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Synthesis and optimization of hyaluronic acid–methotrexate conjugates to maximize benefit in the treatment of osteoarthritis

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ABSTRACT

We previously reported that a conjugate of hyaluronic acid (HA) and methotrexate (MTX) could be a prototype for future osteoarthritis drugs having the efficacy of the two clinically validated agents but with a reduced risk of the systemic side effects of MTX by using HA as the drug delivery carrier. To identify a clinical candidate, we attempted optimization of a lead, conjugate **1**. Initially, in fragmentation experiments with cathepsins, we optimized the peptide part of HA–MTX conjugates to be simpler and more susceptible to enzymatic cleavage. Then we optimized the peptide, the linker, the molecular weight, and the binding ratio of the MTX of the conjugates to inhibit proliferation of human fibroblast-like synoviocytes in vitro and knee swelling in rat antigen-induced monoarthritis in vivo. Consequently, we found conjugate **30** (**DK226**) to be a candidate drug for the treatment of osteoarthritis.

1. Introduction

To provide a novel drug for the treatment of osteoarthritis (OA) which has both the pain-eliminating effect of hyaluronic acid (HA) and the anti-inflammatory effect of methotrexate[1] (MTX), we proposed a drug delivery system (DDS) that localizes MTX at the synovial membrane and uses HA as the carrier. Additionally, we thought the strategy would reduce the risks of the systemic side effects of MTX.[2,3]

In our previous work,[4] we found conjugate **1** (Fig. 1) which showed anti-proliferative effects on human synovial cells stimulated by TNF-α. Moreover, it inhibited knee swelling in an antigen-induced monoarthritis rat model. Conjugate **1** consisted of the following four parts:

1. MTX bonded through α- or γ-carboxylic acid, cleavable by enzymes.
2. A peptide chain recognized and cleaved by intracellular enzymes.
3. Linkers with polyethylene glycol (PEG) to play the role of avoiding the steric hindrance of HA against the approach of enzymes.
4. HA modified through its carboxylic acid because of the diversity of the chemical reaction.

The efficacy of conjugate **1** indicated the importance of the conjugation of HA and MTX because the mixture of HA and MTX did not show efficacy.[4] Although conjugate **1** was promising, it was apparent that further design and modifications were required. The following modifications were considered necessary:

1. For simple and easy scale-up for industrialization, the number of amino acids had to be decreased.
2. To assure better pain-relieving effect, the average molecular weight (Mw) of the HA–MTX conjugates had to be maintained at the same level as native HA.[5–12]
3. To exert comparable efficacy of oral MTX, the binding ratio of MTX had to be sufficiently high.

Considering these requirements, we optimized conjugate **1** to a clinical candidate HA–MTX conjugates in the following steps. First, we optimized the peptide part of conjugate **1** to be simpler and more susceptible to enzymatic cleavage through enzyme recognition experiments in vitro and in arthritic models in vivo. Second, we determined the range of the Mw of HA and the MTX binding ratio from the viewpoint of efficacy and quality control of the conjugates. We also selected a linker for the conjugates that would exert

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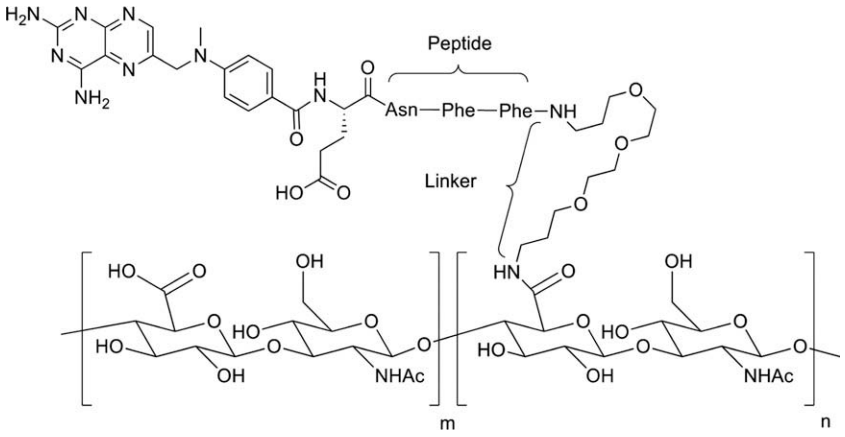


Figure 1. Structure of conjugate **1**. Peptide: AsnPhePhe. Linker: PEG13. MTX binding ratio which means $n/(m+n)$: 1.2%. α-MTX connected (with γ-). HA molecular weight: 1780 kDa.

the best efficacy. Through the optimization process, we identified conjugate **30** as a promising candidate conjugate for a future OA drug.

2. Results and discussion

2.1. Cathepsin fragmentations

First, we examined cathepsin fragmentations of the peptide to determine which pattern connected and which peptide was well recognized by cathepsins. We considered the simplest amino acid sequence recognized by enzymes, especially cathepsins B, D or L because they are typical peptidases found in the synovial tissues.[13–15] We used compounds **2**–**9**, which were models of the HA–MTX conjugates, for a simple analysis of the structures (Fig. 2). Their amino acid sequences were what are known as recognition sites.[16–18] All analyses were performed by LC–MS spectroscope.

Compounds **2**–**9** were synthesized using the solid-phase synthesis method[19,20] (Scheme 1). The α- and γ-connected MTX were provided by applying the method to γ-protected or α-protected glutamic acid. The compounds were treated for 24 h at 37 °C with cathepsins B, D or L. After incubation, media were analyzed to detect the MTX released from the compound. MTX derivatives which bind to one amino acid are also known to have anti-inflammatory effects but less than the MTX itself.[2,3,21–23] Fragment production was determined by the calculated percentage of the UV area (Table 1).

The three peptides with α-connected compounds, GlyPheLeuGly, AsnPhePhe and PhePhe (compounds **2**, **4** and **6**), were well recognized by cathepsins and released MTX or MTX and one or two amino acids. On the other hand, the γ-connected compounds (**3**, **5** and **7**) were tolerant to the enzymes. Some compounds became 'unknowns', with mass spectra showing protonated MTX. We also observed that cathepsin D tended to break between Phe and Phe and cathepsins B and L broke at end of the PhePhe fragment, suggesting that the HA–MTX conjugate must have a PhePhe sequence and be α-connected to MTX.

2.2. Peptide selection

To select the peptides, we introduced amino acids to the conjugates. The synthetic route of conjugate **17**, one of the conjugates used in the evaluation of efficacy, is summarized in Schemes 2 and 3. Compound **16** was the key precursor and the final compound confirmed to be a small molecule. The other precursors were synthesized using the same method as for compound **16** and were used in the conjugation. The MTX binding ratio was calculated by dividing the concentration of the MTX by the concentration of the disaccharide unit (GlcA-GlcNAc). These concentrations were obtained by gel permeation chromatography (GPC) analysis of the conjugates. GPC analysis determined both the binding ratio and the Mw of the conjugates at the same time. As shown in Table 2, conjugates **17**–**24** had 1.3–1.9% MTX binding, PEG13 linkers and Mws of 1380–2190 kDa. All conjugates were single isomers, with the exception of conjugate **1** (AsnPhePhe) and conjugate **20** (Glu). The effects of the HA–MTX conjugates on cell proliferation induced by TNF-α stimulation were examined using human fibroblast-like synoviocytes (HFLS), as shown in Figure 3. Conjugates **1**, **17**, **19** and **23**, which inhibited cell proliferation more effectively than the others, were evaluated in the rat antigen-induced arthritis model.[24–26] The percentages of inhibition for the conjugates com-

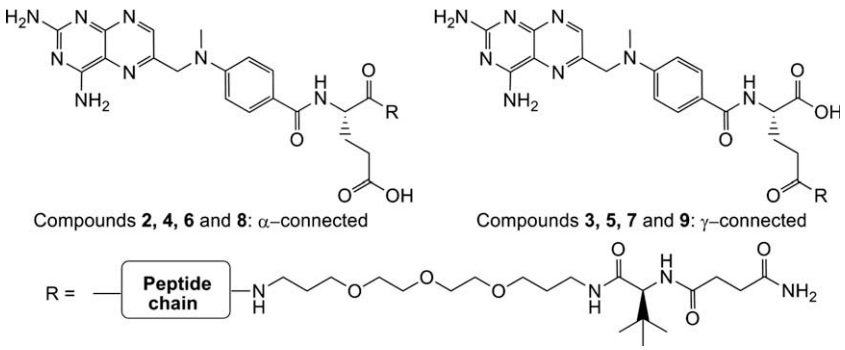
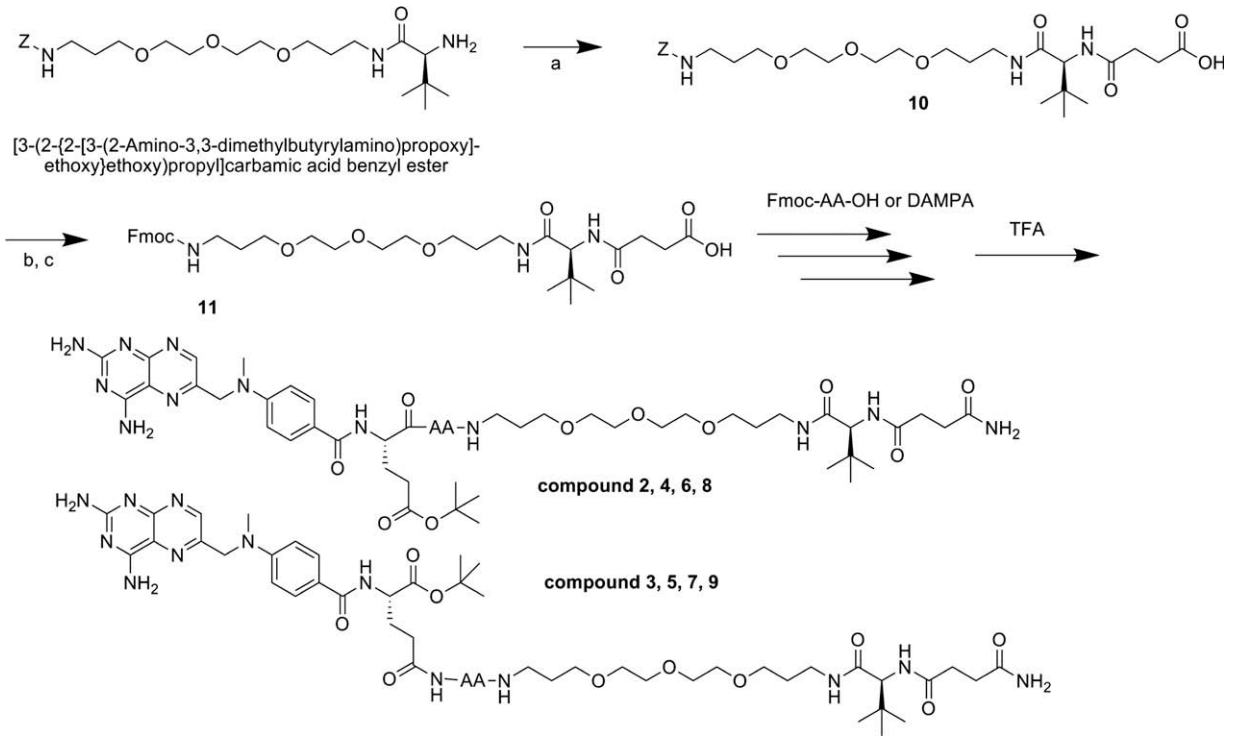


Figure 2. Structure of model compounds **2**–**9**. Peptide chain = -GlyPheLeuGly- (**2**, **3**), -AsnPhePhe- (**4**, **5**), -PhePhe- (**6**, **7**), -Phe- (**8**, **9**).



Scheme 1. Reagents and conditions: (a) Succinic anhydride (1.3 equiv), Et_3N (1.3 equiv), THF, 0 °C to rt, 17 h; (b) H_2, 10% Pd–C (0.08 w/w), MeOH, rt, 3 days; (c) Fmoc-OSu (1.3 equiv), $NaHCO_3$ (2.4 equiv), 1,4-dioxane–H_2O, 0 °C to rt, 9 h, followed by solid-phase synthesis. Fmoc deprotection: Pip/DMF, two times, coupling: DAMPA, Fmoc-AA-OH or Fmoc-AA-OPfp was treated with DIC–HOBt or Pfp ester-NMM or HATU–HOBt protocol, cleavage: 20% TFA/CH_2Cl_2.

pared with the HA-treated groups are shown in Figure 4. The efficacy of conjugates **1** and **17** was almost equal to that of oral MTX treatment, indicated by the bold line. In vitro data for conjugate **17** (PhePhe), conjugate **19** (Phe) and conjugate **23** (Ile) showed strong efficacy. In contrast to conjugates **19** and **23**, conjugate **17** also showed efficacy in vivo. We think the criteria for conjugates for OA drug development includes in vivo efficacy comparable to oral administrated MTX. Consistency with the cathepsin fragmentation experiments supported our decision to select 'PhePhe' for the next optimization.

2.3. Linker selection

To select the linkers, we synthesized conjugates **25–31** using the same method as for conjugate **17** (Schemes 2 and 3) with the peptide fixed to PhePhe (Table 2). During the initial phase, we thought a lengthy PEG13 was necessary to avoid the hindrance of enzyme recognition from the HA backbone; however, shorter linkers played this role well. PEG8, PEG5 and Alkyl2 (conjugates **27**, **28** and **30**) showed anti-proliferative effects on the synovial cells similar to PEG13 (conjugate **17**, Fig. 5). The in vitro results suggested that the efficacy seemed mainly due to the binding ratio of MTX, with the exception of conjugate **31**, which had a Lys branch next to the peptide. On the other hand, the in vivo results suggested that the efficacy of the conjugates was not influenced by the length of the linkers but by the direction or hydrophobicity provided by the linkers. We calculated the log *P* with ISIS Draw software (version 2.5 J) and the order was as follows: PEG8 (−2.167), PEG5 (−1.893), Alkyl2 (−1.618), PEG13 (−1.459), PEG10 (−1.185), Lys(OMe) (−1.169), PEG12 (−0.203), and Alkyl5 (−0.145). The order of the results for the three best conjugates in vitro was PEG8, PEG5, and Alkyl2, and thus high hydrophobicity provided good efficacy in vitro. The in vivo efficacy also supported by the in vitro results (Fig. 6). Consequently, we selected an Alkyl2 linker because it showed strong efficacy in both experiments.

2.4. Molecular weight of conjugates

To select conjugates with the appropriate Mw, we synthesized HA–MTX conjugates **17** (PhePhe, PEG13) and conjugate **30** (PhePhe, Alkyl2) which had Mws ranging between 330 and 2180 kDa (Fig. 7A and B). Control of the Mw was accomplished by the use of HA of various Mws as the starting material. The three classes (330, 800 and 1980 kDa) of Mw of conjugate **17** with 1.1−1.4% MTX inhibited HFLS proliferation (Table 3). However, the conjugates of Mw 330 kDa did not show efficacy in vivo (Fig. 7A). Similar to conjugate **17**, conjugate **30** (Mw 340 kDa) with 1.9% MTX showed insufficient inhibition of knee swelling (Fig. 7B). It is known that high Mw HA tends to stay longer than low Mw HA in the joint cavities and synovial tissues. We thought the low Mw was either insufficient for a conjugate to accumulate at the synovial cells or it metabolized rapidly. In addition, HA (Mw of ca. 2000 kDa) behaves the same as the conventional HA in the human body and so we determined 2000 kDa to be the target Mw.

2.5. Range of binding ratios

To evaluate the range of the binding ratio which would provide sufficient effect on the animal models, we compared in vitro efficacy of conjugates with different MTX binding ratios (Table 4). In the case of conjugate **30** with a 3.8% binding ratio, the in vitro efficacy was as strong as MTX (IC_{50} 0.14 μM). On the other hand, conjugate **30** with lower binding ratios (1.3% and 0.5%) showed weak or no effect (Table 4). In our in vitro assay system (TNF-α-induced proliferation of HFLS), free MTX showed anti-proliferative effect in a dose dependent manner but hyaluronic acid did not. Therefore, the efficacy of the HA–MTX conjugates in this assay system derives from that of MTX. Our preliminary experiments suggested that HA–MTX is incorporated into synovial cells via hyaluronic receptors on the cell surface and degraded by lysosomal enzymes to release MTX inside cells. This indicates that the amount of MTX

Table 1
Cathepsin fragmentation

Compound		Peptide	Cathepsin[a]	Compound remaining[b] (%)	Products (MTX-derivatives)[b] (%)	
					Released MTX	MTX-(amino acids)
2	α	GlyPheLeuGly	B	0	0	100 (Gly)
			D	82	0	18 (GlyPhe)
			L	0	0	100 (Gly)
3	γ	GlyPheLeuGly	B	>90	0	<10 (Gly)
			D	>90	0	<10 (GlyPhe)
			L	20	0	40 (GlyPheLeu), 20 (GlyPheLeuGly), 20 (Gly)
4	α	AsnPhePhe	B	0	0	100 (Asn)
			D	0	0	100 (AsnPhe)
			L	0	63	37 (Asn)
5	γ	AsnPhePhe	B	90	0	10 (Asn)
			D	25	0	70 (AsnPhe), 5 (Asn)
			L	80	0	20 (AsnPhePhe)
6	α	PhePhe	B	0	60	40 (Phe)
			D	0	13	87 (Phe)
			L	0	26	74 (Phe)
7	γ	PhePhe	B	60	0	40 (MTX+1H)
			D	10	0	90 (Phe)
			L	80	0	20 (PhePhe)
8	α	Phe	B	70	15	15 (MTX+1H)
			D	100	0	
			L	45	55	
9	γ	Phe	B	90	0	10 (MTX+1H)
			D	100	0	
			L	100	0	

[a] Enzyme reaction: 37 °C, 24 h.
[b] (Compound remaining, **2**–**9**) + (products) = 100% (calculated from the LC peak area).

released is likely to be proportional to that of the MTX released from a HA–MTX conjugate. We therefore speculate that the amount of MTX conjugated to compound **30** with a low binding ratio (ex. 0.5%) was too small to exert its efficacy in this assay system. Similar results were found with in vivo experiments (Fig. 8A and B). Both in vitro and in vivo effects were stronger in proportion to the increase in the binding ratio, suggesting that the MTX would almost certainly be released from the HA and would act as an anti-inflammation drug. However, the conjugates with higher MTX binding ratios (ex. 4.4%) were no more effective than the 3.8% conjugate, and the Mw of conjugates tended to decrease with increasing MTX binding ratio. So, we concluded that conjugates require at least 1.3%, hopefully around 3.8% of MTX binding ratio, for sufficient efficacy and maintaining desirable Mw. With a weekly dose of MTX, the 3.8% MTX conjugate was only 23.6 mg/week, a mere 1/79 the amount of the MTX of the conjugate that showed obviously stronger effects than oral MTX. The decrease in total dose of MTX in this case suggests a decrease in the risks of systemic side effects.

2.6. Synthesis for industrial development

After the peptide and linker were selected, we reconsidered the process for providing conjugate **30** industrially (Scheme 4). All intermediates were solid, purified without columns and provided yields over 85%. Conjugate **30** obtained from this route was used to evaluate the range of the MTX binding ratios shown above.

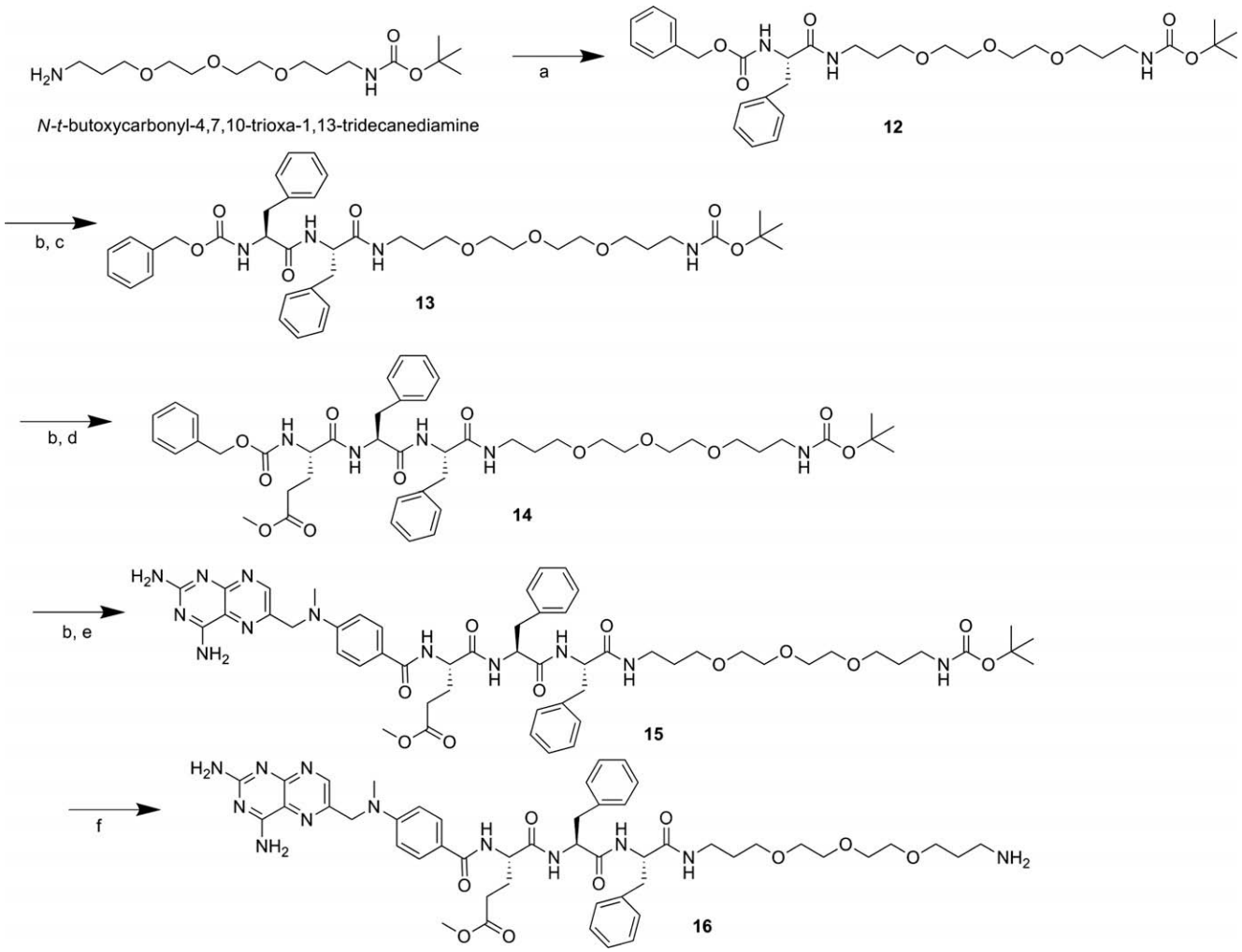
3. Conclusion

We obtained HA–MTX conjugates that showed excellent efficacy including inhibition of the HFLS proliferation induced by TNF-α stimulation and inhibition of knee swelling in rat antigen-induced arthritis. Cathepsin fragmentation experiments suggested that the efficacy of MTX resulted from modification of the α-carboxylic acid of MTX and when 2 or 3 amino acid peptides were well recognized. Conjugate **30** with a modified PhePhe peptide chain and ethylenediamine linker was the best drug candidate for the treatment of OA. The Mw of conjugate **30** was targeted to be 2000 kDa. The binding ratio of MTX was over 1.3% and, at around 3.8%, has sufficient efficacy compared with orally administered MTX. For industrial synthesis, we improved the synthetic route of conjugate **30**: the intermediates mostly crystallized and so there was no need for column purifications. The localization of MTX and reduction of the total amount of MTX administered should reduce the risks of the side effects of MTX. Conjugate **30** is expected to possess the pain-eliminating effect of HA preparations and the anti-inflammatory effect of a MTX derivative. Additionally, conjugate **30** will be a useful and safe drug administered by intra-articular injection and thus avoid the severe systemic risks of MTX. Conjugate **30** (**DK226**) is now under investigation as a drug candidate for the treatment of OA.

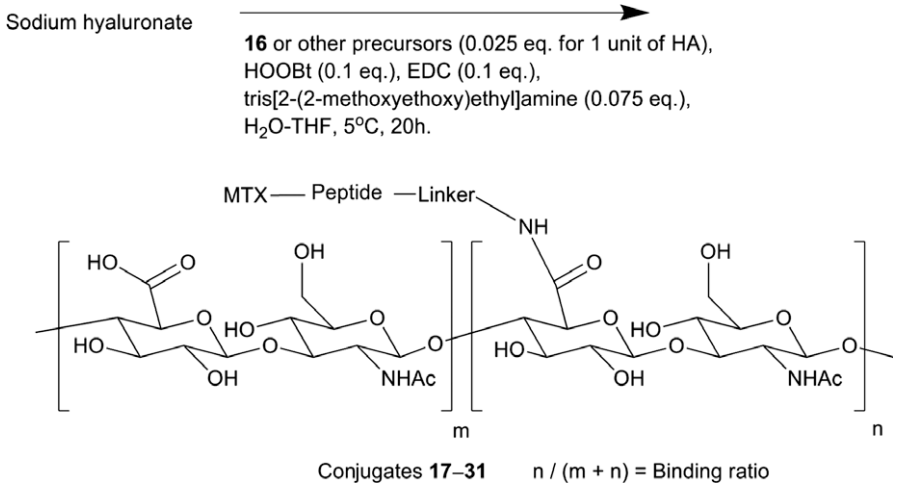
4. Experimental

4.1. Chemistry

All amino acid derivatives, 1-hydroxybenzotriazole monohydrate (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Watanabe Chemical Industries, Ltd. HA was supplied by Denki Kagaku Kogyo, K. K. All solvents were purchased from Kanto Chemical Co., Inc. 4-methylaminobenzoic acid was purchased from Tokyo Chemical Industry Co., Ltd. All other compounds were of the best available commercial grade. ^{1}H NMR spectra were recorded on a JEOL ECP 270 or 500 MHz or a Mercury 300 MHz instrument in $CDCl_3$, DMSO-d_6 or deuterium oxide solutions. Low-resolution mass spectra were determined on LC/PDA/MS (HP 1100/TSP UV 6000/LCQ



Scheme 2. Reagents and conditions: (a) Z-Phe-OH (1.3 equiv), HOBt (1.0 equiv), EDC (1.2 equiv), DMF, 0 °C to rt, 2 days; (b) H_2, 10% Pd–C, rt; (c) Z-Phe-OH (1.6 equiv), HOBt (1.5 equiv), EDC (1.6 equiv), DMF, 0 °C to rt, 2 days; (d) Z-Glu(OMe)-OH (1.1 equiv), HOBt (1.0 equiv), EDC (2.2 equiv), DMF, 0 °C to rt, 16 h; (e) DAMPA (1.5 equiv), HOBt (1.5 equiv), EDC (1.5 equiv), DMF, 0 °C to rt, 2 days; (f) TFA, 0 °C, 40 min.



Scheme 3. Conditions of conjugation.

Classic, LC–MS) system using Cadenza CD-C_{18} (3.0 mm I.D. × 20 mm) column at 35 °C. λ was 190–400 nm total area with retention times evaluated in minutes. The solvents ran as follows: (A) 0.05% TFA, H_2O, (B) 0.05% TFA, MeCN, Gradient (A/B): 95/5–0/100 (9.5 min), 0/100 (2.5 min), Flow rate: 1.0 mL/min. The binding ratio of MTX and the Mw of HA–MTX conjugates were determined by GPC on an LC/PDA/RI (Waters Alliance 2695/2996/2414) system using Shodex OHpak SB-806 HQ (8.0 mm I.D. × 300 mm) column

Table 2
Structure of the conjugates

Conjugate	Peptide	Linker	MTX (%)	Mw (kDa)
1	AsnPhePhe	PEG13	1.2	1780
17	PhePhe	PEG13	1.3–1.4	1380–2190
18	PheGly	PEG13	1.4	1860
19	Phe	PEG13	1.7	1790
20	Glu	PEG13	1.5	1830
21	Trp	PEG13	1.9	1390
22	Tyr	PEG13	1.7	1760
23	Ile	PEG13	1.7	1620
24	βAla	PEG13	1.7	1430
25	PhePhe	PEG12	1.7	1550
26	PhePhe	PEG10	1.6	1620
27	PhePhe	PEG8	2.0	1490
28	PhePhe	PEG5	1.9	1960
29	PhePhe	Alkyl5	1.2	1860
30	PhePhe	Alkyl2	1.9–2.1	1860–2180
31	PhePhe	Lys	1.4	1720

PEG13 = –HN–$(CH_2)_3$–O–$(CH_2)_2$–O–$(CH_2)_2$–O–$(CH_2)_3$–NH–.
PEG12 = –HN–$(CH_2)_3$–O–$(CH_2)_4$–O–$(CH_2)_3$–NH–.
PEG10 = –HN–$(CH_2)_3$–O–$(CH_2)_2$–O–$(CH_2)_3$–NH–.
PEG8 = –NH–$(CH_2)_2$–O–$(CH_2)_2$–O–$(CH_2)_2$–NH–.
PEG5 = –NH–$(CH_2)_2$–O–$(CH_2)_2$–NH–.
Alkyl5 = –NH–$(CH_2)_5$–NH–.
Alkyl2 = –NH–$(CH_2)_2$–NH–.

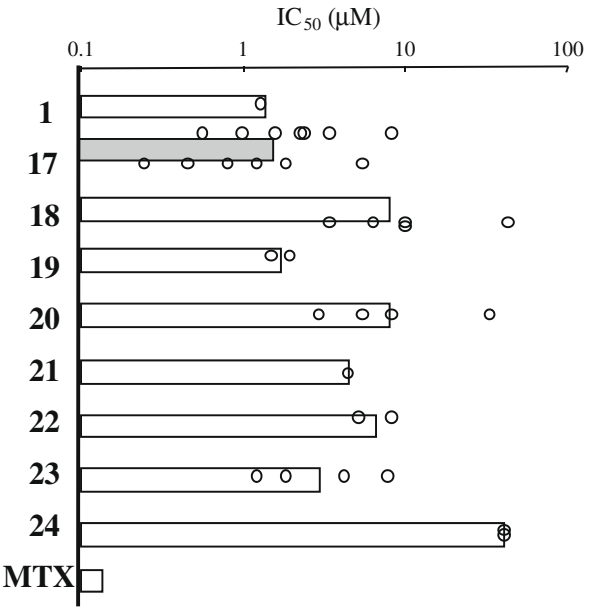


Figure 3. Peptide screening to evaluate the anti-proliferative effect on human fibroblast-like synoviocytes (HFLS).

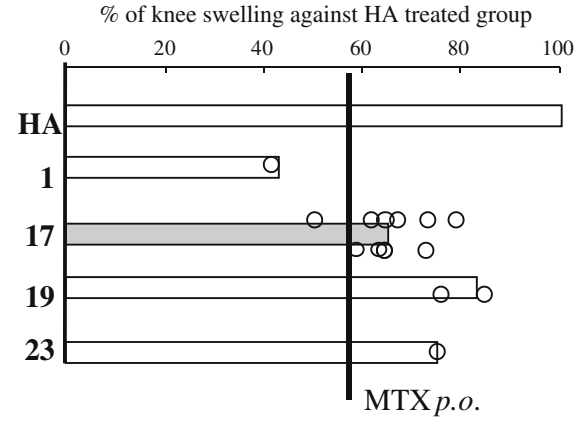


Figure 4. Peptide screening to evaluate the inhibition of knee swelling in antigen-induced arthritic rats. Conjugates **1**, **17**, **19** and **23**: 0.5 mg as HA, once a week, ia MTX: 0.1 mg/kg, five times a week, p.o. for two weeks. Knee swelling, the difference between right and left knee width, was measured on days 0–14. The bold line indicates the average for MTX treatment groups.

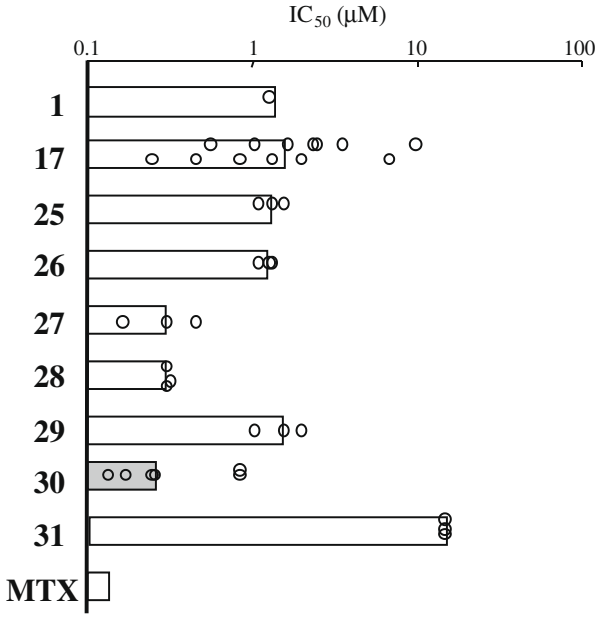


Figure 5. Linker screening, evaluated by anti-proliferative effect on human fibroblast-like synoviocytes (HFLS).

at 40 °C. RI was analyzed at 35 °C. λ was detected at 259 nm. The solvent ran as follows: 50 mM sodium phosphate (pH 6.0), Flow rate: 0.3 mL/min, injection volume: 100 μL.

4.1.1. MTX-α-AsnPhePhe-$NHC_{10}H_{20}O_3NH$-HA (1)

Synthesis was previously reported.[4]

4.1.2. Synthesis of compounds 2–9

4.1.2.1. *N*-[1-(3-{2-[2-(3-Benzyloxycarbonylamino-propoxy)ethoxy]ethoxy}propylcarbamoyl)-2,2-dimethylpropyl]succinamic acid (10). [3-(2-{2-[3-(2-Amino-3,3-dimethylbutyrylamino)-propoxy]ethoxy}ethoxy)propyl]carbamic acid benzyl ester (KNC Laboratories Co., Ltd, 5.0 g, 10.7 mmol) and succinic anhydride (1.28 g, 12.8 mmol) were dissolved in THF (50 mL) at 0 °C. After triethylamine (Et_3N, 1.78 mL, 12.8 mmol) was added, the reaction mixture was warmed to rt. After 17 h, ethyl acetate was added. The organic layer was washed with 1 N HCl solution and brine, dried over sodium sulfate and evaporated. The product was purified by silica gel column chromatography (eluent: dichloromethane/methanol, 20/1) to afford 6.10 g (quant.) of the desired compound **10**. ^1H NMR (270 MHz, $CDCl_3$) δ: 0.971 (s, 9H), 1.75–1.80 (m, 4H), 2.54–2.64 (m, 4H), 3.21–3.68 (m, 16H), 4.24 (d, 1H, J = 9.2 Hz), 5.08 (s, 2H), 5.56 (br, 1H), 7.00–7.35 (m, 7H).

4.1.2.2. *N*-{1-[3-(2-{2-[3-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-propoxy]-ethoxy}-ethoxy)-propylcarbamoyl]-2,2-dimethylpropyl}-succinamic acid (11). Compound **10** was dissolved in 20 mL of methanol. To the mixture, 0.5 g (8% weight of **10**) of 10% Pd–C was added. After stirring under hydrogen atmosphere for 3 days, the reaction mixture was filtered and evaporated. The residue was dissolved in 1,4-dioxane–H_2O (50–30 mL). *N*-(9-Fluorenylmethoxycarbonloxy)succinimide (Fmoc-OSu, 4.33 g, 12.8 mmol) and

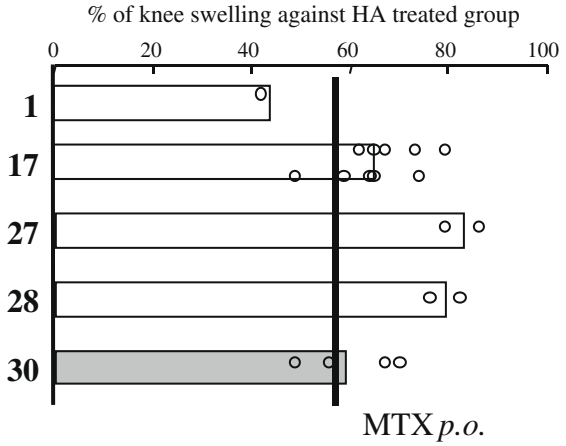


Figure 6. Linker screening to evaluate the inhibition of knee swelling in rat antigen-induced arthritis. Conjugates **27**, **28** and **30**: 0.5 mg as HA, once a week, i.a. MTX: 0.1 mg/kg, five times a week, p.o. for two weeks. Knee swelling, the difference between the right and left knee width, was measured on days 0–14. The bold line indicates the average for MTX treatment groups.

$NaHCO_3$ (1.98 g, 23.5 mmol) were added to the solution and stirred at 0 °C. After 1 h, the reaction mixture was warmed to rt. After 8 h, ethyl acetate was added. The organic layer was washed with 1 N HCl solution and brine, dried over sodium sulfate and evaporated. The product was purified by silica gel column chromatography (eluent: dichloromethane/methanol, 20/1) to afford 1.71 g (2.61 mmol, 24% for two steps) of the desired compound **11**. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 0.97 (s, 9H), 1.75 (br, 4H), 2.55–2.66 (m, 4H), 3.21–3.62 (m, 16H), 4.21–4.40 (m, 4H), 5.51 (br, 1H), 6.95–7.76 (m, 11H). LC–MS *m*/*z*: 656.1 $(M+H)^+$.

4.1.2.3. Solid-phase synthesis of compounds 2–9. Protected amino acids, 20% piperidine in *N*,*N*-dimethylformamide (Pip/DMF) and coupling reagents were purchased from Watanabe Chemical Industries, Ltd.

Fmoc-resin (BACHEM, 0.55 mmol/g, 72.7 mg, 0.04 mmol) was agitated in 1.5 mL of Pip/DMF for 30 min. After washing the resin nine times with 1 mL of DMF, 2.5 equiv of **11**, *N*,*N*′-diisopropylcarbodiimide (DIC) and HOBt and 1.5 mL of DMF were added. After 6 h of agitating for coupling, the resin was washed six times with 1 mL of DMF.

Table 3
Effects of high, middle and low Mw conjugate **17** on human fibroblast-like synoviocytes (HFLS).

Conjugate	MTX (%)	Mw (kDa)	IC_{50} (μM)
1	1.2	1780	1.08
17	1.1	330	12.9
	1.4	800	13.2
	1.4	1980	14.7

Table 4
Effects of high, middle and low binding ratios of MTX for conjugate **30** on human fibroblast-like synoviocytes (HFLS).

Conjugate	MTX (%)	Mw (kDa)	IC_{50} (μM)
1	1.2	1780	1.08
30	0.5	2170	>12.0
	1.3	2090	1.13
	3.8	1910	0.09

4.1.2.3.1. Fmoc deprotection. Washing was carried out twice with 1 mL of Pip/DMF (1 min/time), with 1.5 mL of Pip/DMF (30 min) and nine times with 1 mL of DMF (1 min/time).

4.1.2.3.2. Coupling conditions. DIC–HOBt protocol: *N*-(9-fluorenylmethoxycarboxyl)-L-leucune (Fmoc-Leu-OH), *N*-(9-fluorenylmethoxycarboxyl)-L-glutamic acid γ-*tert*-butyl ester (Fmoc-Glu(O*t*Bu)-OH), *N*-(9-fluorenylmethoxycarboxyl)-L-glutamic acid α-*tert*-butyl ester (Fmoc-Glu(OH)-O*t*Bu) or 4-[*N*-(2,4-diamino-6-pteridinylmethyl)-*N*-methylamino]benzoic acid (DAMPA, 2.0 equiv), DIC (2.0 equiv) and HOBt (2.0 equiv) were agitated under nitrogen in 1.5 mL of DMF for 2 h and for 24 h for DAMPA.

Pfp ester protocol: *N*-(9-Fluorenylmethoxycarboxyl)-L-glycine pentafluorophenyl ester (Fmoc-Gly-OPfp) or *N*-(9-fluorenylmethoxycarboxyl)-L-phenylalanine pentafluorophenyl ester (Fmoc-Phe-OPfp) (2.0 equiv) and *N*-methylmorpholine (NMM, 2.0 equiv) were agitated in 1.5 mL of DMF for 2 h. and with Pip/DMF for 30 min.

HATU–DIPEA protocol: In the case of *N*-(9-fluorenylmethoxycarboxyl)-L-asparagine (Fmoc-Asn-OH) (2.0 equiv), *O*-(7-axabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 2.0 equiv) and diisopropylethylamine (DIPEA, 2.0 equiv) were agitated in 1.5 mL of DMF for 24 h. After each coupling, washing was carried out with 1 mL of DMF (six times, 1 min/time).

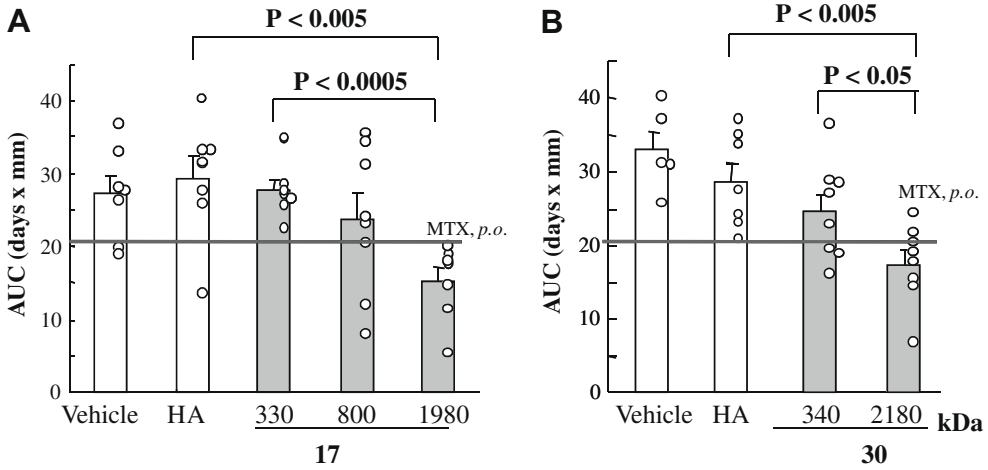


Figure 7. Effects of HA–MTX with different molecular weights of HA on knee swelling in rat antigen-induced arthritis. Knee swelling, the difference between the right and left knee width, was measured on days 0–14, and the AUC for knee swelling was calculated (A and B). Various Mws of conjugate **17**, conjugate **30**, HA (Mw 1900–2500 kDa) and vehicle were i.a. injected once a week. Results are expressed as mean + SEM. P <0.05, compared to HA-treated group. The bold lines indicate the average for the MTX treatment groups.

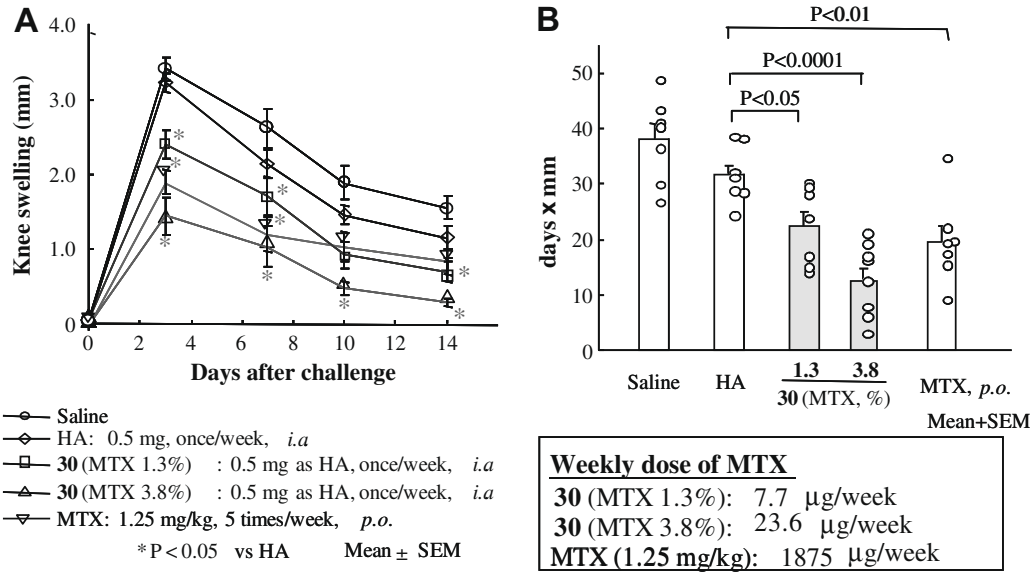
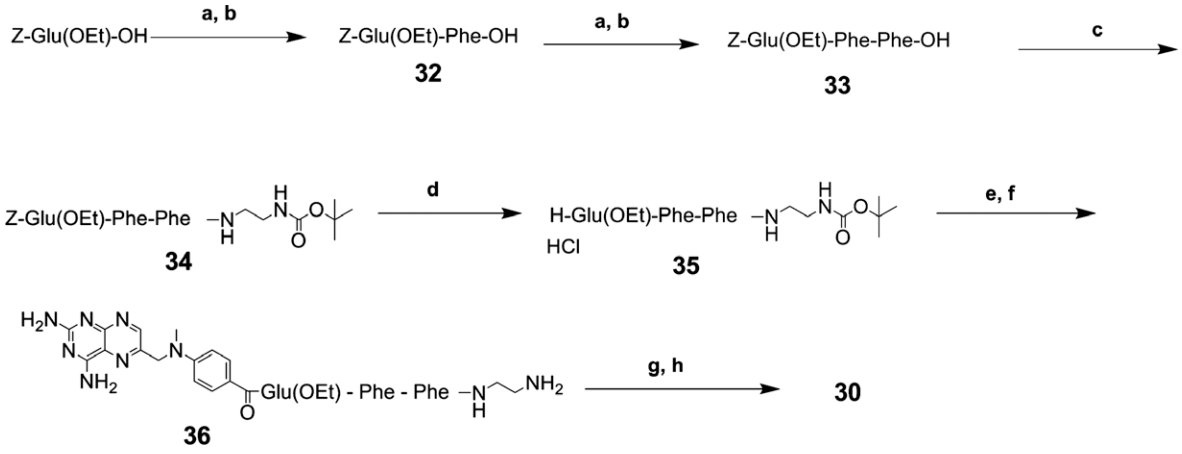


Figure 8. Effects of conjugate **30** with different binding ratios of MTX on knee swelling in rat antigen-induced arthritis. Knee swelling, the difference between the right and left knee width, was measured on days 0–14 (A) and the AUC for knee swelling calculated (B). Results are expressed as mean + SEM. *P* <0.05, compared to HA-treated group.



Scheme 4. Reagents and conditions: (a) H-Phe-O^{t}Bu, EDC–HOBt, NMM, THF, 5–25 °C; (b) H_2SO_4, MeCN, 0–25 °C; (c) *N*-(*tert*-butoxycarbonyl)-1,2-diaminoethane, EDC–HOBt, NMM, THF, 5–25 °C; (d) H_2, Pd/C, DMF–HCl aq, rt; (e) DAMPA, EDC–HOBt, NMP, 55 °C; (f) 7 N HCl–ethanol, DMF, rt; (g) sodium hyaluronate, tris[2-(2-methoxyethoxy)ethyl]amine, EDC–HOOB*t*, THF–H_2O, 5 °C; (h) NaOH aq, 5 °C.

4.1.2.3.3. Cleavage. Peptide resin was washed with dichloromethane and dried. The dried resin was treated for 1 h with 20% TFA/dichloromethane (1.5 mL). Then the resin was filtered off and washed with dichloromethane. The products were purified using the separable HPLC method. (Column: Inertsil ODS-3, 20.0 mm I.D. × 150 mm, GL Science Inc., Mobile Phase: (A) 0.05% TFA, H_2O, (B) 0.05% TFA, MeCN, Gradient (A/B): 90/10–50/50 (25 min), Flow Rate: 10.0 mL/min, Wavelength: 215, 308 nm).

4.1.2.4. Compound 2. Fmoc-Gly-OPfp, Fmoc-Leu-OH, Fmoc-Phe-OPfp, Fmoc-Glu(O*t*Bu)-OH and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (8.9 mg, 15%). LC–MS t_R: 3.8 min, *m*/*z*: 1243.6 $(M+H)^+$.

4.1.2.5. Compound 3. Fmoc-Gly-OPfp, Fmoc-Leu-OH, Fmoc-Phe-OPfp, Fmoc-Glu(OH)-O*t*Bu and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (7.4 mg, 13%). LC–MS t_R: 3.7 min, *m*/*z*: 1243.8 $(M+H)^+$.

4.1.2.6. Compound 4. Fmoc-Phe -OPfp, Fmoc-Asn-OH, Fmoc-Glu(O*t*Bu)-OH and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (8.5 mg, 14%). LC–MS t_R: 3.8 min, *m*/*z*: 1277.5 $(M+H)^+$.

4.1.2.7. Compound 5. Fmoc-Phe-OPfp, Fmoc-Asn-OH, Fmoc-Glu(OH)-O*t*Bu and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (7.2 mg, 12%). LC–MS t_R: 3.7 min, *m*/*z*: 1277.7 $(M+H)^+$.

4.1.2.8. Compound 6. Fmoc-Phe-OPfp, Fmoc-Glu(O*t*Bu)-OH and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (7.3 mg, 13%). LC–MS t_R: 4.0 min, *m*/*z*: 1163.7 $(M+H)^+$.

4.1.2.9. Compound 7. Fmoc-Phe-OPfp, Fmoc-Glu(OH)-O*t*Bu and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (8.9 mg, 16%). LC–MS t_R: 3.9 min, *m*/*z*: 1163.7 $(M+H)^+$.

4.1.2.10. Compound 8. Fmoc-Phe-OPfp, Fmoc-Glu(O*t*Bu)-OH and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (8.8 mg, 18%). LC–MS t_R: 3.6 min, *m*/*z*: 1016.7 $(M+H)^+$.

4.1.2.11. Compound 9. Fmoc-Phe-OPfp, Fmoc-Glu(OH)-O*t*Bu and DAMPA were used for coupling. The desired compound was obtained as a 2·TFA salt (8.8 mg, 18%). LC–MS t_R: 3.5 min, *m*/*z*: 1016.7 $(M+H)^+$.

4.1.3. Synthesis of amine compounds as precursors of the conjugates

4.1.3.1. 4,7,10-Trioxa-13-[*N*-[*N*-[*N*-[4-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-α-(5-methylglutamyl)]phenylalanyl]phenylalanylamino]tridecanylamine: MTX-α-PhePhe-NH–$C_{10}H_{20}O_3$–NH_2 (16).

*4.1.3.1.1. **Z**-Phe-NH–$C_{10}H_{20}O_3$–NH-Boc (**12**).* *N*-Carbobenzoxy-L-phenylalanine (Z-Phe-OH: 852 mg, 2.85 mmol), *N*-*t*-butoxycarbonyl-4,7,10-trioxa-1,13-tridecanediamine (NH_2–$C_{10}H_{20}O_3$–NH–Boc, 760 mg, 2.37 mmol), and HOBt (363 mg, 2.37 mmol) were dissolved in 6 mL of DMF. To the solution EDC (546 mg, 2.85 mmol) was added at 0 °C. The reaction mixture was warmed to rt and stirred for 2 days and then ethyl acetate was added. The organic layer was washed with 10% citric acid, 5% Na_2CO_3 and brine, dried with sodium sulfate and evaporated. The product was purified by silica gel column chromatography (eluent: dichloromethane/methanol, 100/3) to afford 1.35 g (2.24 mmol, 79%) of the desired compound **12** as a colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 1.43 (s, 9H), 1.56–1.74 (m, 4H), 3.06 (d, 2H, *J* = 6.8 Hz), 3.17−3.58 (m, 16H), 4.30–4.39 (m, 1H), 4.98 (br, 1H), 5.08 (s, 2 H), 5.50 (br, 1H), 6.40 (br, 1H), 7.16–7.32 (m, 10H). LC–MS *m*/*z*: 624.3 $(M+Na)^+$.

*4.1.3.1.2. Z-PhePhe-NH–$C_{10}H_{20}O_3$–NH-Boc (**13**).* 1.35 g (2.24 mmol) of compound **12** was dissolved in 12 mL of methanol. To the mixture 200 mg (15% weight of **12**) of 10% Pd−C was added. After stirring under hydrogen atmosphere for 4 h, the reaction mixture was filtered and evaporated. The residue was dissolved in 10 mL of dry DMF. To the solution, Z-Phe-OH (1.07 g, 3.57 mmol, 1.6 equiv), HOBt (514 mg, 3.36 mmol, 1.5 equiv) and EDC (688 mg, 3.59 mmol, 1.6 equiv) were added at 0 °C. The reaction mixture was warmed to rt, stirred for 2 days and then ethyl acetate was added to the mixture. Following the same procedure for **12**, **13** was obtained (1.56 g, 2.08 mmol, 93%) as a white solid generated by the addition of *n*-hexane to the concentrated residue. ^{1}H NMR (270 MHz, $CDCl_3$) δ: 1.43 (s, 9H), 1.60−1.78 (m, 4H), 2.96–3.60 (m, 20H), 4.42–4.59 (m, 2H), 4.96–5.07 (m, 3H), 5.41 (br d, 1H), 6.39 (br, 1H), 6.73 (br d, 1H), 7.08–7.31 (m, 15H). LC–MS *m*/*z*: 771.3 $(M+Na)^+$.

*4.1.3.1.3. Z-Glu(OMe)PhePhe-NH–$C_{10}H_{20}O_3$–NH-Boc (**14**).* Compound **13** (500 mg, 0.668 mmol) was dissolved in 10 mL of methanol. 150 mg (30% weight of **13**) of 10% Pd−C was added to the mixture. After stirring at rt for 24 h under hydrogen atmosphere, the reaction mixture was filtered and evaporated. The residue was dissolved in 5 mL of DMF. *N*-Carbobenzoxy-L-glutamic acid-γ-methyl ester (Z-Glu(OMe)-OH: 217 mg, 0.734 mmol, 1.1 equiv), then HOBt (102 mg, 0.668 mmol, 1.0 equiv) and EDC (141 mg, 0.734 mmol, 2.2 equiv) were added at 0 °C and the reaction mixture stirred at rt for 16 h. Following the same procedure for **12**, the desired residue was obtained followed by silica gel column chromatography (eluent: dichloromethane/methanol, 100/5). **14** was obtained (529 mg, 0.594 mmol, 89%) as a white solid which was generated by the addition of *n*-hexane to the concentrated residue. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.36 (s, 9H), 1.50–1.85 (m, 6H), 2.20 (t, 2H, *J* = 7.9 Hz), 2.70–3.10 (m, 8H), 3.25–3.48 (m, 12H), 3.56 (s, 3H), 3.93–4.02 (m, 1H), 4.20–4.60 (m, 2H), 5.00 (s, 2H), 6.77 (br t, 1H), 7.10–7.45 (m, 16H), 7.82 (br t, 1H, *J* = 6.1 Hz), 7.91 (d, 1H, *J* = 7.9 Hz), 8.22 (d, 1H, *J* = 7.9 Hz). LC–MS *m*/*z*: 914.3 $(M+Na)^+$.

*4.1.3.1.4. MTX-(OMe)-α-PhePhe-NH–$C_{10}H_{20}O_3$–NH-Boc (**15**).*

Compound **14** (514 mg, 0.576 mmol) was suspended in 30 mL of methanol. 100 mg (19% weight of **14**) of 10% Pd−C was added to the mixture. After stirring at rt for 1.5 h under hydrogen atmosphere, the reaction mixture was filtered, evaporated, and the residue was then dissolved in 5 mL of DMF. HOBt (132 mg, 0.864 mmol, 1.5 equiv), EDC (166 mg, 0.864 mmol, 1.5 equiv) and DAMPA (281 mg, 0.864 mmol, 1.5 equiv) were added to this DMF solution at 0 °C. After being stirred at rt for 2 days, 5% Na_2CO_3 was added to the reaction solution, and the generated precipitate was purified by amino silica gel (NH-DM1020, 100–200 mesh, from Fuji Silysia Chemical Ltd) column chromatography (eluent: dichloromethane/methanol, 100/7 and chloroform/methanol, 100/4, two times) to provide 415 mg (0.390 mmol, 68%) of compound **15** as a yellow powder.

^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.36 (s, 9H), 1.48–1.61 (m, 4H), 1.81–1.92 (m, 2H), 2.24 (t, 2H, *J* = 7.9 Hz), 2.70–3.10 (m, 8H), 3.22 (s, 3H), 3.25–3.47 (m, 12H), 3.54 (s, 3H), 4.25–4.50 (m, 3H), 4.79 (s, 2H), 6.61 (br s, 2H), 6.76–6.83 (m, 3H), 7.06–7.24 (m, 10H), 7.45 (br s, 1H), 7.67–7.80 (m, 4H), 7.86 (d, 1H, *J* = 8.1 Hz), 8.09 (d, 1H, *J* = 7.4 Hz), 8.15 (d, 1H, *J* = 8.1 Hz), 8.56 (s, 1H). LC–MS *m*/*z*: 1087.5 $(M+Na)^+$.

*4.1.3.1.5. MTX-(OMe)-α-PhePhe-NH–$C_{10}H_{20}O_3$–NH_2 (**16**).* 3 mL of TFA was added to compound **15** (413 mg, 0.388 mmol) at 0 °C. After stirring for 40 min, the reaction mixture was evaporated and the residue was purifying by aminosilica gel column chromatography (eluent: dichloromethane/methanol, 100/7, two times) to provide 344 mg (0.346 mmol, 89%, 40% for five steps) of compound **16** as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.49–1.95 (m, 4H), 1.81–1.92 (m, 2H), 2.24 (t, 2H, *J* = 7.9 Hz), 2.70–3.10 (m, 8H), 3.22 (s, 3H), 3.25–3.47 (m, 12H), 3.54 (s, 3H), 4.25–4.50 (m, 3H), 4.79 (s, 2H), 6.61 (br s, 2H), 6.76–6.83 (m, 3H), 7.06–7.24 (m, 10H), 7.45 (br s, 1H), 7.83 (br t, 1H, *J* = 5.8 Hz), 8.01 (d, 1H, *J* = 7.9 Hz), 8.09 (d, 1H, *J* = 7.1 Hz), 8.15 (d, 1H, *J* = 7.8 Hz), 8.56 (s, 1H). LC–MS *m*/*z*: 965.5 $(M+H)^+$.

The other precursors were obtained following the same procedures used for **16**.

4.1.3.2. MTX-(OMe)-α-PheGly-NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 18).

Following the same procedure for compound **16** but with *N*-carbobenzoxyglycine (Z-Gly-OH) in place of Z-Phe-OH in the production of **13**, the target compound (528 mg, 0.604 mmol, 54% for five steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.51–1.64 (m, 4H), 1.84–1.94 (m, 2H), 2.21–2.30 (m, 2H), 2.55 (t, 2H, *J* = 6.3 Hz), 2.78–2.92 (m, 1H), 3.03–3.76 (m, 17H), 3.22 (s, 3H), 3.55 (s, 3H), 4.26–4.52 (m, 2H), 4.79 (s, 2H), 6.63 (br s, 2H), 6.82 (d, 2H, *J* = 8.7 Hz), 7.11–7.24 (m, 5H), 7.47 (br s, 1H), 7.62–7.72 (m, 4H), 8.04–8.16 (m, 2H), 8.28 (br t, 1H), 8.56 (s, 1H). LC–MS *m*/*z*: 875.5 $(M+H)^+$.

4.1.3.3. MTX-(OMe)-α-Phe-NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 19).

Following the same procedure for compound **16** but excluding the procedure for 4.1.3.2 (above), the target compound (496 mg, 0.607 mmol, 47% for four steps) was obtained as a yellow powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.49–1.59 (m, 4H), 1.82–1.89 (m, 2H), 2.19–2.27 (m, 2H), 2.55 (t, 2H, *J* = 7.2 Hz), 2.73–3.10 (m, 4H), 3.23 (s, 3H), 3.17–3.48 (m, 12H), 3.55 (s, 3H), 4.21–4.28 (m, 1H), 4.38–4.45 (m, 1H), 4.80 (s, 2H), 6.61 (br s, 2H), 6.83 (d, 2H, *J* = 9.3 Hz), 7.11–7.20 (m, 5H), 7.46 (br s, 1H), 7.66 (br s, 1H), 7.73 (d, 2H, *J* = 9.0 Hz), 7.83 (t, 1H), 7.92 (d, 1H, *J* = 8.4 Hz), 8.12 (d, 1H, *J* = 7.5 Hz), 8.56 (s, 1H). LC–MS *m*/*z*: 818.4 $(M+H)^+$.

4.1.3.4. MTX-(OMe)-α-Glu(OMe)–NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 20).

Following the same procedure for 4.1.3.3 (above) with Z-Glu(OMe)-OH in place of Z-Phe-OH, the target compound (600 mg, 0.738 mmol, 45% in four steps) was obtained as a yellow

powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.50–2.03 (m, 8H), 2.24–2.31 (t, 2H), 2.34–2.40 (t, 2H), 2.49–2.57 (t, 2H), 2.97–3.52 (m, 14H), 3.21 (s, 3H), 3.53 (s, 3H), 3.55 (s, 3H), 4.15–4.36 (m, 2H), 4.78 (s, 2H), 6.61 (br s, 2H), 6.81 (d, 2H, J = 8.7 Hz), 7.46 (br s, 1H), 7.67 (br s, 1H), 7.72 (d, 2H, J = 8.6 Hz), 7.84 (br t, 1H), 7.95 (d, 1H), 8.14 (d, 1H), 8.55 (s, 1H). LC–MS m/z: 814.4 $(M+H)^+$.

4.1.3.5. MTX-(OMe)-α-Trp-NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 21). Following the same procedure for **4.2.3.3** with *N*-carbobenzoxy-L-tryptophan (Z-Trp-OH) in place of Z-Phe-OH, the target compound (171 mg, 0.200 mmol, 20% in four steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.50–1.61 (m, 4H), 1.84–1.97 (m, 2H), 2.23–2.32 (m, 2H), 2.50–2.56 (t, 2H), 2.92–3.15 (m, 4H), 3.22 (s, 3H), 3.29–3.45 (m, 12H), 3.55 (s, 3H), 4.29–4.49 (m, 2H), 4.78 (s, 2H), 6.64 (br s, 2H), 6.80 (d, 2H), 6.92 (t, 1H), 7.04 (t, 1H), 7.10 (s, 1H), 7.26 (d, 1H), 7.44 (br s, 1H), 7.51 (d, 1H), 7.65 (br s, 1H), 7.69 (d, 2H), 7.82 (br t, 1H), 7.93 (d, 1H), 8.10 (d, 1H), 8.55 (s, 1H), 10.80 (s, 1H). LC–MS m/z: 857.5 $(M+H)^+$.

4.1.3.6. MTX-(OMe)-α-Tyr-NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 22). Following the same procedure for **4.2.3.3** with *N*-carbobenzoxy-L-tyrosine (Z-Tyr-OH) in place of Z-Phe-OH, the target compound (133 mg, 0.160 mmol, 62% in four steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.51–1.62 (m, 4H), 1.85–1.95 (m, 2H), 2.23–2.31 (m, 2H), 2.51–2.58 (t, 2H), 2.63–2.91 (m, 2H), 2.95–3.16 (m, 2H), 3.22 (s, 3H), 3.27–3.54 (m, 12H), 3.56 (s, 3H), 4.22–4.35 (m, 2H), 4.79 (s, 2H), 6.57 (d, 2H, J = 8.1 Hz), 6.61 (br s, 2H), 6.82 (d, 2H, J = 8.7 Hz), 6.92 (d, 2H, J = 8.1 Hz), 7.47 (br s, 1H), 7.67–7.88 (m, 5H), 8.13 (d, 1H), 8.55 (s, 1H). LC–MS m/z: 834.4 $(M+H)^+$.

4.1.3.7. MTX-(OMe)-α-Ile-NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 23). Following the same procedure for **4.2.3.3** with *N*-carbobenzoxy-L-isoleucine (Z-Ile-OH) in place of Z-Phe-OH, the target compound (562 mg, 0.717 mmol, 41% in four steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 0.76–0.80 (m, 6H), 0.99–1.10 (m, 1H), 1.36–1.45 (m, 1H), 1.49–1.73 (m, 5H), 1.88–2.07 (m, 2H), 2.33–2.38 (m, 2H), 2.55 (t, 2H, J = 6.6 Hz), 2.98–3.48 (m, 14H), 3.21 (s, 3H), 3.56 (s, 3H), 4.05–4.13 (m, 1H), 4.40–4.48 (m, 1H), 4.78 (s, 2H), 6.60 (br s, 2H), 6.82 (d, 2H, J = 8.4 Hz), 7.46 (br s, 1H), 7.66–7.72 (m, 3H), 7.98 (br t, 1H), 8.12 (d, 1H, J = 7.6 Hz), 8.56 (s, 1H). LC–MS m/z: 784.4 $(M+H)^+$.

4.1.3.8. MTX-(OMe)-α-βAla-NH–$C_{10}H_{20}O_3$–NH_2 (precursor of 24). Following the same procedure for **4.2.3.3** with *N*-carbobenzoxy-β-alanine (Z-βAla-OH) in place of Z-Phe-OH, the target compound (230 mg, 0.310 mmol, 37% for four steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.49–1.62 (m, 4H), 1.79–2.02 (m, 2H), 2.21 (t, 2H, J = 6.9 Hz), 2.32 (t, 2H, J = 7.3 Hz), 2.56 (t, 2H, J = 6.6 Hz), 3.00–3.61 (m, 19H), 3.55 (s, 3H), 4.29–4.38 (m, 1H), 4.78 (s, 2H), 6.61 (br s, 2H), 6.81 (d, 2H, J = 8.6 Hz), 7.43 (br s, 1H), 7.61–7.91 (m, 3H), 7.72 (d, 2H, J = 8.6 Hz), 8.02 (d, 1H, J = 7.8 Hz), 8.55 (s, 1H). LC–MS m/z: 742.4 $(M+H)^+$.

4.1.3.9. MTX-(OMe)-α-PhePhe–NH–$C_{10}H_{20}O_2$–NH_2 (precursor of 25). Following the procedure for compound **16** with *N*-*t*-butoxycarbonyl-4,9-dioxa-1,12-dodecanediamine (NH_2–$C_{10}H_{20}O_2$–NH-Boc) in place of NH_2–$C_{10}H_{20}O_3$–NH-Boc, the target compound (221 mg, 0.233 mmol, 14% in five steps) was obtained as a yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.47–1.60 (m, 8H), 1.80–1.95 (m, 2H), 2.20–2.29 (m, 2H), 2.60 (t, 2H), 2.70–3.10 (m, 6H), 3.22 (s, 3H), 3.25–3.50 (m, 8H), 3.54 (s, 3H), 4.25–4.49 (m, 3H), 4.79 (s, 2H), 6.60 (br s, 2H), 6.81 (d, 2H, J = 8.4 Hz), 7.06–7.20 (m, 10H), 7.45 (br s, 1H), 7.65 (br s, 1H), 7.70 (d, 2H), 7.73 (br t, 1H), 7.83 (d, 1H), 8.10 (d, 1H), 8.11 (d, 1H), 8.55 (s, 1H). LC–MS m/z: 949.5 $(M+H)^+$.

4.1.3.10. MTX-(OMe)-α-PhePhe-NH–$C_8H_{16}O_2$–NH_2 (precursor of 26). Following the procedure for compound **16** with *N*-*t*-butoxycarbonyl-4,7-dioxa-1,10-decanediamine (NH_2–$C_8H_{16}O_2$–NH-Boc) in place of NH_2–$C_{10}H_{20}O_3$–NH-Boc, the target compound (407 mg, 0.442 mmol, 24% for five steps) was obtained as a yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.50–1.57 (m, 4H), 1.85–1.91 (m, 2H), 2.21–2.28 (m, 2H), 2.60 (t, 2H), 2.70–3.13 (m, 6H), 3.22 (s, 3H), 3.25–3.45 (m, 8H), 3.55 (s, 3H), 4.27–4.49 (m, 3H), 4.79 (s, 2H), 6.60 (br s, 2H), 6.82 (d, 2H, J = 8.8 Hz), 7.07–7.21 (m, 10H), 7.43 (br s, 1H), 7.69 (br s, 1H), 7.71 (d, 2H, J = 8.8 Hz), 7.75 (br t, 1H), 7.85 (d, 1H), 8.08 (d, 1H), 8.13 (d, 1H), 8.56 (s, 1H). LC–MS m/z: 921.4 $(M+H)^+$.

4.1.3.11. MTX-(OMe)-α-PhePhe-NH–$C_6H_{12}O_2$–NH_2 (precursor of 27). Following the procedure for compound **16** with *N*-*t*-butoxycarbonyl-3,6-dioxa-1,8-octanediamine (NH_2–$C_6H_{12}O_2$–NH-Boc) in place of NH_2–$C_{10}H_{20}O_3$–NH-Boc, the target compound (148 mg, 0.166 mmol, 23% in five steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.81–1.91 (m, 2H), 2.20–2.25 (m, 2H), 2.61–2.64 (t, 2H), 2.70–2.97 (m, 6H),3.22 (s, 3H), 3.27–3.47 (m, 8H), 3.55 (s, 3H), 4.27–4.47 (m, 3H), 4.79 (s, 2H), 6.62 (br s, 2H), 6.82 (d, 2H, J = 8.7 Hz), 7.06–7.25 (m, 10H), 7.46 (br s, 1H), 7.67 (br s, 1H), 7.71 (d, 2H, J = 8.6 Hz), 7.85 (d, 1H), 7.92 (br t, 1H), 8.07 (d, 1H), 8.15 (d, 1H), 8.56 (s, 1H). LC–MS m/z: 893.6 $(M+H)^+$.

4.1.3.12. MTX-(OMe)-α-PhePhe-NH–C_4H_8O–NH_2 (precursor of 28). Following the procedure for compound **16** with *N*-*t*-butoxycarbonyl-3-oxa-1,5-pentanediamine (NH_2–C_4H_8O–NH-Boc) in place of NH_2–$C_{10}H_{20}O_3$–NH-Boc, the target compound (52 mg, 0.061 mmol, 7% in five steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.84–1.92 (m, 2H), 2.20–2.27 (m, 2H), 2.60–2.64 (t, 2H), 2.71–2.99 (m, 6H), 3.22 (s, 3H), 3.25–3.45 (m, 4H), 3.54 (s, 3H), 4.27–4.50 (m, 3H), 4.79 (s, 2H), 6.61 (br s, 2H), 6.81 (d, 2H, J = 8.4 Hz), 7.05–7.21 (m, 10H), 7.45 (br s, 1H), 7.65 (br s, 1H), 7.70 (d, 2H, J = 8.6 Hz), 7.84 (d, 1H), 7.91 (br t, 1H), 8.07 (d, 1H), 8.15 (d, 1H), 8.55 (s, 1H). LC–MS m/z: 849.4 $(M+H)^+$.

4.1.3.13. MTX-(OMe)-α-PhePhe-NH–C_5H_{10}–NH_2 (precursor of 29). Following the procedure for compound **16** with *N*-*t*-butoxycarbonyl-1,5-pentanediamine (NH_2–C_5H_{10}–NH-Boc) in place of NH_2–$C_{10}H_{20}O_3$–NH-Boc, the target compound (148 mg, 0.175 mmol, 17% in five steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.16–1.56 (m, 6H), 1.81–1.97 (m, 2H), 2.21–2.29 (m, 2H), 2.69–3.06 (m, 6H), 3.23 (s, 3H), 3.55 (s, 3H), 4.25–4.50 (m, 3H), 4.80 (s, 2H), 6.65 (br s, 2H), 6.82 (d, 2H, J = 8.6 Hz), 7.08–7.24 (m, 10H), 7.50 (br s, 1H), 7.60–7.89 (m, 5H), 8.10–8.16 (m, 2H), 8.55 (s, 1H). LC–MS m/z: 847.4 $(M+H)^+$.

4.1.3.14. 2-[*N*-[*N*-[*N*-[4-[[(2,4-Diamino-6-pteridinyl)methyl]methylamino]benzoyl]-α-(5-methylglutamyl)]phenylalanyl]phenylalanylamino]-ethylamine: MTX-(OMe)-α-PhePhe-NH–C_2H_4–NH_2 (precursor of 30). Following the procedure for compound **16** with *N*-*t*-butoxycarbonylethylenediamine (NH_2–C_2H_4–NH-Boc) in place of NH_2–$C_{10}H_{20}O_3$–NH-Boc, the target compound (275 mg, 0.342 mmol, 50% in five steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.80–1.96 (m, 2H), 2.20–2.28 (m, 2H), 2.45 (t, 2H, J = 6.6 Hz), 2.70–3.10 (m, 6H), 3.22 (s, 3H), 3.55 (s, 3H), 4.26–4.52 (m, 3H), 4.79 (s, 2H), 6.61 (br s, 2H), 6.82 (d, 2H, J = 8.7 Hz), 7.06–7.21 (m, 10H), 7.46 (br s, 1H), 7.65–7.73 (m, 3H), 7.85 (d, 1H, J = 8.1 Hz), 8.08–8.16 (m, 2H), 8.56 (s, 1H). LC–MS m/z: 805.3 $(M+H)^+$.

4.1.3.15. MTX-(OMe)-α-PhePhe-Lys-OMe (precursor of 31). Following the same procedure for **16** with *N*-ε-*t*-butoxycarbonyl-L-lysine methyl ester (H-Lys(Boc)-OMe) in place of NH_2–

$C_{10}H_{20}O_3$–NH-Boc, the target compound (178 mg, 0.l97 mmol, 12% in five steps) was obtained as a yellow powder. ^{1}H NMR (270 MHz, DMSO-d_6) δ: 1.25–1.34 (m, 4H), 1.56–1.69 (m, 2H), 1.75–1.90 (m, 2H), 2.18–2.25 (br t, 2H), 2.50–2.60 (m, 2H), 2.65–3.07 (m, 4H), 3.22 (s, 3H), 3.54 (s, 3H), 3.60 (s, 3H), 4.15–4.60 (m, 4H), 4.79 (s, 2H), 6.63 (br s, 2H), 6.81 (d, 2H, J = 8.7 Hz), 7.00–7.25 (m, 10H), 7.45 (br s, 1H), 7.62 (br s, 1H), 7.69 (d, 2H, J = 8.6 Hz), 7.80 (d, 1H), 8.05 (d, 1H), 8.16 (d, 1H), 8.30 (d, 1H), 8.56 (s, 1H). LC–MS m/z: 905.4 $(M+H)^+$.

4.1.4. General procedure for the synthesis of conjugates 17–31

In the following, 'water' refers to extra pure water and HA was treated under sterile conditions.

A solution of 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-benzotriazine (HOOB*t*) (0.125 mmol) and amines including **16** (0.031 mmol) in 20 mL of water–THF (1:1) solvent was added to a suspension of sodium hyaluronate (500 mg, Mw of ca. 2300 kDa) in 10 mL of THF solution. To the mixture, tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1, 0.094 mmol) dissolved in 10 ml of water–THF (1:1) was added, followed by stirring at 5 °C. After 30 min, EDC (0.125 mmol) dissolved in water (10 mL) was added to the mixture, followed by stirring at 5 °C for 20 h. A aqueous solution of 0.09 N NaOH solution (220 mL) was added to the reaction mixture, followed by stirring at 5 °C for 3.5 h. 1 N HCl (20 mL) was added to the reaction mixture for neutralization, then NaCl solution (9 g in 45 mL of water) was added, followed by dropwise addition of ethanol (600 mL). The precipitate which formed was separated by centrifugation. The precipitate was dissolved in water (40 mL) to provide an aqueous solution of the HA–MTX conjugate.

To the conjugate solution, NaCl (6 g) dissolved in 160 mL of water and 400 mL of ethanol were added. The precipitate which formed was separated by centrifugation and dissolved in 500 mL of water, to which NaCl (15 g) was added before filtration using a 0.45 μm filter (Stervex HV: Millipore). Then ethanol (1000 mL) was aseptically added dropwise. The precipitate was filtered and dried in vacuo. The conjugate was dissolved in 40 mL of phosphate buffer solution (2 mM sodium phosphate, 154 mM NaCl, pH 7.2) to provide a sterile aqueous solution of the HA–MTX conjugate. The Mw and the binding ratio of MTX were determined by GPC. The Mw was calculated using a standard curve generated from HA solutions having various Mws (340, 850, 1440, 1760 and 2200 kDa). The binding ratio of MTX was calculated by measuring the ultraviolet absorption (259 nm). The procedure for calculating the binding ratio of MTX was as follows: The concentration of HA in the conjugate was determined by the RI peak area relative to the standard HA solution. Next, the HA concentration was converted to an equivalent concentration of a disaccharide unit with one carboxyl group. The concentration of MTX in the conjugate was determined by the UV peak area relative to the standard MTX solution. The binding ratio of MTX was obtained by dividing the concentration of MTX by the concentration of the disaccharide unit.

4.1.5. MTX-α-PhePhe-NH–$C_{10}H_{20}O_3$–NH-HA (17)

Following the general procedure, an aqueous solution of conjugate **17** was prepared from sodium hyaluronate (500 mg, ca. 2300 kDa) and compound **16** (29.9 mg, 0.031 mmol). The first lot after sterilization had a Mw of ca. 1980 kDa and binding ratio of MTX of 1.4%. The second lot after sterilization had a Mw of ca. 1910 kDa and binding ratio of 1.3%. The third lot after sterilization had a Mw of ca. 1380 kDa and binding ratio of 1.3%. The fourth lot after sterilization had a Mw of ca. 2190 kDa and binding ratio of 1.3%.

When 800 or 320 kDa of sodium hyaluronate was used, the conjugate which Mw of ca. 800 or 330 kDa and binding ratio was 1.4% or 1.1% was obtained, respectively.

^{1}H NMR (500 MHz, D_2O) δ: 1.67 (m), 1.79 (m), 1.84–1.94 (m), 2.02 (br s), 2.12–2.20 (m), 2.59 (m), 2.77 (m), 2.91 (m), 2.99 (m), 3.12–3.25 (m), 3.35 (br s), 3.49 (br s), 3.51 (br s), 3.57 (br s), 3.71 (br s), 3.83 (br s) 4.18 (t), 4.45 (br d), 4.55 (br d), 4.88 (d), 4.96 (d), 6.76 (d), 6.95–7.10 (m), 7.72 (d), 8.68 (s).

4.1.6. MTX-α-PheGly-NH$C_{10}H_{20}O_3$NH-HA (18)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.2. (27.1 mg, 0.031 mmol) provided an aqueous solution of conjugate **18**. After sterilization process, conjugate **18** with a Mw of ca. 1860 kDa and binding ratio of 1.4% was obtained.

4.1.7. MTX-α-Phe-NH–$C_{10}H_{20}O_3$–NH-HA (19)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.3. (25.4 mg, 0.031 mmol) provided an aqueous solution of conjugate **19**. After sterilization, conjugate **19** with a Mw of ca. 1790 kDa and binding ratio of 1.7% was obtained.

4.1.8. MTX-α-Glu-NH–$C_{10}H_{20}O_3$–NH-HA (20)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.4 (25.2 mg, 0.031 mmol) provided an aqueous solution of conjugate **20**. After sterilization, conjugate **20** with a Mw of ca. 1830 kDa and a binding ratio of 1.5% was obtained. ^{1}H NMR (500 MHz, D_2O) δ: *1.57* (m), 1.77 (m), 2.02 (br s), 2.25 (m), 2.37 (t), 3.24 (s), 3.25 (s), 3.35 (br s), 3.51 (br s), 3.56 (br s), 3.71 (br s), 3.83 (br s), *4.13* (m), 4.22 (m), 4.36 (m), 4.46 (br s), 4.55 (br s), 4.91 (s), 6.94 (d), 7.76 (d), 8.66 (s), *8.68* (s).

The terms in italics indicate minor signals. From the signals, conjugate **20** was deduced to be a mixture of α-(major) and γ-(minor) isomers.

4.1.9. MTX-α-Trp-NH–$C_{10}H_{20}O_3$–NH-HA (21)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.5 (26.6 mg, 0.031 mmol) provided an aqueous solution of conjugate **21**. After sterilization, conjugate **21** with a Mw of ca. 1390 kDa and binding ratio of 1.9% was obtained.

4.1.10. MTX-α-Tyr-NH–$C_{10}H_{20}O_3$–NH-HA (22)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.6 (25.9 mg, 0.031 mmol) provided an aqueous solution of conjugate **22**. After sterilization, conjugate **22** with a Mw of ca. 1760 kDa and binding ratio of 1.7% was obtained.

4.1.11. MTX-α-Ile-NH–$C_{10}H_{20}O_3$–NH-HA (23)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.7 (24.3 mg, 0.031 mmol) provided an aqueous solution of conjugate **23**. After sterilization, conjugate **23** with a Mw of ca. 1620 kDa and binding ratio of 1.7% was obtained.

4.1.12. MTX-α-βAla-NH–$C_{10}H_{20}O_3$–NH-HA (24)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.8 (23.0 mg, 0.031 mmol) provided an aqueous solution of conjugate **24**. After sterilization, conjugate **24** with a Mw of ca. 1430 kDa and binding ratio of 1.7% was obtained.

4.1.13. MTX-α-PhePhe-NH–$C_{10}H_{20}O_2$–NH-HA (25)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.9 (29.4 mg, 0.031 mmol) provided an aqueous solution of conjugate **25**. After

sterilization, conjugate **25** with a Mw of ca. 1550 kDa and binding ratio of 1.7% was obtained.

4.1.14. MTX-α-PhePhe-NH–$C_8H_{16}O_2$–NH-HA (26)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.10 (28.5 mg, 0.031 mmol) provided an aqueous solution of conjugate **26**. After sterilization, conjugate **26** with a Mw of ca. 1620 kDa and binding ratio of 1.6% was obtained.

4.1.15. MTX-α-PhePhe-NH–$C_6H_{12}O_2$–NH-HA (27)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.11 (27.7 mg, 0.031 mmol) provided an aqueous solution of conjugate **27**. After sterilization, conjugate **27** with a Mw of ca. 1620 kDa and binding ratio of 2.0% was obtained.

4.1.16. MTX-α-PhePhe-NH–C_4H_8O–NH-HA (28)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.12 (26.3 mg, 0.031 mmol) provided an aqueous solution of conjugate **28**. After sterilization, conjugate **28** with a Mw of ca. 1490 kDa and binding ratio of 1.9% was obtained.

4.1.17. MTX-α-PhePhe-NH–C_5H_{10}–NH-HA (29)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.13 (26.3 mg, 0.031 mmol) provided an aqueous solution of conjugate **29**. After sterilization, conjugate **29** with a Mw of ca. 1960 kDa and binding ratio of 1.2% was obtained.

4.1.18. MTX-α-PhePhe-NH–C_2H_4–NH-HA (30)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.14 (25.0 mg, 0.031 mmol) provided an aqueous solution of conjugate **30**. The first lot, after sterilization, had a Mw of ca. 1860 kDa and binding ratio of 2.1%. The second lot, after sterilization, had a Mw of ca. 2180 kDa and binding ratio of 1.9%. The third lot, after sterilization, had a Mw of ca. 2090 kDa and binding ratio of 1.3%.

When 320 kDa of sodium hyaluronate was used, the conjugate which Mw of ca. 340 kDa and binding ratio was 1.9% was obtained.

4.1.19. MTX-α-PhePhe-Lys-HA (31)

Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa) and the compound obtained in 4.1.3.15 (28.1 mg, 0.031 mmol) provided an aqueous solution of conjugate **31**. After sterilization, conjugate **31** with a Mw of ca. 1720 kDa and binding ratio of 1.4% was obtained.

4.1.20. Synthesis of 32 for industrial improvement

4.1.20.1. Z-Glu(OEt)Phe-OH (32). To a solution of 90.0 g (290 mmol) of *N*-carbobenzoxy-L-glutamic acid-γ-ethyl ester (Z-Glu(OEt)-OH), 74.8 g (290 mmol, 1.0 equiv) of L-phenylalanine-*tert*-butyl ester hydrochloride (H-Phe-O*t*Bu·HCl), 44.4 g (290 mmol, 1.0 equiv) of HOBt in 900 mL (10 v/w) of THF and 90 mL (1 v/w) of H_2O was added 32.3 g (319.0 mmol, 1.1 equiv) of NMM. At 5 °C, EDC (57.9 g, 302 mmol, 1.04 equiv) was added and the reaction mixture was stirred for 1 h at 5 °C then warmed to 25 °C. After 3 h, 900 mL (10 v/w) of H_2O and 900 mL (10 v/w) of ethyl acetate were added to the reaction mixture and stirring for 5 min. The organic layer was washed with 900 mL of 5% $NaHCO_3$ aqueous solution (10 v/w), 10% citric acid/20% NaCl aqueous solution (900 mL and 90 mL, 10 and 1 v/w) and 810 mL (9 v/w) of 20% NaCl aqueous solution then dried over magnesium sulfate (45 g, 0.5 w/w) and evaporated. The product was dissolved in 900 mL (10 v/w) of acetonitrile and evaporated in order to remove ethyl acetate. The product was dried in vacuo and the desired dipeptide ester was obtained (149 g, quant.). To the residue, 745 mL (5 v/w) of acetonitrile was added. At 0 °C, concd. H_2SO_4 (37.3 mL, 0.25 v/w) was added dropwise to the solution. After stirring for 5 h at 0 °C and for 15 min at 25 °C, H_2O 1.49 L (10 v/w) was added dropwise. The precipitate which formed was filtered, washed with 749 mL (5 v/w) of water and dried at 30 °C. The desired dipeptide **32** (132 g, quant.) was obtained as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.16 (t, 2H, J = 7.2 Hz), 1.66–1.92 (m, 2H), 2.27 (t, 2H, J = 8.0 Hz), 2.52–3.08 (m, 2H), 4.00–4.07 (m, 3H), 4.37–4.44 (m, 1H), 5.00 (s, 2H), 7.18–7.41 (m, 10H), 7.40 (d, 1H, J = 8.1 Hz), 8.12 (d, 1H, J = 7.8 Hz). LC–MS m/z: 457.0 $(M+H)^+$.

4.1.20.2. Z-Glu(OEt)PhePhe-OH (33). To a solution of 100 g (219 mmol) of **32**, H-Phe-O*t*Bu (56.3 g, 219 mmol, 1.0 equiv) and HOBt (33.5 g, 219 mmol, 1.0 equiv) in THF–H_2O (1 L and 100 mL, 10 v/w and 1 v/w) was added 24.4 g (241 mmol, 1.1 equiv) of NMM. At 5 °C, EDC (43.5 g, 227 mmol, 1.04 equiv) was added and the reaction mixture was stirred for 1 h then warmed to 25 °C. After 3 h, 1 L (10 v/w) of H_2O and 1 L (10 v/w) of ethyl acetate were added to the reaction mixture. The organic layer was washed with 400 mL of 5% $NaHCO_3$ aqueous solution (1 L, 10 v/w), 10% citric acid/20% NaCl aqueous solution (1 L and 100 mL, 10 and 1 v/w) and 900 mL (9 v/w) of 20% NaCl aqueous solution then dried over $MgSO_4$ (50 g, 0.5 w/w) and evaporated. The product was dissolved in 1 L (10 v/w) of acetonitrile and evaporated in order to remove ethyl acetate. The desired dipeptide ester was obtained (144 g, quant.). To the residue was added 720 mL (5 v/w) of acetonitrile. At 0 °C concd H_2SO_4 (36.0 mL, 0.25 v/w) was added dropwise to the solution. The reaction mixture was kept at 0 °C for 0.5 h and 25 °C for 1.5 h. To the suspension, isopropyl ether (2.16 L, 15 v/w) was added dropwise. After stirring for 1 h, the precipitate was filtered and washed with 720 mL (5 v/w) of isopropyl ether. The precipitate was dried at 30 °C in vacuo and the desired tripeptide **33** (132 g, quant.) was obtained as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.16 (t, 3H, J = 7.2 Hz), 1.60–1.83 (m, 2H), 2.17 (t, 2H, J = 7.7 Hz), 2.69–3.17 (m, 4H), 3.94–4.06 (m, 3H), 4.38–4.48 (m, 1H), 4.54 (br, 1H), 4.96–5.06 (m, 2H), 7.20–7.39 (m, 16H), 7.88 (d, 2H, J = 8.1 Hz), 8.32 (d, 1H, J = 8.1 Hz). LC–MS m/z: 604.0 $(M+H)^+$.

4.1.20.3. Z-Glu(OEt)PhePhe-NH–C_2H_4–NH-Boc (34). To a solution of 60.4 g (100 mmol) of **33**, NH_2–C_2H_4–NH-Boc (16.8 g, 105 mmol, 1.05 equiv) and HOBt (15.3 g, 100 mmol, 1.0 equiv) in DMF (600 mL, 10 v/w) was added 11.1 g (110 mmol, 1.1 equiv) of NMM at 25 °C. The solution was cooled to 5 °C and EDC (20.1 g, 105 mmol, 1.05 equiv) added. After stirring for 4 h at 5 °C, 660 mL (11 v/w) of H_2O was added dropwise and stirred for 30 min at 25 °C. The precipitate which formed was filtered and washed with 600 mL (10 v/w) of water and dissolved in 1.2 L (20 v/w) of ethanol at the temperature of reflux. After stirring for 1.5 h, the solution was cooled to 25 °C and stirred for 2 h. The precipitate which formed was filtered, washed with 300 mL (5 v/w) of ethanol and dried at 30 °C in vacuo. The product **34** (64.6 g, 86.6 mmol, 86.6%, 97.8% purity) was obtained as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.16 (t, 3H, J = 7.2 Hz), 1.36 (s, 9H), 1.62–1.84 (m, 2H), 2.18 (t, 2H, J = 7.8 Hz), 2.72–3.10 (m, 8H), 3.91–4.02 (m, 1H), 4.02 (q, 2H, J = 7.2 Hz), 4.35–4.54 (m, 2H), 5.00–5.06 (m, 2H), 6.68 (br, 1H), 7.20–7.34 (m, 16H), 7.84–7.91 (m, 2H), 8.20 (d, 1H, J = 8.1 Hz). LC–MS m/z: 746.0 $(M+H)^+$, 768.2 $(M+Na)^+$.

4.1.20.4. H-Glu(OEt)PhePhe-NH–C_2H_4–NH-Boc (35). To a solution of 55.0 g (73.7 mmol) of **34** in DMA (550 mL, 10 v/w) under nitrogen atmosphere were added 14.9 mL (0.27 v/w) of concd HCl and 11 g (0.2 w/w) of 10% Pd–C (containing 50% H_2O). After

stirring under hydrogen atmosphere for 2 h, the reaction mixture was filtrated through Celite to remove palladium reagent and washed with 550 mL (10 v/w) of ethyl acetate. To the filtrate was added 1.65 L (30 v/w) of ethyl acetate dropwise. After stirring for 1 h at 5 °C, the precipitate which formed was filtered, washed with 275 mL (5 v/w) of ethyl acetate and dried in vacuo. Compound **35** was obtained as a HCl salt (43.9 g, 67.7 mmol, 91.8%, 98.0% purity). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.17 (t, 3H, J = 7.1 Hz), 1.37 (s, 9H), 1.89–2.00 (m, 2H), 2.32–2.39 (m, 2H), 2.78–3.10 (m, 8H), 3.75 (t, 1H, J = 6.03 Hz), 4.05 (q, 2H, J = 7.1 Hz), 4.44 (q, 1H, J = 6.03 Hz), 4.56–4.62 (m, 1H), 6.71 (br, 1H), 7.15–7.27 (m, 10H), 7.94 (br, 1H), 8.12 (br, 2H), 8.42 (d, 1H, J 8 8.00 Hz), 8.57 (d, 1H, J = 8.0 Hz). LC–MS m/z: 612.1 $(M+H)^+$, 634.2 $(M+Na)^+$.

4.1.20.5. MTX-(OEt)-α-Phe-Phe-NH–C_2H_4–NH_2 (36). To a solution of 13.3 g (41.0 mmol, 0.95 equiv) of DAMPA in *N*-methylpiperidone (NMP, 280 mL, 10 v/w) was added 28.0 g (43.2 mmol) of **35**, 1.99 g (13.0 mmol, 0.3 equiv) of HOBt, 6.55 g (64.8 mmol, 1.5 equiv) of NMM and 10.8 g (56.2 mmol, 1.3 equiv) of EDC. After stirring for 4 h at 55 °C, the reaction mixture was cooled to rt and 420 mL (15 v/w) of water added. After stirring for 0.5 h, the precipitate which formed was filtered and washed with 420 mL of water. The precipitate was dried in vacuo and the desired compound was obtained as a yellow solid (36.7 g, 39.9 mmol, 92.4%). LCMS m/z: 919.2 $(M+H)^+$.

This solid (18.0 g, 19.6 mmol) was suspended in 180 mL (10 v/w) of 7 N HCl–ethanol and stirred for 1 h at rt. To the suspension was added 360 mL of ethanol (20 v/w) and 180 mL of DMF (10 v/w) and the reaction mixture became clear. After 1.5 days, the precipitate which was formed was filtered and washed with 90 mL (5 v/w) of ethanol. The titled compound **36** was dried under reduced pressure at 30 °C and obtained as a yellow solid (3·HCl salt, 12.4 g, 13.4 mmol, 68.2%, 97.8% purity). ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.14 (t, 3H, J = 7.1 Hz), 1.78–1.93 (m, 2H), 2.19–2.24 (m, 2H), 2.49–2.55 (m, 2H), 2.71–3.06 (m, 6H), 3.22 (s, 3H), 4.01 (q, 2H, J = 7.1 Hz), 4.26–4.52 (m, 3H), 4.79 (s, 2H), 6.58 (br, 2H), 6.82 (d, 2H, J = 9.1 Hz), 7.06–7.23 (m, 10H), 7.46 (br, 1H), 7.65 (br, 1H), 7.71 (d, 2H, J = 9.0 Hz), 7.75–7.78 (m, 1H), 7.83 (d, 1H, J = 8.0 Hz), 8.08–8.14 (m, 2H), 8.50 (s, 1H). LC–MS m/z: 820.2 $(M+H)^+$.

4.1.20.6. MTX-α-PhePheNH–C_2H_4–NH-HA (30, high, middle and low binding ratio of MTX). Following the general procedure, sodium hyaluronate (500 mg, ca. 2300 kDa), compound **36** and TDA-1 provided an aqueous solution of conjugate **30**. The amount of compound **36** and TDA-1, and the specs of obtained conjugates were as follows:

Compd **36** (mmol)	TDA-1 (mmol)	Binding ratio (%)	Mw (kDa)
0.008	0.118	0.5	2170
0.020	0.105	1.3	2090
0.063	0.063	3.8	1910

4.2. Biology

4.2.1. Cathepsin fragmentation

5 μL of each compound (40 mM) was mixed with 1 μL cathepsin B (0.6 μg/μL, 3930 U/mg, from Sigma–Aldrich) and 494 μL of acetate buffer (40 mM sodium acetate buffer, pH 5.0) containing 5 mM reduced glutathione and 1 mM EDTA. 5 μL of each compound (40 mM) was mixed with 2 μL cathepsin D (1.3 μg/μL, 381 U/mg, from Sigma–Aldrich) and 493 μL of acetate buffer containing 1 mM EDTA. 5 μL of each compound (40 mM) was mixed with 1 μL cathepsin L (0.3 mg/mL, 1584 mU/mg, from Calbiochem) and 493 μL of acetate buffer containing 5 mM reduced glutathione and 1 mM EDTA. Each enzyme mixture was incubated at 37 °C for 24 h and analyzed by LC–MS (see the general analysis method).

4.2.2. In vitro experiments

HFLS (Cell Applications) was seeded at 5000 cells/well on a 96-well plate (Falcon) and cultured for 3 h in Iscove's modified Dulbecco's medium (IMDM) containing 5% FBS and 1X Antibiotic-Antimycotic (GIBCO BRL). After cellular attachment, TNF-α (recombinant human TNF-α, R&D Systems) (final concentration: 10 ng/mL) and each HA–MTX conjugate at each concentration was added, followed by cultivation for 5 days. Two days before the end of culture, 37 kBq/well of [^{3}H]-deoxyuridine was added to the cells (MORAVEK), followed by determining the uptake quantity (radioactivity) of [^{3}H]-deoxyuridine using a scintillation counter. Cells were recovered by unsticking with 0.05% trypsin-0.2% EDTA.

Radioactivity was calculated as a relative value (% of control), using, as the control, radioactivity in the group of cells cultured without any added test substance. Since the concentration of a free carboxyl group is 2.49×10^{-3} mol/L (1 g/401/L; 401 is the Mw of *N*-acetylglucosamine + glucuronic acid) for each 1 mg/mL of HA, the MTX concentration in each HA–MTX was calculated by multiplying the value by the conjugation rate of MTX. (For 1 mg/mL of HA–MTX conjugate with a conjugation rate of MTX of 1%, the concentration of MTX was 2.49×10^{-5} mol/L.) The value obtained was used to calculate the activity of cell proliferation inhibition (the IC_{50} value) by a 4-parameter logistic method using analysis software (GraphPad Prism 3.02).

4.2.3. In vivo experiments

Six-week-old male LEW/Crj rats were purchased from Charles River Laboratories Japan, Inc. The rats were sensitized with 0.5 mL of an emulsion prepared from a 2-mg/mL mBSA (Calbiochem) aqueous solution and an equal amount of Freund's complete adjuvant (Difco) injected into the flank at 21 and 14 days before inducing arthritis. The arthritis was induced by injecting 50 μL of a 2-mg/mL mBSA aqueous solution into the right knee joint. The left knee joint was untreated and served as the control. Knee joint swelling was assessed by measuring the width of each knee joint with calipers to define the left-right difference. The width of all knee joints was measured twice a week from immediately before inducing arthritis to two weeks after to calculate the area under the curve (AUC) over time for joint swelling. In addition, the AUC was calculated using the value relative to the HA-treated control group (% of control) which HA Mw were in the range of 1900−2500 kDa. HA and HA–MTX conjugates were administered into the right knee joint in an amounts of 50 μL before (7 and 1 day) and after (7 days) inducing arthritis.

At each measurement, the mean and standard deviation of AUC were calculated to perform an unpaired *t*-test between each test substance-treated group and the HA-treated group, and was judged to be significantly different if the probability level was less than 5%. Statistical analysis was performed using SAS version 6.12 (SAS Institute Japan).

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