PREFACE

Microbial conversion or transformation has been used successfully for the production of amino acids, sugars, antibiotics, and other useful pharmaceutical products. The advantages of using microbial reactions are the regiospecificity and stereospecificity of the microbial catalysis and also the mild conditions employed (1). In the field of microbial transformations, the conversion of steroidal compounds has been widely exploited. A microbial process for steroid conversion was first patented in 1937 by Schering (Germany), in which Schoeller and Mamoli reported the reduction of 17-keto group by a yeast. A landmark in steroid transformation was made by the Upjohn Company in 1950. They reported the introduction of a hydroxy group into the 11α-position of progesterone using *Rhizopus* species, though which cortisone production became more convenient and economical. This was followed a few years later by the discoveries of 11β-hydroxylation of Reichstein's compounds S to form hydrocortisone and 11α-hydroxylation of progesterone by bacteria (2,3). Since then, much effort has gone into the microbial transformation of steroidal compounds. In recent decades, thousands of modified steroids have been produced either by a combination of chemical and microbial reactions or simply by microbial reactions.

In the period 1967 to 1982, many reports appeared on the hydroxylation, dehydroxylation, degradation and metabolism of steroidal compounds (4-35). During the same period, however, the structurally related bile acids received much less attention. More recently, interest in the bile acids has increased considerably, principally due to the finding that a naturally occurring bile acid (chenodeoxycholic acid, 3α,7α-dihydroxy-5β-cholanic acid) possesses the therapeutic property of solubilizing cholesterol gallstones (36, 37). At present, gallstone disease is a leading cause of

hospitalisation (38), and in Japan ca. 10% of human adults possess gallstones (39).

Sawada explored a microbial process for supply of the precursor of chenodeoxycholic acid, 12-ketochenodeoxycholic acid (40-42). Lately, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanic acid) has been reported to have similar therapeutic properties (40), and to be superior with respect to side-effects and dose response to chenodeoxycholic acid (44,45). However, the amount of naturally available ursodeoxycholic acid is very low, and a seven-step chemical synthesis of ursodeoxycholic acid has not solved the supply problem due to its low yield (9 to 14%) (46,47). Therefore, a more economical process is required.

Since both chenodeoxycholic acid and ursodeoxycholic acid are dihydroxy derivatives of 5β-cholanic acid, it is possible that other derivatives containing two hydroxy groups may have similar therapeutic effect.

The objective of this work is to study the production of ursodeoxycholic acid and a new dihydroxy derivative of 5β-cholanic acid by microbial conversion.

Chapter 1 describes screening for microorganisms which can transform lithocholic acid (3α-hydroxy-5β-cholanic acid) to ursodeoxycholic acid. A strain thus isolated was identified from its morphological and cultural characteristics as *Fusarium equiseti* M-41. The purified product was identified as ursodeoxycholic acid from its NMR and IR spectra and other physicochemical properties such as melting point and optical rotation. Cultivated in a 10-ℓ jar fermentor, this strain produced ursodeoxycholic acid from lithocholic acid with 35% yield (0.35 g/ℓ) in 112 h.

Part 1 of chapter 2 describes the determination of the optimum conditions for ursodeoxycholic acid production by *Fusarium equiseti* M-41. Dextrin and L-asparagine were found to be the best carbon and nitrogen

sources, respectively. Resting mycelia showed maximum conversion at 28°C, pH 8.0, and dissolved oxygen tension of higher than 60% saturation. The 7β-hydroxylation activity in the mycelia was controlled by dissolved oxygen tension during cultivation: with a dissolved oxygen tension of 15% and over, the activity peak appeared at 25 h of cultivation, but was delayed to 34 and 50 h with dissolved oxygen tensions of 5% and 0%, respectively. After reaching the maximum, the 7β-hydroxylation activity declined rapidly at pH 7.0, but the decline was retarded by increasing the pH to 8.0. Several combinations of operations, such as pH shift from pH 7 to 8, addition of 0.5 M KCl, and dissolved oxygen control, were applied to the production of ursodeoxycholic acid in a jar fermentor, and a much larger amount of ursodeoxycholic acid (1.2 g/ℓ) was produced within 96 h of cultivation.

Part 2 of chapter 2 examines the interaction between mycelia of *F. equiseti* M41 and substrate lithocholic acid. Because lithocholic acid is sparsely soluble in water (ca. 15 mg/ℓ), its interaction with mycelia seemed to be different from that of water soluble substrates, such as amino acids and sugars. The insoluble form of lithocholic acid was found to bind to mycelia with an apparent Km of 1.9 g/ℓ. Also, the 7β-hydroxylation activity in the mycelia showed a correlation with lithocholic acid concentration with an apparent Km value of 1.85 g/ℓ. In the concentration range around 2 g/ℓ, the insoluble form of lithocholic acid was predominant, indicating that its binding to the mycelia played an important role in the conversion. The binding was confirmed to be hydrophobic in nature.

Chapter 3 deals with the transformation of lithocholic acid to a new dihydroxy bile acid by strain ST22. Part 1 deals with the isolation, screening and identification of strain ST22. From its morphological and cultural characteristics, it was identified as *Cunninghamella blakesleeana* (Lendner). Part 2 describes the purification and structural analysis of

the product. From the results of elemental analysis, IR, and ^{13}C-NMR spectroscopy, the product was characterized as a dihydroxy derivative of 5β-cholanic acid, and from the result of two dimensional ^{1}H-NMR spectroscopy, it was identified as 3α,15β-dihydroxy-5β-cholanic acid.

Part 3 describes the biological activity of 3α,15β-dihydroxy-5β-cholanic acid. The capacity of a bile acid to solubilize cholesterol gallstones is known to increase with increasing hydrophilicity of the acid. The hydrophilicity of 3α,15β-dihydroxy-5β-cholanic acid measured by reversed phase HPLC was slightly higher than that of ursodeoxycholic acid; and in vitro cholesterol sulubilization tests showed that the compound was as effective as ursodeoxycholic acid in cholesterol solubilization.

Chapter 4 examines the optimum cultivation conditions for the production of 3α,15β-dihydroxy-5β-cholanic acid by *C. blakesleeana* ST22. Among tested compounds, dextrin was found to be the best carbon source, and L-asparagine the best nitrogen source. With resting mycelia, the optimum temperature was found to be between 33 and 40°C, optimum pH at 8.4. Addition of 2% (v/v) dimethylsulfoxide was found to stimulate the conversion activity two-fold. After optimizing the fermentation conditions, the amount of the product increased from 0.17 g/ℓ to 1.2 g/ℓ.