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ORIGINAL ARTICLE

Enzymatic synthesis of cholecalciferol glycosides using β-glucosidase from sweet almond

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Abstract β-Glucosidase from sweet almond (*Amygdalus communis* var. 'Dulcis') entrapped on to calcium alginate beads catalysed the synthesis of water soluble 17-*O*-(D-glucopyranosyl)cholecalciferol. Optimum conditions for the reaction were: 60% (w/w D-glucose) β-glucosidase, 0.12 mM pH 6 phosphate buffer and 30 h incubation period. β-Glucosidase also catalyzed the reaction with D-glucose **2**, D-galactose **3**, D-mannose **4** and D-fructose **5** with generally low yields in the range of 3–14%. Both α/β anomers of D-glucose **2**, D-galactose **3** and D-mannose **4** reacted, of which the former two formed C6-O derivatives also.

Keywords Cholecalciferol · Cholecalciferol glycosides · Calcium alginate entrapped β-glucosidase · Enzymatic glycosylation · Non-polar media · Water soluble glycosides

Introduction

Vitamin D, belonging to the class of seco-steroids, comprises 10 substances, each with different level of activity (Holick 2004; Maurizio et al 2007). Seco-steroids are those in which one of the ring is broken and in vitamin D by ultra violet B light (UV-B, sunlight). Fat-soluble vitamin D occurs mainly in two active forms: ergocalciferol or activated ergosterol (vitamin D_2) found in plants and irradiated yeast and cholecalciferol or activated 7-dehydrocholesterol (vitamin D_3) formed in human skin after exposure to UV-B rays from the sun (Coburn et al 1996).

Fat-soluble, light sensitive vitamin D_3 (Lehninger 1977) supplementation is very difficult and it can be solved by synthesizing glycosyl derivatives of vitamin D_3 with enhanced solubility and stability. Glycosylation reaction can be effected by many methods—chemical (Du et al. 2004; Sophie et al. 2004) and cell culture (Hamada et al. 2003). Among these, enzymatic synthesis involves milder reaction conditions, simple work-up procedure, easy recovery, less pollution and a cost effective process with good selectivity and yield (Vijayakumar and Divakar 2007). Enzymatic glycosylation can be effected by glucosidases (Sivakumar et al. 2006; Vijayakumar et al. 2006). Glucosidases are hydrolytic in nature, but unusual conditions involving organic solvents with little quantity of water, direct the enzymes towards glycosylation (Ponrasu et al. 2008).

The present study deals with the optimization of reaction conditions (Table 1) for the enzymatic glycosylation of cholecalciferol **1** (vitamin D_3) in terms of enzyme concentration, pH, buffer concentration and incubation period using β-glucosidase from sweet almond. Cholecalciferol glycosides with various carbohydrate molecules were also prepared under the optimum condition worked out (Fig. 1). The results are presented here.

Materials and methods

β-Glucosidase was isolated from sweet almond (*Amygdalus communis* var. 'Dulcis') by tannin precipitation method (Hestrin et al. 1955). About 1 kg of finely powdered defatted sweet almond powder was dispersed in a solution

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Table 1 Optimization of reaction conditions for the synthesis of 17-*O*-(D-glucopyranosyl) cholecalciferol using β-glucosidase

Reaction conditions[a]	Variable parameter[b]	Conversion yields (%)[c]
	pH (0.01 M)	
Cholecalciferol—0.5 mmol	4	10
D-Glucose—1 mmol	5	13
β-Glucosidase (60% w/w D-glucose)	6	14
Buffer concentration—0.12 ml (1.2 ml)	7	12
Incubation period—30 h	8	8
	Buffer concentration (mM)	
Cholecalciferol—0.5 mmol	0.04	11
D-Glucose—1 mmol	0.08	12
β-Glucosidase (60% w/w D-glucose)	0.12	14
pH—6	0.16	13
Incubation period—30 h	0.2	13
	β-Glucosidase concentration (w/w D-glucose)	
Cholecalciferol—0.5 mmol	20	3
D-Glucose—1 mmol	40	10
Buffer concentration—0.12 mM (1.2 ml)	60	14
pH—6	80	12
Incubation period—30 h	100	4
	Incubation (h)	
Cholecalciferol—0.5 mmol	10	11
D-Glucose—1 mmol	20	13
β-Glucosidase (60% w/w D-glucose)	30	14
pH—6	40	13
Buffer concentration—0.12 mM (1.2 ml)	50	12

[a] Initial reaction conditions.
[b] Other variables are the same as the initial reaction conditions, except for the specified ones.
[c] HPLC yields expressed with respect to 0.5 mmol D-glucose employed.

of 50 g of $ZnSO_4.7H_2O$ in 4 l of water and allowed to stand at 0°C for 4 to 5 h. The cold solution was filtered and to the filtrate was cautiously added, a solution of 1.4 g tannin (0.28%) in 500 ml water. The precipitate consisting mostly of impurities was discarded. The bulk of the enzyme was then precipitated slowly by adding 500 ml of 3% tannin solution in water. The precipitate was separated by centrifugation, freed from tannin by repeatedly dispersing it in acetone, dialyzed using 3.5 kDa cut off dialysis bag and finally lyophilized. β-Glucosidase (10.3 g) was obtained from 1 kg of almond powder.

Native β-glucosidase was entrapped on to bead form by the procedure described by Won et al. (2005). A 200 ml of 1 g β-glucosidase solution was mixed with 800 ml of 1% sodium alginate (Fluka, Norway) as sodium salt of alginic acid from brown algae, (*Macrocystis pyrifera*). Mixed solution was then drawn with a syringe into 1 l of 50 mM $CaCl_2$ solution and allowed to harden for 30 min. The beads were then washed twice with 50 mM pH 7.2 tris-HCl buffer and lyophilized. An yield of 3.92 g β-glucosidase was obtained.

Activity of calcium alginate entrapped β-glucosidase measured by Colowick and Kaplan (1976) method was found to be 0.078 mmol /(mg. enzyme. min). Protein content by Lowry's method was found to be 4.2%. D-Glucose and other carbohydrates employed in this work were purchased from SD Fine Chemicals (India.) Ltd, and Sigma-Aldrich Chemical Co. USA. Cholecalciferol and sodium alginate from Sigma-Aldrich Chemical Co. USA. Solvents—di-isopropyl ether, DMF and HPLC grade acetonitrile from SD fine Chemicals (India) Ltd. were used after distilling once.

Glycosylation procedure Glycosylation of cholecalciferol (0.5 mmol) involved refluxing with respective carbohydrates (1 mmol) in an amber coloured 150 ml two necked flat bottom flask fitted with a similar amber coloured condenser containing 100 ml di-isopropyl ether 68°C in presence of 20–100% (w/w carbohydrate) β-glucosidase, pH 4–8, 0.04–0.2 mM (0.4–2 ml of 10 mM) buffer and 10–50 h incubation. Acetate buffer of pH 4.0 and 5.0, phosphate buffer of pH 6 and pH 7 and borate buffer of pH 8 were used for the synthesis. The reactions were conducted under nitrogen atmosphere. After incubation, the solvent was distilled off. Unreacted carbohydrate and the products were dissolved in 15–20 ml of water and extracted with hexane to remove cholecalciferol. The enzyme was removed by filtering off the aqueous extract which was then evaporated to dryness to get the unreacted carbohydrate and product glycosides. Work-up and isolation of the compound was carried out in dark, as

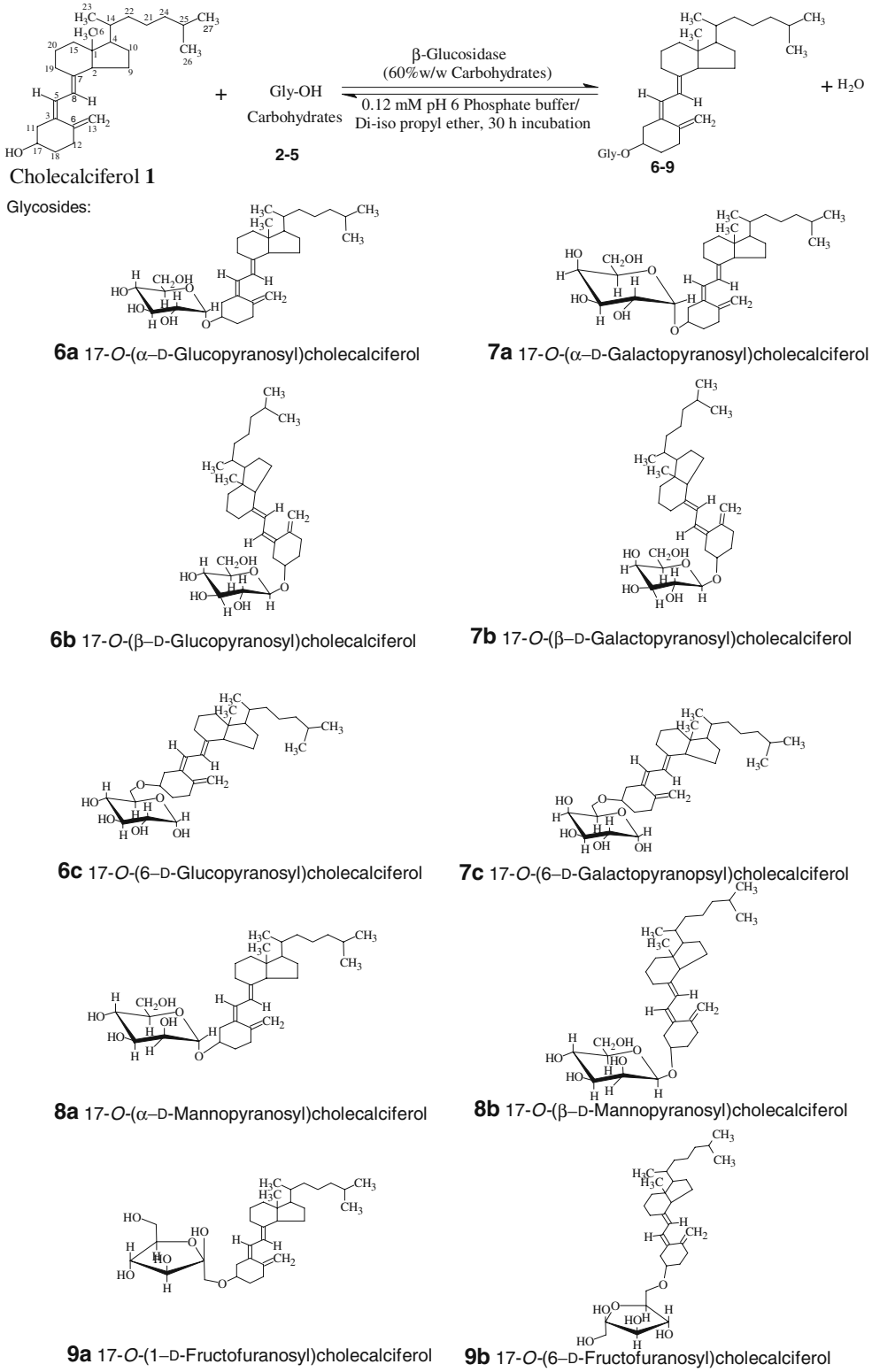

Fig. 1 Synthesis of cholecalciferol glycosides

cholecalciferol is a light sensitive compound. The glycoside was also stored in dark. The dried residue was subjected to HPLC analysis by injecting 20 µl of Phenomenex guard pretreated sample into a 250 mm×4.6 mm aminopropyl column using acetonitrile : water in 70:30 ratio (v/v) as the mobile phase at a flow rate of 1 ml/min and detecting with a refractive index detector. HPLC retention times were: cholecalciferol **1**—3.9 min, D-glucose **2**—6.7 min, D-

Table 2 Spectral data of cholecalciferol-glycosides

Compounds	Spectral data[a]
1. Cholecalciferol-D-glucoside	Solid; UV (H_2O, λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 490.5 M^{-1}), 222.5 nm ($\sigma\rightarrow\pi^*$, $\varepsilon_{222.5}$ 1018 M^{-1}), 265.0 nm ($\pi\rightarrow\pi^*$, ε_{265} – 576 M^{-1}), 293.5 nm ($n\rightarrow\pi^*$, $\varepsilon_{293.5}$ – 576 M^{-1}), 327 nm ($n\rightarrow\pi^*$, $\varepsilon_{327.0}$ 678 M^{-1}). IR (KBr stretching frequency cm^{-1}): 1080 (C-O-C aryl alkyl symmetrical), 1365.7 (C-O-C aryl alkyl asymmetrical), 3382.7 (OH), 1441.8 (aromatic C=C), 2947 (CH). MS (*m/z*) 548 $[M]^+$.
17-*O*-C1α	2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13 MHz) **Glu:** 4.35 (H-1α, d, J=3.5 Hz), 3.49 (H-2), 3.11 (H-4); **Cho:** 6.16 (H-5), 5.95 (H-8), 1.81 (H-9), 1.52 (H-10), 1.92 (H-11), 2.33 (H-12), 0.67 (H-16), 3.56 (H-17), 2.05 (H-19), 1.19 (H-22a), 1.42 (H-22b), 1.12 (H-23), 1.36 (H-24), 0.91 (H-26), 0.91 (H-27); ^{13}C NMR δ_{ppm} (125 MHz) **Glu:** 95.2 (C-1), 71.4 (C-4), 71.8 (C-5); **Cho:** 46.7 (C-1), 54.3 (C-2), 136.1 (C-3), 58.4 (C-4), 128.3 (C-8), 28.0 (C-10), 50.6 (C-11), 30.3 (C-12), 124.1 (C-13), 11.3 (C-16), 60.0 (C-17), 31.6 (C-18), 37.8 (C-22), 19.7 (C-23), 24.5 (C-25).
17-*O*-C1β	^{1}H NMR δ_{ppm} **Glu:** 5.01 (H-1β, d, J=7.5 Hz), 2.76 (H-2), 2.93 (H-3); ^{13}C NMR δ_{ppm} **Glu:** 103.3 (C-1), 76.5 (C-2), 78.6 (C-3).
6-*O*-C1α	^{1}H NMR δ_{ppm} **Glu:** 4.46 (H-1), 3.67 (H-6) ^{13}C NMR δ_{ppm} **Glu:** 68.1 (C-6).
2. Cholecalciferol-D-galactoside	Solid; UV (H_2O, λ_{max}): 192.0 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{192.0}$ – 408.5 M^{-1}), 226.0 nm ($\sigma\rightarrow\pi^*$, $\varepsilon_{226.0}$ 480.8 M^{-1}), 270.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{270.0}$ – 574.5 M^{-1}), 282.0 nm ($n\rightarrow\pi^*$, $\varepsilon_{282.0}$ – 601 M^{-1}), 326.0 nm ($n\rightarrow\pi^*$, $\varepsilon_{326.0}$ 693 M^{-1}). IR (KBr stretching frequency cm^{-1}): 1060 (C-O-C aryl alkyl symmetrical), 1385 (C-O-C aryl alkyl asymmetrical), 3302.8 (OH), 1461.2 (aromatic C=C), 2902 (CH). MS (*m/z*) 548 $[M]^+$.
17-*O*-C1α	2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13 MHz) **Gal:** 4.25 (H-1α, d, J=3.8 Hz), 3.49 (H-2), 3.61 (H-3), 3.71 (H-4); **Cho:** 6.21 (H-5), 6.05 (H-8), 1.72 (H-9), 1.57 (H-10), 1.89 (H-11), 2.33 (H-12), 0.68 (H-16), 3.56 (H-17), 2.15 (H-19), 1.26 (H-22a), 1.21 (H-22b), 1.12 (H-23), 1.38 (H-24), 0.95 (H-26), 0.95 (H-27); ^{13}C NMR δ_{ppm} (125 MHz) **Gal:** 95.7 (C-1), 69.7 (C-3), 71.3 (C-5); **Cho:** 46.8 (C-1), 53.5 (C-2), 137.1 (C-3), 58.4 (C-4), 127.5 (C-8), 25.8 (C-10), 51.2 (C-11), 30.3 (C-12), 125.3 (C-13), 11.8 (C-16), 60.2 (C-17), 32.0 (C-18), 37.2 (C-22), 20.1 (C-23), 23.5 (C-25).
17-*O*-C1β	^{1}H NMR δ_{ppm} **Gal:** 5.01 (H-1β, d, J=6.2 Hz), 3.11 (H-3), 3.41 (H-5); ^{13}C NMR δ_{ppm} **Gal:** 102.1 (C-1), 75.1 (C-3), 76.8 (C-5).
6-*O*-C1α	^{1}H NMR δ_{ppm} **Gal:** 4.61 (H-1), 3.25 (H-6) ^{13}C NMR δ_{ppm} **Gal:** 65.8 (C-6).
3. Cholecalciferol-β-D-mannoside	Solid; UV (H_2O, λ_{max}): 191.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{191.5}$ – 407.4 M^{-1}), 272.0 nm ($\sigma\rightarrow\pi^*$, $\varepsilon_{272.0}$ 578.7 M^{-1}), 291.0 nm ($n\rightarrow\pi^*$, $\varepsilon_{291.0}$ – 619 M^{-1}), 327.5 nm ($n\rightarrow\pi^*$, $\varepsilon_{327.5}$ 696 M^{-1}). IR (KBr stretching frequency cm^{-1}): 1071 (C-O-C aryl alkyl symmetrical), 1355 (C-O-C aryl alkyl asymmetrical), 3326.5 (OH), 1461.4 (aromatic C=C), 2908 (CH). MS (*m/z*) 546 $[M-2]^+$.
17-*O*-C1α	2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13 MHz) **Man:** 5.01 (H-1α, d, J=1.6 Hz), 3.53 (H-2), 3.54 (H-3), 3.51 (H-5); **Cho:** 6.26 (H-5), 5.84 (H-8), 1.92 (H-9), 1.56 (H-10), 1.96 (H-11), 2.35 (H-12), 0.68 (H-16), 3.52 (H-17), 2.02 (H-19), 1.14 (H-22a), 1.46 (H-22b), 1.15 (H-23), 1.32 (H-24), 0.92 (H-26), 0.92 (H-27); ^{13}C NMR δ_{ppm} (125 MHz) **Man:** 96.1 (C-1), 74.1 (C-2), 71.2 (C-3); **Cho:** 47.2 (C-1), 54.3 (C-2), 136.3 (C-3), 58.2 (C-4), 128.2 (C-8), 28.2 (C-10), 51.1 (C-11), 30.5 (C-12), 124.2 (C-13), 11.8 (C-16), 60.1 (C-17), 31.5 (C-18), 37.4 (C-22), 19.5 (C-23), 24.4 (C-25).
17-*O*-C1β	^{1}H NMR δ_{ppm} **Man:** 4.59 (H-1β, d, J=2.8 Hz), 3.35 (H-2), 3.29 (H-3), 3.48 (H-6); ^{13}C NMR δ_{ppm} **Man:** 101.8 (C-1), 71.7 (C-2).
4. Cholecalciferol-D-fructoside	Solid; UV (H_2O, λ_{max}): 190.5 nm ($\sigma\rightarrow\sigma^*$, $\varepsilon_{190.5}$ – 405.3 M^{-1}), 271.5 nm ($\sigma\rightarrow\pi^*$, $\varepsilon_{271.5}$ 577.6 M^{-1}), 291.0 nm ($\pi\rightarrow\pi^*$, $\varepsilon_{291.0}$ – 619 M^{-1}), 327.5 nm ($n\rightarrow\pi^*$, $\varepsilon_{327.5}$ – 696 M^{-1}). IR (KBr stretching frequency cm^{-1}): 1061 (C-O-C aryl alkyl symmetrical), 1391 (C-O-C aryl alkyl asymmetrical), 3308.5 (OH), 1421.7 (aromatic C=C), 2981 (CH). MS (*m/z*) 587 $[M+K]^+$.
17-C1-*O*	2D-HSQCT (DMSO-d_6) ^{1}H NMR δ_{ppm} (500.13 MHz) **Fru:** 3.82 (H-1), 3.62 (H-3), 3.68 (H-5); **Cho:** 5.98 (H-5), 5.90 (H-8), 1.88 (H-9), 1.58 (H-10), 1.97 (H-11), 2.35 (H-12), 0.71 (H-16), 3.49 (H-17), 2.15 (H-19), 1.17 (H-22a), 1.44 (H-22b), 1.14 (H-23), 1.32 (H-24), 0.98 (H-26), 0.98 (H-27); ^{13}C NMR δ_{ppm} (125 MHz) **Fru:** 63.9 (C-1), 104.5 (C-2), 68.53 (C-3), 71.7 (C-4), 71.1 (C-5); **Cho:** 46.6 (C-1), 54.8 (C-2), 136.1 (C-3), 58.5 (C-4), 128.5 (C-8), 28.1 (C-10), 50.4 (C-11), 30.8 (C-12), 124.2 (C-13), 11.4 (C-16), 60.5 (C-17), 31.5 (C-18), 37.2 (C-22), 19.4 (C-23), 24.6 (C-25).
17-C6-*O*	^{1}H NMR δ_{ppm} **Fru:** 3.87, 3.71 (H-5), 3.53 (H-6); ^{13}C NMR δ_{ppm} **Fru:** 64.2 (C-1), 98.1 (C-2), 68.7 (C-3), 72.7 (C-4), 63.2 (C-6).

NMR spectra data were recorded for solutions in DMSO-d_6 at 35 °C.

[a] Chemical shifts are expressed in ppm. Some of the assignments are interchangeable. ^{1}H NMR recorded at 500.13 MHz and ^{13}C NMR at 125 MHz frequencies

Table 3 β-Glucosidase catalyzed syntheses of cholecalciferol glycosides

S.No	β-Glucosidase catalysis	Product proportion[a] (%)	Conversion Yields[b] (%)
1	**6a** 17-*O*-(α-D-Glucopyranosyl)cholecalciferol	17-*O*-α (34)	14
	6b 17-*O*-(β-D-Glucopyranosyl)cholecalciferol	17-*O*-β (48)	
	6c 17-*O*-(6-D-Glucopyranosyl)cholecalciferol	17-*O*-6 (18)	
2	**7a** 17-*O*-(α-D-Galactopyranosyl)cholecalciferol	17-*O*-α (29)	30
	7b 17-*O*-(β-D-Galactopyranosyl)cholecalciferol	17-*O*-β (56)	
	7c 17-*O*-(6-D-Galactopyranosyl)cholecalciferol	17-*O*-6 (15)	
3	**8a** 17-*O*-(α-D-Mannopyranosyl)cholecalciferol	17-*O*-α (52)	26
	8b 17-*O*-(β-D-Mannopyranosyl)cholecalciferol	17-*O*-β (48)	
4	**9a** 17-*O*-(1-D- Fructofuranosyl)cholecalciferol	17-*O*-α (42)	13
	9b 17-*O*-(β-D-Fructofuranosyl)cholecalciferol	17-*O*-β (58)	

[c] Product proportions were determined from the area of respective $^{1}H/^{13}C$ signals. Conversion yields were from HPLC with respect to the carbohydrate. Error in yield measurements is ±5%

galactose **3**—6.9 min, D-mannose **4**—6.5 min and D-fructose **5**—6.3 min, 17-*O*-(D-glucopyranosyl)cholecalciferol **6a-c**—8.2 min, 17-*O*-(D-galactopyranosyl)cholecalciferol **7a-c**—7.7 min, 17-*O*-(D-mannopyranosyl) cholecalciferol **8a-b**—7.8 min and 17-*O*-(D-fructofuranosyl)cholecalciferol **9a-b**—7.0 min. Conversion yields were determined from HPLC peak areas of glycoside and free carbohydrate with respect to the free carbohydrate employed. Error in HPLC measurements will be ±5%. The glycosides formed were separated through size exclusion chromatography using Sephadex G-10 eluting with water. Eventhough the glycosides were separated from unreacted aglycon and carbohydrates, the individual glycosides could not be separated from their reaction mixtures due to same molecular weight of the glycosides formed.

The isolated glycosides were characterized by recording UV, IR, MS and 2D NMR (HSQCT) spectra, which confirmed the product formation. ^{1}H and ^{13}C NMR spectra were recorded on a Brüker DRX-500 MHz spectrometer (500.13 MHz for ^{1}H and 125 MHz ^{13}C). About 40 mg of the sample dissolved in DMSO-d_6 were used for recording the spectra at 35°C. Chemical shift values were expressed in ppm relative to internal tetramethylsilane standard to within ±0.01 ppm. In the NMR data, only resolvable signals are shown. Some assignments are interchangeable. Since, the compounds are surfactant molecules, they aggregate in the solution to result in broad signals, thus, making it difficult to resolve the coupling constant values accurately. NMR data clearly confirmed the formation.

Results and discussion

17-O-(D-Glucopyranosyl)cholecalciferol β-Glucosidase catalyzed synthesis of 17-*O*-(D-glucopyranosyl) cholecalciferol **6a-c** was optimized in terms of pH, buffer concentration, enzyme concentration and incubation period (Fig. 1, Tables 1 and 2). This enzymatic reaction did not take place without the presence of enzymes under the reaction conditions employed. Under optimum conditions 60% (w/w D-glucose) β-glucosidase gave a maximum yield of 14% at 0.12 mM (1.2 ml) pH 6 phosphate buffer in 30 h incubation period. Under the above mentioned optimum conditions, synthesis of cholecalciferol glycosides was attempted with other carbohydrate molecules also to evaluate the propensity of glycosylation by β-glucosidase with diverse carbohydrate molecules, namely: aldohexoses—D-glucose **2**, D-galactose **3** and D-mannose **4**, ketohexose—D-fructose **5**, pentoses—D-ribose and D-arabinose, disaccharides—maltose, lactose and sucrose and sugar alcohols—D-sorbitol and D-mannitol. Of these cholecalciferol **1** reacted only with D-glucose **2**, D-galactose **3**, D-mannose **4** and D-fructose **5** (Table 3).

Spectral characterization

UV spectra of cholecalciferol glycosides, showed shifts in the σ→σ* band in the 190.5 to 192.0 nm (191.0 nm for cholecalciferol) range, σ→π* band in the 222.5–226.5 nm range, π→π* in the 268.5–272.0 nm (291 nm for cholecalciferol) range and n→π* band in the 282.5–327.5 nm (323 nm for cholecalciferol) range. IR spectra showed shifts in the 1060–1080 cm^{-1} range for the glycosidic C-O-C aryl alkyl symmetrical stretching and in the 1355–1391 cm^{-1} range for the asymmetrical stretching frequencies. In 2D-HSQCT spectra, the respective chemical shift values showed glycoside formation (Table 2): from D-glucose **2** C1α glucoside **6a** to C1α at 95.2 ppm and H-1α at 4.35 ppm, C1β glucoside **6b** to C1β at 103.2 ppm and H-1β at 5.01 ppm and 6-*O*-derivatized **6c** C6α at 68.1 ppm and H-6α 3.67; from D-galactose **3** C1α galactoside 7**a** to C1α at 95.7 ppm and H-1α at 4.25 ppm and C1β galactoside 7**b** to C1β at 102.1 ppm and H-1β at 5.01 ppm and 6-*O*-derivatized **7c** to C6α at 65.8 ppm and H-6α at 3.25; from D-mannose **4** C1α mannoside **8a** to C1α at 96.1 ppm and H-1α at 5.01 ppm and C1β mannoside **8b** to C1β at 101.8 ppm and H-1β at

4.59 ppm; from D-ribose **5**C1 fructoside **9a** to C1 at 63.9 ppm and H-1 at 3.82 ppm and C6 fructoside **9b** to C6 at 63.2 ppm and H-1β at 3.53 ppm. The carbon chemical shift value at 60 ppm (59.7 ppm for free cholecalciferol) indicated that glycosylation occurred at the alicyclic OH group at position 17 of cholecalciferol. Mass spectral data also confirmed product formation.

Glycosylation reaction required some amount of water added as buffer and in these reactions 0.4 ml–2 ml of 0.01 M pH 4–8 buffer was added thereby conducting the reaction under low water activity. The reactions were conducted under reflux at the boiling temperature of a non-polar solvent, di-isopropyl ether at 68 °C, as it was found to be the best solvent for this reaction both in terms of boiling point and non-polar nature as it gave the maximum yield under the reaction conditions employed. At a boiling temperature of 68 °C, the enzymes were stable, losing only about 20% of their activity during an incubation period of 50 h. Refluxing di-isopropyl ether during the incubation periods up to 50 h did not also produce peroxides in these glycosylation reactions.

β-Glucosidase isolated from sweet almond was entrapped into calcium alginate beads and used in the glycosylation reaction to facilitate recovery and reuse. In the reaction mixture, the enzymes dissolved in small volume of the buffer employed. They did not get precipitated by the di-isopropyl ether solvent. During workup the solvent in the reaction mixture was distilled off leaving a residue consisting of the enzyme, product and the unreacted substrates. Hence, the amount of the enzyme leached out into the buffer or the di-isopropyl ether solvent could not be determined. Reuse of the calcium alginate bead entrapped β-glucosidase showed 30% reduction in activity, which could be due to loss of the enzyme.

Optimum pH for and β-glucosidase catalyses was found to be 6.0 and 5 at 0.12 mM of buffer. Glucoamylase gave moderate conversion in 30 h. Higher incubation periods resulted in hydrolysis of glycosides. β-Glucosidase 60% (w/w D-glucose) gave 14% conversion.

About 10 glycosides were synthesized enzymatically using both the glucosidases, of which 7 are being reported for the first time (Table 1) namely: 17-*O*-(α-D-galactopyranosyl)cholecalciferol **7a**, 17-*O*-(β-D-galactopyranosyl) cholecalciferol **7b**, 17-*O*-(6-D-galactopyranosyl)cholecalciferol **7c**, 17-*O*-(α-D-mannopyranosyl) cholecalciferol **8a**, 17-*O*-(β-D-mannopyranosyl)cholecalciferol **8b**, 17-*O*-(1-D- fructofuranosyl)cholecalciferol **9a** and 17-*O*-(β-D-fructofuranosyl)cholecalciferol **9b**.

Synthesis of other cholecalciferol glycosides (Table 3) showed that apart from the three aldohexoses and aldopentose employed, D-ribose, D-arabinose, maltose, sucrose, lactose, D-sorbitol and D-mannitol did not undergo glycosylation under the conditions employed. This could be due to inhibitory nature of cholecalciferol **1** to the glucosidases compared to such unreactive carbohydrate molecules. In a competitive inhibition differential binding between cholecalciferol **1** and unreacted carbohydrate molecules could result in no reaction especially if cholecalciferol **1** binds strongly to the enzyme compared to the unreacted carbohydrate molecules. Further carbohydrate binding to the enzyme and transfer of the carbohydrate to the alicyclic nucleophilic OH of cholecalciferol **1** will be prevented, thereby affecting the glycosylation. Although the reactions were conducted with disaccharides maltose, lactose and sucrose, transglycosylation did not occur under the conditions employed. However, the disaccharides were hydrolyzed by the enzymes, the reason for which is not known.

The observed conversion yields were in the 3–14% range. Glycosylation is the reverse of hydrolysis. Even though a non-polar solvent like di-isopropyl ether and a very high concentration of the enzyme were employed, the yields were low. The enzymatic reaction is both kinetically and thermodynamically controlled. The low yields obtained indicate that the substrate, cholecalciferol, is bound strongly to the active site of the enzyme, thereby not only require a high concentration of the enzyme but also result in a low conversion yield.

In case of D-glucose **2** and D-galactose **3** C-6 derivatives were also detected. Anomeric proportions were found to be greatly altered. In D-glucose **2** the proportion of 52:48 is different from that of free D-glucose **2** (60:40). However in D-galactose **3** β anomer has been formed (45:56) compared to 92:8 of free D-galactose **3**. In D-mannose **4**, more of α-glycoside is formed (46:54) compared to (27:73) free D-mannose **4**. The formation of α and β anomeric mixture is due to the α-glucosidase present in sweet almond isolate, which exhibited an activity of 359.7 AU- (μmol/min.mg enzyme preparation).

Thus through glycosylation, a water insoluble cholecalciferol (vitamin D_3) was transformed into a water soluble cholecalciferol glycoside in this study. It is therefore envisaged that the water solubility could enhance the pharmacological applications of vitamin D_3.of glycosides.

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