行政院國家科學委員會補助專題研究計畫成果報告

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計畫類別：■個別型計畫　　□整合型計畫
計畫編號：NSC－89-2113-M-009-036
執行期間：89 年 11 月 01 日至 90 年 07 月 31 日

計畫主持人：邱 顯 泰

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
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□國際合作研究計畫國外研究報告書一份

執行單位：國立交通大學生物科技學系

中　華　民　國 90 年　07 月　31 日

行政院國家科學委員會專題研究計畫成果報告

醣基轉移　在組合式生物合成上之研究與應用

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主持人：邱 顯 泰　國立交通大學生物科技學系

計畫參與人員：陳億霖、余珮君　國立交通大學生物科技研究所

一、中文摘要

醣基轉移 ，在生物多polyketide glycosides 之生物合成路徑中，扮演著重要的角色，。以鼠李醣轉移 為例，鼠李醣的醣基轉移到 polyketide 骨架上，而形成具強效性之生物殺蟲劑，spinosyn。此成果報告，在描述以鼠李醣轉移 之功能性應用在組合式生物合成之研究。在此研究，我們利用化學反應，將 spinosyn 轉化成為 polyketide aglycone 以作為酵素之受質。此研究亦成功的利用鼠李醣生物合成基因群組，表達於大腸桿菌中，進一步來製備酵素的另一鼠李醣。再者，我們使用高效率液相層析，發展出一套精確且靈敏的方法來偵測酵素之活性。最後，亦成功的將本研究之關鍵酵素 ，以可溶性之形式表達於大腸桿菌中。上述結果，將為首次異體表達sugar-to-polyketide之鼠李醣轉移 奠定堅固之基石。此研 應用此醣基轉移 及組合式 藥物之門。

關鍵詞：polyketide aglycone，核 二 磷鼠李醣，醣 基 轉移

Abstract

Glycosyltransferases play a critical role in the biosynthesis of many bioactive polyketide glycosides. Spinosyn rhamnosyltransferase (Rtf), for example, is responsible for the glycosylation of a polyketide with NDP-rhamnose for the biosynthesis a potent insectide, spinosyn. This progress report addresses the current status of our study in the functional expression of Rtf in *E. coli* for potential combinatorial biosynthesis. In the study, the chemical conversion of the spinosyn to the polyketide aglycone and rhamnose has been achieved to obtain one of the enzymatic substrate, the spinosyn aglycone. Another substrate of Rtf, NDP-rhamnose, has also been biosynthesized using a biosynthetic gene cluster of NDP-rhamnose. Furthermore, we have developed an accurate and sensitive detection method for enzymatic activity assays using HPLC (high performance liquid chromatography). Finally, the key enzyme Rtf has also been overexpressed in *E. coli* in a soluble form. Above results provide a strong ground for the first demonstration of the functional expression of the sugar-to-polyketide glycosyltransferase, Rtf, in the near future. The result has further opened an opportunity of diverse applications of the enzyme in combinatorial biosynthesis for drug discovery.

二、緣由與目的

This report describes the current status of our project in the laboratory. The goal of the project is to use interdisciplinary technologies, including molecular genetics, biochemistry and chemistry, to study and engineer the nature's biocatalyst towards making novel bioactive molecules of medical and industrial importance. Our general interest has been in the area of genetic and biosynthetic engineering of important bioactive natural products with strong applications in drug discovery. It has been known that glycosyltransferases are critical biocatalysts responsible for building main structural frame of varieties of bioactive polyketide glycosides by incorporating various sugars. The model biocatalyst being investigated here is the spinosyn rhamnosyltransferase (Rtf) involved in the biosynthesis of an important insecticide, spinosyn. This putative enzyme has been proposed to be responsible for the glycosylation of the spinosyn with NDP-rhamnose based upon the previous genetic evidence. However, the enzyme has not yet been functionally expressed in large quantity for academic and other applications. Our plan has been to clone and express the Rtf for kinetic and mechanistic characterization of the key enzyme to be utilized for making novel organic molecules. The biocatalyst was intended for potential precursor-directed biosynthesis of the related polyketide glycosides of biological importance. Also, the long-term plan has been to engineer the nature biocatalyst for catalyzing the formation of novel bioactive molecules using tools in molecular and protein engineering.

In this report, a brief summary of current accomplishment is addressed. In addition, prospects of the project towards future applications is described in close relationship with our current achievements.

Keywords: polyketide glycoside, glycosyltransferase, NDP-rhamnose, spinosyn.

三、結果與討論

The following goals have been achieved during the period of 89/11/01-90/07/31.

(1) Chemical Conversion of the spinosyns to the aglycone, rhamnose. To carry out functional expression of the putative enzyme in *E. coli*, the enzymatic substrates must be prepared. Spinosyns A and D were isolated from fermentation broth of *S. spinosa* under suitable culture conditions. Chemical degradation of the spinosyns has been achieved to give the spinosyn aglycone and rhamnose in the amount sufficient for the activity assays of the cloned spinosyn rhamnosyltransferase. In particular, the aglycone has been obtained in the mixture form of A and D as well as in pure form of individual A or D. The pure formation of either aglycone was achieved by two major methods: (a) the mixture of the spinosyns A and D was directly subjected to chemical degradation using acidic hydrolysis (H_2SO_4) to obtain the aglycone mixture, followed by careful C18 HPLC separations of the aglycones to give the individual form. (b) alternatively, the spinosyns A and D were first separated by a preparative C18 HPLC method. The pure spinosyn A or D was then chemically degraded to the corresponding aglycone. The HPLC separation and analysis are described in (2). The isolated compounds were identified by NMR (Nuclear Magnetic Resonance) spectrometry and MASS spectrometry. In either way, we have obtained pure aglycone substrate in preparative scale.

(2) HPLC analysis and separation of the spinosyns, the aglycone and sugars. Two main purposes are served by the HPLC experiments: (a) to produce pure substrates for the *in vitro* glycosylation of the spinosyn aglycone by the cloned glycosyltransferase in this study. We have established suitable and accurate HPLC conditions to separate and generate pure spinosyn aglycones A and D, both in preparative and analytical scales. (b) to conduct kinetic analysis of the cloned glycosyltransferases: We have found optimal HPLC conditions for high resolution of the spinosyn aglycones (enzyme substrates), the pseudoaglycones (enzyme products of the first glycosylation), and the spinosyns (the final products of tandem glycosylations). Such high-resolution HPLC conditions are critical for us to analyze and determine the kinetic behavior of both wide-type and engineered glycosyltransferases in a time-dependent manner.

(3) The PCR Cloning and Heterologous Expression of the Rhamnosyltransferase (Rtf) in *E. coli*. We have cloned the Rtf gene using the PCR method on genomic DNA of the producing strain, and ligated the coding gene into both pUC19 and pET based vectors in suitable *E. coli* strains. The cloned Rtf gene in pET with or without a C-terminal Hisx6 tag was transformed to *E. coli* BL21(DE3) to test for protein expression induced by various amounts of IPTG inducer. At this point, we have successfully observed the heterologous overexpression of the spinosyn Rtf in *E. coli* based on the protein SDS electrophoresis page. Current efforts have now been in the process of purifying the expressed Rtf, which should allow us to obtain a large quantity of Rtf for the *in vitro* glycosylation assays with dTDP-rhamnose and the spinosyn aglycone, as well as for the 3D-structural determination by X-ray crystallography.

(4) Biosynthesis and Preparative Purification of dTDP-Rhamnose, the Putative Substrate for Rtf. We have carried out the overexpression of biosynthetic genes of dTDP-rhamnose in *E. coli*, and obtained sufficient quantity of the biosynthetic enzymes in the form of protein extracts for *in vitro* biosynthesis of dTDP-rhamnose using dTDP-D-glucose as the biosynthetic precursor. We are now on the stage of purification of the dTDP-rhamnose resulted from the biosynthetic incubation experiments to serve as the substrate for the rhamnosylation of the spinosyn aglycone.

四、計畫成果自評

In the present status, all the material required for the functional expression of the Rtf gene/enzyme has been obtained. Especially, we have observed relatively large production of the Rtf in *E. coli* in soluble fraction, which is essential for our short-term and long-term goals in the studies of this biologically important enzyme. It should be noted that in the current literature none of any sugar-to-polyketide glycosyltransferase has been functionally overexpressed in *E. coli*. This would be the first demonstration of the key enzyme of this kind for mechanistic and kinetic studies, provided that the later *in vitro* activity can be observed. Since it has been known that polyketide glycosides are important sources of drug leads in nature, the result of current study may open a wide spectrum of future applications in both academic and biomedical fields.

It is worth mentioning that our laboratory was just built this year in an infant stage from an empty classroom which had been used as a storage room. To carry out the research, we have to build everything from a scratch, literally nothing at all. With extremely limited and little resource, we have achieved the above results in as short as a few months while the lab is still in the process of an effort-demanding set-up and the required material and equipments are still terribly in short hands. Thus, we believe we may progress much in the future, provided our lab could be more supported financially.

As addressed earlier, we have made success in cloning and expression of the key enzyme, Rtf, for our intended studies of our project. We have also successfully prepared the required material for testing the activity of the biocatalyst. Current

focus is now on the kinetic and mechanistic characterization of the key player, Rtf. This can be done in relatively short time since all the required assay systems have been established. And the result will greatly facilitate the precursor-directed biosynthesis of novel polyketide glycosides, which may allow us to invent potential novel drugs or bioactive molecules. Therefore, we are actually optimistic towards our ends of this project, i.e. making new molecules in an efficient way for industrial or medical uses.

In summary, the studies involved in this project aim to solve the problem by a combination of techniques in Organic Chemistry and Molecular Genetics. We would like to carry out combinatorial biosynthesis of biologically important natural products by a step-by-step and careful approach starting from understanding the fundamental and molecular action of the key enzymes, glycosyltransferases, in their biosynthesis. Importantly, the possible applications and related studies may thus be initiated and can be unlimited by networking this key player to other enzymatic/genetic components of the similar biosynthetic machinery. The future prospects of our studies on the glycosyltransferases can be expended toward the directed-evolution and engineering of the enzymes. Our ultimate goal is again to make useful and novel drugs or bioactive molecules for industrial and medical uses.