Enzymatic glycosylation of bakuchiol and vanillin in Supercritical carbon dioxide media.

K. Udaya Sankar*, B. Manohar and S. Divakar
[1]Food Engineering Department, Central Food Technological Research Institute, Mysore 570020, Karnataka, India, e-mail: udaya@cftri.res.in

Abstract: Enzymatic syntheses of water soluble bioactive compounds as their glycosides were carried out under supercritical CO_2 ($SCCO_2$) conditions for vanillin, bakuchiol and retinol. With Bakuchiol, under $SCCO_2$ (10 M Pa pressure at 50°C), bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yield range 9% to 46.6%. Antioxidant and ACE inhibitory activity of bakuchiol glucosides showed that 6-O-(6-D-Fructofruranosyl) bakuchiol showed the best antioxidant (1.4mM) and ACE inhibitory activities (0.64 mM). Further vanillin glycoside formed in corresponding yield of 56% with glucose in $SCCO_2$ media.
Keywords: ACE inhibition; Amyloglucosidase; Antioxidant activity; vanillin, vanillin glycoside, Bakuchiol; Bakuchiol glycosides; Glycosylation.

INTRODUCTION

The advantage of using supercritical carbon dioxide as a reaction medium is well documented by several examples in the literature [1,2,3,4]. Recently use of SC CO_2 as a solvent in enzyme-catalysed reactions has been a matter of considerable research interest because of its favorable transport properties that can accelerate mass-transfer-limited enzymatic reactions. Since the first reports on the use of SCF as reaction media, several studies on enzymatic oxidation, hydrolysis, transesterification, esterification, interesterification and enantioselective synthesis have proven the feasibility of enzymatic reactions in supercritical fluids [5,6,7,8,9,10,11]. Frequently the temperature range employed in supercritical carbon dioxide processing is compatible with the use of enzymes as catalysts. An additional benefit of using supercritical fluids along with enzymatic catalysis is that it provides a medium for the recovery of products or reactants and the down stream processing is made facile and economical.

MATERIALS

Amyloglucosidase

Amyloglucosidase (3.2.1.3), from *Rhizopus* mold, a fungal source, with an activity of 22,570 units / g of solid purchased from Sigma Chemical Co., St. Louis, MO, USA was used for the glycosylation. One unit liberates 1.0 mg of glucose from starch, in 3 min at pH 4.5 at 55°C.

Chemicals

D-glucose, D-galactose and D-mannose; ketohexose –maltose from Sigma Chemical Co., St. Louis, MO, USA and D-fructose purchased from SD fine chemicals (Ind.) Ltd.,. Chloroform and di-isopropyl ether was purchased from SD fine Chemicals (Ind.) Ltd. Solvents employed were distilled once before use. HPLC grade acetonitrile purchased from Qualigens fine chemicals Ltd., Vanillin, potassium bromide, Sisco Research Laboratories, Mumbai, sodium acetate, disodium hydrogen phosphate, n-butanol, Ranabaxy Laboratories, New Delhi, di-sodium tetra borate, sodium hydroxide, hydrochloric acid, sulphuric acid, Iodine and silica gel,

SD Fine Chemicals (ind) Ltd., 1-napthol, acetic acid glacial, Loba Chemie Pvt. Ltd., Mumbai, India, Sephadex G-15, Sigma Chemical Co., St. Louis, MO, USA. Whatman filter paper no:1 Whatman International Ltd.,England.

Preparation of buffers

A buffer concentration of 10 mM CH_3COONa for pH 4.0 and 5.0, Na_2HPO_4 for pH 6.0 and 7.0 and $Na_2B_4O_7$ $10H_2O$ for pH 8.0 buffers were prepared by dissolving appropriate quantity of the respective buffer salts in distilled water and the pH was adjusted by adding 0.1M of HCl or NaOH using Control Dynamics pH meter model APX175E/C, India.

Glycosylation procedure-conventional reflux method

Syntheses of bakuchiol glycosides involved refluxing bakuchiol (0.5 mmol) or vanillin with 1.0 mmol carbohydrates in 100 ml di-isopropyl ether in presence of amyloglucosidase (40% w/w carbohydrates), DMF 5.0 ml and 0.1mM (in 100 ml di-isopropyl ether), pH 6.0 buffer for an incubation period of 72 h at 68 °C (Scheme A). After the reaction, the solvent was evaporated and the enzyme denatured at 100 °C by holding in a boiling water bath for 5-10 min. The residue were dissolved in 15-20 mL of water and the reaction mixture extracted with hexane to remove unreacted bakuchiol. The dried residue was subjected to HPLC analysis to determine the extent of conversion. All the reactions were performed in duplicate and the values are expressed as the mean. Unreacted carbohydrate was separated from the product glycosides by size exclusion chromatography using Sephadex G15 column (100 cm x 1 cm), eluting with water at 1mL/h rate. Syntheses of the other bakuchiol glycosides were carried out at the above determined conditions, with bakuchiol and carbohydrates: aldohexoses – D-glucose, D-galactose and D-mannose; ketohexose – D-fructose; pentoses – D-ribose and D-arabinose; disaccharides – maltose, lactose and sucrose; sugar alcohol – D-sorbitol and D-mannitol.

Glycosylation procedure-under $SCCO_2$ condition

Syntheses of the bakuchiol glycosides were carried out with bakuchiol or vanillin and carbohydrates under $SCCO_2$: 10 M Pa pressure at 50°C. The reactor vessel along with the CO_2 supply system is described elsewhere [11] It consists of a reactor of 60 ml capacity with a magnetic stirrer and a recirculating fluid loop by means of a pressure differential for sampling through a Rheodyne valve with 0.5 ml loop for sampling. Total volume of about 60ml of the reactor vessel was thermostatically controlled to maintain a constant temperature. Reaction Process conditions employed are: bakuchiol (0.5 mmol) and carbohydrate (1.0 mmol), amyloglucosidase (40% w/w carbohydrates), DMF 15 ml, and 0.1 mM, pH 6.0 phosphate buffer and 24 h of incubation period. The CO_2 was then released and the reaction products were taken out in 15-20 ml of water, evaporated to dryness and subjected to analyses by HPLC and NMR.

Antioxidant activity measurement

Antioxidant activity of bakuchiol and bakuchiol glycosides were determined by DPPH (2,2 diphenyl-1-picryl hydrazyl) radical scavenging method [12]. The reaction mixture contained 0.1 mL of test sample (5-10mM) and 1.0 mL of DPPH (0.36mM) with the final volume adjusted to 2.0 mL of 0.1M Tris HCl buffer (pH 7.4). The reaction mixture was incubated at room temperature for 20 minutes in the dark and the antioxidant activity was determined by monitoring the decrease in absorbance at 517 nm on an UV-Visible spectrophotometer (Shimadzu, UV 1601). Butylated hydroxy anisole (BHA- 5.6mM) was used as the positive control. IC_{50} value for the antioxidant activity was expressed as the concentration of the

glycoside corresponding to 50% decrease in absorbance value of DPPH from a plot of decrease in absorbance versus concentration of the glycoside. Error in activity measurements is ± 5%.

Angiotensin Converting Enzyme (ACE) inhibition assay

ACE inhibition assay for the bakuchiol and bakuchiol glycosides were performed on to ACE isolated from pig lung by the Cushman and Cheung method [14]. Aliquots of glycoside solutions in the concentration range 0.2 to 1.8 mM (0.1 mL to 0.8 mL of 2.0 mM stock solution) were taken and to this 0.1 mL of ACE solution (0.1% in 0.1 M phosphate buffer, pH 8.3 containing 300 mM NaCl) along with 0.1 mL of 2.5 mM hippuryl-L-histidyl-L-leucine (HHL) were added and incubated in a water bath for 30 min at 37°C. Blanks were performed without the enzyme. Hippuric acid released was estimated from a calibration plot yielding 0.0105 Abs units/nmol hippuric acid. Percentage inhibition was expressed as the ratio of specific activity of ACE in presence of the inhibitor to that in its absence, the latter being considered as 100%. IC_{50} value was expressed as the concentration of the inhibitor required for 50% reduction in ACE specific activity. Error in measurements is ± 5 %.

^{1}H and ^{13}C nuclear magnetic resonance

Two-dimensional Heteronuclear Single Quantum Coherence Transfer spectra (2D HSQCT) were recorded on a Brüker DRX-500 MHz spectrometer operating at 500.13 MHz for ^{1}H and 125 MHz for ^{13}C at 35 °C. Proton and carbon 90° pulse widths were 12.25 and 10.5 μs, respectively. Chemical shifts were expressed in ppm relative to internal tetramethylsilane standard. About 40mg of the glycoside sample dissolved in DMSO-d_6 was used for recording the spectra in magnitude mode with sinusoidal-shaped *z*-gradients of strength 25.7, 15.42 and 20.56 G/cm with a gradient recovery delay of 100 μs to defocus unwanted coherences. Increment of t_1 was in 256 steps with a computer memory size of 4kB. The spectra were processed using unshifted and π/4 shifted sine bell window function in F_1 and F_2 dimensions, respectively.

Product characterization

Isolated glycosides besides measuring melting point and optical rotation were also characterized by recording UV, IR, Mass and 2D-HSQCT NMR spectra which provided good information about the nature and type of products. In 2D-HSQCT some of the assignments are interchangeable. Only resolvable signals are shown. The glycosides, being surfactant molecules tend to aggregate in solution giving rise to broad signals, thus making it difficult to resolve the coupling constant values of some of the signals.

Spectral characterization

Bakuchiol: Solid, UV (λ_{max}): 226.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{226.5}$ – 3481 M^{-1}), 299.0 nm ($n\rightarrow\pi^*$, $\varepsilon_{295.5}$ – 896 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3320 (OH), 1373 (C=C), 2928 (CH); 2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13): 7.27 (H-2), 7.19 (H-3), 6.69 (H-4), 6.71 (H-5), 6.04 (H-7), 6.17 (H-8), 1043 (H-10a), 1.21 (H-10b), 1.95 (H-11), 4.66 (H-12), 1.62 (H-14), 1.52 (H-15), 5.9 (H-16), 1.14 (H-17), 5.08 (H-18a), 5.02 (H-18b); ^{13}C NMR δ_{ppm} (125 MHz): 126.6 (C1), 127.2 (C2), 127.2 (C3), 115.4 (C4), 115.4 (C5), 156.1 (C6), 128.4 (C7), 134.1 (C8), 42.2 (C9), 41.0 (C10), 25.5 (C11), 124.2 (C12), 130.6 (C13), 23.1 (C14), 22.9 (C15), 146.0 (C16), 17.5 (C17), 111.8 (C18).

6-*O*-(D-Glucopyranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 3074 M^{-1}), 229.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{229.5}$ – 1774 M^{-1}), 275.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{275.5}$ – 511 M^{-1}); IR (KBr, Stretching frequency, cm^{-1}): 3371 (OH), 1381 (glycosidic aryl alkyl C-O-C asymmetrical), 1080

(glycosidic aryl alkyl C-O-C symmetrical), 1380 (C=C), 2937 (CH); 2D-HSQCT (DMSO-d_6) **C1α-glucoside**: ^{1}H NMR δ_{ppm} (500.13) **Glu**: 4.77(H-1α, d, J = 2.7 Hz), 3.57 (H-3α), 3.11 (H-4α); **Bakuchiol**: 7.26(H-3), 2.01(H-11), 4.9(H-12), 0.93(H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Glu**: 95.0 (C1α), 75.5 (C3α), 70.8 (C4α), 63.3 (C6α); **Bakuchiol**: 127.9 (C1), 116.1 (C4), 114.9 (C5), 162.5 (C6), 129.8 (C7); **C1β-glucoside**: ^{1}H NMR **Glu**: 4.27 (H-1β, d, J = 6.7 Hz), 3.67 (H-6a), ^{13}C NMR δ_{ppm} **Glu**: 101.8 (C1β), 75.5 (C2β), 76.3 (C3β).

6-*O*-(D-Galactopyranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 2983 M^{-1}), 231.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{231.5}$ – 1123 M^{-1}), 274.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{274.5}$ – 257 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3319 (OH), 1246 (glycosidic aryl alkyl C-O-C asymmetrical), 1064 (glycosidic aryl alkyl C-O-C symmetrical), 1361(C=C), 2917.6 (CH); 2D-HSQCT (DMSO-d_6) **C1α-galactoside**: ^{1}H NMR δ_{ppm} (500.13) **Gal**: 4.28 (H-1α, d, J = 2.7 Hz), 3.67 (H-2α), 3.76 (H-3α), 3.78 (H-4α), 3.66 (H-5α); **Bakuchiol**: 7.14(H-2), 7.16(H-3), 6.50 (H-4), 6.52 (H-5), 6.13 (H-8), 1.14 (H-10), 1.13 (H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Gal**: 95.4 (C1α), 68.4 (C2α), 74.7 (C4α), 62.7 (C6α); **Bakuchiol**: 130.6 (C3), 162.5 (C6), 15.6 (C17); **C1β-galactoside**: ^{1}H NMR **Gal**: 4.63 (H-1α, d, J = 3.4 Hz), 3.29 (H-1β, d, J = 7.2 Hz), 3.33 (H-5β); ^{13}C NMR δ_{ppm} **Gal**: 101.8 (C1β), 70.7 (C3β), 77.5 (C5β).

6-*O*-(D-Mannopyranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 2353 M^{-1}), 225.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{225.5}$ – 120 M^{-1}), 275.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{275.0}$ – 47 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 2925 (OH), 1241 (glycosidic aryl alkyl C-O-C asymmetrical), 1059 (glycosidic aryl alkyl C-O-C symmetrical), 1440 (C=C), 2925 (CH); 2D-HSQCT (DMSO-d_6) **C1α-Mannoside**: ^{1}H NMR δ_{ppm} (500.13) **Man**: 4.95 (H-1α, d, J = 1.6 Hz), 3.29 (H-3α), 3.02 (H-5α), 3.68 (H-6α); **Bakuchiol**: 1.46 (H-10a), 1.47 (H-10b), 0.922 (H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Man**: 94.76 (C1α); **Bakuchiol**: 132.1(C13), 14.3 (C17).

6-*O*-(D-Fructofuranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 2064 M^{-1}), 229.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{229.5}$ – 603 M^{-1}), ($n\rightarrow\pi^*$, $\varepsilon_{256.5}$ – 239 M^{-1}), 288.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{288.5}$ – 222 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3382.8 (OH), 1244 (glycosidic aryl alkyl C-O-C asymmetrical), 1060 (glycosidic aryl alkyl C-O-C symmetrical), 1416 (C=C), 2930.1 (CH); 2D-HSQCT (DMSO-d_6) **C6-fructoside**: ^{1}H NMR δ_{ppm} (500.13) **Fru**: 3.53 (H-3), 3.69 (H-4), 3.57 (H-5), 3.49 (H-6); **Bakuchiol**: 1.42(H-10a), 1.43 (H-10b), 1.13 (H-17); ^{13}C NMR δ_{ppm} (125 MHz): **Fru**: 104.2 (C2), 70.7 (C3), 72.6 (C4), 71.6 (C5), 63.4 (C6); **Bakuchiol**: 35.9 (C9), 38.2 (C10), 27.1 (C11), 14.0 (C17).

6-*O*-(D-Ribofuranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 5015 M^{-1}), 222.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{222.5}$ – 2146 M^{-1}), 260.4 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{260.4}$ – 971 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3350 (OH), 1241 (glycosidic aryl alkyl C-O-C asymmetrical), 1085 (glycosidic aryl alkyl C-O-C symmetrical), 1416 (C=C), 2930 (CH); 2D-HSQCT (DMSO-d_6) **C1α-riboside**: ^{1}H NMR δ_{ppm} (500.13) **Rib**: 4.64 (H-1α, d, J = 3.6 Hz), 3.78 (H-4α); **Bakuchiol**: 7.25 (H-2), 7.1 (H-3), 4.95 (H-12); ^{13}C NMR δ_{ppm} (125 MHz) **Rib**: 96.5 (C1α), 71.1 (C2α); **Bakuchiol**: 127.8 (C2), 130.1 (C7), 26.9 (C11); **C1β-riboside**: ^{1}H NMR δ_{ppm} **Rib**: 4.9 (H-1β, d, J = 7.6 Hz), 3.62 (H-4β); ^{13}C NMR δ_{ppm} **Rib**:101.6 (C1β), 70.9 (C3β): **5-*O*-arylated**: ^{1}H NMR **Rib**: 3.55 (H-1α, d, J = 2.9 Hz); ^{13}C NMR δ_{ppm} **Rib**: 62.1 (C5α).

6-*O*-(D-Arabinofuranosyl)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 6351 M^{-1}), 221.4 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{221.4}$ – 2701 M^{-1}), 259.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{259.5}$ – 1241 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3302 (OH), 1240 (glycosidic aryl alkyl C-O-C asymmetrical), 1085

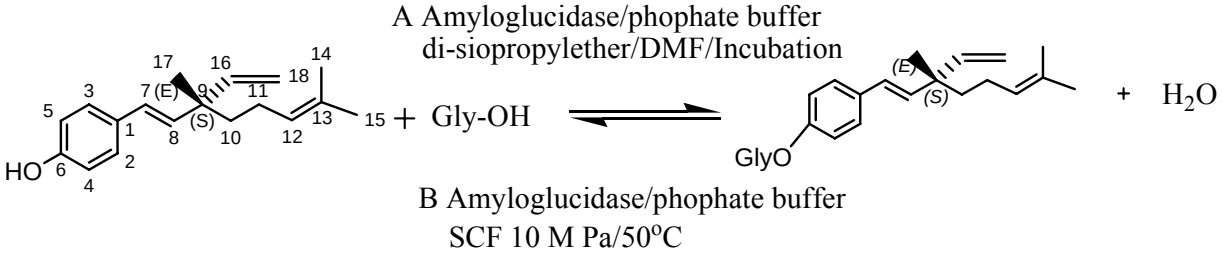
(glycosidic aryl alkyl C-O-C symmetrical), 1404 (C=C), 2926 (CH); 2D-HSQCT (DMSO-d_6) **C1α-arabinoside**: ^{1}H NMR δ_{ppm} (500.13) **Ara**: 5.00 (H-1α, d, J = 3.4 Hz), 3.70 (H-4α), 3.52 (H-2α); **Bakuchiol**: 6.34 (H-8), 0.94 (H-17), 5.09 (H-18a), 5.32 (H-18b); ^{13}C NMR δ_{ppm} (125 MHz): **Ara**: 95.9 (C1α), 75.5 (C2α); **Bakuchiol**: 116.1 (C4), 127.90 (C2), 28.68 (C11); **C1β-arabinoside**: ^{1}H NMR δ_{ppm} **Ara**: 4.96 (H-1β, d, J = 6.2 Hz), 3.40 (H-4β); ^{13}C NMR δ_{ppm} **Ara**:102.1 (C1β), 77.4 (C2), 65.0 (C5β).

6-*O*-(1-D-Sorbitol)bakuchiol: Solid, UV (λ_{max}): 191.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.0}$ – 2092 M^{-1}), 226.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{226.0}$– 446 M^{-1}), 255.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{255.0}$ – 242 M^{-1}); IR (KBr, stretching frequency cm^{-1}): 3384 (OH), 1257 (glycosidic aryl alkyl C-O-C asymmetrical), 1062 (glycosidic aryl alkyl C-O-C symmetrical), 1365 (C=C), 2923 (CH); 2D-HSQCT (DMSO-d_6) **C1-sorbitol**: ^{1}H NMR δ_{ppm} (50013) **Sor**: 3.26(H-1); **Bakuchiol**: 1.87 (H-11), 1.48 (H-14); ^{13}C NMR δ_{ppm} (125 MHz): **Sor**: 60.5 (C1), 74.4 (C2), 71.0 (C3), 73.3 (C4), 72.4 (C5); **Bakuchiol**: 26.7 (C11), 22.2 (C15), 14.0 (C17).

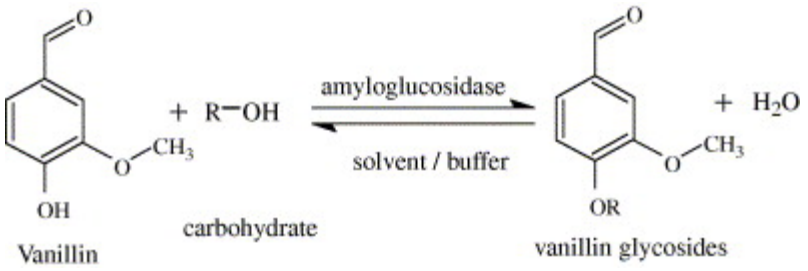
6-*O*-(6-D-Mannitol)bakuchiol: Solid, UV (λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 9000 M^{-1}), 199.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{199.5}$ – 6557 M^{-1}), 209.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{209.0}$ – 6486 M^{-1}), 223.0 nm ($\sigma\rightarrow\pi^*$, $\varepsilon_{223.0}$– 1438 M^{-1}), 269.5 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{263.5}$ – 333 M^{-1}); IR (KBr, stretching frequency, cm^{-1}): 3360 (OH), 1261 (glycosidic aryl alkyl C-O-C asymmetrical), 1076 (glycosidic aryl alkyl C-O-C symmetrical), 1377 (C=C), 2937 (CH); 2D-HSQCT (DMSO-d_6) **C6-mannitol**: ^{1}H NMR δ_{ppm} (500.13) **Mannitol**: 3.61(H-6); **Bakuchiol**: 0.84 (H-17), 5.08 (H-18); ^{13}C NMR δ_{ppm} (125 MHz): **Mannitol**: 72.9 (C2), 70.4 (C3), 70.8 (C4), 72.9 (C5), 65.1 (C6); **Bakuchiol**: 38.2 (C10), 13.9 (C17).

Glycosylation of **vanillin** using amyloglucosidase with d-glucose, maltose, yielded the respective C1 and/or C6 glycosides. The reaction mixtures were analyzed by high performance liquid chromatography (HPLC) in a Shimadzu LC 8A instrument using a μ-Bondapak amino-propyl column (10 μm particle size, 3.9 x 300 mm length) and acetonitrile: water in 70:30 (v/v) as the mobile phase at a flow rate of 1mL/min using refractive index detector. The retention times for carbohydrates were found to be 8.4 to 13.5 min, for riboflavin it was 5.2 min and for the glycosides it was in the 11.5 to 17.5 min range. Conversion yields were determined from the HPLC peak areas of the glycoside and the free carbohydrates with respect to the carbohydrate concentration employed. Error measurements in HPLC yields will be ± 10%.

Mechanism of Glycosylation



Scheme 1: Glycosylation of bakuchiol



Scheme 2: Gl;ycosylation of vanillin

RESULTS AND DISCUSSION

Glycosylation of bakuchiol using conventional reflux method resulted in glycosides of D-glucose, D-ribose and D-arabinose only (Table 1) with yields in the range 9 - 51.4%. Reactions carried out under supercritical CO_2 medium resulted in glycosides with aldohexoses – D-glucose, D-galactose and D-mannose; ketohexose – D-fructose; pentoses – D-ribose and D-arabinose; sugar alcohol – D-sorbitol and D-mannitol. The yields of the glycosides formed under $SCCO_2$ conditions were in the range 9 - 46.6% (Table 1).

Table 1: Conversion yields and proportions of bakuchiol glycosides prepared by the Reflux[a] and SC CO2 media method

S.No.	Glycosides	Product formation		Yield (%)	
		Reflux method[a]	SC CO2 media[b]	Reflux method[a]	SC CO2 media[b]
1	6-*O*-(α-D-Glucopyranosyl)bakuchiol	45	17	9	9
	6-*O*-(β-α-D-Glucopyranosyl)bakuchiol	55	22		
	6-*O*-(5-D-Glucopyranosyl)bakuchiol	ND	61		
2	6-*O*-(α-D-Galactopyranosyl)bakuchiol	ND	29	ND	37.2
	6-*O*-(β-D- Galactopyranosyl)bakuchiol	ND	73		
3	6-*O*-(α-D-Ribofuranosyl)bakuchiol	23	31	51.4	33.3
	6-*O*-(β-D-Ribofuranosyl)bakuchiol	53	69		
	6-*O*-(5-D-Ribofuranosyl)bakuchiol	24	ND		
4	6-O-(6-D-Fructofurnanosyl)bakuchiol	-	ND	ND	31.0
5	6-*O*-(α-D-Arabinofuranosyl)bakuchiol	27	31	42.0	38.0
	6-*O*-(β-D-Arabinofuranosyl)bakuchiol	73	69	-	-
6	6-*O*-(α-D-Mannoopyranosyl)bakuchiol	ND	62	-	32.7
7	6-*O*-(6-D-Mannitol)bakuchiol	ND	-	-	29.1
8	6-*O*-(1-D-Sorbitol)bakuchiol	ND	-	-	46.6

Synthesis of bakuchiol glycosides with carbohydrate molecules showed that except for D-glucose, D-ribose and D-arabinose, the other carbohydrate molecules D-fructose, maltose, sucrose, lactose, D-sorbitol and D-mannitol did not undergo glycosylation under the conventional reflux conditions employed. This could be due to not-so-facile formation of the required oxocarbenium-ion intermediate [13] with the other carbohydrate molecules at 68°C. Since the process conditions under SC-CO2 media are mild, they served as ideal conditions for the formation of glycosides with many carbohydrates. Glycosylation resulted in enhancement of water solubility of bakuchiol.

Spectral characterization

Bakuchiol glycosides were characterized by UV, IR, Mass, Optical rotation and 2DHSQCT NMR. UV spectra of bakuchiol glycosides showed shifts in the $\sigma \rightarrow \sigma^*$band between 191.0nm to 191.5nm, $\sigma \rightarrow \pi^*$ band at 199.5nm to 231.5nm, $\pi \rightarrow \pi^*$ band at 259.5nm to 275nm and $n \rightarrow \pi^*$ band at 288.5nm. IR spectra showed shifts in the OH stretching frequency regain 2925 cm^{-1} - 3397 cm^{-1}, C=C at 1347 cm^{-1} – 1440 cm^{-1}, C-O-C asymmetrical at 1239 cm^{-1} – 1380 cm^{-1}, C-O-C symmetrical stretching at 1049 cm^{-1} – 1085 cm^{-1} and CH at 2923 cm^{-1} – 2937 cm^{-1}. 2DHSQCT NMR confirmed the formation of anomeric C1α and C1β products as well as C6 arylated products, especially C1 and C6 arylated products of D-sorbitol and D-mannitol.

Antioxidant activity

Antioxidant activities of glycosides of bakuchiol and ACE inhibitory activities of bakuchiol glycosides are presented in Table 3 and 4, respectively. Pure bakuchiol showed an antioxidant activity of 1.24 mM (IC_{50} value) as against 0.029 mM for synthetic antioxidant BHA. Various glycosides of bakuchiol showed antioxidant activities ranging from 1.02 to 2.28 mM. Among the 8 glycosides prepared 6-*O*-(D-ribofuranosyl)bakuchiol and 6-*O*-(D-arabinofuranosyl) bakuchiol showed very low IC_{50} values of 1.02 ± 0.102mM and 1.2±0.12mM, while 6-*O*-(D-galactopyranosyl) bakuchiol (1.28 ± 0.128mM) and 6-*O*-(D-glucopyranosyl) bakuchiol (1.34 ± 0.134mM) showed significant IC_{50} values for antioxidant activity. Carbohydrate molecules themselves did not show antioxidant activities. Although phenolic OH group of bakuchiol is modified, it still showed marginally better antioxidant activity better than bakuchiol itself.

ACE inhibition

Bakuchiol glycosides exhibited ACE inhibition almost lesser IC_{50} values for ACE inhibition than bakuchiol itself. Among the different glycosides prepared, 6-*O*-(6-D-fructofuranosyl) bakuchiol, 0.64 ± 0.06mM 6-*O*-(D-ribofuranosyl)bakuchiol, 0.85 ± 0.09mM, 6-*O*-(D-mannopyranosyl) bakuchiol, 0.85 ± 0.09mM and 6-*O*-(6-D-mannitol)bakuchiol, 0.89 ± 0.09mM exhibited better IC_{50} values than the other glycosides. 6-*O*-(D-Arabinofuranosyl) bakuchiol, 1.03 ± 0.10mM, 6-*O*-(1-D-sorbitol)bakuchiol, 1.20 ± 0.12mM, 6-*O*-(D-galactopyranosyl) bakuchiol, 1.22 ± 0.12mM and 6-*O*-(D-glucopyranosyl) bakuchiol, 1.33 ± 0.13mM showed high IC_{50} values for ACE inhibition. Bakuchiol and enalapril showed IC_{50} values of 0.74 ± 0.07mM and 0.071 ± 0.007mM for ACE inhibition respectively. 6-*O*-(6-D-Fructofuranosyl) bakuchiol with IC_{50} value of 0.64± 0.06mM has shown the best ACE inhibition than bakuchiol itself. Modification of the phenolic OH group by the carbohydrate molecule did not affect the ACE inhibition activity.

In contrast, under the supercritical CO_2 atmosphere, glycosides with carbohydrate molecules of carbohydrates D-glucose, D-fructose, D-ribose, D-sorbitol, D-arabinose, D-mannose, and D-mannitol were formed and three disaccharides of maltose, sucrose and lactose were not detected. This could be due to the usefulness of the reaction medium which provided an ideal dielectric medium for the enzymatic reaction to occur with wide variety of carbohydrates. The yield of glycosides were in the range of 9- 46.6%.

The peak with retention time 8.092 minutes is identified as vanillin glucoside and peka with 9.908 identified as vanillin maltoside with authentic samples. The occurrence of the peaks at 8.92 and 9.908 confirm the glycosylation of both glucose and maltose with

vanillin. The glycosylation of glucose and matose with vanillin is completed under the supercritical carbon dioxide conditions is evident from HPLC.

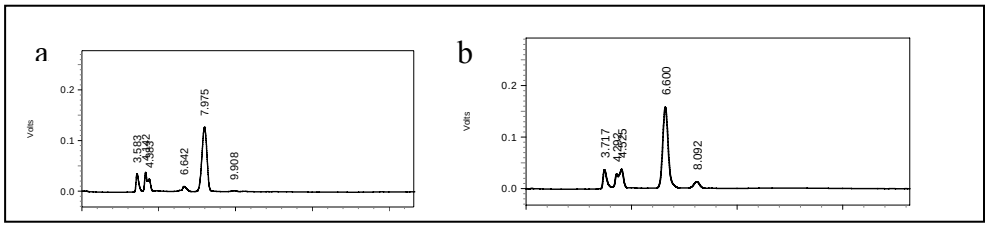


Figure 1: HPLC chromatograms Vanillin-glycosides (a) glucoside (b) maltoside

CONCLUSIONS

Enzymatic syntheses of water soluble Bakuchiol glycosides were reported first time and the reactions were carried in conventional reflux method and under supercritical CO_2 conditions. Out of eleven carbohydrate molecules selected for the reaction, D-glucose, D-ribose and D-arabinose gave glycosides in yields of 9.0% to 51.4% under conventional reflux method. Under supercritical CO_2 atmosphere (100 bar pressure at 50°C), bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yield range 9% to 46.6%. Antioxidant and ACE inhibitory activity of bakuchiol glucosides showed that 6-O-(6-D-Fructofruranosyl) bakuchiol showed the best antioxidant (1.4mM) and ACE inhibitory activities (0.64 mM). The synthesis of vanillin glycosides by enzymatic method using supercritical carbon dioxide has shown precision to a satisfactory degree, accuracy and simplicity. The preparation of vanillin glycosides has been reported by cell suspension culture, chemical and plant cell tissue and organ culture method. So also, steviol-13-O-glucopyranoside produced from the steviol glucosylation was successfully glycosylated to steviolbioside, then to stevioside producing rebaudioside A[14].

REFERENCES

[1] Tai, C.Y., Huang, S.C., Huang, M.S., Liu, H.S., J. Chin. Inst.Chem.Eng. 32 (3) ,**2001**, 269.
[2] Russell,A.J., Beckman, E.J. Enzyme Microb. Technol. 13 (12) ,**1991**, 1007.
[3]Randolph,T.W., Blanch,H.W., Prausnitz, J.M., Wilke,C.R.Biotechnol. Lett. **7(**5),**1985**, 325.
[3] Marty,A., Chulalaksananukul, W., Condoret,J.S., Willemont, R.M.,Durand,G., Biotechnol. Lett. 12 (1),**1990**,11.
[4] Knez, Habulin, M., Krmelj, V. J. Supercrit. Fluids 14, **1998**, 17.
[5] Turner,C., Persson,M., Mathiasson, L., Adlercreutz, P., King, J.W. Enzyme Microb. Technol. 29 (2,3), **2001**, 111.
[6] Yu, H.M, Lin,H.L., Wu,C.Y., Tseng,M.J., Chen,S.T., Jeyashoke, N., Krisnangkura, K. J. Chin. Chem. Soc. 46 (5), **1999**, 647.
[7] Chi, Y.M., Nakamura, K., Yano, T. Agric. Biol. Chem. 52 (6), **1988**, 1541.
[8] Nelson, N.A., Foglia, T.A., Marmer, W.N. J. Am. Oil Chem. Soc. 73(9), **1996**, 1191.
[9] Miller, D.A., Blanch, H.W., Prausnitz,J.M. *Ind Eng Chem Res* 30, **1991**, 939.
[10] Compton, D.L., King, J.W., J. Am. Oil Chem. Soc. 78**(**1), **2001**, 43.
[11] Nagesha, G.K., Manohar, B., Udaya Sankar, K**.,** Enzymatic esterification of free fatty acids of hydrolysed soy deodourized distillate in supercritical CO_2 . **J Super Critical Fluids**. 32, **2004,** 137.
[12] Moon, J.H., Tearo,J., J Agri Food Chem. 48, **1998**, 5062.
[13] Cushman,D.W., Cheung,H.S., Biochem Pharmacol. 20, **1971**, 1637.
[14] Chitradon, L., Mahakhan, P. and Bucke, C. J. Mol. Catal. B. Enzymatic, 10, **2000**, 273.