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## New Flavonoids from the Saudi Arabian Plant *Retama raetam* which Stimulate Secretion of Insulin and Inhibit $\alpha$ -Glucosidase

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*Retama raetam* is a bush which is a member of the family Fabaceae. It is used traditionally in North Africa and Saudi Arabia for the treatment of diabetes. Several flavonoids and alkaloids were already known from this plant. Chromatographic fractionation and purification led to the isolation of three new derivatives of prenylated flavones, retamasin C-E and four new derivatives of prenylated isoflavones, retamasin F-I, in addition to two isoflavones which had not previously been reported in this plant. Particularly interesting structures included isoflavones containing 3,5-dihydro-2H-2,5-methanobenzo[e][1,4]dioxepine and 3a,8b-dihydro-7-hydroxyfuro[3,2-b]benzo[2,1-d]furan units, both of which are new amongst natural product flavonoids. Five new examples (two flavones and three isoflavones) strongly enhanced the glucose-triggered release of insulin by murine pancreatic islets and one isoflavone was a potent inhibitor of  $\alpha$ -glucosidase. This study may rationalise the traditional medicinal use of *R. raetam* and provides new leads for drug design in the treatment of diabetes.

### Introduction

*Retama raetam* (Forsk.) Webb & Berthel is a plant common in North Africa, countries of the Eastern Mediterranean and in the Middle East,<sup>1,2</sup> including Saudi Arabia, and is a member of the family Fabaceae. It is particularly abundant in desert areas and is known locally as R'tm or broom bush. It flourishes in arid desert areas, where it stabilises the land and is a forage plant for camels, goats and sheep.<sup>3</sup> However, ingestion by livestock leads to toxicity.<sup>2</sup> In folk medicine in Saudi Arabia and Morocco, this plant has been used to treat diabetes and hypertension.<sup>4</sup> This traditional therapeutic activity has been borne out in experimental studies in rodents, where aqueous extracts of the plant lowered the concentrations of glucose and lipids in normal and streptozotocin-treated rats<sup>4,5</sup> and caused acute diuresis in normal rats.<sup>6</sup> Anti-oxidant, anti-microbial, anti-viral and insecticidal activities have also been reported.<sup>4,7</sup> One mechanism for the observed hypoglycaemic activity

in rats has been suggested to be inhibition of renal re-uptake of glucose,<sup>8</sup> whereas a methanol extract showed antidiabetic activity in the same species which was attributed to increasing glucose-stimulated release of insulin by the pancreas and to diminution of absorption of glucose in the intestine.<sup>9</sup>

There is a need to identify the antidiabetic / hypoglycaemic compounds present in *R. raetam* for potential development into effective treatments for this major disease, a growing cause of death and disability across the world. Isolation of the active compounds is key, as the plant, crude extracts and folklore preparations have led to significant toxicity to animals<sup>2</sup> and humans.<sup>10</sup> Amongst other classes of compounds, quinolizidine and other alkaloids have been isolated (including cytisine, L-anagryne, sparteine and lupinine), which may be responsible for this toxicity.<sup>11</sup> Pinitol, a cyclitol component of *R. raetam*, is a known hypoglycaemic agent.<sup>12</sup> However, the flavonoids have been most extensively studied as components of this plant. In addition to the simple compounds daidzein, naringenin, apigenin, kaempferol, quercetin and orientin (and their glycosides),<sup>13</sup> luteolin 4'-neohesperidoside has been identified as a more complex flavone-O-glycoside in this plant.<sup>14,15</sup> The flavone-C-glycoside saponarin was characterised by Kassem *et al.*,<sup>15</sup> whereas C-isoprenyl units can be recognised in the *R. raetam* pyranoflavones atalantoflavone<sup>16</sup> and 4',5-dihydroxy-(3'',4''-dihydro-3'',4''-dihydroxy)-2'',2''-dimethylpyrano-(5'',6''':7,8)-flavone,<sup>14</sup> in the furanoflavone retamasin B<sup>16</sup> and in the open-chain flavones licoflavone C<sup>16</sup> and ephedroidin.<sup>16</sup> The isoprenyl unit is truncated in retamasin B.<sup>16</sup> The analogous C-glycosyl isoflavone genistein 8-C-glucoside<sup>15</sup> and the pyrano-isoflavones derrone<sup>16</sup> and hydroxyderrone<sup>16</sup> have also been found in *R. raetam*.

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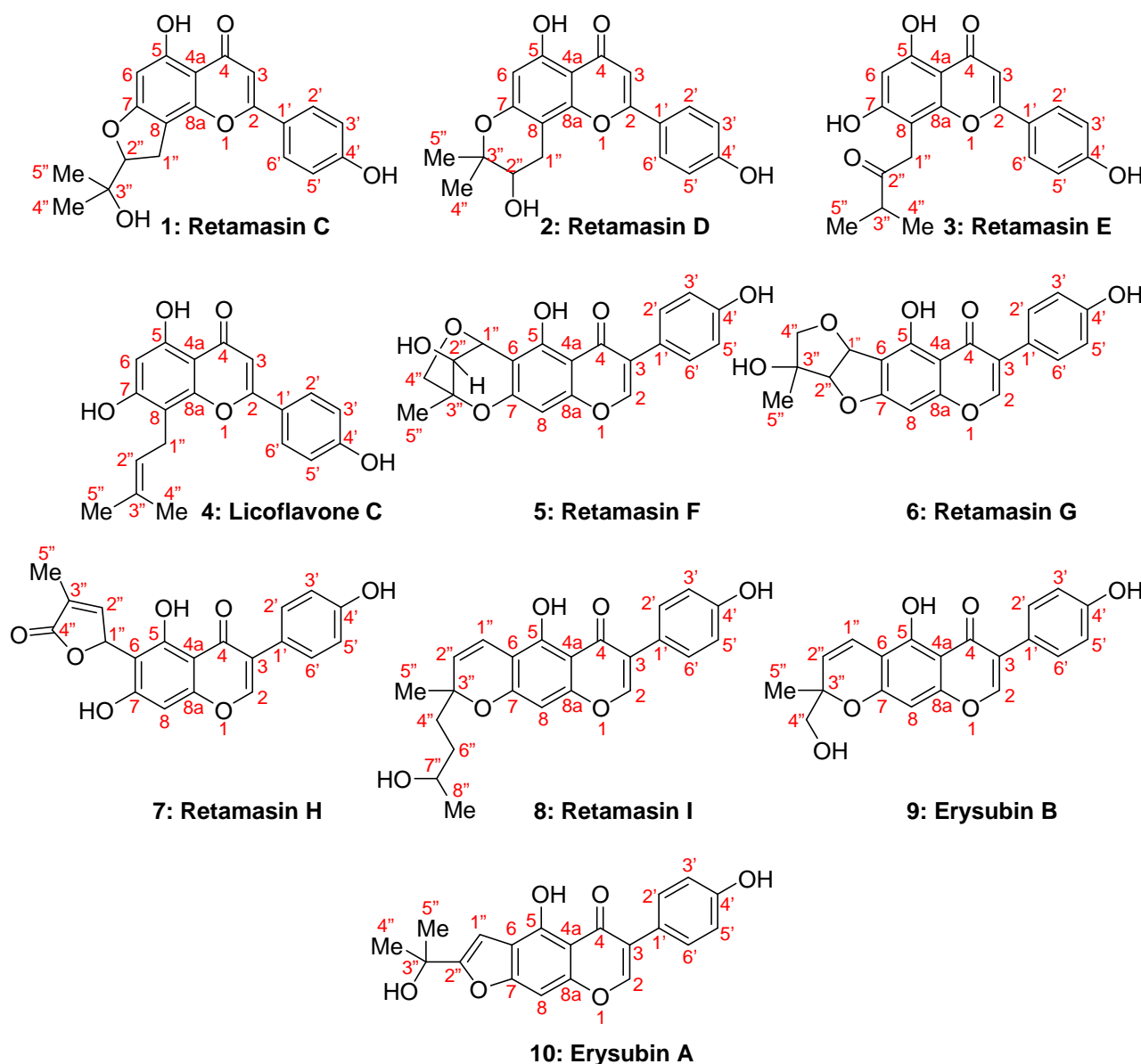
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Electronic Supplementary Information (ESI) available: [Table of NMR data for 4,9,10; <sup>1</sup>H, <sup>13</sup>C, COSY, HSQC, HMBC NMR spectra for 1-10; HRESIMS for 1-3,5-8]. See DOI: 10.1039/x0xx00000x



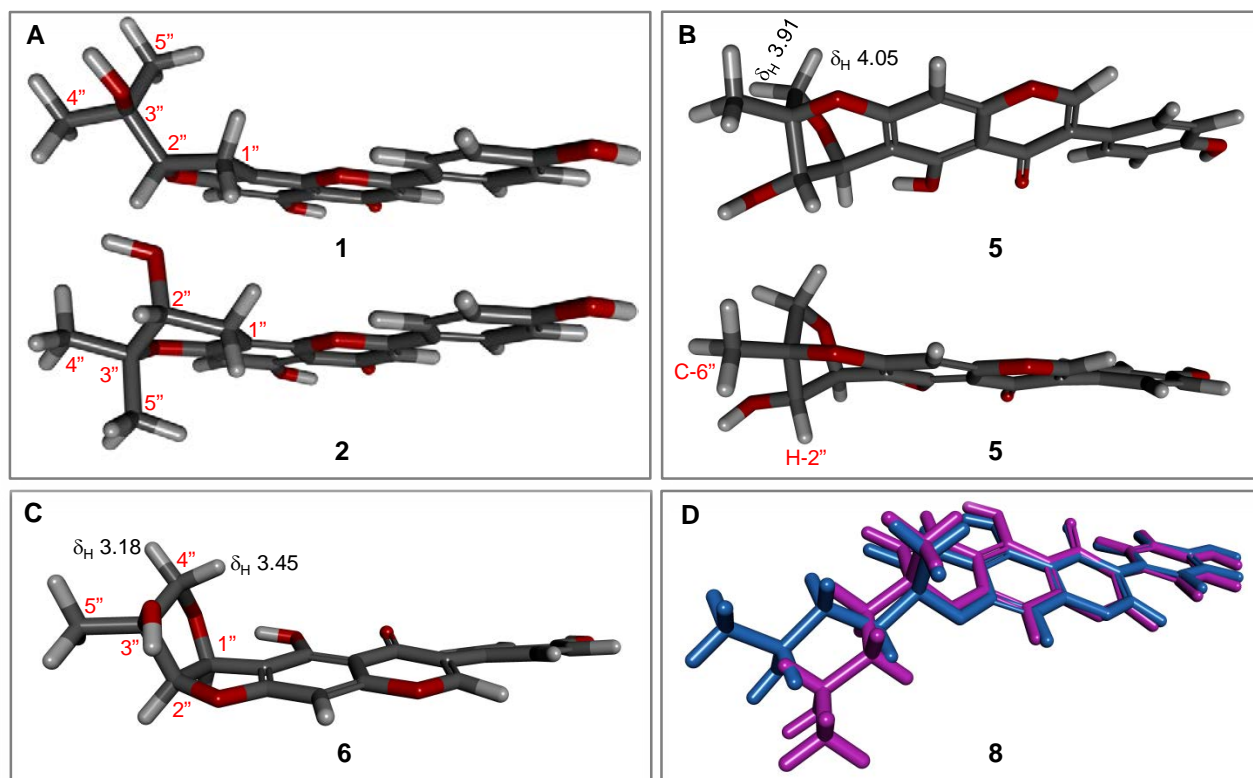
**Figure 1.** Structures of flavonoids newly isolated from *R. raetam*.

Flavonoids have a very wide range of biological and therapeutic activities. In this study, further clarification was sought of the compounds in *R. raetam* which are responsible for its antidiabetic activity. The chloroform extract of the aerial parts of the plant was fractionated chromatographically. Seven new flavonoids (**1-3,5-8**), one known flavone (**4**) and two known isoflavones (**9,10**) (Figure 1) were isolated and characterised spectroscopically, each derived from a flavone or isoflavone carrying a modified C<sub>5</sub> isoprene unit. All compounds were examined for stimulation of secretion of insulin from islets isolated from murine pancreas and seven were selected for assessment for inhibition of  $\alpha$ -glucosidase.

## Results & discussion

The dried aerial parts of *R. raetam* were extracted with chloroform and the solvent was evaporated. This residue was partitioned between hexane and acetonitrile. Chromatography of the acetonitrile fraction, followed by reverse-phase preparative HPLC on a C18 column, afforded ten pure compounds **1-10**. Their structures were elucidated using 1D and 2D NMR and HRESIMS data. The absolute configurations of the chiral compounds **1,2,5-9** cannot be determined from these data.

The HRESIMS of **1** showed a peak in positive mode at  $m/z$  355.1156, corresponding to  $[M + H]^+$  (calcd 355.1181) for the molecular formula C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>. Ions were also seen at  $m/z$  377.0982  $[M + Na]^+$  (calcd 377.1001) and  $m/z$  709.2250  $[2M + H]^+$  (calcd 709.2285). The HRESIMS in negative mode



**Figure 2.** A: MM2-minimised structures of **1** and **2**, showing conformations of the dihydrofuran and dihydropyran rings, respectively. The *pseudo*equatorial location of H-2'' in **2** is also shown. B: Two views of the MM2-minimised structure of the HO-*exo* diastereoisomer of **5**, showing tentative assignments of the  $^1\text{H}$  NMR signals for H-5'' (upper) and the dihedral angle between (H-2'')–(C-2'') and (C-5'')–(C-3'') (lower), suggesting this diastereoisomer. C: Diastereoisomeric identity of **6** demonstrated by NOESY spectroscopy. D: Overlay of the MM2-minimised structures of the two diastereoisomers of **8**.

confirmed the formula, with peaks at  $m/z$  353.1018 [ $\text{M} - \text{H}$ ] $^-$  (calcd 353.1025),  $m/z$  707.2125 [ $2 \text{ M} - \text{H}$ ] $^-$  (calcd 707.2128), and  $m/z$  753.2179 [ $2 \text{ M} + \text{formate}$ ] $^-$  (calcd 753.2183), in addition to a peak at 389 [ $\text{M} + ^{35}\text{Cl}$ ] $^-$ . The NMR spectra showed the appropriate numbers of  $^1\text{H}$  and  $^{13}\text{C}$  signals. The core structure was shown, by a combination of NMR techniques, to be a flavone. Specifically, H-3 ( $\delta_{\text{H}}$  6.80) and H-6 ( $\delta_{\text{H}}$  6.28) both showed HMBC cross-peaks to C-4a ( $\delta_{\text{C}}$  104.68).

The  $^1\text{H}$  NMR spectrum of **1** (Table 1) showed doublets at  $\delta_{\text{H}}$  7.94 ( $J$  8.2 Hz) ( $\delta_{\text{C}}$  128.94) and  $\delta_{\text{H}}$  6.95 ( $J$  8.2 Hz) ( $\delta_{\text{C}}$  116.49), each integrating for 2 H. These signals corresponded to H-2',6' (C-2',6') and H-3',5' (C-3',5'), respectively. The phenolic OH signal at  $\delta_{\text{H}}$  13.19 showed HMBC correlations with C-6 ( $\delta_{\text{C}}$  93.95), C-4a ( $\delta_{\text{C}}$  104.68) and C-5 ( $\delta_{\text{C}}$  161.78), with a weak cross-peak to the ketone ( $\delta_{\text{C}}$  182.30); these located the signal at  $\delta_{\text{H}}$  13.19 as being due to HO-5, which was deshielded by H-bonding to the ketone oxygen. The signal at  $\delta_{\text{H}}$  10.45 was thus due to the phenol at C-4', rationalising the  $\delta_{\text{H}}$  chemical shifts for H-2',3',5',6'. These data, together with the  $\delta_{\text{H}}$  and  $\delta_{\text{C}}$  for ring A, confirmed that the flavone has a 4',5,7-oxygenation pattern. The methylene protons ( $\delta_{\text{H}}$  3.29, m) correlated with the  $^{13}\text{C}$  signal ( $\delta_{\text{C}}$  26.49) in the HSQC spectrum and with C-7 ( $\delta_{\text{C}}$  166.74), C-8 ( $\delta_{\text{C}}$  104.48) and C-8a ( $\delta_{\text{C}}$  152.20) in the HMBC spectrum, which identified this methylene ( $\text{CH}_2$ -1'') as attached at C-8. A COSY cross-peak from H-1'' identified H-2'' ( $\delta_{\text{H}}$  4.80), with HSQC linking this to C-2'' ( $\delta_{\text{C}}$  92.05). H-2'' gave HMBC cross-peaks to C-8 ( $\delta_{\text{C}}$  104.48) and C-7 ( $\delta_{\text{C}}$  166.74) of the

flavone; both of these were three-bond correlations, which confirmed the fusion of the dihydrofuran ring. H-2'' also correlated in HMBC through two bonds with C-1'' ( $\delta_{\text{C}}$  26.49) and quaternary C-3'' ( $\delta_{\text{C}}$  70.53) and through three bonds with the methyl carbons C-4'' ( $\delta_{\text{C}}$  25.35) and C-5'' ( $\delta_{\text{C}}$  26.25). These two methyl groups were diastereotopic; however, although the carbon chemical shifts were different, the  $^1\text{H}$  chemical shifts were identical ( $\delta_{\text{H}}$  1.18). C-3'' also carries an OH group (HO-3''  $\delta_{\text{H}}$  4.74), as shown by the downfield  $^{13}\text{C}$  chemical shift. This OH signal shows HMBC correlations with C-2'', C-3'', C-4'' and C-5''. Together, these spectroscopic data confirmed the structure of **1** (retamasin C), a novel compound.

The positive-ion HRESIMS of **2** showed [ $\text{M} + \text{H}$ ] $^+$  at  $m/z$  355.1161 (calcd 355.1181), showing that it was an isomer of **1**. Ions were also observed at  $m/z$  377.0982 [ $\text{M} + \text{Na}$ ] $^+$  (calcd 377.1001) and  $m/z$  337.1132 [ $\text{M} + \text{H} - \text{H}_2\text{O}$ ] $^+$  (calcd 337.1076); the latter indicated the presence of an aliphatic alcohol. In the negative-ion HRESIMS, ions were detected at  $m/z$  353.1023 [ $\text{M} - \text{H}$ ] $^-$  (calcd 353.1025),  $m/z$  707.2126 [ $2 \text{ M} - \text{H}$ ] $^-$  (calcd 707.2128) and  $m/z$  743.1893 [ $2 \text{ M} + ^{35}\text{Cl}$ ] $^-$  (calcd 743.1900), confirming the formula. The NMR spectra (Table 1) showed the appropriate numbers of  $^1\text{H}$  and  $^{13}\text{C}$  signals. As for **1**, H-3 ( $\delta_{\text{H}}$  6.82, s) and H-6 ( $\delta_{\text{H}}$  6.13, s) both correlated in the HMBC spectrum with C-4a ( $\delta_{\text{C}}$  104.98), confirming **2** as a flavone and not an isoflavone.

The IR spectrum of **2** indicated OH ( $3341 \text{ cm}^{-1}$ ) and a conjugated aryl ketone ( $1645 \text{ cm}^{-1}$ ).  $^{13}\text{C}$  and HSQC NMR

**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for **1-3,5** in  $(\text{CD}_3)_2\text{SO}$  [ $\delta_{\text{H}}$ , multiplicity ( $J$  (Hz));  $\delta_{\text{C}}$ , type]

	<b>1</b>		<b>2</b>		<b>3</b>		<b>5</b>	
Position	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
2		163.8, C <sub>q</sub>		164.0, C <sub>q</sub>		164.2, C <sub>q</sub>	8.39, s	154.9, CH
3	6.80, s	103.2, CH	6.82, s	103.5, CH	6.78, s	103.5, CH		122.8, C <sub>q</sub>
4		182.3, C <sub>q</sub>		182.4, C <sub>q</sub>		182.7, C <sub>q</sub>		181.0, C <sub>q</sub>
4a		104.7, C <sub>q</sub>		105.0, C <sub>q</sub>		104.00, C <sub>q</sub>		105.5, C <sub>q</sub> <sup>b</sup>
5		161.8, C <sub>q</sub>		159.3, C <sub>q</sub> <sup>a</sup>		160.2, C <sub>q</sub>		157.6, C <sub>q</sub> <sup>b</sup>
6	6.28, s	93.995, CH	6.13, s	99.5, CH	6.28, s	9.99, CH		109.8, C <sub>q</sub>
7		166.7, C <sub>q</sub>		159.4, C <sub>q</sub> <sup>a</sup>		161.8, C <sub>q</sub>		160.2, C <sub>q</sub>
8		104.5, C <sub>q</sub>		99.8, C <sub>q</sub>		101.4, C <sub>q</sub>	6.49, s	94.1, CH
8a		152.2, C <sub>q</sub>		154.8, C <sub>q</sub>		155.5, C <sub>q</sub>		157.7, C <sub>q</sub> <sup>b</sup>
1'		121.5, C <sub>q</sub>		121.7, C <sub>q</sub>		122.0, C <sub>q</sub>		121.4, C <sub>q</sub>
2',6'	7.94, d, $J$ 8.1	128.9, CH	7.92, d, $J$ 8.8	128.8, CH	7.85, d, $J$ 8.8	129.0, CH	7.39, d, $J$ 8.4	130.6, CH
3',5'	6.95, d, $J$ 8.1	116.5, CH	6.93, d, $J$ 8.8	116.5, CH	6.91, d, $J$ 8.8	116.4, CH	6.83, d, $J$ 8.4	115.6, CH
4'		162.3, C <sub>q</sub>		161.8, C <sub>q</sub>		164.0, C <sub>q</sub>		158.0, C <sub>q</sub> <sup>b</sup>
1''	3.29, m	26.5, CH <sub>2</sub>	2.73, dd, $J$ 16.1, 6.8; 3.05 dd, $J$ 16.1, 5.2	25.3, CH <sub>2</sub>	3.95, s	35.2, CH <sub>2</sub>	5.02, s	73.0, CH
2''	4.80, dd, $J$ 8.6, 8.1	92.0, CH	3.75, brt, $J$ 6	67.3, CH		211.5, C <sub>q</sub>	4.17, d, $J$ 3.8	73.9, CH
3''		70.5, C <sub>q</sub>		79.4, C <sub>q</sub>	2.84, m	~40, CH		88.4, C <sub>q</sub>
4''	1.18, s	25.3, CH <sub>3</sub>	1.32, s	21.5, CH <sub>3</sub>	1.07, d, $J$ 6.9	18.8, CH <sub>3</sub>	3.91, d, $J$ 10.3; 4.05, d, $J$ 10.3	77.0, CH <sub>2</sub>
5''	1.18, s	26.2, CH <sub>3</sub>	1.24, s	25.6, CH <sub>3</sub>	1.07, d, $J$ 6.9	18.8, CH <sub>3</sub>	1.50, s	16.4, CH <sub>3</sub>
HO-5	13.19, s		12.70, s		12.96, s		13.37, s	
HO-7					10.7, br			
HO-3'			5.29, br					
HO-4'	10.45, br		10.4, br		10.4, br		9.64, s	
HO-2''							5.90, d, $J$ 3.8	
HO-3''	4.74, br							

<sup>a</sup> Assignments could be reversed. <sup>b</sup> Assignments could be interchanged.

showed five methine signals, one methylene signal, two methyl signals and ten quaternary signals. The diastereotopic CH<sub>2</sub> protons resonated at  $\delta_{\text{H}}$  2.73 and  $\delta_{\text{H}}$  3.05, correlating in HSQC with the methylene C-1'' ( $\delta_{\text{C}}$  25.32). HMBC cross-peaks between these H-1'' signals and the downfield aromatic carbon signals at  $\delta_{\text{C}}$  159.3 (C-7) and  $\delta_{\text{C}}$  154.82 (C-8a) linked this methylene to C-8, supported by weaker two-bond HMBC cross-peaks from these H-1'' methylene protons to C-8. The COSY spectrum showed cross-peaks between these H-1'' signals and the signal at  $\delta_{\text{H}}$  3.75 (HSQC to  $\delta_{\text{C}}$  67.28). These chemical shifts indicated that this carbon (C-2'') also carried an oxygen, confirmed as OH by the COSY cross-peak to HO-2'' ( $\delta_{\text{H}}$  5.29). Three-bond HMBC correlations linked both H-1'' to a quaternary carbon signal ( $\delta_{\text{C}}$  79.44) for C-3'' linked to another oxygen. H-2'' was linked by HMBC to two methyl signals (C-4''  $\delta_{\text{C}}$  21.53 and C-5''  $\delta_{\text{C}}$  25.61). Weak four-bond HMBC cross-peaks from H-4'' ( $\delta_{\text{H}}$  1.32) and H-5'' ( $\delta_{\text{H}}$  1.24) to C-7 ( $\delta_{\text{C}}$  159.3) confirmed the six-membered dihydropyran ring fused at C-7 and C-8. NOESY cross-peaks showed that H-2'' is close in space to both H-4'' and H-5''; this is only possible if H-2'' is in a *pseudo*equatorial position. This conformation was supported

by the  $^1\text{H}$  NMR signals for both H-1'' being broad doublets, with only the  $^2J$  value being large (>4 Hz); hence there were no *pseudo*axial / *pseudo*axial interactions with H-2'' and it adopted a *pseudo*equatorial conformation (Figure 2A). The geminal methyl groups in **2** resonated at different chemical shifts in both  $^1\text{H}$  and  $^{13}\text{C}$  spectra, which reflect their axial and equatorial positions in the *pseudo*chair of the dihydropyran conformation (Figure 2A). This contrasted with the almost planar conformation of the dihydrofuran in **1**, in which the CMe<sub>2</sub> unit was exocyclic and the signals for the diastereotopic methyl groups were accidentally coincident in both  $^1\text{H}$  and  $^{13}\text{C}$  spectra. These spectroscopic data confirmed the structure of **2** (retamasin D), a new compound. The CD spectrum (Supplementary Information) showed a positive peak in the region 260 nm – 400 nm; using the analysis of Wei et al.<sup>17</sup> suggests that **2** may have *R* configuration.

The positive-ion HRESIMS of **3** showed an  $[\text{M} + \text{H}]^+$  peak at  $m/z$  355.1159 (calcd 355.1181) for the molecular formula C<sub>20</sub>H<sub>18</sub>O<sub>6</sub>. The negative-ion spectrum contained ions at  $m/z$  353.1020 ( $[\text{M} - \text{H}]^-$  calcd 353.1025),  $m/z$  707.2126 ( $[2 \text{M} - \text{H}]^-$  calcd 707.2129) and  $m/z$  743.1894 ( $[2 \text{M} + ^{35}\text{Cl}]^-$  calcd



743.1895). The  $^{13}\text{C}$  NMR spectrum (Table 1) contained seventeen discrete signals, of which ten were for quaternary carbons, five for methine carbons, one for a methylene carbon and one for two magnetically equivalent methyl carbons. The carbonyl resonance at  $\delta_{\text{C}}$  211.53 showed a dialkyl ketone. The IR spectrum showed absorbances for OH (3347  $\text{cm}^{-1}$ ), a conjugated aryl ketone carbonyl (1654  $\text{cm}^{-1}$ ) and a H-bonded dialkyl ketone carbonyl (1697  $\text{cm}^{-1}$ ).

Analysis of the  $^1\text{H}$ ,  $^{13}\text{C}$ , COSY, HSQC and HMBC NMR spectra of **3** indicated a structure similar to that of apigenin (4',5,7-trihydroxyflavone). C-8 ( $\delta_{\text{C}}$  101.38) was quaternary (no HSQC correlations), showing that the substituent was attached to the apigenin core at this position. Three phenolic OH signals were seen: HO-5 ( $\delta_{\text{H}}$  12.96, s), HO-4' ( $\delta_{\text{H}}$  10.4, br) and HO-7 ( $\delta_{\text{H}}$  10.7, br) H-bonded to the dialkyl ketone. The methylene protons (H-1'') resonated as a singlet ( $\delta_{\text{H}}$  3.95), thus the compound is achiral. This signal showed three-bond HMBC cross-peaks to C-7 ( $\delta_{\text{C}}$  161.79) and C-8a ( $\delta_{\text{C}}$  155.52) and a two-bond cross-peak to C-8 ( $\delta_{\text{C}}$  101.38), confirming that  $\text{CH}_2\text{-1''}$  is attached at C-8 of the flavone. A weaker two-bond HMBC correlation connected the dialkyl ketone signal C-2'' ( $\delta_{\text{C}}$  211.53) to H-1''. The presence of an isopropyl group was shown by a doublet, integrating for 6 H, at  $\delta_{\text{H}}$  1.07 (H-4'' / H-5'') and a multiplet signal for H-3'' at  $\delta_{\text{H}}$  2.84. Three-bond HMBC cross-peaks were observed between the signals for H-4'' / H-5'' and the signal for the C-2'' ketone. Thus **3** (retamasin E, a novel compound) is identified as a 8-prenylated apigenin oxidised to a ketone at C-2''.

Compound **4** was identified as licoflavone C by comparison of the NMR spectra (Table S1, ESI) and melting point with the data reported for synthetic and natural product samples.<sup>18,19</sup> It should be noted that the NMR data should be compared with compound 10 in Al-Maharik and Botting<sup>19</sup> and not compound 6, as the spectroscopic data for these two compounds were erroneously transposed in that paper. IR data were also consistent with the structure.

The positive-ion HRESIMS of **5** showed that the molecular formula is  $\text{C}_{20}\text{H}_{16}\text{O}_7$ , with  $[\text{M} + \text{H}]^+$  observed at  $m/z$  369.0953 (calcd 369.0974),  $[\text{M} + \text{Na}]^+$   $m/z$  391.0772 (calcd 391.0794),  $[2\text{M} + \text{H}]^+$   $m/z$  737.1833 (calcd 737.1870) and  $[2\text{M} + \text{Na}]^+$   $m/z$  759.1652 (calcd 759.1690). This was confirmed by negative-ion HRMS peaks at  $m/z$  367.0818  $[\text{M} - \text{H}]^-$  (calcd 367.0818),  $m/z$  403.0585  $[\text{M} + ^{35}\text{Cl}]^-$  (calcd 403.0585),  $m/z$  413.0874  $[\text{M} + \text{HCO}_2]^-$  (calcd 413.0873),  $m/z$  771.1491  $[2\text{M} + ^{35}\text{Cl}]^-$  (calcd 771.1481) and  $m/z$  781.1791  $[2\text{M} + \text{HCO}_2]^-$  (calcd 781.1769).

Compound **5** was an isoflavone, as shown by the downfield chemical shift of the  $^1\text{H}$  NMR singlet for H-2 ( $\delta_{\text{H}}$  8.39) (Table 1), which had HSQC connectivity with C-2 ( $\delta_{\text{C}}$  154.93). This proton showed a strong HMBC cross-peak with the signal for the carbonyl C-4 ( $\delta_{\text{C}}$  180.96) and a weaker two-bond correlation with quaternary C-3 ( $\delta_{\text{C}}$  122.85) but not with C-4a ( $\delta_{\text{C}}$  105.51). The latter was clearly identified by its HMBC correlation with H-8 ( $\delta_{\text{H}}$  6.49). H-8 made a two-bond correlation with C-8a ( $\delta_{\text{C}}$  157.63 / 157.66 / 158.02), which formed a three-bond connection with H-2. The 4-hydroxyphenyl unit at C-3 was demonstrated by HMBC correlations from H-2' / H-6' ( $\delta_{\text{H}}$  7.39) to C-3 ( $\delta_{\text{C}}$  122.85) and from H-2 to C-1' ( $\delta_{\text{C}}$  121.41). HO-4' ( $\delta_{\text{H}}$  9.64) was identified by a HMBC cross-peak with C-3' / C-5' ( $\delta_{\text{C}}$

115.56). In the IR spectrum, peaks were seen for the OH (3333  $\text{cm}^{-1}$ ) and conjugated aryl ketone (1643  $\text{cm}^{-1}$ ) groups. C-6 ( $\delta_{\text{C}}$  109.85) was quaternary and the chemical shift indicated substitution with a  $\text{sp}^3$ -hybridised carbon. It made a three-bond correlation with H-8 and a good two-bond correlation with H-1'' ( $\delta_{\text{H}}$  5.02). C-1'' was a methine, with the  $^{13}\text{C}$  NMR signal at  $\delta_{\text{C}}$  73.03. H-1'' also showed a two-bond HMBC correlation with C-2'' ( $\delta_{\text{C}}$  73.90) and three-bond correlations with C-3'' ( $\delta_{\text{C}}$  88.38) and C-4'' ( $\delta_{\text{C}}$  77.01). Of these, C-3'' was quaternary, C-2'' was a methine and C-4'' was a methylene; the chemical shifts of each indicated an attached oxygen. Both of the diastereotopic H-4'' protons formed HMBC cross-peaks with C-1'' and C-2'' through three bonds, but only the signal at  $\delta_{\text{H}}$  3.91 formed a cross-peak with the methyl signal at  $\delta_{\text{C}}$  16.39 (C-5''). The lack of cross-peak from  $\delta_{\text{H}}$  4.05 was likely to be due to the dihedral angle along (C-3'')–(C-4'') between the (C-4'')–H bond to this proton and the (C-3'')–(C-5'') bond being *ca.* 90°. Thus the diastereotopic H-4''  $^1\text{H}$  NMR signals were tentatively assigned as shown in Figure 2B. A similar argument can be used to suggest that HO-2'' may be located in the *exo* position (Figure 2B), as the dihedral angle between the (H-2'')–(C-2'') bond and the (C-3'')–(C-5'') bond was also *ca.* 90° in this diastereoisomer, whereas it was *ca.* 30° in the other (HO-2'' *endo*) structure. Further evidence was provided by H-2'' being coupled only to HO-2'' and not to H-1'' and the lack of a corresponding COSY cross-peak; this was only possible when HO-2'' was located *exo* and the dihedral angle between (H-1'')–(C-1'') and (H-2'')–(C-2'') is *ca.* 90°. The HO-2''-*exo* diastereoisomer was confirmed by the observation of a strong NOESY cross-peak from the HO-2'' ( $\delta_{\text{H}}$  5.90) to the H-4'' signal ( $\delta_{\text{H}}$  4.05) and a weaker one to H-4'' ( $\delta_{\text{H}}$  3.91). This observation also assigned the signals for the diastereotopic H-4'', with H-4'' ( $\delta_{\text{H}}$  4.05) being on the face towards the OH. The methyl group (5'') was located as being attached at C-3'', as it was a singlet and through a two-bond HMBC cross-peak from H-5'' ( $\delta_{\text{H}}$  1.50) to C-3'' and a weak four-bond cross-peak to C-7 ( $\delta_{\text{C}}$  160.19). Taken together, these correlations demonstrated that there is a bicycle fused to the flavone core at C-6 and C-7 and that the structure of **5** (retamasin F, a novel compound) was as shown. From the positive value in the 260 nm – 400 nm region of the CD spectrum (Supplementary Information), we tentatively assign the configuration of **5** as 1''S, 2''S, 3''R.<sup>17</sup>

In positive-ion mode, HRESIMS of **6** showed a peak for  $[\text{M} + \text{H}]^+$  was seen at  $m/z$  369.0970 (calcd 369.0974), corresponding to the molecular formula  $\text{C}_{20}\text{H}_{16}\text{O}_7$ . Supporting this assignment were peaks for  $[\text{M} + \text{Na}]^+$  at  $m/z$  391.0772 (calcd 391.0794),  $[2\text{M} + \text{H}]^+$  at  $m/z$  737.1832 (calcd 737.1870) and  $[2\text{M} + \text{Na}]^+$  at  $m/z$  759.1651 (calcd 759.1690). Negative ions were observed for  $[\text{M} + ^{35}\text{Cl}]^-$  at  $m/z$  403.0585 (calcd 403.0585),  $[\text{M} + \text{HCO}_2]^-$  at  $m/z$  413.0873 (calcd 413.0873) and  $[2\text{M} + ^{35}\text{Cl}]^-$   $m/z$  771.1478 (calcd 771.1481). It is an isomer of **5**. The  $^{13}\text{C}$  NMR spectrum (Table 2) showed eighteen discrete resonances, of which two were each for two carbons in magnetically equivalent environments. There were signals for ten quaternary carbons (including one carbonyl), eight methine carbons, one methylene carbon and one methyl carbon.

As for **5**, this compound gave NMR resonances characteristic of an isoflavone substituted at C-6 ( $\delta_{\text{C}}$  109.10) with a  $\text{sp}^3\text{-C}$  substituent. C-2 was a methine and resonated downfield at  $\delta_{\text{C}}$  154.86. The H-2 signal was also downfield ( $\delta_{\text{H}}$  8.38) and was identified by HMBC cross-peaks to C-1' ( $\delta_{\text{C}}$  121.38), C-4 ( $\delta_{\text{C}}$  181.30) and C-8a ( $\delta_{\text{C}}$  159.86). The 3-(4-hydroxyphenyl) unit was also located with a strong HMBC correlation from H-2' / H-6' ( $\delta_{\text{H}}$  7.36) to C-3 ( $\delta_{\text{C}}$  122.76). C-8 ( $\delta_{\text{C}}$  89.44) is a methine and H-8 ( $\delta_{\text{H}}$  6.59) formed two-bond HMBC correlations with C-7 ( $\delta_{\text{C}}$  167.38) and C-8a ( $\delta_{\text{C}}$  159.86) and three-bond correlations with C-4a ( $\delta_{\text{C}}$  106.22) and C-6 ( $\delta_{\text{C}}$  109.10). Two phenolic OH signals were present in the  $^1\text{H}$  NMR spectrum, H-4' at  $\delta_{\text{H}}$  9.58 and the intramolecularly H-bonded HO-5 at  $\delta_{\text{H}}$  13.47. One aliphatic OH signal was observed at  $\delta_{\text{H}}$  5.30, which was shown by HSQC to be a tertiary alcohol attached at C-3'' ( $\delta_{\text{C}}$  77.08). The IR spectrum contained peaks for the OH ( $3363\text{ cm}^{-1}$ ) and conjugated aryl ketone ( $1655\text{ cm}^{-1}$ ) groups, consistent with a hydroxylated isoflavone. Attached to C-6 was a methine carbon C-1'' ( $\delta_{\text{C}}$  78.68), with H-1'' forming an HMBC cross-peak with C-7 ( $\delta_{\text{C}}$  167.38). This carbon comprised one atom of a tetrahydrofuran, which was demonstrated as follows. H-1'' correlated with C-4'' ( $\delta_{\text{C}}$  72.79) in the HMBC spectrum and one of the methylene protons H-4'' ( $\delta_{\text{H}}$  3.45) correlates back with C-1'', joining the bridge across the oxygen. This H-4'' also formed a three-bond correlation with C-2'' ( $\delta_{\text{C}}$  91.91) and H-2'' ( $\delta_{\text{H}}$  4.76) showed a cross-peak with C-4''. The final carbon of the tetrahydrofuran, quaternary C-3'', was identified through two-bond HMBC correlations with both H-4'' methylene protons ( $\delta_{\text{H}}$  3.18 and  $\delta_{\text{H}}$  3.45) and with H-2''. The substituents at C-3'' were an alcohol (as noted above) and a methyl group. C-5'' ( $\delta_{\text{C}}$  25.12) of the methyl group correlated in HMBC with both H-4'' methylene protons, with HO-3'' and with H-2'', whereas the H-5'' protons formed cross-peaks with C-2'', C-4'' and, more weakly, with C-3''. As C-2'' was a methine and there were only two phenolic OH signals, then C-2'' must be attached to the oxygen at C-7 of the isoflavone core and the 6,7-substitution must be a tetrahydrofuranofuran. Thus **6** (retamasin G) had the new structure shown.

This structure has three chiral centres and thus four diastereoisomers were possible. NOESY cross-peaks between H-1'' and H-2'' confirmed that the tetrahydrofuran / dihydrofuran ring-fusion in **6** had *cis* configuration (Figure 2C). The coupling constant between these two protons  $^3J_{1''-2''}$  was 5.6 Hz, which is close to the corresponding  $^3J_{\text{cis}}$  in the tetrahydrofuran / dihydrofuran fused system in panacene<sup>20</sup> (6.0 Hz) and in a related *cis*-3a,8b-dihydrofuro[3,2-*b*]-benzofuran-2(3*H*)-one (5.8 Hz).<sup>21</sup> Correlation was also seen in the NOESY spectrum between H-2'' and H-5'', which shows that this methyl is also *cis* to H-2''. The diastereotopic methylene proton signals were assigned by NOESY correlations HO-3''  $\rightarrow$  H-4'' ( $\delta_{\text{H}}$  3.18) and H-5''  $\rightarrow$  H-4'' ( $\delta_{\text{H}}$  3.45), as shown in Figure 2C.

The benzo-fused furanofuran unit in **6** is hitherto unknown in natural-product flavones and isoflavones, although it is present in the hexahydrofuranobenzofurans (+)-panacene (from the sea hare *Aplysia brasiliensis*),<sup>22</sup> platypodantherone (from *Platypodanthera melissaefolia*),<sup>23</sup> and (+)-gynunone (from *Gynura elliptica*).<sup>24</sup> The motif is seen fused to coumarin in

compounds from *Aegle marmelos*<sup>25</sup> and *Peucedanum praeruptorum*,<sup>26</sup> and to xanthenes in psorofebrin and related compounds from *Psorospermum febrifugum*.<sup>27</sup> In all cases, the two furans are *cis*-fused, as in **6**. However, amongst compounds with Me and OH at the equivalent of C-3'', two examples have the same relative configuration as **6** (with Me *cis* to the ring-junction-H).<sup>25,26</sup> Two others have the opposite configuration (Me *trans* to ring-junction-H), although one lacks the OH.<sup>24,27</sup>

The positive-ion HRESIMS of **7** contained a peak for  $[\text{M} + \text{H}]^+$   $m/z$  367.0800 (calcd 367.0818), corresponding to the molecular formula  $\text{C}_{20}\text{H}_{14}\text{O}_7$ . A peak for  $[2\text{M} + \text{Na}]^+$  was seen at  $m/z$  755.1344 (calcd 755.1377). The negative-ion spectrum contained peaks at  $m/z$  365.0659 ( $[\text{M} - \text{H}]^-$ , calcd 365.0661),  $m/z$  731.1399 ( $[2\text{M} - \text{H}]^-$ , calcd 731.1401) and  $m/z$  767.1164 ( $[2\text{M} + ^{35}\text{Cl}]^-$ , calcd 767.1168). The  $^{13}\text{C}$  NMR spectrum (Table 2) contained eighteen discrete resonances, with two of the methine signals representing two equivalent carbons each. There were signals for eleven quaternary carbons, eight methine carbons and one methyl carbon. The  $^1\text{H}$  spectrum contained signals for three phenolic OH groups, HO-4' ( $\delta_{\text{H}}$  9.65), H-5 ( $\delta_{\text{H}}$  13.73) and HO-7 ( $\delta_{\text{H}}$  11.5).

Compound **7** was an isoflavone, with the singlet for H-2 at  $\delta_{\text{H}}$  8.37, the methine carbon C-2 at  $\delta_{\text{C}}$  154.70 and the quaternary C-3 at  $\delta_{\text{C}}$  122.97. The 3-(4-hydroxyphenyl) substituent was shown by signals at  $\delta_{\text{H}}$  7.40 (H-2' / H-6') and  $\delta_{\text{H}}$  6.84 (H-3' / H-5'). The former correlated in the HMBC spectrum with C-4' ( $\delta_{\text{C}}$  157.72 or 158.00) and with C-3. An HMBC cross-peak from H-3' / H-5' identified C-1' ( $\delta_{\text{C}}$  121.44). IR peaks were observed for the OH ( $3322\text{ cm}^{-1}$ ) and conjugated aryl ketone ( $1630\text{ cm}^{-1}$ ) groups, consistent with a hydroxylated isoflavone. H-8 ( $\delta_{\text{H}}$  6.46) showed HMBC cross-peaks with C-4a ( $\delta_{\text{C}}$  104.11 or 104.40), 6-C ( $\delta_{\text{C}}$  104.11 or 104.40), C-7 ( $\delta_{\text{C}}$  167.59) and C-8a ( $\delta_{\text{C}}$  161.73) and weakly with C-2 through four bonds. The  $^1\text{H}$  NMR signal at  $\delta_{\text{H}}$  6.36 was identified as being attached to  $\text{sp}^3$ -hybridised C-1'' ( $\delta_{\text{C}}$  74.16) and hence to C-6 by HMBC cross-peaks to C-5 ( $\delta_{\text{C}}$  157.72 or 158.00), C-6 and C-7. The unusually downfield chemical shift of this proton was consistent with its benzylic environment, with lactone oxygen and alkene groups adjacent. A strong two-bond HMBC cross-peak of H-1'' to C-2'' ( $\delta_{\text{C}}$  149.15) identified the alkene and the downfield chemical shift of the C-2'' signal indicated that the alkene was conjugated to a carbonyl. A weak three-bond cross-peak was seen between H-1'' and alkene carbon C-3'' ( $\delta_{\text{C}}$  128.48). H-2'' ( $\delta_{\text{H}}$  7.35) correlated in HMBC with the lactone carbonyl C-4'' ( $\delta_{\text{C}}$  175.03). Finally, the methyl-5'' was located as being attached to the lactone at C-3'' by HMBC correlations from H-5'' to C-3'', C-4'' and C-2''. Thus the structure of **7** (retamasin H) was confirmed as a novel isoflavone carrying a  $\gamma$ -lactone substituent at C-6.

Compound **8** gave a  $[\text{M} + \text{H}]^+$  peak in the HRESIMS at  $m/z$  395.1473 (calcd 395.1495), corresponding to the molecular formula  $\text{C}_{23}\text{H}_{22}\text{O}_6$ . A further peak was seen for  $[2\text{M} + \text{H}]^+$  at  $m/z$  789.2872 (calcd 789.2911). The negative-ion HRMS confirmed the formula with a plethora of peaks for  $[\text{M} - \text{H}]^-$  at  $m/z$  393.1340 (calculated 393.1338),  $[\text{M} + ^{35}\text{Cl}]^-$  at  $m/z$  429.1105 (calcd 429.1105),  $[\text{M} + \text{HCO}_2]^-$   $m/z$  at 439.1394 (calculated 439.1393),  $[2\text{M} - \text{H}]^-$  at  $m/z$  787.2752 (calcd 787.2755),  $[2\text{M} +$

**Table 2.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for **6-8** in  $(\text{CD}_3)_2\text{SO}$  [ $\delta_{\text{H}}$ , multiplicity ( $J$  (Hz));  $\delta_{\text{C}}$ , type]

	<b>6</b>		<b>7</b>		<b>8</b>	
Position	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$
2	8.38, s	154.9, CH	8.37, s	154.7, CH	8.37, s	154.7, CH
3		122.8, C <sub>q</sub>		123.0, C <sub>q</sub>		122.9, C <sub>q</sub>
4		181.3, C <sub>q</sub>		181.0, C <sub>q</sub>		180.9, C <sub>q</sub>
4a		106.2, C <sub>q</sub>		104.1, C <sub>q</sub> <sup>a</sup>		105.8, C <sub>q</sub>
5		158.9, C <sub>q</sub>		157.7, C <sub>q</sub> <sup>b</sup>		157.2, C <sub>q</sub>
6		109.1, C <sub>q</sub>		104.4, C <sub>q</sub> <sup>a</sup>		105.0, C <sub>q</sub>
7		167.4, C <sub>q</sub>		167.6, C <sub>q</sub>		159.7, C <sub>q</sub>
8	6.59, s	89.4, CH	6.46, s	93.9, CH	6.50, s	94.8, CH
8a		159.9, C <sub>q</sub>		161.7, C <sub>q</sub>		158.0, C <sub>q</sub>
1'		121.4, C <sub>q</sub>	6.36, br	74.2, CH		121.5, C <sub>q</sub>
2',6'	7.36, d, $J$ 8.5	130.7, CH	7.40, br	130.7, C <sub>q</sub>	7.39, d, $J$ 8.3	130.6, CH
3',5'	6.81, d, $J$ 8.5	115.6, CH	6.84, br	115.6, C <sub>q</sub>	6.83, d, $J$ 8.3	115.4, CH <sup>c</sup>
4'		158.0, C <sub>q</sub>		158.0, C <sub>q</sub> <sup>b</sup>		156.4, C <sub>q</sub>
1''	5.71, d, $J$ 5.6	78.7, CH	5.71, d, $J$ 5.6	78.7, CH	6.66, brd, $J$ 10.3	115.5, CH <sup>c</sup>
2''	4.76, d, $J$ 5.6	91.9, CH	7.35, br	149.1, CH	5.74, 0.5 H, d, $J$ 10.1; 5.75, 0.5 H, d, $J$ 10.1	128.6, CH
3''		77.1, C <sub>q</sub>		128.5, C <sub>q</sub>		81.1, C <sub>q</sub>
4''	3.18, d, $J$ 8.5 3.45, d, $J$ 8.5	72.8, CH <sub>2</sub>		175.0, C <sub>q</sub>	1.62, 0.5 H, m; 1.67, 0.5 H, m; 1.81, 0.5 H, m; 1.87, 0.5 H, m	36.9, CH <sub>2</sub>
5''	1.29, s	25.1, CH <sub>3</sub>	1.87, s	10.8, CH <sub>3</sub>	1.41, s	27.4, CH <sub>3</sub>
6''					1.4, m	33.7, CH <sub>2</sub>
7''					3.56, septet, $J$ 6.0	66.2, CH
8''					1.03, d, $J$ 6.0	24.2, CH <sub>3</sub>
HO-5	13.47, s		13.73, s		13.38, s	
HO-7			11.5, br			
HO-4'	9.58, br		9.65, br		9.62, s	
HO-3''	5.30, br					
HO-7''					4.407, 0.5 H, d, $J$ 4.4; 4.414, 0.5 H, d, $J$ 4.4	

<sup>a</sup> Assignments could be reversed. <sup>b</sup> Assignments could be reversed. <sup>c</sup> Assignments could be reversed.

$^{35}\text{Cl}^-$  at  $m/z$  823.2519 (calcd 823.2518) and  $[2\text{ M} + \text{HCO}_2]^-$  at  $m/z$  833.2807 (calculated 833.2809). Twenty-one discrete  $^{13}\text{C}$  NMR signals were recorded (Table 2), with two resonances corresponding to two equivalent carbons each. Ten signals were for quaternary carbons (all  $\text{sp}^2$ ); there were nine methines (eight  $\text{sp}^2$ , one  $\text{sp}^3$ ), two methylenes and two methyls. In the IR spectrum, peaks were seen for OH ( $3311\text{ cm}^{-1}$ ) and aryl ketone ( $1630\text{ cm}^{-1}$ ), consistent with a hydroxylated isoflavone.

The core of **8** was an isoflavone, with NMR signals for H-2 at  $\delta_{\text{H}}$  8.37 and for methine C-2 at  $\delta_{\text{C}}$  154.73. The singlet signal for H-8 ( $\delta_{\text{H}}$  6.50) correlated in the HMBC spectrum with C-4a ( $\delta_{\text{C}}$  105.76), C-6 ( $\delta_{\text{C}}$  105.02), C-7 ( $\delta_{\text{C}}$  159.72) and C-8a ( $\delta_{\text{C}}$  157.96), with a weaker four-bond cross-peak with the carbonyl C-4 ( $\delta_{\text{C}}$  180.91).  $^1\text{H}$  NMR signals for two phenols were recorded: HO-4' ( $\delta_{\text{H}}$  9.62) and H-5 ( $\delta_{\text{H}}$  13.38); thus the oxygen at the 7-position must be substituted. C-6 was shown to be quaternary, so a ring system connecting C-6 and O-7 was expected. H-1'' ( $\delta_{\text{H}}$  6.66) resonated as a broad doublet, coupled to H-2'' ( $\delta_{\text{H}}$  5.74, 0.5 H;  $\delta_{\text{H}}$  5.75, 0.5 H), as confirmed

by a cross-peak in the COSY spectrum. Both protons formed cross-peaks in the HMBC spectrum with C-3'' ( $\delta_{\text{C}}$  81.10). Moving outwards in the substituent, C-3'' formed two-bond HMBC cross-peaks with both diastereotopic protons of the CH<sub>2</sub>-4'' methylene ( $\delta_{\text{H}}$  1.65 m and  $\delta_{\text{H}}$  1.80 m;  $\delta_{\text{C}}$  36.95); another cross-peak linked C-4'' with H-2''. Strong COSY cross-peaks showed that this methylene was adjacent to another methylene, with both H-6'' protons resonating as a complex multiplet ( $\delta_{\text{H}}$  1.4) and the signal for C-6'' appearing at  $\delta_{\text{C}}$  33.66. Further COSY correlation showed that H-6'' is coupled to a methine proton (H-7'') at  $\delta_{\text{H}}$  3.56. This chemical shift, together with the chemical shift for C-7'' ( $\delta_{\text{C}}$  66.23), suggested an electronegative substituent here. Correspondingly, HO-7'' ( $\delta_{\text{H}}$  4.41) resonated as a doublet ( $J$  2.9 Hz), with a COSY connection to H-7'' and with HMBC connections to C-6'', C-7'' and a methyl (C-8'';  $\delta_{\text{C}}$  24.17). The structure of this side-chain was tied together through further HMBC cross-peaks H-7''  $\rightarrow$  C-4'' and H-8''  $\rightarrow$  C-6''. The remaining methyl carbon was identified as C-5'' ( $\delta_{\text{C}}$  27.40) through HMBC correlations with H-2''; both H-4'' and H-5'' correlated with C-3''. Thus **8** (retamasin I) had the



**Table 3.** Percentage inhibition of the activity of  $\alpha$ -glucosidase by flavonoids isolated from *R. raetam*.

Compound	% Inhibition of $\alpha$ -glucosidase activity at 50 $\mu$ M
<b>1</b>	62.9 $\pm$ 1.9
<b>2</b>	43.6 $\pm$ 0.4
<b>5</b>	33.2 $\pm$ 1.6
<b>6</b>	61.0 $\pm$ 0.6
<b>7</b>	96.3 $\pm$ 0.2
<b>9</b>	46.7 $\pm$ 0.9
<b>10</b>	67.9 $\pm$ 0.7
<b>Acarbose (positive control)</b>	98.7 $\pm$ 0.9

structure shown. The detail of the  $^1\text{H}$  and COSY NMR spectra showed that there were two overlapping signals for each of H-2'', H-6'' and HO-7'', each integrating for 0.5 H. This may indicate that the novel compound **8** had been isolated as an equimolar mixture of diastereoisomers. Figure 2D shows an overlay of the MM2-minimised structures of these diastereoisomers and reveals that the environments of these protons were the most significantly different between these forms. The positive value in the 260 nm – 400 nm region of the CD spectrum (Supplementary Information) suggests that the configuration of **8** may be 3''S,<sup>17</sup> with a 50:50 mixture of epimers at C-7''.

Compounds **9** and **10** were identified as erysubin B and erysubin A, respectively. Their  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra (Table S1, ESI) were almost identical to those reported for compounds isolated from the wood of *Erythrina suberosa* var. *glabrescens*.<sup>28</sup> The assignments were supported by COSY, HSQC and HMBC data and by comparison of mp with literature values.

C-Prenylation of flavones is well known in plants, although a review by Botta *et al.* suggested that C-prenylated isoflavones were less common, as isoflavone synthase and prenyltransferases were less widespread in nature.<sup>29</sup> The C-prenyl group is also found cyclised onto an *ortho*-phenol to form extensive ranges of furanoflavones and pyranoflavones.<sup>30</sup> One may speculate that the isomeric furanoflavone **1** and pyranoflavone **2** (also atalantoflavone<sup>16</sup>) arise through 5-*exo* and 6-*endo* cyclisations of a common oxidised derivative of **4**, such as 8-(3,3-dimethyloxiranylmethyl)-4',5,7-trihydroxyflavone. Retamasin A and retamasin B, previously reported in *R. raetam*,<sup>16</sup> are furanoflavones which may also derive from **4**.

In the isoflavone series, **5**, **6**, **7**, **9** and **10** can all be identified as derivatives of a 6-prenylated isoflavone which have been oxidised and cyclised in different modes. The 6-prenyl unit can also be identified as C-1'', C-2'', C-3'', C-4'' and C-5'' in **8** but a three-carbon extension (C-6'', C-7'' and C-8'') is also present. The biosynthetic origin of these three carbons is unclear but they may be the stump of another prenyl unit which has been partly trimmed down. The benzo-fused bicyclic system in **5** is new amongst natural products but has been identified synthetically as the product of treatment of 2-(1-hydroxy-2-methyl-2-(oxiran-2-yl)ethyl)phenols (oxiranes

derived from an arylisoprene) with acid at low temperature.<sup>31</sup> More vigorous treatment with acid triggers an elimination reaction to give 2-hydroxymethylchromenes. It may be that **5** is biosynthesised from a 6-prenylisoflavone and subsequently undergoes acid-catalysed elimination to give **9**.

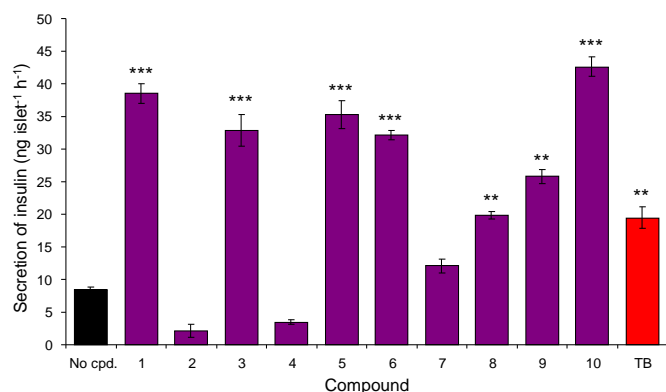
Type-2 diabetes is a long-term metabolic disease in which the ability of peripheral cells to take up glucose from the blood in response to insulin is decreased ("insulin resistance"), leading to an increase in the concentration of glucose in the blood and thence to a range of pathologies. There is also some loss of glucose-stimulated release of insulin by the  $\beta$ -cells of the Islets of Langerhans in the pancreas. Clinically used drugs for treatment of this disorder mostly address either the production of insulin by the  $\beta$ -cells (sulfonylureas, *e.g.* tolbutamide, glibenclamide) or increase the sensitivity of the peripheral cells to insulin (stimulating uptake of glucose, *e.g.* metformin). Type-1 diabetes also involves loss of glucose-stimulated release of insulin by the  $\beta$ -cells. Thus the flavonoids **1–10** isolated from *R. raetam* were evaluated for their ability to increase the secretion of insulin by isolated murine pancreatic islets.

Figure 3 shows the amount of insulin secreted per islet in the presence of the compounds (200  $\mu$ M), with tolbutamide (200  $\mu$ M, positive control) and in the absence of test compound (negative control). The insulin secretory activity of control islets was 8.5  $\pm$  0.38 ng islet<sup>-1</sup> h<sup>-1</sup> upon stimulation by glucose (16.7 mM). This secretion was enhanced significantly ( $p < 0.01$ ) in the presence of the standard sulfonylurea drug tolbutamide (19.49  $\pm$  1.69 ng islet<sup>-1</sup> h<sup>-1</sup>). Isoflavone **7** had little effect and flavones **2** and **4** inhibited the release of insulin under these conditions. However, the remaining flavonoids stimulated the glucose-triggered release of insulin significantly, with **8** and **9** raising the production of insulin *ca.* 3-fold and **1,3,5,6,10** raising secretion by *ca.* 5-fold. There is no clear *prima facie* structure-activity relationship distinguishing potent stimulators from weaker stimulators or inhibitors and the biochemical target is unknown but these flavones / isoflavones represent interesting new lead structures for further development of drugs to treat Type-2 diabetes and may explain the traditional use of this plant.<sup>4</sup>

Inhibitors of  $\alpha$ -glucosidase, a key enzyme in the digestion of polysaccharides, are also used in the management of diabetes. These inhibitors have diverse structures. Acarbose, a mimic of a tetrasaccharide, is particularly effective in populations with a diet high in carbohydrate.<sup>32</sup> 2-(3-Ethoxy-4-hydroxyphenyl)quinazolin-4-one was the most potent inhibitor of  $\alpha$ -glucosidase amongst a series of quinazolinones<sup>33</sup> and the natural product sarcoviolin  $\beta$  is potentially inhibitory.<sup>34</sup> Table 3 lists the percentage inhibition of  $\alpha$ -glucosidase activity by flavonoids **1,2,5-7,9,10** at 50  $\mu$ M; acarbose was used as a positive control. All test compounds showed some activity. The most potent flavonoid **7** showed IC<sub>50</sub> = 36.3  $\pm$  1.7  $\mu$ M for inhibition of this enzyme.

## Conclusions

This paper reports major extension of the number of flavonoids isolated and characterised from *R. raetam*. Three new 8-



**Figure 3.** Glucose-stimulated secretion of insulin from murine pancreatic islets by compounds **1–10**. Freshly isolated islets were incubated for 1 h at 37°C in KRB buffer containing glucose (16.7 mM) in the absence (negative control) or presence of different test compounds and the amount of insulin secreted was measured. Values are mean  $\pm$  SEM from three independent experiments. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , compared with the value for the negative control. TB = tolbutamide.

prenylated flavone derivatives **1–3** and four new 6-prenylated isoflavone derivatives **5–8** are reported, along with identification of two known isoflavones **9,10** newly observed in this plant. The fused bicyclic heterocycles in **5** and **6** are demonstrated in naturally occurring flavonoids for the first time. Although only modest activity was seen in many of the new flavonoids in inhibition of the catalytic activity of  $\alpha$ -glucosidase, **7** showed similar potency to acarbose and may be worthy of further investigation. More importantly, five of the new flavonoids (**1,3,5,6,10**) showed strong stimulation of the glucose-triggered release of insulin from pancreatic islets, with effects much greater than that of the widely used antidiabetic drug tolbutamide. Although they are isomers, **1** and **2** showed very different activities in the insulin secretion assay (Figure 3); this reflects the very different conformations of the saturated heterocycles and the presentation of the functional groups to the receptor(s). These biological observations rationalise the traditional use of *R. raetam* in folk medicine and provides new structural leads for drugs development for treatment of this rapidly increasing major worldwide disease, adding to the range of therapeutic activities known for prenylated flavonoids.<sup>35</sup>

## Experimental

### General experimental procedures

IR spectra were recorded on a Perkin-Elmer FTIR 600 series spectrometer. UV spectra were obtained from an HP 8453 diode array spectrophotometer. The 1D and 2D NMR (COSY, HSQC, HMBC) spectra using standard pulse programs were recorded at room temperature on a Bruker Avance DRX 700 spectrometer operating at 700 MHz (<sup>1</sup>H) and 175 MHz (<sup>13</sup>C), on a Bruker Avance 500 FT spectrometer at 500 MHz (<sup>1</sup>H) and 125 MHz (<sup>13</sup>C), and on an Agilent 400 spectrometer at 400 MHz (<sup>1</sup>H) and 100 MHz (<sup>13</sup>C). Chemical shifts ( $\delta$ ) are reported in parts per million (ppm) relative to the internal standard SiMe<sub>4</sub>

and coupling constants ( $J$ ) are given in Hz. HRESIMS were obtained on an Agilent Series 1100 SL mass spectrometer. Column chromatography was performed using normal-phase silica gel (Merck; 230–400  $\mu$ m) and reversed-phase silica gel (LiChroprep RP-18, Merck; 40–63  $\mu$ m). Preparative HPLC was performed on a Shimadzu system (Kyoto, Japan), consisting of two LC-6AD Semi-Preparative Solvent Delivery pumps connected with Rheodyne manual injector, communications bus module CBM-20A, a multi-wavelength photo-diode array detector (SPD-M20A), FRC-10A fraction collector, and Shimadzu LC solution software. The column was a Shim-pack PREP-ODS(H) Kit 250 mm  $\times$  20 mm, with 5  $\mu$ m particles. The gradient elution used a mobile phase consisting of acetonitrile and water (2:3) programmed linearly to 100% MeCN over 25 min at the flow rate 20 mL min<sup>-1</sup>. The UV detection wavelength was 254 nm. Analytical HPLC was performed using the same system but with a Shim-pack PREP-ODS(H) Kit (A) 250 mm  $\times$  4.6 mm with 5  $\mu$ m particles and flow rate 1.0 mL min<sup>-1</sup>. TLC was carried out on silica gel sheets (Alugram Sil G/UV254, Macherey-Nagel, Germany) and reversed-phase plates (RP-18 F254S, Merck, Germany), with visualisation by 10% aq. H<sub>2</sub>SO<sub>4</sub>, followed by heating.

### Plant material

The aerial parts of *Retama raetam* were collected in April 2010 near Al-looz Mountain, Tabook, Saudi Arabia and were identified by Dr. M. Yusuf, taxonomist, College of Pharmacy, King Saud University (KSU), Riyadh, Saudi Arabia. A voucher specimen (No.15567) has been deposited at the herbarium of the College of Pharmacy, KSU.

### Extraction and isolation

The air-dried aerial parts (1.0 Kg) of *Retama raetam* were percolated at room temperature with CHCl<sub>3</sub> and the solvent was evaporated to yield a dark brown residue (30.0 g). This extract was partitioned between hexane and MeCN to afford hexane fraction (10.0 g) and MeCN fraction (14.0 g). The MeCN fraction (13.0 g) was subjected to silica gel chromatography (5.5  $\times$  50 cm) with a linear gradient elution of methanol and CHCl<sub>3</sub> to give fractions A–T. Fraction D (150 mg) was separated by reversed-phase silica gel column chromatography (MeOH / H<sub>2</sub>O, 4:1  $\rightarrow$  MeOH) to give **1** (8.0 mg), **2** (5.0 mg) and **3** (2.1 mg). Fraction E (414 mg) was separated by reversed-phase chromatography on a C<sub>18</sub> column (MeCN / H<sub>2</sub>O, 13:7  $\rightarrow$  MeCN) to yield **4** (2.7 mg), **5** (4.1 mg) and **6** (1.2 mg), Fraction F (495 mg) was separated by reversed-phase silica gel column chromatography (MeOH / H<sub>2</sub>O, 3:1  $\rightarrow$  MeOH) to give **7** (4.1 mg), **8** (2.1 mg) and **9** (4.0 mg). Fraction H (241 mg) was further purified by reversed-phase chromatography on a C<sub>18</sub> column (MeOH / H<sub>2</sub>O, 7:3  $\rightarrow$  MeOH) to yield **10** (0.8 mg).

**Retamasin C (1):** yellow powder; mp 195–196°C; [ $\alpha$ ]<sub>D</sub><sup>25</sup> –18.6 (c 0.7, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 311 (3.68), 263 (3.62) nm; IR (KBr)  $\nu_{\max}$  3315, 1650, 1601, 1589, 1511 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data, see Table 1; HRESIMS:  $m/z$  355.1156 [M + H]<sup>+</sup> (calcd for C<sub>20</sub>H<sub>19</sub>O<sub>6</sub>, 355.1181).

**Retamasin D (2):** yellow powder; mp 204–206°C; [ $\alpha$ ]<sub>D</sub><sup>25</sup> –10.9 (c 0.11, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 324 (3.60), 263

(3.54) nm; IR (KBr)  $\nu_{\max}$  3341, 1645, 1544, 1473  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 1; HRESIMS:  $m/z$  355.1161  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{19}\text{O}_6$ , 355.1181).

**Retamasin E (3):** yellow powder; mp 231–232°C; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 333 (3.62), 263 (3.52) nm; IR (KBr)  $\nu_{\max}$  3347, 1697, 1654, 1416  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 1; HRESIMS:  $m/z$  355.1159  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{19}\text{O}_6$ , 355.1181).

**Licoflavone C (4):** powder; mp 172–174°C (lit.<sup>19</sup> mp 173–174°C); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 326 (3.62), 305 (3.56) nm; IR (KBr)  $\nu_{\max}$  3267, 1651, 1423  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Supplementary Information.

**Retamasin F (5):** yellow powder; mp 224–225°C;  $[\alpha]_D^{25}$  –22.6 (c 0.7, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 315 (3.64), 263 (3.63); IR (KBr)  $\nu_{\max}$  3333, 1643, 1608, 1574, 1521  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 2; HRESIMS:  $m/z$  369.0953  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{17}\text{O}_7$ , 369.0974).

**Retamasin G (6):** yellow powder; mp 233–234°C;  $[\alpha]_D^{25}$  –26.0 (c 0.08, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 333 (3.61), 263 (3.55) nm; IR (KBr)  $\nu_{\max}$  3363, 1655, 1530, 1425  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 2; HRESIMS:  $m/z$  369.0970  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{17}\text{O}_7$ , 369.0974).

**Retamasin H (7):** yellow powder; mp 247–248°C;  $[\alpha]_D^{25}$  –41.8 (c 0.23, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 321 (3.62), 263 (3.52); IR (KBr)  $\nu_{\max}$  3322, 1630, 1525, 1412  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 2; HRESIMS:  $m/z$  367.0800  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{20}\text{H}_{15}\text{O}_7$ , 367.0818).

**Retamasin I (8):** yellow powder; mp 218–219°C;  $[\alpha]_D^{25}$  –2.8 (c 0.10, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 325 (3.62), 263 (3.52) nm; IR (KBr)  $\nu_{\max}$  3311, 1621, 1534, 1433  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Table 2; HRESIMS:  $m/z$  395.1473  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{23}\text{H}_{23}\text{O}_6$ , 395.1495).

**Erysubin B (9):** powder; mp 248–249°C (lit.<sup>32</sup> mp 247–249°C);  $[\alpha]_D^{25}$  –17.0 (c 0.10, MeOH); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 284 (3.56), 225 (3.62); 205 (3.65) nm; IR (KBr)  $\nu_{\max}$  3342, 1621, 1427  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Supplementary Information.

**Erysubin A (10):** powder; mp 231–232°C (lit.<sup>32</sup> mp 231–233°C); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 353 (3.62), 267 (3.60); 212 (3.57) nm; IR (KBr)  $\nu_{\max}$  3355, 1598, 1419  $\text{cm}^{-1}$ ;  $^1\text{H}$  and  $^{13}\text{C}$  NMR data, see Supplementary Information.

#### Sources of materials for biological assays

Tolbutamide (TB), collagenase V and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Mouse Ultrasensitive Insulin ELISA kit was purchased from Crystal Chem Inc. (IL, USA).

#### Isolation of pancreatic islets

Primary pancreatic islets were isolated from male BALB/c mice (28–36 g) from the International Center for Chemical and Biological Sciences (ICCBS) Animal Research Facility, University of Karachi, Pakistan. The handling of animals and harvesting of tissues were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and with prior approval from the Animal Use Committee of ICCBS

(Protocol number: 2015-0020). Pancreatic islets were isolated by digestion with collagenase V and islet-picking methods as described earlier.<sup>36</sup> Briefly, mice were anaesthetised with sodium thiopental (30 mg  $\text{Kg}^{-1}$ ) and the distended whole pancreas was digested at 37°C in collagenase solution (1.0 mg  $\text{mL}^{-1}$ ) for 15 min. The digested islets were further purified by centrifugation at 1000 rpm for 1.0 min, followed by filtration using a pre-wetted 70  $\mu\text{m}$  cell strainer. Finally, the islets were manually picked with a siliconised Pasteur pipette under a NIKON SMZ-745 stereomicroscope. The isolation and purification medium used was Hank's Balanced Salt Solution (HBSS) without calcium, magnesium and phenol red. Diameters were measured using a camera mounted on a stereomicroscope.

#### Insulin secretion assay

Freshly isolated islets ( $n = 3$ ) of similar diameter (120–160  $\mu\text{m}$ ) were pre-incubated for 45 min at 37°C in Krebs–Ringer bicarbonate (KRB) buffer solution containing BSA (0.1%) and glucose (3.0 mM) in Eppendorf tubes. Each was performed in triplicate. The islets were washed with KRB buffer before being transferred into KRB buffer (300  $\mu\text{L}$ ) containing glucose (16.7 mM), supplemented with either test compound (pre-dissolved in DMSO, 200  $\mu\text{M}$ ) or tolbutamide (pre-dissolved in DMSO, 200  $\mu\text{M}$ ; a standard insulin secretagogue used as a positive control) and were incubated at 37°C for 1.0 h. At the end of incubation, an aliquot (200  $\mu\text{L}$ ) was placed on ice-cold water and kept at –40°C until the insulin was assayed. Concentrations of insulin in the collected aliquots were measured using the mouse ultrasensitive insulin ELISA kit according to the high-range protocol. The reading is the concentration of insulin in  $\text{ng mL}^{-1}$  of medium. After calculation of the absolute amount of insulin in the medium, this amount was divided by three (the number of islets in the tube).

#### Assay of inhibition of $\alpha$ -glucosidase

The assays of inhibition of  $\alpha$ -glucosidase were conducted according to the method reported elsewhere<sup>33</sup> with slight modification. The reaction mixture contained sodium phosphate buffer (50 mM, pH 6.8) in a total volume of 300  $\mu\text{L}$ .  $\alpha$ -Glucosidase (40 mU) from *Saccharomyces cerevisiae* (Sigma Chemical Co., St. Louis, MO, USA) was incubated with target compounds for 15 min at 37°C. Compounds were dissolved in DMSO; the final concentration of this solvent in the assay mixture was 3%. After addition of 4-nitrophenyl- $\alpha$ -D-glucopyranoside (0.7 mM), the enzyme reaction was continuously monitored at 400 nm in 96-well microplates using SpectraMax Plus 384 microplate reader (Molecular Devices, CA, USA). Acarbose was used as a positive control. The activity of the compounds was measured as percent inhibition of  $\alpha$ -glucosidase activity relative to controls with no test compounds. The assays were performed in quadruplicate and the mean percentage inhibitions were calculated, along with their respective SEM (standard error of the mean) values.

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