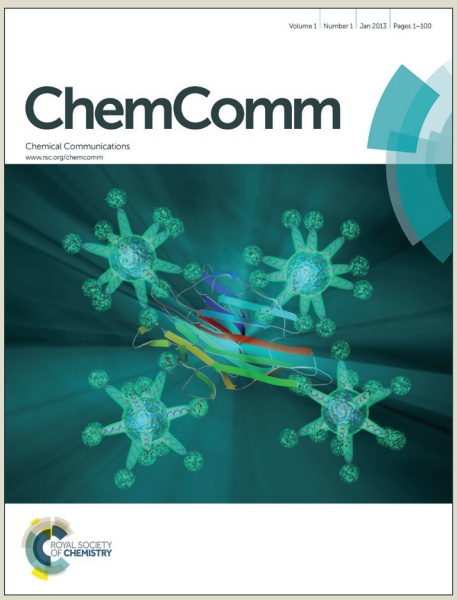
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Systematic synthesis of sulfated oligofucosides and their effect on breast cancer MCF-7 cells

Shinsuke Arafuka, Nozomi Koshiba, Daisuke Takahashi* and Kazunobu Toshima*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

DOI: 10.1039/b000000x

Sulfated tetrafucosides 2-5 with different sulfation patterns, and a non-sulfated tetrafucoside 6, were designed and systematically synthesized from the common key intermediate 12. In addition, their anti-proliferative activities and apoptosis-inducing activities against human breast cancer MCF-7 cells were evaluated. Our results demonstrated that the sulfated tetrafucosides 2-4 reduced the number of MCF-7 cells in a dose dependent manner, and of these, 3,4-O-sulfated type 4 showed the highest anti-proliferative activity, comparable to the activity of fucoidan 1 isolated from Fucus vesiculosus. Furthermore, it was revealed that both 1 and 4 exhibited apoptosis-inducing activities through activation of caspase-8 on MCF-7 cells.

In recent decades, the development of structurally-defined ultralow molecular weight (ULMW; ca 1500 Da) derivatives of naturally occurring and biologically active sulfated polysaccharides has been of increasing interest in the fields of chemistry, biology, and medicine.[1,2] Fucoidan is a sulfated polysaccharide extracted from marine brown algae. The structure and composition of fucoidan varies among different brown seaweed species. However, fucoidan generally possesses two types of homofucose backbone chains (Figure 1). The first chains contain repeated (1→3)-linked α-L-fucopyranosyl residues (type I), and the second chains contain alternating (1→3)- and (1→4)-linked α-L-fucopyranosyl residues (type II).[3] It is also known that fucoidan exhibits a wide range of biological activities such as anti-inflammatory, anticoagulant, and antitumor activities.[3,4] In particular, the antitumor activity of fucoidan has attracted much attention because fucoidan has been reported to effectively inhibit tumor growth of lymphoma[5] and mouse breast cancer,[6] and to show anti-angiogenesis activity against Lewis lung carcinoma and B16 melanoma[7] *in vivo*. Moreover, *in vitro* studies have led to the postulation of several mechanisms underlying the antitumor activities.[8] Recently, fucoidan **1** was isolated from *Fucus vesiculosus*, shown to possess a type II backbone chain, and found to induce apoptosis in human HS-Sultan cells[9] and human breast cancer MCF-7 cells.[10] Thus, fucoidan holds promise as a potential antitumor agent with few side effects. However, little is known about how the length and sulfation pattern of oligofucosides determine their biological properties. This lack of information is due to their structural complexity, heterogeneity, and uniformity of sulfation patterns. In this context, to date several studies of oligofucosides with different sulfation patterns have been reported.[11] However, detailed structure-activity relationships and the mode of action of the antitumor properties of fucoidan have yet to be elucidated. Here we describe the design and systematic synthesis of the tetrafucosides **2-6** with different sulfation patterns (2,3,4-*O*-sulfated type **2**, 2,3-*O*-sulfated type **3**, 3,4-*O*-sulfated type **4**, 4-*O*-sulfated type **5**, and non-sulfated type **6**) consisting of a type II backbone chain. We also evaluated the antitumor activities of these tetrafucosides against MCF-7 cells.

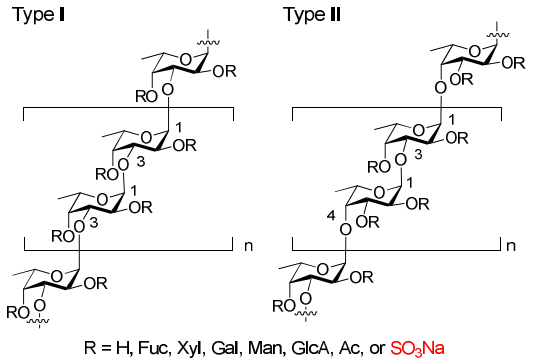


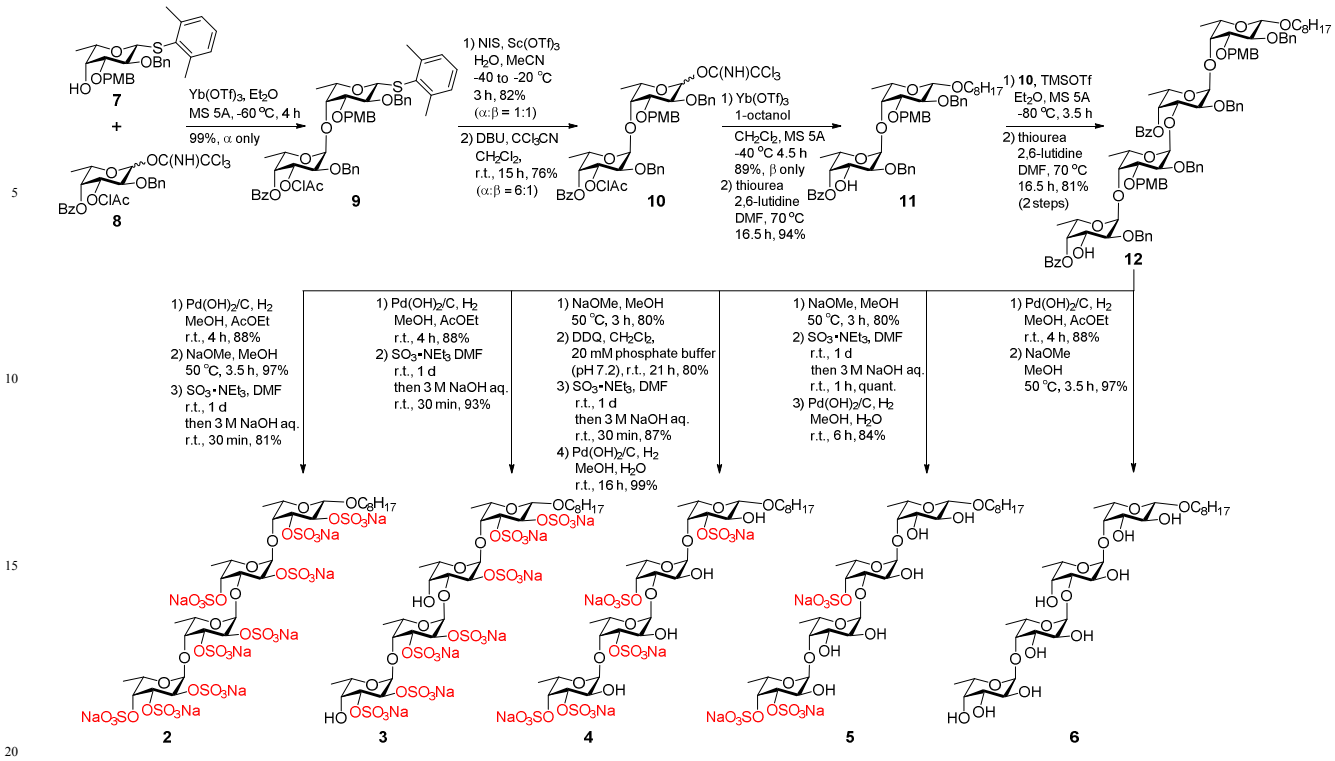
Figure 1. Two types (I and II) of homofucose backbone chains in brown seaweed fucoidans

As illustrated in the synthetic scheme for **2-6** (Scheme 1), we developed an orthogonal deprotection strategy utilizing the common key intermediate **12**. Compound **12** has three different protecting groups, benzyl (Bn), benzoyl (Bz), and *p*-methoxybenzyl (PMB), at the appropriate positions to minimize the number of synthetic steps and to afford sufficient quantities of oligofucosides for biological assays.

Monosaccharide glycosyl acceptor **7** was prepared from L-fucose in 8 steps (see Scheme S1 in ESI). Chemoselective glycosylation of **7** with **8**[11c] using a catalytic amount of $Yb(OTf)_3$ in Et_2O at −60 °C for 4 h proceeded smoothly to provide the desired disaccharide **9** as a single isomer in excellent yield. In this reaction, the use of the

Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-8522 (Japan). E-mail: dtak@applc.keio.ac.jp, toshima@applc.keio.ac.jp; Fax: (+81) 45-566-1576.

† Electronic supplementary information (ESI) available: See DOI: 10.1039/b000000x/



Scheme 1. Synthesis of non-sulfated and sulfated tetrafucosides.

2,6-dimethylphenylthio group in the acceptor **7** was critical for increasing the chemical yield.[12] For elongation of the carbohydrate chain, **9** was readily converted to a suitable glycosyl donor and acceptor pair (**10** and **11**). Hydrolysis of **9** using NIS/Sc(OTf)$_3$, followed by introduction of a trichloroacetimidate group, provided **10** in good yield. Compound **10** was condensed with 1-octanol using Yb(OTf)$_3$ in CH_2Cl_2 at −40 °C for 4.5 h to afford octyl fucoside, whose ClAc group was removed by treatment with thiourea to provide **11** in high yield. At this stage, it was confirmed by ^{1}H-NMR analysis that the fucoside **11** adopts a β configuration ($J_{1,2}$=7.8 Hz). Glycosylation of **11** with **10** using TMSOTf as an activator in Et_2O at −80 °C, followed by deprotection of the ClAc group, provided desired key intermediate **12** as a single isomer in 81% yield in 2 steps. Next, designed sulfated and non-sulfated tetrafucosides **2**-**6** were synthesized from the common intermediate **12**. Deprotection of the Bn and PMB groups in **12** under hydrogenolysis conditions, followed by saponification, provided the non-sulfated **6**. Sulfation of **6** using SO_3•NEt_3 complex in DMF gave the 2,3,4-*O*-sulfated tetrasaccharide **2**. In addition, deprotection of the Bn and PMB groups in **12**, followed by sulfation and saponification, provided 2,3-*O*-sulfated tetrasaccharide **3**. On the other hand, saponification of **12**, followed by sulfation and hydrogenolysis, provided 4-*O*-sulfated tetrasaccharide **5**. Finally, saponification of **12**, followed by deprotection of the PMB groups with DDQ, sulfation, and hydrogeolysis afforded the desired 3,4-*O*-sulfated tetrafucoside **4**.

With the designed tetrafucosides in hand, the effects of **2**-**6** on the proliferation of MCF-7 breast cancer cells were examined using the MTT assay. The cells were treated with different doses (10-800 μM) for 96 h. The results are shown in Figure 2a. 4-*O*-sulfated type **5** and non-sulfated **6** provided only low anti-proliferation activities, even at 800 μM. In sharp contrast, 2,3,4-*O*-sulfated type **2**, 2,3-*O*-sulfated type **3**, and 3,4-*O*-sulfated type **4** effectively reduced the number of MCF-7 cells in a dose-dependent manner, and of these, **4** showed the highest anti-proliferative activity. These results provide the first demonstration that homogeneous and structurally-defined sulfated tetra

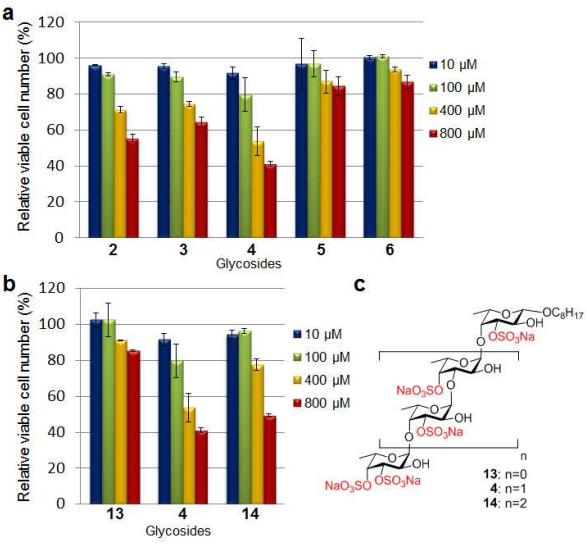


Figure 2. Effects of a) **2**-**6** and b) **4**, **13** and **14** on MCF-7 cell proliferation. MCF-7 cells were seeded into 96-well plates (1×10^3 cells/well). After 24 h, compounds were added at the indicated concentrations and the cells were incubated for 96 h at 37 °C in 5% CO_2 in air. MTT reagent was then added to each well, and the cells were incubated for up to 3 additional hours. The absorbance at 540 nm was read on a plate reader. c) Chemical structures of 3,4-*O*-sulfated oligofucosides **4**, **13**, and **14**.

fucosides show anti-proliferative activity against human breast cancer MCF-7 cells. Moreover, these results suggest that slight differences in the pattern of sulfate groups on **2-5** affect their anti-proliferative activity.

Next, to elucidate the minimum carbohydrate chain-length required for anti-proliferative activity, 3,4-*O*-sulfated type disaccharide **13** and hexasaccharide **14** were designed (Figure 2c). After chemical synthesis of **13** and **14** (see Scheme S3 in the Supporting Information), we examined their anti-proliferative activities against MCF-7 cells under the same conditions described above. As shown in Figure 2b, disaccharide **13** showed lower anti-proliferative activity than **4** or **14**. In addition, the tetrasaccharide **4** showed slightly higher activity than that of the hexasaccharide **14**. These results clearly indicate that **4** is the minimum structure required for anti-proliferative activity against MCF-7 cells. Furthermore, we carried out MTT assays using **4**, fucoidan **1** (isolated from *F. vesiculosus*; 600 kDa), and fucoidan **15** (isolated from *Chorda filum*; 350 kDa) possessing a type I backbone chain in order to compare their anti-proliferative activities against MCF-7 cells. In this assay, the cells were treated with different doses (10-1000 μg/mL) for 96 h (Figure 3a). Interestingly, we found that the fucoidan **1** showed higher anti-proliferative activity than fucoidan **15**; in addition, the activity of **1** was almost the same as that of **4**. These results suggest that the 3,4-*O*-sulfated oligofucoside structure with a type II backbone chain is one of the key components for fucoidan to show anti-proliferative activity against MCF-7 cells. Furthermore, it was clearly confirmed that **4** exhibited neither cytotoxicity nor anti-proliferative activity against normal human lung fibroblast WI-38 cells (Figure 3b).

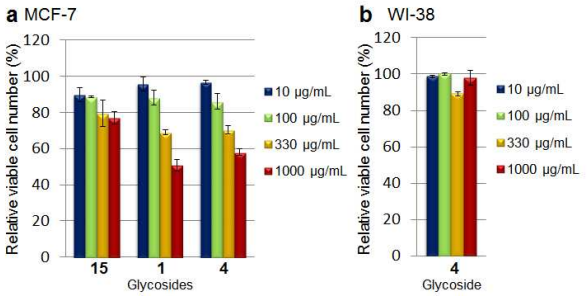


Figure 3. a) Effects of fucoidans and **4** on MCF-7 cell proliferation. MCF-7 cells were seeded into 96-well plates (1×10^3 cells/well). After 24 h, the compounds were added at the indicated concentrations. The cells were incubated for 96 h at 37 °C in 5% CO_2 in air, then MTT reagent was added to each well and the cells were incubated for up to 3 additional hours. The absorbance at 540 nm was read on a plate reader. b) Effect of **4** on WI-38 cell proliferation. WI-38 cells were seeded into 96-well plates (2.5×10^3 cells/well). After 24 h, **4** was added at the indicated concentrations. The cells were incubated for 96 h at 37 °C in 5% CO_2 in air, then MTT reagent was added to each well and the cells were incubated for up to 3 additional hours. The absorbance at 540 nm was read on a plate reader.

Next, to reveal whether the fucoidan **1** and the tetrafucoside **4** could induce apoptotic cell death, we evaluated the morphology of the nucleus in each cell by staining with Hoechest 33342[13] after treatment with **1** or **4**. As shown in Figure 4, the treatment of MCF-7 cells with **1** or **4** resulted in the induction of chromatin condensation, which was visualized as intense blue-white fluorescence within the cell nucleus (Figures 4b and c). In addition, it was confirmed that when **1** or **4** was exposed to the cells together with Z-VAD-fmk[9] (Z-VAD), a general caspase inhibitor, the number of apoptotic cells significantly decreased, as shown in Figures 4d and e. These results clearly indicate that not only **1**, but also **4**, induces caspase-dependent apoptosis in MCF-7 cells.

To confirm the involvement of caspases in **1**- or **4**-induced apoptosis, we evaluated the activation of caspase-8 and 9. It is known that caspase-8 and 9 are essential for executing the major extrinsic and intrinsic pathways of apoptosis, respectively.[14] MCF-7 cells were treated with **1** or **4** (330 μg/mL) for 1-4 d. The results are summarized in Figure 5. No cleavage of caspase-9 was detected. On the other hand, the active fragments of caspase-8 (43 and 41 kDa) were clearly observed only after treatment with **1** or **4**. Caspase-8 is known to play a crucial role in apoptosis triggered by the interaction of ligand with integrins[15] or death receptors such as Fas, the tumor necrosis factor (TNF) receptor, and TNF-related apoptosis-inducing ligand receptor.[16] Thus, these results may suggest that not only the fucoidan **1**, but also the ultralow molecular weight **4**, interact with target receptor proteins on the MCF-7 cell membrane. In addition, these results provide the first demonstration that a homogeneous and structurally-defined sulfated tetrafucoside epitope can be sufficient for the induction of apoptosis in MCF-7 cells through activation of caspase-8.

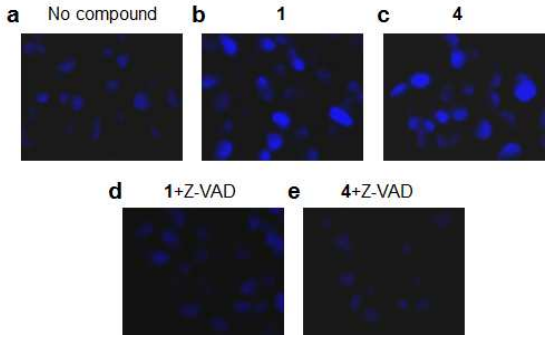


Figure 4. The degree of apoptosis represented as fluorescent images of the cell nucleus by fluorescence microscopy. MCF-7 cells were seeded into 12-well plates (3×10^4 cells/well). After 24 h, the cells were treated with (a) no compound, (b) **1**, (c) **4**, (d) **1** and Z-VAD, and (e) **4** and Z-VAD for 48 h at 37 °C in 5% CO_2 in air. The cells were stained with the DNA-specific fluorescent dye, Hoechst 33342.

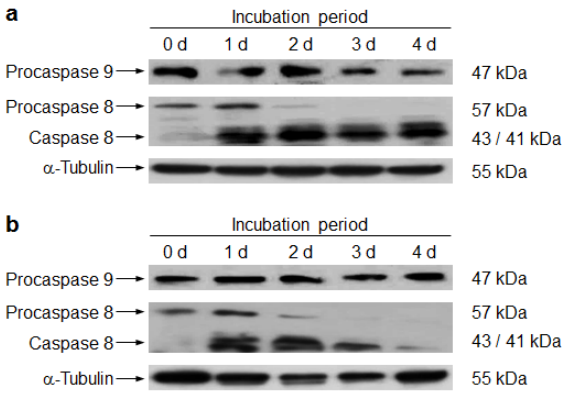


Figure 5. Western blot analysis of the effects of a) **1** and b) **4** on protein levels in MCF-7 cells. Cells were treated with each compound for 0-4 d at 37 °C in 5% CO_2 in air. Each sample was analyzed using tricine-SDS-PAGE and immunoblotting with appropriate monoclonal antibodies. α-Tubulin levels are shown as a protein loading control.

In conclusion, we have shown that the designed and synthesized sulfated tetrafucoside, 3,4-*O*-sulfated type **4**, effectively reduced the number of MCF-7 cells in a dose-dependent manner without causing cytotoxicity towards normal WI-38 cells. In addition, the tetrafucoside epitope was found to be the minimum structure required for anti-proliferative activity. Furthermore, it was revealed that not only the fucoidan **1**, but also **4**, induced apoptosis in MCF-7 cells through activation of caspase-8. On the basis of these findings, we anticipate that the results presented here contribute to the development of sulfated oligosaccharide-based antitumor agents exhibiting fewer side effects. The identification of the target protein of **4** is now under investigation in our laboratories.

We wish to thank Prof. Dr. Siro Simizu, Faculty of Science and Technology, Keio University, for helpful discussions about biological assay. This research was supported in part by the MEXT-supported Program for the Strategic Research Foundation at Private Universities, 2012-2016, from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) and by Mizuho Foundation for the Promotion of Sciences.

Notes and references

1. For examples of ULMW derivatives of heparin: (a) M. Petitou, J.-P. Hérault, A. Bernat, P.-A. Driguez, P. Duchaussoy, J.-C. Lormeau and J.-M. Herbert, *Nature*, 1999, **398**, 417; (b) Y. Xu, S. Masuko, M. Takieddin, H. Xu, R. Liu, J. Jing, S. A. Mousa, R. J. Linhardt and J. Liu, *Science*, 2011, **334**, 498.
2. For examples of ULMW derivatives of chondroitin sulfates: (a) S. E. Tully, R. Mabon, C. I. Gama, S. M. Tsai, X. Liu and L. C. Hsieh-Wilson, *J. Am. Chem. Soc*., 2004, **126**, 7736; (b) C. I. Gama, S. E. Tully, N. Sotogaku, P. M. Clark, M. Rawat, N. Vaidehi, W. A. Goddard III, A. Nishi and L. C. Hsieh-Wilson, *Nat. Chem. Biol*., 2006, **2**, 467.
3. A. Cumashi, N. A. Ushakova, M. E. Preobrazhenskaya, A. D'Incecco, A. Piccoli, L. Totani, N. Tinari, G. E. Morozevich, A. E. Berman, M. I. Bilan, A. I. Usov, N. E. Ustyuzhanina, A. A. Grachev, C. J. Sanderson, M. Kelly, G. A. Rabinovich, S. Iacobelli and N. E. Nifantiev, *Glycobiology*, 2007, **17**, 541.
4. (a) O. Berteau and B. Mulloy, *Glycobiology*, 2003, **13**, 29R; (b) B. Li, F. Lu, X. Wei and R. Zhao, *Molecules*, 2008, **13**, 1671; (c) V. H. Pomin and P. A. S. Mourão, *Glycobiology*, 2008, **18**, 1016; (d) M. T. Ale, J. D. Mikkelsen and A. S. Meyer, *Mar. Drugs*, 2011, **9**, 2106; (e) W. A. J. P. Wijesinghe and Y.-J. Jeon, *Carbohydr. Polym.*, 2012, **88**, 13; (f) M. T. Ale and A. S. Meyer, *RSC Adv*. 2013, **3**, 8131.
5. H. Maruyama, H. Tamauchi, M. Iizuka and T. Nakano, *Planta Med.*, 2006, **72**, 1415.
6. M. Xue, Y. Ge, J. Zhang, Q. Wang, L. Hou, Y. Liu, L. Sun and Q. Li, *PLoS One*, 2012, **7**, e43483.
7. S. Koyanagi, N. Tanigawa, H. Nakagawa, S. Soeda and H. Shimeno, *Biochem. Pharmacol*., 2003, **65**, 173.
8. K. Senthilkumar, P. Manivasagan, J. Venkatesan and S.-K. Kim, *Int. J. Biol. Macromol.*, 2013, **60**, 366.
9. Y. Aisa, Y. Miyakawa, T. Nakazato, H. Shibata, K. Saito, Y. Ikeda and M. Kizaki, *Am. J. Hematol.*, 2005, **78**, 7.
10. (a) Y. Yamasaki-Miyamoto, M. Yamasaki, H. Tachibana and K. Yamada, *J. Agric. Food Chem.*, 2009, **57**, 8677; (b) Y. Yamasaki, M. Yamasaki, H. Tachibana and K. Yamada, *Biosci. Biotechnol. Biochem*., 2012, **76**, 1163.
11. (a) N. Ustyuzhanina, V. Krylov, A. Grachev, A. Gerbst and N. Nifantiev, *Synthesis*, 2006, 4017; (b) C. Zong, Z. Li, T. Sun, P. Wang, N. Ding and Y. Li, *Carbohydr. Res.*, 2010, **345**, 1522; (c) V. B. Krylov, Z. M. Kaskova, D. Z. Vinnitskiy, N. E. Ustyuzhanina, A. A. Grachev, A. O. Chizhov and N. E. Nifantiev, *Carbohydr. Res.*, 2011, **346**, 540.
12. When the ethyl thioglycoside was used as an acceptor instead of **7**, aglycon transfer reaction proceeded as a side reaction; Z. Li and J. C. Gildersleeve, *J. Am. Chem. Soc.*, 2006, **128**, 11612.
13. G. Lizard, S. Fournel, L. Genestier, N. Dhedin, C. Chaput, M. Flacher, M. Mutin, G. Panaye and J.-P. Revillard, *Cytometry*, 1995, **21**, 275.
14. (a) S. Nagata, *Cell*, 1997, **88**, 355; (b) K. Kuida, T. F. Haydar, C. Y. Kuan, Y. Gu, C. Taya, H. Karasuyama, M. S.-S. Su, P. Rakic and R. A. Flavell, *Cell*, 1998, **94**, 325.
15. (a) D. L. Brassard, E. Maxwell, M. Malkowski, T. L. Nagabhushan, C. C. Kumar and L. Armstrong, *Exp. Cell Res.*, 1999, **251**, 33; (b) D. G. Stupack, X. S. Puente, S. Boutsaboualoy, C. M. Storgard and D. A. Cheresh, *J. Cell Biol.*, 2001, **155**, 459.
16. A. Ashkenazi and V. M. Dixit, *Science*, 1998, **281**, 1305.