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Methylcelluloses End-Functionalized with Peptides as Thermoresponsive Supramolecular Hydrogelators

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18   **Abstract** This paper describes the synthesis of methylcelluloses end-

19   functionalized with peptides and an investigation into their functions. We found

20   that aqueous solutions of methylcellulose end-functionalized not only with

21   carbohydrates but also with peptide segments, such as di(arginine) and

22   di(glutamic acid), behave as thermoresponsive supramolecular hydrogelators at

23   human-body temperature. The slow drug release from thermoresponsive

24   hydrogels of methylcelluloses end-functionalized with peptides is attributed to

25   ionic interactions between model drugs and peptide segments in these hydrogels.

26   Reactions of methylated cellobiose with di(arginine) and di(glutamic acid) were

27   used to determine optimum reaction conditions for the synthesis of

28   methylcelluloses end-functionalized with these peptide residues). The surface

29   activities, zeta potentials, thermal properties, hydrogelation behavior, and

30   cytotoxicities of these peptide-functionalized methylcelluloses are also discussed.

31

Highlights:

• Methylcelluloses end-functionalized with peptides were synthesized.

• Peptides-end-functionalized methylcelluloses behave as thermoresponsive
supramolecular hydrogelators at human-body temperature.

• The slow drug release from thermoresponsive hydrogels of methylcelluloses
end-functionalized with peptides was achieved.

Graphical Abstract:
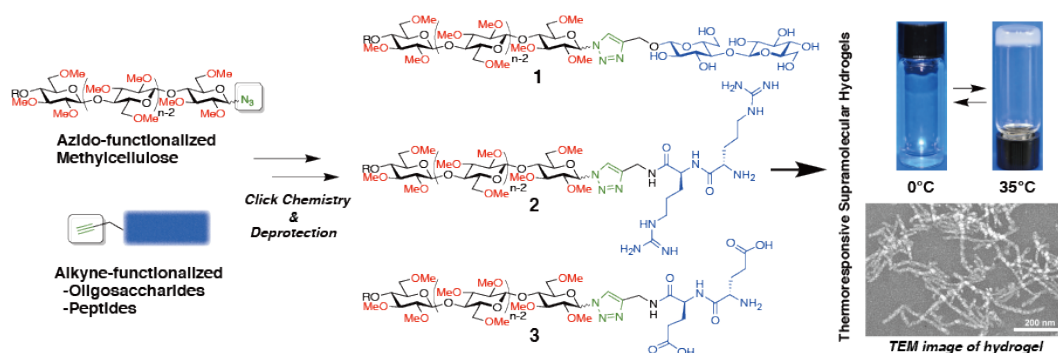
Introduction

Thermoresponsive hydrogels have received increased attention in recent years. In particular, thermoresponsive supramolecular hydrogels (Du et al. 2015) with lower critical solution temperatures (LCSTs) (Yamagami et al. 2018) enable the development of biomedical applications such as injectable drug-delivery technology (Baumann et al. 2009). While poly(N-isopropylacrylamide) (Fundueanu et al. 2009) is a well known thermoresponsive polymer derived from fossil resources, methylcellulose (MC) is a thermoresponsive material from renewable resources.

The degree of substitution (DS) of industrially produced MC is 1.8, and its aqueous solution exhibits thermoreversible hydrogelation at approximately 60 °C. The physical properties of aqueous MC solutions have received considerable academic attention (Desbrieres et al. 1998; Heymann 1935; Rees 1972; Savage 1957). Kato et al. concluded that the network junction points in MC gels are between 4 and 8 units long (Kato et al. 1978).

Our studies have focused on structure-property-function relationships of methylcellulose (Kamitakahara et al. 2009a; Kamitakahara et al. 2009b; Kamitakahara et al. 2008a; Kamitakahara et al. 2012; Kamitakahara and Nakatsubo 2010; Kamitakahara et al. 2006; Kamitakahara et al. 2007; Kamitakahara et al. 2009c; Kamitakahara et al. 2008b; Karakawa et al. 2002; Nakagawa et al. 2011a; Nakagawa et al. 2011b; Nakagawa et al. 2012a; Nakagawa et al. 2012b; Nakagawa et al. 2012c). Diblock methylcellulose bearing a sequence of at least ten 2,3,6-tri-O-methylglucosyl units and an unmodified cellobiosyl unit plays a crucial role in the thermoreversible hydrogelation of an aqueous MC solution (Nakagawa et al. 2011a).

67   Our detailed study on the structure-property relationships of methylcellulose

68   with a sequence of over twenty 2,3,6-tri-*O*-methylglucosyl units revealed the

69   thermoreversible hydrogelation properties of an aqueous diblock methylcellulose

70   solution at human-body temperature (Nakagawa et al. 2011a). In addition, Bodvik

71   et al. reported that MC forms fibrillar aggregates that were observed by cryogenic

72   transmission electron microscopy (Cryo-TEM) (Bodvik et al. 2010); we also

73   reported the same morphology (Nakagawa et al. 2012c), as did the group of

74   Lodge (Lott et al. 2013a; Lott et al. 2013b). Moreover, we found that well-defined

75   diblock methylcellulose self-assembles thermoresponsively into ribbon-like

76   nanostructures in water to form a thermoreversible hydrogel at human-body

77   temperature (Nakagawa et al. 2012c). The intermolecular interactions in the

78   fibrillar nanostructure of commercial MC in aqueous solution at LCST and in the

79   ribbon-like nanostructure of well-defined diblock methylcellulose are essentially

80   the same; hydrophobic interactions between a sequence of 2,3,6-tri-*O*-

81   methylglucosyl units, and hydrogen bonding between less-methylated glucosyl

82   units both play crucial roles during the aggregation of methylcellulose molecules

83   at LCST.

84   This finding prompted us to explore methylcellulose analogues that can be

85   synthesized by more simple and straightforward methods than glycosylation

86   (Nakagawa et al. 2011b). We selected the Huisgen 1,3-dipolar cycloaddition

87   reaction for the development of methylcellulose analogues (Nakagawa et al.

88   2012b). An aqueous solution of the newly synthesized methylcellulose analogue,

89   which included hydrophobic and hydrophilic segments connected by 1,2,3-

90   triazoles, exhibited thermoreversible hydrogelation properties (Nakagawa et al.

91   2012b) equivalent to that of a well-defined diblock methylcellulose (Nakagawa et

92   al. 2011b). Consequently, we developed a synthetic method for the end-

93   functionalization of methylcellulose to produce methylcellulosyl azide and

94   propargyl methylcelluloside (Kamitakahara et al. 2016). Moreover, not only did

95   nonionic segments, such as cellobiosyl units (as hydrophilic blocks) induce

96   thermoreversible hydrogelation, but ionic segments did as well (Yamagami et al.

97   2018), although it was crucial that the concentrations of the diblock

98   methylcellulose analogues in aqueous media remain at 4 wt%.

99   Our recent results suggested that it might be possible to install any functional

100   group at the methylcellulose end and retain thermoreversible hydrogelation

101   behavior at human-body temperature. Hence, we explored the end-

102   functionalization of methylcellulose with peptides. Peptides exhibit potent

103   biological activities due to the functional diversity of their amino-acid chains, and

104   play crucial roles in organisms that differ from those of oligo- and

105   polysaccharides. Therefore, the end-functionalizations of polysaccharide

106   derivatives with peptides are expected to yield a variety of functional materials

107   that exhibit thermoresponsive properties. An oligosaccharide-based synthetic

108   glycoprotein (Bonduelle and Lecommandoux 2013) has been reported by the

109   group of Lecommandoux. However, polysaccharide-derivative-*block*-

110   oligosaccharides (Breitenbach et al. 2017; de Medeiros Modolon et al. 2012) or

111   polysaccharide-derivative-*block*-oligopeptides have not received much attention

112   from researchers. Carbohydrate-based block copolymers with polyester (Fajardo

113   et al. 2014; Liu and Zhang 2007), poly(methyl methacrylate) (Dax et al. 2013;

114   Togashi et al. 2014), poly(styrene) (Loos and Müller 2002; Otsuka et al. 2013;

115   Yagi et al. 2010), poly(*N*-isopropylacrylamide) (Dax et al. 2013; Otsuka et al.

116   2012), poly(γ-benzyl-L-glutamate) (Kamitakahara et al. 2014), and poly(3-

117   hexylthiophene) (Sakai-Otsuka et al. 2017) polyisoprene (Hung et al. 2017), and

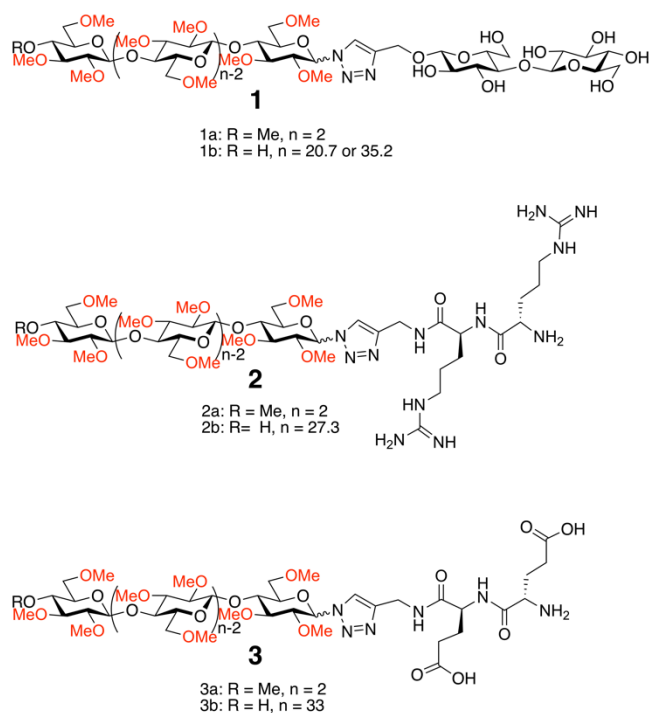118   poly(ethyleneoxide) (Akiyoshi et al. 1999) have been reported. Shoichet and her

119 colleagues focused on a physical blend of hyaluronan and methylcellulose

120 covalently linked to peptides for tissue-engineering purposes (Parker et al. 2016).

121 They modified methylcellulose with peptides by the "grafting to" method, in

122 which peptide moieties were randomly introduced onto the methylcellulose

123 backbone. In contrast, our method gives thermoresponsive hydrogels composed of

124 only methylcellulose and peptides, and are devoid of other polysaccharides. Our

125 new peptide-end-functionalized methylcelluloses are linear polysaccharide

126 derivatives with blocky structures that exhibit a broad range of new properties,

127 including thermoreversible hydrogelation at temperatures close to that of the

128 human body, formation of ribbonlike supramolecular nanostructures by self-

129 assembly, surface activities, and slow drug release from thermoresponsive

130 supramolecular hydrogel.

131   Herein, we describe the end-functionalization of tri-*O*-methylcellulose with

132 cationic di(arginine) and anionic di(glutamic acid) units as peptide segments in

133 order to develop new functionality (Chart 1). The synthesis, characterization, and

134 thermal properties of aqueous solutions of these materials, as well as their zeta

135 potentials, surface activities, in vitro cytotoxicities, and drug-release behavior

136 from their supramolecular hydrogel matrices, are discussed.

137

138

Chart 1. Compounds **1–3**.

1a: R = Me, n = 2
1b: R = H, n = 20.7 or 35.2

2a: R = Me, n = 2
2b: R= H, n = 27.3

3a: R = Me, n = 2
3b: R = H, n = 33

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141

Results and discussion

Synthesis of Peptide Segments

Peptide segments were synthesized following standard Fmoc methodology, as shown in Scheme 1. For side-chain protection, we used the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (pbf) group for the guanidine group, while the γ carboxylic acid group of glutamic acid was protected as a *tert*-butyl (*t*Bu) ester.

Propargylamine was reacted with *N*α-Fmoc-*N*ω-Pbf-L-arginine (**11**) to give the alkyne-functionalized *N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*-propargylamide (**12**) in 89% yield. The amide bond was successfully formed with DMT-MM (Kunishima et al. 1999a; Kunishima et al. 1999b) as the condensation reagent. The Fmoc group of compound **12** was removed to afford *N*ω-Pbf-L-arginine-*N*-

propargylamide (**13**) in 82% yield. *N*α-Fmoc-*N*ω-Pbf-L-arginine (**11**) was

coupled with *N*ω-Pbf-L-arginine-*N*-propargylamide (**13**) to produce *N*α-Fmoc-

*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-propargylamide (**9**) in 89% yield.

Fmoc-Glu(O*t*-Bu)-OH (**14**) was also reacted with propargylamine to produce

the alkyne-functionalized Fmoc-Glu(O*t*-Bu)-*N*-propargylamide (**15**) in

quantitative yield. Removal of the Fmoc group from compound **15** afforded

Glu(O*t*-Bu)-*N*-propargylamide (**16**) in quantitative yield. Compounds **14** and **16**

were coupled with DMT-MM to produce *N*α-Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-

propargylamide (**10**) in 95% yield.

Compounds **9** and **10**, bearing alkyne groups, are peptide-containing segments

for the end-functionalization of methylcellulose.
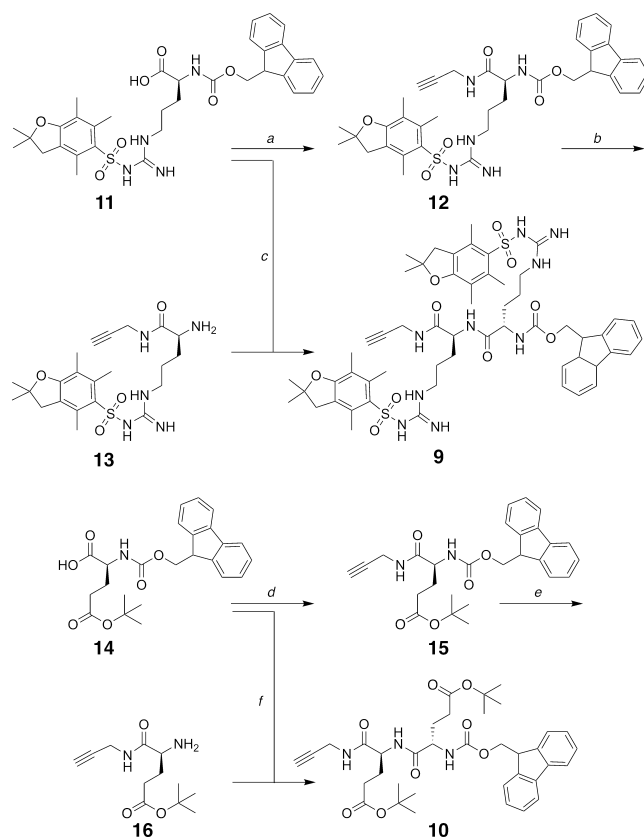

Scheme 1. Synthesis of peptide segments **9** and **10**. (a) propargylamine / DMT-MM / MeOH /r.t. / 3 h / 89%; (b) piperidine/CH₂Cl₂ / r.t. / 1 / 82%; (c) DMT-MM / MeOH / r.t. / 4 h / 89%; (d) propargylamine / DMT-MM / MeOH / r.t. 3 h / quantitative yield; (e) piperidine/CH₂Cl₂ / r.t. / 1 h / quantitative yield; (f) DMT-MM / MeOH / r.t. / 1 h / 95%.

Peptide-End-Functionalized Methylcelluloses by Huisgen 1,3-Dipolar Cycloadditions

Scheme 2 displays the synthetic routes to methylcelluloses end-functionalized

with peptides, as well as the control compounds. Trehalose-type methylated

cellobiose derivative **1a** and diblock methylcellulose analogues **1b** (Yamagami et

al. 2018) are control compounds for peptide-functionalized methylated cellobiose

derivatives **2a** and **3a**, and peptide-end-functionalized methylcelluloses **2b** and **3b**,

respectively.
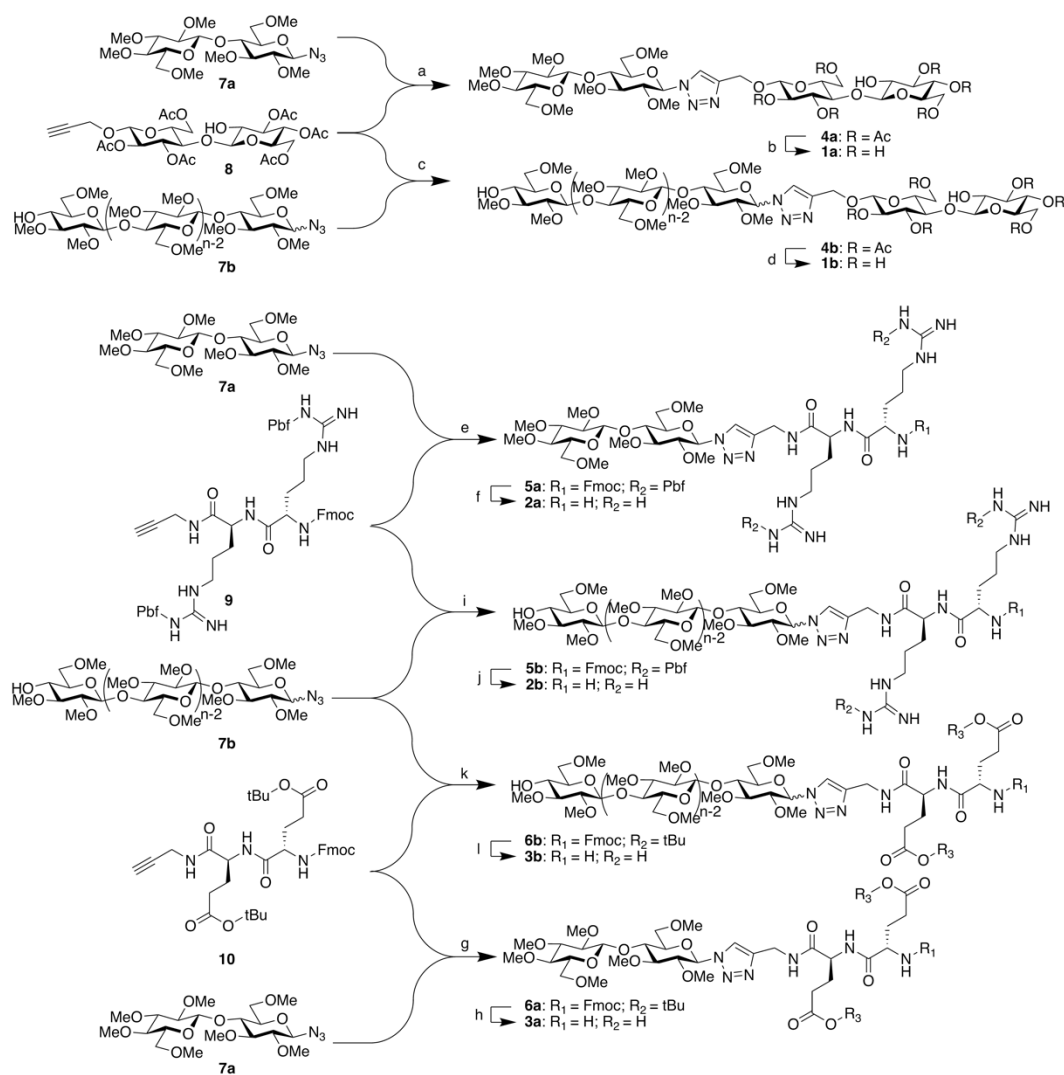
Scheme 2. Synthesis of methylcelluloses end-functionalized with peptides

a) Cu(I)Br / sodium ascorbate / PMDETA / DMF / r.t. / 21 h / 56%; b) 28% $NaOCH_3$ in MeOH / MeOH/THF / r.t. / 3 h / quantitative yield; c) CuBr / sodium ascorbate / PMDETA / MeOH/CH_2Cl_2/ r.t. / 4 d / 78.5%; d) 28% $NaOCH_3$ in MeOH / MeOH/THF / r.t. / overnight / quantitative yield; e) Cu(I)Br / sodium ascorbate / MeOH/CH_2Cl_2/ r.t. / 2 h/ 85%; f) piperidine /

185 CH$_2$Cl$_2$ / r.t. / 1 h / 65%; TFA/H$_2$O /37 °C / 4 h / 52%; g) CuBr / sodium ascorbate / MeOH /
186 CH$_2$Cl$_2$ / r.t. / 2 h / 87%; h) piperidine / CH$_2$Cl$_2$ / r.t. / 1 h / 47%; TFA/H$_2$O/ r.t. / 4 h / 58%; i)
187 CuSO$_4$·H$_2$O / sodium ascorbate / MeOH/CH$_2$Cl$_2$ / r.t. / 14 h/ quantitative yield; j)
188 piperidine/CH$_2$Cl$_2$ / r.t. / 4 h / 89%; TFA/H$_2$O /37 °C / 4 h/ 76%; k) CuSO$_4$·H$_2$O / sodium ascorbate
189 / MeOH/CH$_2$Cl$_2$ / r.t. / 14 h / 93%; l) piperidine/CH$_2$Cl$_2$ / r.t. / 4 h / 84%; TFA/H$_2$O /r.t. / 4 h / 52%.

190

191   Methylated cellobiose derivatives **2a** and **3a** were prepared in order to optimize

192 reaction conditions for the methylcellulose derivatives. Copper-assisted azide-

193 alkyne cycloaddition (CuAAC, the "click reaction") of 2,3,4,6-tetra-*O*-methyl-β-

194 D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl azide (**7a**) and

195 propargylated peptide segments **9** and **10** afforded methylated cellobiose

196 derivatives **5a** (85% yield) and **6a** (87% yield) bearing peptide residues,

197 respectively. Subsequent deprotections of the peptide segments gave methylated

198 cellobioses **2a** and **3a** end-functionalized with peptides.

199   The optimized reaction conditions for the cellobiose derivatives allowed us to

200 synthesize the peptide-functionalized methylcelluloses **2b** and **3b**. The CuAAC

201 reactions of tri-*O*-methylcellulosyl azide (**7b**) and propargylated peptide segments

202 **9** and **10** afforded methylcelluloses **5b** and **6b** end-functionalized with protected

203 peptides, respectively; deprotection of the peptide segments of these compounds

204 afforded peptide-functionalized methylcelluloses **2b** and **3b**.

205   While tri-*O*-methylcellulosyl azide (**7b**) is a mixture of both α- and β-anomers,

206 the cellobiosyl azide **7a** is only the β-anomer. Moreover, each methylcellulose

207 derivative **7b**, **4b**, **5b**, **6b**, **1b**, **2b**, and **3b** bears a single hydroxyl group at the C-4

208 position of its methylated glucosyl residue furthest from the azide. In contrast,

209 cellobiosyl azide derivative **7a** has no such hydroxyl group. We have reported the

210 synthesis of blockwise alkylated (1→4) linked trisaccharides, and found that the

211 anomeric configuration between the hydrophobic and hydrophilic segments

212 affects surface activity of the aqueous solution (Nakagawa et al. 2011c).

213

Characterization

Figure 1 displays the ^{1}H-NMR spectra of cellobiose derivatives **1a**, **2a**, and **3a** acquired in deuterium oxide. Proton resonances are assigned on the basis of two-dimensional NMR experiments (see experimental section). The triazole protons of compounds **1a**, **2a**, and **3a** appear at 8.24, 8.19, and 8.24 ppm, respectively. Carbon resonances of compounds **1a**, **2a**, and **3a** have also been assigned (see experimental section). Interestingly, the triazole proton of compound **3a** appears as a broad singlet. The methylene protons of compound **3a** adjacent to the triazole ring also appear as a broad peak at about 4.40–4.61 ppm. In addition, the C-1 proton of the cellobiosyl residue appears as a broad doublet at 5.75 ppm.

The transverse relaxation times T_2s of triazole proton and C1 proton adjacent to the triazole of anionic compound **3a** would be shorter than that of compounds **1a** and **2a**, although molecular weights of compounds **1a**, **2a**, and **3a** are similar. The transverse relaxation times T_2s of protons of an anionic surfactant, sodium dodecyl sulfate (SDS), depend on their positions; T_2s of the internal protons are shorter than that of methyl protons and methylene protons adjacent to the sulfate group (Yu et al. 2017). The transverse relaxation time T_2s of the protons of anionic compound **3a** showed the same tendency as the anionic surfactant, SDS.
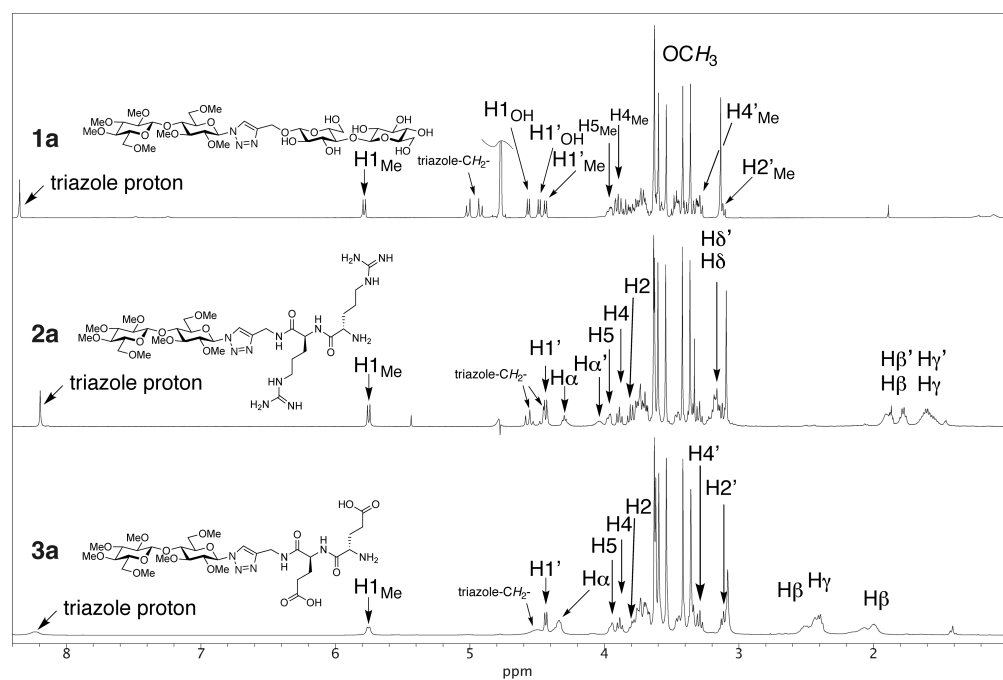
Figure 1. 500-MHz ^{1}H-NMR spectra of compounds **1a**, **2a**, and **3a** in D_2O.

Figure 2 displays the MALDI-TOF MS spectra of compounds **1a**, **2a**, and **3a**. Pseudomolecular sodium $[M+Na]^+$ and potassium $[M+K]^+$ adduct-ion peaks corresponding to compound **1a** appear at m/z 868.6 and 884.6, respectively. The pseudomolecular pr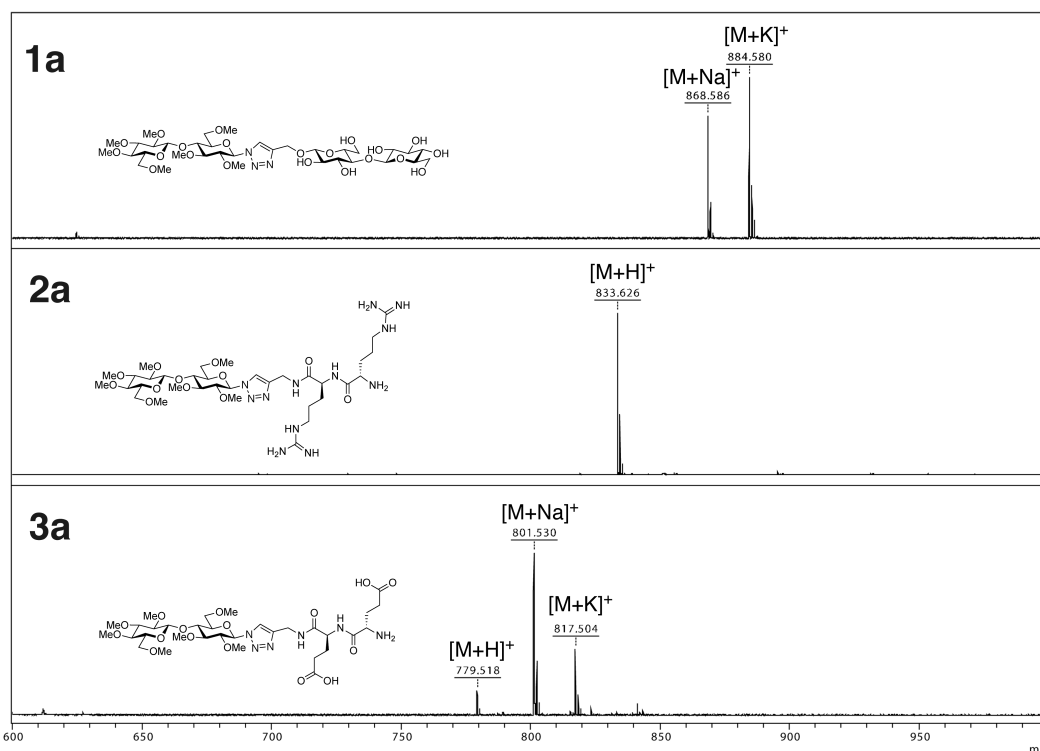oton adduct-ion peak $[M+H]^+$ of compound **2a** appears at m/z 833.6, while pseudomolecular proton $[M+H]^+$, sodium $[M+Na]^+$, and potassium $[M+K]^+$ adduct-ion peaks of compound **3a** appear at m/z 779.5, 801.5, and 817.5, respectively.

Figure 2. MALDI-TOF MS spectra of compounds **1a**, **2a**, and **3a**.

In summary, the NMR and MALDI-TOF MS data for cellobiose derivatives **1a**, **2a**, and **3a** reveal that the conditions for the CuAAC reactions and the deprotections of the peptide residues are appropriate. Hence, the reaction conditions developed for these cellobiose derivatives were used to synthesize the peptide-functionalized methylcelluloses **1b**, **2b**, and **3b**.
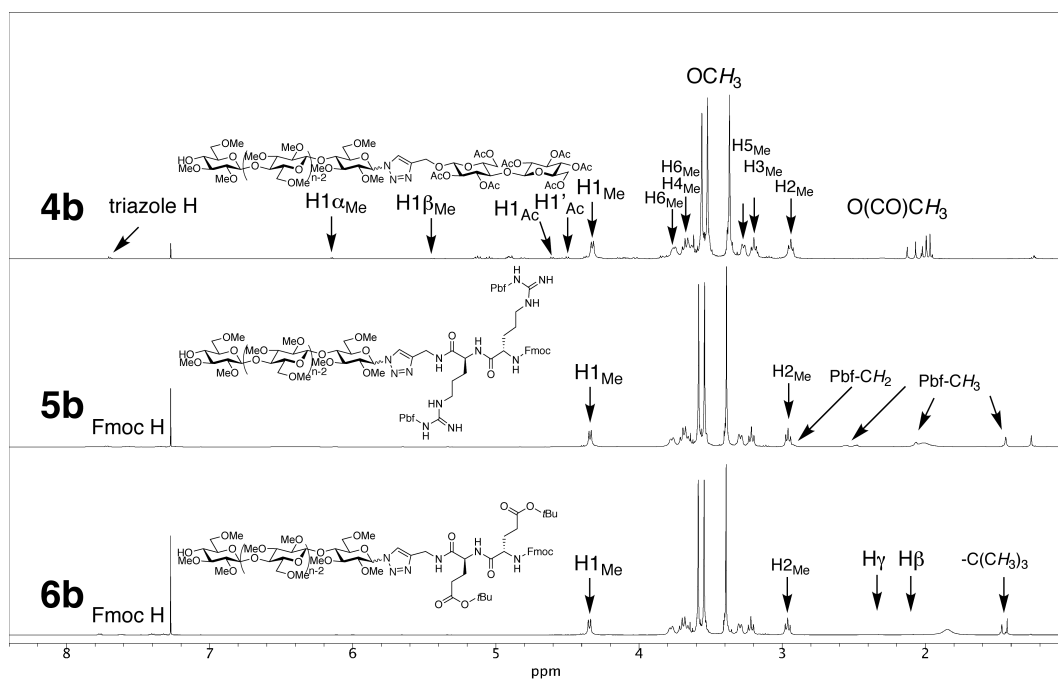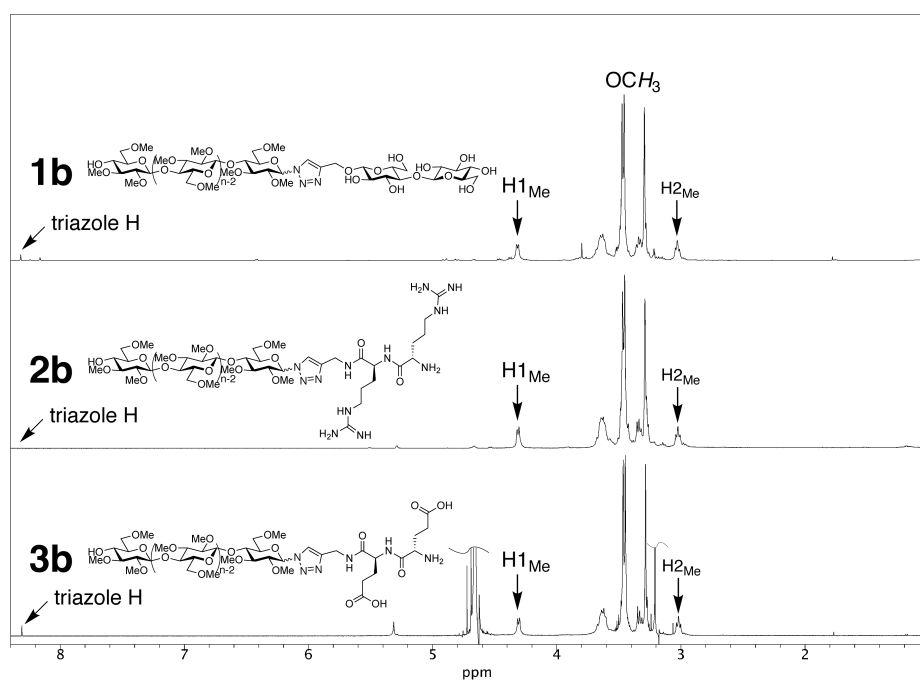
Figure 3. ^{1}H-NMR spectra of compounds **4b**, **5b**, and **6b** in CDCl$_3$.

Compounds **5b** and **6b** as well as the trehalose-type diblock methylcellulose analogue **4b** (Yamagami et al. 2018), as an authentic sample of a methylcellulose end-functionalized with a peptide, were synthesized according to the optimized reaction conditions for cellobiose derivatives **5a** and **6a**, as well as **4a**. Figure 3 displays the ^{1}H-NMR spectra of compounds **4b**, **5b**, and **6b** acquired in CDCl$_3$. The triazole proton of compound **4b** appears at 7.69 (β-anomer) and 7.70 (α-anomer) ppm (α/β ratio = 2/1) (Yamagami et al. 2018), while the triazole protons of compounds **5b** and **6b** were unable to be identified due to overlapping resonances associated with the aromatic protons of their Fmoc groups, although proton resonances of the peptide side-chains were observed.

The same deprotection procedures used for the cellobiosyl compounds **1a**, **2a**, and **3a** were used for the polymeric compounds, to give methylcellulose derivatives **1b**, **2b**, and **3b**. Figure 4 displays the ^{1}H-NMR spectra of

267   methylcellulose derivatives **1b**, **2b**, and **3b** acquired in D$_2$O. Resonances

268   corresponding to the peptide moieties at the methylcellulose ends are not clearly

269   evident in their spectra because of the higher DP of the methylcellulose residues,

270   compared to the cellobiosyl compounds **1a**, **2a**, and **3a**, as shown in Figure 1.

271   However, the triazole protons of compounds **2b** and **3b** appear at 8.42 and 8.43

272   ppm, respectively, although those of compound **1a** appear at 8.42, 8.35, and 8.27

273   ppm. This fact indicates that compounds **2b** and **3b** are end-functionalized with

274   peptide residues. The zeta potential of compounds **1b**, **2b**, and **3b** summarized in

275   Table 1 also indicate that the deprotections of compounds **2b** and **3b** were

276   successful.

277
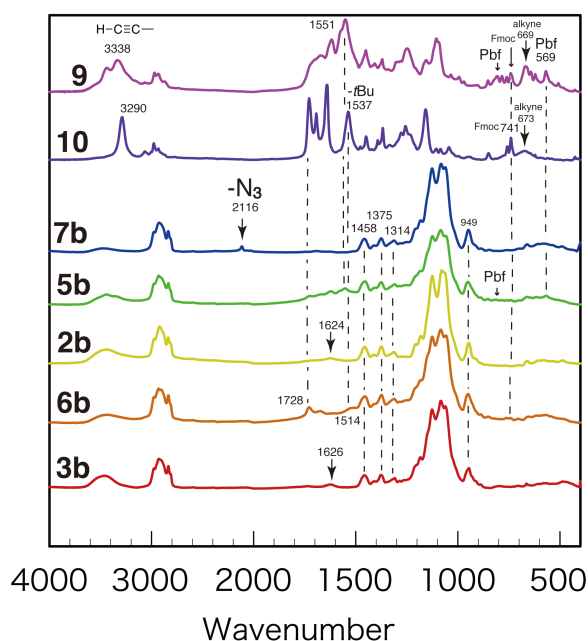

278   **Figure 4.** ^{1}H-NMR spectra of compounds **1b**, **2b**, and **3b** in D$_2$O.

279

280   Figure 5 displays the FT-IR spectra of compounds **2b**, **3b**, **5b**, **6b**, **7b**, **9**, and **10**.

281   Infrared absorption peaks corresponding to the protected di(arginine) and

282   di(glutamic acid) segments **9** and **10** appear in the spectra of methylcelluloses **5b**

283 and **6b** end-functionalized with the di(arginine), and di(glutamic acid) derivatives,

284 respectively. The infrared absorption peak associated with methylcellulosyl azide

285 **7b** was observed at 2116 cm^{-1}. After the Huisgen 1,3-dipolar cycloaddition of **7b**

286 with the di(arginine) or di(glutamic acid) derivatives **9** or **10**, infrared absorption

287 peaks associated with the azido group were not evident in the spectra of **5b** and **6b**,

288 which indicates that the Huisgen 1,3-dipolar cycloadditions were successful. The

289 infrared spectra of compounds **5b** and **6b** changed during deprotection to give **2b**

290 and **3b**. The infrared absorption peaks corresponding to Pbf and Fmoc groups of

291 MC-*b*-(Arg(Pbf))$_2$-Fmoc **5b** were not evident in the spectrum of MC-*b*-ArgArg **2b**.

292 The infrared absorption peaks associated with the *t*Bu and Fmoc groups of MC-*b*-

293 (Glu(O*t*Bu))$_2$-Fmoc **6b** were not evident in the spectrum of MC-*b*-GluGlu **3b**. The

294 FT-IR analyses of the end-functionalized methylcellulose derivatives provide us

295 with additional experimental data regarding the polymer reactions that occur at the

296 ends of these macromolecules. We conclude that methylcelluloses **2b** and **3b** end-

297 functionalized with peptides were successfully synthesized.

298

299 **Figure 5.** FT-IR spectra of compounds **2b**, **3b**, **5b**, **6b**, **7b**, **9**, and **10**.

Physical properties of compounds 1b, 2b, and 3b

301

Table 1 summarizes the structures, solution surface tensions, and zeta potentials

302

of compounds **1b**, **2b**, and **3b**, while Figure 6 shows the surface tensions of

303

solutions of compounds **1b**, **2b**, and **3b** as functions of concentration, measured at

304

23 °C. Compounds **2b** and **3b** exhibited similar surface-tension curves; the critical

305

micelle concentrations (CMCs) of the arginine-containing compound **2b** and the

306

glutamic-acid-containing compound **3b** are both 0.0035 mg/mL, slightly lower

307

than that of the cellobiose derivative **1b** (0.008 mg/mL). An ionic peptide residue

308

at the end of the methylcellulose unit improves its surface activity compared to

309

that of methylcellulose end-functionalized with the nonionic cellobiosyl residue.

310

In contrast, the CMCs of methylcelluloses **2b** and **3b** end-functionalized with

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peptides (0.0035 mg/mL) are clearly lower than that of commercial SM-4

312

methylcellulose (0.1 mg/mL).

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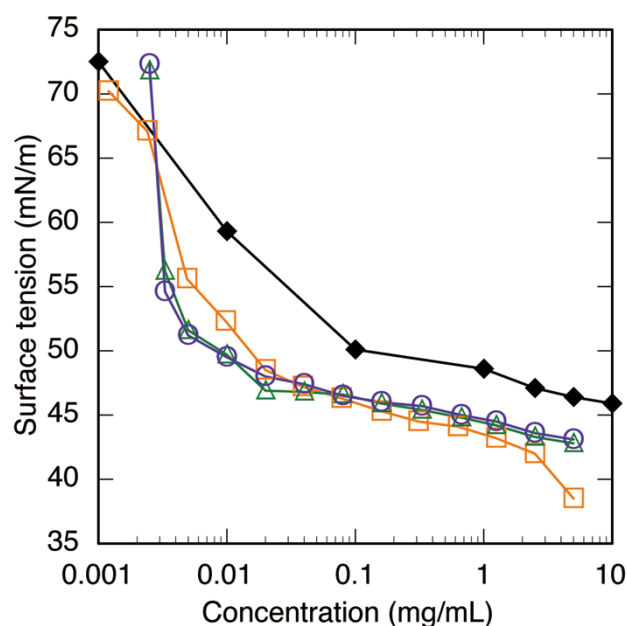
Figure 6. Surface tensions of compounds **1b**, (orange open squares), **2b** (green open triangles), and **3b** (purple open circles), and commercial MC (black solid diamonds), as functions of concentration.
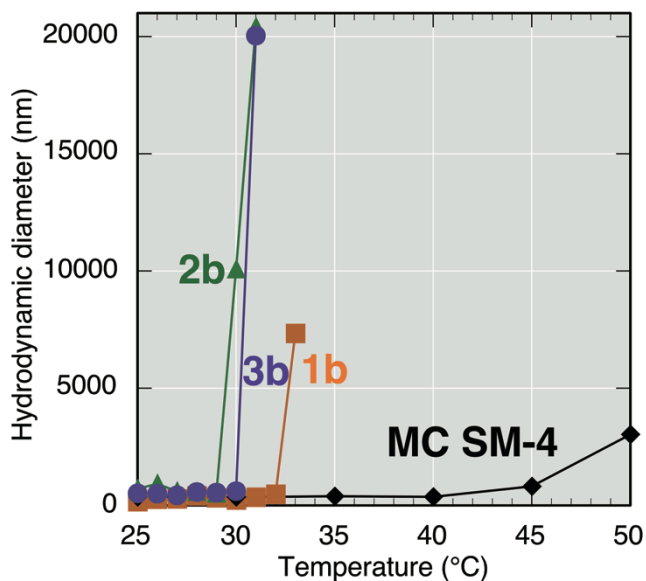
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317

318   **Table 1**. Surface tensions and zeta potentials of compounds **1b**, **2b**, and **3b**.

Comp. No.	Hydrophilic segment	DP_n of hydrophobic segment	DS	Surface tension (mN/m) at CMC	Critical micelle concentration (mg/mL)	Zeta potential (mV)
MC			1.8	50.1	0.1	
1b	Cellobiose	20.7	2.65	49.0	0.008	−11.5
2b	Arginine dimer	27.3	–	47.3	0.0035	−7.5
3b	Glutamic acid dimer	32.8	–	49.3	0.0035	−14.1

319

320   Figure 7 displays the temperature-dependence of the supramolecular

321   aggregation behavior of compounds **1b**, **2b**, and **3b** in water, obtained by dynamic

322   light scattering (DLS) experiments. The hydrodynamic diameters of compounds

323   **1b**, **2b**, and **3b** at 25 °C were determined to be 138, 706, and 476 nm, respectively.

324   Although the hydrodynamic diameter of commercial MC SM-4 gradually

325   increased at temperatures above approximately 45 °C, those of compounds **1b**, **2b**,

326   and **3b** increased dramatically at 33 °C, 30 °C, and 31 °C, respectively.

327

331

332 Figure 8 shows the DSC thermograms of 2 wt% aqueous solutions of

333 compounds **2b** and **3b**. Endothermic peaks appear at 31.5 °C and 32.6 °C for

334 compounds **2b** and **3b**, respectively. The endothermic temperatures of compounds

335 **2b** and **3b** are closely related to the supramolecular aggregation temperatures

336 determined by DLS. The endothermic peaks observed by DSC are attributed to

337 dehydration around the peptide-end-functionalized MCs **2b** and **3b**. Dehydration

338 around these cellulosic molecules promotes their supramolecular aggregation, as

339 shown in Figure 7.

340
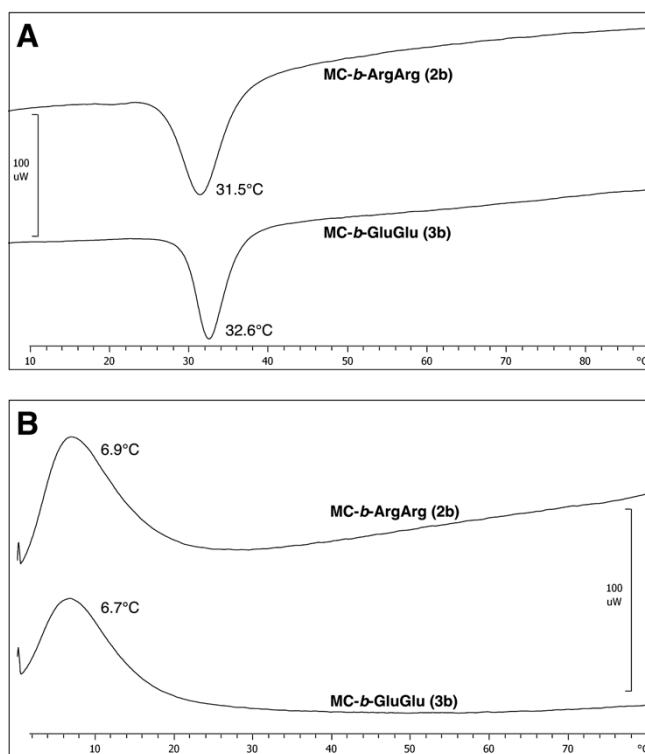
Figure 8. DSC thermograms of 2.0 wt% aqueous solutions of compounds **2b** and **3b**: (A) heating curves (3.5 °C/min) and (B) cooling curves (3.5 °C/min).

Table 2 displays images of 2.0 and 4.0 wt% aqueous solutions of compounds **1b**, **2b**, and **3b** at 0 and 35 °C. The 2 wt% aqueous solution of the cellobiose-functionalized MC **1b** forms a hydrogel at 35 °C. In contrast, the peptide-functionalized MCs **2b** and **3b** do not form hydrogels at 35 °C, rather their solutions became turbid at this temperature. In other words, the 2.0 wt% aqueous solutions of **2b** and **3b** phase separate at 35 °C. However, 4.0 wt% aqueous solutions of **1b**, **2b**, and **3b** form hydrogels at 35 °C. The concentrations of the thermally induced supramolecular structures of **2b** and **3b** are the keys to forming thermo-reversible hydrogels because 4.0 wt% aqueous solutions of the peptide-functionalized MCs **2b** and **3b** form hydrogels at 35 °C.

356 **Table 2.** Images of 2.0 and 4.0 wt% aqueous solutions of compounds **1b**, **2b**, and **3b**.

357

358 **Figure 9.** SEM images of the hydrogels of (a, b) **2b** and (c, d) **3b**. (e, f) TEM images of the
359 hydrogel of **2b**. Insets (b) and (d) are enlargements of regions in panels (a) and (c).

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Lyophilized hydrogels from compounds 2b and 3b

361

362   SEM images of lyophilized hydrogels of **2b** and **3b** are shown in Figures 9a–d.

363   We previously reported that methylcellulose end-functionalized with cellobiose

364   exhibits a layered structure (Yamagami et al. 2018). In contrast, the peptide-

365   functionalized methylcelluloses **2b** and **3b** form a three-dimensional mesh

366   structure (**2b**) and a spongy, foam-like structure (**3b**), indicating that the

367   hydrophilic segments at the ends of the methylcellulose units of the lyophilized

368   hydrogels have different nanostructures.

TEM images of the nanostructure of the thermoresponsive supramolecular hydrogel of 2b

369
370

371   TEM images of the hydrogel of compound **2b** are shown in Figures 9e–f, in

372   which regular stick-like structures with orthogonal widths of approximately 12 nm

373   and 15 nm, can be seen. The widths of the stick-like structures are constant,

374   although their lengths vary widely. The molecular length of compound **2b** is ~16

375   nm, which suggests that the longer width of a single rectangular structure is likely

376   to correspond to the molecular length of compound **2b**. The average thickness of

377   these structures was determined by atomic force microscopy to be approximately

378   10 nm (data not shown). Rectangular self-assemblies appear to form stick-like

379   structures, and their entanglements produce a macroscopic hydrogel.

Drug release from thermoresponsive supramolecular hydrogel matrices

380
381

382   The thermo-reversible supramolecular hydrogels of MCs **2b** and **3b** end-

383   functionalized with cationic and anionic peptides, respectively, are expected to

384   interact with anionic and cationic compounds, respectively. Therefore, we

385   investigated the ionic drug-release behavior of these hydrogels. To that end,

386   diclofenac sodium and imipramine were selected as model anionic and cationic

387   drugs, respectively.

388   Figure 10 displays the drug-release behavior of the thermoresponsive

389   supramolecular hydrogel matrices of **1b**, **2b**, and **3b**. These matrices exhibit

390   almost the same release behavior for diclofenac sodium (DFNa) at 37 °C at the

391   start of release testing. Approximately 28–29% of the DFNa was released from

392   hydrogel matrices **1b**, **2b**, and **3b** after 1 h. After 12 h, ~81% of the DFNa was

393   released from cationic hydrogel matrix **2b**, while ~100% of the DFNa was

394   released from the nonionic and anionic hydrogel matrices **1b** and **3b**. This

395   observation indicates that the cationic di(arginine) segment at the end of the MC

396   affects the release behavior of the anionic DFNa from the thermoresponsive

397   supramolecular hydrogel matrix.

398   In contrast, cationic imipramine interacts with anionic supramolecular hydrogel

399   matrix **3b**. At the beginning of imipramine-release testing (after 1 h of incubation)

400   27%, 28%, and 22% of the imipramine was released from hydrogel matrices **1b**,

401   **2b**, and **3b**, respectively. After 12 h, 98%, 96%, and 81% of the imipramine was

402   released from hydrogel matrices **1b**, **2b**, and **3b**, respectively, which indicates that

403   the anionic di(glutamic acid) segment at the end of the MC interacts with the

404   cationic imipramine. Ionic interactions between the cationic imipramine and the

405   anionic hydrogel matrix **3b** promote the relatively slow release of imipramine

406   from the thermoresponsive supramolecular hydrogel matrix.

24

407
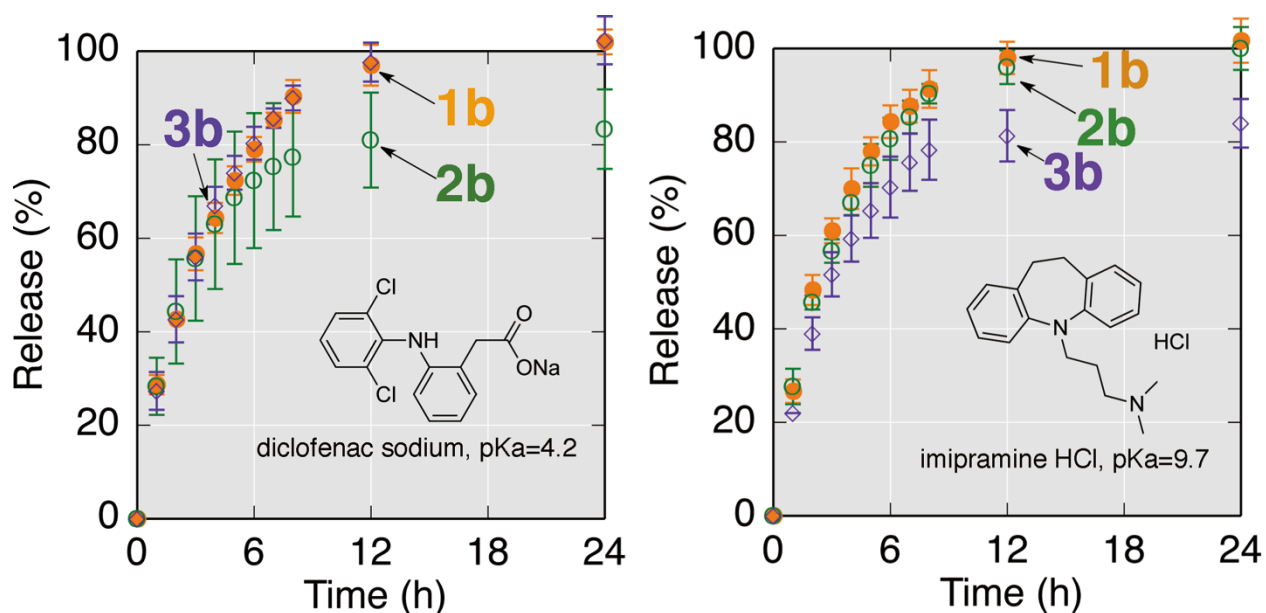
Figure 10. Drug-release behavior from the thermoresponsive supramolecular hydrogel matrices of
1b (orange solid circles), **2b** (green open circles), and **3b** (purple open diamonds).

To gain deep insight into the interaction between the model drugs and the MCs end-functionalized with peptides, we performed ^{1}H-NMR experiments involving DFNa, methylated cellobiose derivative **2a** (a model compound of the MC end-functionalized with di(arginine) **2b**), and a mixture of DFNa and **2a**, in deuterium oxide.

Figure 11 reveals changes in the chemical shifts corresponding to both compounds, namely DFNa and **2a**, following mixing. After mixing anionic DFNa and cationic **2a** in D_2O, the proton resonances of DFNa, numbered 1 to 8, were observed to shift downfield by 0.007, 0.010, 0.020, 0.033, 0.019, 0.019, 0.019, and 0.009 ppm, respectively, which indicates that all of the protons in DFNa became deshielded through the removal of electron density. In contrast, the methylene protons of the arginine side-chain appear at higher magnetic fields following mixing, which indicate that these protons have became shielded due to an increase in electron density. In addition, the triazole proton of **2a**, which

425   resonated at 8.19 ppm prior to mixing, also appeared at slightly higher magnetic

426   field following mixing with DFNa.

427   These observations indicate that supramolecular hydrogels of methylcellulose

428   end-functionalized with ionic peptides are expected to not only be

429   thermoresponsive but also pH responsive. This temperature/pH dual-

430   responsivenesses of compounds **2b** and **3b** are currently under investigation.

431
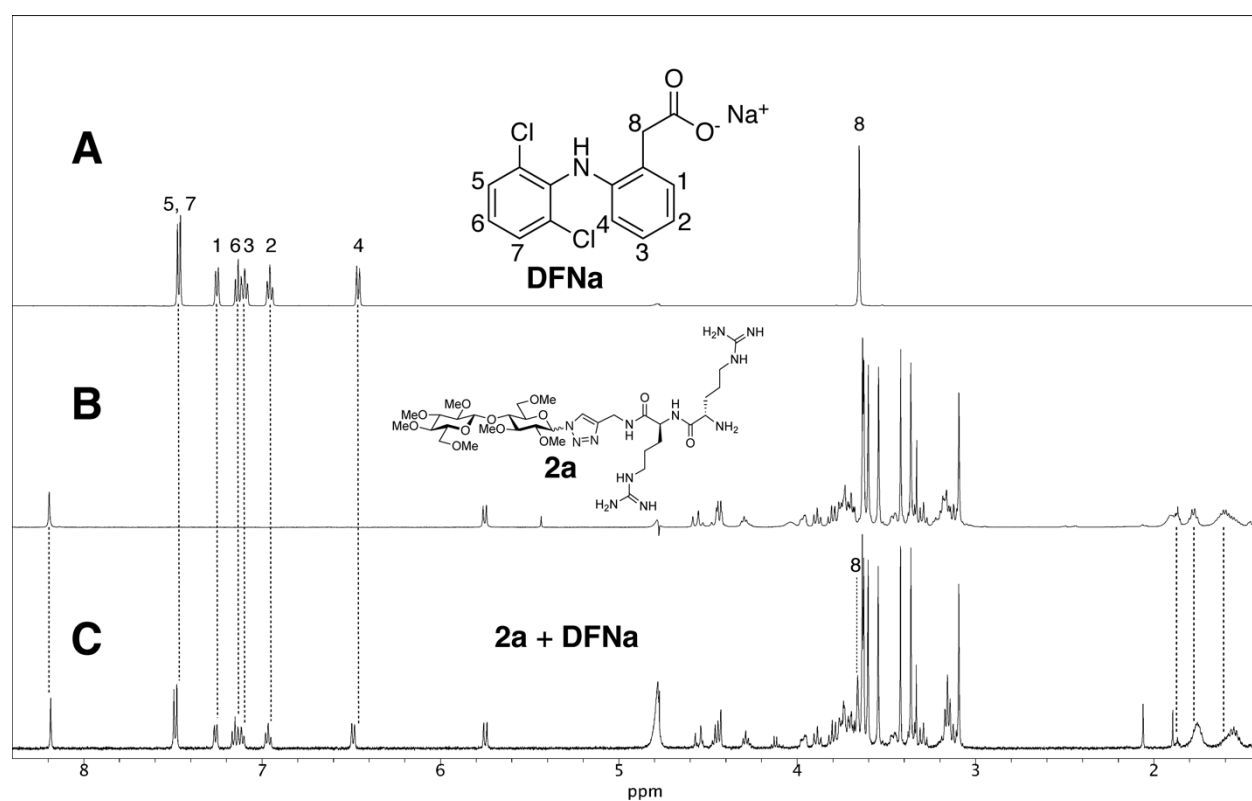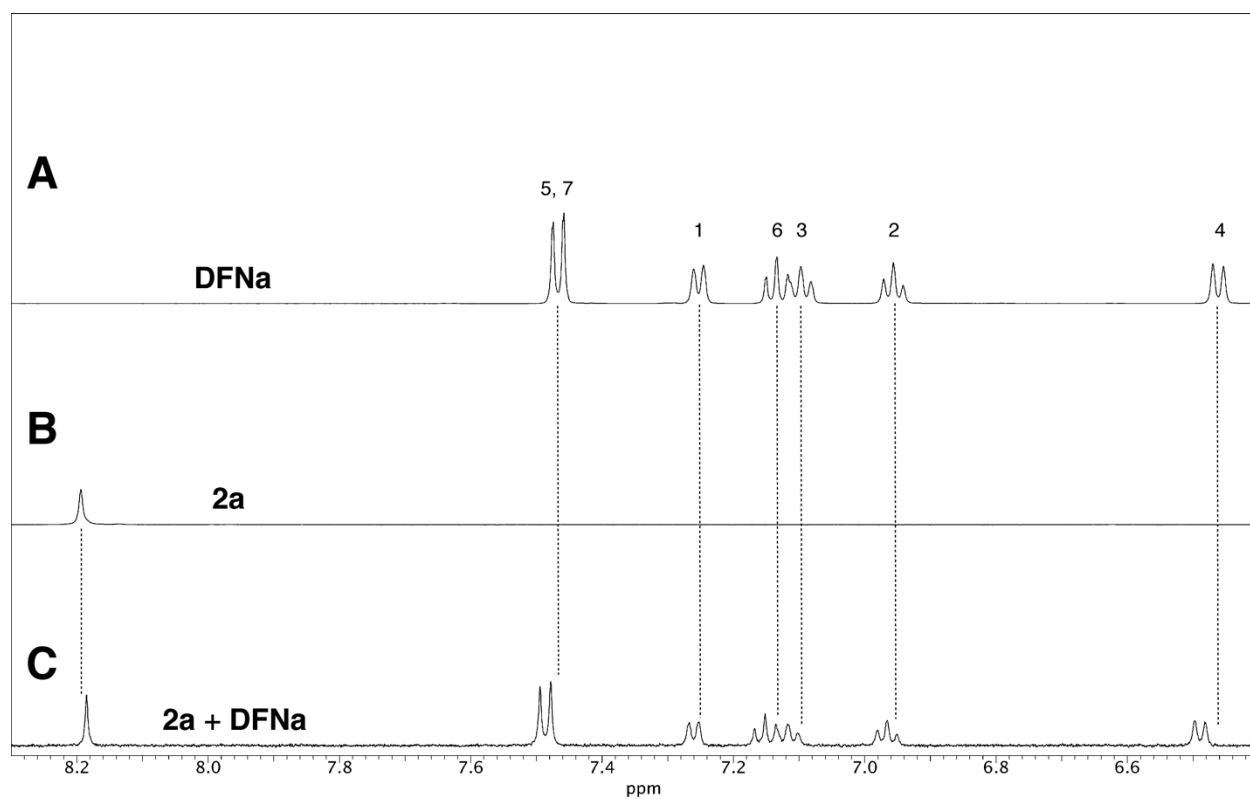
Figure 11. ^{1}H-NMR analyses of the interactions between compound **2b** and diclofenac sodium.
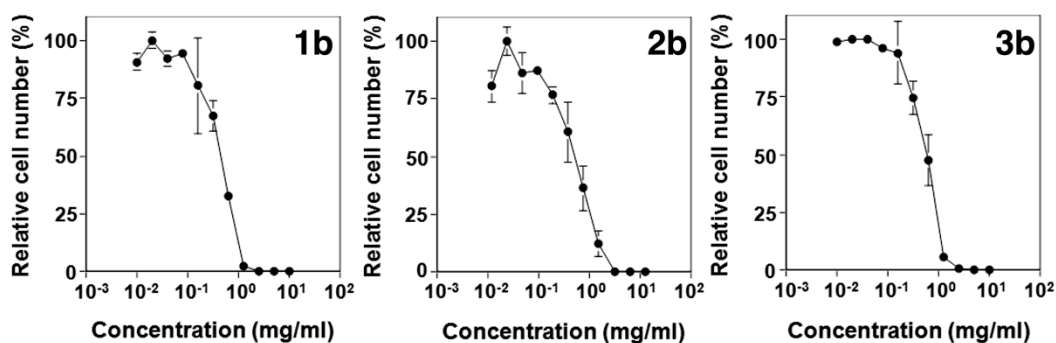
Figure 12. Inhibition of U937 cell growth by compounds **1b**, **2b**, and **3b**.

Cytotoxicities of compounds 1b, 2b, and 3b

ATP-based U937 histocytoma cell-viability assays reveal that the half maximal inhibitory concentrations (IC$_{50}$ values) of compounds **1b**, **2b**, and **3b** were 470, 560, and 600 μg/mL, respectively, as shown in Figure 12. As previously noted, 4 wt% aqueous solutions (40 mg/mL) of compounds **2b** and **2c** become supramolecular thermoresponsive hydrogels at 37 °C (see Table 2). The hydrogelation of an aqueous solution of compound **2b** or **2c** is likely to inhibit cell growth of suspension-type cells such as U937. However, the cytotoxicities of aqueous solutions of compounds **2b** and **2c**, determined by IC$_{50}$ values, have been evaluated correctly, despite some supramolecular structures potentially present in the aqueous solutions. In summary, methylcelluloses end-functionalized with peptides are essentially nontoxic to U937 histocytoma cells because IC$_{50}$ values of 0.6 wt% are considerably high.

Conclusion

Aqueous solutions of methylcelluloses end-functionalized with peptides exhibit supramolecular thermoresponsive hydrogelation behavior equivalent to those of block-functionalization methylcelluloses (Nakagawa et al. 2011a; Nakagawa et al. 2012c; Yamagami et al. 2018). The tri-*O*-methylcellulose block, as a hydrophobic segment (Kamitakahara et al. 2016), is a key structure for supramolecular

28

456 thermoresponsive hydrogelation. Investigation into the relationships between the

457 anomeric configuration at the reducing-end of the tri-*O*-methylcellulose block and

458 physico-chemical properties in solution is now in progress. We were able to not

459 only introduce carbohydrates (Yamagami et al. 2018) but also peptides as

460 hydrophilic segments at the ends of hydrophobic tri-*O*-methylcellulose units,

461 resulting in the syntheses of a pool of diblock methylcellulose analogues with

462 biological functions that retain thermoresponsive hydrogelation behavior at

463 human-body temperature. As methylcellulose is produced from cellulose, which is

464 a natural resource, methylcelluloses end-functionalized with peptides are expected

465 to be used in biomedical applications as, for example, comparatively safe

466 injectable hydrogels.

467

Experimental Section

Materials.

470   *N*α-(9-Fluorenylmethoxycarbonyl)-*N*ω-(2,2,4,6,7-

471 pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine   (*N*α-Fmoc-*N*ω-Pbf-L-

472 arginine) was purchased from Watanabe Chemical Industries, Ltd., Japan. *N*α-(9-

473 Fluorenylmethoxycarbonyl)glutamic acid γ-tert-butyl ester monohydrate (Fmoc-

474 Glu(O*t*-Bu)-OH) was purchased from Peptide Institute, Inc., Japan. Other

475 chemicals were purchased from Nacalai Tesque, Wako Pure Chemicals, Tokyo

476 Chemical Industry, and Sigma-Aldrich. All reagents and solvents were of

477 commercial grade and were used without further purification.

Synthesis.

479   ***N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*-propargylamide** (**12**)

480   *N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*-propargylamide (**12**) (Yang et al. 2011) was

481   prepared from *N*α-Fmoc-*N*ω-Pbf-L-arginine (**11**) (89% yield). Briefly, to a

482   solution of *N*α-Fmoc-*N*ω-Pbf-L-arginine (**11**, 200 mg) in methanol (5 mL) was

483   added propargylamine (19 μL) and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-

484   methylmorpholinium chloride (DMT-MM, 102 mg). The reaction mixture was

485   stirred for 3 h under nitrogen. The crude product was purified by preparative

486   silica-gel thin-layer chromatography (PTLC, eluent: 10% MeOH/CH_2Cl_2) to give

487   compound **12** (188 mg, 89% yield).

488   ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.42 (6H, CH$_3$, Pbf), 1.55-1.58 (m, 2H, Hγ),

489   1.85 (m, 2H, Hβ), 2.07 (s, 3H, CH$_3$, Pbf), 2.14 (t, 1H, *J*=2.0 Hz, CH$_2$CC<u>H</u>), 2.50

490   (s, 3H, CH$_3$, Pbf), 2.57 (s, 3H, CH$_3$, Pbf), 2.91 (s, 2H, CH$_2$, Pbf), 3.28 (m, 2H, Hδ),

491   3.98 (m, 2H, C<u>H$_2$</u>CCH), 4.14 (t, 1H, *J*=7.0 Hz, CH, Fmoc), 4.27 (m, 1H, Hα),

492   4.34 (d, 2H, *J*=7.0 Hz, Fmoc), 7.24-7.74 (arom. Fmoc)

493   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 12.4 (CH$_3$, Pbf), 17.9 (CH$_3$, Pbf), 19.3 (CH$_3$,

494   Pbf), 25.2 (Cγ), 28.5 (CH$_3$, Pbf), 29.1 (<u>C</u>H$_2$CCH), 30.1 (Cβ), 40.5 (Cδ), 43.1 (<u>C</u>H$_2$,

495   Pbf), 46.9 (<u>C</u>H, Fmoc), 54.1 (Cα), 67.1 (<u>C</u>H$_2$, Fmoc), 71.3 (CH$_2$C<u>C</u>H), 79.4

496   (CH$_2$<u>C</u>CH), 86.4 (C, Pbf), 117.5 (arom. Pbf), 119.9 (arom. Fmoc), 124.7 (arom.

497   Pbf), 125.1 (arom. Fmoc), 127.0 (arom. Fmoc), 127.7 (arom. Fmoc), 132.3 (arom.

498   Pbf), 138.4 (arom. Pbf), 141.1 (arom. Fmoc), 143.6 (arom. Fmoc), 143.7 (arom.

499   Fmoc), 156.3, 156.6 (CO Fmoc, quaternary C of guanidino group of arginine),

500   158.9 (-O-<u>C</u> arom. Pbf), 172.1 (Arg, Cα-<u>C</u>O-NH-)

501   ***N*ω-Pbf-L-arginine-*N*-propargylamide (13)**

502   *N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*-propargylamide (**12**, 200 mg) was dissolved in

503   50% piperidine/dichloromethane (2 mL). The reaction mixture was stirred for 1 h

504   at room temperature under nitrogen, after which it was concentrated to dryness.

505   The crude product was extracted with ethyl acetate, washed with water and brine,

506   dried over sodium sulfate, and concentrated to dryness. The residue was purified

507   by silica-gel column chromatography (eluent: 15% methanol/dichloromethane,

508   v/v) to afford compound **13** (111 mg, 82% yield).

509   ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.47 (6H, CH$_3$, Pbf), 1.56-1.63 (m, 2H, Hγ),

510   1.80 (m, 2H, Hβ), 2.09 (s, 3H, CH$_3$, Pbf), 2.22 (t, 1H, J=2.0 Hz, CH$_2$CC$\underline{H}$), 2.50

511   (s, 3H, CH$_3$, Pbf), 2.57 (s, 3H, CH$_3$, Pbf), 2.96 (s, 2H, CH$_2$, Pbf), 3.22 (m, 2H, Hδ),

512   3.45 (t, 1H, Hα), 3.98 (m, 2H, C$\underline{H}_2$CCH),

513   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 12.4 (CH$_3$, Pbf), 17.9 (CH$_3$, Pbf), 19.3 (CH$_3$,

514   Pbf), 25.4 (Cγ), 28.5 (CH$_3$, Pbf), 28.8 ($\underline{C}$H$_2$CCH), 31.9 (Cβ), 40.6 (Cδ), 43.2 ($\underline{C}$H$_2$,

515   Pbf), 54.2 (Cα), 71.3 (CH$_2$C$\underline{C}$H), 79.6 (CH$_2$$\underline{C}$CH), 86.4 (C, Pbf), 117.5 (arom.

516   Pbf), 124.6 (arom. Pbf), 132.2 (arom. Pbf), 138.2 (arom. Pbf), 156.4 (quaternary

517   C of guanidino group of arginine), 158.9 (-O-$\underline{C}$ arom. Pbf), 175.1 (Arg, Cα-$\underline{C}$O-

518   NH-)

519   ***N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-propargylamide**   **(9)**

520   (Morelli and Matile 2017)

521   To a solution of *N*α-Fmoc-*N*ω-Pbf-L-arginine (**11**, 101 mg, 1.2 equiv.) and *N*ω-

522   Pbf-L-arginine-*N*-propargylamide (**13**, 60 mg, 1.0 equiv.) in methanol (5 mL) was

523   added DMT-MM (43 mg, 1.2 equiv.). The reaction mixture was stirred for 4 h at

524   room temperature under nitrogen, and then concentrated to dryness. The crude

525   product was purified by PTLC (eluent: 10% methanol/dichloromethane) to afford

526   compound **9** as colorless crystals (126 mg, 89% yield).

527   ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.42 (12H, CH$_3$, Pbf), 1.63 (m, 4H, Hγ), 1.78

528   (m, 2H, Hβ), 1.92 (m, 2H, Hβ), 2.05 (s, 3H, CH$_3$, Pbf), 2.06 (s, 3H, CH$_3$, Pbf),

529   2.09 (1H, CH$_2$CC$\underline{H}$), 2.48 (s, 3H, CH$_3$, Pbf), 2.49 (s, 3H, CH$_3$, Pbf), 2.55 (s, 3H,

530   CH$_3$, Pbf), 2.57 (s, 3H, CH$_3$, Pbf), 2.90 (s, 2H, CH$_2$, Pbf), 2.91 (s, 2H, CH$_2$, Pbf),

531   3.21 (m, 4H, Hδ), 3.91-3.96 (m, 2H, CH$_2$CCH), 4.09 (t, 1H, J=7.0 Hz, CH, Fmoc),

532   4.27 (broad t, 1H, Hα), 4.32-4.36 (d, d, 1H, 1H, J=7.0 Hz, Fmoc), 7.17-7.73

533   (arom. Fmoc)

534   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 12.4 (CH$_3$, Pbf), 17.9 (CH$_3$, Pbf), 19.3 (CH$_3$,

535   Pbf), 25.6 (Cγ), 28.5 (CH$_3$, Pbf), 29.1 (CH$_2$CCH), 29.1 (Cβ), 40.0 (Cδ), 40.6 (Cδ),

536   43.1 (CH$_2$, Pbf), 46.9 (CH, Fmoc), 53.5 (Cα), 55.1 (Cα), 67.3 (CH$_2$, Fmoc), 71.3

537   (CH$_2$CCH), 79.4 (CH$_2$CCH), 86.4 (CH, Fmoc), 117.6 (arom. Pbf), 119.9 (arom.

538   Fmoc), 124.7 (arom. Pbf), 125.1 (arom. Fmoc), 127.0 (arom. Fmoc), 127.7 (arom.

539   Fmoc), 132.3 (arom. Pbf), 138.4 (arom. Pbf), 141.1 (arom. Fmoc), 143.7 (arom.

540   Fmoc), 156.4, 157.0 (CO Fmoc, quaternary C of guanidino group of arginine),

541   158.9 (-O-C arom. Pbf), 172.0 (Arg, Cα-CO-NH-), 173.4 (Arg, Cα-CO-NH-)

542   MALDI-TOF MS (m/z): calcd for C$_{56}$H$_{73}$N$_9$O$_{10}$S$_2$, 1095.49; found, [M+Na]$^+$ =

543   1116.2

544   FT-IR (KBr): 3440, 3338, 2970, 2934, 1670, 1618, 1551, 1450, 1369, 1248, 1155,

545   1105, 853, 806, 783, 760, 741 (Fmoc), 669, 642, 621, 569 (Pbf, -SO$_2$NH-) cm^{-1}

546

547   **Fmoc-Glu(Ot-Bu)-N-propargylamide** (**15**) (Aagren et al. 2006)

548   To a solution of Fmoc-Glu(Ot-Bu)-OH (**14**, 300 mg) in methanol (5 mL) was

549   added propargylamine (52 μL) and DMT-MM (102 mg). The reaction mixture

550   was stirred for 3 h under nitrogen. The crude product was purified by PTLC

551   (eluent: 10% MeOH/CH$_2$Cl$_2$) to give compound **15** as colorless crystals (324 mg,

552   quantitative yield).

553 ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.47 (s, 9H, CH$_3$, *t*Bu), 1.96 (m, 1H, Hβ), 2.09

554 (m, 1H, Hβ), 2.22 (t, 1H, *J*=2.0 Hz, CH$_2$CC<u>H</u>), 2.33 (m, 1H, Hγ), 2.43 (m, 1H,

555 Hγ), 4.05 (broad s, 2H, C<u>H$_2$</u>CCH), 4.21 (2H, CH Fmoc, Hα), 4.41 (m, 2H, Fmoc),

556 5.78 (d, 1H, *J*=7.5 Hz, NH), 6.70 (broad s, 1H, NH), 7.32 (dt, 2H, *J*=1.0 Hz, *J*=7.5

557 Hz, Fmoc), 7.32 (dt, 2H, *J*=1.0 Hz, *J*=1.0 Hz, Fmoc), 7.41 (dt, 2H, *J*=1.0 Hz,

558 Fmoc), 7.77 (d, 2H, *J*=7.5 Hz, Fmoc),

559 ^{13}C-NMR (125 MHz, CDCl$_3$): δ 28.0 (<u>C</u>H$_2$CCH), 28.0 (CH$_3$, *t*Bu), 39.2 (Cβ),

560 31.6 (Cγ), 47.1 (<u>C</u>H, Fmoc), 54.1 (Cα), 67.1 (<u>C</u>H$_2$, Fmoc), 71.8 (CH$_2$C<u>C</u>H), 79.1

561 (CH$_2$<u>C</u>CH), 81.2 (quaternary C of *t*Bu), 120.0 (arom. Fmoc), 125.0 (arom. Fmoc),

562 127.1 (arom. Fmoc), 127.7 (arom. Fmoc), 141.3 (arom. Fmoc), 143.7 (arom.

563 Fmoc), 156.3 (CO Fmoc), 171.0 (Glu, -Cα-<u>C</u>O-NH-), 173.0 (Glu, <u>C</u>δOO *t*Bu)

564 MALDI-TOF MS (*m/z*): calcd for C$_{27}$H$_{30}$N$_2$O$_5$, 462.22; found, [M+Na]$^+$ = 485.2,
565 [M+K]$^+$ = 501.2

566

567 **Glu(O*t*-Bu)-*N*-propargylamide** (**16**) (Aagren et al. 2006)

568 Fmoc-Glu(O*t*-Bu)-*N*-propargylamide (**15**, 308 mg) was dissolved in 50%

569 piperidine/dichloromethane (2 mL). The reaction mixture was stirred for 1 h at

570 room temperature under nitrogen, after which it was concentrated to dryness. The

571 crude product was extracted with ethyl acetate, washed with water and brine,

572 dried over sodium sulfate, and concentrated to dryness. The residue was purified

573 by silica-gel column chromatography (eluent: dichloromethane; eluent: 5%

574 methanol/dichloromethane, v/v) to afford compound **16** (166 mg, quantitative

575 yield).

576 ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.45 (s, 9H, CH$_3$ (*t*Bu)), 1.83 (m, 1H, Hβ), 2.09

577 (m, 1H, Hβ), 2.23 (t, 1H, *J*=2.0 Hz, CH$_2$CC<u>H</u>), 2.35 (m, 2H, Hγ), 3.44 (t, 1H, Hα),

578 4.05 (broad s, 2H, C<u>H$_2$</u>CCH),

579 ^{13}C-NMR (125 MHz, CDCl$_3$): δ 28.0 (CH$_3$ (tBu)), 28.8 (CH$_2$CCH), 30.1 (Cβ),

580 31.9 (Cγ), 54.4 (Cα), 71.4 (CH$_2$CCH), 79.6 (CH$_2$CCH), 80.7 (C(CH$_3$)$_3$), 172.8

581 (Glu, -Cα-CO-NH-), 174.1 (Glu, CδOO tBu)

582 MALDI-TOF MS (m/z): calcd for C$_{12}$H$_{20}$N$_2$O$_3$, 240.15; found, [M+Na]$^+$ = 263.9

583

584 **Nα-Fmoc-Glu(Ot-Bu)-Glu(Ot-Bu)-N-propargylamide (10)**

585 To a solution of Fmoc-Glu(Ot-Bu)-OH (**14**, 328 mg, 1.2 equiv.) and Glu(Ot-

586 Bu)-N-propargylamide (**16**, 148 mg, 1.0 equiv.) in methanol (5 mL) was added

587 DMT-MM (204 mg, 1.2 equiv.). The reaction mixture was stirred for 1 h at room

588 temperature under nitrogen, and then concentrated to dryness. The crude product

589 was purified by PTLC (eluent: 5% methanol/dichloromethane) to afford

590 compound **10** as colorless crystals (378 mg, 95% yield).

591 ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.43 (s, 9H, CH$_3$ (tBu)), 1.47 (s, 9H, CH$_3$ (tBu)),

592 1.96-2.15 (m, 4H, Hβ), 2.13 ((t, 1H, J=2.0 Hz, CH$_2$CCH), 2.33-2.47 (m, 4H, Hγ),

593 3.96-4.03 (broad s, 2H, CH$_2$CCH), 4.20 (1H, H (Fmoc)), 4.20-4.24 (1H, Hα),

594 4.38-4.44 (m, 2H, CH$_2$ (Fmoc)), 4.48-4.49 (m, 1H, Hα), 7.30-7.77 (8H, arom.

595 Fmoc)

596 ^{13}C-NMR (125 MHz, CDCl$_3$): δ 27.0 (Cβ'), 27.4 (Cβ), 28.0 (CH$_3$ (tBu)), 29.1

597 (CH$_2$CCH), 30.1 31.7 (Cγ'), 31.8 (Cγ), 52.6 (Cα'), 55.2 (Cα), 67.3 (CH$_2$, Fmoc),

598 71.3 (CH$_2$CCH), 79.4 (CH$_2$CCH), 81.1 (C(CH$_3$)$_3$), 81.3 (C(CH$_3$)$_3$), 120.0, 125.1,

599 127.0, 127.7, 141.2, 143.7, 156.5, 170.6 (Glu, -Cα-CO-NH-), 171.6 (Glu, -Cα-

600 CO-NH-), 173.0 (Glu, CδOO tBu), 173.2 (Glu, CδOO tBu)

601 MALDI-TOF MS (m/z): calcd for C$_{36}$H$_{45}$N$_3$O$_8$, 647.32; found, [M+Na]$^+$ = 670.4

602 FT-IR (KBr): 3290, 2978, 1728, 1694, 1639, 1537 (tBu), 1450, 1368, 1258,

603 1157, 851, 758, 741 (Fmoc), 673 cm^{-1}

604

2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

glucopyranosyl azide (7a)

A 60% suspension of sodium hydride in mineral oil (1.97 g, 82.1 mmol, 14.0

equiv.) was added to a solution of β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl

azide (Schamann and Schafer 2003; Ying and Gervay-Hague 2003) (1.29 g, 3.51

mmol) in DMF (30 mL) at 0 °C. The mixture was stirred at 0 °C for 1 h under

nitrogen. Methyl iodide (3.1 mL, 49.8 mmol, 14.2 equiv.) was then added to the

reaction mixture at 0 °C. After 2 h, the mixture was warmed to room temperature

and stirred for 2 h. The reaction was monitored by analytical thin-layer

chromatography (TLC). Methanol (0.43 mL) was added to deactivate the sodium

hydride. The mixture was concentrated and the crude product was extracted with

ethyl acetate, washed with distilled water, brine, dried over Na_2SO_4, and

concentrated to dryness. The residue was purified by silica-gel column

chromatography (eluent: 2:1 (v/v) ethyl acetate/*n*-hexane) to give colorless

crystals (**7a**, 0.799 g, 49% yield).

^{1}H-NMR (500 MHz, CDCl$_3$): δ 2.86 (dd, 1H, *J*=8.0 Hz, *J*=8.5 Hz, H2'), 1H,

2.90 (t, 1H, *J*=9.0 Hz, H3'), 3.06 (t, 1H, *J*=9.5 Hz, H3'), 3.12 (t, 1H, *J*=9.5 Hz,

H4'), 3.16 (ddd, 1H, *J*=2.0 Hz, *J*=4.0 Hz, *J*=9.5 Hz, H5'), 3.19 (t, 1H, *J*=9.0 Hz,

H3), 3.32 (s, 3H, OC*H*$_3$), 3.34 (s, 3H, OC*H*$_3$), 3.36 (ddd, 1H, *J*=2.0 Hz, *J*=4.0 Hz,

J=10.0 Hz, H5), 3.46 (s, 3H, OC*H*$_3$), 3.48 (s, 3H, OC*H*$_3$), 3.52 (dd, 1H, *J*=4.0 Hz,

J=11.0 Hz, H6), 3.53 (s, 6H, OC*H*$_3$), 3.56 (s, 3H, OC*H*$_3$), 3.56 (dd, 1H, *J*=2.0 Hz,

J=10.5 Hz, H6'), 3.62 (dd, 1H, *J*=9.0 Hz, *J*=10.0 Hz, H4), 3.63 (dd, 1H, *J*=2.0 Hz,

J=11.0 Hz, H6), 3.67 (dd, 1H, *J*=4.0 Hz, *J*=11.0 Hz, H6), 4.23 (d, 1H, *J*=7.5 Hz,

H1'), 4.40 (d, 1H, *J*=8.5 Hz, H1)

629   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 103.2 (C1'), 89.9 (C1), 86.9 (C3'), 84.9 (C3),

630   84.0 (C2'), 82.8 (C2), 79.3 (C4'), 77.2 (C4), 76.9 (C5), 74.7 (C5'), 71.2 (C6'), 70.1

631   (C6), 60.8, 60.6, 60.6, 60.6, 60.3, 59.3, 59.2 (OCH$_3$)

632

633   Tri-O-methylcellulosyl azide (**7b**) Compound **7b** was prepared according to our

634   previous paper (Kamitakahara et al. 2016).

635   FT-IR (KBr): 3478, 2926, 2836, 2116 (N$_3$), 1458, 1375, 1314, 1182, 1061, 949,

636   664 cm^{-1}

637

638   **2-Propynyl 2,3,6-tri-O-acetyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-acetyl-**

639   **β-D-glucopyranoside (8)** Compound **8** was prepared according to our previous

640   paper (Yamagami et al. 2018).

641

642   **1-[2,3,4,6-Tetra-O-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-O-methyl-β-**

643   **D-glucopyranosyl]-4-[2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl-(1→4)-2,3,6-**

644   **tri-O-acetyl-β-D-glucopyranosyloxymethyl]-1H-1,2,3-triazole (4a)**

645   Azide **7a** (117 mg, 0.252 mmol) and glycoside **8** (170 mg, 0.252 mmol) were

646   dissolved in DMF (9 mL). Copper(I) bromide (361.5 mg, 2.52 mmol, 10 equiv.),

647   sodium ascorbate in water (998.5 mg/1.26 mL, 20 equiv.), and N,N,N',N'',N''-

648   pentamethyldiethylenetriamine (PMDETA, MW = 173.3, d = 0.83 g/mL, 0.5 mL,

649   10 equiv.) were added to the solution at room temperature. The reaction mixture

650   was stirred for 21 h. The insoluble component was then removed by filtration and

651   washed with dichloromethane. The washings and filtrate were combined and

652   concentrated, and the DMF was azeotropically removed with ethanol. The crude

653   product was purified by silica-gel column chromatography (eluent: 10%

36

654 MeOH/CH$_2$Cl$_2$) to give 1-[2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-

655 2,3,6-tri-*O*-methyl-β-D-glucopyranosyl]-4-[2,3,4,6-tetra-*O*-acetyl-β-D-

656 glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxymethyl]-1*H*-

657 1,2,3-triazole (**4a**, 161.3 mg, 56% yield).

658 ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.97, 1.99, 2.01, 2.01, 2.04, 2.09, 2.15 (COC*H*$_3$),

659 2.95 (t, 1H, *J*=8.5 Hz, H2'$_{Me}$), 3.15 (t, 1H, *J*=9.0 Hz, H3'$_{Me}$), 3.19 (s, 3H, OC*H*$_3$),

660 3.21 (t, 1H, *J*=9.0 Hz, H4'$_{Me}$), 3.26 (ddd, 1H, *J*=2 Hz, *J*=3.5 Hz, *J*=9.5 Hz, H5'$_{Me}$),

661 3.33 (s, 3H, OC*H*$_3$), 3.43 (s, 3H, OC*H*$_3$), 3.44 (t, 1H, *J*=9.0 Hz, H3$_{Me}$), 3.55 (s, 3H,

662 OC*H*$_3$), 3.57 (s, 3H, OC*H*$_3$), 3.64 (s, 6H, OC*H*$_3$), 3.6-3.7 (H6'$_{Me}$, H2$_{Me}$, H5$_{Me}$, H5$_{Ac}$,

663 H5'$_{Ac}$), 3.75 (dd, 1H, *J*=4.0 Hz, *J*=11.0 Hz, H6$_{Me}$), 3.79 (t, 1H, *J*=9.5 Hz, H4$_{Ac}$),

664 3.82 (dd, 1H, *J*=9.0 Hz, *J*=10.0 Hz, H4$_{Me}$), 4.05 (dd, 1H, *J*=2.5 Hz, *J*=12.5 Hz,

665 H6$_{Ac}$), 4.11 (dd, 1H, *J*=5.0 Hz, *J*=12.0 Hz, H6'$_{Ac}$), 4.34 (d, 1H, *J*=8.0 Hz, H1'$_{Me}$),

666 4.36 (dd, 1H, *J*=4.0 Hz, *J*=12.0 Hz, H6$_{Ac}$), 4.52 (d, 1H, *J*=8.0 Hz, H1'$_{Ac}$), 4.56 (dd,

667 1H, *J*=2.0 Hz, *J*=12.0 Hz, H6'$_{Ac}$), 4.62 (d, 1H, *J*=8.0 Hz, H1$_{Ac}$), 4.81 (d, 1H,

668 *J*=13.0 Hz, OC*H*$_2$-triazole), 4.92 (d, 1H, *J*=13.0 Hz, OC*H*$_2$-triazole), 4.9-4.95

669 (H2$_{Ac}$, H2'$_{Ac}$), 5.07 (t, 1H, *J*=9.5 Hz, H4'$_{Ac}$), 5.15 (t, 1H, *J*=9.0 Hz, H3$_{Ac}$) , 5.15 (t,

670 1H, *J*=9.0 Hz, H3'$_{Ac}$), 5.47 (d, 1H, *J*=9.5 Hz, H1$_{Me}$), 7.69 (s, 1H, triazole)

671 ^{13}C-NMR (125 MHz, CDCl$_3$): δ 20.5, 20.6, 20.6, 20.9 (CO*C*H$_3$), 59.0 (O*C*H$_3$),

672 59.4 (O*C*H$_3$), 60.3 (O*C*H$_3$), 60.4 (O*C*H$_3$), 60.7 (O*C*H$_3$), 60.7 (O*C*H$_3$), 60.8

673 (O*C*H$_3$), 61.5(C6'$_{Ac}$), 61.7 (C6$_{Ac}$), 62.8 (O*C*H$_2$-triazole), 67.8 (C4'$_{Ac}$), 70.0 (C6$_{Me}$),

674 71.2 (C6'$_{Me}$), 71.4 (C2$_{Ac}$ or C2'$_{Ac}$), 71.6 (C2$_{Ac}$ or C2'$_{Ac}$), 72.0 (C5$_{Ac}$), 72.4 (C5'$_{Ac}$),

675 72.8 (C3$_{Ac}$ or C3'$_{Ac}$), 72.9 (C3$_{Ac}$ or C3'$_{Ac}$), 74.8 (C5$_{Me}$), 76.3(C4$_{Ac}$), 77.9 (C4$_{Me}$),

676 79.3 (C4'$_{Me}$), 82.1 (C2$_{Me}$), 84.0 (C2'$_{Me}$), 85.2 (C3$_{Me}$), 87.0 (C3'$_{Me}$), 87.3 (C1$_{Me}$),

677 99.6 (C1$_{Ac}$), 100.7 (C1'$_{Ac}$), 103.3 (C1'$_{Me}$), 122.0 (triazole *C*H), 144.3 (O-CH$_2$-*C*=),

678 169.0, 169.3, 169.6, 169.7, 170.2, 170.5 (*C*OCH$_3$)

679   **1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-**

680   **D-glucopyranosyl]-4-[β-D-glucopyranosyl-(1→4)-β-D-**

681   **glucopyranosyloxymethyl]-1*H*-1,2,3-triazole (1a)**

682   Sodium methoxide (28%) in methanol (0.01 mL, 1.4 equiv.) was added at room

683   temperature to a solution of 1-[2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-

684   (1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl]-4-[2,3,4,6-tetra-*O*-acetyl-β-D-

685   glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxymethyl]-1*H*-

686   1,2,3-triazole (**4a**, 146 mg, 0.128 mmol) in MeOH (1 mL) and THF (1 mL). The

687   mixture was stirred for 3 h at room temperature. The solution was neutralized with

688   Amberlyst H⁺. The Amberlyst H⁺ was removed by filtration and washed with

689   MeOH. The combined filtrate and washings were concentrated to dryness to give

690   1-[2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

691   glucopyranosyl]-4-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyloxymethyl]-

692   1*H*-1,2,3-triazole (**1a**, 108.3 mg, quantitative yield).

693 ^{1}H-NMR (500 MHz, D$_2$O): δ 3.01 (t, 1H, *J*=9.5 Hz, H2'$_{Me}$), 3.03 (s, 3H, OCH$_3$),

694   3.18 (t, 1H, *J*=10.0 Hz, H4'$_{Me}$), 3.21 (H2$_{OH}$), 3.21 (H2'$_{OH}$), 3.25 (t, 1H, *J*=10.0 Hz,

695   H3'$_{Me}$), 3.25 (s, 3H, OCH$_3$), 3.28 (H4'$_{OH}$), 3.31 (s, 3H, OCH$_3$), 3.33-3.38 (m, 1H,

696   H5$_{OH}$), 3.36 (t, 1H, *J*=9.0 Hz, H3'$_{OH}$), 3.38 (H5'$_{Me}$), 3.43 (s, 3H, OCH$_3$), 3.49 (s,

697   3H, OCH$_3$), 3.5 (H3$_{OH}$), 3.50-3.56 (H4$_{OH}$), 3.52 (s, 6H, OCH$_3$), 3.56-3.7 (H6$_{Me}$ and

698   H6'$_{Me}$), 3.58-3.86 (H6$_{OH}$ and H6'$_{OH}$), 3.62 (H3$_{Me}$), 3.73 (t, 1H, *J*=9.0 Hz, H2$_{Me}$),

699   3.79 (t, 1H, *J*=10.0 Hz, H4$_{Me}$), 3.86 (m, 1H, H5$_{Me}$), 4.33 (d, 1H, *J*=8.0 Hz, H1'$_{Me}$),

700   4.38 (d, 1H, *J*=8.5 Hz, H1'$_{OH}$), 4.46 (d, 1H, *J*=7.5 Hz, H1$_{OH}$), 4.81 (d, 1H, *J*=13.0

701   Hz, OC*H*$_2$-triazole), 4.90 (d, 1H, *J*=13.0 Hz, OC*H*$_2$-triazole), 5.68 (d, 1H, *J*=9.0

702   Hz, H1$_{Me}$), 8.24 (s, 1H, triazole C*H*)

703   ^{13}C-NMR (125 MHz, D$_2$O): δ 61.0 (O$\underline{C}$H$_3$), 61.1 (O$\underline{C}$H$_3$), 62.3 (O$\underline{C}$H$_3$), 62.6

704   (O$\underline{C}$H$_3$), 62.6 (C6$_{OH}$ or C6'$_{OH}$), 62.7 (O$\underline{C}$H$_3$), 63.2 (O$\underline{C}$H$_3$), 63.2 (C6$_{OH}$ or C6'$_{OH}$),

705   64.6 (OC$\underline{H}_2$-triazole), 72.1 (C4'$_{OH}$), 72.4 (C6$_{Me}$ or C6'$_{Me}$), 73.1 (C6$_{Me}$ or C6'$_{Me}$),

706   75.4 (C2'$_{OH}$), 75.8 (C2$_{OH}$), 76.1 (C3'$_{OH}$), 76.9 (C3$_{OH}$), 77.4 (C5$_{OH}$), 78.1 (C5$_{OH}$ or

707   C5'$_{Me}$), 78.6 (C4$_{Me}$), 78.7 (C5$_{OH}$ or C5'$_{Me}$), 79.3 (C5$_{Me}$), 81.1 (C4$_{OH}$), 81.4 (C4'$_{Me}$),

708   83.9 (C2$_{Me}$), 85.4 (C2'$_{Me}$), 86.0 (C3$_{Me}$), 87.6 (C3'$_{Me}$), 88.9 (C1$_{Me}$), 104.1 (C1$_{OH}$),

709   105.1 (C1'$_{Me}$), 105.2 (C1'$_{OH}$), 127.5 (triazole CH), 146.9 (O-CH$_2$-C=)

710   MALDI-TOF MS (*m/z*): calcd for C$_{34}$H$_{59}$N$_3$O$_{21}$, 845.36; found, [M+Na]$^+$ =

711   868.586, [M+K]$^+$ = 884.580

712

713   **1-(2,3,6-Tri-*O*-methyl-cellulosyl)-4-[2,3,4,6-tetra-*O*-acetyl-β-D-**

714   **glucopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyloxymethyl]-1*H*-**

715   **1,2,3-triazole (4b)**

716   Compound **4b** was prepared according to our previous paper (Yamagami et al.

717   2018). GPC analysis: $M_n = 7.4 \times 10^3$, $M_w / M_n = 1.4$, $DP_n = 34.8$.

718

719   **1-(2,3,6-Tri-*O*-methyl-cellulosyl)-4-[β-D-glucopyranosyl-(1→4)-β-D-**

720   **glucopyranosyloxymethyl]-1*H*-1,2,3-triazole (1b)**

721   Compound **1b** was prepared according to our previous paper (Yamagami et al.

722   2018). GPC analysis: $M_n = 6.8 \times 10^3$, $M_w / M_n = 1.4$, $DP_n = 33$.

723

724   **1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-**

725   **D-glucopyranosyl]-4-(*N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-**

726   **methyl)-1*H*-1,2,3-triazole (5a)**

727   To a solution of 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-

728   methyl-β-D-glucopyranosyl azide (**7a**, 25 mg, 0.054 mmol) and Fmoc-Arg(Pbf)-

729   Arg(Pbf)-NH-CH$_2$CCH (**9**, 59 mg, 0.054 mmol) in 3 mL of

730   methanol/dichloromethane (1/4, v/v) were added CuBr (77 mg, 0.054 mmol, 10

731   equiv.) and aqueous sodium ascorbate (213 mg/269 μL). The reaction mixture

732   was stirred under nitrogen at room temperature for 2 h. The mixture was purified

733   by preparative TLC (eluent: 1:9 (v/v) methanol/dichloromethane) to give 1-

734   [2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

735   glucopyranosyl]-4-(*N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-methyl)-

736   1*H*-1,2,3-triazole (**5a**, 71 mg, 0.045 mmol, 85% yield).

737   ^{1}H-NMR (500 MHz, CDCl$_3$): δ: 1.44 (C*H*$_3$, Pbf), 1.59 (Hγ), 1.85 (Hβ), 2.06-

738   2.09 (CH$_3$, Pbf), 2.49-2.59 (CH$_3$, Pbf), 2.92 (CH$_2$, Pbf), 2.94 (CH$_2$, Pbf), 2.92 (t,

739   1H, *J*=9.0 Hz, H2'$_{Me}$), 3.11 (OC*H*$_3$), (3.12 (t, 1H, *J*=9.0 Hz, H3'$_{Me}$), 3.15 (t, 1H,

740   *J*=9.0 Hz, H4'$_{Me}$), 3.21-3.25 (m, 1H, H5'$_{Me}$), 3.22 (OC*H*$_3$), 3.26 (Hδ), 3.40 (OC*H*$_3$),

741   3.35 (t, 1H, *J*=8.5 Hz, H3$_{Me}$), 3.38 (OC*H*$_3$), 3.52 (OC*H*$_3$), 3.53 (OC*H*$_3$), 3.59

742   (OC*H*$_3$), 3.62 (OC*H*$_3$), 3.5-3.7 (H6'$_{Me}$, H2$_{Me}$, H5$_{Me}$), 3.6-3.75 (2H, H6$_{Me}$), 3.75 (t,

743   1H, *J*=9.5 Hz, H4$_{Me}$), 4.1-4.35 (broad s, Hα), 4.18-4.24 (1H, H (Fmoc)), 4.30

744   (H1'$_{Me}$), 4.32-4.36 (C*H*$_2$, Fmoc), 4.43-4.60 (broad d, broad d, 2H, NH-C*H*$_2$-

745   triazole), 5.41 (H1$_{Me}$), 5.60 (NH), 6.0-6.6 (NH), 7.2-7.8 (arom. H, Fmoc)

746   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 12.5 (CH$_3$, Pbf), 17.9 (CH$_3$, Pbf), 19.3 (CH$_3$,

747   Pbf), 25.6 (Cγ), 28.6 (CH$_3$, Pbf), 29.7 (Cβ), 40.8 (Cδ), 43.2 (*C*H$_2$, Pbf), 47.0 (*C*H,

748   Fmoc), 53.6 (Cα), 54.4 (Cα), 58.7, 59.3, 60.4, 60.6, 60.8 (O*C*H$_3$), 67.3 (*C*H$_2$,

749   Fmoc), 70.1 (C6), 71.2 (C6'), 74.7 (C5'), 77.2 (C4), 77.4 (C5), 79.3 (C4'), 81.8

750   (C2), 84.0 (C2'), 85.2 (C3), 86.4 (CH, Fmoc), 86.4 (CH, Fmoc), 86.9 (C3'), 87.0

751   (C1), 103.3 (C1'), 117.6 (arom. Pbf), 119.9 (arom. Fmoc), 120.0, 122.5 (triazole

752 *C*H), 124.6 (arom. Pbf), 124.9 (arom. Fmoc), 125.2 (arom. Fmoc), 127.1 (arom.

753 Fmoc), 127.6 (arom. Fmoc), 132.3 (arom. Pbf), 132.7 (arom. Pbf), 138.3 (arom.

754 Pbf), 141.1 (arom. Fmoc), 143.7 (arom. Fmoc), 144.5 (O-CH$_2$-*C*=), 156.4 (arom.

755 Pbf), 158.7 (-O-$\underline{C}$ arom. Pbf), 172.2 (Arg, Cα-$\underline{C}$O-NH-), 173.5 (Arg, Cα-$\underline{C}$O-NH)

756   MALDI-TOF MS (*m/z*): calcd for C$_{75}$H$_{108}$N$_{12}$O$_{20}$S$_2$, 1560.72; found, [M+H]$^+$ =

757 1559.755, [M+Na]$^+$ = 1581.722, [M+K]$^+$ = 1597.671

758

759   **1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-**

760   **D-glucopyranosyl]-4-(Arg-Arg-NH-CH$_2$)-1*H*-1,2,3-triazole (2a)**

761   1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

762 glucopyranosyl]-4-(*N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-methyl)-

763 1*H*-1,2,3-triazole (**5a**, 61 mg, 0.039 mmol) was dissolved in

764 piperidine/dichloromethane (1/1, v/v, 1 mL). The reaction mixture was stirred

765 under nitrogen at room temperature for 1 h and then concentrated to dryness. The

766 crude product was purified by preparative TLC (eluent: 15:85 (v/v)

767 methanol/dichloromethane) to give 1-[2,3,4,6-tetra-*O*-methyl-β-D-

768 glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl]-4-(*N*ω-Pbf-L-

769 arginine-*N*ω-Pbf-L-arginine-*N*-methyl)-1*H*-1,2,3-triazole (34 mg, 0.025 mmol,

770 65% yield; MALDI-TOF MS (*m/z*): calcd for C$_{60}$H$_{96}$N$_{12}$O$_{18}$S$_2$, 1336.64; found,

771 [M+Na]$^+$ = 1359.5).

772   1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

773 glucopyranosyl]-4-(*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-methyl)-1*H*-1,2,3-

774 triazole (31 mg, 0.023 mmol) was dissolved in TFA/distilled water (8/2, v/v, 1.0

775 mL) and stirred under nitrogen at 37 °C for 4 h. The reaction mixture was

776 concentrated to dryness and the crude product was purified by gel-filtration

column chromatography (LH-20, eluent: methanol) to give 1-[2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl]-4-(Arg-Arg-NH-CH$_2$)-1*H*-1,2,3-triazole (**2a**, 10 mg, 0.012 mmol, 52% yield).

^{1}H-NMR (500 MHz, D$_2$O): δ 1.5-1.7 (m, 4H, Hγ and Hγ'), 1.73-1.83 (m, 2H, Hβ), 1.85-1.95 (m, 2H, Hβ'), 3.09 (s, 3H, OC*H$_3$*), 3.12 (t, *J*=9.5 Hz, H2'), 3.12-3.25 (m, 4H, Hδ and Hδ'), 3.29 (t, *J*=9.0 Hz, H4'), 3.36 (t, *J*=9.0 Hz, H3'), 1H, 3.36 (s, 3H, OC*H$_3$*), 3.42 (s, 3H, OC*H$_3$*), 3.46 (m, 1H, H5'), 3.54 (s, 3H, OC*H$_3$*), 3.60 (s, 3H, OC*H$_3$*), 3.63 (s, 3H, OC*H$_3$*), 3.63 (s, 3H, OC*H$_3$*), 3.67-3.75 (2H, H6'), 3.70 (t, 1H, *J*=9.5 Hz, H3), 3.72-3.78 (2H, H6), 3.81 (t, 1H, *J*=9.0 Hz, H2), 3.89 (t, 1H, *J*=9.0 Hz, H4), 3.97 (m, 1H, H5), 4.04 (broad s, 1H, Hα'), 4.30 (t, 1H, *J*=6.5 Hz, Hα (near sugar residue)), 4.44 (d, 1H, *J*=8.0 Hz, H1'), 4.44 (d, 1H, *J*=14.5 Hz, NHC*H$_2$*-triazole), 4.57 (d, 1H, *J*=15.5 Hz, OC*H$_2$*-triazole), 5.75 (d, 1H, J=9.5 Hz, H1), 8.19 (s, 1H, C*H*, triazole)

^{13}C-NMR (125 MHz, D$_2$O): δ 26.0 (Cγ'), 26.9 (Cγ), 30.6 (Cβ or Cβ'), 30.8 (Cβ or Cβ'), 36.8 (NHC*H$_2$*-triazole), 43.0 (Cδ or Cδ'), 43.1 (Cδ or Cδ'), 51.5 (Cα'), 56.6 (Cα (near sugar residue)), 61.0 (OC*H$_3$*), 61.1 (OC*H$_3$*), 62.4 (OC*H$_3$*), 62.5 (OC*H$_3$*), 62.6 (OC*H$_3$*), 62.7 (OC*H$_3$*), 63.2 (OC*H$_3$*), 72.5 (C6), 73.1 (C6'), 76.1 (C5'), 78.7 (C4), 79.3 (C5), 81.4 (C4'), 84.0 (C2), 85.4 (C2'), 86.0 (C3), 87.8 (C3'), 88.9 (C1), 105.1 (C1'), 126.2 (CH of triazole), 147.5 (quaternary C of triazole). 159.4 (quaternary C of guanidino group of arginine), 159.4 (quaternary C of guanidino group of arginine), 175.8 (Cα-*C*O-NH-), 175.9 (Cα'-*C*O-NH-)

MALDI-TOF MS (*m/z*): calcd for C$_{34}$H$_{64}$N$_{12}$O$_{12}$, 832.48; found, [M+H]$^+$ = 833.63

801   1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-

802   D-glucopyranosyl]-4-[Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1*H*-1,2,3-

803   triazole (6a)

804   To a solution of 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-

805   methyl-β-D-glucopyranosyl azide (**7a**, 25 mg, 0.054 mmol) and Fmoc-

806   Glu(O*t*Bu)-Glu(O*t*Bu)-NH-CH$_2$CCH (**10**, 35 mg, 0.054 mmol) in 3 mL of

807   methanol/dichloromethane (1/4, v/v) were added CuBr (77 mg, 0.054 mmol, 10

808   equiv.) and aqueous sodium ascorbate (213 mg/269 μL). The reaction mixture

809   was stirred under nitrogen at room temperature for 2 h, after which it was purified

810   by PTLC (eluent: 1:9 (v/v) methanol/dichloromethane) to give 1-(2,3,4,6-tetra-*O*-

811   methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl)-4-

812   [*N*α-Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1*H*-1,2,3-triazole (52 mg, 0.047

813   mmol, 87% yield).

814   ^{1}H-NMR (500 MHz, CDCl$_3$): δ: 1.42 (s, 9H, CH$_3$ (*t*Bu)), 1.46 (s, 9H, CH$_3$ (*t*Bu)),

815   1.90-2.02 (m, 2H, Hβ), 2.02-2.20 (m, 2H, Hβ), 2.26-2.48 (m, 4H, Hγ), 2.96 (t, 1H,

816   *J*=8.5 Hz, H2'$_{Me}$), 3.14 (t, 1H, *J*=9.0 Hz, H3'$_{Me}$), 3.15 (s, 3H, OC*H*$_3$), 3.20 (t, 1H,

817   *J*=8.5 Hz, H4'$_{Me}$), 3.23-3.28 (H5'$_{Me}$), 3.30 (s, 3H, OC*H*$_3$), 3.39 (t, 1H, *J*=8.5 Hz,

818   H3$_{Me}$), 3.42 (s, 3H, OC*H*$_3$), 3.54 (s, 3H, OC*H*$_3$), 3.56 (s, 3H, OC*H*$_3$), 3.62 (s, 3H,

819   OC*H*$_3$), 3.63 (s, 3H, OC*H*$_3$), 3.54-3.58 (m, H5$_{Me}$), 3.58-3.67 (3H, H6$_{Me}$, H6'$_{Me}$),

820   3.64 (t, 1H, *J*=9.0 Hz, H2$_{Me}$), 3.6-3.75 (dd, 1H, *J*=4.5 Hz, *J*=11.0 Hz, H6$_{Me}$), 3.79

821   (t, 1H, *J*=9.5 Hz, H4$_{Me}$), 4.12 (m, 1H, Hα), 4.20 (t, 1H, *J*=4.5 Hz, C*H* (Fmoc)),

822   4.32 (d, 1H, *J*=8.0 Hz, H1$_{Me}$), 4.35 (broad d, 2H, *J*=7.0 Hz, CH$_2$ (Fmoc)), 4.43 (m,

823   1H, Hα), 4.54 (broad d, *J*=5.5 Hz, NH-C*H*$_2$-triazole), 5.39 (d, 1H, *J*=9.0 Hz, H1$_{Me}$),

824   6.07 (d, *J*=6.0 Hz, N*H*-Cα), 7.28-7.78 (8H, arom. Fmoc), 7.74 (broad s, triazole

825   H)

826   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 27.0 (triazole-CH$_2$-NH-Cα-Cβ-), 27.3 (Cβ-Cα-

827 NH-Fmoc), 28.0 (CH$_3$ (tBu)), 28.0 (CH$_3$ (tBu)), 31.8 (triazole-CH$_2$-NH-Cα-Cβ-

828 Cγ), 31.8 (Cγ-Cβ-Cα-NH-Fmoc), 35.1 (triazole-CH$_2$-NH-), 47.1 (CH, Fmoc),

829 53.0 (triazole-CH$_2$-NH-CO-Cα-NH-), 55.3 (-CO-Cα-NH-Fmoc), 59.0 (OCH$_3$),

830 59.3 (OCH$_3$), 60.3 (OCH$_3$), 60.3 (OCH$_3$), 60.6 (OCH$_3$), 60.7 (OCH$_3$), 60.8 (OCH$_3$),

831 67.1 (CH$_2$, Fmoc), 70.0 (C6), 71.1 (C6'), 74.7 (C5'), 77.1 (C4), 77.8 (C5), 79.3

832 (C4'), 81.1 (C(CH$_3$)$_3$), 81.3 (C(CH$_3$)$_3$), 81.9 (C2), 84.0 (C2'), 85.3 (C3), 86.9 (C3'),

833 87.2 (C1), 103.2 (C1'), 119.9 (arom. Fmoc), 121.6 (triazole CH), 125.2 (arom.

834 Fmoc), 127.1 (arom. Fmoc), 127.1 (arom. Fmoc), 127.7 (arom. Fmoc), 141.2

835 (arom. Fmoc), 141.3 (arom. Fmoc), 143.7 (arom. Fmoc), 143.9 (arom. Fmoc),

836 144.8 (O-CH$_2$-C=), 156.6 (CO Fmoc), 171.0 (Glu, triazole-CH$_2$-NH-CO-Cα-NH-),

837 171.6 (Glu, -CO-Cα-NH-Fmoc), 173.1 (Glu, Cδ), 173.1 (Glu, Cδ)

838   MALDI-TOF MS (*m/z*): calcd for C$_{55}$H$_{80}$N$_6$O$_{18}$, 1112.55; found, [M+Na]$^+$ =

839 1135.615, [M+K]$^+$ = 1151.583

840

841   **1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-**

842 **D-glucopyranosyl]-4-(Glu-Glu-NH-CH$_2$)-1*H*-1,2,3-triazole (3a)**

843   1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

844 glucopyranosyl]-4-[*N*α-Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1*H*-1,2,3-

845 triazole (**6a**, 46 mg, 0.034 mmol) was dissolved in piperidine/dichloromethane

846 (1/1, v/v, 1 mL). The reaction mixture was stirred under nitrogen at room

847 temperature for 1 h, and then concentrated to dryness. The crude product was

848 purified by PTLC (eluent: methanol/dichloromethane (15/85, v/v)) to give 1-

849 [2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-

glucopyranosyl]-4-[Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1*H*-1,2,3-triazole (24 mg, 0.016 mmol, 47% yield).

1-[2,3,4,6-Tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl]-4-(Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl)-1*H*-1,2,3-triazole (66 mg, 0.079 mmol) was dissolved in TFA/dichloromethane (9/1, v/v, 1 mL) and stirred under nitrogen at room temperature for 4 h. The crude product was purified by gel-filtration chromatography (LH-20, eluent: methanol) to give 1-[2,3,4,6-tetra-*O*-methyl-β-D-glucopyranosyl-(1→4)-2,3,6-tri-*O*-methyl-β-D-glucopyranosyl]-4-(Glu-Glu-*N*-methyl)-1*H*-1,2,3-triazole (**3a**, 36 mg, 0.046 mmol, 58% yield).

^{1}H-NMR (500 MHz, D$_2$O): δ 1.9-2.1 (m, 2H, Hβ), 2.2-2.3 (m, 2H, Hβ), 2.3-2.6 (m, 4H, Hγ), 3.12 (t, 1H, *J*=8.5 Hz, H2'$_{Me}$), 3.29 (t, 1H, *J*=9.5 Hz, H4'$_{Me}$), 3.36 (t, 1H, *J*=8.5 Hz, H3'$_{Me}$), 3.36 (s, 3H, OCH$_3$), 3.42 (s, 3H, OCH$_3$), 3.43-3.48 (H5'$_{Me}$), 3.54 (s, 3H, OCH$_3$), 3.60 (s, 3H, OCH$_3$), 3.62 (s, 3H, OCH$_3$), 3.63 (s, 6H, OCH$_3$), 3.7 (H3$_{Me}$), 3.80 (t, 1H, *J*=9.5 Hz, H2$_{Me}$), 3.89 (t, 1H, *J*=9.5 Hz, H4$_{Me}$), 3.93-3.98 (m, 1H, H5$_{Me}$), 3.65-3.83 (H6'$_{Me}$, H6$_{Me}$), 3.79 (t, 1H, *J*=9.5 Hz, H4$_{Me}$), 3.86 (m, 1H, H5$_{Me}$), 4.2-4.5 (broad s, 1H, Hα), 4.34 (d, 1H, *J*=8.0 Hz, H1'$_{Me}$), 4.4-4.61 (NH-C*H*$_2$-triazole), 5.75 (broad d, 1H, *J*=7.0 Hz, H1$_{Me}$), 8.24 (broad s, triazole C*H*)

^{13}C-NMR (125 MHz, D$_2$O): δ 27.9 (Cβ'), 29.0 (Cβ), 31.9 (Cγ and Cγ'), 37.1 (NH*C*H$_2$-triazole), 56.1 (Cα (near sugar residue)), 59.4 (Cα'), 61.1 (O*C*H$_3$), 61.2 (O*C*H$_3$), 62.2 (O*C*H$_3$), 62.5 (O*C*H$_3$), 62.6 (O*C*H$_3$), 62.7 (O*C*H$_3$), 63.2 (O*C*H$_3$), 72.5 (C6 or C6'), 73.1 (C6 or C6'), 76.1 (C5'), 78.7 (C4), 79.4 (C5), 81.5 (C4'), 84.0 (C2), 85.5 (C2'), 86.0 (C3), 87.8 (C3'), 89.1 (C1), 105.2 (C1'), 126.6 (CH of triazole), 177.6 (Cα-*C*O-NH-, and Cα'-*C*O-NH-), 184.9 (Cγ-(*C*O)OH, Cγ'-(*C*O)OH)

874   MALDI-TOF MS (*m/z*): calcd for $C_{32}H_{54}N_6O_{16}$, 778.36; found, $[M+H]^+$ =

875   779.518, $[M+Na]^+$ = 801.530, $[M+K]^+$ = 817.504

876

877   **1-(Tri-*O*-methylcellulosyl)-4-(*N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-**

878   **arginine-*N*-methyl)-1*H*-1,2,3-triazole (5b)**

879   Sodium ascorbate (227 mg/287 μL, 1.15 mmol, 20 equiv., 4 M in H_2O) and

880     $CuSO_4 \cdot 5H_2O$ (143 mg, MW = 249.69, 0.573 mmol, 10 equiv.) were added to a

881   solution of 2-propynyl *N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-

882   propargylamide (**9**, 100 mg, MW = 788.8, 0.127 mmol, 2.2 equiv.) and tri-*O*-

883   methylcellulosyl azide (**7b**, 419 mg, M_n = 7.34 × 10³, DP_n = 35.8, 0.0571 mmol,

884   1.0 equiv.) in methanol/dichloromethane (1/4, v/v, 10 mL). The reaction mixture

885   was stirred at room temperature for 14 h under nitrogen, after which it was

886   concentrated and passed through a silica-gel chromatography column (eluent:

887   20% MeOH/CH_2Cl_2) to give the crude product. The crude product was purified by

888   gel-filtration column chromatography (LH-60, eluent: 20% MeOH/CH_2Cl_2) to

889   give   1-(tri-*O*-methylcellulosyl)-4-(*N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-

890   arginine-*N*-methyl)-1*H*-1,2,3-triazole (**5b**, 420 mg, quantitative yield; GPC

891   analysis: M_n = 5.6 × 10³, M_w / M_n = 1.8).

892   ¹H-NMR (500 MHz, $CDCl_3$): δ: 1.44 (CH_3, Pbf), 2.07 (CH_3, Pbf), 2.47-2.56

893   (CH_3, Pbf), 2.92 (CH_2, Pbf), 2.96 (t, *J*=8.5 Hz, H2$_{Me}$ (internal)), 3.21 (t, 1H, *J*=9.0

894   Hz, H3$_{Me}$ (internal)), 3.29 (m, *J*=9.0 Hz, H5$_{Me}$ (internal)), 3.39 (s, OCH_3), 3.54 (s,

895   OCH_3), 3.58 (s, OCH_3), 3.69 (t, *J*=9.0, H4$_{Me}$ (internal)), 3.6-3.73 (m, H6$_{Me}$

896   (internal)), 3.77 (m, H6$_{Me}$ (internal)), 4.34 (d, *J*=8.0 Hz, H1 $_{Me}$ (internal)), 6.12

897   (H1α$_{Me}$), 6.1-6.7, 7.24 (Fmoc), 7.35 (Fmoc), 7.57 (Fmoc), 7.62 (d, *J* = 7.0 Hz,

898   Fmoc), 7.82.

899   ^{13}C-NMR (125 MHz, CDCl$_3$): δ 12.5 (CH$_3$, Pbf), 17.9 (CH$_3$, Pbf), 19.3 (CH$_3$,

900   Pbf), 25.5 (Cγ), 28.6 (CH$_3$, Pbf), 40.9 (Cδ), 43.2 (CH$_2$, Pbf), 47.0 (CH, Fmoc),

901   59.1 (OCH$_3$), 60.3 (OCH$_3$), 60.5 (OCH$_3$), 67.3 (CH$_2$, Fmoc), 70.3 (C6), 74.8 (C5),

902   77.4 (C4), 83.5 (C2), 85.0 (C3), 86.4 (CH, Fmoc), 103.1 (C1), 117.6 (arom. Pbf),

903   119.9 (arom. Fmoc), 124.7 (arom. Pbf), 125.2 (arom. Fmoc), 127.1 (arom. Fmoc),

904   127.7 (arom. Fmoc), 132.3 (arom. Pbf), 132.7 (arom. Pbf), 138.3 (arom. Pbf),

905   141.1 (arom. Fmoc), 143.7 (arom. Fmoc), 156.4 (arom. Pbf), 158.8 (-O-C arom.

906   Pbf)

907   FT-IR (KBr): 3442, 2930, 2836, 1722, 1663, 1622, 1551, 1454, 1375, 1310,

908   1125, 1184, 951, 853, 812, 785, 761, 741 (Fmoc), 700, 662, 567 (Pbf, -SO$_2$NH-)

909   cm^{-1}

910

911   **1-(Tri-*O*-methylcellulosyl)-4-(L-arginine-L-arginine-*N*-methyl)-1*H*-1,2,3-**

912   **triazole (2b)**

913   1-(Tri-*O*-methylcellulosyl)-4-(*N*α-Fmoc-*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-

914   arginine-*N*-methyl)-1*H*-1,2,3-triazole (**5b**, 293 mg) was dissolved in

915   piperidine/dichloromethane (1/1, v/v, 4 mL). The reaction mixture was stirred at

916   room temperature for 4 h under nitrogen, after which it was concentrated and

917   purified by gel-filtration column chromatography (LH-60, eluent: 20%

918   MeOH/CH$_2$Cl$_2$) to give 1-(tri-*O*-methylcellulosyl)-4-(*N*ω-Pbf-L-arginine-*N*ω-Pbf-

919   L-arginine-*N*-methyl)-1*H*-1,2,3-triazole (260 mg, 89% yield; GPC analysis: M_n =

920   6.7 × 10^3, M_w / M_n = 1.7).

921   1-(Tri-*O*-methylcellulosyl)-4-(*N*ω-Pbf-L-arginine-*N*ω-Pbf-L-arginine-*N*-

922   methyl)-1*H*-1,2,3-triazole (244 mg) was dissolved in trifluoroacetic acid/distilled

923   water (1/4, v/v, 2 mL) and stirred at 37 °C for 4 h under nitrogen. The mixture

924　was concentrated, purified by gel-filtration column chromatography (LH-60,

925　eluent: 20% MeOH/CH_2Cl_2), and further purified by PTLC (eluent: 10%

926　MeOH/CH_2Cl_2). The purified 1-(tri-*O*-methyl-cellulosyl)-4-(L-arginine-L-

927　arginine-*N*-methyl)-1*H*-1,2,3-triazole was dispersed in water. Water-soluble

928　component was collected by removal of the water-insoluble component by

929　filtration through cotton wool, which was then concentrated to give 1-(tri-*O*-

930　methylcellulosyl)-4-(L-arginine-L-arginine-*N*-methyl)-1*H*-1,2,3-triazole (**2b**, 186

931　mg, 76% yield, GPC analysis: $M_n = 6.0 \times 10^3$, $M_w / M_n = 1.6$).

932　^{1}H-NMR (500 MHz, D_2O): δ 1.5-2.0 (m, Hγ and Hγ', Hβ, Hβ'), 3.13 (t, *J* = 8.5

933　Hz, $H2_{Me}$), 3.40 (s, OMe), 3.45 (t, *J* = 9.5 Hz, $H3_{Me}$), 3.56 (s, OMe), 3.58 (s, OMe),

934　3.55-3.60 ($H5_{Me}$), 3.68-3.80 ($H4_{Me}$, $H6_{Me}$), 4.42 (d, *J* = 7.5 Hz, $H1_{Me}$), 4.65 (d, *J* =

935　8.0 Hz), 4.99 (d, *J* = 3.5 Hz), 5.40 (d, *J* = 3.5 Hz), 5.62 (broad s), 8.42 (s, C*H*,

936　triazole)

937　FT-IR (KBr): 3446, 2922, 2836, 1624, 1456, 1375, 1314, 1125, 1078, 947, 768,

938　702, 662, 606, 581, 538, 488 cm^{-1}

939

940　**1-(Tri-*O*-methyl-cellulosyl)-4-[Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-**

941　**1*H*-1,2,3-triazole (6b)**

942　Sodium ascorbate (109 mg/137 μL, 0.55 mmol, 20 equiv., 4 M in H_2O) and

943　CuSO$_4$·5H$_2$O (68 mg, MW = 249.69, 0.27 mmol, 10 equiv.) were added to a

944　solution of *N*α-Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-propargylamide (**10**, 53 mg,

945　MW = 647.77, 0.082 mmol, 3.0 equiv.) and tri-*O*-methylcellulosyl azide (**7b**, 200

946　mg, M_n = 7.34 × 10^3, DP_n = 35.8, 0.027 mmol, 1.0 equiv.) in

947　methanol/dichloromethane (1/4, v/v, 5 mL). The reaction mixture was stirred at

948　room temperature for 14 h under nitrogen, after which it was concentrated and

949 passed through a silica-gel chromatography column (eluent: 20% MeOH/CH$_2$Cl$_2$).

950 The crude product was purified by gel-filtration column chromatography (LH-60,

951 eluent: 20% MeOH/CH$_2$Cl$_2$), and then by PTLC (eluent: 10% MeOH/CH$_2$Cl$_2$) to

952 give 1-(tri-*O*-methylcellulosyl)-4-[Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1*H*-

953 1,2,3-triazole (186 mg, 93% yield, GPC analysis: $M_n = 7.4 \times 10^3$, $M_w / M_n = 1.6$).

954 ^{1}H-NMR (500 MHz, CDCl$_3$): δ 1.43 (s, CH$_3$ (*t*Bu)), 1.47 (s, CH$_3$ (*t*Bu)), 2.0-2.2

955 (m, 4H, Hβ), 2.25-2.450 (m, 4H, Hγ), 2.96 (t, *J*= 8.5 Hz, H2$_{Me}$), 3.22 (t, *J*= 9.0 Hz,

956 H3$_{Me}$), 3.29 (m, H5$_{Me}$), 3.39 (s, OMe), 3.54 (s, OMe), 3.59 (s, OMe), 3.64-3.74

957 (H4$_{Me}$), 3.64-3.82 (H6$_{Me}$), 4.1-4.2 (1H, Hα), 4.25-4.35 (2H, C*H*$_2$ (Fmoc)), 4.35 (d,

958 *J*= 7.5 Hz, H1$_{Me}$), 7.32 (t, *J*= 7.5 Hz, arom., Fmoc), 7.40 (t, *J*= 7.5 Hz, arom.,

959 Fmoc), 7.62 (broad d, *J*= 7.0 Hz, arom., Fmoc), 7.76 (d, *J*= 7.5 Hz, arom., Fmoc)

960 ^{13}C-NMR (125 MHz, CDCl$_3$): δ 28.0 (CH$_3$ (tBu)), 28.0 (CH$_3$ (tBu)), 31.9

961 (triazole-CH$_2$-NH-$\underline{C}$α-Cβ-$\underline{C}$γ), 35.2 (triazole-$\underline{C}$H$_2$-NH-), 47.1 ($\underline{C}$H, Fmoc), 59.1

962 (O$\underline{C}$H$_3$), 59.6, 60.1, 60.3 (O$\underline{C}$H$_3$), 60.4, 60.5 (O$\underline{C}$H$_3$), 60.8, 67.2 ($\underline{C}$H$_2$, Fmoc),

963 70.3 (C6), 72.2, 73.2, 73.2, 74.9 (C5), 77.4 (C4), 81.2 ($\underline{C}$(CH$_3$)$_3$), 83.5 (C2), 85.0

964 (C3), 86.1, 103.1 (C1), 120.0 (arom. Fmoc), 125.1 (arom. Fmoc), 127.1 (arom.

965 Fmoc), 127.7 (arom. Fmoc), 141.3 (arom. Fmoc), 143.8 (arom. Fmoc), 144.4

966 (arom. Fmoc)

967 FT-IR (KBr): 3430, 2932, 2904, 2836, 1728, 1672, 1514 (*t*Bu), 1454, 1373,

968 1312, 1125, 1084, 1059, 951, 889, 851, 762, 743 (Fmoc), 704, 656, 615, 577 cm^{-1}

969

970 **1-(Tri-*O*-methyl-cellulosyl)-4-(Glu-Glu-*N*-methyl)-1H-1,2,3-triazole (3b)**

971 1-(Tri-*O*-methyl-cellulosyl)-4-[Fmoc-Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1*H*-

972 1,2,3-triazole (161 mg) was dissolved in piperidine/dichloromethane (1/1, v/v, 2

973 mL). The reaction mixture was stirred at room temperature for 4 h under nitrogen,

974 after which it was concentrated and purified by gel-filtration column

975 chromatography (LH-60, eluent: 20% MeOH/CH$_2$Cl$_2$), and then by PTLC (eluent:

976 10% MeOH/CH$_2$Cl$_2$) to give 1-(tri-*O*-methylcellulosyl)-4-[Glu(O*t*-Bu)-Glu(O*t*-

977 Bu)-*N*-methyl]-1H-1,2,3-triazole (136 mg, 84% yield; GPC analysis: M_n =

978 8.0×10^3, M_w / M_n = 1.6).

979 1-(Tri-*O*-methylcellulosyl)-4-[Glu(O*t*-Bu)-Glu(O*t*-Bu)-*N*-methyl]-1H-1,2,3-

980 triazole (124 mg) was dissolved in trifluoroacetic acid/distilled water (9/1, v/v, 1

981 mL). The reaction mixture was stirred at room temperature for 4 h under nitrogen

982 atmosphere, concentrated, and then purified by gel-filtration column

983 chromatography (LH-60, eluent: 20% MeOH/CH$_2$Cl$_2$), and further purified by

984 PTLC) eluent: 10% MeOH/CH$_2$Cl$_2$). The water-soluble component was collected

985 by removal of the water-insoluble component by filtration, and concentrated to

986 give 1-(tri-*O*-methylcellulosyl)-4-(Glu-Glu-*N*-methyl)-1*H*-1,2,3-triazole (**3b**, 64

987 mg, 52% yield; GPC analysis: M_n = 7.0×10^3, M_w / M_n =1.6).

988 ^{1}H-NMR (500 MHz, D$_2$O): δ 3.14 (t, J = 9.0 Hz, H2$_{Me}$), 3.40 (s, OMe), 3.45 (t, J

989 = 9.0 Hz, H3$_{Me}$), 3.57 (s, OMe), 3.58 (s, OMe), 3.57-3.62 (H5$_{Me}$), 3.60-3.82 (H4$_{Me}$,

990 H6$_{Me}$), 4.43 (d, J = 8.0 Hz, H1$_{Me}$), 4.48 (d, 5.40 (d, J = 3.0   Hz), J = 3.5 Hz), 5.43

991 (broad s), 8.43 (s, triazole C*H*)

992 FT-IR (KBr): 3460, 2928, 2836, 1732, 1626, 1458, 1377, 1310, 1126, 1084,

993 1061, 945, 800, 704, 664, 571, 486 cm^{-1}

994 **Characterization**

995 *General*

996 ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian 500 NMR (500 MHz) or

997 Varian INOVA300 (300 MHz) spectrometer in chloroform-*d* with

998 tetramethylsilane as the internal standard, or in deuterium oxide with 3-

999 (trimethylsilyl)-1-propanesulfonic acid sodium salt as the external standard.

1000 Chemical shifts (δ) and coupling constants (J) are given in ppm and Hz,

1001 respectively. Matrix-assisted laser-desorption/ionization time-of-flight mass

1002 spectrometry (MALDI-TOF MS) was performed on a Bruker MALDI-TOF

1003 Autoflex III mass spectrometer in positive ion and reflector or linear modes. A

1004 smartbeam laser was used for ionization. All spectra were acquired in linear mode

1005 and calibrated externally. 2,5-Dihydroxybenzoic acid was used as the matrix in

1006 MALDI-TOF MS experiments. Shimadzu components, namely the liquid

1007 chromatography injector (LC-10ATvp), column oven (CTO-10Avp), ultraviolet-

1008 visible detector (SPD-10Avp), refractive index detector (RID-10A),

1009 communication bus module (CBM-10A), and LC workstation (CLASS-LC10),

1010 were used for HPLC separations, with Shodex columns (KF802, KF802.5, and

1011 KF805). Number- and weight-averaged molecular weights (M_n, M_w) and

1012 polydispersity indices (M_w/M_n) were determined using polystyrene standards

1013 (Shodex). A flow rate of 1 mL/min at 40 °C was chosen, and chloroform was used

1014 as the eluent.

1015 *Differential scanning calorimetry (DSC)*

1016 DSC thermograms were recorded on a DSC823^e instrument (Mettler Toledo,

1017 Zurich, Switzerland) with an HSS7 sensor under nitrogen during (0 → 90 → 0 °C)

1018 heating/cooling cycles, with heating and cooling rates of 3.5 °C/min. Each

1019 temperature cycle was repeated three times in order to ensure reproducibility.

1020 Sample concentrations of 2.0 wt% were used in DSC experiments.

1021 *Dynamic light scattering (DLS) experiments*

1022 DLS experiments were performed with an ELS-Z zeta-potential and particle-

1023 size analyzer (Otsuka Electronics Co., Ltd, Osaka, Japan) and conducted in the

1024 10–90 °C temperature range. Sample solutions were maintained at the required

1025 temperature for 5 min prior to each experiment. Sample concentrations of 0.2 wt%

1026 were used in these experiments.

1027 *Surface-tension measurements*

1028 Surface tensions were measured with a CBVP-A3 surface tensiometer (Kyowa

1029 Interface Science, Co. Ltd., Tokyo, Japan) at 23 °C using the Wilhelmy method.

1030 A Teflon cell containing 700 μL of the required solution was used in these

1031 experiments. Surface tensions gradually decreased with time, and stable values

1032 were recorded after 30 min.

1033 *Scanning electron microscopy (SEM) and transmission electron*
1034 *microscopy (TEM)*

1035 The three hydrogels from aqueous solutions containing compounds **1b**, **2b**, and

1036 **3b** were frozen in liquid nitrogen, lyophilized, and cut with a razor blade. The cut

1037 surfaces of the hydrogels were sputter-coated with gold with an ion-coater (JFC-

1038 1100E, JEOL, Tokyo, Japan) and examined by scanning electron microscopy

1039 (SEM, JSM-6060, JEOL) at an accelerator voltage of 5 kV.

1040 A drop of an aqueous dispersion of compound **2b** was mounted on a copper grid

1041 with an elastic carbon-support film (Oken Shoji, Tokyo, Japan) and examined by

1042 transmission electron microscopy (TEM, JEM1400, JEOL) at an accelerator

1043 voltage of 100 kV after negative staining with uranyl acetate.

1044 *Release of model drugs from the thermoresponsive hydrogels*

1045 Compounds **2b** and **3b** (2 or 4 mg) were respectively added to glass vials

1046 containing solutions of diclofenac sodium (DFNa) or imipramine hydrochloride

1047 (IMC) (0.025 wt%) in PBS (100 μL), and the compounds were dissolved at about

1048 0 °C. The glass vial was then heated at 37 °C for 10 min while left to stand. After

1049   a hydrogel had formed, a fresh PBS solution (500 μL), which had been pre-heated

1050   to 37 °C, was carefully poured onto the hydrogel surface. The glass vial was

1051   shaken at 60 rpm in a water-bath shaker (Eyela NTS-4000, Tokyo Rikakikai Co.,

1052   Ltd.). The aqueous layer (500 μL) was then collected and filtered through a

1053   membrane filter (pore size: 0.45 μm). The UV absorbance of the aqueous solution

1054   was recorded at 260 nm in a 96-well microplate using a SpectraMax Plus 384

1055   Microplate Reader (Molecular Devices). The amount of released drug was

1056   evaluated from the UV absorbance.

1057   *Cytotoxicity assays*

1058   Compounds **1b**, **2b**, and **3b** were dispersed in PBS at a concentration of 20

1059   mg/mL and serially diluted by factors of two in a 96-well flat-bottomed plate (50

1060   μL/well (Corning Inc., Corning, NY). The human histocytoma U937 cell line was

1061   suspended in complete RPMI1640 medium at 1×10^4 cells/mL and the cell

1062   suspension was added to the 96-well flat bottom plate (5×10^2 cells/50 μl/well).

1063   The plate was incubated at 37 °C in a 5%-CO_2 atmosphere for 4 d. The plate was

1064   allowed to stand at room temperature for 30 min, after which 100 μL of CellTiter-

1065   Glo Luminescent Cell Viability Assay reagent (Promega Corp., Madison, WI)

1066   was added to each well. After thorough mixing, the contents of each well were

1067   transferred into an Optiplate™-96 multi-well plate (Perkin Elmer, Waltham, MA).

1068   Cell viability was determined by measuring luminescence with an ARVO™ SX

1069   Delfia 1420 Multilabel Counter (Perkin Elmer Life and Analytical Sciences,

1070   Shelton, CT).

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