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Novel trivalent anti-influenza reagent

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ABSTRACT

We designed and synthesized novel trivalent anti-influenza reagents. Sialyllactose was located at the terminal of each valence which aimed to block each receptor-binding site of the hemagglutinin (HA) trimer on the surface of the virus. Structural analyses were carried out with a model which was constructed with a computer simulation. A previously reported cyclic glycopeptide blocker [Ohta, T.; Miura, N.; Fujitani, N.; Nakajima, F.; Niikura, K.; Sadamoto, R.; Guo, C.-T.; Suzuki, T.; Suzuki, Y.; Monde, K.; Nishimura, S.-I. *Angew. Chem. Int. Ed.*, **2003**, *42*, 5186] bound to the HA in the model. The analyses suggest that the glutamine residue in the cyclic peptide bearing Neu5Acα2,3Galβ1,4Glc trisaccharide via a linker interacts with the Gln189 in HA through hydrogen bonding. The present anti-influenza reagents likely interact with a glutamine residue included in the vicinity of Gln189. A plague reduction assay of the influenza virus, A/PR/8/1934 (H1N1), was performed in MDCK cells to evaluate for the synthesized compounds to inhibit viral replication. One of the compounds showed approximately 85% inhibition at the concentration of 400 μM at 4 °C.

An influenza outbreak poses a significant threat to public the health worldwide as highlighted by the novel swine origin influenza A (H1N1). Since the first detection in April 2009 until November 2009, the human case of infection with the virus has been confirmed over 6770 deaths in 206 countries by the WHO.[1] The pandemic of novel H1N1 virus hasten the development of new anti-virus reagents that are effective for various subtypes of influenza A virus. Oseltamivir and zanamivir as neuraminidase (NA) inhibitors are now widely used as effective anti-influenza drugs. However, strains resistant to these inhibitors have been selected mutations of NA result in inhibitor resistance.[2,3] Therefore, novel inhibitors of influenza virus are strongly required.

Influenza virus expresses the other glycoprotein, hemagglutinin (HA), which plays a fundamental role in the initial step of the infection process.[4] HA binds to the receptor carbohydrate chain terminated with neuraminic acid on the host cell-surface. Thus, HA is a potential target of anti-influenza virus reagents. Inhibition of the binding between HA and the receptor on the cell-surface should prevent humans from being infected by virus. HA blocker is expected to act as a prophylactic reagent. The structure of the HA has been analyzed in detail by extensive biochemical and crystallographic studies.[5] These studies indicated that the HA has three identical receptor-binding sites on the top of the trimer that protruded from the viral envelope.[6] In addition, sialyllactose binds to the HA of influenza virus more strongly than one neuraminic acid unit.[7] It is important to efficiently occupy the binding sites of the HA. We have reported that a trivalent glycopeptide inhibits efficiently hemagglutination mediated by viral hemagglutinin.[8] Several groups also demonstrated that synthetic polymers bearing multiple neuraminic acids showed greatly enhanced affinity to influenza HA.[9] In recent years, multivalent blockers designed with polyamino acid have also been developed.[10] Since multivalent glycoconjugate blockers have densely displayed neuraminic acids at the terminal of the sugar chain, it is thought to amplify the affinity between the HA and the blockers. Since syntheses of glycoproteins and glycopeptides are quite expensive, complicated and difficult to complete in short period of time with the large quantity, it is defective for HA blockers as preventive medicines. In this Letter, we report the synthesis and evaluation of the inhibitory effect of novel trivalent blockers against influenza virus A/PR/8/1934 (H1N1).

We have developed much more simple and practical trivalent blockers with siallyllactose at each terminal of valence (Fig. 1). In our previous Letter, we reported that the monovalent glycopeptide showed no inhibition of hemagglutination. Therefore, in this study, we only designed trivalent blockers. These blockers were

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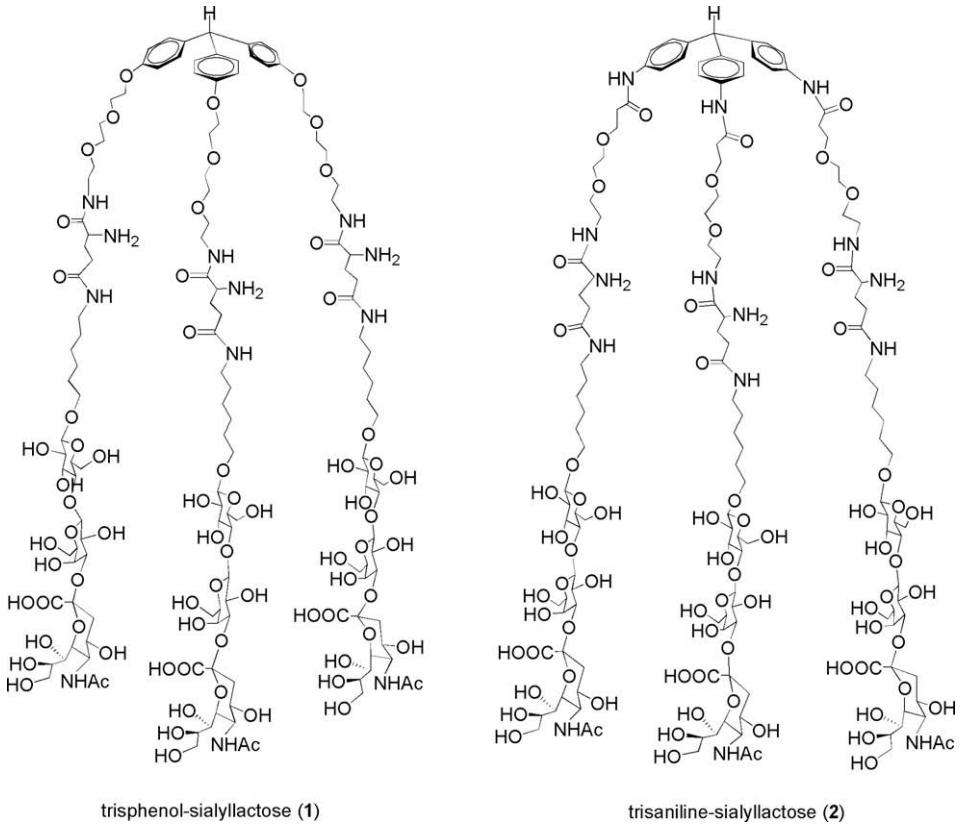


Figure 1. Chemical structures of novel trivalent anti-influenza reagetns.

designed with structure-based methods shown in Figure 2a and b. Geometrical observation was performed with the model structures which were constructed with the HA (PDBID is 1HGG) and our previously developed glycopeptides. The major character of the binding between HA and the blocker were kept in the model structure. Then novel alternative core component were

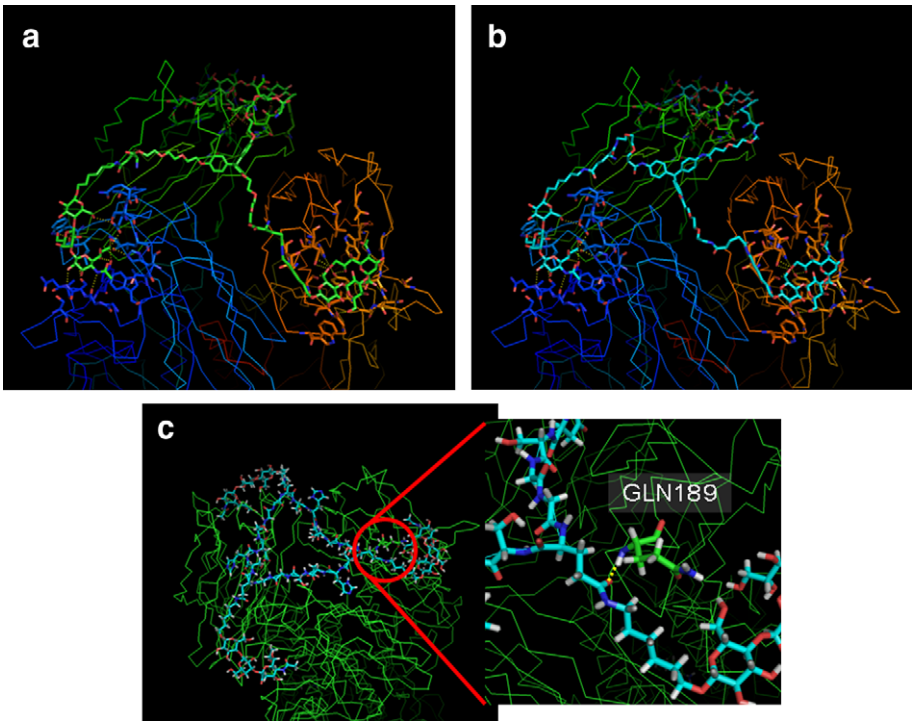


Figure 2. Model structures of binding between trivalent trivalent anti-influenza reagents and HA. (a) trisphenol type, (b) trisaniline type, and (c) previously synthesized HA blocker (Ref. 8).

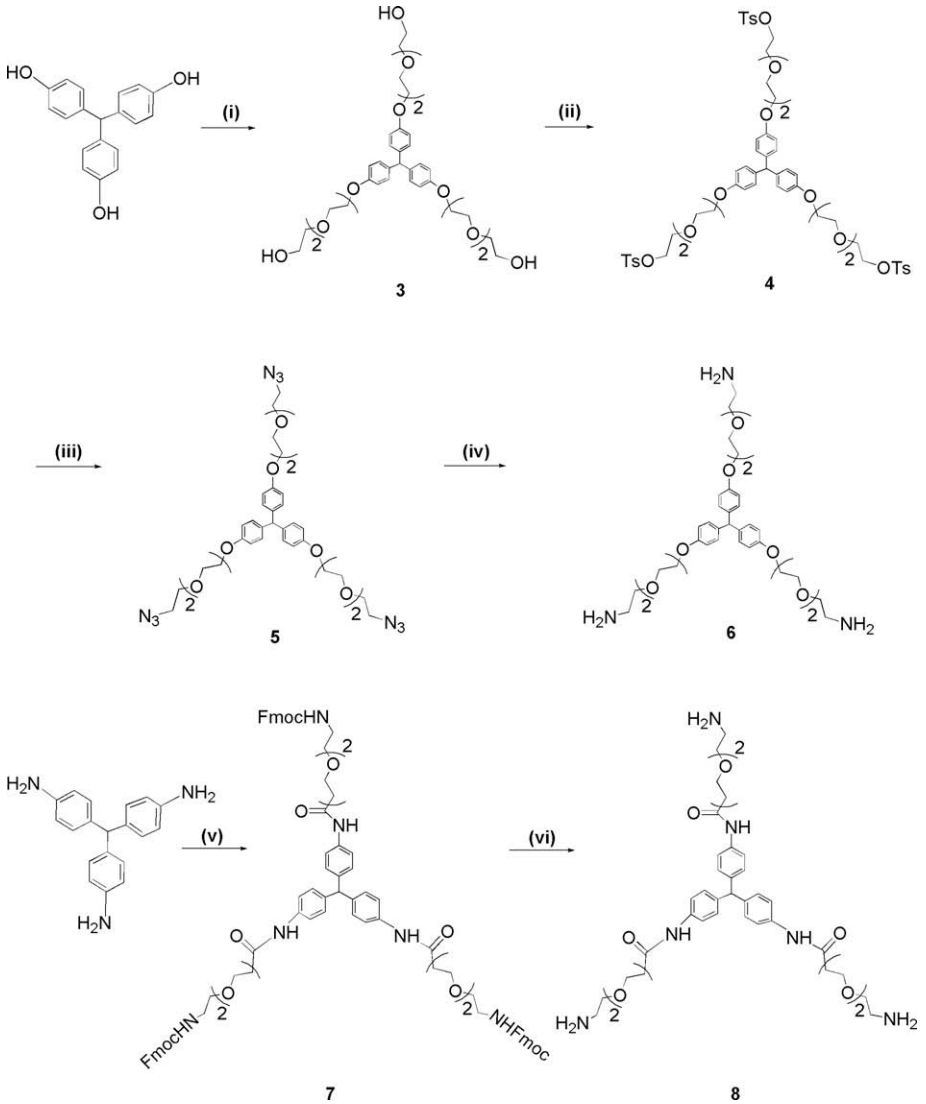
examined. The length of the linker was determined and the trivalent blockers were designed and synthesized. In order to induce an interaction with the Q189 residue as shown in Figure 2c, glutamine residue was introduced in the linker part as connectors between aglycon core and trisaccharides in addition to the interaction between sialyllactose and the HA receptor site.

Our designed compounds bound to the HA (PDB ID:1HGG) on model structures with computer simulations. We carried out short molecular dynamics simulations (200 ps) at 1000 K with the time step of 1 fs by using MMFF94S force fields. During the simulation, the geometrical position of sialylllactoses and the HA were fixed. The conformation with the lowest potential energy as shown in Figure 2 was selected and observed. This observation suggests that the lengths of the linkers and the position of the glutamine residue were appropriate and the designed compounds could bind efficiently with HA.

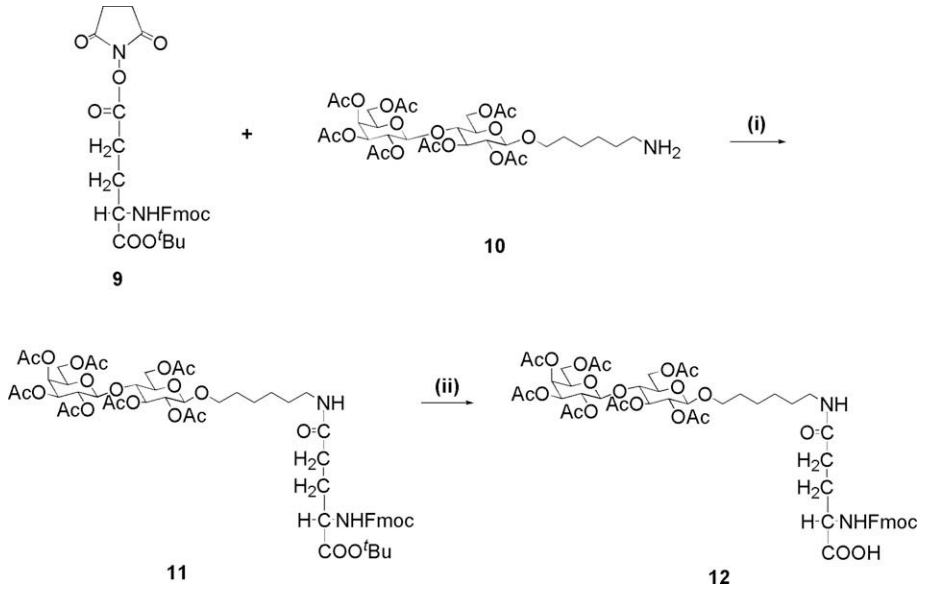
Chemical synthesis of the core parts of the HA blockers is outlined in Scheme 1. Trisphenol and trisaniline skeleton were adopted as the starting materials because both have rigid conformations because of the existence of a center sp^3 carbon, so three valences can spread out evenly. The flexible hydrophilic linker between trisaccharide and the core was built from oligoethyleneglycol structure derived from 2-[2-(2-chloroethoxy)ethoxy]ethanol and 3-{2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy] -ethoxy}-propionic acid. First, **3** and **7** were synthesized according to the above design. Then the tosyl group was introduced into **3** as a leaving group in order to convert it to the azide compound **5** which was transformed to the trisamine **6** by a single reduction reaction. Another core aglycon **8** was built briefly in two steps by normal deprotection of Fmoc in **7**.

N-α-Fluorenylmethoxycarbonyl-*O*-*t*-butyl L-glutamic acid (Fmoc-Glu-O^{t}Bu) was activated into its *N*-succinimidyl ester intermediate **9** and coupled with the full-protected lactose derivative **10**[11] to give the key intermediate **11** which was converted effectively into the corresponding acid form **12** by TFA (Scheme 2).

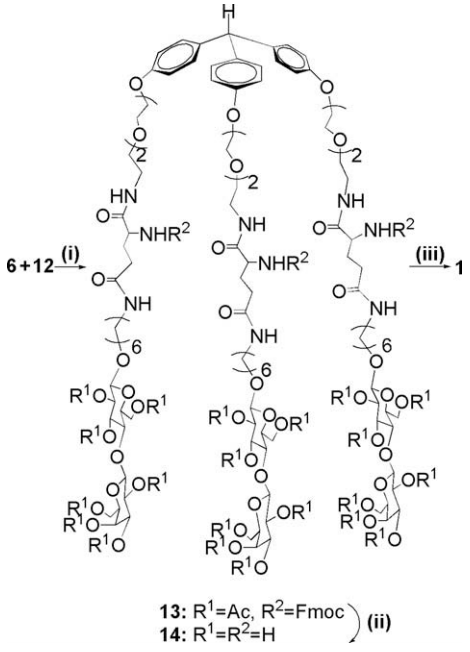
As depicted in Schemes 3 and 4, the key condensation reactions between trisamine (**6** or **8**) and acid **12** were carried out by the use of condensing agents EDC and DPPA, respectively. After the Fmoc protecting group at amino group and the acetyl group on sugar were removed smoothly, sialic acid was introduced by



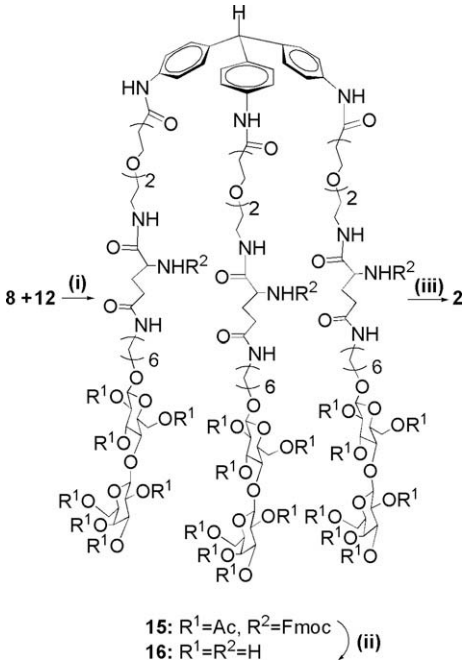
Scheme 1. Reagents and conditions: (i) 2-[2-(2-chloroethoxy)ethoxy]ethanol, K_2CO_3, KI, DMF, 100 °C, 46 h, 65%; (ii) TsCl, TEA, CH_2Cl_2, rt, 3 h, 67%; (iii) NaN_3, DMF, 60 °C, 9 h, 94%; (iv) H_2 gas, 10% Pd–C, MeOH/EtOAc = 2:1, rt, 2 h, 91%; (v) 3-{2-[2-(9*H*-fluoren-9-ylmethoxycarbonylamino)-ethoxy]- ethoxy}-propionic acid, DCC, DMAP, CH_2Cl_2, rt, 6 h, 83%; (vi) piperidine, CH_3CN–$CHCl_3$, rt, 1.5 h, 60%.



Scheme 2. Reagents and conditions: (i) TEA, $CHCl_3$, rt., 24 h, 56%); (ii) TFA, CH_2Cl_2, rt, 3 h, 94%.



Scheme 3. Reagents and conditions: (i) EDC, DMAP, CH_2Cl_2, rt, 22 h, 59%; (ii) (a) piperidine, CH_3CN, rt, 1.5 h; (b) NaOMe–MeOH, rt, 1 h, 87% (two steps); (iii) α2,3-(*N*)-sialyltransferase, CMP-NANA, cacolylate buffer, $MnCl_2$, 37 °C, 28 h, 45%.



Scheme 4. Reagents and conditions: (i) DPPA, TEA, DMF, rt, 18 h, 32%; (ii) (a) piperidine, CH_3CN, rt, 1.5 h; (b) NaOMe–MeOH, rt, 1 h, 80% (two steps); (iii) α2,3-(*N*)-sialyltransferase, CMP-NANA, cacolylate buffer, $MnCl_2$, 37 °C, 64 h, 46%.

an enzymatic method with α2,3-sialyltransferase under a mild condition to give the target trisphenol–sialyllactose **1** and trisaniline–sialyllactose **2**. Since α2,3-(*N*)-sialyltransferase was easy to purchase, we used the enzyme and synthesized the compounds with the Neu5Ac-α2,3Gal terminal. The final target compounds were purified by reverse phase high-performance liquid chromatography, followed by lyophilization. All of the structures of the synthesized compounds were confirmed by ^1H, ^{13}C NMR and MS measurements.[12]

Four synthesized substances, **1**, **2**, **14** and **16** showed higher water solubility, thus the test was facilitated in an aqueous system. The inhibition effects against human influenza A virus infection were investigated by means of inhibition of the cytopathic effect on MDCK cells.[13] The employed virus strain, A/PR/8/1934 (H1N1), recognized the receptor with saccharide terminated in Neu5Ac-α2,3Gal and Neu5Ac-α2,6Gal.[14] Since the amount of the synthesized compounds **1** and **2** is quite small, the inhibition activity against the concentration of the compounds could not be observed. Thus, we could not evaluate the IC_{50} of the inhibition. We evaluated the inhibition effect with MDCK cells at a certain concentration of 400 μM. As the results are shown in Table 1, **1** showed a significant inhibitory effect, particularly at lower temperature 4 °C, as the number of plaques was reduced and approximately 85% inhibition of plaque formation while **2** gave a weaker effect than **1**. Although **14** and **16** possess the same trivalent skeleton

Table 1
Blocking effects of novel trivalent blockers on virus replication

	Numbers of plagues at different adsorption temperature (percentage of plaque [%])	
	4 °C	35 °C
Trisphenol–sialyllactose (**1**)	9 × 10 (15.5)	38 × 10 (45.6)
Trisaniline–sialyllactose (**2**)	39 × 10 (67.2)	47 × 10 (56.6)
Trisphenol–lactose (**14**)	42 × 10 (72.4)	64 × 10 (77.1)
Trisaniline–lactose (**16**)	45 × 10 (77.6)	68 × 10 (81.9)
Virus only	58 × 10 (100.0)	83 × 10 (100.0)
No virus (inoculation)	0 (0.0)	0 (0.0)

as **1** and **2** with no neuraminic acid they indicated the small blocking effect. It suggests that there are no-specific interaction between **14** or **16** and virus. **1** showed a higher activity at a low temperature (4 °C) than it was at 35 °C. This suggests that neuraminidase activity was suppressed at this temperature. Terminal neuraminic acid might be partly cleaved by neuraminidase at 35 °C.

Unexpectedly, **1** and **2** exhibited no hemagglutination-inhibition assay (HI) activity (data not shown). In the molecular simulations, we investigated a geometrical aspect of the complex between the compounds and hemagglutinin. In this study, we challenged to simplify the complex HA blocker which previously designed. The compounds **1** and **2** were quite simple compared with the previously designed glycopeptides type HA blocker. Our compounds can suppress the replication of the influenza virus. It is necessary to elucidate the interaction between virus and the anti-influenza reagents. This study was the first step of such a computer assisted drug design. We could not perform the experiment to evaluate the interaction between virus and our synthesized compounds, because the amount of obtained compounds was quite small. We also could not determine the reason why **2** had less virus replication activity than **1**. The two compounds carried three equivalent neuraminic acid sugar units. They only differed in core structure. Exceedingly, the freedom of the sugar moieties in **2** was different from that of **1** because of the stiff amido bond, hence the trisaccharide in **2** was unable to approach the viral HA binding sites efficiently. Perhaps some other steric factors were limited the conformational flexibility of **2**.

In conclusion, novel trivalent anti-influenza reagents were designed and constructed effectively. The inhibition effects were examined in the virus replication of the A/PR/8/34 (H1N1) virus strain in MDCK cells. Based on the significant activity of trisphenol–sialyllactose **1** at 400 μM, this may be a potential candidate for anti-influenza drug. It is impressed that our compounds has inhibition of virus replication without HAI activity. It suggests that the further investigation to understand the detail of interaction between the anti-influenza blocker and virus were indispensable to better design of novel anti-influenza reagents. The temperature dependence of the MDCK assay indicated that the NA activity of the influenza virus removes the terminal neuraminic acid in each valence. Therefore, unnatural glycosidic bonds have to be examined to prevent the neuraminic acid being removed by NA. On the basis of the results from this study, we are examining the more effective anti-influenza reagents.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.04.060.

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