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The Selective Enzymatic Synthesis of Lipophilic Esters of Swainsonine

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Abstract—The potent and specific inhibitor of Golgi α-mannosidase II, swainsonine (SW) has been isolated in high yield from *Swainsona procumbens* and derivatised by regiospecific enzymatic reactions. In this study the regioselectivity of three commercially available enzymes, subtilisin Carlsberg, porcine pancreatic lipase (PPL) and *Candida cylindracea* lipase was determined for the acylation of swainsonine in predominantly anhydrous organic medium. The use of subtilisin in pyridine facilitated the single step synthesis of 2-*O*-butyryl-SW in a 23% yield, whilst catalysis by PPL in tetrahydrofuran gave 2-*O*-butyryl-SW (6%) and 1,2-di-*O*-butyryl-SW (31%).

Introduction

Swainsonine (I), an indolizidine alkaloid first isolated from *Swainsona canescens*,[1] has received interest due to its potentially therapeutic biological activities. Swainsonine is a potent and specific inhibitor of lysosomal acid and cytosolic α-mannosidases[2] as well as Golgi α-mannosidase II.[3] Its inhibition of the latter enzyme leads to the accumulation of hybrid-type oligosaccharides and a decrease in glycoproteins containing complex side chains.

During the malignant transformation of murine and human cells the degree of GlcNAc-β1-6Man-α1-6Man-β-branching in asparagine linked oligosaccharides increases. Swainsonine affects the expression of β1–6 branched chain oligosaccharides leading to the inhibition of tumour cell invasion and metastasis.[4] Its activity has been shown to reduce the growth of human melanoma cells (MeWo) by 50%.[5] Swainsonine modulates the effect of immuno-suppressive factors produced in the serum of tumour bearing mice and reverses the effect of Concanavalin A leading to stimulated lymphocyte proliferation.[6] The alkaloid confers a protective effect against high-dose chemotherapy in mice by inducing recovery from myelosuppression,[7] and enhances natural killer (NK) cell activity in vivo leading to the inhibition of metastasis.[8]

A recent, phase IB clinical trial of swainsonine established the maximum tolerated oral dose as 300 μg/kg/day.[9] Reported side effects include edema, elevated serum aspartate amino transferase, anorexia, abdominal pains and fatigue. Previous research on swainsonine found that substitution of the C-2 hydroxyl with hydrophobic functional groups such as 2-*O*-butyryl and 2-*O*-*p*-nitrobenzoyloxy produced compounds with 300-fold lower inhibition of Jack Bean α-mannosidase in vitro.[10] In contrast, the in vivo biological activity of the compounds were comparable to that of swainsonine when measured as inhibitors of oligosaccharide processing. Indirect evidence suggests that the hydrolysis of the swainsonine derivatives by esterases was essential for their in vivo activity.[10]

Although biological availability data for swainsonine or its derivatives has not been reported, uptake studies of the related alkaloid castanospermine have been published. Using ^{14}C-labelled castanospermine and 6-*O*-butyrylcastanospermine it was found that the lipophilic properties of the later compound facilitated its absorption into mouse melanoma and human T cells (30-fold higher than castanospermine).[11] Studies in mice given a single oral dose of either compound showed that 6-*O*-butyrylcastanospermine was preferentially absorbed from the gastrointestinal tract and the compound was rapidly converted to castanospermine in the blood and intracellularly.[12] Multiple oral doses were also effective in obtaining higher concentrations of the prodrug in the target organs. Similarly, the increased lipid solubility of some esters of swainsonine would be expected to increase their intestinal and cellular absorption. Therefore, lower oral doses of prodrug, compared to swainsonine, would achieve equivalent plasma and tissue levels of swainsonine. In addition, the lower inhibition by swainsonine esters prior to hydrolysis, may help

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alleviate some of the dose dependent side effects that occur with oral swainsonine treatment, thereby making them attractive candidates for prodrug development. The aim of this study was to develop enzymatic methods for the regioselective synthesis of ester derivatives of swainsonine. Three commercially available enzymes, Subtilisin Carlsberg, porcine pancreatic lipase and *Candida cylindracea* lipase were used in a predominantly anhydrous medium. The use of enzymes in synthetic organic chemistry has been recently reviewed.[13] In addition, we provide conclusive evidence of considerable accumulation of swainsonine in the seeds of *Swainsona procumbens*.

Experimental

Swainsona procumbens seeds were obtained from Nindethana Seed Service, Woogenilup, Western Australia. Subtilisin Carlsberg, porcine pancreatic lipase, *C. cylindracea* lipase, Jack Bean α-mannosidase, 2,2,2-trichloroethanol, acetic anhydride and butyric anhydride were purchased from Sigma; bovine serum albumin was obtained from Miles Laboratories. All reagents were of analytical grade unless otherwise stated. Organic solvents were redistilled, then dried by standing for at least 24 h with 3Å molecular sieves prior to use.

Analytical TLC was performed using 0.2 mm silica gel 60 F_{254} (Machery-Nagel, Germany) and compounds were visualised using permanganate.[14] Centrifugal silica gel chromatography was performed on a Chromatotron (Model 7924T, Harrison Research, USA) using 2 mm silica gel PF_{254} (Merck) on a glass rotor. RP-HPLC was performed on a Waters system consisting of two pumps (Model 6000A and M45), solvent programmer (Model 660), injector (Model U6K) and detector (Model 450). Analytical separations were achieved on a Waters C-18 Nova-Pak column (8×100 mm). Preparative separations used a Waters C-18 Prep Nova-Pak HR column (25×100 mm). ^{1}H NMR spectra were recorded at 300 MHz on a Bruker AC 300 spectrometer or at 500.1 MHz on a Bruker DMX 500 spectrometer. Chemical shifts (δ) are given with respect to the chemical shift of TMS or residual $CHCl_3$ for samples dissolved in $CDCl_3$. Infrared analyses were performed on a dual beam Perkin–Elmer 298 infrared spectrophotometer in chloroform solution. Optical rotations were recorded on a Jasco dip-1000 digital polarimeter with readings measured at 589 nm (Na) and 25.0 °C using a 1mL solution cell in either dichloromethane or water depending on solubility. Mass measurements were made using a VG Autospec Q mass spectrometer operating at a resolving power of 6000 (10% valency definition). The instrument was operated in the positive electron ionisation mode and samples were introduced using a heated direct insertion probe. In vitro activity of swainsonine, and related compounds were determined using the method previously described.[15]

2,2,2-Trichloroethylethanoate was prepared by mixing 2,2,2-trichloroethanol (50 mmol), acetic anhydride (105 mmol) and pyridine (80 mmol) in a screw capped vessel for 24 h (25 °C). The reaction was terminated by the addition of 1 M hydrochloric acid (55 mL) and extracted with diethyl ether (3×50 mL). The combined organic phase was washed with 5% (w/v) sodium hydrogen carbonate (50 mL) followed by water (3×50 mL) and dried over anhydrous sodium sulphate. The diethyl ether was evaporated under reduced pressure (40 °C) leaving a light yellow oil. Vacuum distillation gave 4.58 g (48%) of 2,2,2-trichloroethylethanoate, a colourless liquid; bp 72 °C at 20 mmHg, lit. 71 °C at 18 mmHg.[16] 2,2,2-Trichloroethylbutanoate (TCEB) was synthesised as for 2,2,2-trichloroethylethanoate but with butyric anhydride. Yield of 2,2,2-trichloroethylbutanoate was 7.56 g (69%); bp 110–112 °C at 20 mmHg, lit. 119.8–120 °C at 48 mmHg.[17]

Enzymes were conditioned prior to use in organic solvent.[18] Subtilisin Carlsberg (100 mg) was lyophilised from 1 ml of 0.1 M potassium phosphate buffer, pH 7.8. *C. cylindracea* lipase (1 g) was dissolved in 2 mL of 0.05 M potassium phosphate buffer, pH 7.0 and cooled to 4 °C. The mixture was dried in vacuo at room temperature for 72 h. Porcine pancreatic lipase (5 g) was dissolved in 15 mL of 0.05 M glycine buffer, pH 8.4 and cooled to 4 °C. An equal volume of chilled (−20 °C) acetone was added, and the mixture stirred at 4 °C for 30 min. The precipitated enzyme was recovered by centrifugation (14 000 *g*; 20 min) and washed with cold (−20 °C) acetone, dried under a stream of nitrogen, with further drying in vacuo at room temperature for 72 h.

Isolation of swainsonine

Powdered *S. procumbens* seed (200 g) was extracted with methanol for 48 h. Following evaporation of methanol, the residue was suspended in water (200 mL) and extracted three times with an equal volume of petroleum ether (bp 80–100 °C). The aqueous fraction was passed through a SP Sephadex-C25 ion exchange column in the ammonium form (bed volume 400 mL). The column was washed with three volumes of water and eluted with three volumes of 1 M ammonium hydroxide. The eluate was evaporated and the residue dried in vacuo over silica gel. The residue was redissolved in water (50 mL) and extracted with chloroform in a liquid:liquid extractor for 72 h. The organic phase was evaporated and the residue vacuum sublimed (90 °C, 0.1 mmHg). The sublimate was crystallised from chloroform to give colourless needles of swainsonine (342 mg; 0.17% w/w). Mp 144–145 °C, $[\alpha]_D^{25}$ −89.3° (*c* 1, H_2O), [lit. mp 144–146 °C, $[\alpha]_D^{20}$ −87.2°[19]]. ^{1}H NMR (D_2O, 500 MHz) δ 4.39 (ddd, J=2.2, 6.3, 7.8 Hz, H-2), 4.30 (dd, J=4.2, 6.2 Hz, H-1), 3.87 (apptd, J=4.7, 10.3 Hz, H-8), 2.95 (m, H-5eq), 2.93 (m, H-3), 2.60 (1H, dd, J=7.8, 11.1 Hz, H-3′), 2.11 (m, H-7eq), 2.01 (td, J=2.9, 11.9 Hz, H-5ax), 1.96 (dd, J=4.0, 13.9 Hz, H-8a), 1.78 (m, H-6eq), 1.59 (qt, J=4.2, 13.5 Hz, H-6ax), 1.29 (dq, J=4.5, 12.2 Hz, H-7ax). Proton assignments are based on those given by Kardono et al.[20]; EIMS *m*/*z* (relative intensity) 173 (27.5), 155 (36.0), 138 (18.8), 129 (6), 113 (100), 100 (17.9), 96 (56), 84 (8), 72 (26); high-resolution mass spectra *m*/*z* 173.105044, calcd for $C_8H_{15}NO_3$, 173.105194.

Synthesis of 1,2,8-tri-*O*-acetylswainsonine

To swainsonine (0.1 mmol) dissolved in pyridine (3 mL) was added acetic anhydride (2.5 mmol). The reaction was stirred in a screw capped vessel (25 °C) under nitrogen for 48 h at which time the reaction was terminated by the addition of water (5 mL) and extracted with ethyl acetate (4×5 mL). The combined organic phase was washed with an equal volume of water, dried over anhydrous sodium sulfate, and evaporated under reduced pressure (40 °C). The residue was purified by analytical HPLC using acetonitrile:water (20:80; v/v) at 1 mL/min, giving 25 mg (84%) of swainsonine triacetate as a colourless oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 5.53 (dd, J = 4.3, 6.5 Hz, H-1), 5.22 (ddd, J = 2.0, 6.2, 7.8 Hz, H-2), 4.96 (ddd, J = 4.8, 10.3, 12.7 Hz, H-8), 3.17 (dd, J = 2.1, 11.2 Hz, H-3) 3.06 (m, H-5eq), 2.59 (dd J = 7.6, 11.2 Hz, H-3′), 2.14 (dd, J = 4.3, 9.6 Hz, H-8a), 1.97–1.85 (m, H-7eq, H-5ax), 1.80–1.75 (m, H-6ax, H-6eq), 1.24 (m, H-7ax), 2.09 (s, CH_3CO-2), 2.06 (s, CH_3CO-1), 2.00 (s, CH_3CO-8); EIMS m/z (relative intensity) 256 (2), 239 (40), 180 (37), 137 (45), 120 (100); high resolution mass spectra m/z 256.118327; calcd for $C_{12}H_{18}NO_5$ $[M-Ac]^+$, 256.118498. Proton assignments are based on those given by Kardono et al.[20]

Synthesis of 1,2,8-tri-*O*-butyrylswainsonine

This synthesis was as for swainsonine triacetate but, with butyric anhydride. The residue was chromatographed by preparative HPLC using aqueous 75% methanol at 4 mL/min. The solvent was removed under reduced pressure (40 °C) to yield 35 mg (92%) of 1,2,8-tri-*O*-butyrylswainsonine as a pale-yellow oil. $[\alpha]_D^{25}$ −5.5° (c 1, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 300 MHz) 5.40 (dd, J = 4.1, 7.1 Hz, H-1), 5.16 (ddd, J = 2.3, 6.2, 7.2 Hz, H-2), 4.85 (ddd, J = 4.7, 10.3, 10.3 Hz, H-8), 3.04 (dd, J = 2.3, 11.1 Hz, H-3) 2.96 (m, H-5eq), 2.52 (dd, J = 7.8, 11.1 Hz, H-3′), 2.15 (m, H-8a) 2.07 (m, H-7eq), 1.84 (m, H-5ax), 1.64 (m, H-6ax, H-6eq), 1.17 (m, H-7ax), 2.12 (t, J = 7.4 Hz, $CH_3CH_2CH_2CO$), 1.52 (sextet, $CH_3CH_2CH_2CO$), 0.84 (t, 7.4 Hz, $CH_3CH_2CH_2CO$); IR, ν, cm^{-1} ($CHCl_3$), 3600–3400, 2800 (Bohlmann bands), 1740 (C=O), 1350 (CH_3), 1275 (C–O), 950; EIMS m/z (relative intensity) 384 (0.7), 383 (0.5), 312 (1.2), 296 (14.5), 295 (50.1), 208 (37.3), 137 (65), 136 (14.9), 120 (100); high resolution mass spectra m/z 383.223779; calcd for $C_{20}H_{33}NO_6$, 383.230788.

Synthesis of 2-*O*-butyrylswainsonine using subtilisin Carlsberg

To swainsonine (0.1 mmol) dissolved in pyridine (3 mL) was added 2,2,2-trichloroethylbutanoate (1 mmol) and subtilisin (232 units). The reaction was shaken at 200 rpm and 45 °C with product formation monitored by silica gel TLC (dichloromethane:ethanol, (85:15; v/v). The reaction was terminated (72 h) by centrifugation (3500 g; 10 min) and removal of the enzyme, followed by evaporation of the supernatant under a stream of nitrogen. The residue was purified by centrifugal silica gel chromatography using 150 mL of dichloromethane: ethanol (8.5:1.0; v/v), collecting 2 mL fractions at a flow rate of 1 mL/90 s. Selected fractions were further purified by analytical HPLC using an aqueous acetonitrile gradient (acetonitrile, linear 0–100% in 30 min; 1 mL/min) to give 6 mg (25%) of 2-*O*-butyrylswainsonine (II) (R_f 0.50) as a waxy semi-solid. $[\alpha]_D^{25}$ −49.0° (c 0.46, H_2O), ^{1}H NMR ($CDCl_3$, 300 MHz): δ 5.09 (ddd, J = 2.0, 6.0, 7.7 Hz, H-2), 4.46 (dd, J = 4.1, 6.2 Hz, H-1), 3.87 (ddd, J = 4.6, 10.0, 12.3 Hz, H-8), 3.10 (dd, J = 2.0, 11.1 Hz, H-3), 2.97 (m, H-5eq), 2.51 (dd, J = 7.6, 11.0 Hz, H-3′), 2.07 (m, H-7eq), 1.90 (td, J = 3.6, 11.1 Hz, H-5ax), 1.81 (dd, J = 4.1, 8.9 Hz, H-8a), 1.69 (m, H-6eq), 1.60 (m, H-6ax) 1.20 (m, H-7ax), 2.37 (t, J = 7.4 Hz, $CH_3CH_2CH_2CO$) 1.68 (sextet, $CH_3CH_2CH_2CO$), 0.96 (t, J = 7.4 Hz, $CH_3CH_2CH_2CO$). IR, ν, cm^{-1} ($CHCl_3$), 3650, 3600–3300 (OH), 2780–2820 (Bohlmann bands), 1730 (C=O), 1375 (CH_3), 1250 (C–O), 1135. EIMS: m/z (relative intensity) 243 (1.8), 172 (5), 155 (95.9), 138 (100.0), 120 (13.4), 113 (13.4); high-resolution mass spectra m/z 243.146016; calcd for $C_{12}H_{21}NO_4$, 243.147058.

Synthesis of 2-*O*-butyrylswainsonine and 1,2-di-*O*-butyrylswainsonine using porcine pancreatic lipase

To swainsonine (0.1 mmol) dissolved in tetrahydrofuran (3 mL) was added 2,2,2-trichloroethylbutanoate (1 mmol) and porcine pancreatic lipase (690 units). The reaction was shaken at 175 rpm and 37 °C with product formation monitored by silica gel TLC (dichloromethane:ethanol, 85:15 (v/v)). The reaction was terminated (16 h) by centrifugation (3500 g; 10 min) and removal of the enzyme, followed by evaporation of the supernatant under a stream of nitrogen. The residue was purified by centrifugal silica gel chromatography using 250 mL of dichloromethane:ethanol (80:20; v/v), collecting 5 mL fractions at a flow rate of 1 mL/min. Two products were isolated:

Product 1. 2-*O*-Butyrylswainsonine (II) (R_f 0.50) was isolated as a waxy semi-solid in a 6% (w/w) yield (25% with subtilisin). $[\alpha]_D^{25}$ −49.0° (c 0.46, H_2O), ^{1}H NMR ($CDCl_3$, 500 MHz) δ 5.09 (ddd, J = 1.8, 6.1, 7.7 Hz, H-2), 4.47 (dd, J = 4.3, 6.2 Hz, H-1), 3.86 (ddd, J = 4.6, 10.1, 12.3 Hz, H-8), 3.09 (dd, J = 1.6, 11.1 Hz, H-3), 2.96 (m, H-5eq), 2.50 (dd, J = 7.5, 11.0 Hz, H-3′), 2.07 (m, H-7eq), 1.89 (td, J = 3.1, 11.5 Hz, H-5ax), 1.81 (dd, J = 4.2, 9.0 Hz, H-8a), 1.69–1.60 (m, H-6eq, H-6ax), 1.25 (m, H-7ax), 2.37 (t, J = 7.4 Hz, $CH_3CH_2CH_2CO$), 1.67 (dd, J = 7.4, 14.4 Hz, $CH_3CH_2CH_2CO$), 0.95 (t, J = 7.4 Hz, $CH_3CH_2CH_2CO$); IR, ν, cm^{-1} ($CHCl_3$), 3650, 3600–3300 (OH), 2780–2820 (Bohlmann bands), 1730 (C=O), 1375 (CH_3), 1250 (C–O), 1135. EIMS m/z (relative intensity) 243 (2.1), 225 (2.7), 172 (6.2), 156 (33.1), 155 (85.3), 138 (100), 120 (13.4), 113 (18.5); high-resolution mass spectra m/z 243.146926; calcd for $C_{12}H_{21}NO_4$, 243.147058.

Product 2. 1,2-di-*O*-Butyrylswainsonine (III) (R_f 0.74): Following silica gel chromatography selected fractions were further purified by preparative HPLC using aqueous 85% methanol (v/v) at a flow rate of 5 mL/min. One peak was collected and evaporation of the solvent gave 9.6 mg (31%) of 1,2-di-*O*-butyrylswainsonine as a

pale-brown residue. Mp 60–62 °C, $[\alpha]_D^{25}$ −50.0° (*c* 1, CH_2Cl_2); ^1H NMR ($CDCl_3$, 300 MHz) δ 5.47 (dd, *J* = 3.4, 6.3 Hz, H-1), 5.15 (ddd, *J* = 2.6, 6.1, 7.4 Hz, H-2), 3.40 (ddd, *J* = 4.7, 9.8, 12.2 Hz, H-8), 3.19 (dd, *J* = 2.7, 11.0 Hz, H-3), 3.01 (ddd, *J* = 3.4, 7.1, 10.8 Hz, H-5eq), 2.62 (dd, *J* = 8.4, 11.0 Hz, H-3′), 2.06 (m, H-7eq), 1.95 (td, *J* = 3.5, 11.1 Hz, H-5ax), 1.88 (dd, *J* = 3.5, 8.9 Hz, H-8a), 1.74–1.57 (m, H-6eq, H-6ax) 1.23 (m, H-7ax), 2.31 (t, *J* = 7.4 Hz, $CH_3CH_2CH_2CO$), 1.65 (sextet, $CH_3CH_2CH_2CO$), 0.94 (t, *J* = 7.4 Hz, $CH_3CH_2CH_2CO$); IR, ν, cm^{-1} ($CHCl_3$), 3650, 3600–3400 (O–H), 1725 (C=O), 1300 (CH_3), 1150 (C–O),1100; EIMS *m*/*z* (relative intensity) 313 (1.8), 225 (14), 138 (100), 120 (15); high resolution mass spectra *m*/*z* 313.175789; calcd for $C_{16}H_{27}NO_5$, 313.188844.

Results and Discussion

To date there has only been limited evidence for the presence of swainsonine in *S. procumbens*.[15] The extraction of *S. procumbens* seeds led to the isolation of swainsonine (I) in a 0.17% (w/w) yield. As anticipated, the isolated sample was a potent inhibitor of Jack Bean α-mannosidase (IC_{50} = 5.8 μM[15]), which is consistent with the documented biological activity of swainsonine.[2] The identification of the alkaloid was confirmed by the preparation and characterisation of peracylated derivatives of the alkaloid. The isolation of a significant quantity of swainsonine provided an opportunity for the novel synthesis of 2-*O*-butyrylswainsonine. In this study the regioselectivity of three commercially available enzymes, subtilisin Carlsberg, porcine pancreatic lipase and *C. cylindracea* lipase, was investigated with respect to swainsonine.

Subtilisin has a broad substrate specificity in many organic solvents.[13] In anhydrous pyridine subtilisin demonstrates a selectivity for the C-2 hydroxyl group of swainsonine and reaction with TCEB afforded 2-*O*-butyrylswainsonine (II) in an overall yield of 23%. No other substitutions were detected with subtilisin.

The use of tetrahydrofuran as a reaction medium facilitated catalysis by porcine pancreatic lipase (PPL) and *C. cylindracea* lipase (CCL). In the presence of TCEB, both lipases catalysed the mono and diesterification of swainsonine. However, in comparison to PPL the activity of the CCL was far lower in tetrahydrofuran, as indicated by the TLC monitoring of the reaction. As the activity of PPL was higher it was deemed to be the most appropriate biocatalyst for the preparative scale acylation of swainsonine. In anhydrous tetrahydrofuran PPL acylates the C-2 position of swainsonine (6%). In contrast to catalysis by subtilisin, the use of PPL also resulted in the esterification of the C-1 hydroxyl group to give 1,2-di-*O*-butyrylswainsonine (III) (31%). No other products were detected by silica gel TLC (Fig.1).

Initial reactions with PPL and swainsonine were attempted in pyridine at 45 °C and 200 rpm. Under these conditions products were not detected by TLC after four days, but swainsonine was still detected. This suggests the catalytic efficiency of PPL is effected more by the nature of the organic solvent than by subtle changes in temperature and mixing conditions.

In vitro analysis of 2-*O*-butyrylswainsonine and 1,2-di-*O*-butyrylswainsonine determined that both compounds were less active than swainsonine at inhibiting Jack Bean α-mannosidase (data not shown). However, in vivo studies of 6-*O*-butyryl-castanospermine have shown that it is both better tolerated than castanospermine and is taken up more rapidly by cells.[11] Previously, Dennis and colleagues[10] reported the potential

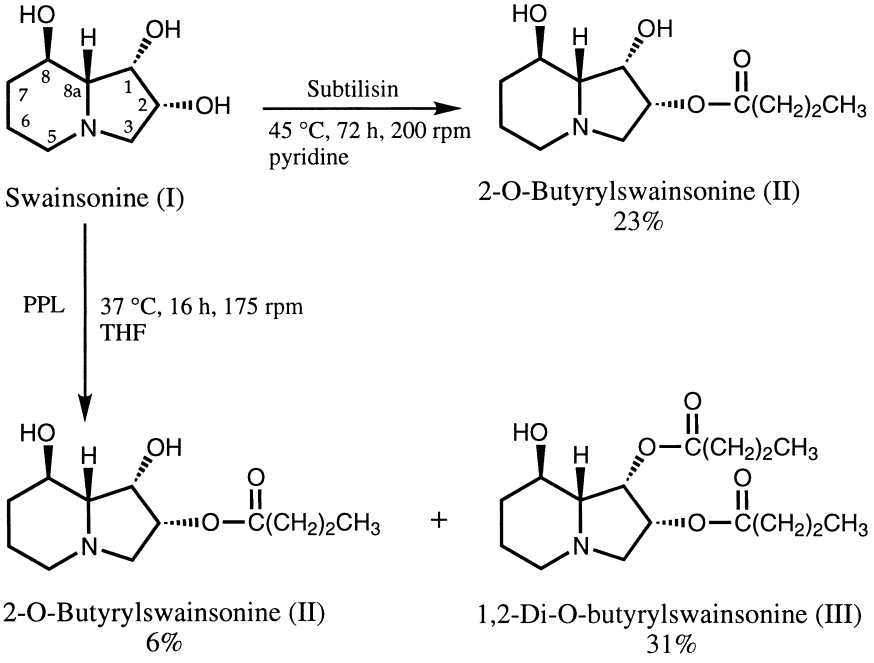

Figure 1. Enzymatic conversion of swainsonine to 2-*O*-butyrylswainsonine and 1,2-di-*O*-butyrylswainsonine.

therapeutic value of derivatives of swainsonine, particularly 2-*O*-butyrylswainsonine. Perhaps some of the reported in vivo side effects of swainsonine treatment[9] may be reduced by the administration of lypophilic esters of swainsonine. The regioselectivity of enzymatic catalysis in organic medium facilitates the single step synthesis of 2-*O*-acyl and 1,2-di-*O*-acyl analogues of swainsonine. The procedures described here, including the isolation of significant quantities of swainsonine from *S. procumbens*, are capable of being conveniently scaled up to produce amounts of compounds for pharmacological studies.

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