Research Article

Journal of PeptideScience

Received: 6 April 2011 Revised: 28 July 2011 Accepted: 1 August 2011 Published online in Wiley Online Library: 3 November 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1415

Sialic acid and sialyl-lactose glyco-conjugates: design, synthesis and binding assays to lectins and swine influenza H1N1 virus

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The terminal parts of the influenza hemagglutinin (HA) receptors α2,6- and α2,3-sialyllactoses were conjugated to an artificial carrier, named sequential oligopeptide carrier (SOC_4), to formulate human and avian receptor mimics, respectively. SOC_4, formed by the tripeptide unit Lys-Aib-Gly, adopts a rigid helicoids-type conformation, which enables the conjugation of biomolecules to the Lys-$N^{\epsilon}H_2$ groups. By doing so, it preserves their initial conformations and functionalities of the epitopes. We report that SOC_4-glyco-conjugate bearing two copies of the α2,6-sialyllactose is specifically recognized by the biotinylated *Sambucus nigra* (elderberry) bark lectin, which binds preferentially to sialic acid in an α2,6-linkage. SOC_4-glyco-conjugate bearing two copies of the α2,3-sialyllactose was not recognized by the biotinylated *Maackia amurensis* lectin, despite its well-known α2,3-sialyl bond specificity. However, preliminary immune blot assays showed that H1N1 virus binds to both the SOC_4-glyco-conjugates immobilized onto nitrocellulose membrane. It is concluded that Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 5 and Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 6 mimic the HA receptors. These findings could be useful for easy screening of binding and inhibition assays of virus–receptor interactions. Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: influenza virus; sequential oligopeptide carrier (SOC_4); chemoselective oxime ligation; SOC_4-sialo-conjugates; HA-binding receptor mimics; lectin binding assays; immune blot binding of H1N1

Introduction

The possibility of an outbreak of H5N1 avian influenza virus is not considered a remote possibility if uncontrolled. Therefore, there is a great deal of interest in examining the various factors important for entry of the virus into host cells, the interspecies transmission and the host restrictions of influenza virus [1].

Human and avian influenza A viruses differ in their recognition of host cell receptors: the former preferentially recognize receptors with saccharides terminating in sialic acid-α2,6-galactose (SAα2,6Gal), whereas the latter prefer those ending in SAα2,3Gal. It is the receptor-binding site of hemagglutinin (HA) protein that recognizes SAα2,3Gal or SAα2,6Gal on the host cell surface and initiates the virus attachment. A conversion from SAα2,3Gal to SAα2,6Gal recognition is thought to be one of the changes that must occur before avian influenza viruses can replicate efficiently in humans and acquire the potential to cause a pandemic [2–4].

Computational studies based on the crystal structure of H5N1 HA showed that SAα2,3Gal has multiple strong hydrophobic and hydrogen bond interactions in its *trans* conformation with HA, whereas the SAα2,6Gal only shows weak interactions in a *cis*-type conformation [5]. In an another study, it was shown that a characteristic structural topology – and not the α2,6-linkage itself – enables specific binding of HA to α2,6-sialylated glycans and that recognition of this topology may be critical for adaptation of HA to bind glycans in the upper respiratory tract of humans [6]. In agreement with this study, it was suggested that the distribution and detection of SAα2,3Gal and SAα2,6Gal-linked receptors within the respiratory tract might not be as clear-cut as has been reported [7,8].

Carbohydrate recognition is crucial in numerous biological properties such as stability, activity, binding, affinity and specificity for other biomolecules [9]. The importance of HA binding to the host receptor at the sialyl-saccharide termination has led to an ongoing interest in the formation of *N*-linked glycopeptides. Several approaches to the chemical synthesis of glycopeptides have been reported. For example, peptides have been glycosylated with synthetic or naturally isolated oligosaccharides, and glycosylated amino acids have been isolated from naturally occurring glycoproteins and used in chemical conjugation of polypeptides. The hydroxyl groups of the carbohydrate moiety have been protected or left unprotected. After removal of the C-terminal protection, the glycosyl amino acids have been used as active esters or with the use of *in situ* coupling reagents. Recent developments in the ligation of unprotected peptide fragments by chemoselective methods have opened new routes to

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the synthesis of glycopeptides. However, the regiospecific chemical synthesis of glycopeptides remains a difficult task because of the chemical lability of the glycosidic linkage [9–11].

Our objective here is to design, synthesize and use models of the terminal segment of the receptor to validate their binding to different lectins as well as to appropriate influenza viruses. For this purpose, sialic acid (SA), 3′-sialyllactose (3′SL) and 6′-sialyllactose (6′SL) were linked independently, in two and four copies, to SOC_4, a synthetic carrier developed in our laboratory, which is formed by having three repeats of the tripeptide unit Lys-Aib-Gly in tandem. The saccharides were conjugated to the Lys-$N^{\varepsilon}H_2$ groups of the carrier via a chemoselective ligation approach, which generates an oxime bond between the H_2N-O-groups of the modified lysine residues and the reducing end of each saccharide. ^1H NMR, computational and CD studies have shown that the carrier adopts a helical conformation, which is retained even after the attachment of various biomolecules to the Lys-$N^{\varepsilon}H_2$ groups of SOC_4. Moreover, it was found that the attached biomolecules do not interact with the carrier or with each other and preserve their conformation and initial bioactivity [12–14].

The SOC_4 carriers, H-SOC_4(Aoa)$_4$-NH_2 **1** and Ac-SOC_4[(Ac)$_2$,(Aoa)$_2$]-NH_2 **2** [15], bearing four or two amino-oxyacetyl (Aoa) groups, respectively, were synthesized. The following SOC_4-glyco-conjugates: H-SOC_4(SA-Aoa)$_4$-NH_2 **3**, Ac-SOC_4[(Ac)$_2$,(SA-Aoa)$_2$]-NH_2 **4**, Ac-SOC_4[(Ac)$_2$,(3′SL-Aoa)$_2$]-NH_2 **5** and Ac-SOC_4[(Ac)$_2$,(6′SL-Aoa)$_2$]-NH_2 **6** were also prepared. Their conformational properties were studied by CD, and their ability to recognize lectins was tested in a microtiter plate binding assay. Because of their specificity to different carbohydrates, lectins are often used as tools for the structural and functional study of glycans [16].

Our study pointed out that Ac-SOC_4[(Ac)$_2$,(6′SL-Aoa)$_2$]-NH_2 **6**, bearing two copies of the 6′-sialyllactose, is specifically recognized by the biotinylated *Sambucus nigra* (SNA) lectin, which binds preferentially to sialic acid attached to α2,6Gal – the terminal part of the human HA receptor. However, SOC_4[(Ac)$_2$,(3′SL-Aoa)$_2$]-NH_2 **5** is not recognized by *Maackia amurensis* (MAL II) biotinylated lectin, although its known specificity to the α2,3-sialyl bond. However, both compounds bind to H1N1 virus in an immune blot experiment.

Materials and Methods

Chemicals

N^{α}-Fmoc protected amino acids, 2-(1H-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophospate (HBTU), HOBt and Rink Amide AM resin were purchased from GL Biochem (Shanghai, China). DIEA was from Merck-Schuchardt (Munich Germany). Triisopropylsilane (TIS) was obtained from Sigma (St. Louis, MO, USA), whereas HPLC-grade acetonitrile, DCM and DMF were from LABSCAN (Dublin, Ireland). Piperidine, DIC, Boc-amino-oxyacetic acid (Boc-Aoa), TFA, TFE and sialic acid (*N*-acetyl-neuraminic acid, Neu5Ac) were from Fluka (Buchs, Switzerland). 6′-sialyllactose (6′-*N*-acetyl-neuraminyl-lactose, Neu5Acα2,6Galβ1,4Glc) and 3′-sialyllactose (3′-*N*-acetyl-neuraminyl-lactose, Neu5Acα2,3Galβ1,4Glc) ammonium salts were obtained from IsoSep (Tullinge, Sweden). Biotinylated lectins *Maackia amurensis* Lectin II (MAL II) and SNA were purchased from Vector Laboratories (Burlingame, CA, USA). Microtiter plates (NUNC Immuno Plates) were from NUNC A/S (Roskilde, Denmark) (Denmark), whereas ExtrAvidin/alkaline phosphatase solution and *p*-nitrophenyl phosphate were from Sigma.

Peptide Synthesis

Solid phase peptide synthesis [17,18] was carried out manually following the Fmoc/tBu methodology on a Rink Amide AM resin (0.67 mmol/g). Fmoc groups were removed using 20% piperidine/DMF. The protected fragment of the SOC_4 carrier, where SOC_4 is (K-Aib-G)$_4$, was synthesized by coupling each Fmoc-amino acid (3 mol equiv.) in the presence of HBTU/HOBt/DIEA (2.9 : 3 : 6 molar ratio) in a DMF/DCM mixture. Completion of couplings and deprotection reactions were monitored using the Kaiser ninhydrin test. The synthesized compounds are shown in Table 1.

For the synthesis of compound **1**, lysines were introduced as Fmoc-Lys(Mtt)-OH. The Mtt groups were removed by 1.8% TFA/DCM, whereas Boc-amino-oxyacetic acid was coupled to the Lys-$N^{\varepsilon}H_2$ groups using DIC and HOBt (three molar excess). Deprotection of the *N*-terminal Lys of the carrier (Fmoc removal) was also performed. Compound **2** was synthesized as previously reported [15]. Briefly, lysines at the fourth and tenth positions were introduced as Fmoc-Lys(Ac)-OH, whereas lysines at the first and seventh positions were introduced as Fmoc-Lys(Mtt)-OH. The *N*-terminal group of the carrier was acetylated using acetic anhydride in pyridine.

The side chain deprotection and the resin cleavage were achieved by treatment of each peptidyl resin with TFA/TIS/H_2O (95/2.5/2.5, v/v/v, 4 h). The resin was removed by filtration, the filtrate was evaporated under reduced pressure and the product was precipitated with cold diethyl ether. The precipitate was

Table 1. Parameters of the synthesis, purification and characterization of the synthesized compounds **1–6**

No.	Compound	Yield (%)[a]	HPLC gradient elution (% B)[b]	t_R (min)	ESI-MS		
					Molecular ion	Calculated	Found
1	H-SOC_4(Aoa)$_4$-NH_2	45	5–70	13	$[M+H]^+$	1391.6	1392.3
2	Ac-SOC_4[(Ac)$_2$,(Aoa)$_2$]-NH_2[c]	38	10–50	13	$[M+H]^+$	1371.6	1372.4
3	H-SOC_4(SA-Aoa)$_4$-NH_2	25	5–70	11.5	$[M-2H]^{-2}$	1276.8	1276.9
4	Ac-SOC_4[(Ac)$_2$,(SA-Aoa)$_2$]-NH_2	56	10–50	11	$[M+2H]^{+2}$	977.6	978.1
5	Ac-SOC_4[(Ac)$_2$,(3′SL-Aoa)$_2$]-NH_2	18	10–50	15	$[M+2H]^{+2}$	1301.8	1302.2
6	Ac-SOC_4[(Ac)$_2$,(6′SL-Aoa)$_2$]-NH_2	29	10–50	15	$[M+2H]^{+2}$	1301.8	1302.3

[a]The purity of the final products was analyzed according to HPLC peak integrals at 214 nm on analytical HPLC and was estimated >95%.
[b]Eluent B: CH_3CN/0.1% TFA; eluent A: H_2O/0.1% TFA.
[c]Reference [15].

filtered, dissolved in 2 N aqueous acetic acid and lyophilized to obtain the crude peptide.

Crude peptides were purified by semipreparative HPLC. Acetonitrile was evaporated, and the fractions were lyophilized to obtain the pure peptide. The purity of the peptides was checked using analytical HPLC and characterized using ESI-MS. The calculated molecular mass-to-charge ratios were in agreement with those found using ESI-MS (Table 1).

Chemoselective Ligation with Sialic Acid, 3′-sialyllactose and 6′-sialyllactose

SOC_4-glyco-conjugates **3–6** (Table 1) were synthesized in the liquid phase using the chemoselective ligation approach [10,19,20], which leads to the formation of an oxime bond between each aminooxy group of the SOC_4 carrier (**1** and **2**) and the corresponding saccharide moiety (Figure 1).

For compound **3**, sialic acid (10 μmol) was added to the stirred compound **1** solution (20 mM in 0.1 M acetate buffer pH 4.0), and the reaction was allowed to proceed at 35–40 °C for 27 h. For compounds **4–6**, a solution of each carbohydrate (50 mM in 0.1 M acetate buffer pH 4.0) was added dropwise to the stirred compound **2** solution (10 mM in the previous buffer), and the reaction was allowed to proceed at 35–40 °C for 24, 72 and 48 h, respectively (Figure 1).

The glyco-conjugates **3–6** were purified by semipreparative HPLC, acetonitrile was evaporated and the fractions were lyophilized to obtain the pure product. Purity was checked by analytical HPLC and characterization was performed by ESI-MS (Figure 2). The calculated molecular mass-to-charge ratios were in agreement with those found using ESI-MS (Table 1).

Analytical HPLC

Analytical HPLC of the synthesized compounds **1–6** (Table 1) was performed using a Supelco (Sigma Aldrich, Bellefonte, USA) Discovery C18 (25 cm × 4.6 mm, 5 μm) reverse phase column with a Waters (Milford, USA) instrument equipped with a Waters 616 pump and a Waters 2487 dual λ absorbance detector. Eluent A was 0.1% TFA in water, and eluent B was 0.1% TFA in acetonitrile. A linear gradient for compounds **1** and **3** was 5%–70% B at a flow rate of 1 ml/min for 30 min and for compounds **2**, **4**, **5** and **6** was 10%–50% B at a flow rate of 1 ml/min for 30 min.

Semipreparative HPLC

Semipreparative HPLC purification was carried out on a SHIMADZU (Shimadzu Corporation, Tokyo, Japan) semipreparative instrument equipped with a SHIMADZU LC-10 AD VP pump and a SHIMADZU SPD-10A VP UV-VIS detector, using a Supelco Discovery C18 (25 cm 10 mm, 5 μm) reverse phase column, with the same linear gradient as for analytical HPLC at a flow rate of 4.7 ml/min. Detection was carried out at $\lambda = 214$ nm.

Mass Spectrometry

Positive or negative ion ESI-MS analyses of synthesized compounds **1–6** were performed on a Micromass Platform (Waters Corporation, Milford, USA) Quandrupole LC-MS. Capillary and cone voltages were set to 3 kV and 35–75 V, respectively. Samples were dissolved in water/acetonitrile/trifluoroacetic acid (1/1/0.05 v/v/v).

Circular Dichroism

The CD spectra were recorded at 25 °C on a Jasco (Jasco Corporation, Japan) J-815 spectropolarimeter equipped with a thermoelectric temperature controller. Spectra were obtained using a quartz cell of 1 mm path length, and the concentration of the tested compounds was 10^{-4} M. Experiments were performed in TFE/H_2O mixtures (0%, 50% and 100%). Spectra were obtained with a 1 nm bandwidth, a scan speed of 50 nm/min and a response of 1 s. The signal-to-noise ratio was improved by accumulating three scans. All CD spectra are reported in terms of mean residue

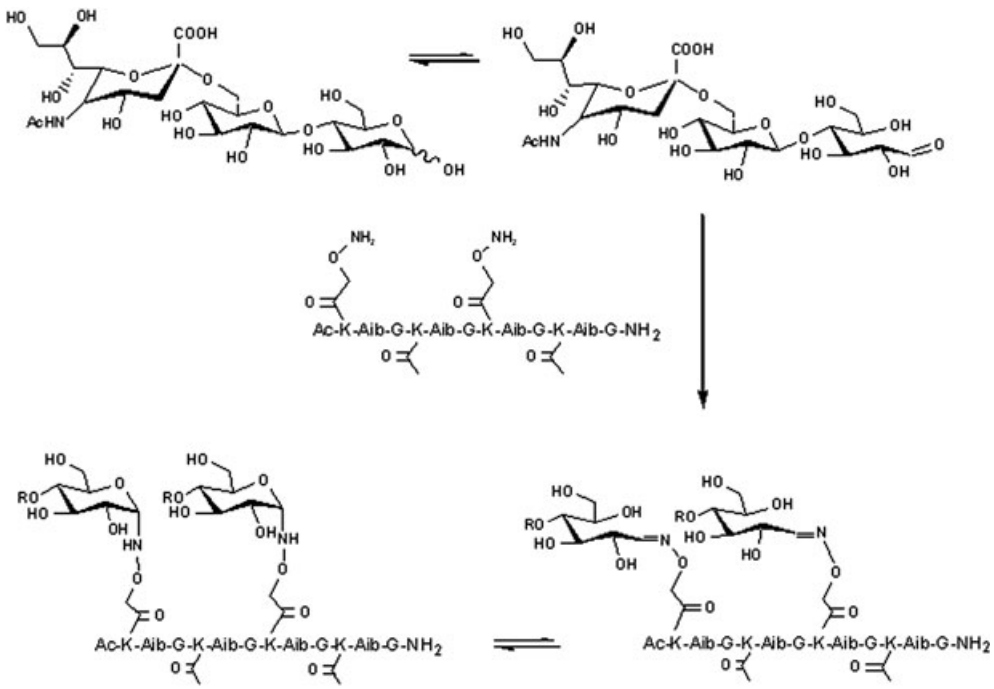

Figure 1. Coupling reaction of 6′-sialyllactose to Ac-SOC_4[$(Ac)_2$,$(Aoa)_2$]-NH_2 **2**. R = SAα2,6Gal.

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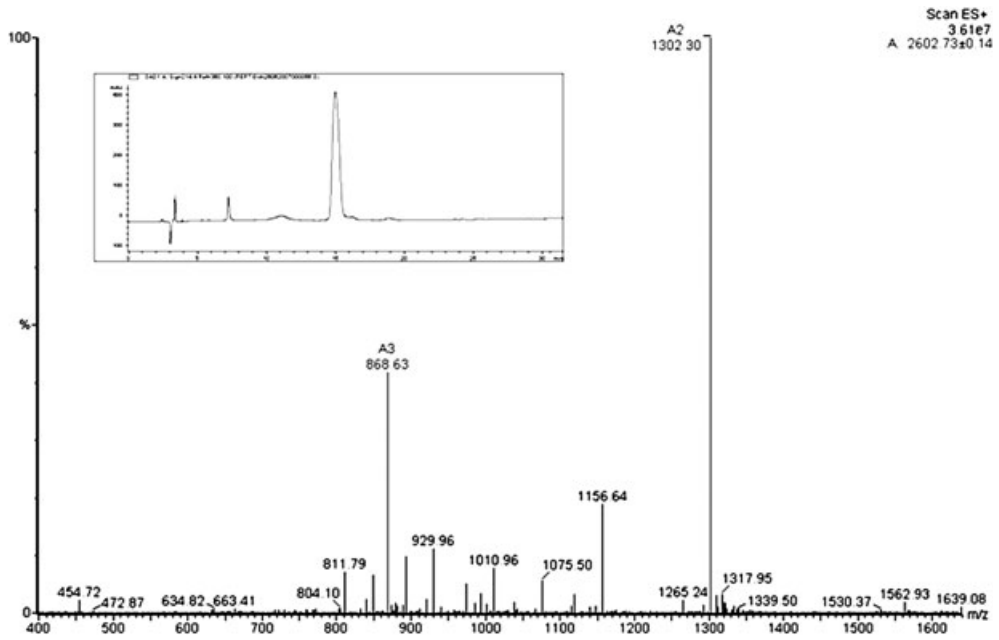

Figure 2. ESI-MS mass spectrum and analytical HPLC of the purified Ac-SOC_4[$(Ac)_2$,(6′SL-$Aoa)_2$]-NH_2 **6**.

molar ellipticity $[\Theta]_R$ in degrees $cm^2/dmol^{-1}residue^{-1}$. The percentage helical content was estimated on the basis of the $[\Theta]_{222}$ nm values, in different environments, as described by Chen *et al.* [21].

Microtiter Plate Binding Assay of Lectins to SOC_4-glyco-conjugates

The 96-well polystyrene plates were coated overnight at 4 °C with 5 μg/ml of each compound in carbonate buffer, pH 9.6 (50 μl/well). After washing of the plates with Tris Buffered Saline (TBS) (0.05 M Tris-HCl buffer/0.15 M NaCl, pH 7.4) containing 0.05% Tween 20 (TBS-T), biotinylated lectins (MAL II and SNA) were added and incubated at room temperature for 30 min (50 μl/well). The plates were washed, and the ExtrAvidin/alkaline phosphatase solution (diluted 1 : 10 000 in TBS-T) was added (50 μl/well). After 1 h at room temperature, the plates were extensively washed and incubated with *p*-nitrophenyl phosphate in carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ (1 mg/ml, 50 μl/well). The absorbance was read at 405 nm in a microtiter plate reader after 30 min incubation with the substrate at 37 °C [22,23].

Binding Assay of H1N1 Virus to Immobilized SOC_4-glyco-conjugates

The attachment of virus to SOC_4-glyco-conjugates **5** and **6** was performed using immune blot assay as previously described with some modifications [24]. Briefly, nitrocellulose membrane was washed, and SOC_4-glyco-conjugates **5** and **6** as well as acetylated SOC_4 carrier (control) were immobilized by adding 2 μl of the solution containing 100 μg/ml of each compound for 15 min and blocked with 1% blocking buffer (BSA in Phosphate Buffered Saline (PBS)) for 1 h at 37 °C. After several washes with PBS-Tween20, 0.1% membrane was further incubated with 1 : 100 dilutions of the H1N1 virus for 1 h at 37 °C, washed and incubated with swine influenza H1N1-positive serum (dilution 1 : 50) for 2 h at 37 °C. Peroxidase conjugated secondary anti-pig antibody (1 : 20 000) was used in chemiluminesence ECL system (GE Healthcare Munich, Germany) to detect the binding of virus to SOC_4-glyco-conjugates.

Results

Sequential oligopeptide carriers (SOCn, $n = 2–7$), a novel foldamer class of artificial carriers, was introduced by our laboratory as a tool for the construction of reconstituted protein mimics with various functionalities [25–28]. Here, the terminal parts, sialic acid and sialyllactose, of the HA receptor, critical for the recognition of the influenza virus, were linked independently to SOC_4 to reconstitute mimics of the receptor. The SOC_4-glyco-conjugates were synthesized in two steps: (i) solid phase synthesis of the SOC_4 carrier bearing two or four amino-oxy-acetyl (H_2N-O-CH_2CO-) groups and (ii) chemoselective ligation of the saccharide through the formation of an oxime bond. Derivatization reactions were carried out in acetate buffer (0.1 M) at pH 4.0 and in excess of the carbohydrate. All final products were obtained in sufficient yields and high purity as confirmed by HPLC and ESI-MS (Table 1).

The CD spectra of the amino-oxy-acetyl SOC_4 carrier, Ac-SOC_4[$(Ac)_2$,$(Aoa)_2$]-NH_2 **2**, in TFE/H_2O (50/50 v/v) and 100% TFE, exhibited a positive band at 192 nm and two negative bands at 205 and 222 nm typical of helical structure. The helical content of the carrier, estimated on the basis of the $[\Theta]_{222}$ value, was found 12% and 10% at 50% and 100% TFE, respectively [20]. These helical features, in agreement with our previous studies, were conserved even after the attachment of the saccharides to the carrier. For example, Ac-SOC_4[$(Ac)_2$,(SA-$Aoa)_2$]-NH_2 **4** and Ac-SOC_4[$(Ac)_2$,(6′SL-$Aoa)_2$]-NH_2 **6** exhibited 15% and 17% helical conformation at 50% TFE and 21% and 16% at 100% TFE, respectively, confirming the persistence of the helical structure of the carrier in its conjugated form (Figure 3).

The binding of lectins to SOC_4-glyco-conjugates was tested in a biotin/avidin-mediated binding assay. SNA lectin binds preferentially to sialic acid attached to terminal α2,6Gal, whereas MAL II lectin appears to bind sialic acid in an α2,3Gal linkage. Lectins were used to differentiate the functional properties of SOC_4-glyco-conjugates because of their carbohydrate specificity. Figure 4 represents the binding of lectins to microtiter plates coated with sialic acid conjugates and sialyllactose conjugates.

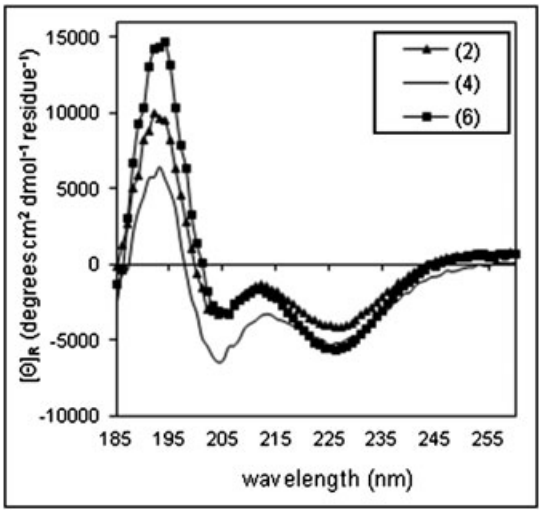

Figure 3. CD spectra of Ac-SOC_4[$(Ac)_2$,$(Aoa)_2$]-NH_2 **2**, Ac-SOC_4[$(Ac)_2$,$(SA\text{-}Aoa)_2$]-NH_2 **4** and Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6** in 50% TFE/H_2O.

SOC_4-conjugates bearing four and two copies of sialic acid (**3** and **4**) were not recognized by the lectins. The same is true for SOC_4-conjugate **5** with two copies of 3′-sialyllactose. On the contrary, compound Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6**, which incorporates two copies of 6′-sialyllactose, was well recognized by SNA lectin that is specific for α2,6Gal. More important, the binding of SNA lectin to compound **6** is dose-dependent (10, 100, and 200 μg/well of lectin) indicating the specificity of the synthesized glyco-conjugate. A similar pattern was obtained when microtiter plates were coated with different concentrations of glyco-conjugate **6** ranging from 1 to 10 μg/ml.

Preliminary immune blot assays showed that the SOC_4-glyco-conjugates **5** and **6** are both well recognized by H1N1 virus onto nitrocellulose membrane, whereas the acetylated SOC_4 carrier is not recognized (Figure 5). These findings indicate that the virus binds both α2,6 and α2,3Gal bonds contrary to lectins.

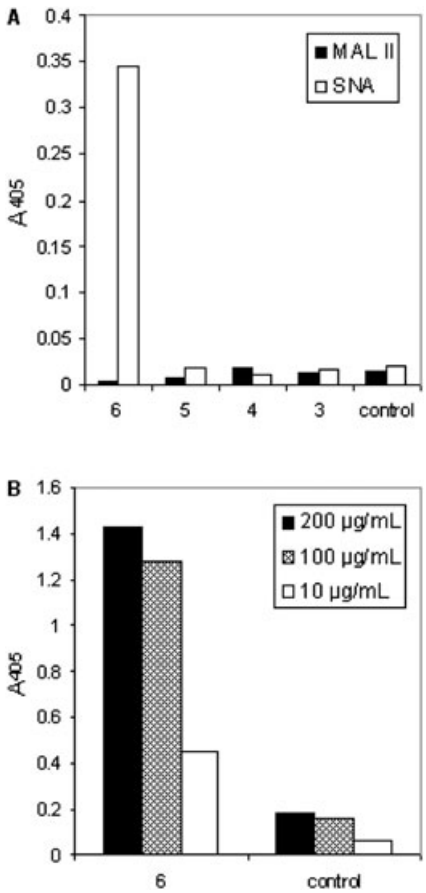

Figure 4. (A) Binding of H-SOC_4($SA\text{-}Aoa)_4$-NH_2 **3**, Ac-SOC_4[$(Ac)_2$,$(SA\text{-}Aoa)_2$]-NH_2 **4**, Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 **5** and Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6** to biotinylated SNA and MAL II lectins (10 μg/ml). (B) Binding of serially diluted biotinylated SNA lectin to Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6**. Concentration of substrates: 5 μg/ml. Control: acetylated SOC_4 carrier.

Discussion

Sequential oligopeptide carrier, SOC_4, is an artificial carrier introduced by our laboratory with the aim of optimizing the

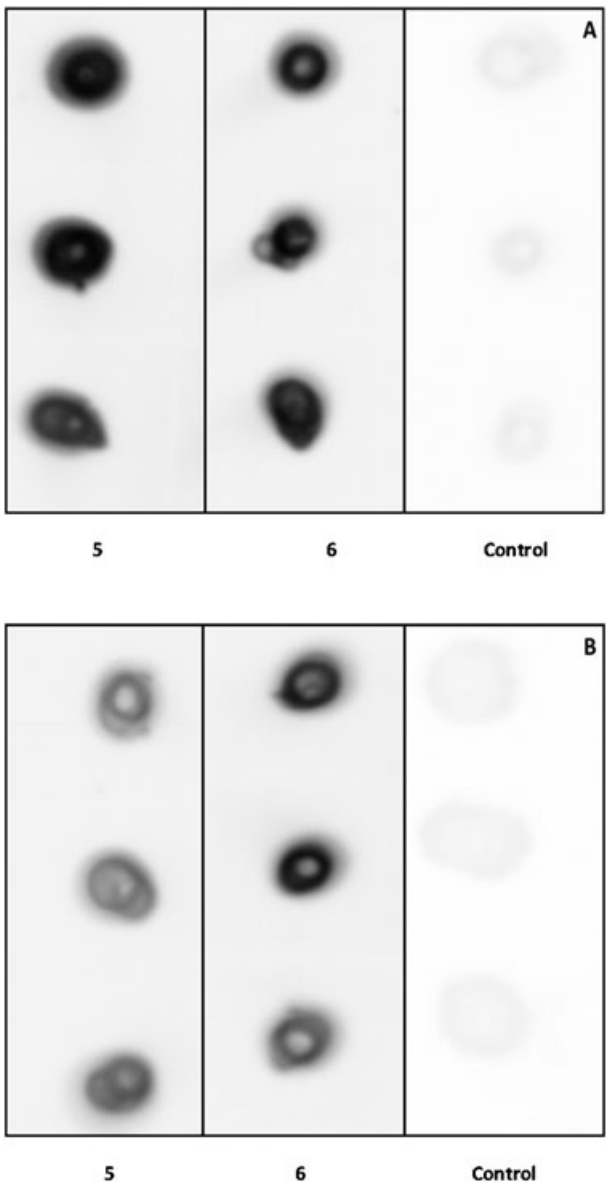

Figure 5. Immune blot with immobilized SOC_4-glyco-conjugates **5** and **6** showing binding with H1N1 virus (A) and without H1N1 virus (B) onto nitrocellulose membrane. Concentration of substrates: 100 μg/ml. Acetylated SOC_4 carrier with and without the H1N1 virus are indicated as control.

presentation of the anchored biomolecules and help in the reconstitution of protein mimics. SOC_4 is a sterically constrained scaffold comprising α-aminoisobutyric acid (Aib), a strongly helicogenic C^{α}-tetrasubstituted amino acid. It was found that the beneficial structural elements of SOC_4 (helical conformation), attributed mainly to the inclusion of Aib, induce a favorable arrangement of the conjugated biomolecules, which retain their initial 'active' conformation and their potential for site-specific interactions [25–28]. Sialic acid, 3′-sialyllactose and 6′-sialyllactose, which are the terminal and most critical parts of the HA receptor for its recognition of influenza viruses, were coupled to SOC_4 to formulate simplified reconstituted mimics of the receptors.

Conformational studies by CD pointed out that the helical features of the carrier are retained even after the attachment of the saccharides to the carrier. It is expected that the coupled terminal parts of HA receptor might preserve, in agreement with our previous results, their initial topology, which is critical for the virus recognition.

With the aim to test the potential of SOC_4-glyco-conjugates as simplified models of the human and avian HA receptor, their binding to lectins was studied. Lectins are very often used as tools to investigate the carbohydrate specificity. Sialic acid conjugates were not recognized by lectins indicating that sialic acid alone is not sufficient for interacting with lectins. Although *Maackia amurensis* (MAL) II lectin is thought to be highly specific for the α2,3Gal linkage, Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 **5** did not bind to this lectin. It seems likely that the α2,3Gal linkage is not sufficient for binding to MAL II lectin and that some other structural features might be required, as for example hydrophobic and hydrogen bond interactions in a *trans*-type conformation with lectin. However, in contrast to what is observed for compound **5**, SOC_4-conjugate that bears two copies of 6′-sialyllactose, Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6**, is well recognized by SNA lectin confirming the carbohydrate specificity of this lectin for the α2,6Gal bond. Apparently, the predetermined conformation of compound **6** fulfils the *cis*-type structural requirements for a specific interaction with SNA lectin [5,6]. Moreover, these findings indicate that Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6** adopts the appropriate orientation that allows the coupled 6′-sialyllactoses to obtain a characteristic structural topology that enables the specific binding to SNA lectin.

The binding of H1N1 virus onto immobilized Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 **5** suggests a relatively low sensitivity of *Maackia amurensis* (MAL II) lectin toward the α2,3Gal bond. However, H1N1 virus showed a specific binding both to Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6** as well as to Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 **5**. We assume that this discrepancy of virus binding to both the SOC_4-glyco-conjugates could be because of the form of virus propagation because it was obtained initially from allantoic cavity of Specific Pathogen Free (SPF) eggs. Virus propagation in the eggs may have shifted the binding of H1N1 virus also toward Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 **5**. Similar observation has been reported previously [29,30]. Our results indicate a potential use of Ac-SOC_4[$(Ac)_2$,$(3'SL\text{-}Aoa)_2$]-NH_2 **5** and Ac-SOC_4[$(Ac)_2$,$(6'SL\text{-}Aoa)_2$]-NH_2 **6** in easy screening of binding and inhibition assays of virus–receptor interactions.

Acknowledgments

This study was supported by the EU (FP6 «EUROFLU» project, SP5B-CT-2007-044098). It was also supported in part by the INIA (National Institute of Research and Food Technology), RTA2010-00084-C02-02 (to A.D). It was also supported in part by the INIA (National Institute of Research and Food Technology), RTA2010-00084-C02-02 (to A.D).

References

1 Longini IM, Nizam A, Xu K, Ungchusak K, Hanshaowarakul W, Cummings DA, Halloran ME. Containing pandemic influenza at the source. *Science* 2005; **309**: 1083–1087.
2 Rogers GN, Paulson LC. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 haemagglutinin based on species of origin. *Virology* 1983; **127**: 361–373.
3 Yamada S, Suzuki Y, Suzuki T, Le MQ, Nidom CA, Sakai-Tagawa Y, Muramoto Y, Ito M, Kiso M, Horimoto T, Shinya K, Sawada T, Kiso M, Usui T, Murata T, Lin Y, Hay A, Haire LF, Stevens DJ, Russell RJ, Gamblin SJ, Skehel JJ, Kawaoka Y. Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors. *Nature* 2006; **444**: 378–382.
4 Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* 1983; **304**: 76–78.
5 Li M, Wang B. Computational studies of H5N1 haemagglutinin binding with SAα2,3Gal and SAα2,6Gal. *Biochem. Biophys. Res. Commun.* 2006; **347**: 662–668.
6 Chandrasekaran A, Srinivasan A, Raman R, Viswanathan K, Raguram S, Tumpey TM, Sasisekharan V, Sasisekharan R. Glycan topology determines human adaptation of avian H5N1 virus haemagglutinin. *Nat. Biotechnol.* 2008; **26**: 107–113.
7 Nicholls JM, Chan RWY, Russell RJ, Air GM, Peiris JSM. Evolving complexities of influenza virus and its receptors. *Trends Microbiol.* 2008; **16**: 149–157.
8 Qi L, Kash C, Dugan VG, Wang R, Jin G, Cunningham RE, Taubenberger JK. Role of sialic acid binding specificity of the 1918 influenza virus heamagglutinin protein in virulence and pathogenesis for mice. *J. Virol.* 2009; **83**: 3754–3761.
9 Jimenez-Castells C, de la Torre BG, Andreu D, Gutierrez-Gallego R. Neo-glycopeptides: the importance of sugar core conformation in oxime-liked glycoprobes. *Glycoconj. J.* 2008; **25**: 879–887.
10 Cervigni SE, Dumy P, Mutter MS. Synthesis of glycopeptides and lipopeptides by chemoselective ligation. *Angew. Chem. Int. Ed Engl.* 1996; **35**: 1230–1232.
11 Christiansen-Brams I, Meldal M, Bock K. Protected-mode synthesis of *N*-linked glycopeptides: single-step preparation of building blocks as peracetyl glycosylated N^{α}-Fmoc asparagine Opfp esters. *J. Chem. Soc. Perkin Trans. 1* 1993; **13**: 1461–1472.
12 Tsikaris V, Sakarellos C, Sakarellos-Daitsiotis M, Orlewski P, Marraud M, Cung MT, Vatzaki E, Tzartos S. Construction and application of a new class of sequential oligopeptide carriers (SOC_n) for multiple anchoring of antigenic peptides-application to the acetylcholine receptor (AChR) main immunogenic region. *Int. J. Biol. Macromol.* 1996; **19**: 195–205.
13 Krikorian D, Panou-Pomonis E, Voitharou Ch, Sakarellos C, Sakarellos-Daitsiotis M. A peptide carrier with a built-in vaccine adjuvant: construction of immunogenic conjugates. *Bioconjug. Chem.* 2005; **16**: 812–819.
14 Kargakis M, Zevgiti S, Krikorian D, Sakarellos-Daitsiotis M, Sakarellos C, Panou-Pomonis E. A palmitoyl-tailed sequential oligopeptide carrier for engineering immunogenic conjugates. *Vaccine* 2007; **25**: 6708–6712.
15 Skarlas Th, Zevgiti S, Droebner K, Panou-Pomonis E, Planz O, Sakarellos-Daitsiotis M. Influenza virus H5N1 hemagglutinin (HA) T-cell epitope conjugates: design, synthesis and immunogenicity. *J. Pept. Sci.* 2011; **17**: 226–232
16 Hirabayashi J. Lectin based structural glycomics, glycoproteomics, and glycan profiling. *Glycoconj. J.* 2004; **21**: 35–40.
17 Bodansky M, Bodansky A. Activation and coupling. In *The Practice of Peptide Synthesis*, 2nd edn, Bodansky M, Bodansky A (eds). Springer-Verlag: Berlin, Heidelberg, 1994; 75–125.
18 Giralt E, Albericio F. Synthesis of peptides on solid supports. In *Synthesis of Peptide and Peptidomimetics, Volume E22a: Synthesis of Peptides*, Goodman M, Felix A, Moroder L, Toniolo G (eds). Georg Thieme Verlag: Stuttgard, NY, 2003; 665–824.
19 Zhao Y, Kent SBH, Chait BT. Rapid, sensitive structure analysis of oligosaccharides. *Proc. Natl. Acad. Sci. U.S.A.* 1997; **94**: 1629–1633.

20 Carrasco MR, Nguyen MJ, Burnell DR, MacLaren MD, Hengel SM. Synthesis of neoglycopeptides by chemoselective reaction of carbohydrates with peptides containing a novel *N*′-methyl-aminooxy amino acid. *Tetrahedron Lett.* 2002; **43**: 5727–5729.
21 Chen YH,Yang JT, Martinez H. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispertion. *Biochemistry* 1972; **11**: 4120–4131.
22 Wu AM, Wu JH, Liu J-H, Singh T. Recognition profile of Bauhinia purpurea agglutinin (BPA). *Life Sci.* 2004; **74**: 1763–1779.
23 Duk M, Lisowska E, Wu JH, Wu AM. The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. *Anal. Biochem.* 1994; **221**: 266–272.
24 Ferraz N, Carnevia D, Nande G, Rossotti M, Miguez M, Last J, Gonzalez-Sapienza G. Specific immunoassays for endocrine disruptor monitoring using recombinant antigens cloned by degenerated primer PCR. *Anal. Bioanal. Chem.* 2007; **389**: 2195–2202.
25 Alexopoulos Ch, Sakarellos-Daitsiotis M, Sakarellos C. Synthetic carriers: sequential oligopeptide carriers SOCn-I and SOCn-II as an innovative and multifunctional approach. *Curr. Med. Chem.* 2005; **12**: 1469–1479.
26 Sakarellos-Daitsiotis M, Alexopoulos Ch, Sakarellos C. Sequential oligopeptide carriers, SOCn, as scaffolds for the reconstitution of antigenic proteins: applications in solid phase immunoassays. *J. Pharm. Biomed. Anal.* 2004; **34**: 761–769.
27 Voitharou C, Krikorian D, Sakarellos C, Sakarellos-Daitsiotis M, Panou-Pomonis E. A complementary La/SSB epitope anchored to sequential oligopeptide carrier regulates the anti-La/SSB response in immunized animals. *J. Pept. Sci.* 2008; **14**: 1069–1076.
28 Sakarellos-Daitsiotis M, Krikorian D, Panou-Pomonis E, Sakarellos C. Artificial carriers: a strategy for constructing antigenic/immunogenic conjugates. *Curr. Top. Med. Chem.* 2006; **6**: 1715–1735.
29 Ito T, Suzuki Y, Takada A, Kawamoto A, Otsuki K, Masuda H, Yamada M, Suzuki T, Kida H, Kawaoka Y. Differences in sialic acid-galactose linkages in the chicken egg amnion and allantois influence human influenza virus receptor specificity and variant selection. *J. Virol.* 1997; **71**: 3357–3362.
30 Gambaryan AS, Karasin AI, Tuzikov AB, Chinarev AA, Pazynina GV, Bovin NV, Matrosovich MN, Olsen CW, Klimov AI. Receptor-binding properties of swine influenza viruses isolated and propagated in MDCK cells. *Virus Res.* 2005; **114**: 15–22.

