Enzyme mediated-transesterification of verbascoside and evaluation of antifungal activity of synthesised compounds

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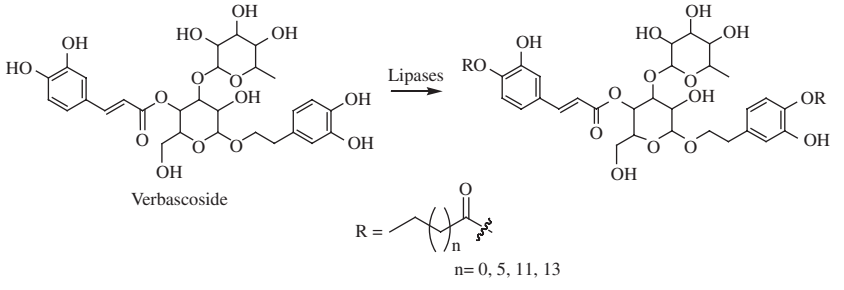


Enzymatic acylation of verbascoside, a polyhydroxylated natural product, has been reported in this study using five different commercial lipases and taking *p*-nitrophenyl alkanoates as acyl donors. Out of these enzymes, the immobilised *Candida antarctica* lipase B was found as the enzyme of choice. Mono- and di-acylated products were formed, with mono as major product indicating high regioselective nature of such transformations. A series of acyl esters of verbascoside have been synthesised by this enzymatic transesterification methodology. The lipophilicity of the synthesised analogues was also checked. The analogues were further subjected to synergistic antifungal activity with amphotericin B (AmB) against *Candida albicans.* Fourfold reduction in minimum inhibitory concentration of AmB was observed with few synthesised analogues such as verbascoside 4″-octanoate (**3b**), verbascoside 4″-palmitate (**3d**) and verbascoside 4″,4′-dipalmitate (**4d**) at a concentration of 0.5 μg/mL.

Keywords: transesterification; verbascoside; acylation; lipophilicity; antifungal activity

1. Introduction

Phenylethanoid glycosides constitute an important class of natural products possessing wide range of biological activities (Fu et al. 2008). Verbascoside (Scarpati & Delle 1993) also named as acteoside was first isolated from the flowers of *Syringa vulgaris* (Birkofer et al. 1968); it is a phenylethanoid glycoside and is widely distributed in plants such as *Ligstrum purpurascens* (Wong et al. 2001), *Callicarpa dichotoma* (Koo et al. 2006; Lee et al. 2006), *Cistanche deserticola* and *Boschniakia rossica* (Wu et al. 2006), *Scrophularia ningpoenis* (Huang et al.

2008) and *Rehmannia glutinosa* (Li et al. 2006). It is a highly water soluble polyphenolic molecule. *In vitro* and *in vivo* pharmacological studies have shown that verbascoside possesses various pharmacological activities, such as anti-inflammatory (Diaz et al. 2004), hepatoprotective (Xiong et al. 1999), anti-apoptotic, antioxidative (Chiou et al. 2004), anticancer (Kyung et al. 2007) and neuroprotective (Sheng et al. 2002; Koo et al. 2006; Li et al. 2008). Verbascoside and its analogues have also been reported to effectively scavenge 1,1-diphenyl-2-picrylhydrazyl and nitric oxide *in vitro* (Chen et al. 2009; Liu et al. 2013)

Besides the above-mentioned biological properties of verbascoside, it is also reported that verbascoside exhibits synergistic antifungal activity with antifungal drug amphotericin B (AmB) against various pathogenic *Candida* species (Ali et al. 2011). AmB, an antifungal antibiotic used for the treatment of invasive fungal infections from the 1950s till date, has various drawbacks and has become imperative to search for safe and more-effective chemotherapeutic approaches for the treatment of invasive fungal infections. Combination therapy has been found to be an important strategy, as synergistic interactions can potentially increase antifungal efficacy, reduce toxicity, cure faster, prevent the emergence of resistance and provide broader-spectrum antifungal activity than monotherapy regimens (Richardson 2005). While exploring verbascoside for antifungal activity, when synergistically used along with AmB it potentiated the membrane disruption activity of AmB against a wide range of pathogenic strains of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus* resulting in a strong fungicidal effect (Ali et al. 2011).

Recently, verbascoside has been found to show a very low oral bioavailability (BA) of 0.12% (Wu et al. 2012; Qia et al. 2013). It also shows a high aqueous solubility owing to its polyhydroxylated structure. However, a proper hydrophilic–lipophilic balance is very important for sustained release of a molecule in the biological system. In order to increase the structural diversity, and to improve physical and chemical properties for better pharmacological and pharmacokinetic attributes, acylation of natural and synthetic organic compounds is a common synthetic protocol (Singh et al. 2013). Therefore, transesterification was chosen for selective acylation of verbascoside with acyl groups of varying side chains to improve its BA and subsequent antifungal potentiating effect. Catalysis of the transesterification reaction is generally carried by chemical and enzymatic methods. Currently, the chemical transesterification methods are the major industrial approach, however; due to its obvious drawbacks, such as formation of non-specific products and unwanted by-products and a limited choice of reagents needed to accomplish a selective acylation, it is challenging (Richardson 2005). Enzymatic acylation of polyhydroxylated compounds has been proved to be one of the most attractive alternatives to the conventional chemical methods due to its high selectivity and simplicity (Richardson 2005). Enzyme catalysed reactions offer a highly efficient process under mild conditions, diminish undesired side reactions, and facilitate more pure product recovery. Furthermore, enzymes are proteins, and as such they are completely biodegradable and environment friendly (Singh et al. 2009). As a result, this method has been extensively used for the esterification of carbohydrates, nucleosides and other natural products. The use of lipases as biocatalysts for selective acylation of polyhydroxylated natural products such as flavonoids, saponins and other polyphenolic compounds have been extensively studied (Svendsen 1994; Akimoto et al. 1999; Ferrero & Gator 2000; Kontogianni et al. 2001; Riva 2002; Park et al. 2003; Intra et al. 2004; Gonzalez-Sabín et al. 2011). Lipases accept a wide array of complex molecules as substrates and catalyse reactions with high enantio- and regioselectivity. Keeping in view the tremendous biological activity and potentiating fungicidal effect of verbascoside on AmB against wide range of invasive fungal pathogens, this study was aimed to synthesise a focused library of selectively acylated analogues of verbascoside with different HLB by employing commercially available lipases for the evaluation of synergistic antifungal activity with AmB against *C. albicans* (ATCC 9002).

2. Results and discussion

2.1. *Chemical acylation*

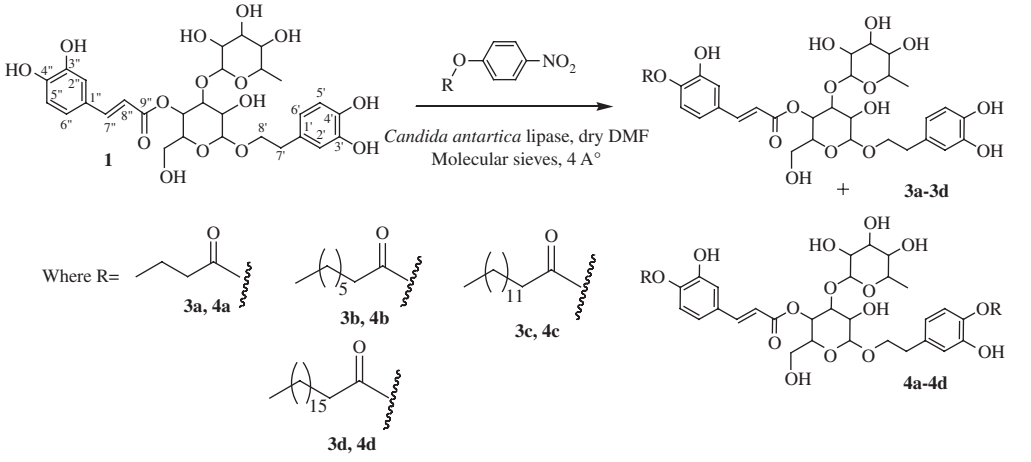
Chemical acylation of verbascoside was carried through its reaction with two anhydrides, namely acetic anhydride and butyric anhydride in dry DCM/DIMAP (dichloro methane/dimethylamino pyridine) (Liljeblad et al. 2010). Acylation through this method resulted in peracylated products which were characterised by spectral analysis. These peracylated compounds when studied for change in lipophilicity using Schrodinger software were found to show drastic increase in C log P (Table S1). Thus, direct chemical acylation of verbascoside was not appropriate, due to lack of suitable reagents and protocols to discriminate among specific hydroxyl groups of the same molecule and to acylate regioselectively one over several hydroxyl groups present on the scaffold. Furthermore, this molecule being highly sensitive to acidic and thermal conditions, the acid-sensitive protection–deprotection strategies cannot be employed while designing its regioselective acylation. Therefore, we switched towards the enzymatic transesterification using various commercial lipases and a suitable labile ester as acyl transfer agent.

2.2. *Enzymatic acylation*

Enzymes are attractive bio-catalysts due to their cost-effective production, stereo- and regioselectivity, simple and fast reaction and rational alteration of properties. Therefore initially, transesterification of the verbascoside was studied using a panel of commercially available lipases and *p*-nitropalmitate was used as an acyl donor.

Five commercial lipases, namely *Candida rugosa* lipase, lipase acrylic resin from *Candida antarctica* (lipase B), lipase from wheat germ, *Candida cylindracea* lipase and porcine pancreatic lipase, were used in this study to investigate the regioselective acylation of verbascoside using *p*-nitrophenyl palmitate in dry organic solvent. These commercial enzymes were screened to evaluate their potential in terms of selectivity and yields.

The substrate solubility was assayed in different solvents, owing to the poor solubility of verbascoside in less polar solvents, dimethylformamide (DMF) was found to be suitable solvent for carrying out the enzymatic reaction. For each lipases used, two acylated products of verbascoside were formed, with mono-acylated in major yield (Scheme 1). It was very interesting to note that all the five enzymes showed same specificity in transesterification; however, they only differed in the percentage conversion of the reaction. While with soluble



Scheme 1. Acylation of verbascoside using *p*-nitrophenyl alkanoate.

lipases, reaction conversion was lower ranging from 12% (porcine pancreatic lipase) to 21% (lipase from wheat germ); whereas higher conversion of approximately 50% was achieved with resin-bound immobilised *C. antarctica* lipase B. Owing to the higher conversion rate, resin-bound immobilised *C. antarctica* lipase B was the catalyst of choice for transesterification. Various acylated products thus obtained were purified through column chromatography and their structures determined by using ^{1}H NMR, ^{13}C NMR and mass spectra (MS). After introducing various long fatty acid chains, change in lipophilicity was calculated using Schrodinger software (Table S1).

Using *C. antarctica* lipase B, and different long-chain *p*-nitrophenyl alkanoates as acyl donors, two types of products, mono- (**3a**–**3d**) and di-acylated (**4a**–**4d**) compounds were observed with mono-acylated compounds in major yields (35%) indicating high regioselectivity of such transformations. In all the cases, acylation was taking place on phenolic hydroxyls. The structures of the compounds were confirmed by using ^{1}H NMR in which a shift of the protons attached to the carbon adjacent to the carbon whose hydroxyl was getting acylated was observed, as in the case of 'verbascoside 4″-butyrate'; ^{1}H NMR signal at δ 7.26 ($J = 8.50$ Hz) corresponding to H-5″ of the caffeic acid moiety appeared downfield (0.45 ppm) compared with its parent δ 6.8 ($J = 8.5$ Hz). Simultaneous little downfield shift was also observed in H-6″ signal of caffeic acid moiety, which showed that acylation has taken place on the hydroxyl attached to C-4″ carbon of caffeic acid moiety. While in 'verbascoside 4″,4′-butyrate', acylation was taking place at two places. In one place, ^{1}H NMR signal at δ 6.4 ($J = 8.45$ Hz) corresponding to H-5′ proton of hydroxyltyrosol was shifted downfield to δ 6.8 ($J = 8.45$ Hz) which reveals that hydroxyl group attached to C-4′ of hydroxyltyrosol has got acylated. Simultaneous downfield shift was also observed in H-6′ of hydroxyltyrosol. Another shift was same as observed in mono-acylated analogues that are on C-4″ hydroxyl of caffeic acid moiety. Same pattern was found for other cases also in which same signal shifts were observed for mono- and di-acylated products, and no shift in sugar signals was observed. Confirmation was also obtained from ^{13}C as additional peaks were observed at 170–175 ppm corresponding to the additional carbonyls.

2.3. *Synergistic action of verbascoside analogues with AmB*

Verbascoside and its analogues synthesised were assayed to test whether these possess synergistic effect with AmB against '*C. albicans*.' Interestingly, all the analogues showed a promising potentiating AmB activity. It was observed that at lower concentration range of 1.5 and 0.75 μg/mL most of the derivative compounds were strongly potentiating the activity of AmB than the parent verbascoside (Table 1). Few analogues such as verbascoside 4″-octanoate (**3b**), verbascoside 4″-palmitate (**3d**) and verbascoside 4″,4′-palmitate (**4d**) with increased lipophilicity showed fourfold reduction in minimum inhibitory concentration (MIC) of AmB at a concentration of 1.5 μg/mL than parent verbascoside. These analogues could be attractive prototypes that can be used to modulate the currently available antifungal antibiotics with reduced toxicity.

3. Experimental

3.1. *Measurements*

NMR (^{1}H and ^{13}C) spectra were recorded at 500 and 100 MHz on Bruker DPX200 instrument (Bruker, Billerica, MA, USA) in CD_3OD/DMSO/$CDCl_3$ with TMS as internal standard for protons and solvent signals as internal standard for carbon spectra. Chemical shift values were expressed in δ (ppm), and coupling constants were given in Hz. MS were recorded on MALDI mass spectrometer. Melting points were recorded on Buchi Melting point apparatus D-545

Table 1. *In vitro* antifungal activity in of verbascoside derivatives screened for their synergistic activity with AmB.

Compound	AmB + 0[a]	AmB + 50	AmB + 25	AmB + 12.5	AmB + 6.15	AmB + 3.12	AmB + 1.5	AmB + 0.75
Acteoside	0.5[b]	0.12	0.12	0.12	0.25	0.25	0.5	0.5
3a	0.5	0.12	0.12	0.12	0.12	0.25	0.5	0.5
4a	0.5	0.12	0.25	0.25	0.25	0.25	0.25	0.5
3b	0.5	0.12	0.12	0.12	0.12	0.12	0.12	0.25
4b	0.5	0.12	0.12	0.25	0.25	0.25	0.25	0.5
3c	0.5	0.12	0.12	0.12	0.25	0.25	0.25	0.5
4c	0.5	0.12	0.12	0.12	0.25	0.25	0.25	0.25
3d	0.5	0.12	0.12	0.25	0.12	0.12	0.12	0.25
4d	0.5	0.12	0.12	0.12	0.12	0.12	0.12	0.25

[a] Concentration of verbascoside and its analogues in μg/mL.
[b] MIC values.

(Buchi, Postfach, Meierseggstrasse, Switzerland); IR spectra (KBr discs) were recorded on Bruker Vector 22 instrument (Bruker, Billerica, MA, USA). Silica gel-coated aluminium plates of thickness 0.25 mm (Merck) were used for TLC. Reagents and solvents used were LR grade. The chromatograms were visualised under UV (254–366 nm) and MeOH/H_2SO_4.

3.2. *Experimental procedure for chemical and enzymatic transesterification of verbascoside*

In a typical experimental procedure for chemical transesterification, a solution of verbascoside (0.112 g, 1 mmol) in dry DCM (10 mL) was taken, to which the anhydrides (acetic or butyric anhydride, 4 mmol), DMAP (10 mg) were added and stirred for 24 h at ambient temperature. After the completion of reaction, solvent was evaporated *in vacuo* and the crude product was subjected to column chromatography (silica gel, 100–200 mesh, elution; *n*-chloroform/MeOH (90:10) gradient) to obtain pure products. The pure products were characterised on the basis of ^{1}H NMR, ^{13}C NMR and MS given in compound characterisation (supporting information).

A typical experimental procedure for the enzymatic transesterification is illustrated in Scheme 1. The reaction was initiated by dissolving the substrate (verbascoside, 0.1 mmol) in dry organic solvent (DMF) in the presence of the lipase (*C. antarctica* lipase B, 20% weight equivalent), an acyl donor (*p*-nitrophenyl alkanoate, (0.2 mmol), pre-activated molecular sieves (4 Å) and left for shaking on digital shaker at 120 rpm. The reaction mixture was worked up in H_2O and Et_2O. The organic layer was separated and evaporated *in vacuo* and the crude product was subjected to column chromatography as described earlier. The acylated product was purified by using column chromatography and the structure was determined by using MS and NMR spectra (^{1}H, ^{13}C, and by comparison with spectra of their parent molecule) as shown in compound characterisation **S2**. *p*-Nitro alkanoates were synthesised using known procedure involving esterification reaction between *p*-nitrophenol (1 mmol), and corresponding acids (1.3 mmol) in the presence of DCC (6.0 mmol) and DMAP (10 mg, catalytic amount) under dry conditions gave the crude product. The crude product was purified by using column chromatography (silica gel, 60–120 mesh, eluent *n*-hexane/EtOAc gradient). Lipase from *C. rugosa*, porcine pancreas type II, wheat germ lipase and *C. antarctica* were purchased from Sigma, and *C. cylindracea* lipase from Fluka acrylic resin. All the commercial enzymes were used as such. Verbascoside was procured from Natural Product Division of the institute (CSIR-IIIM, Jammu) and isolated from its natural resource by known procedure (Steglich & Höfle 1969). Enzymatic reactions were carried out on IKA digital shaker at room temperature and 120 rpm.

3.3. *Bioassay for antifungal activity*

The fungicidal effect of AmB in the presence and absence of verbascoside and its analogues was performed against '*C. albicans* ATCC 90028' as described previously (Bjorkling et al. 1991). The adjusted inoculum suspension ($\sim 5 \times 10^6$ CFU mL^{-1}) was diluted 10-fold in medium containing AmB at a concentration of 0.25 × MIC and in combination with increasing concentrations of verbascoside and analogues ranging from 0.75 to 50 μg/mL. AmB alone at 4 × MIC was used as a control. Culture flasks were incubated with an agitation of 200 rpm at 35°C for 24 h, and the number of CFU was determined on Sabouraud dextrose agar plates with lecithin and polysorbate 80 (Becton Dickinson, Becton Drive, NJ, USA) using a serial dilution method. Plates were then incubated at 35°C for 24–48 h. The lower limit of accurate and reproducible detectable colony counts was 10 CFU mL^{-1}. Synergy was defined as a 100-fold or greater decrease in colony count at 24 h by the combination of agents with reference to the starting inoculum and also when compared with the most active single agent. The MIC of AmB was determined for *C. albicans* in the absence and presence of increasing concentrations of verbascoside analogues as described previously. The synergistic interaction between AmB and verbascoside analogues against '*C. albicans*' was calculated based on the fractional inhibitory concentration (FIC) index, obtained by adding the FICs of AmB and verbascoside analogues. The FIC was calculated by dividing the MIC of the combination of AmB and verbascoside analogues by the MIC of AmB or verbascoside analogues alone. All experiments were conducted in duplicate three times on separate occasions.

4. Conclusion

Selective acylation of verbascoside was achieved using resin-bound immobilised *C. antarctica* lipase B in the presence of *p*-nitro butyrate, *p*-nitro octanoate, *p*-nitro myristylate and *p*-nitro palmitate as acyl donors. Mono- and di-acylated products were formed which were subjected to synergistic antifungal activity with AmB. The analogues such as verbascoside 4″-octanoate (**3b**), verbascoside 4″-palmitate (**3d**) and verbascoside 4″,4′-palmitate (**4d**) were found to reduce the MIC of AmB to fourfold from 0.5 μM to 0.12 μM compared with the parent verbascoside in the concentration range of 1.5–0.75 μg/mL.

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