鯊烯環化酵素之產物多樣性與開發寡核酸先導藥物之研究

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計畫參與人員：張程翔，柯震宇，張琬琳，陳京瑤，劉媛婷，王裕國

一、中文摘要

本計畫對於研究氧化鯊烯環化酵素之產物多樣性與其環化反應機制間之關係已獲得初步結果。經由針對由阿拉伯芥之氧化鯊烯環化酵素(CAS)進行雙點突變以觀察其功能之變化與演化之關係，並進一部探討其環化/重組機制、以及產物之專一性與多樣性。結果顯示在 29 個 針對酵素活性區域表面之胺基丙酸突變株中有 16 個突變株經置換後無法獲得羊毛硬脂醇之產物以互補酵母菌之生存。這 29 個突變株中 Y118、L124、G127、L179、T215、G366、P367、V368、L372、C484、E548、Y616 與 C730，對於 CAS 環化重組的機制沒有顯著的影響；F123、W217、W221、M254、H257、V261、Y262、W416、F472、F550、I553、W610、F726、I732、Y734 與 Y737 在 CAS 催化的過程中，可能協助受質環化與重組，擔任決定酵素活性有無的關鍵角色。

在分析 CAS 含雙點突變之產物的過程中，利用打破具雙點突變之酵母菌，以萃取的方式從酵母菌細胞中擷取出非可皂化的脂質。利用薄層色層分析片 (TLC) 了解產物的分佈、以矽膠的管柱 (Silica column) 將產物純化分離、氣相色層分析 (GC-MS) 推其分子量，再利用核磁共振光譜學 (NMR) 分析產物的結構。由產物分析結果發現，包含活性區域靠近受質接受區下方之 M254、H257、V261 的雙點突變效應下，有相同的新產物生成。新產物分別是 24-Methlene 24,25 -Dihydrolanosterol、4,4-Dimethyl fecosterol 與 4-Methyl Fecosterol，分子量分別為 440、 426 與 412。我們推測 $CAS^{Y410C\ M254A}$、$CAS^{Y410C\ H257A}$ 與 $CAS^{Y410C\ V261A}$ 突變效應，失去環化的活性而轉變成固醇類甲基轉移酶的功能性 (Sterol Methyl Transferase, **SMT**)，使得羊毛硬脂醇改變代謝途徑產生 24-Methlene 24, 25-Dihydrolanosterol，隨即經細胞中 C-14α 的去甲基酶作用後，進而形成 4,4-Dimethyl Fecosterol、4-Methyl Fecosterol 與 Fecosterol，進入野生型麥角脂醇生合成途徑的下游，最後形成終產物麥角脂醇 (Ergosterol)。

關鍵詞：分子模擬，胺基丙酸掃描，定點突變，氧化鯊烯環化酵素，羊毛硬脂醇，重複質譜

Abstract

Oxidosqualene-lanosterol cyclase (OSC) and oxidosqualene-cycloartenol synthase (CAS) catalyze the complex cyclization/rearrangement of (3*S*)2,3-oxidosqualene into lanosterol in mammal and fungi versus cycloartenol in algae and photosynthetic plants. To study evolutionary relationships between OSC and CAS, cyclization / rearrangement mechanism, as well as product specificity and/or diversity, twenty-nine non-alanine residues located on the putative active site cavity surface of CAS^{Y410C} mutant, a CAS mutant which changes its enzymatic activity from CAS to OSC, were mutated to alanine and assayed for their ability to complement the OSC deficiency and to study the effect of double mutations. All of the mutations were verified by restriction mapping and DNA sequencing, followed by genetic counter-selection. Among them, 16 out of 29 mutations failed to complement the ERG7 disruption indicating that these residues are crucial for

the catalytic function of the enzyme.

The nonsaponifiable lipids of inactive mutants were isolated and characterized by TLC, GC-MS and NMR. The $CAS^{V261AY410C}$ mutant 、 $CAS^{M254AY410C}$ mutant and $CAS^{H257AY410C}$ mutant, which alone failed to complement the viability of cyclase-deficient yeast strain but accumulated 24-methylene-24,25-dihydrolanosterol (eburicol), 4,4-dimethyl fecosterol and 4-methyl fecosterol, when both wild-type OSC and CAS double mutations were present, were isolated. The results indicated that a dramatic enzymatic activity change from oxidosqualene cyclase to sterol methyl transferase and concomitantly generated an alternative ergosterol biosynthetic pathway, were derived from the above-mentioned double mutations. In addition, the discovery here will provide important information with which to understand the active site topology for both enzymes and have significant consequences in relation to rational drug design and to the molecular engineering of the ergosterol biosynthetic pathway.

Keywords: oxidosqualene-lanosterol cyclase, site-directed mutagenesis, plasmid shuffling, cation-π interaction, alanine-scanning, tandem-MS-MS.

二、緣由與目的

The family of triterpene cyclase enzymes catalyzes highly diverse and complex cyclization/rearrangement reactions (*1-3*). In fungi and mammals oxidosqualene-lanosterol cyclase (ERG7, EC 5.4.99.7) mediates the conversion of (3*S*)-2,3-oxidosqualene into lanosterol, whereas this same substrate is elaborated to cycloartenol through the agency of oxidosqualene-cycloartenol synthase (CAS, EC 5.4.99.8) in algae and photosynthetic plants. In prokaryotes, squalene-hopene cyclase (SHC) catalyzes the conversion of squalene to pentacyclic triterpenoids (Fig. 1).

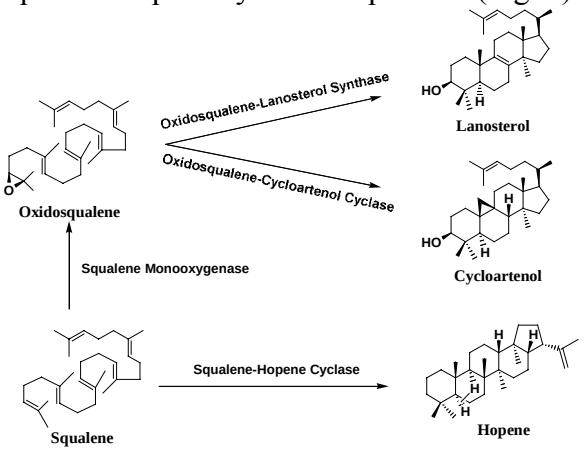

Figure 1. **Triterpene cyclization reactions.**

This enzymatic reaction represents one of the most remarkable and fascinating biotransformations in nature and has inspired many scientists to study its structure-function correlations (*4-12*). Moreover, over 90 triterpenes with $C_{30}H_{50}O$ formula have been derived from (oxido)squalene cyclization (*13*). In parallel, the oxidosqualene cyclases are also attractive targets in designing antifungal and anticholesteremic drugs (*14-18*). Elegant biogenetic and bioorganic investigations, including studies of the structures of novel products formed from designed alternate substrates, have provided a detailed model for cyclase-mediated substrate transformation (*19-25*). In this model, enzymes initiate cyclization cascades by protonating the terminal double bond of squalene or the oxirane moiety of oxidosqualene. The enzymes then promote cyclization cascades leading, in the case of ERG7 and CAS, to an intermediate protosterol cation which undergoes a series of hydride and methyl shifts. Lanosterol

results from C-9 deprotonation of a C-8 cation while cycloartenol forms after a further hydride migration and deprotonation of the C-10 methyl group (**Figure 2**)

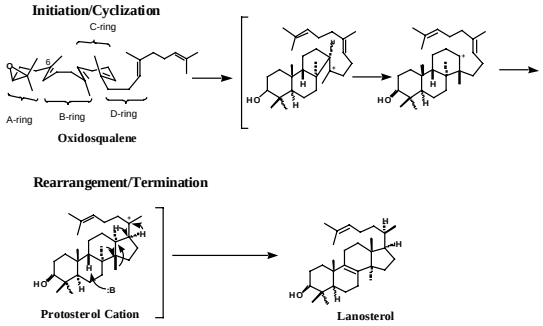

Figure 2. Complex Cyclization/Rearrangement of Oxidosqualene to Lanosterol.

The speculation of the parallel active site sueface location may also be important in determining substrate recognition, cyclization/rearrangement cascade, or changing product specificity for all cyclases, the corresponding region in *S. cerevisiae* was subjected to investigation. Threfore, twenty-nine non-alanine residues located on the putative active site surface of CAS from *A. thaliana* were also mutated and assayed for their ability to complement the cyclase-deficient, ERG7 knockout, *S. cerevisiae* strain. These results will further extend our understanding about the mechanism of oxidosqualene cyclization/rearrangement cascade and provide basis for further elucidation of the biological origins as well as to develop novel OSC inhibitors for antifungal and hypocholesteremic purposes.

三、結果與討論

Generation of Active Site Site-Directed Mutants in the Single Mutant of *A. thaliana cas* Gene.

Twenty-nine non-alanine residues were constructed through replacement of the single mutant cyclase gene with the corresponding alanine substitution. Mutants were generated by polymerase chain reaction (PCR), restriction digested with endonuclease restriction enzyme, followed by cloning into a vector to obtain a recombinant plasmid containing a single DNA fragment incorporating the desired mutation sequence and the new restriction site (Table 1). All of the mutant clones were confirmed through DNA sequencing by using ABI PRISM BigDye Terminator Cycle Sequencing Reaction kit on an Applied Biosystems 373 DNA Sequencer available in the Institute.

Characterization of Site-Directed Mutagenesis Effects on Oxidosqualene Cyclase Activity via Plasmid Shuffle.

Haploid strain CBY57[pZS11] (ERG7 △::LEU2 ade2-101 his3-△200 leu2-△1 lys2-801 trp1-△63 [pZS11]), a yeast strain bearing both a genomic ERG7-disrupted gene and a URA3 centromeric plasmid with wild type *S. cerevisiae* cyclase gene, allowed the use of a plasmid shuffle to analyze the effects of mutations in an ERG7 knockout background. As negative and positive controls for the plasmid shuffle, CBY57[pZS11] was transformed by electroporation with plasmids pRS314 and pTKOSCRS314WT, TRP1 centromeric plasmids bearing no insert and the wild type *S. cerevisiae* oxidosqualene-lanosterol cyclase gene, respectively. Transformants were selected on SD media containing adenine, lysine, histidine and uracil, and pRS314 and pTKOSCRS314WT were recovered from cell-free lysates grown in this media. CBY57[pZS11], CBY57[pZS11][pRS314] and

CBY57[pZS11][pTKOSCRS314WT] were then plated on complete media containing 5-fluoroorotic acid (5'-FOA) to counterselect for pZS11. As expected, growth was only observed on plates inoculated with CBY57[pZS11][pTKOSCRS314WT].

CBY57[pZS11] was transformed by electroporation with 29 site-directed CAS mutants and selected for growth in the media containing adenine, lysine, histidine and uracil, then re-selected for growth in the presence of 5'-FOA. Following the counterselection with 5'-FOA, F123A、W217A、W221A、M254A、H257A、V261A、Y262A、W416A、F472A、F550A、I553A、W610A、F726A、I732A、Y734A and Y737A mutations failed to complement the ERG7 disruption, indicating that these residues are crucial for the catalytic function of the enzyme (**Table 1**).

pWLC_RS313CAS*AY410C**	**NMR**	**GC mass**	**New product**
W2 = pWLC2RSCAS **F123A**Y410C	√	√	-
W7 = pWLC7RSCAS**W217A**Y410C	√	√	-
W8 = pWLC8RSCAS**W221A**Y410C	√	√	-
W9 = pWLC9RSCAS**M254A**Y410C	√	√	√
W10 = pWLC10RSCAS**H257A**Y410C	√	√	√
W11 = pWLC11RSCAS **V261A**Y410C	√	√	√
W12 = pWLC12RSCAS **Y262A**Y410C	√	√	-
W17 = pWLC17RSCAS Y410C**W416A**	√	√	-
W18 = pWLC18RSCASY410C **F472A**	√	√	-
W21 = pWLC21RSCAS Y410C **F550A**	√	√	-
W22 = pWLC22RSCAS Y410C **I553A**	√	√	-
W23 = pWLC23RSCAS Y410C**W610A**	√	√	-
W25 = pWLC25RSCAS Y410C **F726A**	√	√	-
W27 = pWLC27RSCAS Y410C **I732A**	√	√	-
W28 = pWLC28RSCAS Y410C **Y734A**	√	√	-
W29 = pWLC29RSCAS Y410C **Y737A**	√	√	-
Wild type CAS	√	√	Cycloartenol
Wild type OSC (CBY57)	√	√	Lanosterol

Table 1. Complementation results of site-directed mutations of double mutations of oxidosqualene-cycloartenol synthase gene in CBY58 strain.

Structure Characterization of New Products Produced by CAS Double Mutants We generated site-specific mutants and performed plasmid shuffle selection to identify amino acid residues crucial for cyclization/rearrangement mechanism and product diversity, as previously described.[2,3] Among several inactive mutants identified, the products distribution of W9, W10, W11 and W25 within yeast strain, which contains both wild type ERG7 and CAS double mutant within the strain, were analyzed. Preliminary GC revealed three new membraneous products, in addition to ergosterol. Two of these products showed identical chromatographic migration on TLC to that of lanosterol (LA), and one migrated between LA and ergosterol. Following isolation by column chromatography and characterization by GC-MS, a new major compound was identified that has identical molecular mass (*m/z* = 426) but has a different structure compared to LA. The 150 MHz ^{13}C NMR spectrum of this compound revealed the presence of one di-quaternary substituted and one quaternary-quaternary substituted double bonds (105.93 156.67 and 127.82, 135.79 ppm). The 600 MHz ^{1}H NMR spectrum also showed the presence of olefinic protons at 4.67 and four quaternary methyl groups (0.59, 0.80, 0.97, and 0.98, each s, 3H). The HMQC spectrum showed that the methyl protons at 0.59 are attached to the carbon at 11.22 ppm and that the methyl protons at 0.80 are attached to the carbon at 15.35 ppm. For the olefinic protons, the HMQC

spectrum established that the methylene protons at 4.67 are attached to the carbon at 105.93 ppm. However, detailed structure of the major compound could not be elucidated with ^{1}H and ^{13}C NMR, DEPT, HMBC, HMQC, and even INADEQUATE spectra. A crystal subjected to X-ray diffraction analysis revealed the three-dimensional structure to be 4,4-dimethyl fecosterol (4,4-DMF), as shown in Figure 3.

Fig. 3. Structure of 4,4-dimethyl fecosterol as determined by X-ray diffraction. Selected distances (Å): C(8)-C(9) = 1.341; C(24)-C(28) = 1.380; C(3)-O(1) = 1.436.

Various amounts of another compound, which showed the M^{+} at m/z = 440, were also detected from W9, W10, W11 and W25 mutants. GC-MS analysis and comparison to published data revealed the trace compound to be 24-methylene-24,25-dihydrolanosterol (eburicol).[5,6] Analysis of the ^{1}H and ^{13}C NMR signals for the minor component revealed that this compound also contains two double bonds and is one carbon less than that of 4,4-DMF, implying that this compound was possibly derived from 4,4-DMF. Detailed analysis of the ^{1}H and ^{13}C NMR, DEPT, HMBC, HMQC spectra as well as comparison to that of 4,4-DMF confirmed the identification of this compound as 4-methyl fecosterol (4-MF). The W25 mutant containing both Y410C and F726A produced lanosterol in conjunction with eburicol, 4,4-DMF and 4-MF as shown in Fig. 4.

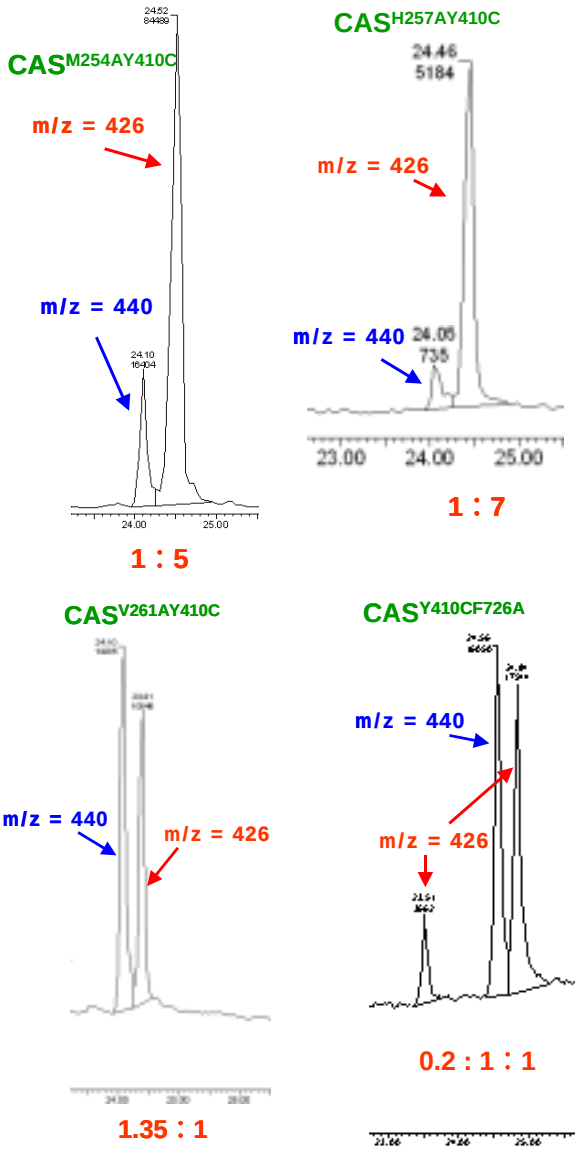

Figure 4. GC spectra of W9, W10, W11, and W25.

In *S. cerevisiae*, the consensus opinion for LA conversion to ergosterol has been that three consecutive rounds of demethylation occurred at the C-14 and C-4 positions of LA, yielding zymosterol. This is then methynylated by $\Delta^{24(25)}$ sterol methyl transferase (ERG6) to produce fecosterol.[5,9] Analyses of sterol composition of *S. cerevisiae* also revealed the presence of LA, zymosterol, fecosterol, episterol, and ergosterol, but not the C-31 sterol derivative eburicol.[5,10] The main sterols accumulated in this study are also different from that

obtained from fission yeast *Schizosaccharomyces pombe* and *Schizosaccharomyces octosporus*, as well as from ketoconazole-treated *Trypanosoma cruzi*, all of which accumulate eburicol as a major end product.[5,11] Thus, the isolation of eburicol, 4,4-DMF, and 4-MF in this study was seemingly inconsistent with previous observation for *S. cerevisiae* ergosterol biosynthetic pathway. However, this can be resolved if both an additional route from LA to ergosterol exists and a new sterol methyl transferase activity occurred upon CAS double mutant transformation of the CBY57[pZS11] strain. In the presence of both ERG7 and CAS double mutants, two pathways may exist from LA to fecosterol, as shown in Fig. 5. 1) In the classical pathway, LA is demethylated at positions C-14 and C-4 to yield zymosterol and then methynylated by ERG6 to produce fecosterol. 2) In the alternative pathway, the formation of the eburicol from LA is followed by consecutive C-14 and C-4 demethylations to yield 4,4-DMF and 4-MF, followed by a third demethylation to yield fecosterol.

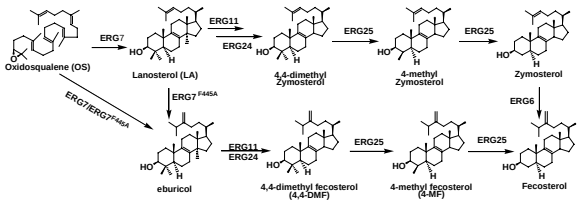

Figure 5. **Proposed sterol transformations and encoding genes, from oxidosqualene to fecosterol, in the presence of *ERG7* or *ERG7/ERG7*^{F445A}, in *Saccharomyces cerevisiae*. *ERG7*, oxidosqualene-lanosterol cyclase; *ERG11*, lanosterol C-14 demethylase; *ERG24*, sterol C-14 reductase; *ERG25*, sterol C-4 methyl oxidase; *ERG6*, sterol C-24 methyl transferase.**

Detailed comparison of putative reaction mechanism and active site topography revealed high similarities between oxidosqualene cyclase and sterol methyl transferase.[3a,12,13] Both enzymes are commonly hypothesized to form a stereochemically prefolded substrate conformation and then generate a carbocation intermediate, which undergoes 1,2-hydride migration and deprotonation. Analysis of protein sequences for both enzymes also revealed the aromatic amino acids involved in substrate binding or catalysis. The phenylalanine to alanine substitution may cause minor changes in the topography of the active site and affect the substrate (or product) affinity, the relationship between binding energy and catalysis, or the recognition of the terminal side chain. An alternative biosynthetic pathway to ergosterol from lanosterol was then generated.

In conclusion, we identified four CAS double mutants that fail to complement the viability of cyclase-deficient yeast strain but accumulates eburicol, 4,4-DMF and 4-MF when present with wild type ERG7. These CAS mutants derived from A. thaliana, which was demonstrated for the first time in *S. cerevisia*e, showed that a double amino acid residues substitution in the primary sequence of oxidosqualene cyclase can alter the ergosterol biosynthetic pathway and result in the accumulation of novel 24-methylene sterols end products. Finally, this discovery may provide important information for understanding the active site topology for both CAS and ERG6 enzymes and significantly benefit rational drug design and molecular engineering of the ergosterol biosynthetic pathway.

四、計劃成果自評

We have successfully accomplished the preliminary objective to study the active site architecture and cyclization/rearrangement reaction mechanism of oxidosqualene-lanosterol cyclase as well as

to expand cyclase product diversity. New data and insights have been obtained to facilitate the understanding of the oxidosqualene cyclization reaction, specially in the initiation step and substrate specificity and protein evolution. Based on the active site residues site-directed mutagenesis results and novel product isolation, an evolutionary model for the oxidosqualene cyclase enzyme could be proposed. The obtained result has been advanced in elucidating the possible role of some amino acid residues present in the enzyme, specially for the residues involved in the initiation of cyclization or high energy transition state intermediate. Therefore, the scientific value of this research result provides important viewpoint relevant to understand the active site architecture and the cyclization/rearrangement mechanism of oxidosqualene cyclase.

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