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学位論文全文に代わる要約
Extended Summary in Lieu of Dissertation

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学位論文題目：
Title of Dissertation

Evaluation of Anti-Proliferative Activity of Rare Aldohexoses, and Development of D-Allose Derivatives with Biological Activities
(希少アルドヘキソースのがん細胞増殖抑制活性の評価および生物活性を有するD-アロース誘導体の開発)

学位論文要約：
Dissertation Summary

General introduction

Sugars are important as energy source and component material for living organisms including human. Rare sugars are defined as monosaccharides and their derivatives rarely existing in nature by International Society of Rare Sugars. Recently, rare sugars have attracted attention owing to their biological activities. For example, D-allulose (D-Alu, Figure 1), a C-3 epimer of D-fructose, induced the up-regulation of defense-related genes, and showed antihyperglycemic and anti-obesity activities. D-Allose (D-All, Figure 1), a C-3 epimer of D-glucose (D-Glc), showed plant growth-regulatory, antioxidant, neuroprotective, and anti-proliferative activity. Thus, rare sugars are regarded as a promising source for the development of pharmaceutical drugs and food additives owing to their unique biological activities.

D-All showed anti-proliferative activity against several cancer cell lines (ovarian, prostate, liver, leukemia, and tongue) without affecting normal cells. D-All is incorporated into cells and phosphorylated by hexokinases to generate D-allose-6-phosphate (A6P, Figure 2). A glucose-responsive transcription factor, MondoA, senses A6P and activates the transcription of thioredoxin-interacting protein (TXNIP). It was reported that TXNIP involved in various cellular processes such as redox balance, apoptosis, and glucose homeostasis. TXNIP expression is induced in cancer cell lines in which D-All exhibits significant anti-proliferative activity. Thus, TXNIP plays an important role in the anti-proliferative activity of D-All. As known as the Warburg effect, cancer cells rely on glycolysis for producing ATP even under normal oxygen level. It was reported that cancer cells increase the glycolytic system more than thirty times and consume at least ten times more glucose than normal cells to produce the energy required for cell proliferation. In addition, glucose uptake was mediated by GLUTs, and it was reported that GLUTs are overexpressed in cancer cell lines such as HeLa, Caco-2, HL-60, HepG2, and HTB63. Therefore, glucose metabolism can be considered as a target for anticancer therapy.

On the other hand, various type of structure modification of monosaccharides have been conducted because sugars are an important substrate for living organisms. For example, 2DG (Figure 3), C-2 deoxy derivative of D-Glc, is one of the most well-known sugar derivatives and it acts as a competitive inhibitor of glucose metabolism. 2DG inhibits the initial step of glucose metabolism, it partially interferes with both glycolysis and OXPHOS, and exhibit cytotoxicity caused by suppression of ATP production and inducing cell death. Thus, 2DG is attracting attention as an anticancer agent and has been approved for phase II clinical trials as an adjuvant chemotherapy agent. In addition, mitobronitol is dibrominated analog of D-mannitol and one of alkylating agents (Figure 3). However, the clinically used of 2DG and mitobronitol were limited by its serious side effects. Few studies have evaluated the biological activity by modifying the structure of rare sugars. Afach *et al*. synthesized 6-*O*-octanoyl-D-All (Figure 3) and reported that it showed six-times higher growth inhibitory activity against lettuce seedings. Chowdhury *et al*. reported that D-All and 2-deoxy-D-All (Figure 3) showed similar growth inhibitory activity to D-All. Furthermore, Yoshihara *et al*. synthesized eight 1-deoxyketohexose stereoisomers and reported that 1-deoxy-D-Alu (Figure 3) only showed significant growth inhibitory activity against *C. elegans*. Yanagita *et al*. reported 6-*O*-dodecanoyl-D-All (Figure 3) showed approximately 30-times stronger anti-proliferative activity than D-All against a MOLT-4F human leukemia T-cell line and the length of the acyl chain of D-All esters was important for the activity. These researches suggest that the biological activity of D-All might be improved by derivatization.

As mentioned above, rare sugars exhibit various biological activities. D-All is expected as a seed anti-cancer compound. Despite the promising activity of D-All, the concentration required to exhibit the activity is relatively high. Thus, the author decided to investigate the anti-proliferative activity of rare aldohexoses and develop D-All derivative.

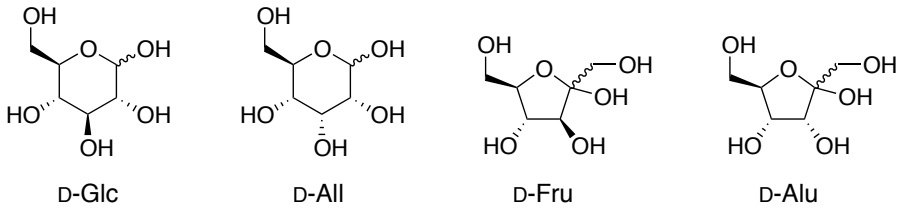


Figure 1. The structures of D-Glc, D-All, D-Fru, and D-Alu.

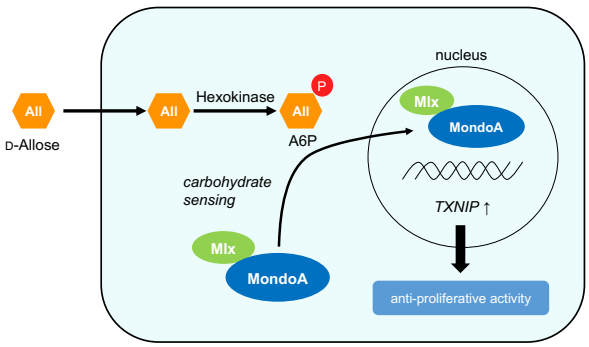


Figure 2. Mechanism of action of anti-proliferative activity of D-All.

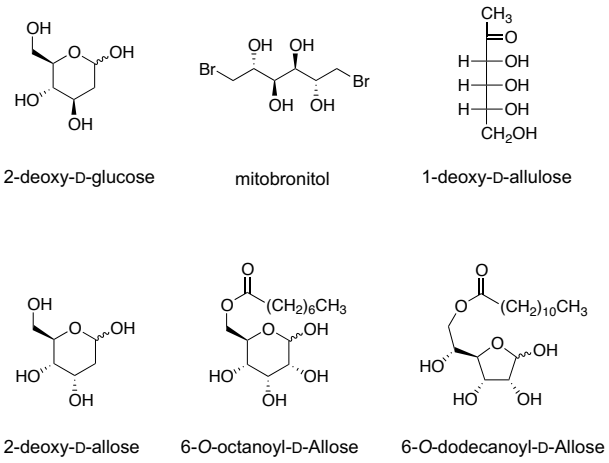


Figure 3. Structures of monosaccharide derivatives.

Synthesis of D-Allose fatty acid esters with nematocidal activity

More than 1.5 billion people worldwide are infected by intestinal nematodes, which causes a negative impact on human health and productivity in developing countries. In veterinary medicine, they pose a very severe threat to livestock. However, anthelmintic drugs are few in number and most of them were developed decades ago. Therefore, it is desirable to develop an anthelmintic with a novel mechanism of action. Sato *et al*. reported that the D-All shows weak growth-inhibitory activity against *C. elegans* (GI_{50}, 200 mM). However, D-allose itself was not considered to be an anthelmintic because of its lack of nematicidal activity even at a concentration of 200 mM. As mentioned above, it has also reported that D-allose has weak inhibitory activities on the growth of plants and human leukemia cells, and medium-chain fatty acid esterification of D-All largely enhances its activities. Thus, the author synthesized D-All fatty acid esters with different carbon chain lengths for the seed compound of novel anthelmintic drugs.

The author synthesized D-All fatty acid esters for the evaluation of the nematocidal activities. In addition, to reveal the mechanism of action, the author also synthesized the fatty acid ester of D-Glc and nonhydrolyzable alkoxy analogs of D-All. The nematocidal activity of D-All derivatives against *C .elegans* were measured. Among D-All fatty acid esters **1**–**4**, only **3** showed nematocidal activity, and the lethality rates were 42.3 and 97.4% at concentrations of 0.2 and 1 mM, respectively (Table 1). The other D-All fatty acid esters showed no activity at all. The activity of **3** was dose-dependent at concentrations between 0.18 and 1 mM, and its 50% lethal concentration (LC_{50}) value was estimated to be 0.24 mM (Table 1). The strength of the nematocidal activity of **3** is approximately 1/10th of the common anthelmintic albendazole in *C. elegans*. However, D-Glc octanoic acid ester **5** was found to be inactive the D-All moiety might be essential for the nematocidal activity of **3**. On the other hand, octanoic acid showed weak activity (LC_{50}, 3.88 mM). Thus, to investigate whether the nematocidal activity of **3** was caused by the toxic octanoic acid formed by

hydrolysis in the body of *C. elegans*, the nematocidal activities of nonhydrolyzable alkoxy analogs **6** and **7** were examined. Compound **6** having the same carbon chain length as **3** exhibited almost the same activity as **3** (LC_{50}, 0.20 mM). These results suggest that **3** is not hydrolyzed within the body of *C. elegans* and exerts toxicity in its intact form. In addition, **7** having the same carbon chain length as **4** showed no activity.

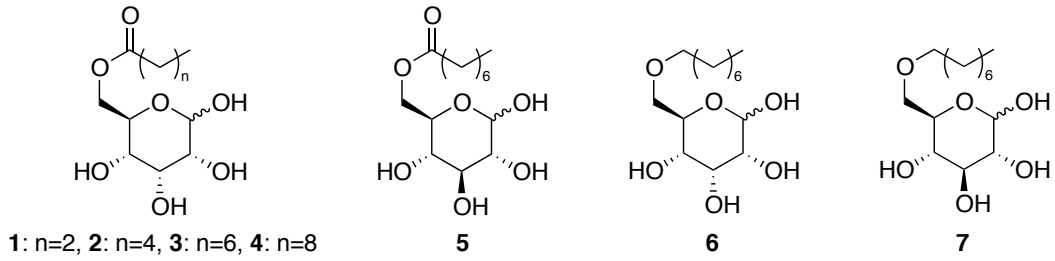


Figure 4. The structures of **1**-**7**

Table 1. Nematicidal activity of acyl- and alkoxyl derivatives of D-All.

	% lethality ± SD (n = 3)		IC_{50} [95% confidence interval]
	0.2 mM	1 mM	(mM)
1		0	
2		0	
3	42.3 ± 5.7	97.4 ± 4.4	0.24 [0.21–0.27]
4		0	
5		0	
6	47.6 ± 13.2	98.1 ± 3.2	0.20 [0.19–0.21]
7		0	
Octanoic acid	0	23.1 ± 8.0	3.88 [2.76–8.68]

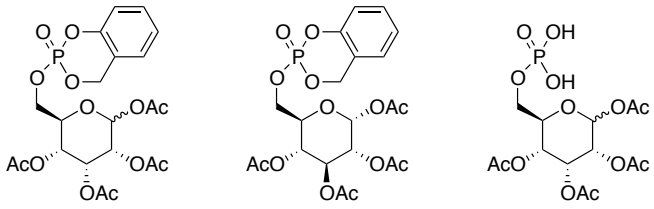
Design and synthesis of membrane-permeable derivative of D-allose-6-phosphate with anti-proliferative activity against MOLT-4F cells.

As mention above, the concentration required to exhibit the activity of D-All is relatively high, which might be caused by the low affinity to GLUTs and hexokinases. To develop a superior D-All derivative, the author focused on the metabolism and its mechanism of action. Like D-Glc, D-All is incorporated into cells through GLUTs and passive diffusion and is phosphorylated by hexokinases to generate A6P. A6P was considered to be important for the activity of D-All. Therefore, treating cells with A6P represents a promising strategy, but A6P probably cannot pass though cell membranes because of its negative charge and high hydrophilicity. To overcome this interference, the author carried out the prodrug modification of A6P to synthesis membrane-permeable A6P derivative **11**.

The author designed A6P derivative **11** (Figure 5), where the hydroxyl groups and the phosphate group are protected by acetyl groups and a cycloSal group, respectively. These

protecting group can be hydrolyzed to A6P in cells and exhibit higher activity than D-All. The anti-proliferative activity of **11** as well as D-All was evaluated using a growth inhibition assay against the MOLT-4F human leukemia cell line, and the GI_{50} values were estimated from dose-response curves. Compound **11** inhibited the growth of the MOLT-4F cells in a concentration-dependent manner (Figure 6). The GI_{50} value of **11** was 5.2 μM, which was approximately 250-fold and 8-fold lower than those of D-All (GI_{50}, 1300 μM) and 6-*O*-decanoyl-D-All (GI_{50}, 44 μM), respectively. However, unlike D-All inducing cell cycle arrest but not cell death in the MOLT-4F cells, **11** showed cytotoxicity and completely killed the cells at 30 μM. This result was contrary to our design intention, which was that the mechanism of action of **11** would be similar to that of D-All because **11** can release A6P within the cell.

It was reported that D-All strongly induces expression of the TXNIP protein, which is responsible for the anti-proliferative activity of D-All. Thus, to examined whether TXNIP expression was involved in the activity of **11**, the author conducted the western blotting using the anti-TXNIP antibody (Figure 7). The expression of the TXNIP protein induced by **11** was unexpectedly low compared with that induced by D-All, which implies the involvement of a TXNIP-independent pathway in the activity of **11**. Furthermore, to investigate the effect of derivatization on the activity of **11**, the author synthesized and evaluated the anti-proliferative activity of G6P derivative **12** which has the same protecting group as **11** and tetraacetyl-A6P. compound **12** and **13** showed weak and no activity, respectively. These results suggest that protecting of phosphate group was necessary for the membrane-permeability and the cytotoxicity was caused by derivatization.



11 **12** **13**

Figure 5. The Structures of **11**–**13**.

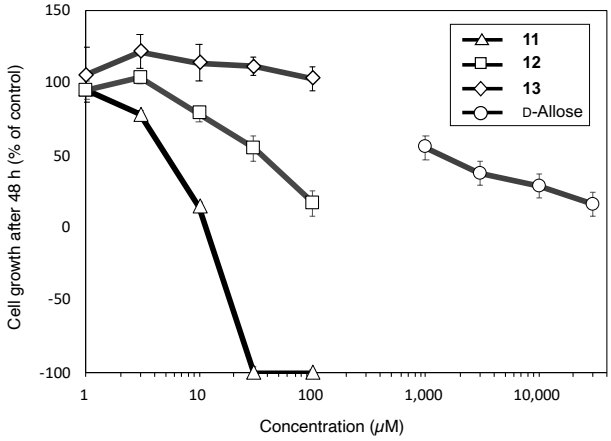


Figure 6. Anti-proliferative activities of **11**–**13** at 1–100 μM and D-All at 1–30 mM against the MOLT-4F cell line. Values are means ± standard deviation (n = 3).

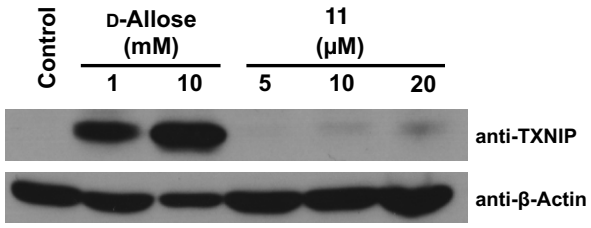


Figure 7. TXNIP expression induced by D-All (1 and 10 mM) and **11** (5, 10, 20 μM). MOLT-4F cells were incubated with each concentration of D-All or **11** for 48 h, and then the expressions of TXNIP and β-actin protein were analyzed by western blotting.

Screening of rare aldohexose: anti-proliferative activity against MOLT-4F and DU-145 cells and structure–activity relationship of D-Idose.

There are sixteen stereoisomers of aldohexoses: eight D-forms and eight L-forms. Among the aldohexoses, only three members, D-Glc, D-Man, and D-Gal, are abundantly found in nature. The other thirteen members are classified as rare sugars (Figure 8). However, the anti-proliferative activities of the other rare aldhexoses have not been examined yet. Thus, the author evaluated the anti-proliferative activity of the rare aldohexoses.

MOLT-4F cells were incubated in the presence of different concentrations of the rare aldohexoses (1, 3, 5, 10, and 20 mM for D-All, D-Alt, D-Gul, D-Tal, L-All, L-Alt, L-Glc, L-Man, L-Gul, L-Gal, and L-Tal, each and 1, 3, and 5 mM each for D-Ido and L-Ido) and the cell viability was measured. Among the D-form rare aldohexoses, D-Ido showed significant inhibitory activity at 5 mM, while D-Tal and D-Alt showed activity at 20 mM (Figure 9). However, among the L-forms of aldohexoses, only L-Tal showed significant inhibitory activity at 20 mM. Cell growth in the presence of 5 mM D-Ido (60%) was comparable to that in the 5 mM D-All-treated group (46%). The author next examined the anti-proliferative activity of rare aldohexoses against DU-145 human prostate cancer cell line, which is also

known to be susceptible to D-All. While MOLT-4F cells were reported to undergo cell cycle arrest upon treatment of D-All, DU-145 cells were reported to undergo apoptosis by 20 or 40 mM of d-All. We evaluated the effect of the 5 or 20 mM of the rare aldohexoses against DU-145 cells. Among 5 mM of the aldohexoses, only L-Ido (77%) inhibited cell growth to less than 80%. On the other hand, at 20 mM, not only D-All but also the rest of the rare aldohexoses, except for D-Ido and L-Ido due to the sample concentration limit, showed low to moderate anti-proliferative activity. This result implies that the anti-proliferative activity of those rare aldohexoses against DU-145 cells are partly caused by physicochemical factors such as osmotic stress rather than biochemical processes specific to type of sugars. In light of these results, the author used MOLT-4F cells which seems to be sensitive to D-Ido for the further analysis of the anti-proliferative activity of D-Ido.

Although D-Ido showed anti-proliferative activity comparable to D-all against MOLT-4F cells, the report which the biological activity of D-Ido was little or none. Thus, the author conducted the structure–activity relationship study of D-Ido. α-D-idopyranose was reported to be relatively unstable owing to its 1,3-diaxial interaction and adopts chair and twisted-boat conformations. Because of its instability, D-Ido can isomerize relatively easily into D-sorbose (D-Sor) in acidic or basic conditions via Lobry de Bruyn-van Ekenstein transformation. In addition, while phosphorylation of the hydroxy group at the C-6 position of D-All is crucial for its anti-proliferative activity, it was unclear that whether D-Ido was phosphorylated in cells. To examine the role of the C-6 hydroxy group of D-Ido on its bioactivity, the author tested the anti-proliferative activity of 6-deoxy-D-Ido and L-xylose (L-Xyl) that have the same configurations as D-Ido at the C-1 to C-4 positions. 6-Deoxy-D-Ido. D-Sor, 6-deoxy-D-Ido, and L-Xyl did not show significant activity against MOLT-4F cells (Figure 8). These results suggest that the aldose structure and C-6 hydroxy group of D-Ido might be important for the activity.

Although D-Ido showed anti-proliferative activity, there was no information on its mechanism of action. Thus, the author first examined effect of D-Ido treatment on cellular glucose uptake by using the 2DG Uptake Measurement Kit (Cosmo Bio). The 2DG uptakes of 5 mM D-Ido- and D-All-treated groups were 78% and 65% of the untreated group, respectively, which has some correlation to the suppression of cell growth in the presence of 5 mM of D-Ido or D-All (60% and 46%, respectively). Therefore, the anti-proliferative activity of D-Ido might be ascribable to the inhibition of cellular glucose uptake and consequent depletion of intracellular glucose and metabolites of glycolytic pathway. D-All was reported to inhibit glucose uptake via induction of TXNIP expression and subsequent downregulation of GLUT. Therefore, to clarify whether the TXNIP expression is involved in the anti-proliferative activity of D-Ido, the author examined the TXNIP expression in MOLT-4F cells treated with D-Ido. Western blotting experiment showed that D-Ido did not induce TXNIP expression at 5 mM. This result suggests that the TXNIP pathway was not involved in the activity of D-Ido.

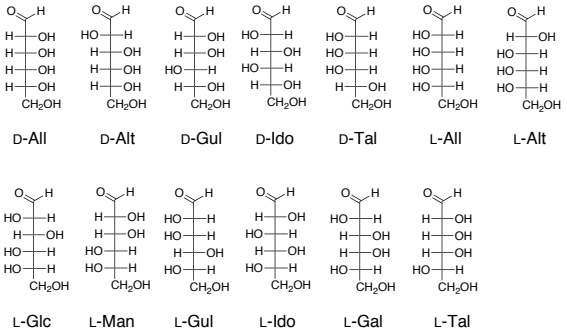


Figure 8. The structure of rare aldohexoses: D-allose (D-All), D-altrose (D-Alt), D-Gulose (D-Gul), D-Talose (D-Tal), L-Allose (L-All), L-Altrose (L-Alt), L-Glucose (L-Glc), L-Mannose (L-Man), L-Gulose (L-Gul), L-Galactose (L-Gal), and L-Talose (L-Tal).

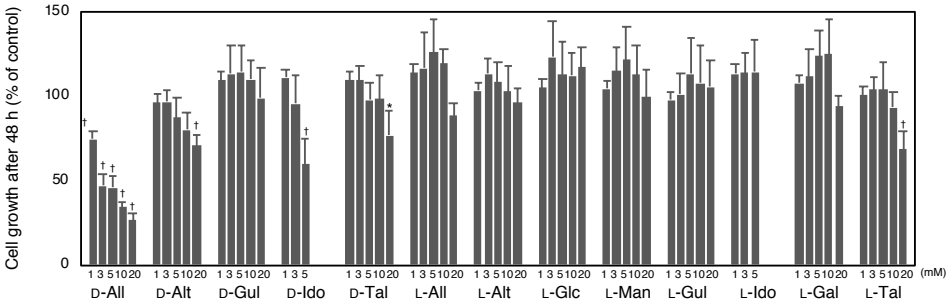


Figure 9. Anti-proliferative activities of D-All, D-Alt, D-Gul, D-Tal, L-All, L-Alt, L-Glc, L-Man, L-Gul, L-Gal, and L-Tal at 1–20 mM each and D-Ido and L-Ido at 1–5 mM against the MOLT-4F cell line. Values are represented as means ± standard deviation (n = 6 or 21). † $p < 0.001$, * < 0.01 (Dunnett's test).

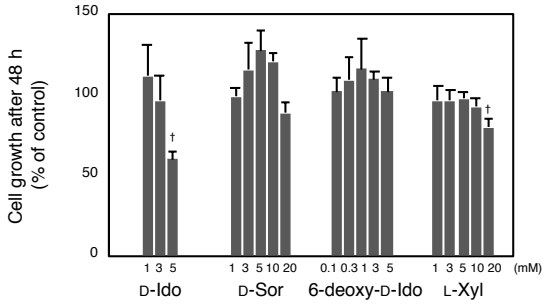


Figure 8. Anti-proliferative activities of D-Ido at 1–3 mM, D-Sor and L-Xyl at 1–20 mM, and 6-deoxy-D-Ido at 0.1–5 mM concentration against the MOLT-4F cell line. Values are means ± standard deviation (n = 6 or 21). † $p < 0.001$ (Dunnett's test).

Summary and Conclusion

The author first synthesized D-All fatty acid esters (**1**-**4**), 6-*O*-Octanoyl-D-Glc (**5**), and nonhydrolyzable alkoxy analog of D-All (**5**, **6**). Compound **3** showed nematocidal activity, and

the optimum length of fatty acid chain was found to be C8. In addition, **6** not hydrolyzed within the body of *C. elegans* and exerts toxicity in its intact form. Further studies are in progress to identify the mechanisms responsible for the toxicity induced by these D-All derivatives, which may be lead compound of a novel class of anthelmintics having different mechanisms of action from those of conventional drugs.

The author next carried out the prodrug modification of A6P to synthesis membrane-permeable A6P derivative. A6P derivative **11** was designed its hydroxyl group and phosphate group are protected by acetyl group and cycloSal group, respectively, and these protecting groups are hydrolyzed in the cells by enzymatically and non-enzymatically steps. Compound **11** showed 250-fold higher anti-proliferative activity against MOLT-4F cell lines than that of D-All. Although it was reported that D-All induced cell cycle arrest but not cell death in the MOLT-4F cells, **11** showed cytotoxicity and completely killed the cells at 30 μM. The expression of the TXNIP protein induced by **11** was unexpectedly low compared with that induced by D-All, which shows the involvement of a TXNIP-independent pathway in the activity of **11**. On the other hand, G6P derivative **12** and **13** showed weak and no activity, respectively. Thus, these results suggest that protecting of phosphate group was necessary for the membrane-permeability and the cytotoxicity was caused by derivatization. This study shows that the biological activity of D-All can be enhanced or altered by derivatization of its phosphate. Similar derivatization of phosphates of other rare monosaccharides could be a promising strategy for enhancing their biological activities.

Finally, to investigate rare sugars that showed anti-proliferative activity, the author evaluated the activity of rare aldohexoses. Among tested rare aldohexoses, D-All and D-Ido showed at 5 mM inhibited cell proliferation by 60% and 46%, respectively. To clarify the contribution of the D-Sor which isomerized product of D-Ido and the importance of the hydroxy group at C-6 of D-Ido, the activity of D-Sor, 6-deoxy-D-Ido and L-Xyl were evaluated. D-Sor, 6-deoxy-D-Ido, and L-Xyl did not showed significant activity against MOLT-4F cells. These results suggest that the aldose structure and C-6 hydroxy group of D-Ido might be important for the activity. It was reported that the mechanism of action of D-All differs depending on the cell type. Thus, evaluation of the anti-proliferative activity of these rare aldohexoses against other types of cancer cell lines might be worthwhile.

This study revealed that a derivatization of rare sugar can be promising strategy to improve the biological activity and demonstrated the possibility of anti-cancer activity of rare sugars which had not been well investigated could be used as anti-cancer agent. This finding also indicates that drugs design based on monosaccharides may be possible in the development of anticancer drugs with novel mechanisms of action.