行政院國家科學委員會專題研究計畫　成果報告

細胞中電子傳遞鏈 complex I (NADH:ubiquinone oxidoreductase)酵素活抑制劑 Pterulone 其最佳衍生物的合成

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中文計畫摘要:

在粒線體電子傳遞鏈已知為細胞中產能的最重要過程,在傳遞過程中主要包括了四個複合體(Complexs I, II, III, IV),其中 Complex I (NADH:Ubiquinone oxidoreductase)為高能分子 NADH 進入電子傳遞鏈的入口。而細胞中可以轉換出 ATP 的高能分子除了 NADH 外另一個則是 $FADH_2$,而 Complex II 則是 $FADH_2$ 高能分子的入口處,在細胞中 NADH 扮演了主要提供能量的角色,而 $FADH_2$ 則較為少量且次要。

Pterulon 為真菌 *Pterula sp*82618 所代謝出來具有抗其他真菌的代謝物質,根據研究發現此化合物具有抑制 complex I 酵素活性,部分屬於 complex I 抑制劑的化合物已被應用於農業上作為殺蟲劑方面的用途。近年來在一些報告中發現這類抑制劑對正常細胞的細胞毒性相當得低(IC_{50}=36 ì M)但卻可以有效的抑制癌細胞的生長,雖然完整的機制尚未清楚瞭解但已知主要的途徑是藉由抑制促進癌細胞生長的酵素 ODC (orthine decarboxylase,是癌細胞中特有的酵素)的活性而達到抑制癌細胞生長的目的。

因此本研究擬利用經全合成的天然物 Pterulone 及其衍生物對純化出來的細胞粒線體電子傳遞鏈中 Complex I 酵素活性的抑制效果進行探討。本研究結果中發現目前所合成的這些化合物其抑制酵素活

性的效果仍未達理想，即抑制酵素活性 IC_{50} 仍是太高介於 4~55 μM 之間接近於 Pterulone 的抑制活性與 Rotenone 的 0.5 nM 相差甚遠，因此還需進行結構式的修改及酵素活性的測試。

關鍵詞：粒線體、電子傳遞鏈抑制劑、NADH:Ubiquinone oxidoreductase。

英文計畫摘要:

Complex I is the first of three large enzyme complexes located in the inner mitochondrial membrane. This enzyme forms the electron transport chain that carries electron from NADH to molecular oxygen during oxidative phosphorylation.

There are a wide variety of natural and synthetic inhibitors of complex I which have found multiple applications. Recently, it has been shown that inhibition of complex I cause concomitant reduction in the activity of orthine decarboxylase (ODC). Orthine decarboxylase (ODC) is responsible for the biosynthesis of polyamine growth factors required for cellular proliferation, and induction of ODC activity has been associated with tumor promotion. Since the over expression of ODC in tumor cell contributes to aberrant proliferation, the ability of complex I inhibitors to reduce ODC activity makes them promising candidates as next generation antitumor agents.

The fungal metabolites pterulone (**1**) and its analogue **2** were isolated from fermentations of a *Pterula sp* 82168 species, and *Mycena galopus*, respectively. Pterulone (**1**) exhibited significant antifungal activity, and it is a highly potent inhibitor of complex I with an IC_{50} value of 36 μM. Although the pharmacological profile of **2** has not yet been reported, compound **2** is structurally related to **1**; therefore, it is believed that **2** will exhibit similar biological activity as pterulone (**1**). The goal of this study is to identify novel lead complex I inhibitors through structure-activity relationship (SAR) study based on compound **2**. These lead compounds synthesized through this SAR study would be more amenable to further synthetic modification as required for optimization of physical and pharmacological properties. So far, seven compounds synthesized in this project, they exhibit the IC_{50} between 4 μM to 55 μM; they are much less potent as compared with rotenone (IC_{50} = 0.5 nM). The antagonistic effects of the analogues herein were only with little improvement as compared with pterulone (IC_{50}=36 μM). From these preliminary

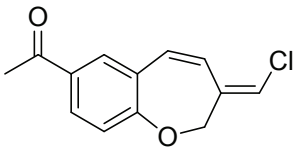
SAR study, the data suggest that the binding environment for these inhibitors are composed with hydrophobic amino acid, and the dimension of binding site are large enough to tolerated large halogen group. Detail on studying the binding domain by variation on the inhibitors is currently under way.

Keywords : Mitochondria, Complex I inhibitor, NADH:Ubiquinone oxidoreducatse

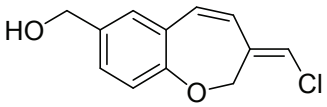
Introduction

NADH:Ubiquinone oxidoreductase comprises the first phosphorylation site of mitochondria and is the energy-conserving enzyme complex that is commonly known as "complex I".[1] There are a wide variety of natural and synthetic inhibitors of complex I which have found multiple applications.[2] Complex I inhibitors have been used to elucidate the role of this enzyme in normal cell physiology and also have been used to mimic complex I deficiencies in order to study mitochondrial diseases.[3] Inhibitors of complex I have also been a preferred targeted for the development of commercial insecticides and acaricides for years.[4] Recently, it has been shown that inhibition of complex I causes concomitant reduction in the activity of orthine decarboxylase (ODC).[5] ODC is responsible for the biosynthesis of polyamine growth factors required for cellular prolification.[6] Since the overexpression of ODC in tumor cell contributes to aberrant proliferation, the ability of complex I inhibitors to reduce ODC activity makes them promising candidates as next generation antitumor agents.[7]

The fungal metabolites pterulone (**1**) and its analogue **2** were isolated from fermentations of a *Pterula sp* 82168 species, and *Mycena galopus*, respectively.[8, 9] The structures of both **1** and **2** were assigned based on their physical and spectral characteresitcs.[9,10] The architectural framework that is common to **1** and **2** is a monochlorinated 2,3-dihydro-1-benzoxepine ring skeleton. The differences between **1** and **2** are found in the substitution at the 7-position and in the geometric configuration of the vinyl chloride. Pterulone (**1**) bears an acetyl group at the 7-position and its vinyl chloride is in the *E*-configuration. On the other hand, compound **2** bears a hydroxymethyl group at the 7-position and its vinyl chloride is in the *Z*-configuration. Pterulone (**1**) exhibited significant antifungal activity, and it is a highly potent inhibitor of complex I with an IC_{50} value of 36 μM.[8] The pharmacological profile of **2** has not yet been reported. Since **2** is structurally related to **1**, it is believed that **2** will exhibit similar biological activity as pterulone (**1**).[9]



pterulone (1)



2

The goal of this study is to identify potent complex I inhibitors through

structure-activity relationship (SAR) study based on compound **2**. Since the total syntheses of **1** and **2** were achieved in 4 and 5 steps respectively, it would be a simple modification based on current synthetic approach to construct more lipophilic analogue of **2**.[11] In order to completed this SAR project, there are three tasks need to be overcome. The first task will be the complex I isolation. The second task is to setting up the reliable enzyme assay system for the analogues. The last task is the synthesis of the analogue to optimize the antagonist. In this report, complex I isolation and establishing a reliable assay system had successfully completed in this group. The pharmacological profile of **2** and its analogues will also be reported. These lead compounds synthesized through this SAR study would be more amenable to further synthetic modification as required for optimization of physical and pharmacological properties.

Material and Method

Mitochondria purification

The mitochondria purification is according the methods of Pharo et al.,[12] and Smith.[13] Minced bovine heart is homogenized for 45 sec in 750g lots in 2,250 ml of a medium containing 0.25 M sucrose, 13 mM Tris-HCl, pH 7.8, 13 mM K_2HPO_4, and 0.1 mM EDTA in blender. Following centrifugation for 15 min at 1,000xg to remove the debris, the supernatant fluid is filtered through several layers of cheese-cloth into a chilled beaker and kept cold. The residue is homogenized again with one d for 10 min as above, and the supernatant fluid is collected in the same beaker as before. The combined supernatant fraction is adjusted pH to 7.8 with 1 M KOH or 2.0 M unneutralized Tris and the suspension is centrifuged for 15 min at 26,000xg.

Enzyme Assay

The NADH:NBQ (nonylubiquinone) activity is measured using a Beckman DU-70 spectrophotometer in dual wavelength mode by following NADH oxidation at 340/400 nm at 30℃.[4] 100 μM NADH and 50 μg of purified complex I is added to buffer containing 50 mM Tris/HCl, pH 7.4, 5 μM Kresoxim and 2 mM KCN. The catalytic reaction is started by addition of 60 μM NBQ. The inhibition of residual rate is assay with complex I (**2**) and its analog dissolved in ethanol which are added before starting the catalytic reaction with 60 μM NBQ.

Synthesis of analogues

General. Proton and carbon NMR were obtained on a Bruker AMX-500

spectrometer. NMR spectra were recorded in $CDCl_3$ solution, expect as otherwise stated. Chemical shifts were reported in ppm relative to tetramethylsilane (δ units). Fast atom bombardment (FAB) mass spectra and elemental analyses were recorded on a Micromass ZAB spectrometer and Perkin-Elmer 2400 elemental analyzer repetitively at the Analytical Facility of The National Taiwan University. IR spectra were obtained on Perkin Elmer Spectrum RXI FT-IR system. Silica gel TLC was performed on 60F-254 pre-coated sheets (E. Merck) and column chromatography was done on silica gel (60-120 mesh). All of chemicals were used directly as purchased from Acros, Aldrich, or TCI unless otherwise noted. Compounds **1**, **2**, **8**, **9**, and **10** were synthesized according to the previous published procedure.[11]

Mesolyate **3**. To a stirred solution of alcohole **2** (0.33 mmol) in CH_2Cl_2 (3 mL) in ice bath, of a solution of MsCl (0.5mmol) and Et_3N(0.6 mmol) in CH_2Cl_2 (1 mL) was added. The mixture was stirred at 0°C for 1 hour. The mixture was diluted with CH_2Cl_2 and washed with water. The organic extract was dried ($MgSO_4$) and concentrated under reduced pressure. Chromatography of the residue eluting with ethyl acetate/hexanes mixture gave 1.47 g (99%) of **3** as yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.94 (s, 3H), 4.61 (s, 2H), 4.79 (s, 2H), 6.24 (s, 1H), 6.23 (d, 1H, J= 11.5 Hz), 6.38 (d, 1H, J= 11.9 Hz), 6.52 (s, 1H), MS (EI) m/z: 300.

General Procedure for the Synthesis of **4-7** The NaH (0.05 mmol) were added at 0 °C to a well stirred solution of appropriated alcohol (0.04 mmol) in anhydrous THF (1 mL) under nitrogen atmosphere. After the resulting suspension was stirred at 0 °C for 30 min, then **3** (23.93 mg, 0.04 mmol) in anhydrous THF (1 mL) was added dropwised into mixture under nitrogen atmosphere at 0°C. The corresponding reaction mixture was stirred at 0°C for 1 hour. Then, the mixture was quenched with 1 N HCl at 0 °C and extracted with ethyl acetate. The combine organic extract was dried ($MgSO_4$) and concentrated under reduced pressure. Chromatography of the residue eluting with ethyl acetate/hexanes mixture gave analytically pure compounds. Spectroscopic and analytical data of **4-7** follow.

Benzyl ether **4** ^{1}H NMR: δ 4.54 (s, 2H) , 4.61 (s, 2H), 4.63(s, 2H), 6.23 (d, 1H, J = 11.5 Hz), 6.38 (d, 1H, J= 11.9 Hz), 6.52 (s, 1H), 7.02 (d, 1H, J= 8.0 Hz), 7.19-7.25 (m 5H) 7.44 (dd, 1H, J= 8.0 Hz, 2.4Hz), 7.50 (d, 1H, J= 2.4 Hz). ^{1} MS (EI) m/z: 312

Benzyl ether **5** ^{1}H NMR: δ 3.24 (s 6H). 3.30 (s. 3H), 4.51 (s, 2H) , 4.59 (s, 2H), 4.62 (s, 2H), 6.23 (d, 1H, J= 11.5 Hz), 6.38 (d, 1H, J= 11.9 Hz), 6.52 (s, 1H), 7.09 (d, 1H, J= 8.6 Hz), 7.21 (s 2H), 7.44 (dd, 1H, J= 8.6 Hz, 2.4Hz), 7.55 (d, 1H, J= 2.4Hz). ^{1} MS (EI) m/z: 451

Benzyl ether **6** ^{1}H NMR: δ 3.24 (s 6H). 4.54 (s, 2H) , 4.60 (s, 2H), 4.65 (s, 2H), 6.27 (d, 1H, J= 11.5 Hz), 6.38 (d, 1H, J= 11.9 Hz), 6.52 (s, 1H), 7.07 (d, 1H, J= 8.6 Hz), 7.19 (s, 1H), 7.21 (d, 1H, J= 8.0 Hz) 7.26(d, 1H J= 8.0 Hz), 7.44 (dd, 1H, J=

8.6 Hz, 2.4Hz), 7.50 (d, 1H, J= 2.4 Hz). [1] MS (EI) m/z: 404.

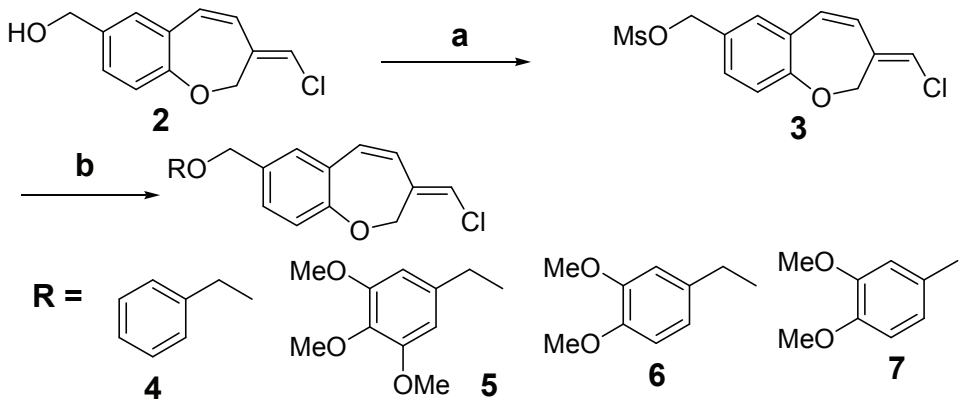
Benzyl ether **7** ^{1}H NMR: δ 3.24 (s 6H). 3.30 (s. 3H), 4.54 (s, 2H) , 4.61 (s, 2H), 4.65 (s, 2H), 6.27 (d, 1H, J= 11.5 Hz), 6.38 (d, 1H, J= 11.9 Hz), 6.52 (s, 1H), 7.07 (d, 1H, J= 8.6 Hz), 7.19 (s 2H), 7.44 (dd, 1H, J= 8.6 Hz, 2.4Hz), 7.50 (d, 1H, J= 2.4 Hz). [1] MS (EI) m/z: 437

Results

Inhibitor Synthesis

The goal of this study is to identify potent complex I inhibitors through structure-activity relationship (SAR) study based on compound **2**. Various literature studies identified potent complex I inhibitors composed large hydrophobic functional group within the molecule.[2.3] The initial approach for design the inhibitors was to increase of the hydrophobocity of **2**. The synthetic of the various aromatic derivatized **2** was shown in scheme I. The synthesis began with fictionalized the known benzyl alcohol **2**.[11] The benzyl alcohol in **2** was activated for various nucleophile attack with installation of methansulfonyl group. Treatment of the methansulfonyl ether **3** with various alkoxide generated in situ gave ether **4-7** in rang of 78 to 90% yield.

Scheme I



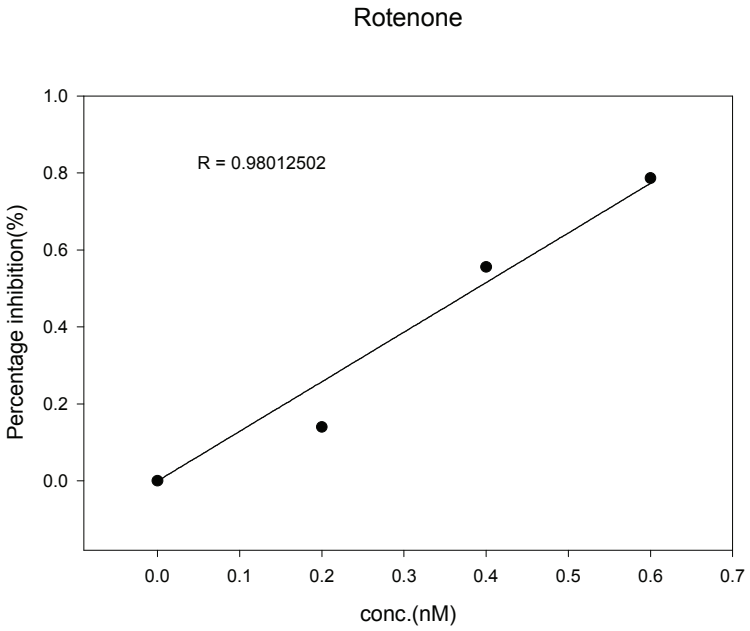
Condition: (a) MsCl, Et_3N, CH_2Cl_2 90%; (b) Alkoxide, NaH, THF

Complex I isolation

The mitochondria purification is according the methods of Pharo et al.,[12] and Smith.[13] A Batch of 600g minced bovine heart yield 126 mg of beef heart mitochondria. The purified beef heart mitochondria (BHM) was assayed for NADH oxidation activities according to Okun procedure[14] with 2.4 mg beef heart mitondria/ml yield 0.1 unit/ min. The BHM was also exam for the sensitivity toward known complex I inhibitors, rotenone. The inhibition cure of BHM against rotenone

was shown in figure. The IC_{50} value of rotenone was 0.39 nM which was comparable to known result (IC_{50} = 0.5 nM).

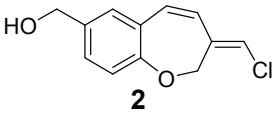
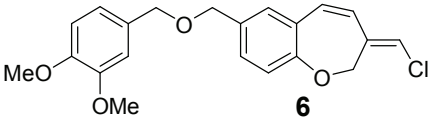
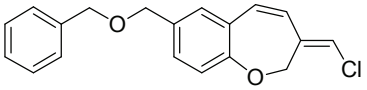
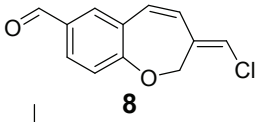
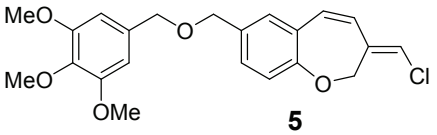
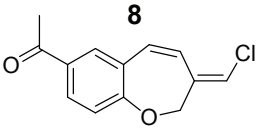
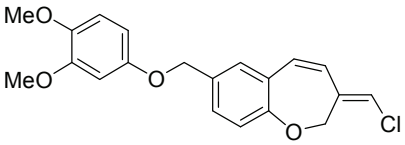
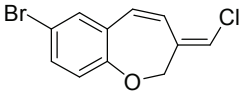
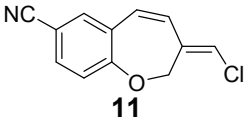
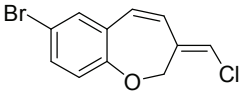
Figure



Complex I analogues

The inhibitory activities of various analogues against BHM were listed in table below. The most potent inhibitors tested were dimethoxyphenylether **7** IC_{50} value of 4 μM. The natural product **2** displayed IC_{50} value of 23 μM against the BHM. The IC_{50} value of pterulone (**8**) was reported as 36 μM,[8] and the aldehyde analogue **8** exhibited IC_{50} value of 34 μM.

Table

Inhibitors	$IC_{50}(\mu M)$	Inhibitors	$IC_{50}(\mu M)$
 **2**	23	 **6**	9.4
 **4**	20	 **8**	34
 **5**	8.5	 **1**	36[8]
 **7**	4	 **9**	50
 **11**	30	 **10**	55

Discussion

The three major tasks of this project, mitochondria purification, establishing reliable NADH assay, and inhibitors synthesis for this study had been complete. The tasks had been fulfilled in this project. The mitochondria were purified according to the published procedure. Although the total protein yield from the mitochondria purification were not very efficient, it was sufficient enough for establish the reliable NADH assay system, and assay the inhibition effect of the synthetic inhibitor.

The complex I is a mutilsubuints proteins which composes 40 different subunits.[1] Upon till this date, there was no completed structural information on this protein, and much of the structural information were from the SAR studies of various know complex I inhibitors. Most of known complex I inhibitors composed large hydrophobic region.[2,3] From this SAR study, the potency of the inhibitors was also increased as the inhibitors increased the hydrohobicity. The natural product **2** composes a hydrophilic hydroxyl moiety with IC_{50} value in mid 20 μM, in contrast; the IC_{50} values decreased to the single digit region as the hydroxyl moiety in **2** was alkylated with hydrophobic methoylated benzyl group. The vinyl chloride configuration within the inhibitors did not affect the inhibition potency. The IC_{50}

value of **10** with *Z* vinyl chloride configuration was comparable with the reported IC_{50} value of **9** with *E* vinyl chloride configuration. From these preliminary SAR study, the data suggest that the binding environment for these inhibitors are composed with hydrophobic amino acid, and the dimension of binding site are large enough to tolerated large halogen group. Detail on studying the binding domain by variation on the inhibitors is currently under way.

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