行政院國家科學委員會專題研究計畫 期中進度報告

二磷酸核 醣之組合式生物合成在藥物研發之應用(1/3)

計畫類別：個別型計畫
計畫編號：NSC91-2113-M-009-013-
執行期間：91 年 08 月 01 日至 92 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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報告類型：精簡報告

處理方式：本計畫涉及專利或其他智慧財產權，2 年後可公開查詢

中 華 民 國 92 年 6 月 3 日

國科會 NSC 91-2113-M-009-013 期中精簡報告；計畫主持人：邱顯泰

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中文摘要

自然界許多具生物活性的天然物(包括多醣與二次代謝物)含有特殊去氧醣。這些去氧醣的結構常為決定它們生物活性與功能的重要因素。本三年期計畫旨在應用功能性的基因表現與酵素活性鑑定來研究 spinosyns 結構中 dimethylforosamine 的生合成途徑基因群，並且結合其他物種的特殊去氧醣生合成酵素，來進行細胞外組合式生合成各種特殊去氧醣。並再進一步應用 spinosyn 醣基轉移酵素，來將獲得的各類特殊去氧醣併接到天然物的化學結構中，創造多樣的天然物結構，以作為藥物研發之用。在這多年期計畫的第一期，我們已成功達到預期目標，歸納如下：(一)成功將 *gtt*、 *gdh*、*spnO*、 *spnN* 等四個基因轉殖，並在大腸桿菌中大量表現並純化其蛋白質。(二)成功測得前兩酵素的活性，並應用高效率層析法測得相關動力學資訊。而酵素的產物亦被高解析核磁共振光譜與質譜儀鑑定完成。(三)成功發現並證明 Gtt 可將各類一磷酸醣(sugar-1-phosphate)轉變成相關二磷酸核? 醣。(四)成功連結 Gtt 與 Gdh 在生物體外的生合成途徑，證明應用各類特殊去氧醣酵素組合式生合成各種特殊去氧醣的可行性。總之，我們已達成第一期計畫目標，且所得成果具指標性。所建立的分子轉殖、酵素純化與活性鑑定分析系統，可完整應用在將來目標的執行，繼續向本計畫的總目標邁進。

關鍵詞：天然物、去氧醣、二次代謝物、生物合成、酵素學、藥物研發

英文摘要(Abstract)

Many bioactive natural products, including polysaccharides and secondary metabolites, require deoxysugars as their structural counterparts to target specific biological targets to present their biological activities and functions. In this three-year project, we proposed to characterize the biosynthetic pathway of the dimethylforosamine involved in the biosynthesis of spinosyns from *Saccharopolyspora spinosa* by functional expression and kinetic characterization of each enzyme of the dimethylforosamine pathway. In combination with other deoxysugar enzymes from other pathways, we proposed to utilize spinosyn glycosyltransferases to demonstrate the possibility to engineer the bioactive natural products by incorporating various biosynthesized deoxysugars into their chemical skeletons. During the first period (08/01/2002~05/30/2003) of the project, we have successfully achieved the intended goals, including: (1) The four target genes, *gtt*, *gdh*, *spnO* and *spnN*, involved in the dimethylforosaime biosynthesis from *S. spinosa* were cloned and overexpressed in *E. coli*. (2) The enzymatic activities of the first two

enzymes Gtt and Gdh of the pathway were determined kinetically by the HPLC (High Performance Liquid Chromatography) method developed in this project. Their enzymatic products were made in preparative scale and structurally characterized by NMR (Nuclear Magnetic Resonance) and MASS spectrometric methods. (3) Gtt was found to catalyze the conversion of various sugar-1-phosphates to NDP-sugars in the presence of NTP. (4) Tandom multi-enzymatic synthesis using Gtt and Gdh was realized in our *in vitro* system. In summary, the promising experimental results obtained in the first period of the project have proved our idea of the proposed goals. The methods and analytical system can now be applied to functionally express and characterize other biosynthetic enzymes, and can be utilized for the aims of combinatorial biosynthesis and natural product engineering.

關鍵詞 (Keywords): Natural Products, Deoxysugars, Biosynthesis, Secondary Metabolites, Enzymology, Drug Discovery

報告內容：

The presence of deoxysugars in many bioactive natural products, particularly secondary metabolites, is crucial for their corresponding biological activities. The various functional groups displayed on the saccharides dictate unique molecular recognition of the molecules by specific biological targets. Deoxysugars have also been recognized as important immunological determinants and are known to contribute to the serological specificity of many immunologically active structures. In microorganisms, deoxygenated sugars other than 2-deoxy-D-ribose can be found as elements of lipopolysaccharides (LPS), extracellular polysaccharides (EPS) in addition to many antibiotics they produced. LPS are constituents of the gram-negative bacterial cell wall and represent the endotoxin of these organisms. They exhibit pyrogenicity and lethal toxicity in higher organisms but are also very important for bacterial survival. For EPS, examples are capsular polysaccharides, lipochitooligosaccharides (Nod Factors) and colanic acid, all of which exhibit various potent and interesting biological activities. No matter what kind of architecture the polysaccharides adapt, sugar constituents of many of them have been determined. They also contain sugars of wide structural diversity, including neutral sugars, amino sugars, sugar acids, and many different deoxysugars.

Although the contribution of deoxysugars to biological activities is well recognized, the knowledge concerning the biosynthesis of deoxysugars and their subsequent assembly into bioactive glycosides and oligosaccharides is still very limited. For example, exact biosynthetic pathways leading to the formation of the deoxysugars need further discovery, identification and characterization. Many pathways known so far are speculated based on chemical sense of organic transformation, and has, in most cases, not yet been firmly defined based on experimental evidence. To resolve this problem, genes and enzymes probably involved in the biosynthesis of the deoxysugars need to be functionally expressed and characterized.

Moreover, the key building block of the bioactive glycosides, sugar nucleotide diphosphate (NDP-sugar), needs to be efficiently synthesized. Since the carbohydrate portion of the bioactive secondary metabolites often determines their biological activities, one may try to incorporate various sugar moieties into the bioactive products to modify their functions or activities using appropriate glycosyltransferases. This requires availability of the activated sugar nucleotides, NDP-sugars. However, due to the structural complexity and instability of the NDP-sugars, they are difficult to synthesize in sufficient yield. And only very few of them are available in market and are very expensive. Therefore, the

utilization of enzymes for the synthesis of carbohydrates has gained an increasing interest because products can be obtained in high yield and in a one-step procedure with high regio- and stereoselectivity and without protection and deprotection of reactive groups. One can also combine chemical synthesis and appropriate enzymatic systems to do chemo-enzymatic synthesis of deoxysugars. For example, precursor-directed biosynthesis of the natural and unnatural 6dOHs and bioactive glycosides can be utilized using the natural biosynthetic enzymes.

In this project, we have been engaged in the combinatorial biosynthetic studies of various deoxysugars using biosynthetic enzymes from biosynthetic pathways of spinosyns and polysaccharides, as well as kinetic characterization of some interesting enzymes. We also proposed a model that may utilize spinosyn glycosyltransferases to incorporate various deoxysugars generated from this project and so as to "engineer" bioactive natural products, e.g. spinosyns, for application of drug discovery. The project was divided into three stages/years of specific aims. Here we report our progress obtained in the first stage during the period of 08/01/2002~05/30/2003, described as follows.

(1) Molecular Cloning of the Four Target Genes, *gtt*, *gdh*, *spnO* and *spnN*, Involved in the NDP-Sugar Biosynthesis from *Saccharopolyspora spinosa*. The four genes have been successfully cloned from the spinosyn-producing organism, *S. spinosa*. The genes were cloned into the cloning vector using the PCR (Polymerase Chain Reaction) method on the genomic DNA isolated from the microorganism. The coding DNA fragments were subsequently cloned into the pET19 or pET21 expression vector in frame with an N- or C-terminal His-tag coding DNA sequence, respectively. The cloned DNA fragments were sequenced and confirmed to be the target genes.

(2) Heterologous Expression and Large-Scale Purifictaion of the Four Biosynthetic Enzymes (Gtt, Gdh, SpnO and SpnN) in *E. coli*. The four proteins have been successfully overexpressed in *E. coli* and purified to near homogeneity in high yield. Each of the four expression plasmids obtained above was individually transformed into *E. coli* BL21(DE3) for protein expression induced by IPTG at various concentrations (0~1mM) at various induction temperatures (e.g. 4~30℃) to obtain the highest yield of soluble proteins. The best induction condition was found to be 15℃/1mM IPTG/24hr, 15℃/0.25mM IPTG/10hr, 15℃/0.25mM IPTG/36hr and 30℃/1mM IPTG/8hr for Gtt, Gdh, SpnO and SpnN, respectively. Each protein was then subjected to large-scale protein purification by the Ni-NTA column chromatography (Qiagen) to obtain purified proteins for enzymatic assays. **Figure A** illustrates the expression and purification results for Gtt and Gdh.

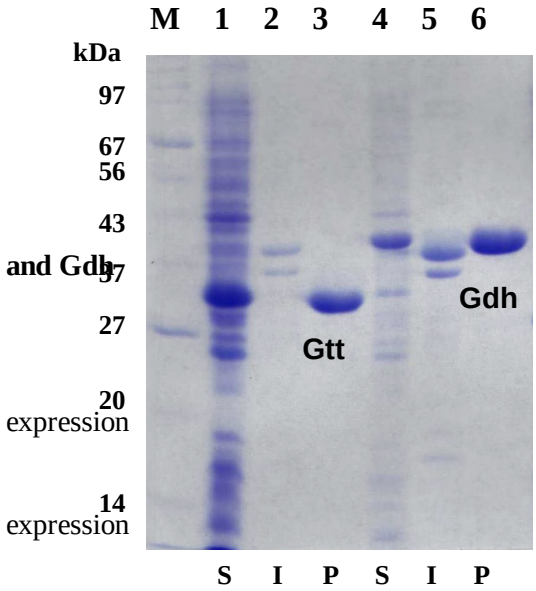


Figure A.

Heterologous Expression of Gtt and Gdh

M: Protein Standard Marker

Lane 1: soluble fraction of Gtt expression

Lane 2: insoluble fraction of Gtt expression

Lane 3: purified fraction of Gtt expression

Lane 4: soluble fraction of Gdh expression

Lane 5: insoluble fraction of Gdh expression

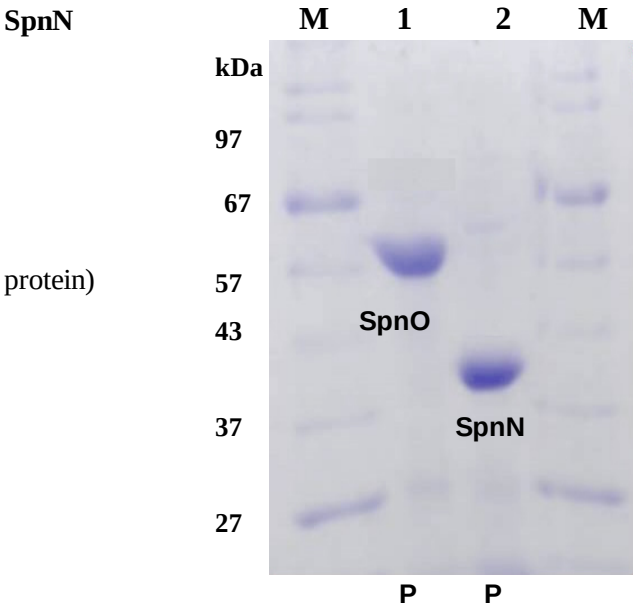
Lane 6: purified fraction of Gdh expression

S: soluble; I: insoluble; P: purified protein

As shown in **Figure A**, Gtt and Gdh have been purified to near homogeneity in large quantity (17 mg/L culture and 20 mg/L culture, respectively). In a similar fashion, SpnO and SpnN were also obtained in sufficient purity and quantity (3.8 mg/L culture and 3.0 mg/L culture, respectively) as shown in **Figure B**.

Figure B.

The SDS-PAGE of SpnO and SpnN



M: Protein Standard Marker

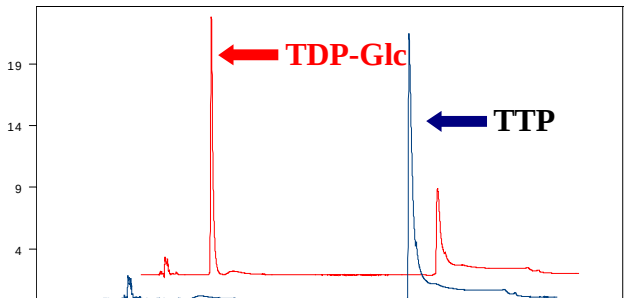
Lane 1: purified fraction of SpnO

Lane 2: purified fraction of SpnN

(10%SDS-PAGE; P: purified protein)

(3) Functional Characterization and Enzymatic Assays of Each of the Four Biosynthetic Enzymes. Since all the purified proteins have been available, we immediately proceeded to carry out enzymatic assays to functionally characterize each protein. Along the studies, we have also developed a suitable method and optimal conditions for both analytical analysis of the enzymatic reaction mixture as well as the large-scale preparation protocols for isolation of desired NDP-sugar products using HPLC (high performance liquid chromatography).

(3a) Gtt. Gtt catalyzes the conversion of glucose-1-phosphate (G1P) to NDP-glucose (NDP-Glc) in the presence of NTP. We carried out the incubation of G1P and TTP with Gtt, and observed a time-dependent production of TDP-Glc based on analytical HPLC. The identity of the enzymatic product TDP-Glc was found to be identical to commercial authentic TDP-Glc as judged by NMR and MASS spectroscopy. In **Figure C** was shown the HPLC analysis profile of the Gtt reaction.



After Reaction

Before Reaction

Figure C. Enzymatic Conversion of G1P to TDP-Glc by Gtt

The co-substrate TTP was consumed after the reaction, whereas TDP-Glc was formed.

(3b) Gdh. Gdh was proposed to carry out the conversion of NDP-Glc to 4KD6G (4-keto-6-deoxy-D-glucose). The enzymatic incubation of Gdh with TDP-Glc in the presence or absence of $NAD(P)^+$ was examined with HPLC. The HPLC result of the Gdh reaction is shown in **Figure D**. As shown in **Figure D**, a time-dependent consumption of TDP-Glc took place concurrently with a time-dependent production of KDG, and the reaction went to completion. The reaction did not require exogenous $NAD(P)^+$. The product 4K6DG was isolated in preparative scale using ion-exchange HPLC and was subjected to and confirmed by NMR and MASS characterization.

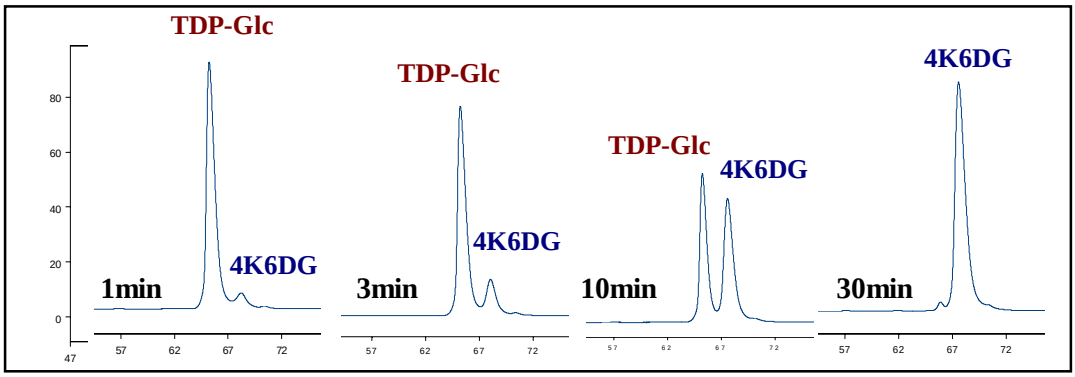


Figure D. The time-dependent transformation of TDP-Glc to 4K6DG catalyzed by Gdh.

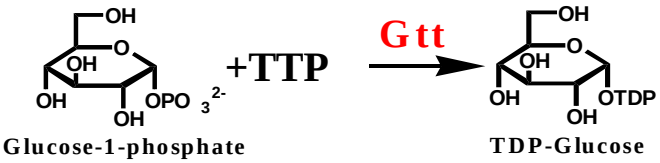
(3c) Kinetic Characterization of Gdh. The kinetic parameters (*Km* and k_{cat})

were also determined for the enzyme Gdh. The Gdh reaction was examined at various concentrations of TDP-Glc based on 4K6DG absorption using a UV-Vis spectrometer. The *Km* and k_{cat} were estimated to be 0.28 mM and 0.38 s^{-1} at 30℃, respectively.

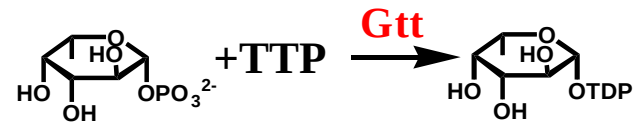
(3d) Tandom multi-enzymatic synthesis of 4K6DG. In the proposal, we intended to realize the *in vitro* biosynthesis of NDP-sugars using various biosynthetic enzymes of NDP-sugars. We thus tested our idea with the reaction of G1P and TTP to which Gtt and Gdh were co-added or Gtt was added first before the addition of Gdh. In either case, we were able to generate the desired product, 4K6DG, as judged by HPLC. The detailed kinetic profile for either condition is being further characterized. The similar observation was also obtained in our *in vitro* studies of the NDP-rhamnose biosynthetic pathway.

(4) Novel Enzymatic Transformation of Fucose-1-Phosphate (F1P) to TDP-Fucose by the Gtt Enzyme. As mentioned earlier in this progress report, we have successfully demonstrated the enzymatic activity of Gtt. We thus proceeded to carry out the pilot-test of the specificity of Gtt using other sugar-1-phosphates as substrate analogues. F1P differs from G1P in both stereochemistry and function groups of the sugar moiety in substantial degree. As we incubated Gtt with F1P and TTP, we observed a time-dependent enzymatic production of a new compound, which presumably is TDP-fucose. The NMR and MASS characterizations are in progress to identify the compound. The enzymatic conversion of sugar-1-phosphates to NDP-sugars by Gtt is illustrated in **Scheme A**. Other sugar-1-phosphates have also been tested for the enzymatic transformation. Preliminary results have shown that Gtt was also capable to convert various sugar-1-phosphates to UDP-sugars and TDP-sugars in the presence of UTP and TTP, respectively. The enzymatic products and kinetic characterization of these various reactions are now in progress.

Scheme A. The Enzymatic Conversion of Sugar-1-Phosphates to corresponding TDP-Sugars by Gtt.



Glucose-1-phosphate **TDP-Glucose**



Fucose-1-phosphate **TDP-Fucose**

In this progress report, we summarize our experimental progress that was achieved within the first period (08/01/2002~05/30/2003) of the project. As we are moving towards the coming second year (**STAGE II**, 08/01/2003~07/31/2004), we feel very confident because the results obtained so far strongly support our original idea and plan. At this point, we have, as planned, successfully cloned the 4 target genes, overexpressed them in *E. coli*, and purified the corresponding proteins to near homogeneity in preparative scale. As proposed, the first two enzymes Gtt and Gdh were demonstrated to be active and their enzymatic products were identified and characterized by HPLC, NMR and MASS. The Mechalis-Menton kinetics was also demonstrated on Gdh, whereas its kinetic parameters were also determined. Moreover, we successfully demonstrated the relaxed specificity of Gtt, which would allow us to generate various NDP-sugars as proposed. As mentioned in the proposal, the NDP-sugars can serve as universal substrates for glycosyltrasnefrases in the biosynthesis of many bioactive natural product glycosides and polysaccharides. Most importantly, the *in vitro* functional reconstitution of the NDP-sugar biosynthetic pathway was also found feasible. Some results presented here are being summarized in manuscript and submitted for publication.

In summary, the current result of the first four genes *gtt*, *gdh*, *spnO* and *spnN* of the **STAGE I** has paved the smooth and promising road, by building up a well-established system, for the related studies of the last four genes, spnQ, spnR, spnS and spnP of the **STAGE II** to be carried out in the second year (08/01/2003~07/31/2004). The developed experimental protocols in the cloning, expression, protein purification, enzymatic assays, product isolation and characterization, and kinetic examination shall serve as an solid base and model for the goals to be achieved in the second year (**STAGE II**, 08/01/2003~07/31/2004), and certainly for those in the third year (**STAGE III**, 08/01/2004~07/31/2005) as well.

計畫成果自評部份

Carbohydrates are important components of many natural products produced by

all living organisms. By nature, one thus needs to understand the biosynthetic origins of the carbohydrates and their conjugates in order to manipulate and utilize them. NDP-hexoses have been found to be important precursors for the biosynthesis of all glycoconjugates, including low molecular glycosyl secondary metabolites. In particular, the glycosyl moieties of many bioactive secondary metabolites are key elements absolutely required for their biological activities, as shown many previous studies. Therefore, manipulations or modification of the glycosyl moieties of the bioactive molecules may provide an exciting opportunity to modify or change their biological activities, and have thus become a new approach in drug discovery. Also, many pathogenic bacteria possess unique hexoses that are not present in higher organisms like mammals. The enzymes involved in their biosynthesis have clearly become increasingly interesting targets for therapeutic inhibition. Besides, synthesis of NDP-hexoses has been an important, but difficult, subject in glycobiology and carbohydrate research. To make functional variations presented by various natural or designed NDP-hexoses, one often has to suffer from tedious procedures involving protection and deprotection of reactive group in traditional chemical synthesis, which is also a limiting factor for future large-scale production of NDP-hexoses in industry.

Taking all the factors into considerations, the study of this project proposed to provide new insights into NDP-hexose biosynthesis and a new way to make them in a relatively efficient fashion as well. Their critical roles in biological systems deserve no doubt. Overall speaking, the study may provide an important method to understand and characterize enzymes and their relationship involved in the carbohydrate biosynthesis, which may further provide endless opportunities for scientists to utilize them for studies in glycobiology or make material more efficiently.