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## Anti-inflammatory marine cyclic peptide stylissatin A and its derivatives inhibit differentiation of murine preadipocytes †

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**Stylissatin A, an anti-inflammatory cyclic heptapeptide, and its derivatives potently inhibited the differentiation of preadipocytes and reduced triglyceride accumulation in mature adipocytes, with little cytotoxicity. Our studies might contribute to the development of leads for new anti-inflammatory and anti-obesity agents.**

Naturally occurring cyclic peptides have broad biological profiles as well as structural and conformational diversity, and have been widely used as drug leads and pharmacological tools.<sup>1</sup> These compounds have recently inspired medicinal chemists to design therapeutic agents due to their favorable properties, such as high bioavailability, binding affinity, target selectivity, proteolytic stability, and low toxicity.<sup>2</sup> Notably, these compounds include proline-rich cyclic peptides of marine origin.<sup>3</sup> In our continuing search for bioactive compounds from marine invertebrates, a proline-rich cyclic heptapeptide, stylissatin A (SA, **1**), was isolated from the Papua New Guinean marine sponge *Stylissa massa*.<sup>4</sup> Through the use of 1D and 2D NMR spectroscopic analysis, Marfey's method, and MS/MS analysis, its structure was determined to be cyclo-[L-Tyr<sup>1</sup>-L-Phe<sup>2</sup>-L-Pro<sup>3</sup>-L-Ile<sup>4</sup>-L-Pro<sup>5</sup>-L-Phe<sup>6</sup>-L-Ile<sup>7</sup>] (Fig. 1).<sup>5</sup> The structure of **1** including its absolute stereochemistry was established by comparison of its spectroscopic data with those of synthetic peptides prepared in the solid- and solution-phases.<sup>6</sup> The natural and synthetic **1** showed comparable anti-inflammatory activities [facilitated the reduction of nitrogen oxide (NO) production in lipopolysaccharide (LPS)-stimulated murine

RAW264.7 macrophage cells] ( $EC_{50}$  = 87 and 73  $\mu$ M). Recently, Shaheen *et al.* also synthesized **1** and determined its proline conformations as *cis*-Pro<sup>3</sup> and *trans*-Pro<sup>5</sup>.<sup>7</sup> Several variants of **1** replaced with L-Ala and L-Glu residues were also prepared, which inhibited inflammatory cytokine IL-2 production in RAW264.7 cells.

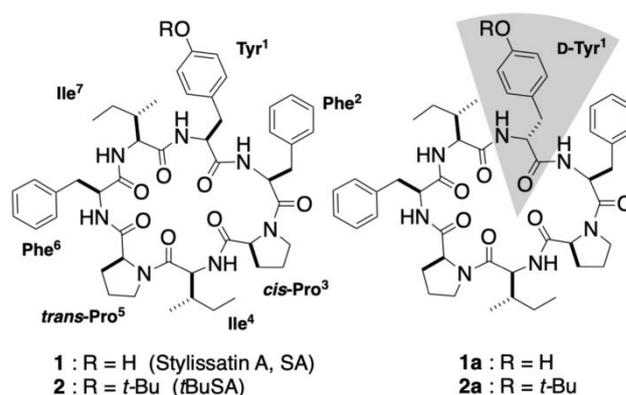


Figure 1 Structures of stylissatin A (SA) and its derivatives.

Meanwhile, we recently found that the activities of a *tert*-butyl ether analog of stylissatin A (*t*BuSA, **2**) and its *D*-*allo*-Ile<sup>4</sup> epimer **2d**, an unexpected side-product in solution-phase synthesis, were approximately six times more potent than SA (**1**) ( $EC_{50}$  = 12 and 13  $\mu$ M), while they did not affect cell viability, at least up to 200  $\mu$ M (Table 1).<sup>6</sup> Several antioxidants having phenol groups, such as coumaric acids (phenolic acids) and rutin (flavonoids), show anti-inflammatory activities.<sup>8</sup> Thus, the finding that protection of the tyrosine hydroxy group of **1** facilitated anti-inflammatory activity was surprising, and motivated us to further investigate the mode of action of SA. Here we report structure-activity relationship (SAR) studies of **1**, which led to the discovery of new anti-inflammatory SA derivatives that also potently inhibit differentiation in murine

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3T3-L1 preadipocytes and reduce fat accumulation in mature adipocytes.

**Table 1** Inhibition of NO production by SA derivatives after 24 hours in murine macrophage RAW264.7 cells.

compound	R	D-amino acids	EC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)	selectivity index
SA ( <b>1</b> )	H	–	73	>200	>2.7 <sup>a</sup>
<b>1a</b>	H	Tyr <sup>1</sup>	60	85	1.4
<b>1b</b>	H	Phe <sup>2</sup>	>200	170	NA
<b>1c</b>	H	Pro <sup>3</sup>	>200	>200	NA
<b>1d</b>	H	<i>allo</i> -Ile <sup>4</sup>	140	>200	>1.4 <sup>a</sup>
<b>1e</b>	H	Pro <sup>5</sup>	>200	>200	NA
<b>1f</b>	H	Phe <sup>6</sup>	>200	>200	NA
<b>1g</b>	H	<i>allo</i> -Ile <sup>7</sup>	>200	>200	NA
<b>1h</b>	H	Pro <sup>3,5</sup>	>200	>200	NA
<b>2</b>	<i>t</i> Bu	–	12	>200	>16 <sup>a</sup>
<b>2a</b>	<i>t</i> Bu	Tyr <sup>1</sup>	12	>200	>16
<b>2b</b>	<i>t</i> Bu	Phe <sup>2</sup>	11	11	1.0
<b>2c</b>	<i>t</i> Bu	Pro <sup>3</sup>	15	16	1.1
<b>2d</b>	<i>t</i> Bu	<i>allo</i> -Ile <sup>4</sup>	13	>200	>15 <sup>a</sup>
<b>2e</b>	<i>t</i> Bu	Pro <sup>5</sup>	3	15	5.0
<b>2f</b>	<i>t</i> Bu	Phe <sup>6</sup>	19	60	3.2
<b>2g</b>	<i>t</i> Bu	<i>allo</i> -Ile <sup>7</sup>	20	180	9.0
<b>2h</b>	<i>t</i> Bu	Pro <sup>3,5</sup>	17	>200	>11
<b>3</b>	propargyl	–	16	30	1.9
<b>4</b>	Me	–	17	30	1.8
<b>5</b>	Bn	–	14	18	1.2
<b>6</b>	Ac	–	190	>200	>1.1
<b>7</b>	Glc(OBn)	–	>200	>200	NA
<b>8</b>	Glc	–	>200	>200	NA
indomethacin	–	–	50	>200	>4.0
hydrocortisone	–	–	>200	>200	NA

Average of two reproducible runs are shown. Glc = D-glucosyl.  
<sup>a</sup> Data taken from Ref. 6.

First, the tyrosine hydroxy group of SA was modified, based on the high potency of *t*BuSA (**2**) (Scheme S1). Alkylation of **1** using propargyl bromide, methyl iodide, or benzyl bromide gave propargyl, methyl, and benzyl ethers **3–5**, respectively. Similarly, acetate **6** and tetra-*O*-benzyl β-glucoside **7** were prepared from **1** by using acetic anhydride or Mukaiyama's formimidate reagent.<sup>9</sup> Hydrogenolysis of the benzyl groups from **7** provided glucoside **8**. Since SA (**1**) contains only L-amino acids, the introduction of D-amino acid(s) was expected to induce large differences in both conformation and activity compared to the original compound, e.g. D-*allo*-Ile<sup>4</sup>-*t*BuSA (**2d**). Thus, linear heptapeptides containing one or two D-amino acids, including D-Tyr<sup>1</sup>, D-Phe<sup>2</sup>, D-Pro<sup>3</sup>, D-*allo*-Ile<sup>4</sup>, D-Pro<sup>5</sup>, D-Phe<sup>6</sup>, D-*allo*-Ile<sup>7</sup>, and D-Pro<sup>3,5</sup> analogs, were synthesized on solid-phase resin (Scheme S2). Subsequent macrolactamization afforded *t*BuSA D-amino acid analogs **2a–2h**. Finally, cleavage of the *tert*-butyl group readily provided SA D-amino acid analogs **1a–1h**.

With the 23 synthetic SA derivatives in hand, we investigated their inhibitory effects on NO production in LPS-stimulated murine RAW264.7 macrophages along with their cytotoxicity (Table 1). The amount of NO liberated in the culture medium was measured using the Griess method,<sup>10</sup> and

cytotoxicity was calculated by the MTT assay,<sup>11</sup> and each is expressed as EC<sub>50</sub> and IC<sub>50</sub> values, respectively. For the Tyr<sup>1</sup>-modified analogs, alkoxy derivatives **3–5** had EC<sub>50</sub> values similar to that of **2**, but their selectivity indexes (IC<sub>50</sub>/EC<sub>50</sub>) were low. Acetate **6** and two glucosides **7** and **8** were not effective. Thus, protection of the tyrosine residue by *tert*-butyl ether gave the best results. Among the eight SA analogs **1a–1h**, only D-Tyr<sup>1</sup>-SA (**1a**) showed a higher anti-inflammatory effect (EC<sub>50</sub> = 60 μM) than **1**, with moderate cytotoxicity. Meanwhile, for the *t*BuSA analogs **2a–2h**, D-Tyr<sup>1</sup>-, D-*allo*-Ile<sup>7</sup>-, and D-Pro<sup>3,5</sup>-*t*BuSA (**2a**, **2g**, **2h**) potently inhibited NO production (EC<sub>50</sub> = 12, 20, 17 μM) with low cytotoxicity, comparable to **2** and **2d**. The anti-inflammatory activities of these five SA analogs were all stronger than that of indomethacin (EC<sub>50</sub> = 50 μM). Four other *t*BuSA analogs, D-Phe<sup>2</sup>-, D-Pro<sup>3</sup>-, D-Pro<sup>5</sup>-, and D-Phe<sup>6</sup>-*t*BuSA (**2b**, **2c**, **2e**, **2f**), had EC<sub>50</sub> values similar to **2**, but were all cytotoxic. Therefore, among the tested compounds, **2** and **2a** showed the most desirable anti-inflammatory activities.

Chronic inflammation in adipose tissue is closely associated with metabolic disorders such as obesity, insulin resistance, and type 2 diabetes.<sup>12</sup> Obesity can often trigger serious lifestyle-related diseases such as cancer, cardiovascular disease, hypertension, hyperlipemia, and diabetes. Activation of inflammatory signaling pathways, such as TLR4-mediated activation of Mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB), is considered to be an important link between such disorders.<sup>13</sup> Thus, we examined the inhibitory effects of SA and its derivatives with anti-inflammatory activity on adipocyte differentiation.

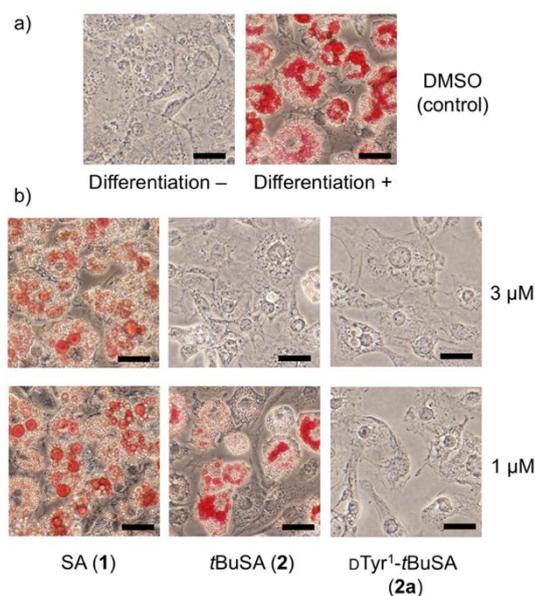
Murine 3T3-L1 fibroblasts differentiate to mature adipocytes under treatment with insulin-like growth factor I, glucocorticoid, fatty acids, and an agent that increases the intracellular cAMP level.<sup>14</sup> Adipocytes are the primary site for energy storage and accumulate triglycerides under conditions of excess nutrition. Thus, the 3T3-L1 system has been widely used to investigate the basic cellular mechanisms underlying obesity-related disorders. Both SA (**1**) and *t*BuSA (**2**) inhibited the differentiation of murine 3T3-L1 preadipocytes (EC<sub>50</sub> = 9.1 and 1.9 μM) (Table 2, Fig. 2). Seven *t*BuSA D-amino acid analogs, except for **2c**, were also effective at 10 μM, and notably, D-Tyr<sup>1</sup>-*t*BuSA (**2a**) had the most potent inhibitory effect (EC<sub>50</sub> = 440 nM) (Figs. S1, S2). The adipocyte differentiation-inhibitory activities of these SA derivatives were more potent than that of hydrocortisone (EC<sub>50</sub> = 155 μM). As in the Griess assay, protection of the tyrosine hydroxy group as a *tert*-butyl ether in *t*BuSA (**2**) and its D-Tyr<sup>1</sup> analog **2a** resulted in a 3.3- to 4.3-fold increase in the inhibition of triglyceride accumulation with respect to **1** and its D-Tyr<sup>1</sup> analog **1a**, respectively. The cytotoxicity pattern for 3T3-L1 preadipocytes resembled that for RAW264.7 macrophages: compounds **1**, **1a**, **2a**, **2d**, and **2h** were not toxic at 200 μM, while *t*BuSA (**2**) and compounds **2b**, **2c**, and **2e** were moderately toxic (IC<sub>50</sub> = 15–110 μM). Thus, D-Tyr<sup>1</sup>-*t*BuSA (**2a**) had the largest selectivity index value (>450) toward preadipocytes. Furthermore, SA (**1**), *t*BuSA (**2**), and its D-Tyr<sup>1</sup> isomer **2a** potently reduced triglyceride accumulation in mature adipocytes following 4 days of treatment (EC<sub>50</sub> = 6.1, 1.9, 7.0 μM, respectively) (Fig. S3). While the roles of SA and its

derivatives in the inhibition of adipocyte differentiation and fat consumption in mature adipocytes are still unclear, specific inflammatory signalling pathways might be suppressed to inhibit adipogenesis and/or activate lipogenesis.

**Table 2** Inhibition of differentiation of murine preadipocytes (3T3-L1) by SA derivatives.

compound	EC <sub>50</sub> (μM)	IC <sub>50</sub> (μM)	selective index
SA (1)	9.1	>200	>22
1a	2.8	>200	>71
2	1.9	21	11
2a	0.44	>200	>450
2b	4.7	15	3.2
2c	27	110	4.1
2d	6.8	>200	>29
2e	4.2	98	23
2f	4.3	190	44
2g	5.7	170	29
2h	5.0	>200	>40
indometacin	>200	>200	NA
hydrocortisone	155	>200	>1.3

Average of two reproducible runs are shown. EC<sub>50</sub> and IC<sub>50</sub> values were evaluated by the treatment of compounds for 7 and 3 days, respectively.



**Figure 2** Inhibition of adipocyte differentiation on murine fibroblast 3T3-L1 cells by SA analogs. Scale bar = 32 μm. Accumulated triglycerides were visualized with Oil red O stain. a) Undifferentiated and differentiated cells. b) Treatment of differentiating preadipocytes with compounds **1**, **2**, and **2a** at 3 μM (top) and 1 μM (bottom) for 7 days.

As the related cyclic peptide studies, the labs of Uemura and Taunton both studied the highly *N*-methylated cyclic heptapeptide (–)-ternatin. This compound was isolated from the mushroom *Coriolus versicolor* as a potent inhibitor of fat-accumulation in 3T3-L1 adipocytes (EC<sub>50</sub> = 61 nM),<sup>15</sup> and also inhibits HCT116 cancer cell proliferation (IC<sub>50</sub> = 71 nM).<sup>16</sup>

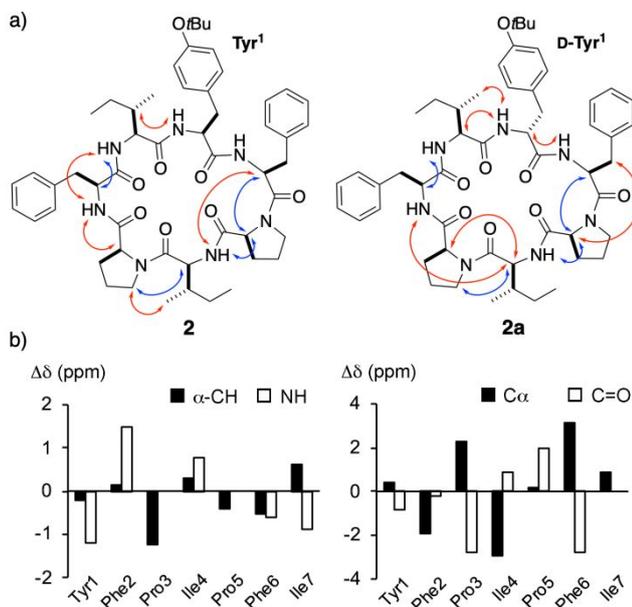
Despite its low selectivity index value (1.6), (–)-ternatin significantly suppressed the increase in body weight and fat accumulation in mice fed a high-fat diet at 5 mg kg<sup>-1</sup> day<sup>-1</sup>. Interestingly, replacement of the *D*-(*N*Me)Ala<sup>5</sup>–*L*-(*N*Me)Ala<sup>6</sup> moiety in (–)-ternatin by the *L*–*D* stereoisomer resulted in a complete loss of activity. Thus, we evaluated the conformations of **2** and **2a** to explain their differences in biological activity.

NMR studies revealed that both stylissatin A (**1**) and tBuSA (**2**) had almost single conformers.<sup>4,6</sup> In contrast, *D*-Tyr<sup>1</sup>-tBuSA (**2a**) was observed as ca. 1:1 and 3:1 mixtures of two conformers in CD<sub>3</sub>OD and DMSO-*d*<sub>6</sub>, respectively. Based on the 1D and 2D NMR analyses, the protons including five NH protons and the carbons of **2** and **2a** (major conformer) were fully assigned (Table S1). Based on a comparison of the chemical shift differences Δ<sub>βγ</sub> (δ C<sub>β</sub> – δ C<sub>γ</sub>) of two Pro<sup>3,5</sup> residues with the literature,<sup>17</sup> we established that both compounds **2** and **2a** had *cis*-Pro<sup>3</sup> and *trans*-Pro<sup>5</sup> conformations in DMSO-*d*<sub>6</sub>, as with those of SA (**1**) (Table S2).<sup>7</sup>

To establish whether the *D*-amino acid replacement affects the macrocycle conformations of **2** and **2a**, we compared their NMR spectra. The proton and carbon chemical shifts of the main chain between **2** and **2a** were highly different for the overall amino acid residues, especially Pro<sup>3</sup>-H<sub>α</sub>, Tyr<sup>1</sup>-NH, and Phe<sup>2</sup>-NH (|Δδ<sub>H</sub>| > 1 ppm), and Pro<sup>3</sup>-C<sub>α</sub>, Ile<sup>4</sup>-C<sub>α</sub>, Phe<sup>6</sup>-C<sub>α</sub>, Pro<sup>3</sup>-C=O, and Phe<sup>6</sup>-C=O (|Δδ<sub>C</sub>| > 2 ppm) (Fig. 3). Especially, the NH protons of Phe<sup>2</sup> and Ile<sup>4</sup> residues were down-field shifted in **2**, whereas those of Tyr<sup>1</sup> and Ile<sup>7</sup> were up-field shifted. In the NOESY spectra, nine and ten key correlations between the different amino acid residues were observed for **2** and **2a**, respectively. Among them, only four NOEs (Phe<sup>2</sup>-H<sub>α</sub>/Pro<sup>3</sup>-H<sub>α</sub>, Pro<sup>3</sup>-H<sub>α</sub>/Ile<sup>4</sup>-NH, Ile<sup>4</sup>-H<sub>α</sub>/Pro<sup>5</sup>-H<sub>δ</sub>, and Phe<sup>6</sup>-H<sub>α</sub>/Ile<sup>7</sup>-NH) were common, and the others were different: Tyr<sup>1</sup>-NH/Ile<sup>7</sup>-H<sub>β</sub>, Phe<sup>2</sup>-H<sub>α</sub>/Ile<sup>4</sup>-NH, Ile<sup>4</sup>-H<sub>γ</sub>/Pro<sup>5</sup>-H<sub>δ</sub>, Pro<sup>5</sup>-H<sub>α</sub>/Phe<sup>6</sup>-NH, and Phe<sup>6</sup>-NH/Ile<sup>7</sup>-NH for **2**, and Tyr<sup>1</sup>-NH/Ile<sup>7</sup>-H<sub>α</sub>, Tyr<sup>1</sup>-NH/Ile<sup>7</sup>-H<sub>γ</sub>, Tyr<sup>1</sup>-H<sub>α</sub>/Phe<sup>2</sup>-NH, Phe<sup>2</sup>-H<sub>β</sub>/Pro<sup>3</sup>-H<sub>α</sub>, Ile<sup>4</sup>-H<sub>α</sub>/Pro<sup>5</sup>-H<sub>α</sub>, and Ile<sup>4</sup>-H<sub>α</sub>/Phe<sup>6</sup>-NH for **2a**. These results suggested that the macrocycle conformations and hydrogen bond patterns of **2** and **2a** were different with each other, which could enhance the activities of *D*-isomers against macrophages and preadipocytes.<sup>18</sup> It is reasonable to consider that the primary target biomacromolecules of **2** and **2a** are common, since their inhibitory effects against two cell lines are similar. Meanwhile, the macrocycle conformations of two cyclic peptides bound to target molecules would be different, which might induce or inhibit the interactions with another target molecule(s) in a different way, like protein–protein interaction modulators. Such difference in the interactions with multiple cellular targets could explain why **2a** has better inhibitory activity than **2**.

In summary, we showed that an anti-inflammatory marine cyclic peptide, stylissatin A (SA), and its derivatives potently inhibited the differentiation of preadipocytes. To the best of our knowledge, there are no short or cyclic peptides that show both anti-inflammatory and adipocyte differentiation-inhibitory activities. Due to the similar activity profiles against 3T3-L1 and RAW264.7 cells, the target molecules and cellular signalling mechanism of SA derivatives for preadipocytes might be similar to those for macrophages. While the detailed mechanisms of

the inhibitory effects of SA and its derivatives are still unknown, our SAR studies might contribute to the development of leads for new anti-inflammatory and anti-obesity agents. Further studies on stylissatin A, including identification of its target on adipocytes and macrophages using functionalized SA derivatives and its effects on cellular signaling, and *in vivo* experiments using mouse models of inflammation and obesity, are in progress.



**Figure 3** Spectral comparison of tBuSA (**2**) and D-Tyr<sup>1</sup>-tBuSA (**2a**) in DMSO-*d*<sub>6</sub>. a) Key NOE correlations between the different amino acid residues of **2** and **2a**. Common and different NOEs for two compounds are shown in blue and red, respectively. b) Difference between the chemical shifts in  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra. Chemical shifts of  $\alpha\text{-CH}$ , NH,  $\text{C}\alpha$ , and  $\text{C=O}$  atoms in **2** minus those in **2a** (major conformer) are shown.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

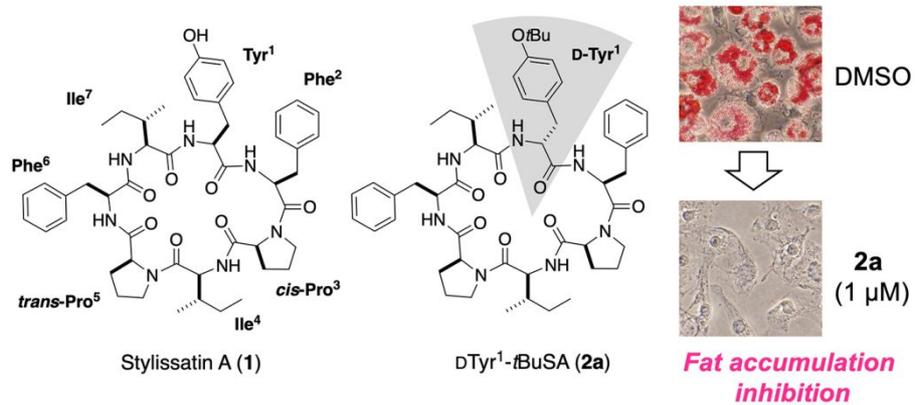
We thank Mr. Kazushi Koga for his valuable suggestions regarding cryoprobe NMR measurement, Ms. Mikiko Hirukawa for the bioassay of control samples, and Drs. Baro Gise and Tito Akindele for their preliminary SAR studies. This work is supported in part by JSPS grants (18H04613 and 19H02839 to M.K. and 26242073 to H.K.) and JSPS A3 Foresight Program. Supports were also provided by PRESTO, JST (JPMJPR1535), and the Naito Foundation.

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- We examined molecular modeling studies of **2** and **2a** based on the restrictions of key NOEs and/or the dihedral angles of *cis*-Pro<sup>3</sup> and *trans*-Pro<sup>5</sup>. However, coupling constants of NH/ $\alpha$ -CH on the most stable models were far different from the observed ones, and thus we were unable to provide their satisfied calculated conformers.

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Stylissatin A, an anti-inflammatory cyclic heptapeptide, and its derivatives inhibited the differentiation of preadipocytes and the triglyceride accumulation in adipocytes.