

# 行政院國家科學委員會專題研究計畫 成果報告

## 利利利利利利利利利利利利利利利利利肽/核酸 Aptamer 之研究

計畫類別：個別型計畫

計畫編號：NSC93-2113-M-009-012-

執行期間：93年08月01日至94年07月31日

執行單位：國立交通大學生物科技研究所

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## 利用氧化鯊烯環化酵素作為篩選胜肽/核酸 Aptamer 之研究

執行期限：93 年 08 月 01 日至 94 年 07 月 31 日

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### 一、中文摘要

膽固醇合成途徑中負責關鍵中間環化步驟反應之酵素-氧化鯊烯環化酵素，由於其在生化及醫學中之學術重要性與作為發展降低膽固醇及抗黴菌藥物之重要標的之潛力，而成為學術與藥廠中研發之重要題目。本研究利用氧化鯊烯環化酵素作為標的以尋找可與其進行交互作用之核酸片段與胜肽序列，希望未來發展成為藥物。核酸片段之篩選經由經由配位體指數遞增演化之技術，結合聚合酶連鎖反應與膠體遲滯電泳技術，經過數個重複篩選、放大、清洗之過程，已得到數段核酸片段，經由 DNA 定序儀已測定其序列。另外在胜肽序列之篩選，利用兩種噬菌體胜肽庫，七個及十二個隨機胜肽的胜肽庫，對氧化鯊烯環化酵素進行配位基的篩選。經由五次循環之鍵結、洗滌，亦已獲得五個對氧化鯊烯環化酵素具有親和性的噬菌體胜肽序列，由西方墨點法測定得到 1 個七胜肽序列和 4 個十二胜肽序列具有反應。

**關鍵詞：**配位體指數遞增演化，氧化鯊烯環化酵素，羊毛硬脂醇，重複質譜，聚合酶連鎖反應，膠體遲滯電泳，隨機胜肽庫

### Abstract

Oxidosqualene cyclase fulfills a unique role in designing new antifungal, hypocholesteremic, and phytotoxic drugs, due to its great biological and medicinal significance and potential. In this study, the mechanistic insights and structure-function relationships of oxidosqualene cyclase enzyme is extended and the enzyme is further explored for protein-DNA and protein-peptide interactions study to search for OSC-binding DNA and peptide search. We have applied systematic evolution of ligands by exponential enrichment (SELEX) technology to identify OSC-binding aptamer from a DNA pool of more than  $10^{15}$  individual nucleic acid molecules. Following the amplification of the selected oligonucleotides by PCR and successive iteration of the selection procedure, oligonucleotides with high binding affinity and specificity were obtained. In parallel, two peptide libraries, one heptapeptide and one dodecapeptide library, were subjected to

biopanning for OSC binding ligand identification. Following five biopanning process of OSC-binding selection and DNA sequencing of selected phages, five OSC-bound phages were selected. Among them, one heptapeptide and four dodecapeptides, showing OSC-binding affinity were identified from Western blotting experiments.

Keywords: systematic evolution of ligands by exponential enrichment (SELEX), oxidosqualene-lanosterol cyclase, lanosterol, tandem mass, electrophoresis mobility shift assay (EMSA), random peptide library

### 二、緣由與目的

The world's population continues to be seriously plagued by pathogenic organisms, which cause serious health problems. On the other hand, the relationships between increase of coronary heart disease and elevated plasma LDL cholesterol are well established. The development of cholesterol lowering drugs mainly focused on inhibiting HMG-CoA reductase activity will be very beneficial for prevention of this disease. Both cholesterol and fungal sterol biosyntheses share a common biosynthetic pathway until oxidosqualene, the substrate which is catalyzed by oxidosqualene cyclase to form the sterol and related triterpenoid core structure.

Oxidosqualene cyclase enzymes (OSCs) constitute a family of enzymes which catalyze the enzymatic conversion of oxidosqualene into a diverse array of sterols and related triterpenes. The multiple pathways for cyclization, rearrangement and oxidation of (oxido)squalene into structural diverse sterols and triterpenoids represent one of the most remarkable and fascinating biotransformations found in nature. The structure-function relationships and product profiles of (oxido)squalene cyclase enzymes have fascinated both chemists and biochemists for more than half a century because of the complexity of the enzymatic reactions and the diverse products distribution as well as their unique role in designing antifungal, hypocholesterolemic, and phytotoxic drugs.

In our laboratory, we have successfully applied random mutagenesis coupled with plasmid shuffle strategies to change the product specificity and

characterize the putative functional motif, important in determining substrate recognition, cyclization/rearrangement cascade, or changing product specificity for all cyclases. Several critical amino acid residues (Tyr410Cys, Ala464Val, His477Tyr, Ile481Thr, and Tyr532His derived from oxidosqualene-cycloartenol cyclase (CAS) gene of *A. thaliana*) involved in determining product specificity, especially in the final termination of carbocation between lanosterol and cycloartenol formation, have been identified. The result suggested that a single amino acid change is sufficient to alter the product specificity in the course of evolution. We have also successfully purified OSC from bovine liver to homogeneity as a single band under silver stain determination. These results provide bases for further understanding the biological origins and structure-function relationships of OSC as well as for developing novel OSC inhibitors for antifungal and hypocholesteremic purposes. Moreover, three independent regions on ERG7 of *S. cerevisiae* and 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. Three amino acid residues (Trp443, Phe445, and Lys448 in yeast ERG7 gene) were also identified using alanine scanning coupled with plasmid shuffle strategies and demonstrated the catalytic or structural role of aforementioned region in determining the substrate's entrance, cyclization/rearrangement mechanism and/or product specificity on oxidosqualene-lanosterol cyclase, oxidosqualene-cycloartenol synthase, and other cyclase enzymes. In addition, Phe-104-Thr, Trp-232-Arg, Trp-390-Ala, Phe-510-Arg, Trp-587-Ala, Phe-699-Ala, and Tyr-707-Ala failed to complement the ERG7 disruption were identified from the ERG7 counterselection process, indicating that these residues are also crucial for the catalytic function of the enzyme.

For the last decade, exploitation of nucleic acid's shape- or sequence-specific recognition properties as well as the *in vitro* polymerase chain amplification has greatly contributed to the identification of novel nucleic acid molecules for specific bio-molecular interactions with a variety of binding partners. SELEX, systematic evolution of ligands by exponential enrichment, is now widely applied to screen RNA or DNA oligos for binding against target small molecule organic dyes, nucleic acid binding proteins, or nucleic acids. The technology involves the synthesis of random oligonucleotide library, *in vitro* hybridization between target and library, enrichment of binding sequence via PCR amplification, iteration of selection cycle for binding affinity improvement, and identification of binding sequence. The novel SELEX technology is gaining more and more importance as an extremely useful screening tool, since a far larger population of molecules ( $>10^{13}$ ) can be rapidly screened in short time. Moreover, functional

molecules can be selected from the mainly non-functional pool of RNA or DNA by column chromatography or other selection techniques that are suitable for the enrichment of any desired property.

In parallel, peptide aptamer, oligopeptides that can specifically recognize and strongly bind to a protein of interest, has become another new tools for molecular medicine search. Peptide aptamers are small oligopeptide, which have the potential to dominantly interfere with specific activity of their target proteins and, therefore, could be used as *in vivo* inhibitors. In addition, peptide aptamers also provide a basis for the development of novel diagnostic and therapeutic strategies, with implications for a broad variety of different disease entities, including metabolic disorders, infections, and cancer. For the diagnostic purposes, peptide aptamers could be employed as antibody-like molecules for the detection of the target, such as in western blot analyses. Following the immobilization of the target protein on a membrane, the peptide aptamer which specifically interact with the target protein could be visualized with secondary antibodies against the scaffold or against a tag sequence fused to the aptamer, which will become an alternative to the development of monoclonal antibodies.

### 三、結果與討論

#### Generation of Degenerate Single Strand DNA Library

A pool of 72-mer oligonucleotides which contain a degenerate 36-nucleotide sequence flanked by two 18-nucleotide regions was synthesized using the solid-phase phosphoramidite chemistry. The nucleotide sequences for the flanking regions are as follow: 5'-CGTACGGTCGACGCTAGC-3' and 5'-biotin-GGATCCGAGCTCCACGTG-3'. The synthetic DNA was purified by PAGE and amplified by PCR to generate DNA library. The resulting biotinylated double stranded DNA was applied to an avidin-agarose column pre-equilibrated with buffer A. After the binding of the biotinylated DNA to the avidin beads, the unbound DNA was washed with five times of buffer A. Matrix-bound double strand DNA denatured with NaOH and the free DNA was precipitated. Following centrifugation, the DNA library was re-dissolved in the selection buffer and stored at -20 °C for selection.

#### Selection Using Proteins Immobilized on Microtiter Plate

To perform the aptamer selection, the ssDNA library was "panned" against protein immobilized on the surface of a microtiter plate. Different concentration of OSC was loaded onto the surface of the microtiter plate and incubated overnight at room temperature. After overnight incubation, linkers that are not derivatized with OSC were blocked with bovine serum albumin and incubated at 37 °C for 3

hr, followed by thrice washes with KPi/Tween 20. DNA library was first denatured at 95 °C for 5 min and then placed on ice for 5 min. The ssDNA pool was added to a BSA-only well and then incubated at room temperature for 1 hr. Next, the solution was transferred to the “OSC target” well and incubated for 1 hr before washed thrice with selection buffer. The bound aptamer was eluted with guanidine thiocyanate after the plate was heated at 80 C for 15 min and precipitated. The eluted DNA was PCR amplified and subjected for next round of selection. After 3 to 5 rounds of selection the recovered PCR products were visualized by gel electrophoresis and compared to the ones obtained from the control wells.

### Selection Using Protein Immobilized on Filters

To ensure the most accessible folding or stable structure of DNAs, samples were pre-thermally equilibrated and then passed through filters to remove species not bound to the filters. Bound DNAs were extracted from the filters by 7M urea and heat incubation. Following the incubation of DNA with OSC-bound filter, proteinase K was added to digest the protein portion. The digested protein was extracted with phenol/chloroform and the DNA portion was precipitated with NaOAc/Ethanol solution. The collected DNA was PCR amplified and subjected for next round of selection. Again, after 3 to 5 rounds of selection the recovered PCR products were visualized by gel electrophoresis and compared to the ones obtained from the control wells.

### Filter Binding Assay

Results from measurement of the amount of radioactively labeled DNA showed that only the DNA interact with the OSC was observed from filter binding assay. DNA did not interact with BSA as shown in Figure 1.

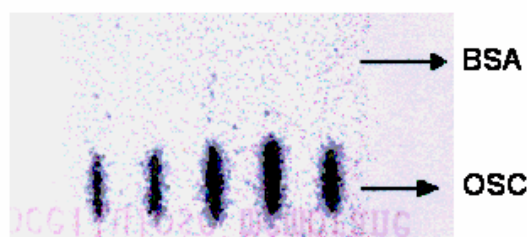


Figure 1. Filter binding assay of DNA with various concentrations of OSC ranging from 0.25, 0.5, 1.0, 2.0 to 4.0 μmol.

### Electrophoretic Mobility Shift Assay (EMSA)

EMSA was applied to determine the later rounds of SELEX. A specific DNA-protein complex was observed as an up-shift band at position of 156 kDa as shown in Figure 2. DNA was eluted from DNA-protein complex and cloned into pBluescript II SK(+) for sequence analysis.

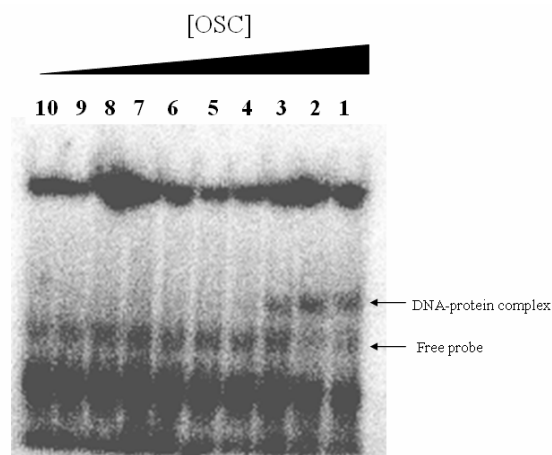


Figure 2. EMSA results of OSC-bound DNA.

### DNA Sequencing

DNA sequencing of SELEX plasmids isolated through the OSC-bound targets was carried out by the dideoxy chain-termination method using ABI PRISM BigDye Terminator Cycle Sequencing Reaction kits on an Applied Biosystems 3100 DNA Sequencer. After several rounds of selection, 9 aptamers which showed binding affinity to OSC target were selected, cloned, and sequenced. Following analysis of the sequences using the NCBI Blast program, the results were shown in Figure 3.

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2-7-1      GGTGGCCTTCGTTGGCCTCGCCCGAGCGGTCATCAACCGCTGTG-66C
1-13-1     GTTAGTTGAGGATTAGTAGGAGTTTACCAGGGGTAGCCCTGTTC
2-14       TAGTTAGAGGTTTAGTAGGAGTCTGGGTTTGTTCGAC
1-7-1      GTTGAACGATGTTTCGCTCGTATGTGTGAGGAT ATGCCTGGC
2-3-2      TCAGGTGTGTTTAAACGAGGAGGCCGCGGAAATTAGGTGGGGGGAG
2-5-1      RCGCCTGGTGTGGCGCCAAAGGGGGTCAAGCTGATCTA
1-10-2     ACTCGACTAGCCATGCTAGCGAATATCATATGGCGARTGAC
4-19       TCCACTACGGCTCTGCTAGCCCATGCTGAGAAAC
2-4-5      CACAGGGTGACTGTGTGCCCACTCGCGTTGAGGTTCTCTGTGTACGCCTGC

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Figure 3. DNA sequences of OSC aptamers.

### Biopanning Screening of OSC-Binding Peptide

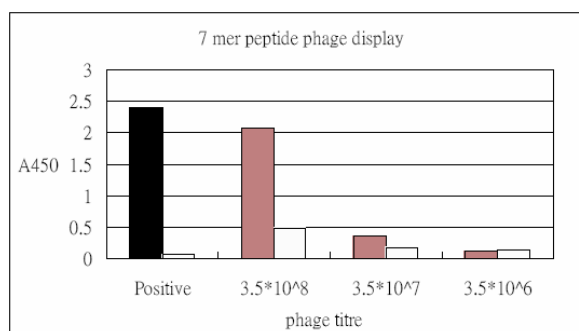
Prepare a solution of 100 μg/mL of OSC in 5 mM KPi (add 1 mM DTT and 0.2 %Triton X-100)(pH 7.4). Add 150 μL of OSC solution to each microtiter well. Store plates at 4°C overnight. At the time, inoculate 10 mL LB medium with ER2738 at 37 °C. Next day pour off the coating solution from each well and washing each well rapidly 6X with TBST (TBS + 0.1% [v/v] Tween-20). Fill each well completely with blocking buffer. Incubate at least 1 hour at 4°C. Discard the blocking solution and wash each well rapidly 6X with TBST buffer. Dilute 4 x 10<sup>10</sup> phage (10 μL of original library) with 100 μL of TBST. Pipet onto coated plate and rock gently for 10–60 minutes at room temperature. Discard nonbinding phage by pouring off and slapping plate face-down onto a clean paper towel. Wash plates 20 times with TBST. Elute bound phage with 200 μL of an elution buffer and rock gently for 10 minutes at room temperature. Pipet eluate into a microcentrifuge tube of 15 mL 1 M Tris-HCl (pH 9.1). Titer a small amount (~1 μL) of the eluate.

### ELISA Assay of OSC-Binding Peptide

For each clone to be characterized, inoculate 20 mL of LB medium with ER2738 and incubate at 37°C until slightly turbid. Alternatively, dilute an overnight culture of ER2738 1:100 in 20 mL LB. Add 5 µL of phage to each culture and incubate at 37°C with vigorous aeration for 4.5 hours. Transfer the culture to a centrifuge tube and spin 10 minutes at 10,000 rpm. Transfer supernatant to a fresh tube and re-spin. Pipet the upper 80% of the supernatant to a fresh tube and add 1/6 volume of PEG/NaCl. Allow phage to precipitate at 4°C for overnight. Spin PEG precipitation 15 minutes at 10,000 rpm at 4°C. Decant supernatant, re-spin briefly, and remove residual supernatant with a pipette. Suspend the pellet in 1 mL TBS. Transfer the suspension to a microcentrifuge tube and spin for 5 minutes at 4°C to pellet residual cells. Transfer the supernatant to a fresh microcentrifuge tube and re-precipitate with 1/6 volume of PEG/NaCl. Incubate on ice 60 minutes. Microcentrifuge for 10 minutes at 4°C. Discard supernatant, re-spin briefly, and remove residual supernatant with a micropipet. Suspend the pellet in 50 µL TBS. Titer the sample.

Coat one row of ELISA plate wells for each clone to be characterized with 100–200 µL of 100 µg/mL of OSC in 5 mM KPi buffer. Incubate at 4°C overnight. Shake out excess target solution and wash 6X with TBST ( TBS+0.1% tween-20 ). Fill each well completely with blocking buffer. Additionally, one row of uncoated wells per clone to be characterized should also be blocked in order to test for binding of each selected sequence to BSA-coated plastic. Incubate the blocked plates at 4°C, 1 hours. Shake out the blocking buffer and wash each plate 6 times with 1X TBST, slapping the plate face-down onto a clean section of paper towel each time. Carry out ten serial dilutions of the phage in 200 µL of TBS/Tween per well, starting with  $10^{12}$  virions in the first well of a row and ending with  $10^5$  virions in the well. Incubate at room temperature for 1 hours with agitation. Wash plate 6 times with 1X TBST. Dilute HRP-conjugated anti-M13 antibody 1:5,000 in blocking buffer. Add 200 µL of diluted conjugate to each well and incubate at room temperature for 1 hour. Wash 6 times with 1X TBST. Add 1 µL 30% H<sub>2</sub>O<sub>2</sub> to 10 mL of ABTS stock solution per plate to be analyzed. Add 200 µL substrate solution to each well, incubate at room temperature for 10–60 minutes. Read plates using a microplate reader set at 405 nm.

(a)



(b)

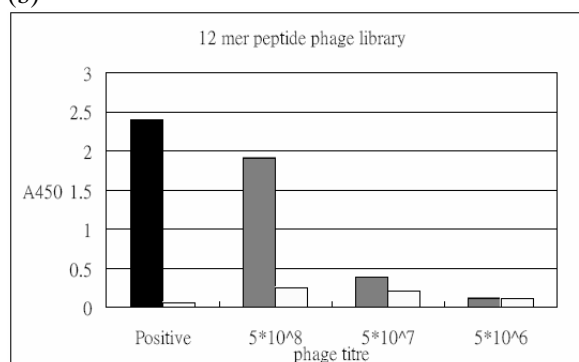


Figure 4. Biopanning results of (a) 7-mer peptide phage library and (b) 12-mer peptide phage library

Sample Number	Sequence
α8	tcttctcattgggtgggtgggagatgaggggttatgatgggt S S H W W W W D A R G Y D G
21	tcttttcattgctgagtggtgctactccttctccttggccgggt S F H A E W P T P S P W P G
α5	tcttttcattgggtggaatggtgggtattattagatgacctgggt S F H W W N V G Y Y Q M P G
α3	tcttttcattgggtggaatggtgggtattattagatgacctgggt S F H W W N V G Y Y Q M P G
ε7	tcttttcattgggtcgtggtatactcctagtaggcccgtcgggt S F H W S W Y T P S R P S G

Figure 5. OSC-binding peptide sequences

### 四、計劃成果自評

We have successfully accomplished the preliminary objective to select the OSC binding aptamers. The microtiter plate binding selection and electrophoretic mobility shift assay were proven to be useful for binding affinity selection. New materials have been obtained to regulate the oxidosqualene cyclization reactivity. The obtained result will be used to design new OSC inhibitor and regulate the OSC activity though interfering the binding affinity and specificity. Besides, the application of phage display libraries for OSC-binding peptide screening have resulted in obtaining one heptapeptide and four dodecaheptides which showed binding affinity to OSC enzyme. Further experiments on OSC affinity binding and specificity will be examined using the

monoclonal antibody generated from the OSC enzyme. Therefore, this research result opens new avenue for future development of DNA or peptide-based antifungal and hypocholesteremic drug for therapeutical purposes.

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