Amyloglucosidase Catalyzed Syntheses of Bakuchiol Glycosides in Supercritical Carbon Dioxide

Balaraman Manohar,* Soundar Divakar,† and Kadimi Udaya Sankar*

Food Engineering Department, Central Food Technological Research Institute, Mysore 570020, India
**E-mail: manoharmysore@yahoo.co.in or manoharb@cftri.res.in*
†Fermentation Technology and Bioengineering Department,Central Food Technological Research Institute, Mysore 570020, India
Received January 31, 2009, Accepted June 23, 2009

Enzymatic syntheses of water soluble Bakuchiol glycosides were carried out in di-isopropyl ether organic media using amyloglucosidase from *Rhizopus* mold. The reactions were carried out under conventional reflux conditions and in supercritical CO_2 atmospheric conditions. Out of the eleven carbohydrate molecules employed for the reaction, D-glucose, D-ribose and D-arabinose gave glycosides in yields of 9.0% to 51.4% under conventional reflux conditions. Under supercritical CO_2 atmosphere (100 bar pressure at 50 ^{o}C), bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yields ranging from 9% to 46.6%. Out of the bakuchiol glycosides prepared, 6-*O*-(6-D-fructofruranosyl)bakuchiol showed the best antioxidant (1.4 mM) and ACE inhibitory activities (0.64 mM).

Key Words: ACE inhibition, Antioxidant activity, Bakuchiol, Bakuchiol glycosides, Glycosylation

Introduction

Bakuchiol (I) is a biologically active mono-terphenic phenolic compound having a single hydroxyl group on the aromatic ring and an unsaturated hydrocarbon chain. Bakuchiol is isolated from the seeds of Chiba *Psoralea corylifolia* L. distributed all over the subcontinent extending well into Southeast Asia. The seed-oil is used externally for the treatment of leucoderma, psoriasis and leprosy in Indian folk medicine.[1] The plant, known as Bakuchi in Sanskrit, has been used in Ayurvedic medicinal system as a cardiac tonic, vasodilator and pigmentor. It is widely used in Chinese medicine to treat a variety of diseases and possesses antitumor, antibacterial, cytotoxic and antihelmenthic properties.[2] Thermally sensitive bakuchiol, psoralen and isosporalen, the major components present in the seed possess important therapeutic properties.

Earlier studies on the principal components of Chiba seed have shown significant antibacterial and antioxidant activities and a great potential for use in food additives and mouthwash for preventing and treating dental caries.[3,4] Synthetic antioxidants such as butylated hydroxyl-anisole (BHA) widely used in food industries are known to cause liver damage and carcinogenesis.[5,6] Hence, development of effective non-toxic antioxidants from natural sources is very much desired.

Bakuchiol exhibits poor water solubility, stability and absorbability. Glycosylation improves the pharmacological property by increasing the water solubility of Bakuchiol. One-step enzymatic glycosylation is useful for the preparation of glycosides rather than chemical glycosylation, which requires large number of protection-deprotection steps.

Production of fine chemicals results in generation of considerable solvent waste as synthesis generally includes number of steps. Typically one kg of end product leads to generation of 15 kg of wastes. Most of them are solvents and by-products. Therefore, several reactions were performed in water or in supercritical fluids (SCF) recently. Manufacturing processes in liquid and supercritical fluids (SCF) are advantageous in terms of energy reduction, ease of product recovery, lesser cost of downstream processing and reduction in side reactions.

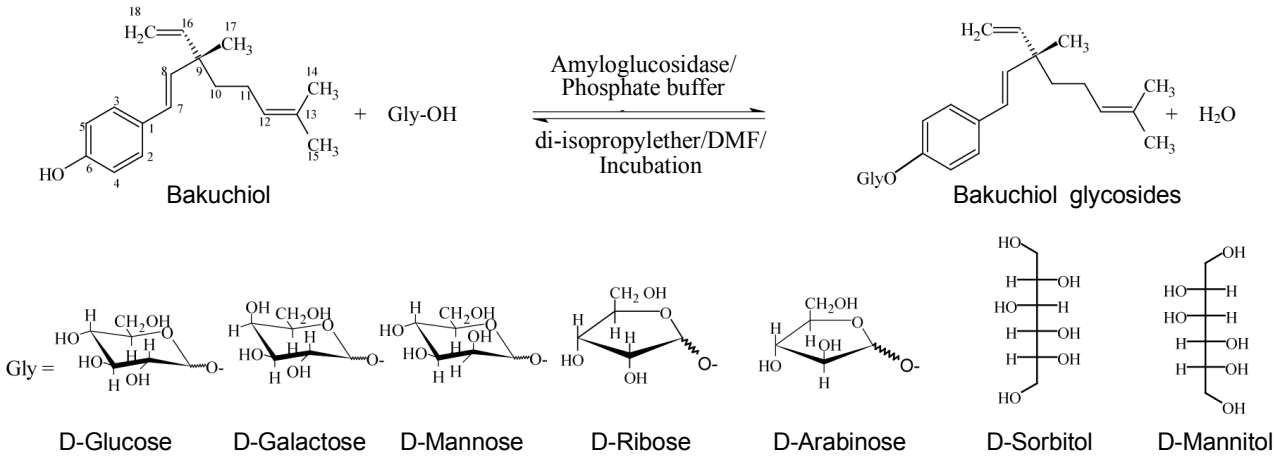
The advantage of using supercritical carbon dioxide as a reaction medium is well documented.[7-10] Recently, use of supercritical CO_2 ($SCCO_2$) as a solvent in enzyme-catalysed reactions has been a matter of considerable research interest because of its favorable transport properties that can accelerate mass-transfer-limited enzymatic reactions. Since the first reports on the use of SCF as reaction media in 1980s, several studies on enzymatic oxidation, hydrolysis, transesterification, esterification, interesterification and enantioselective synthesis have proven the feasibility of enzymatic reactions in supercritical fluids.[11-17] The temperature range employed in supercritical carbon dioxide processing is favorable for the use of enzymes as catalysts besides providing a medium for the recovery of products or reactants without many unwanted process steps.

Enzymatic glycosylation of bakuchiol with different carbohydrates in supercritical fluid media has not been reported in literature. Hence, the present study is attempted to prepare water soluble bakuchiol glycosides enzymatically using amyloglucosidase from *Rhizopus* mold utilizing different carbohydrate molecules in two different environments: one under conventional reflux conditions and another under $SCCO_2$ conditions.

Experimental Section

Enzymes. Amyloglucosidase from *Rhizopus* sp. was purchased from Sigma Company, St. Louis, MO, USA. Amyloglucosidase activity[18] was found to be 11.2 activity units (AU- μmol/(mg. enzyme. min)).

Chemicals and reagents. D-Galactose and D-fructose were



Scheme 1. Syntheses of Bakuchiol glycosides

procured from HiMedia Pvt. Ltd, India. D-Glucose and sucrose purchased from SD Fine Chemicals (India) Ltd., D-mannose, D-arabinose, D-ribose, D-sorbitol and D-mannitol, from Loba Chemie Pvt. Ltd., India and maltose from Sigma Chemical Co., St. Louis, MO, USA were employed. Lactose, HPLC grade acetonitrile and di-isopropyl ether were from Sisco Research Laboratories Pvt. Ltd., India. Di-isopropyl ether was distilled once before use.

Glycosylation procedure-conventional reflux method. Syntheses of bakuchiol glycosides involved refluxing bakuchiol (0.5 mmol) 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 1). 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 containing unreacted bakuchiol, carbohydrates, along with the product glycosides 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 triplicate and the mean values are shown in tables. Unreacted carbohydrate was separated from the product glycosides by size exclusion chromatography using Sephadex G15 column (100 cm × 1 cm), eluting with water at 1 mL/h rate. Individual glycosides could not be separated satisfactorily, due to similar polarity of the glycosides formed.

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. The conditions employed with the enzymes are: bakuchiol (0.5 mmol) and carbohydrate (1.0 mmol), amyloglucosidase (40% w/w carbohydrates), 0.2 mM, pH 6.0 phosphate buffer and 72 h of incubation period.

Glycosylation procedure at SCCO$_2$ conditions. Syntheses of the bakuchiol glycosides were carried out with bakuchiol and carbohydrates in supercritical CO_2 atmosphere of 100 bar pressure at 50 °C. The reactor vessel along with the CO_2 supply system is shown schematically in Figure 1. It consists of a reactor of 120 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 50 mL 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.

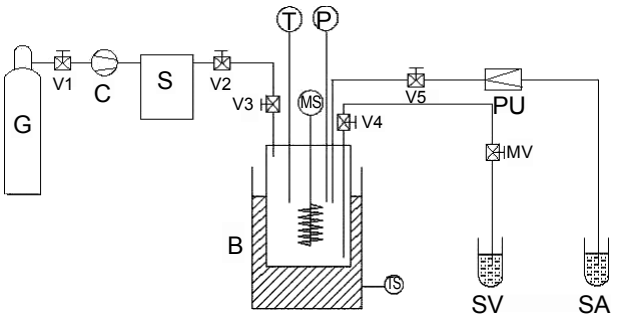


Figure 1. Process schematic diagram of experimental set up to carry out the reactions in SCF CO_2. G, Gas cylinder; C, Compressor; S, Surge tank; B, Berghof autoclave; T, Temperature indicator; P, Pressure indicator; MS Magnetic stirrer; V1-V5, High pressure needle valve; MV, Micrometer valve; SV, Sampling valve; SA, Saline solution; TS, Thermostat; PU, Pump.

Antioxidant activity measurement. Antioxidant activity of bakuchiol and bakuchiol glycosides were determined by DPPH (2,2 diphenyl-1-picryl hydrazyl) radical scavenging method.[19] The reaction mixture contained 0.1 mL of test sample (5 ~ 10 mM) and 1.0 mL of DPPH (0.36 mM) with the final volume adjusted to 2.0 mL of 0.1 M 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- 5.6 mM) 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 with ACE isolated from pig lung by the Cushman and Cheung method.[20] 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 40 mg 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 4 kB. 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); MS (m/z) 419$[M+1]^+$. 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); MS (m/z) 419$[M+1]^+$. 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); **C1 β-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); MS (m/z) 419$[M+1]^+$. 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); MS (m/z) 419$[M+1]^+$. 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); MS (m/z) 388$[M]^+$. 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*-aryl-ated**: ^{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); MS (m/z) 427 $[M+K]^+$. 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).

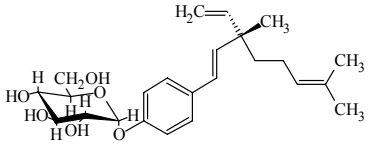
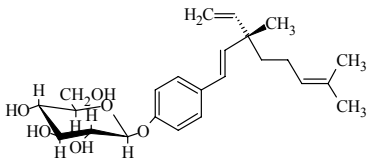
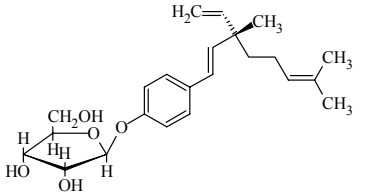
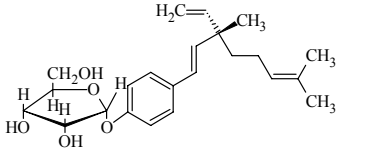
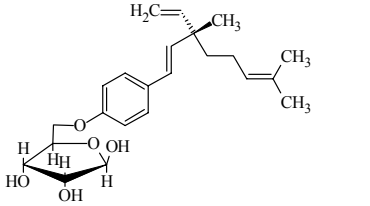
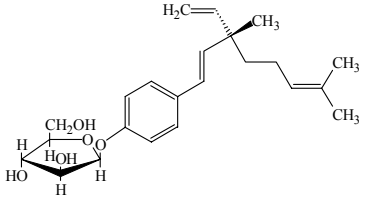
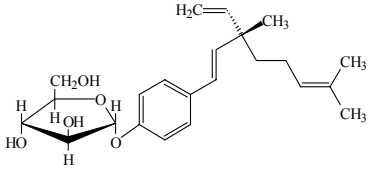
6-*O*-(1-D-Sorbitol)bakuchiol: Solid, UV (λ_{max}): 191.0 nm ($\sigma \rightarrow \sigma^*$, $\varepsilon_{191.0}$ − 2092 M^{-1}), 226.0 nm ($\sigma \rightarrow \sigma^*$, $\varepsilon_{226.0}$ − 446 M^{-1}), 255.0 nm ($\pi \rightarrow \pi^*$, $\varepsilon_{255.0}$ − 242 M^{-1}); IR (KBr, stretching 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); MS (m/z) 419$[M+1]^+$. 2D-HSQCT (DMSO-d_6) **C1-sorbitol**: ^{1}H NMR δ_{ppm} (500.13) **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); MS (m/z) 419$[M+1]^+$. 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).

Results and Discussion

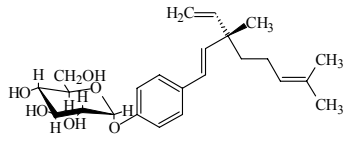
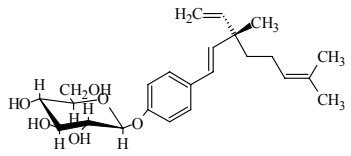
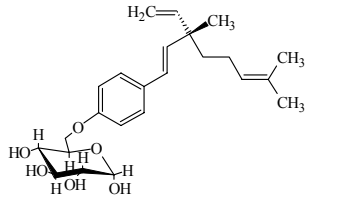
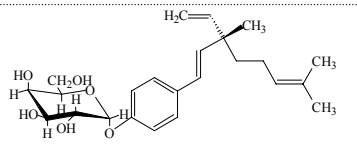
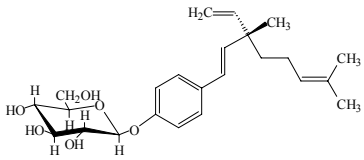
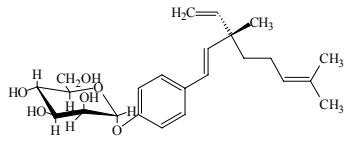
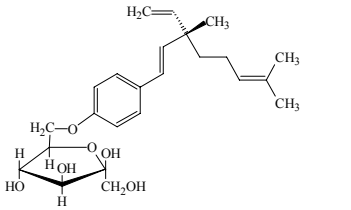
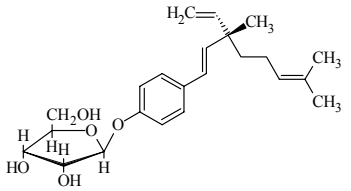
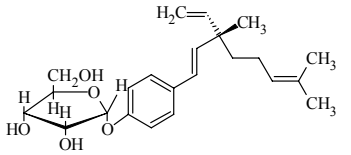
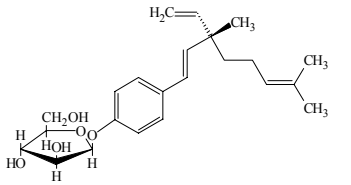
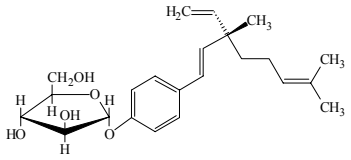
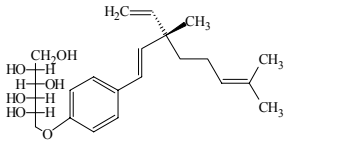
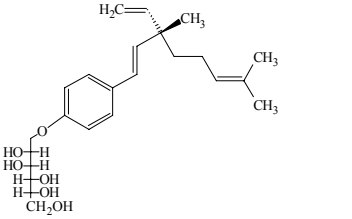
Glycosylation of bakuchiol using conventional reflux method resulted in glycosides of D-glucose, D-ribose and D-arabinose only (Table 1) with yields in the range 9 ~ 51.4%. Reactions carried out in supercritical CO_2 media resulted in glycosides with aldohexoses − D-glucose, D-galactose and D-mannose; 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 ranging from 9 to 46.6% (Table 2). Since, the $SCCO_2$ conditions are mild, they are used as ideal conditions for the formation of glycosides

Table 1. Conversion yields and proportions of bakuchiol glycosides prepared by the Reflux method[a]

No.	Glycosides	Amyloglucosidase catalysis[b] Product (% proportions)[c]	Yield (%)[d]
1	 6-*O*-(α-D-Glucopyranosyl)bakuchiol	6-*O*-α (45)	9.0
	 6-*O*-(β-α-D-Glucopyranosyl)bakuchiol	6-*O*-β (55)	
2	 6-*O*-(α-D-Ribofuranosyl)bakuchiol	6-*O*-α (23)	51.4
	 6-*O*-(β-D-Ribofuranosyl)bakuchiol	6-*O*-β (53)	
	 6-*O*-(5-D-Ribofuranosyl)bakuchiol	6-*O*-5 (24)	
3	 6-*O*-(α-D-Arabinofuranosyl)bakuchiol	6-*O*-α (27)	42.0
	 6-*O*-(β-D-Arabinofuranosyl)bakuchiol	6-*O*-β (73)	

[a]Reaction refluxed at 68 °C in di-isopropyl ether solvent at atmospheric pressure; [b]Bakuchiol − 0.5 mmol and carbohydrate 1.0 mmol; enzyme concentration 40% w/w carbohydrates; solvent − di-isopropyl ether, DMF − 5.0 mL; 0.1 mM (1.0 mL) pH 6.0 phosphate buffer; incubation period − 72 h; [c]The product proportions were calculated from the area of respective carbon signals. [d]Conversion yields were from HPLC with respect to the carbohydrate. Error in yield measurements is ± 5%.

Table 2. Conversion yields and proportions of bakuchiol glycosides prepared under $SCCO_2$ condition[a]

No.	Glycosides	Amyloglucosidase catalysis[b] Product (% proportions)[c]	Yield (%)[d]
1	 6-*O*-(α-D-Glucopyranosyl)bakuchiol	6-*O*-α (17)	9
	 6-*O*-(β-D-Glucopyranosyl)bakuchiol	6-*O*-β (22)	
	 6-*O*-(5-D-Glucopyranosyl)bakuchiol	6-0-6 arylated (61)	
2	 6-*O*-(α-D-Galactopyranosyl)bakuchiol	6-*O*-α (29)	37.2
	 6-*O*-(β-D-Galactopyranosyl)bakuchiol	6-*O*-β (73)	
3	 6-*O*-(α-D-Mannoopyranosyl)bakuchiol	6-*O*-α	32.7
4	 6-*O*-(6-D-Fructofuranosyl)bakuchiol	6-*O*-6	31.0
5	 6-*O*-(α-D-Ribofuranosyl)bakuchiol	6-*O*-α (31)	33.3
	 6-*O*-(β-D-Ribofuranosyl)bakuchiol	6-*O*-β (69)	
6	 6-*O*-(α-D-Arabinofuranosyl)bakuchiol	6-*O*-α (38)	38
	 6-*O*-(β-D-Arabinofuranosyl)bakuchiol	6-*O*-β (62)	
7	 6-*O*-(1-D-Sorbitol)bakuchiol	6-*O*-1	46.6
8	 6-*O*-(6-D-Mannitol)bakuchiol	6-*O*-6	29.15

[a]Reaction conducted in supercritical CO_2 (100 bar at 50 °C). [b]Bakuchiol − 0.5 mmol and carbohydrate 1.0 mmol; enzyme concentration 40% w/w carbohydrates; solvent − DMF − 15.0 mL; 0.1 mM (1.0 mL) pH 6.0 phosphate buffer; incubation period − 24 h. [c]The product proportions were calculated from the area of respective carbon signals. [d]Conversion yields were from HPLC with respect to the carbohydrate. Error in yield measurements is ± 5%.

Table 3. IC_{50} values for Antioxidant activities of bakuchiol glycosides[a]

Compound	IC_{50} value (mM)
Butylated Hydroxy Anisole	0.029
Bakuchiol	1.24
6-*O*-(D-Glucopyranosyl)bakuchiol	1.34
6-*O*-(D-Galactopyranosyl)bakuchiol	1.28
6-*O*-(D-Mannoopyranosyl)bakuchiol	2.13
6-*O*-(6-D-Fructofuranosyl)bakuchiol	1.40
6-*O*-(D-Ribofuranosyl)bakuchiol	1.02
6-*O*-(D-Arabinofuranosyl)bakuchiol	1.20
6-*O*-(1-D-Sorbitol)bakuchiol	2.28
6-*O*-(6-D-Mannitol)bakuchiol	1.80

[a]Antioxidant activity values determined by DPPH radical scavenging method. Error in measurements is ± 5%.

with the above mentioned monosaccharides.

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[19] with the other carbohydrate molecules at 68 °C. Since the process conditions under $SCCO_2$ 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 191.0 nm to 191.5 nm, $\sigma \rightarrow \pi^*$ band at 199.5 nm to 231.5 nm, $\pi \rightarrow \pi^*$ band at 259.5 nm to 275 nm and $n \rightarrow \pi^*$ band at 288.5 nm. 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 ± 0.102 mM and 1.2 ± 0.12 mM, while 6-*O*-(D-galactopyranosyl) bakuchiol (1.28 ± 0.128 mM) and 6-*O*-(D-glucopyranosyl) bakuchiol (1.34 ± 0.134 mM) 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 were also tested for ACE inhibition. Bakuchiol glycosides exhibited almost lesser IC_{50} values for ACE inhibition than bakuchiol itself. Among the different glycosides prepared, 6-*O*-(6-D-fructofuranosyl) bakuchiol, 0.64 ± 0.06 mM 6-*O*-(D-ribofuranosyl)bakuchiol, 0.85 ± 0.09 mM, 6-*O*-(D-mannopyranosyl)bakuchiol, 0.85 ± 0.09 mM and 6-*O*-(6-D-mannitol)bakuchiol, 0.89 ± 0.09 mM exhibited better IC_{50} values than the other glycosides. 6-*O*-(D-Arabinofuranosyl)bakuchiol, 1.03 ± 0.10 mM, 6-*O*-(1-D-sorbitol) bakuchiol, 1.20 ± 0.12 mM, 6-*O*-(D-galactopyranosyl)bakuchiol, 1.22 ± 0.12 mM and 6-*O*-(D-glucopyranosyl)bakuchiol, 1.33 ± 0.13 mM showed high IC_{50} values for ACE inhibition. Bakuchiol and enalapril showed IC_{50} values of 0.74 ± 0.07 mM and 0.071 ± 0.007 mM for ACE inhibition respectively. 6-*O*-(6-D-Fructofuranosyl) bakuchiol with IC_{50} value of 0.64 ± 0.06 mM 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.

Table 4. IC_{50} values for Angiotensin Converting Enzyme Inhibitory activities of bakuchiol glycosides[a]

Compound	IC_{50} value (mM)
Enalapril	0.071
Bakuchiol	0.74
6-*O*-(D-Glucopyranosyl)bakuchiol	1.33
6-*O*-(D-Galactopyranosyl)bakuchiol	1.22
6-*O*-(D-Mannoopyranosyl)bakuchiol	0.85
6-*O*-(6-D-Fructofuranosyl)bakuchiol	0.64
6-*O*-(D-Ribofuranosyl)bakuchiol	0.85
6-*O*-(D-Arabinofuranosyl)bakuchiol	1.03
6-*O*-(1-D-Sorbitol)bakuchiol	1.20
6-*O*-(6-D-Mannitol)bakuchiol	0.89

[a]Angiotensin Converting Enzyme Inhibitory activity determined by Cushman and Cheung method.[20] Error in measurements is ± 5%.

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%.

Among the various carbohydrate molecules employed, particularly the glycosylation of aldo-hexoses like D-glucose, D-galactose and ketohexose D-fructose, aldo-pentoses like D-ribose, D-arabinose and sugar alcohol D-mannitol with phenolic OH group of bakuchiol converted bakuchiol into a freely water soluble compounds as well as enhance its biological activities also.

Conclusions

Enzymatic syntheses of water soluble Bakuchiol glycosides were reported first time. The reactions were carried in two different media: one by conventional reflux conditions and the other in supercritical CO_2. Out of the eleven carbohydrate

molecules employed 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 conditions (100 bar pressure at 50 ^{o}C), bakuchiol formed glycosides with D-glucose, D-galactose, D-mannose, D-fructose, D-ribose, D-arabinose, D-sorbitol and D-mannitol in yield ranging from 9% to 46.6%. Out of the bakuchiol glycosides prepared, 6-*O*-(6-D-fructofruranosyl)bakuchiol showed the best antioxidant (1.4 mM) and ACE inhibitory activities (0.64 mM).

Acknowledgments. Authors thank project assistants Mr. T. Ponrasu and Mr. R. E. Charles for the help during the experiment.

References

1. Kondo, Y.; Kato, A.; Kubota, Y.; Nozoe, S. *Heterocycles* **1990**, *31*, 187-190.
2. Latha, P. G.; Evans, D. A.; Panikkar, K. R.; Jayawardhanan, K. K. *Fitoterpia*. **2000**, *3*, 223-231.
3. Katsura, H.; Tsutikiyama, R.; Suzuki, A.; Kobayashi, M. *Anti Microbial Agents Chemother*. **2001**, *45*(11), 3009-3013.
4. Haraguchi, H.; Inoue, J.; Tamura, Y.; Mizutani, K. *Phytotherapy Research* **2002**, *16*, 39-544.
5. Grice, H. C. *Food and Chemical Toxicology* **1986**, *24*, 1127-1130.
6. Wichi, H. P. *Food and Chemical Toxicology* **1988**, *26*, 717- 723.
7. Tai, C. Y.; Huang, S. C.; Huang, M. S.; Liu, H. S. *J. Chin. Inst. Chem. Eng.* **2001**, *32*(3), 269-275.
8. Russell, A. J.; Beckman, E. J. *Enzyme Microb. Technol.* **1991**, *13*(12), 1007.
9. Randolph, T. W.; Blanch, H. W.; Prausnitz, J. M.; Wilke, C. R. *Biotechnol. Lett.* **1985**, *7*(5), 325-328.
10. Marty, A.; Chulalaksananukul, W.; Condoret, J. S.; Willemont, R. M; Durand, G. *Biotechnol. Lett.* **1990**, *12*(1), 11-16.
11. Knez, M. H.; Krmelj ,V. *J. Supercrit. Fluids* **1998**, *14*, 17-29.
12. Turner, C.; Persson, M.; Mathiasson, L.; Adlercreutz, P.; King, J. W. *Enzyme Microb. Technol.* **2001**, *29*, 111-121.
13. Yu, H. M.; Lin, H. L.; Wu, C. Y.; Tseng, M. J.; Chen, S. T.; Jeyashoke, N.; Krisnangkura, K. *J. Chin. Chem. Soc*. **1999**, *46*(5), 647-650.
14. Chi, Y. M.; Nakamura, K.; Yano, T. *Agric. Biol. Chem*. **1988**, *52*(6), 1541-1550.
15. Nelson, L. A.; Foglia, T. A.; Marmer, W. N. *J. Am. Oil Chem. Soc.* **1996**, *73*(9), 1191-1195.
16. Miller, D. A.; Blanch, H. W.; Prausnitz, J. M. *Ind. Eng. Chem. Res.* **1991**, *30*, 939-946.
17. Compton, D. L.; King, J. W. *J. Am. Oil Chem. Soc.* **2001**, *78*(1), 43-47.
18. Sumner, J. B.; Sisler, E. B. *Arch. Biochem.* **1944**, *4*, 333-336.
19. Moon, J. H.; Tearo, J. *J. Agri. Food Chem*. **1998**, *48*, 5062-5065.
20. Cushman, D. W.; Cheung, H. S. *Biochem. Pharmacol*. **1971**, *20*, 1637-1638.