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Phenolic group on A-ring is key for dracoflavan B as a selective noncompetitive inhibitor of α-amylase

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ABSTRACT

A high throughput assay was applied to guide the isolation of a new pancreatic α-amylase inhibitor, dracoflavan B, from the dragon's blood resin from *Daemonorops draco*. Applying C18 column, we successfully isolated both diastereomers and their structures verified by ^{1}H NMR spectra in comparison with the literature values. Their activity in inhibition of pancreatic α-amylase with comparable IC_{50} values of 23 μM (A type) and 27 μM (B type) that are similar to that of acarbose. Dracoflavan B shows much weaker activity in inhibiting bacterial α-amylase and no activity towards fungal α-amylase. Moreover, both isomers show no inhibitory activity towards mammalian α-glucosidase. Kinetic analysis revealed that using starch as the substrate, dracoflavan B was a non-competitive α-amylase inhibitor with a K_i value of 11.7 μM. Lack of α-amylase inhibition for proanthocyanidin A2 dimer demonstrated that dracoflavan B hydrophobic nature of the B, A′, C′ and B′ rings are important for its α-amylase inhibition. In addition, selective chemical modification studies revealed that the phenolic group is also vital to dracoflavan B for its pancreatic α-amylase inhibition activity. Without the A ring phenolic hydrogen bond donor, the derivatives of dracoflavan B showed detrimental α-amylase inhibition. On the contrary, galloylation on the A ring phenolic OH group enhanced the activity as shown by the low IC_{50} (12 μM) against α-amylase which is 56% more potent as compared to dracoflavan B.

1. Introduction

Type II diabetes is one of the most serious chronic diseases associated with alarmingly increased rate due to aging population, unhealthy diets, and lifestyle.[1,2] For diabetic patients, controlling postprandial hyperglycemia is one of the main therapeutic targets. One way to do so is to reduce starch digestion rates by consuming low glycemic index (GI) foods or intake of starch hydrolase inhibitors.[3,4] Extensive research efforts have been focused on discovery of plant based starch hydrolase inhibitor as active ingredients for low GI foods.[5,6] Pancreatic α-amylase is a key enzyme in the digestive system in catalyzing the initial step in hydrolysis of starch to maltodextrin and oligosaccharides, which are degraded to glucose by brush border α-glucosidase in the small intestine.[7] Modulation of pancreatic α-amylase activity through the therapeutic use of inhibitors would be of considerable medical relevance in prevention of postprandial hyperglycemia. Depending on the chemical nature, there are two types of α-amylase inhibitors, proteinaceous[8,9] and nonproteinaceous. The former are polypeptides isolated from plant, microbes, and animal and normally have high inhibition potency with IC_{50} in nanomolar concentration (cf. μM range for non-proteinaceous ones).[10] However, the application of the proteinaceous inhibitors in mitigating postprandial hyperglycemia is challenged by their sensitivity to conformational changes due to denaturalization and protease digestion in the gastrointestinal tract. On the other hand, nonproteinaceous inhibitors, particularly pseudosugars such as acarbose, voglibose and miglitol are stable in the gastrointestinal tract and have found clinical application as anti-diabetic drug.[11] They are also potent inhibitors of brush border α-glucosidase and have side effects of flatulence and diarrhea.[12–14] Mechanistically, pseudosugar based inhibitors bind to the active sites of α-amylase and α-glucosidase and thus are competitive or mixed inhibitors as revealed by Michaelis–Menten kinetics analysis.[15]

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Rarely reported are selective α-amylase inhibitors of non-proteinaceous nature. We hypothesize that, selective inhibitor of α-amylase instead of α-glucosidase may reduce the accumulation of fermentable oligosaccharides, which would be a feed stock of gut microflora as it move down the gastrointestinal track and lead to flatulence. Botanicals, particularly traditional medicine, is rich sources of bioactive compounds including starch hydrolase inhibitors. During our search for novel inhibitors from a few hundreds of commercially available botanical materials for traditional medicine use, we found that the crude extract of dragon's blood belonging to *Daemonorops draco* exhibited selective pancreatic amylase inhibitory activity. It is thus an objective of this work in discovering such inhibitors from botanical sources using high throughput screening assay-guided fractionation and isolation of active compounds.

Starch digestion rate by α-amylase are most commonly measured by the dinitrosalicylic acid (DNSA) assay, which relies on the reduction of one of the nitro groups by reducing sugar generated due to starch hydrolysis. The DNSA assay could not be conveniently adapted to a high throughput fashion because the assay requires high temperature (95 °C) and basic media.[16] Therefore, samples of the starch hydrolysis reaction need to be taken during the time course of the kinetic study and measured separately. In addition, recent research work has shown that tea catechins (reducing agent or antioxidants) cause interference in the DNSA assay.[17] Because plant extracts typically contain polyphenolic compound, DNSA assay is not suitable for quantitation of α-amylase inhibition activity of botanical samples. We recently developed a high throughput assay on a 96-well microplate by monitoring turbidity changes during corn starch hydrolysis and applied the assay in studying starch hydrolase inhibition activity of polyphenolic compounds.[18,19] In the process of screening large amount of botanical materials for their potentials as starch hydrolase inhibitors, we discovered a new class of highly selective α-amylase inhibitor isolated from a resin called dragon's blood.

Dragon's blood is a common name used for resins and saps obtained from four plant genera; *Croton* (Euphorbiaceae), *Dracaena* (Dracaenaceae) mainly found in China, *Daemonorops* (Palmaceae), and *Pterocarpus* (Fabaceae).[20] In Southeast Asian countries, the dragon's blood resin is from *Daemonorops draco*. The chemical constituents of dragon blood are very complex,[21] yet there is no study on the activity in starch hydrolase inhibition.

2. Results and discussion

2.1. Assayed-guided isolation of dracoflavan B

We identified the active compounds in *Daemonorops draco* as dracoflavan B (Fig. 1) by high throughput assay-guided fractionation and identification. By applying semi-preparative column chromatography, we were able to separate two isomers with baseline resolution using achiral C_{18} column (Supplementary Fig. 1). The structures of the compounds were identified by comparing their ^{1}H and ^{13}C NMR spectra with that of literature report.[21]

2.2. Activity and selectivity of dracoflavan B on starch hydrolase inhibition

Figure 2 showed the time courses of starch digestion in the presence or absence of dracoflavan B (mixture of two diastereomers) by α-amylase and α-glucosidase, respectively. While good activity is observed for α-amylase, there is no detectable activity for α-glucosidase even at concentration as high as 370 μM. To The area under the kinetic curve is used to illustrate concentration response and with IC_{50} of 23 μM (A type isomer) and 27 μM (B type isomer) (Fig. S2). This values is comparable to that of acarbose (IC_{50} of 28 μM, for α-amylase) measured under the same conditions) (Fig. S3). However, acarbose is stronger inhibitor for intestinal α-glucosidase. In addition, while acarbose acts as competitive inhibitor for α-amylase, dracoflavan B is a noncompetitive inhibitor (vide infra).

It is known that α-amylase from different biological species have significantly different structural motif and an inhibitor that work on mammal α-amylase may not be active towards α-amylases from other species. Therefore, we determined the inhibition activity of dracoflavan B towards bacteria (*Bacillus subtilis*) and fungal (*Aspergillus oryzae*) α-amylase. The results showed that dracoflavan B is a little weaker inhibitor for bacterial α-amylase (IC_{50} = 42 μM) and completely inactive towards fungal α-amylase (Fig. S4). This indicates that dracoflavan B may inhibit gut microflora in digestion of starch and hence further reduce the digestibility of starch. Our results further highlight the importance of choosing the relevant enzymes for screening of α-amylase inhibitors as bioactive constituents for human use as anti-diabetic medicine.

2.3. Inhibition kinetics and mechanism

From the inhibition kinetics of dracoflavan B on pancreatic α-amylase, dracoflavan B showed a non-competitive inhibition against α-amylase (Fig. 3) with the inhibition constant (K_i) value was determined to be 11.7 μM. The structure of hydrophobic dracoflavan B does not bear any similarity to that of the hydrophilic starch molecule and thus it is reasonable for dracoflavan B not acting as a competitive inhibitor against α-amylase. Dracoflavan B has a ring skeleton of A type proanthocyanidin (e.g., A2 dimer) which are present in large amount in plant kingdom. However, A2 dimer has excessive phenolic hydroxyl groups on the B, A′, C′ and B′ rings as compared to dracoflavan B (Fig. 1). Our investigation showed that A2 dimer has no inhibition towards α-amylase. This highlights that the hydrophobic nature of B, A′, C′ and B′ rings of dracoflavan B are essential for its binding with the α-amylase.

2.4. Molecular docking study

To further understand the binding site of α-amylase for dracoflavan B, we carried out molecular docking study by using Sitemap to locate the allosteric binding sites (BSs) using the α-amylase enzyme-substrate complex. An ideal SiteScore of 1.0 indicates that the binding site possesses the greatest probability and suitability as a binding site. Four sites were located and a SiteScore was assigned to each BS. BS 1 obtained the highest score of 0.857 and therefore was selected for further molecular docking studies (Fig. 4).

Docking of dracoflavan B in BS 1 was conducted using the XP scheme of Glide where the various interactions (Table 1) between the ligand and the protein are explicitly calculated to obtain information on the possible major interactions that might aid in binding affinity. The lower the values for the docking score or the intermolecular interactions, the more favorable the interaction is.

From Table 1, it is observed that B type dracoflavan B binds to the protein with a little greater affinity than A. From the values obtained from the various intermolecular interactions, both diastereomers exhibit similar extents of hydrogen bonding and vdW interactions. However, B type isomer possesses less coulombic interactions with the protein but compensated by having less solvent exposure. The additional coulombic interaction formed by A type dracoflavan B can be seen from Figure 5 where one of the phenyl moiety forms a π–π interactions with Phe348 whereas such an interaction was not observed in B type dracoflavan B. Experimentally, however, there is no measurable difference on

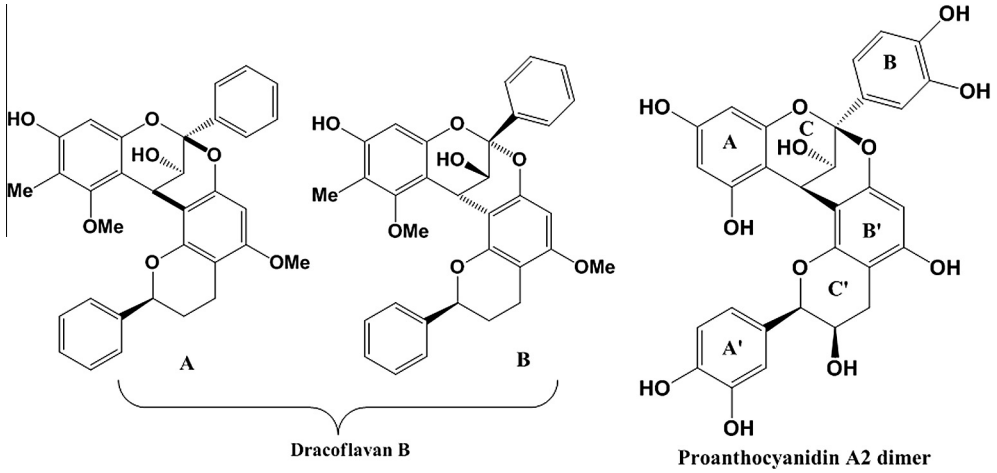

Figure 1. Chemical structures of dracoflavan B (diastereomer A and B) and proanthocyanidin A2 dimer, which is more commonly found in nature and shares the same ring skeleton.

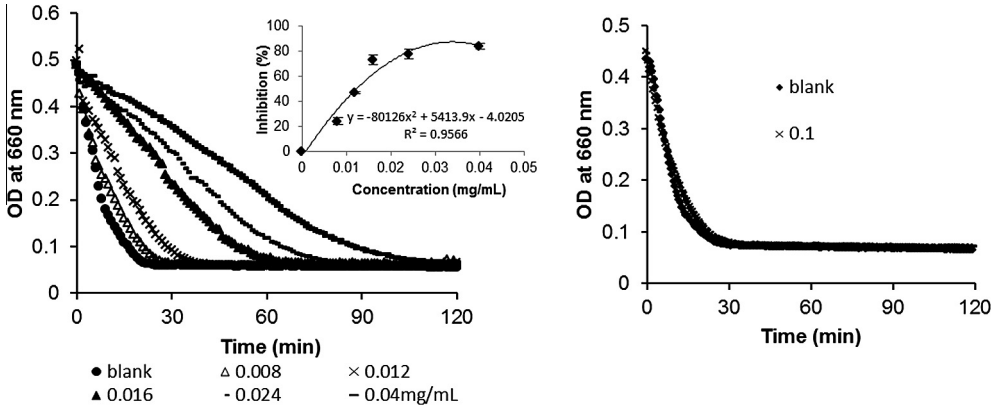

Figure 2. Turbidity change at different concentrations of dracoflavan B (mixtures of two diastereomers) that reflects dracoflavan B's inhibitory effect on starch hydrolysis by left: α-amylase; and right: α-glucosidase.

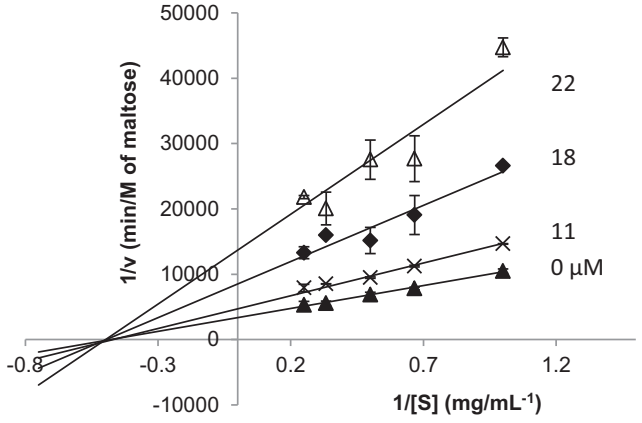

Figure 3. Lineweaver–Burk plots of the inhibition kinetics of porcine pancreatic α-amylase by dracoflavan B under various concentrations.

the inhibitory activity between the two diastereomers. Nonetheless, docking study provides information on the binding sites of dracoflavan B which is in agreement with that of kinetic study showing that dracoflavan B is a non-competitive inhibitor. The hydrophobic nature dictates its binding with α-amylase as revealed by docking study.

Dracoflavan B has a same ring skeleton as that of proanthocyanidin A2, which is present in large amount in plant kingdom. However, proanthocyanidin A2 does show measurable α-amylase inhibitory activity. Molecular docking study on interaction of proanthocyanidin A2 and that of α-amylase revealed that proanthocyanidin A2 does not bind in the same pocket as that of dracoflavan B. Instead, proanthocyanidin A2 interacts with a domain C of the enzyme (Fig. 6) through hydrogen bonding interactions between the catecholic B′ ring with TYR468 and GLN476 and the C(7)-OH group at A ring with SER478 and hydrophobic interactions. The binding apparently does not compromise the enzyme activity.

2.5. Preparation and characterization of dracoflavan B derivatives

Reaction of dracoflavan B with respective acylating reagents resulted in **1**–**5**, respectively (Fig. 7). Their structures were characterized by ^{1}H NMR spectra and high resolution mass spectra (shown in Supporting information). Compound **1** showed methyl protons at 2.26–2.28 (s) ppm. Compound **2** exhibited new aromatic protons due to benzoyl group at 7.59–7.61 (t), 7.71–7.72 (t) and 8.18–8.21 (d) ppm. For **4**, new methyl and aromatic protons corresponding to acetyl gallo group were observed at 2.32–2.36 (s) and 7.95–7.98 (s) ppm respectively. After deprotecting acetyl groups of

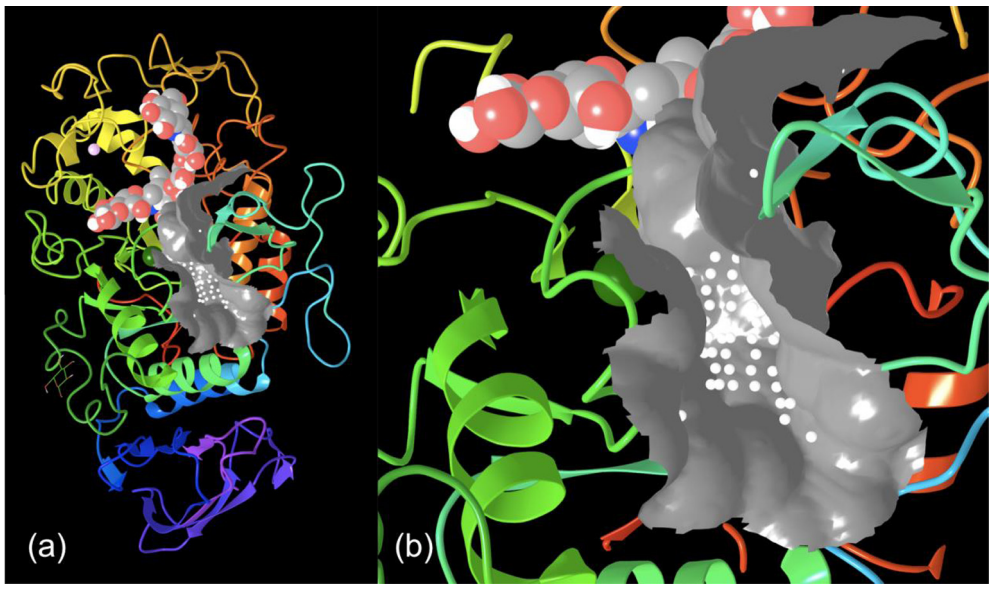

Figure 4. (a) Location of BS 1 within α-amylase. (b) A zoomed-in view of BS 1. Dummy atoms are shown in white to illustrate the depth of BS 1.

Table 1
Docking score of dracoflavan B in BS 1

	Diastereomer	Docking score	Hydrogen bonding	vdW	Coulombic interactions	Solvent exposure
Site 1	A	−3.62	−1.0	−30.0	−6.1	0.52
	B	−4.47	−1.3	−31.4	−3.9	0.26

All calculated values are in kcal mol^{-1}.

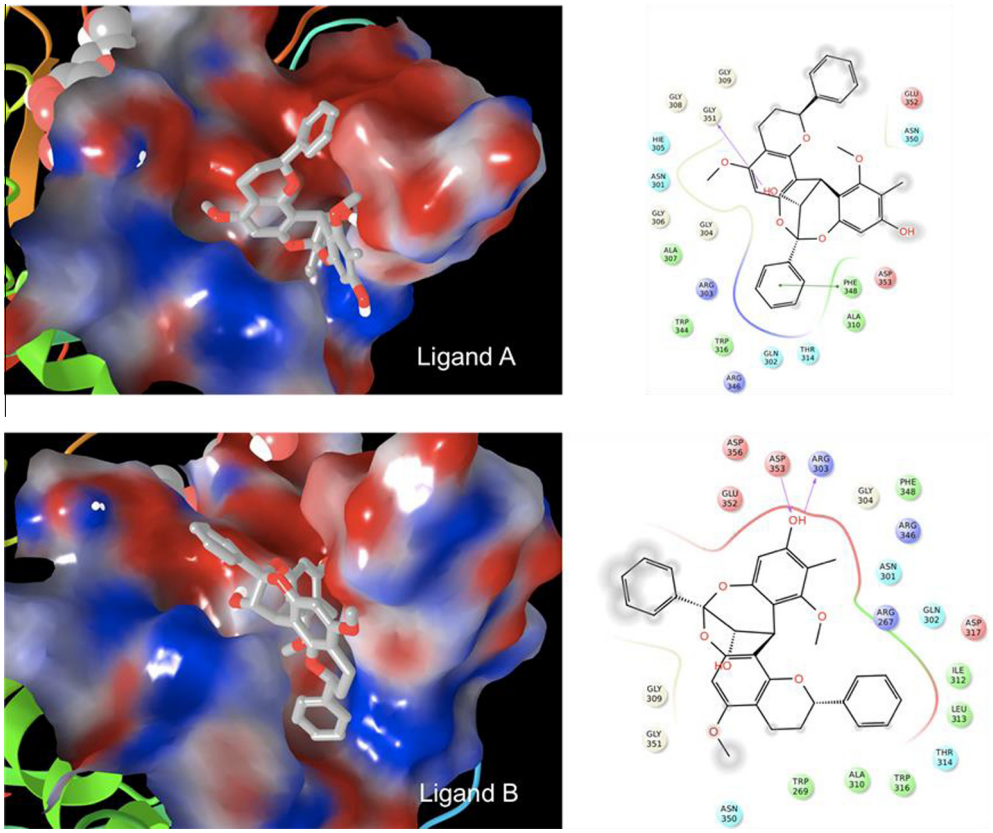

Figure 5. (Left) Binding conformation of dracoflavan B diastereomers in BS 1 shown as molecular surface. Regions in red represent electronegative areas, regions in blue represent electropositive areas, and white areas are neutral. (Right) 2-D ligand interaction diagrams of dracoflavan B diastereomers in BS 1.

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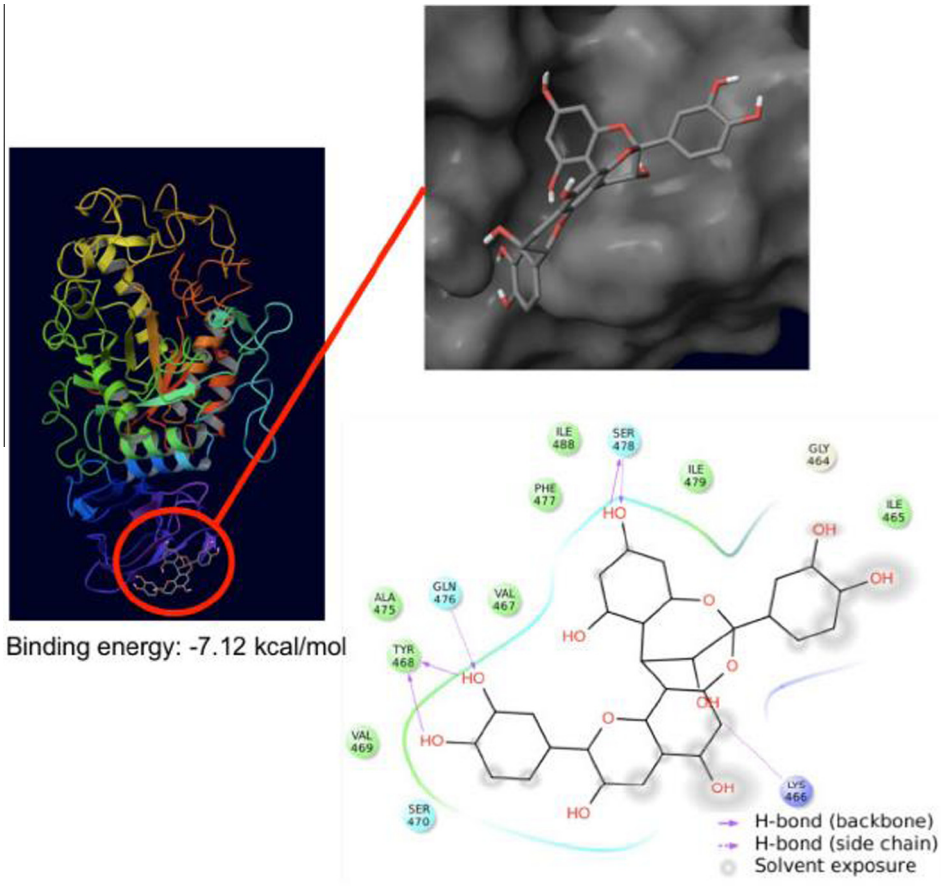

Figure 6. Molecular docking results of binding sites of proanthocyanidin A2 with α-amylase shows that the binding site is different from that of dracoflavan B.

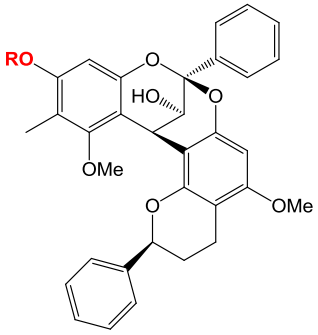

Figure 7. Chemical structures of dracoflavan B derivatives. **1**, RO = CH_3CO_2; **2**, RO = $PhCO_2$; **3**, RO = SO_4^-; **4**, RO = 3,4,5-triacetylbenzoyl; 5, RO = galloyl.

4, **5** was obtained and its phenol protons on the galloyl group was observed at 7.26–7.28 (s) ppm.

2.6. Structural and activity relationship of dracoflavan B and its α-amylase inhibition

To probe the structural activity relationship between dracoflavan B and its α-amylase inhibition, potential interaction group on dracoflavan B in particular the phenolic hydroxyl was modified to a more hydrophobic ester **1**, **2** or more hydrophilic with a charged sulfate group in **3**. To our surprise, **1**, **2** and **3** completely lose inhibitory activity against α-amylase inhibition in Figure 8. The result demonstrated that phenolic group is essential for the α-amylase inhibition of dracoflavan B. Apparently, dracoflavan B uses its phenolic group as hydrogen bond donor to interact with hydrogen bonding acceptors of α-amylase binding site and this binding is critical for its inhibition activity. With this finding, we hypothesize that, if more OH group is attached through the phenol group, we may be able to increase the inhibition activity, provided the steric factor does not have negative effect on binding to the enzyme. To verify our hypothesis, dracoflavan B's phenolic hydroxyl group was further esterified with gallic acid to give **5**. Indeed, the IC_{50} value of **5** against α-amylase was determined to be 12 μM which is 56% more potent in comparison to dracoflavan B. In sharp contrast, precursor **4** shows no measurable α-amylase inhibition activity (Fig. 8). The result highlighted that the hydrogen bond donors on the galloyl moiety of **5** are crucial for improving the α-amylase inhibition.

Mother Nature is crafty in making α-amylase and α-glucosidase inhibitors with diverse structural motif. The most intensively studies ones are those with pseudo-sugars such as acarbose, 1-deoxynojirimycin and its structural analogs, and kotalanol. The common features of these pseudosugar inhibitor is that they are all competitive inhibitor and highly potent towards α-glucosidase. They not only mimic the substrate structure (such as sucrose) but, under physiological pH, are positively charged, and enhance the charge attraction with catalytic active sites of starch hydrolases, which are negatively charge due to the presence of deprotonated carboxylate. The inhibitory activity of kotalanol is highly sensitive to the stereochemistry of sugar side chain.[22] Although there are proteinaceous α-amylase inhibitors,[23] it is very rare to have selective inhibitor of α-amylase (in comparison with α-glucosidase inhibition) of secondary metabolites isolated so far. Dracoflavan B represents a new type of inhibitor for starch hydrolysis and

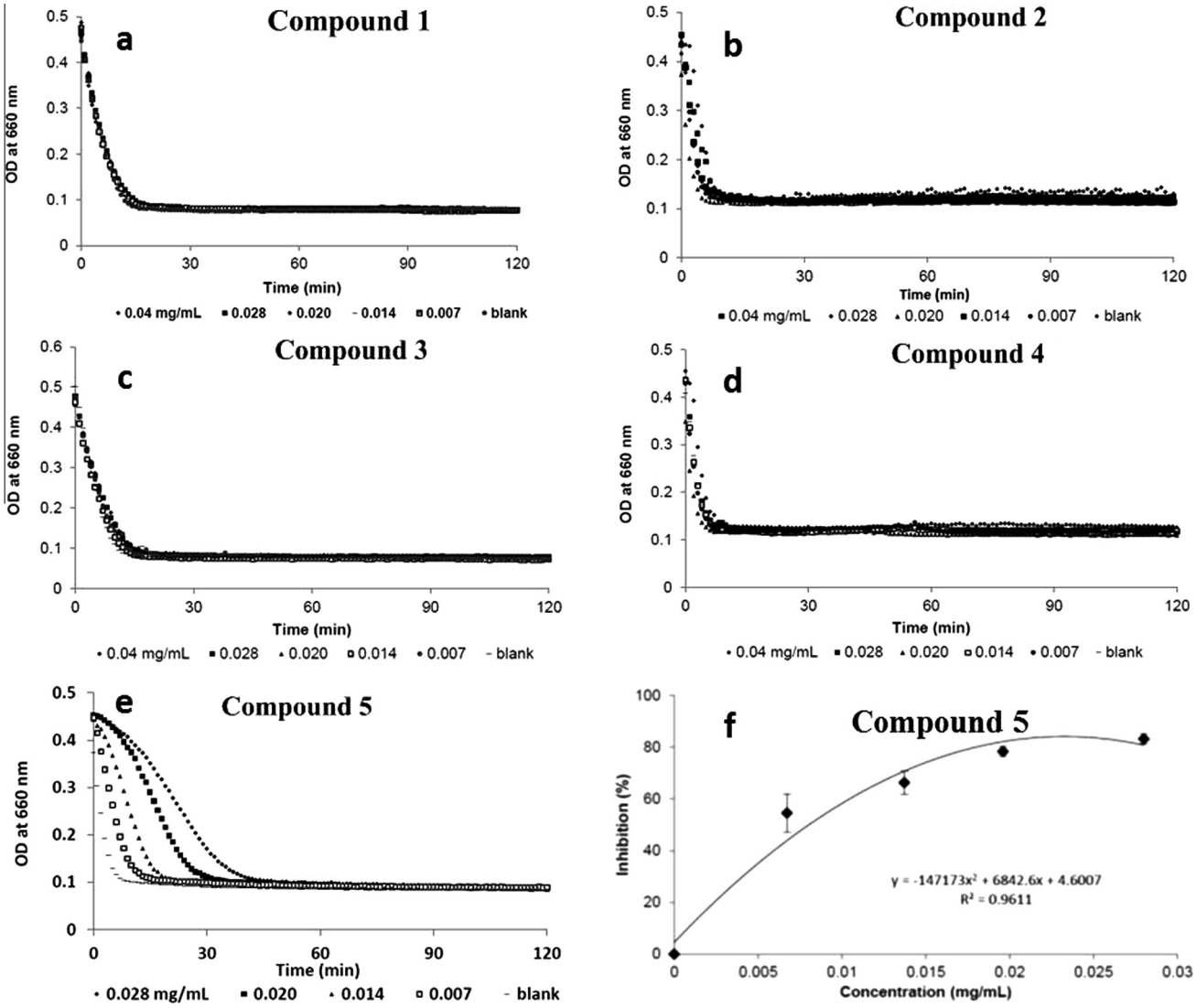

Figure 8. Dracoflavan B derivatives, as a mixture of a and b, inhibition against porcine pancreatic α-amylase. a, compound **1**; b, compound **2**; c, compound **3**; d, compound **4**; e, compound **5**, f, dose response of compound **5** against α-amylase inhibition.

warrants further study to realize its potential as bioactive compound for controlling postprandial blood glucose concentration in a normal range.

3. Experimental

3.1. Materials and instruments

Dragon's blood resin was purchased from the local medical hall. Porcine pancreatic α-amylase, α-glucosidase in the form of rat intestine acetone powder, corn starch, 3,5-dinitrosalicyclic acid, acarbose and maltose were obtained from Sigma–Aldrich Chemical Co. (St Louis, MO). ^{1}H NMR was recorded using Advance 300 MHz Bruker spectrometer in $(CD_3)_2CO$. Column chromatography was performed on silica gel 60 (Merck 40–60 nm, 230–400 mesh) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). The electrospray ionization mass spectra were obtained from a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The heated capillary and voltage were maintained at 250 °C and 4.5 KV, respectively. The full-scan mass spectra from *m*/*z* 50–2000 were recorded. UV–vis absorption spectra were recorded with a Shimadzu UV-1601 UV–visible spectrophotometer and they were acquired at room temperature in a 1-cm path length 1.5 mL quartz cuvette.

3.2. Preparation of dragon's blood extract

Prior to extraction, the samples were ground into powder. For the methanol extract, dragon's blood (100 g) was added to methanol (3 × 1 L) with shaking at room temperature for 6 h. The solids were then removed by filtration.

3.3. Isolation of dracoflavan B with α-amylase inhibitory activity

The methanol extract from dragon's blood was dried at 40 °C by rotary evaporation. Subsequently, 3 g of methanol extract was fractionated by silica gel chromatography (gradient eluent/chloroform-acetone-methanol) to obtain nine fractions (Fr A–I). Each fraction was tested on their α-amylase inhibition activity. Only Fr C (chloroform: acetone; 10:1) exhibited the desirable α-amylase inhibitory activities. Hence, fraction C was further separated by silica gel chromatography (gradient eluent/hexane-chloroform). Twelve fractions were obtained and Fr 10 (chloroform/hexane;

10:1) was the only active fraction. The Fr 10 was further purified by Sephadex LH-20 column (isocratic chloroform/methanol; 1:1) to obtain dracoflavan B (15 mg). Structural determination of the A and B type dracoflavan B (Fig. 1) were made by HR-ESI-MS and ^{1}H NMR, which matches nicely with literature values of the same compound isolated from *Daemonorops draco*.[21,24]

3.4. Separation of the dracoflavan B diastereomers

Dracoflavan B diastereomers were purified using 250 mm × 10 mm id., 5 μM, YMC C-18 semi-preparative HPLC column. The A and B type dracoflavan B were separated using an isocratic eluents with solvent mixture of H_2O (25%) and methanol (75%) at 2.0 mL/min. The A and B type dracoflavan B were eluted at 46 and 44 min respectively. A type dracoflavan B: white crystal; UV λ_{max} 208 and 281 nm; HR-ESI-MS *m*/*z* 537.1935 $[M-H]^-$ (calculated for $C_{33}H_{29}O_7$, 537.1919); ^{1}H NMR (300 MHz, acetone) δ 8.27 (s, 1H, H-7), 7.74 (d, *J* = 1.6 Hz, 2H, H-10,14), 7.71 (d, *J* = 7.6 Hz, 2H, H-10,14′), 7.59–7.30 (m, 6H, H-11,11′,12,12′,13,13′), 6.38 (s, 1H, H-8), 6.17 (s, 1H, H-6′), 5.03 (dd, *J* = 10.4, 1.8 Hz, 1H, H-2′), 4.67 (d, *J* = 3.4 Hz, 1H, H-4), 4.30 (d, *J* = 5.4 Hz, 1H, H-3-R), 4.16 (dd, *J* = 5.3, 3.4 Hz, 1H, H-3), 3.77 (s, 3H, H-5′-OR), 3.54 (s, 3H, H-5), 2.75–2.64 (m, 2H, H-4′a,4′b), 2.39–2.18 (m, 1H, H-3′a), 2.09 (s, 3H, H-15), 2.03–1.95 (m, 1H, H-3′b). B type dracoflavan B: ^{1}H NMR (300 MHz, acetone) δ 8.20 (s, 1H, H-7), 7.73 (d, *J* = 1.6 Hz, 2H, H-10,14), 7.62 (d, *J* = 7.6 Hz, 2H, H-10′,14′), 7.51–7.29 (m, 6H, H-11,11′,12,12′,13,13′), 6.36 (s, 1H, H-8), 6.16 (s, 1H, H-6′), 5.11 (dd, *J* = 10.4, 2.2 Hz, 1H, H-2′), 4.72 (d, *J* = 3.3 Hz, 1H, H-4), 4.35 (d, *J* = 5.3 Hz, 1H, H-3-R), 4.21 (dd, *J* = 5.2, 3.5 Hz, 1H, H-3), 3.76 (s, 3H, H-5′-OR), 3.37 (s, 3H, H-5), 2.75–2.59 (m, 2H, H-4′a,4′b), 2.32–2.21 (m, 1H, H-3′a), 1.97 (s, 3H, H-15), 1.87–1.80 (m, 1H, H-3′b).

3.5. Acylated dracoflavan B, 1

Dracoflavan B (30 mg, 0.0557 mmol) was first dissolved in 760 μL pyridine. Next, acetic anhydride (28.4 mg, 0.279 mmol) was added to dracoflavan B and the mixture was stirred overnight at room temperature. **1** was purified using 250 mm × 10 mm id., 5 μM, YMC C-18 semi-preparative HPLC column. The A and B type **1** were separated using an isocratic eluents with solvent mixture of H_2O (20%) and methanol (80%) at 2.0 mL/min. The A and B type **1** were collected at 40 and 38 min respectively. A type **1**: White crystals; UV λ_{max} 211 and 275 nm; HR-ESI-MS *m*/*z* 603.1994 $[M+Na]^+$ (calculated for $C_{35}H_{32}NaO_8$, 603.1989); ^{1}H NMR (300 MHz, acetone) δ 7.72 (d, *J* = 1.6 Hz, 2H, H-10,14), 7.67 (d, *J* = 7.3 Hz, 2H, H-10′,14′), 7.51–7.31 (m, 6H, H-11,11′,12,12′,13,13′), 6.59 (s, 1H, H-8), 6.18 (s, 1H, H-6′), 5.05 (dd, *J* = 10.2, 1.9 Hz, 1H, H-2′), 4.76 (d, *J* = 3.5 Hz, 1H, H-4), 4.52 (d, *J* = 5.1 Hz, 1H, H-3-R), 4.27 (dd, *J* = 5.0, 3.5 Hz, 1H, H-3), 3.77 (s, 3H, H-5′-OR), 3.55 (s, 3H, H-5), 2.72–2.61 (m, 2H, H-4′a,4′b), 2.28 (s, 3H, H-16), 2.25–2.19 (m, 1H, H-3′a), 2.02 (s, 3H, H-15), 2.02–1.93 (m, 1H, H-3′b). B type **1**: ^{1}H NMR (300 MHz, acetone) δ 7.73 (d, *J* = 1.6 Hz, 2H, H-10,14), 7.62 (d, *J* = 7.1 Hz, 2H, H-10′,14′), 7.51 – 7.33 (m, 6H, H-11,11′,12,12′,13,13′), 6.57 (s, 1H, H-8), 6.17 (s, 1H, H-6′), 5.10 (dd, *J* = 10.5, 2.1 Hz, 1H, H-2′), 4.81 (d, *J* = 3.4 Hz, 1H, H-4), 4.57 (d, *J* = 5.1 Hz, 1H, H-3-R), 4.31 (dd, *J* = 5.0, 3.5 Hz, 1H, H-3), 3.77 (s, 3H, H-5′-OR), 3.38 (s, 3H, H-5), 2.78–2.54 (m, 2H, H-4′a,4′b), 2.26 (s, 3H, H-16), 2.27–2.19 (m, 1H, H-3′a), 1.92 (s, 3H, H-15), 1.89–1.76 (m, 1H, H-3′b).

3.6. Benzoylated dracoflavan B, 2

Dracoflavan B (20 mg, 0.0372 mmol) was dissolved in 1 mL dichloromethane. Thereafter, triethylamine (TEA) (24 mg, 0.0372 mmol) was added to dracoflavan B and the deprotonation reaction was performed at room temperature for 30 min. Subsequently, benzoyl chloride (34 mg, 0.0372 mmol) was added and the mixture was allowed to react overnight at room temperature. Finally, **2** was purified using 250 mm × 10 mm id., 5 μM, YMC C-18 semi-preparative HPLC column. The products were eluted using an isocratic eluents with solvent mixture of H_2O (12.5%) and methanol (87.5%) at 2.0 mL/min. The A and B type **2** were collected at 28 and 26 min, respectively. A type **2**: White crystals; UV–Vis λ_{max} = 206 nm and 274 nm; HR-ESI-MS *m*/*z* 643.2320 $[M+H]^+$ (calculated for $C_{40}H_{35}O_8$, 643.2326); ^{1}H NMR (300 MHz, acetone) δ 8.21 (d, *J* = 8.5 Hz, 2H, H-18), 7.75 (d, *J* = 1.5 Hz, 2H, H-10,14), 7.72 (t, *J* = 1.3 Hz, 1H, H-16), 7.69 (d, *J* = 7.0 Hz, 2H, H-10′,14′), 7.61 (t, *J* = 7.5 Hz, 2H, H-17), 7.51 – 7.31 (m, 6H, H-11,11′,12,12′,13,13′), 6.78 (s, 1H, H-8), 6.20 (s, 1H, H-6′), 5.07 (dd, *J* = 10.2, 1.6 Hz, 1H, H-2′), 4.81 (d, *J* = 3.5 Hz, 1H, H-4), 4.54 (d, *J* = 5.0 Hz, 1H, H-3-R), 4.30 (dd, *J* = 5.3, 3.4 Hz, 1H, H-3), 3.79 (s, 3H, H-5′-OR), 3.59 (s, 3H, H-5), 2.72–2.64 (m, 2H, H-4′a,4′b), 2.29–2.21 (m, 1H, H-3′a), 2.09 (s, 3H, H-15), 2.04–1.95 (m, 1H, H-3′b). B type **2**: ^{1}H NMR (300 MHz, acetone) δ 8.18 (d, *J* = 8.5 Hz, 2H, H-18), 7.77 (d, *J* = 1.8 Hz, 2H, H-10,14), 7.71 (t, *J* = 1.3 Hz, 1H, H-16), 7.63 (d, *J* = 7.0 Hz, 2H, H-10′,14′), 7.59 (t, *J* = 7.5 Hz, 2H,, H-17), 7.53–7.33 (m, 6H, H-11,11′,12,12′,13,13′), 6.75 (s, 1H, H-8), 6.19 (s, 1H, H-6′), 5.12 (dd, *J* = 10.5, 2.0 Hz, 1H, H-2′), 4.86 (d, *J* = 3.5 Hz, 1H, H-4), 4.59 (d, *J* = 5.3 Hz, 1H, H-3-R), 4.35 (dd, *J* = 5.3, 3.7 Hz, 1H, H-3), 3.78 (s, 3H, H-5′-OR), 3.42 (s, 3H, H-5), 2.74–2.60 (m, 2H, H-4′a,4′b), 2.33–2.19 (m, 1H, H-3′a), 1.98 (s, 3H, H-15), 1.97–1.87 (m, 1H, H-3′b).

3.7. Sulfated dracoflavan B, 3

Dracoflavan B (15 mg, 0.0279 mmol) was first dissolved in 400 μL anhyd THF. Next, 30 % methanolic solution was prepared by dissolving sodium methoxide (300 mg, 5.55 mmol) in 885 μL methanol. Thereafter, 30 % methanolic solution (8.9 μL, 0.0558 mmol) was added to the dracoflavan B and the mixture was stirred at room temperature for 30 min. Subsequently, sulfur trioxide triethylamine complex (4 mg, 0.0221 mmol) was added and the mixture was stirred overnight at room temperature. Finally, **3** was purified using 250 mm × 10 mm id., 5 μM, YMC C-18 semi-preparative HPLC column. The products were eluted using an isocratic eluents with solvent mixture of H_2O (20%) and methanol (80%) at 2 mL/min. Subsequently, **3** were collected at 14 min. A type **3**: White crystal; UV λ_{max} 206 and 279 nm; HR-ESI-MS *m*/*z* 617.1491 $[M-H]^-$ (calculated for $C_{33}H_{29}O_{10}S$, 617.1487); ^{1}H NMR (300 MHz, acetone) δ 7.77 (d, *J* = 1.6 Hz, 2H, H-10, 14), 7.70 (d, *J* = 7.9 Hz, 2H, H-10′, 14′), 7.49–7.32 (m, 6H, H-11, 11′, 12, 12′, 13, 13′), 7.13 (s, 1H, H-8), 6.15 (s, 1H, H-6′), 5.03 (dd, *J* = 10.3, 1.7 Hz, 1H, H-2′), 4.71 (d, *J* = 3.7 Hz, 1H, H-4), 4.19 (d, *J* = 5.0 Hz, 1H, H-3-R), 3.76 (s, 1H, H-5′-OR), 3.53 (s, 3H, H-5), 2.73–2.63 (m, 2H, H-4′a, 4′b), 2.28–2.20 (m, 1H, H-3′a), 2.09 (s, 3H, H-15), 2.00–1.95 (m, 1H, H-3′b). B type **3**: ^{1}H NMR (300 MHz, acetone) δ 7.75 (d, *J* = 1.4 Hz, 2H, H-10, 14), 7.62 (d, *J* = 7.3 Hz, 2H, H-10′, 14′), 7.49–7.32 (m, 6H, H-11, 11′, 12, 12′, 13, 13′), 7.10 (s, 1H, H-8), 6.14 (s, 1H, H-6′), 5.10 (dd, *J* = 10.6, 2 Hz, 1H, H-2′), 4.76 (d, *J* = 3.3 Hz, 1H, H-4), 4.24 (d, *J* = 5.3 Hz, 1H, H-3-R), 3.75 (s, 1H, H-5′-OR), 3.35 (s, 3H, H-5), 2.73–2.63 (m, 2H, H-4′a, 4′b), 2.28–2.20 (m, 1H, H-3′a), 2.07 (s, 3H, H-15), 1.89–1.81 (m, 1H, H-3′b).

3.8. Acetyl-protected galloylated dracoflavan B, 4

The hydroxyl group of gallic acid (1 g, 5.88 mmol) was protected by reacting with acetic anhydride (3.4 mL, 35.28 mmol) in the presence of catalytic amount of sulfuric acid (32 μL) at 75 °C for 1 min. Subsequently, a clear yellow solution was obtained and cooled to room temperature. Next, 10 mL of water was added and stirred at room temperature for 2.5 h to obtain a white crystal product. Later, the acetyl-protected gallic acid was collected using

vacuum filtration. Thereafter, the acetyl-protected gallic acid was dried overnight in the vacuum oven. The acetyl-protected gallic acid was then reacted with excess thionyl chloride (118 mg, 0.992 mmol) in chloroform at 62 °C for 90 min. Later, 20 mL of toluene was added and evaporated to remove the unreacted thionyl chloride. The 3,4,5-triacetoxybenzyol chloride obtained was a white crystal. Next, dracoflavan B (40 mg, 0.0743 mmol) and triethylamine (49 mg, 0.0743 mmol) was dissolved in dichloromethane and then stirred in the ice bath for 30 min. Subsequently, 3,4,5-triacetoxybenzyol chloride was added to the mixture. The reaction was performed at room temperature overnight. Finally, **4** was purified using 250 mm × 10 mm id., 5 μM, YMC C-18 semi-preparative HPLC column. The products were eluted using an isocratic eluents with solvent mixture of H_2O (17.5%) and methanol (82.5%) at 2 mL/min. The A and B type **4** were obtained at 32 and 31 min respectively. A type **4**: White crystals; UV λ_{max} 208 and 274 nm; HR-ESI-MS *m*/*z* 815.2354 $[M-H]^-$ (calculated for $C_{46}H_{39}O_{14}$, 815.2345); ^1H NMR (300 MHz, acetone) δ 7.98 (s, 2H, H-16), 7.75 (d, *J* = 1.4 Hz, 2H, H-10,14), 7.68 (d, *J* = 7.5 Hz, 2H, H-10′,14′), 7.54–7.33 (m, 6H, H-11, 11′, 12, 12′, 13, 13′), 6.83 (s, 1H, H-8), 6.20 (s, 1H, H-6′), 5.07 (dd, *J* = 10.3, 2.3 Hz, 1H, H-2′), 4.80 (d, *J* = 3.8 Hz, 1H, H-4), 4.29 (d, *J* = 5.5 Hz, 1H, H-3-R), 3.78 (s, 3H, H-5′-OR), 3.58 (s, 3H, H-5), 2.73–2.61 (m, 2H, H-4′a,4′b), 2.36 (s, 3H, H-18), 2.33 (s, 6H, H-17,19), 2.31–2.23 (m, 1H, H-3′a), 2.09 (s, 3H, H-15), 2.04–1.92 (m, 1H, H-3′b). B type **4**: ^1H NMR (300 MHz, acetone) δ 7.95 (s, 2H, H-16), 7.75 (d, *J* = 1.4 Hz, 2H, H-10,14), 7.62 (d, *J* = 8.0 Hz, 2H, H-10′,14′), 7.54–7.32 (m, 6H, H-11, 11′, 12, 12′, 13, 13′), 6.81 (s, 1H, H-8), 6.18 (s, 1H, H-6′), 5.11 (dd, *J* = 9.8, 2.0 Hz, 1H, H-2′), 4.85 (d, *J* = 3.8 Hz, 1H, H-4), 4.34 (d, *J* = 5.4 Hz, 1H, H-3-R), 3.78 (s, 3H, H-5′-OR), 3.42 (s, 3H, H-5), 2.76–2.66 (m, 2H, H-4′a,4′b), 2.35 (s, 3H, H-18), 2.32 (s, 6H, H-17,19), 2.29–2.25 (m, 1H, H-3′a), 1.98 (s, 3H, H-15), 1.89–1.81 (m, 1H, H-3′b).

3.9. Galloylated dracoflavan B, 5

Compound **4** (18 mg, 0.0226 mmol) was first dissolved in 1 mL aqueous methanol (1:4 v/v water/methanol). Next, ammonium acetate (7 mg, 0.0904 mmol) was added and the mixture was stirred for 27 h at room temperature to obtain **5**. Finally, the product was purified using 250 mm × 10 mm id., 5 μM, YMC C-18 semi-preparative HPLC column. **5** were purified using an isocratic eluents with solvent mixture of H_2O (20%) and methanol (80%) at 2 mL/min. The retention time for **5** was observed at 21 min. A type **5**: White crystals; UV λ_{max} 208 and 274 nm; HR-ESI-MS *m*/*z* 689.2039 $[M-H]^-$ (calculated for $C_{40}H_{33}O_{11}$, 689.2028); ^1H NMR (300 MHz, acetone) δ 7.81–7.74 (m, 3H, H-10, 14, 16), 7.70 (d, *J* = 7.4 Hz, 2H, H-10′,14′), 7.56–7.35 (m, 6H, H-11, 11′, 12, 12′, 13, 13′), 7.28 (s, 3H, H-17,18, 19), 5.08 (dd, *J* = 10.0, 1.7 Hz, 1H, H-2′), 4.80 (d, *J* = 3.4 Hz, 1H, H-4), 4.30 (d, *J* = 5.5 Hz, 1H, H-3-R), 3.79 (s, 3H, H-5′-OR), 3.59 (s, 3H, H-5), 2.78–2.64 (m, 2H, H-4′a, 4′b), 2.33–2.20 (m, 1H, H-3′a), 2.10 (s, 3H, H-15), 1.96–1.90 (m, 1H, H-3′b). B type **5**: ^1H NMR (300 MHz, acetone) δ 7.81–7.74 (m, 3H, H-10, 14, 16), 7.64 (d, *J* = 7.1 Hz, 2H, H-10′, 14′), 7.56–7.35 (m, 6H, H-11, 11′, 12, 12′, 13, 13′), 7.26 (s, 3H, H-17, 18, 19), 5.13 (dd, *J* = 10.8, 1.7 Hz, 1H, H-2′), 4.85 (d, *J* = 3.4 Hz, 1H, H-4), 4.34 (d, *J* = 5.4 Hz, 1H, H-3-R), 3.79 (s, 3H, H-5′-OR), 3.42 (s, 3H, H-5), 2.78–2.64 (m, 2H, H-4′a, 4′b), 2.33–2.20 (m, 1H, H-3′a), 1.97 (s, 3H, H-15), 1.90–1.82 (m, 1H, H-3′b).

3.10. α-Amylase and α-glucosidase inhibitory activity of dracoflavan B

The inhibition activities of dracoflavan B on α-amylase and α-glucosidase were monitored and quantified based on the turbidity measurement according to previous work.[18] Acarbose was prepared in sodium phosphate buffer (0.1 M, pH 6.9). Dracoflavan B was first dissolved in methanol and then diluted by sodium phosphate buffer to appropriate concentrations.

3.11. Kinetic analysis

The mode of inhibition of pancreatic α-amylase by dracoflavan B was determined by using Michaelis–Menten and Lineweaver–Burk equations. Corn starch, in the concentration range of 0.25–1 mg/mL was used as substrate for α-amylase. The concentrations of dracoflavan B used for the inhibitory kinetics of α-amylase were 11 μM, 18 μM, and 22 μM. The initial velocity *v* was obtained from the concentration of liberated reducing sugar in the reaction for 5 min at 37 °C, determined using the 3, 5-dinitrosalicyclic acid (DNSA) method. DNSA reagent was prepared as described by Bernfeld.[1] Dracoflavan B solution (20 μL) with serial concentrations and starch solution (60 μL) were pipetted into centrifuge vials and incubated at 37 °C for 15 min. Enzyme solution (20 μL) was added to each vial. The reaction was quenched at 5 min by addition of DNSA reagent (100 μL). Sample vials were further immersed in a boiling water bath for 5 min for color development. A control sample with identical procedures was also prepared by replacing the dracoflavan B solution with phosphate buffer solution. An aliquot of 200 μL from each vial was pipetted into 96-well plate. Absorbance at 540 nm was measured by a microplate reader. The Michaelis constant K_m and maximal velocity V_{max} of α-amylase were determined from Lineweaver–Burk plots. The inhibition constant K_i of the non-competitive inhibitor was calculated using the following equation: $1/v = K_m(1+[I]/K_i)/(V_{max}[S])+(1+[I]/K_i)/V_{max}$.

3.12. Molecular docking study

SiteMap1 of the Maestro suite was used to determine the probable binding sites of the α-amylase enzyme-inhibitor complex. Probes were placed inside a protein cavity to obtain the area information for van der Waals (vdW) and electrostatic interactions. The information obtained is then translated to grid maps and illustrated as hydrophobic and hydrophilic regions of the cavity.

Docking was done using Glide2-5 of the Maestro suite in extra-precision (XP) mode since the XP mode provides an extensive range of choices pertaining to various intermolecular interactions in order to determine the most accurate binding conformation of the ligand possible.[25] A docking score was assigned to every binding conformation. The lower the docking score, the more favorable is the binding conformation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.11.008.

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