Fine specificities of two lectins from *Cymbosema roseum* seeds: a lectin specific for high-mannose oligosaccharides and a lectin specific for blood group H type II trisaccharide

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The legume species of *Cymbosema roseum* of Diocleinae subtribe produce at least two different seed lectins. The present study demonstrates that *C. roseum* lectin I (CRL I) binds with high affinity to the "core" trimannoside of *N*-linked oligosaccharides. *Cymbosema roseum* lectin II (CRL II), on the other hand, binds with high affinity to the blood group H trisaccharide (Fucα1,2Galα1-4GlcNAc-). Thermodynamic and hemagglutination inhibition studies reveal the fine binding specificities of the two lectins. Data obtained with a complete set of monodeoxy analogs of the core trimannoside indicate that CRL I recognizes the 3-, 4- and 6-hydroxyl groups of the α(1,6) Man residue, the 3- and 4-hydroxyl group of the α(1,3) Man residue and the 2- and 4-hydroxyl groups of the central Man residue of the trimannoside. CRL I possesses enhanced affinities for the Man5 oligomannose glycan and a biantennary complex glycan as well as glycoproteins containing high-mannose glycans. On the other hand, CRL II distinguishes the blood group H type II epitope from the Lewis^x, Lewis^y, Lewis^a and Lewis^b epitopes. CRL II also distinguishes between blood group H type II and type I trisaccharides. CRL I and CRL II, respectively, possess differences in fine specificities when compared with other reported mannose and fucose recognizing lectins. This is the first report of a mannose-specific lectin (CRL I) and a blood group H type II-specific lectin (CRL II) from seeds of a member of the Diocleinae subtribe.

Introduction

Glycan-binding proteins (lectins) of the Leguminosae family comprise one of the largest and well-studied groups of homologous proteins (Strosberg et al. 1986). Despite similarities in their physicochemical properties and their relatively conserved primary sequences, leguminosae lectins display considerable diversity in their carbohydrate-binding specificities (Sharon and Lis 1990). Although the biological functions of plant lectins remain largely speculative, it appears that endogenous lectins can potentially protect the plants from predators and pathogens (Etzler and Mohnen 2009). Due to their inherent toxicity, some plant lectins are capable of deterring predatory insects. Enzymatic activities of some lectins may contribute to plant defense. For example, *Dolichos biflorus* seed lectin is a lipoxygenase, an enzyme required for wound induced defense pathways in plants (Etzler and Mohnen 2009). However, considering their abilities to interact with a range of glycan structures, a potential function of plant lectins is pathogen recognition through their surface glycans. Glycans comprise a good portion of the so-called "pathogen-associated molecular patterns", the surface molecules of pathogens recognized by host receptors including lectins. It is already shown that endogenous lectins play important roles in the innate immune system of animals (van Kooyk and Rabinovich 2008). Similarly, plant lectins can potentially serve as innate pathogen surveillance molecules in plants.

Plant tissues, like their animal counterpart, often possess lectins with different glycan specificities indicating their involvement in a multitude of biological functions (Van Damme et al. 1997). The nodules of *Arachis hypogaea* contain a Man-binding lectin and a Gal-binding lectin. Two lectins, jacalin (Galβ1-3GalNAc-specific) and Artocarpin (Man-specific), have been isolated from the seeds of *Artocarpus integrifolia*. Two species of the genus *Cytisus* produce two seed lectins specific for GlcNAc oligomers and Gal, respectively. Beside the GalNAc-binding seed lectin named DBA, *D. biflorus* synthesizes two other lectins in other tissues which recognize yet unknown complex carbohydrate structures. Four lectins with different

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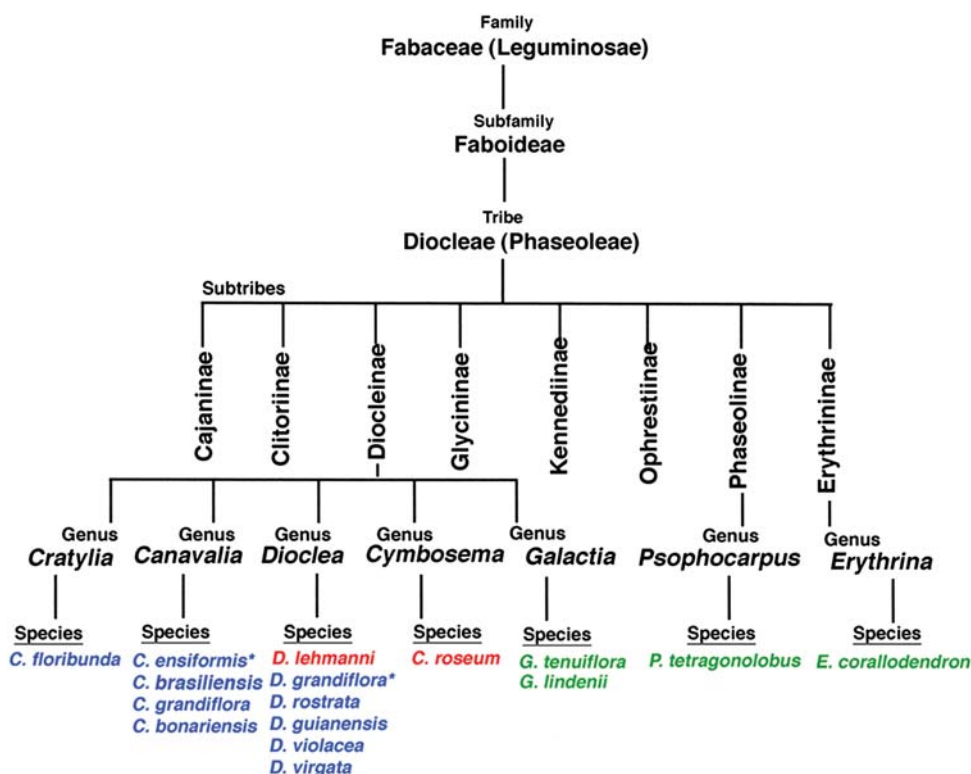
Scheme I. Occurrence of mannose specific and fucosylated glycan-binding lectins in Diocleae tribe and Diocleinae subtribe. Several species (in blue) of Diocleinae subtribe possess mannose specific lectins, whereas two species (in red) contain a second lectin specific for fucosylated glycans, along with the mannose-specific lectin (Pérez 1998; Almanza et al. 2004). Beside the well-characterized mannose-binding lectin, both *C. ensiformis* and *D. grandiflora* (asterisks) have been indicated to have the second lectin specific for fucosylated glycans (Almanza et al. 2004). Fucosylated glycan-binding lectins have been isolated from two other Diocleinae species, namely, *G. tenuiflora* and *G. lindenii* (green; Du et al. 1994; Almanza et al. 2004). Beside Diocleinae subtribe, species from other subtribes of Diocleae tribe also contain a fucosylated glycan-binding lectin. The species include *P. tetragonolobus* (Srinivas et al. 1999) and *E. corallodendron* (Moreno et al. 1997; green).

carbohydrate-binding properties have been reported from *Griffonia simplicifolia* seeds. Carbohydrate ligands of these lectins include blood group A, blood group B as well as Lewis b antigens and GlcNAc oligomers. *Phaseolus vulgaris* seed lectins, PHA-E and PHA-L, are specific for closely related complex carbohydrate structures. The pokeweed, *Phytolacca americana*, produces at least six GlcNAc oligomer-binding lectins. Winged bean agglutinins WBA-I and WBA-II bind to blood group A and blood group H(O) antigens, respectively. The seeds of *Ulex europaeus* contain three lectins with different glycan-binding properties: the Fuc-binding UEA-I is very specific for blood group H(O) substances, whereas UEA-II and UEA-III recognize chitobiose and lactose, respectively. Three *Vicia villosa* seed lectins show specificities for blood group A antigen, Tn antigen and mannose/glucose, respectively. A GalNAc-specific lectin and a Man-specific lectin have been purified from *Wistaria floribunda* (Van Damme et al. 1997).

From a phylogenetic view, the Diocleae tribe of the Fabaceae (Leguminosae) family (Scheme I) has been the source of many well-characterized lectins with a variety of glycan-binding specificities. The tribe Diocleae is divided into several subtribes (Scheme I) of which the subtribe Diocleinae has been extensively investigated for lectins. Interestingly,

different species of Diocleinae subtribe (Scheme I) produce closely related lectins with conserved binding sites that recognize Man oligosaccharides. It is becoming apparent that some of the same species co-synthesize a second lectin with a conserved binding site most complementary to blood group H type II trisaccharide.

In previous studies, we have reported the carbohydrate-binding specificities of lectins from 10 phylogenetically related species from Diocleinae subtribe (Scheme I; Dam, Cavada, et al. 1998; Dam, Oscarson, et al. 1998). These Man-specific proteins, known as Diocleinae lectins, show enhanced affinity for core trimannoside, 3,6-di-*O*-(α-D-mannopyranosyl)-D-mannose, the core structure of all *N*-linked glycans (**1** in Figure 1) and Man5 oligomannose glycan (**13** in Figure 1), but differential specificities for a complex glycan (**14** in Figure 1; Dam, Cavada, et al. 1998).

Despite their phylogenetic proximity, apparently conserved amino acid sequences and similar carbohydrate specificities, the mannose-specific Diocleinae lectins show subtle differences in fine carbohydrate-binding properties and demonstrate different levels of biological activities when used as external stimuli for immunobiological functions such as histamine release from rat peritoneal mast cells (Gomes et al. 1994), lymphocyte proliferation and interferon γ production
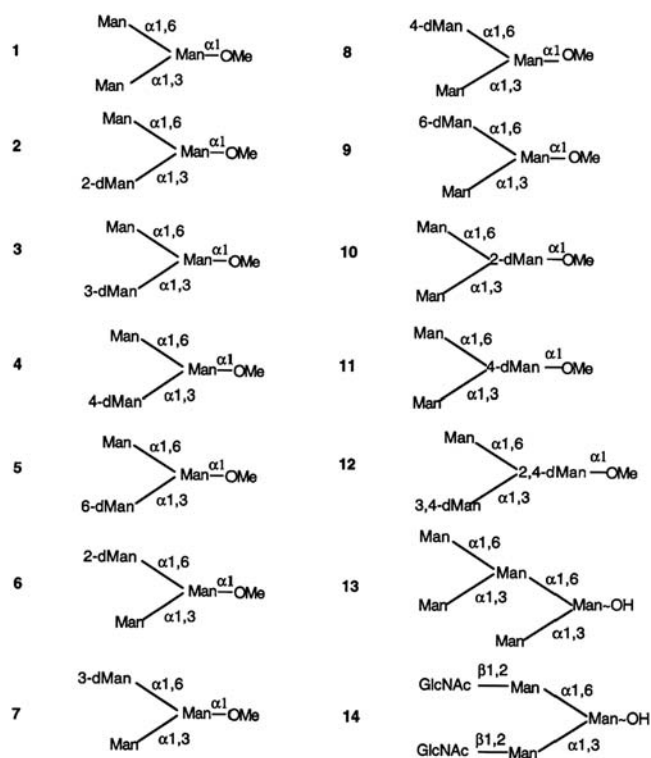
Fig. 1. Structures of the core trimannoside (**1**) and its deoxy analogs (**2–12**), Man5 oligomannose (**13**) and a complex pentasaccharide (**14**).

(Barral-Netto et al. 1992), peritoneal macrophage stimulation and inflammatory reaction (Rodrigues et al. 1992) as well as induction of paw edema and peritoneal cell immigration in rats (Bento et al. 1993). Subtle differences in carbohydrate-binding activities may contribute to the above-mentioned differential stimulatory abilities of Diocleinae lectins. In fact, the relative affinities of Diocleinae lectins for complex carbohydrate **14** (Figure 1) correlated with their abilities to release histamine in rat peritoneal mast cells (Dam, Cavada, et al. 1998).

The present study reports the glycan-binding properties of two new lectins from the seeds of *Cymbosema roseum*, another species of Diocleinae subtribe (Scheme I). These two lectins have been designated as *C. roseum* lectin I (CRL I) and *C. roseum* lectin II (CRL II). CRL I, like other Man-specific Diocleinae lectins, binds to Man oligosaccharides, whereas CRL II recognizes the blood group H type II trisaccharide (Fucα1,2Galβ1-4GlcNAc-). We herein present hemagglutination inhibition and isothermal titration calorimetry (ITC) data for the binding of CRL I to a variety of mono- and oligosaccharides including trimannoside **1**, deoxy analogs **2–12**, Man5 oligomannose carbohydrate **13**, biantennary complex carbohydrate **14** (Figure 1) and CRL II to different fucosylated glycans (Figure 2). Binding properties of CRL I and CRL II have been compared with other Man-specific Diocleinae lectins and Fuc-specific plant lectins, respectively. This is the first report of a lectin (CRL II) from a member of the Diocleinae subtribe that recognizes the blood group H trisaccharide and a lectin (CRL I) that recognizes Man residues

in the same tissue. This study provides a molecular basis of glycan recognition by CRL I and CRL II. The implication of the occurrence of two lectins with specificities for Man- and Fuc-glycans, respectively, from the same source is discussed.

Results

Two lectins (CRL I and CRL II) have been purified by affinity chromatography from the seeds of *C. roseum*, a species of Diocleinae subtribe. Preliminary studies suggested that CRL I was a Man-specific lectin and CRL II a lactose-specific lectin (Cavada et al. 2006; Rocha et al. 2009). We herein report the fine carbohydrate recognition properties of both lectins using hemagglutination inhibition and ITC measurements.

Binding properties of CRL I

Hemagglutination inhibition data in Table I shows that CRL I possesses enhanced affinity for core trimannoside **1** relative to methyl α-mannopyranoside (MeαMan), a feature observed with other Man-specific Diocleinae lectins including concanavalin A (ConA) and *Dioclea grandiflora* lectin (DGL; Dam, Cavada, et al. 1998). ITC data shown in Table II demonstrate that the K_a and $-\Delta H$ values of CRL I for **1** are greater than those obtained with MeαMan. The K_a for **1** is ~40 times higher than that of MeαMan, whereas the $-\Delta H$ for **1** is 7.3
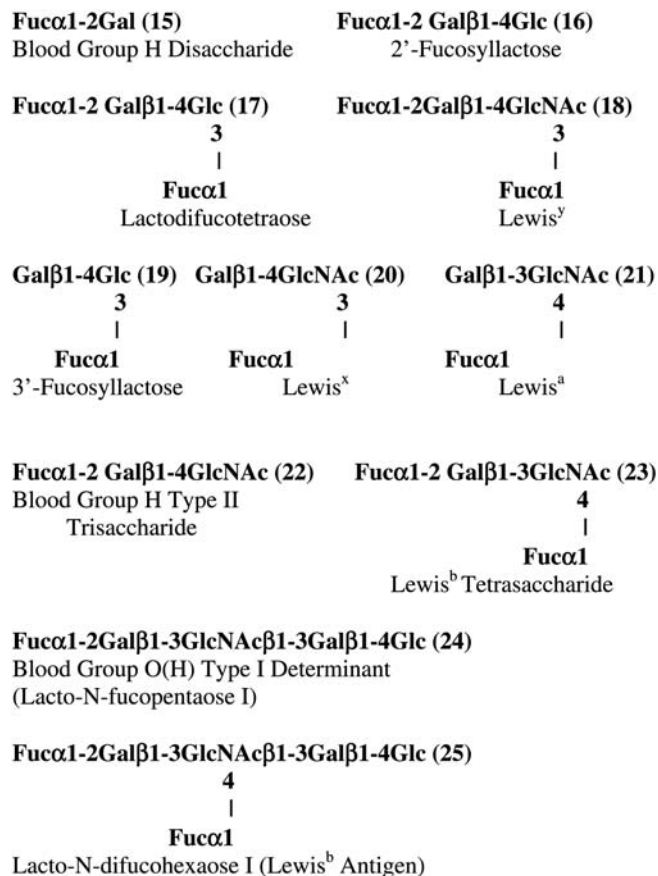

Fig. 2. Structures of fucosylated glycans.

Table I. Inhibitory potencies of trimannoside **1** and its deoxy analogs for CRL I-mediated hemagglutination of rabbit erythrocytes

Carbohydrates (μM)	Minimum concentration required for inhibition
MeαMan	11 000
1 Trimannoside	9
2 α(1-3)2-deoxy	45
3 α(1-3)3-deoxy	NI at 1200 μM
4 α(1-3)4-deoxy	165
5 α(1-3)6-deoxy	32
6 α(1-6)2-deoxy	27
7 α(1-6)3-deoxy	NI at 1500 μM
8 α(1-6)4-deoxy	NI at 1600 μM
9 α(1-6)6-deoxy	NI at 1600 μM
10 "Core" 2-deoxy	940
11 "Core" 4-deoxy	730
12 Tetradeoxy	5800
13 Man-5	6
14 Gn2 Man3	52

NI, non-inhibitory.

Table II. Thermodynamic-binding parameters obtained from the titration of CRL I at pH 7.2 with saccharides at 27°C

Carbohydrates	K_a^a $(M^{-1} \times 10^{-4})$	$-\Delta G^b$ (kcal mol^{-1})	$-\Delta H^c$ (kcal mol^{-1})	$-T \Delta S^d$ (kcal mol^{-1})
MeαMan	0.13	4.3	5.2	0.9
1 Trimannoside	36.7	7.6	12.9	5.3
2 α(1-3)2-deoxy	31.4	7.5	11.7	4.2
3 α(1-3)3-deoxy	1.4	5.7	8.6	2.9
4 α(1-3)4-deoxy	9.3	6.8	10.8	4.0
5 α(1-3)6-deoxy	33.8	7.6	11.5	3.9
6 α(1-6)2-deoxy	29.1	7.5	11.2	3.7
7 α(1-6)3-deoxy	1.1	5.5	7.4	1.9
8 α(1-6)4-deoxy	1.5	5.7	8.4	2.7
9 α(1-6)6-deoxy	1.3	5.6	8.1	2.5
10 "Core" 2-deoxy	7.9	6.7	10.2	3.5
11 "Core" 4-deoxy	5.8	6.5	9.7	3.2
12 Tetradeoxy	0.86	5.4	6.8	1.4
13 Man-5	41.4	7.7	14.6	6.9
14 Gn2 Man3	32.3	7.5	8.1	0.6

n values are between 0.95 and 1.07. Errors in n are less than 5%.
[a]Errors in K_a are 2–7%.
[b]Errors in ΔG are 1%.
[c]Errors in ΔH are 1–5%.
[d]Errors in $T \Delta S$ are 2–8%.

Table III. Inhibitory potencies of various saccharides for CRL II-mediated hemagglutination of rabbit erythrocytes

Carbohydrates	Minimum concentration required for inhibition (μM)
D-Galactose	15 000
D-Fucose	17 000
L-Fucose	NI at 25 mM
Lactose	3500
Galβ1-3GlcNAc	4300
Galβ1-4GlcNAc	2500
2′-Fucosyllactose (**16**)	40
3′-Fucosyllactose (**19**)	NI at 20 mM
Lewis^y trisaccharide (**18**)	NI at 18 mM
Lewis^x trisaccharide (**20**)	NI at 15 mM
Lewis^a trisaccharide (**21**)	NI at 15 mM
Blood group H disaccharide (**15**)	1000
Blood group H type II trisaccharide (**22**)	18
Lacto-N-fucopentaose I (H type I) (**24**)	450
Lactodifucotetraose (**17**)	2000
Lacto-N-difucosylhexaose I (Lewis^b antigen) (**25**)	8000
Lewis^b tetrasacccharide (**23**)	5000

NI, non-inhibitory.

are α(1-3)3-deoxy (**3**), α(1-6)3-deoxy (**7**), α(1-6)4-deoxy (**8**), α(1-6)6-deoxy (**9**), "core" 2-deoxy (**10**) and "core" 4-deoxy (**11**). α(1-3)4-deoxy (**4**) is also less potent than **1**. These results indicate the involvements of the 3-, 4- and 6-hydroxyls of the α(1-6) Man, the 3- and 4-hydroxyls of the α(1-3) Man and the 2- and 4-hydroxyls of the central Man of trimannoside **1** in binding. Data obtained with the tetradeoxy analog **12** further confirm this finding. In **12**, 3- and 4-hydroxyl groups of α(1-3)-linked mannose and 2- and 4-hydroxyl groups "core" mannose are modified. In the absence of these hydroxyl groups on α(1-3)-linked and "core" mannose that potentially make contact with CRL I-binding site, **12** would interact with CRL I only through α(1-6)-linked mannose. Therefore, functionally the affinity of **12** would be similar to that of the monosaccharide mannose. Indeed, the inhibitory potency of **12** is comparable with that of MeαMan (Table I). It should be noted that other reported Man-specific Diocleinae lectins interact with **1** through the same set of hydroxyl groups (Dam, Oscarson, et al. 1998; Dam et al. 2000). These results indicate the presence of a highly conserved binding site for **1** in Man-specific Diocleinae lectins.

Thermodynamics of CRL I binding to trimannoside 1

ITC data in Table II show that CRL I binds to **1** with a change in enthalpy (ΔH) of -12.5 kcal mol^{-1} and a K_a of 24.7×10^4 M^{-1}. The affinity constant of CRL I for **1** is ∼43-fold greater than that of MeαMan and the ΔH for **1** is -7.3 kcal mol^{-1} greater than that of the monosaccharide (Table II). The greater $-\Delta H$ value of **1** indicates that the high affinity of CRL I for **1**, relative to MeαMan, is due to extended site interactions. It has been shown that the magnitude of the $-\Delta H$ value is often related to the size of the binding site of a lectin (Gupta et al. 1997; Dam, Oscarson, et al. 1998). The present results can be compared with ITC data obtained with other mannose-binding Diocleinae lectins, which show greater $-\Delta H$ values for **1** than those of MeαMan,

kcal mol^{-1} greater than that of the monosaccharide. The $-\Delta H$ values for other Diocleinae lectins binding to **1** are 5–7 kcal mol^{-1} greater than that for MeαMan, similar to the differences observed for CRL I (Table II). These data strongly suggest that like other Man-binding Diocleinae lectins, CRL I possesses an extended binding site that accommodates trimannoside **1**.

In order to determine which hydroxyl groups of **1** are involved in binding to CRL I, hemagglutination inhibition experiments were performed using monodeoxy analogs (**2–11**) and a tetradeoxy analog (**12**) of **1** (Figure 1). Specific hydroxyl group(s) of the mannose residues of **1** has been modified in these analogs (Figure 1). The inhibition data (Table I) show that CRL I interacts with certain monodeoxy analogs with reduced affinity compared with **1**. These analogs

and higher K_a values for **1** compared with the monosaccharide (Mandal et al. 1994; Gupta et al. 1997; Dam, Oscarson, et al. 1998; Dam et al. 2000). Thus, it appears that all Diocleinae lectins possess extended glycan-binding sites that can accommodate trimannoside **1**.

Thermodynamics of CRL I binding to monodeoxy analogs of trimannoside 1

Hemagglutination inhibition data in Table I have shown that specific hydroxyl groups of **1** interact with the binding site of CRL I. Other Diocleinae lectins have similar patterns of binding toward deoxy trimannoside analogs **2–12** in Figure 1. In order to further characterize the binding epitopes of **1** with CRL I, ITC has been used to determine the thermodynamics of binding of monodeoxy analogs **2–11** and tetradeoxy analog **12** with the lectin.

Interactions of the α(1-3) arm of trimannoside **1** with CRL I were determined using the α(1-3)2-deoxy (**2**), α(1-3) 3-deoxy (**3**), α(1-3)4-deoxy (**4**) and α(1-3)6-deoxy (**5**) derivatives (Figure 1). Table II shows that the K_a values of **2** (2.1×10^5 M^{-1}) and **5** (2.0×10^5 M^{-1}) are slightly less than that of **1** (2.5×10^5 M^{-1}) and that the ΔH values for **2** (-11.7 kcal mol^{-1}) and **5** (-11.5 kcal mol^{-1}) are also only slightly reduced from that of **1** (-12.5 kcal mol^{-1}). On the other hand, **3** shows about 18-fold decrease in K_a (1.4×10^4 M^{-1}) and -3.9 kcal mol^{-1} less ΔH value compared with **1**. Analog **4** shows a ΔH of -10.8 kcal mol^{-1}, and a moderate reduction in K_a (1.6×10^5 M^{-1}). These results suggest substantial binding of the 3-OH of the α(1-3) arm of **1** with CRL I, and possible binding of the 4-OH on the same arm. The K_a values derived from the ITC measurements agree with hemagglutination inhibition studies which show affinities of CRL I for **2**, **4** and **5** to be similar to **1**, but the lectin binds to **4** with slightly reduced affinity whereas **3** is a very poor inhibitor.

ITC data for binding of monodeoxy analogs of the α(1-6) arm of **1** are also shown in Table II. The K_a value (1.7×10^5 M^{-1}) for the α(1-6)2-deoxy analog (**6**) is slightly lower than that of **1**, and its ΔH is -11.2 kcal mol^{-1}. The K_a values for **7**, **8** and **9** are nearly 22-fold lower than that of **1**, and their $-\Delta H$ values are considerably lower (-7.4, -8.2 and -8.1 kcal mol^{-1}, respectively) than **1**. These data suggest the involvement in binding of 3-, 4- and 6-hydroxyl groups of the α(1-6) Man residue of **1**.

ITC data for the 2-deoxy (**10**) and 4-deoxy (**11**) derivatives of the central Man residue of **1** (Table II) show reductions in K_a for both analogs (7.9×10^4 and 5.8×10^4 M^{-1}, respectively), relative to **1**. The ΔH values of **10** and **11** are -10.2 and -9.7 kcal mol^{-1}, respectively. These data indicate the involvement of the 2- and 4-OH of the "core" or central Man residue of **1** in binding to CRL I.

Binding of tetradeoxy analog of 1

The α(1-3)3,4-deoxy,"core" 2,4-deoxy analog of **1** (**12**) (Figure 1) was synthesized and its thermodynamic-binding parameters determined for CRL I. In the absence of the interacting OH groups on α(1-3)-linked mannose (3- and 4-OH) and the "core" or central Man residue (2- and 3-OH), **12** would interact with CRL I only via the α(1-6)-linked mannose residue. Indeed, the thermodynamic data indicate that functionally **12** is a monosaccharide. Table II shows that CRL I binds to **12** with K_a and ΔH values comparable with those of MeαMan. The data obtained with **12** indicate that the α(1-6)-linked mannose residue of **1** binds to the primary binding site of CRL I. The binding thermodynamics of **12** further confirm the observations that 3-, 4-OH of α(1-3)-linked mannose, 3-, 4-, 6-OH of α(1-6)-linked mannose and 2-, 4-OH of the "core" or central mannose of the trimannoside **1** make contacts with the binding site of
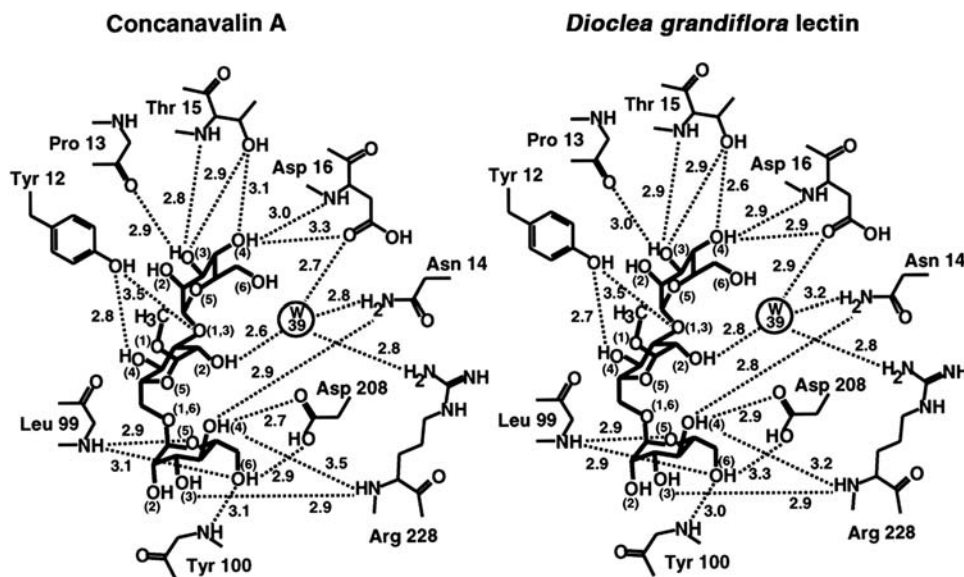
Fig. 3. Schematic diagrams of the binding site amino acid residues of ConA and DGL in contact with trimannoside **1**. The sphere represents conserved water molecule W39, and the dotted lines represent hydrogen bond contacts. The contact distances are labeled in Å. The data are from Dam et al. (2000). Thermodynamic data presented here suggest that CRL I recognizes the same set of OH groups of **1**.

CRL I. CRL I essentially recognizes the same set of OH groups of **1** as do the other reported Diocleinae lectins including ConA and DGL. X-ray crystallographic data showing the contacts between the OH groups of **1** and ConA/DGL-binding site are shown in Figure 3. A comparison of deoxy sugar-binding data of CRL I and ConA is shown in Figure 4.

Binding of Man5 oligomannose carbohydrate (13)

Hemagglutination inhibition data in Table I show that the CRL I binds Man5 oligosaccharide **13** with almost the same affinity as **1**. This indicates that the trimannoside moiety on the $\alpha(1\text{-}6)$ arm is the primary epitope for interaction, as observed for other Diocleinae lectins (Dam, Oscarson, et al. 1998). ITC studies confirm the hemagglutination data and show that the K_a (3.1×10^5 M^{-1}) and ΔH (-14.6 kcal mol^{-1}) values of **13** are slightly higher than those of **1** (Table II).

Binding of biantennary complex oligosaccharide 14

Hemagglutination inhibition data in Table I show that the affinity of **14** for CRL I is comparable with that of trimannoside **1**. ITC data for CRL I binding to **14** are shown in Table II. The K_a value is 2.2×10^5 M^{-1} when compared with 2.5×10^5 M^{-1} for **1**. Interestingly, the $-\Delta H$ value for **14** is 4.4 kcal mol^{-1} less than the $-\Delta H$ value of **1**, and $T \Delta S$ of **14** is 4.1 kcal mol^{-1} more positive than the corresponding value of **1**. The data suggest that CRL I essentially binds to the core trimannoside portion of **14**; however, the overall binding thermodynamics are very different than that of **1**. Addition of GlcNAc residues to $\alpha(1\text{-}3)$- and $\alpha(1\text{-}6)$-linked mannose residues of **1**, as in **14**, results in an entropically favored binding interaction. Elongation of $\alpha(1\text{-}3)$- and $\alpha(1\text{-}6)$-linked mannose residues of the core trimannoside of **14** with GlcNAc residues may facilitate diffusion of **14** to CRL I and thus reduces the entropic cost.
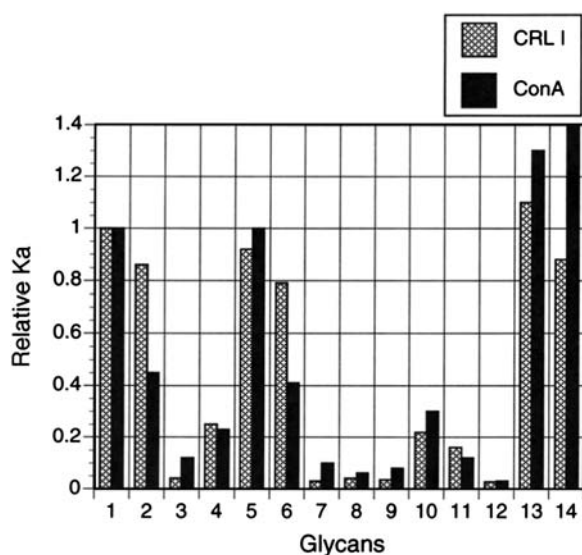
Although all Diocleinae lectins show comparable enhanced affinities and conserved binding mechanism for **1**, their affinities for **14** are non-identical. Among the Diocleinae lectins tested, ConA, *Canavalia brasiliensis*, *Dioclea guianensis* and *Dioclea virgata* show higher affinity for **14**, compared with that of **1**; whereas DGL, *Canavalia bonariensis*, *Cratylia floribunda*, *Dioclea rostrata* and *Dioclea violacea* bind to **14** with poor affinity (Dam, Cavada, et al. 1998). In this regard, CRL I belongs to the first subgroup. The structural basis for this difference has been investigated (Rozwarski et al. 1998). Importantly, the ability of these nine Diocleinae lectins to induce histamine release from rat peritoneal mast cells (Gomes et al. 1994) was shown to correlate with the relative affinities of the proteins for **14** (Dam, Cavada, et al. 1998).

Glycan-binding specificities of CRL II

Hemagglutination inhibition data in Table III show that CRL II interacts only weakly with the monosaccharides D-Gal and D-Fuc. D-Man failed to bind to the lectin under these conditions. However, the affinity of blood group H disaccharide (Fucα1-2Gal, **15**) is 15–17 times stronger than that of the constituent monosaccharides (Table III). The disaccharides lactose and type I and II LacNAc also bind somewhat better than the above monosaccharides, which explain the initial isolation of CRL II on a lactose affinity column (Rocha et al. 2009).

Fucosylation of lactose and LacNAc at the non-reducing end dramatically improves the binding affinities to CRL II. For example, 2′-fucosyllactose (Fucα1-2Galβ1-4Glc, **16**) shows ~100-fold enhancement in affinity compared with lactose, and blood group H type II trisaccharide (Fucα1-2Galβ1-4GlcNAc, **22**) is ~150 times more potent as an inhibitor than LacNAc II (Table III). However, fucosylation of the reducing-end Glc residue of lactose (3′-fucosyllactose, **19**) and the GlcNAc residue of LacNAc I and II, respectively, (Lewis[x], **20**, and Lewis[a], **21**, respectively) completely diminishes binding activity. Addition of an $\alpha(1\text{-}3)$-linked fucose (as in Lewis[y] tetrasaccharide, **18**) or an $\alpha(1\text{-}4)$-linked

Fig. 4. Bar graphs showing the comparison of relative K_a values of ConA and CRL I obtained with the glycans in Figure 1.
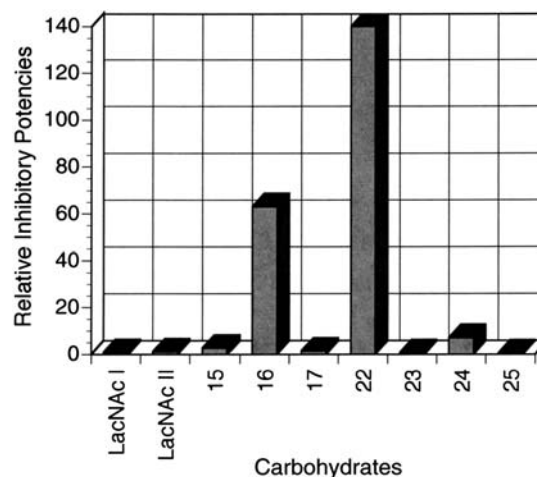

Fig. 5. Bar graphs showing the relative potencies of various glycans for inhibiting CRL II-induced agglutination.

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Fuc (as in Lewis[b] tetrasaccharide, **23**) to the reducing-end GlcNAc of blood group H type II trisaccharide (Fucα1-2Galβ1-4GlcNAc, **22**) turns the glycans virtually unrecognizable by CRL II. When an α(1-3)-linked Fuc is added to the reducing-end glucose of 2′-fucosyllactose, as in lactodifucotetraose, **17**, the binding affinity is reduced by 50-fold. Elongation of blood group H type II trisaccharide (**22**) with a lactose residue at the reducing end (as in blood group O(H) type I determinant, lacto-*N*-fucopentaose I, **24**) lowers the affinity by 25-fold. When the GlcNAc residue of **24** is fucosylated, as in lacto-*N*-difucohexaose I (**25**), the glycan could not bind to CRL II. Figure 5 shows bar graphs representing the relative inhibitory potencies of different glycans.

Thermodynamics of CRL II binding to fucosylated and non-fucosylated glycans

Thermodynamic data obtained by ITC measurements for CRL II binding to some of the glycans in Figure 2 are shown in Table IV. Due to low affinities, the monosaccharides in Table III did not give ITC data.

The thermodynamic data in Table IV are consistent with the hemagglutination inhibition data in Table III. The stoichiometry of binding of the glycans in Table IV show n values close to 1 that indicate a single binding site per monomer of CRL II. Blood group H type II trisaccharide (**22**) exhibits the highest K_a of 1.2×10^5 M^{-1} The ΔG of **22** is -6.9 kcal mol^{-1}, which is comprised of a favorable ΔH of -9.1 kcal mol^{-1} and an unfavorable $T\Delta S$ of -2.2 kcal mol^{-1} (Table IV). 2′-Fucosyllactose (**16**) differs from **22** with a slightly lower K_a of 8.4×10^4 M^{-1}, and a lower ΔH of -7.9 kcal mol^{-1} and more favorable $T\Delta S$ of -1.2 kcal mol^{-1}. Hence, the GlcNAc moiety of **22** is slightly more favorable for binding than the Glc moiety of **16**. The Fuc moiety makes a significant contribution to the K_a value of **22** when compared with that of

Galβ1-4GlcNAc, due mostly to differences in the ΔH values of the two glycans (-6.9 kcal mol^{-1} for **22** vs -4.5 kcal mol^{-1} for the disaccharide). This suggests binding of the Fuc moiety of **22** with CRL II. The GlcNAc residue of Galβ1-4GlcNAc provides a slightly higher K_a for the disaccharide compared with lactose, which is consistent with the differences observed between **16** and **22**. The large increase in affinity of the disaccharides compared with Gal in Table IV is consistent with binding of the GlcNAc (and Glc) residues of the disaccharides to CRL II. Thus, the binding site of CRL II appears to consist of three subsites that recognize the three sugar residues of **16** and **22**.

The lower K_a values of **17**, **23**, **24** and **25** are evidence of the specificity of CRL II for **22** when compared with the above closely related blood group oligosaccharides, including blood group H type I trisaccharide (**24**).

Comparison of blood group H type II trisaccharide-binding thermodynamics of CRL II with two other fucose-binding lectins

Binding of blood group H type II trisaccharide to two other well-studied Fuc-binding lectins, namely *Lotus tetragonolobus* agglutinin (LTA) and *U. europaeus* agglutinin I (UEA-I; Du et al. 1994; Gohier et al. 1996; Mollicone et al. 1996), was studied by ITC. Table V shows the comparative blood group H type II trisaccharide-binding data obtained with CRL II, LTA and UEA-I. The stoichiometry of binding (n) is similar for all three lectins. CRL II shows the highest affinity and LTA possesses the weakest affinity for the trisaccharide. The K_a of CRL II is ~10 and ~2 times higher than those of LTA and UEA-I, respectively.

Discussion

The *C. roseum* seed lectins CRL I and CRL II recognize Man oligosaccharides and the blood group H type II trisaccharide, respectively. CRL I binds to the core trimannoside structure of *N*-linked glycans (**1**), high Man structures such as pentasaccharide Man5 (**13**) and a biantennary complex carbohydrate structure (**14**). The α(1-6)-linked Man residue of **1** binds to the primary binding site, whereas the α(1-3) and the central or core Man residues of **1** interact with the extended area of the binding site. The 3-, 4-, 6-OH groups of the α(1-6)-linked Man, 3-, 4-OH of the α(1-3)-linked Man and 2-, 4-OH of the

Table IV. Thermodynamic-binding parameters obtained from the titration of CRL II at pH 7.2 with saccharides at 27°C

Carbohydrates	K_a^{a} (M^{-1} × 10^{-4})	$-\Delta G^{b}$ (kcal mol^{-1})	$-\Delta H^{c}$ (kcal mol^{-1})	$-T\Delta S^{d}$ (kcal mol^{-1})	n^{e}
D-Galactose*					
D-Fucose*					
Lactose	0.12	4.2	6.9	2.7	1.10
Galβ1-3GlcNAc	0.10	4.1	7.1	3.0	1.06
Galβ1-4GlcNAc	0.18	4.5	7.5	3.0	0.95
2′-Fucosyllactose (**16**)	8.4	6.7	7.9	1.2	0.97
H disaccharide (**15**)	0.27	4.7	6.7	2.0	1.06
H type II trisaccharide (**22**)	12.2	6.9	9.1	2.2	1.03
Lacto-*N*-fucopentaose I (**24**)	0.31	4.8	7.6	2.8	0.94
Lactodifucotetraose (**17**)	0.21	4.5	6.4	1.9	1.09
Lacto-*N*-difucosyl-hexaose I (**25**)	0.24	4.6	5.9	1.3	0.99
Lewis[b] tetrasacccharide (**23**)	0.51	5.1	6.5	1.4	1.06

*Affinity too weak for reliable ITC data.
[a]Errors in K_a are 3–6%.
[b]Errors in ΔG are 2%.
[c]Errors in ΔH are 1–5%.
[d]Errors in $T\Delta S$ are 2–7%.
[e]Errors in n are less than 5%.

Table V. Thermodynamic-binding data derived from the binding of blood group H type II trisaccharide (**22**) to CRL II, LTA and UEA-I lectins

Lectins	K_a^{a} (M^{-1} × 10^{-4})	$-\Delta G^{b}$ (kcal mol^{-1})	$-\Delta H^{c}$ (kcal mol^{-1})	$-T\Delta S^{d}$ (kcal mol^{-1})	n^{e}
CRL II	12.2	6.9	9.1	2.2	1.03
LTA	0.75	5.3	3.6	-1.7	1.05
UEA-I	4.3	6.3	9.7	3.4	1.01

[a]Errors in K_a are 3–6%.
[b]Errors in ΔG are 2%.
[c]Errors in ΔH are 1–5%.
[d]Errors in $T\Delta S$ are 2–7%.
[e]Errors in n are less than 5%.

central Man make direct contact with the CRL I-binding site. In contrast, CRL II prefers glycans with very specific fucosylation. Among all the glycan tested, the blood group H type II trisaccharide (**22**) interacts with CRL II with highest affinity. Addition of more Fuc residues to this structure or elongation of this trisaccharide with additional sugar residues impairs the binding activities.

Man and Fuc residues are expressed in abundance on pathogen- and self-associated glycan structures (Springer et al. 1961; Peumans and Van Damme 1995; Staudacher et al. 1999; Varki et al. 2009). Synthesis of Man- and Fuc-specific lectins in the same tissue can facilitate interactions with a diverse array of glycoproteins/glycolipids of self and non-self origin. Expression of Man- and Fuc-specific lectins in the same plant tissue may be a primordial strategy for expanding functional diversity that might have served as an impetus for the evolution of lectins with dual specificities. For example, the mammalian lectin DC-SIGN (dendritic cell-specific ICAM-grabbing non-integrin) with its dual specificities for Man- and Fuc-containing glycans can interact with numerous self- and pathogen-associated glycoconjugates (Cambi and Figdor 2003; van Kooyk and Geijtenbeek 2003; Geijtenbeek et al. 2004; van Kooyk and Rabinovich 2008; Garcia-Vallejo and van Kooyk 2009). The present results also indicate that other species of the Diocleinae subtribe may express Fuc-specific lectins along with Man-binding lectins. Indeed, *Dioclea lehmanni*, a species of Diocleinae subtribe (Scheme I), contains two lectins specific for Man- and Fuc-containing oligosaccharides, respectively (Pérez 1998; Almanza et al. 2004). Two other Diocleinae species namely, *Canavalia ensiformis* and *D. grandiflora*, also contain a Fuc-specific lectin along with their Man-specific lectins, ConA and DGL, respectively (Almanza et al. 2004). The Fuc-binding lectins in other species of Diocleinae subtribe may have escaped detection due to low expression levels.

Materials and methods
Glycans and lectins
Trimannoside **1**, Man5 oligomannose (**13**), biantennary complex carbohydrate (**14**) and the fucosylated oligosaccharides (**15–25**) were purchased from Sigma Chemicaal company, Dextra Laboratories Ltd, UK, Calbiochem (San Diego, CA) and V-Labs (Covington, LA). Synthesis of oligosaccharides **2–12** have been described previously (Oscarson and Tedebark 1995). The concentrations of carbohydrates were measured by the phenol-sulfuric acid method using an appropriate mixture of Man, Glc and Gal as the standard (Dubois et al. 1956; Saha and Brewer 1994). Purity of the oligosaccharides was checked by ^{1}H NMR spectroscopy (Oscarson and Tedebark 1995).

Cymbosema roseum seeds were obtained from the State of Amazonas, Brazil. Lectins were purified by a single-step affinity chromatography. CRL I was purified from the seed extract using mannose–Sepharose-4B column equilibrated with a solution of 0.15 M NaCl, 5 mM $CaCl_2$ and 5 mM $MnCl_2$ (Cavada et al. 2006). After removing the unbound material, the lectin was eluted with 0.1 M glycine, 0.15 M NaCl, pH 2.6. The run-off was collected and applied on a lactose–Sepharose-4B

column, and the bound CRL II was also eluted with 0.1 M glycine, 0.15 M NaCl, pH 2.6 (Rocha et al. 2009).

Hemagglutination inhibition assay
The assay was performed at room temperature using a 2-fold serial dilution technique (Osawa and Matsumoto 1972) and 3% (v/v) rabbit erythrocytes in Hepes buffer (0.1 M Hepes, 0.15 M NaCl, 1 mM $CaCl_2$ and 1 mM $MnCl_2$, pH 7.2). The minimum concentration of saccharide required for complete inhibition of four hemagglutination doses was determined by visual inspection.

Isothermal titration microcalorimetry
ITC experiments were performed using MCS and VP-ITC instruments from Microcal, Inc. (Northampton, MA). In individual titrations, injections of 4 μL of carbohydrate were added from the computer-controlled 250 or 100 μL microsyringe at an interval of 4 min into the lectin solution (cell volume = 1.3424 mL for MCS and 1.43 mL for VP-ITC) dissolved in the same buffer as the saccharide, while stirring at 350 rpm. Control experiments performed by making identical injections of saccharides into a cell containing buffer with no protein showed insignificant heats of dilution. The experimental data were fitted to a theoretical titration curve using software supplied by Microcal, with ΔH (enthalpy change in kcal mol^{-1}), K_a (association constant in M^{-1}) and n (number of binding sites per monomer), as adjustable parameters. The quantity $c = K_a M_t(0)$, where $M_t(0)$ is the initial macromolecule concentration, is of importance in titration microcalorimetry (Wiseman et al. 1989). All experiments were performed with c values $1 < c < 200$. The instrument was calibrated using the calibration kit containing ribonuclease A (RNase A) and cytidine 2′-monophosphate supplied by the manufacturer. Thermodynamic parameters were calculated from the equation:

$$\Delta G = \Delta H - T\Delta S = -RT \ln K_a$$

where ΔG, ΔH and ΔS are the changes in free energy, enthalpy and entropy of binding, respectively. T is the absolute temperature (K) and $R = 1.98$ cal mol^{-1} K^{-1}.

Conflict of interest statement
None declared.

Abbreviations
ConA, concanavalin A; DGL, *Dioclea grandiflora* lectin; CRL I, *Cymbosema roseum* lectin I; CRL II, *Cymbosema roseum* lectin II; ITC, isothermal titration calorimetry; LTA, *Lotus tetragonolobus* agglutinin; MeαMan, methyl α-mannopyranoside; UEA-I, *Ulex europaeus* agglutinin I; WBA, winged bean agglutinins.

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