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(Article begins on next page)

1 **Potent antitumor activity of anti-HER2 antibody-topoisomerase I inhibitor conjugate**
2 **based on self-immolative dendritic dimeric-linker**

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29

30 **Keywords**

31 Antibody-Drug Conjugate (ADC), Belotecan, Breast Cancer, Dendritic Linker, Dimeric
32 Prodrug, Exatecan, HER2, Trastuzumab

33 **Abstract**

34 Antibody-drug conjugates (ADCs) are a rapidly expanding class of anticancer
35 therapeutics, with 14 ADCs already approved worldwide. We developed unique linker
36 technologies for the bioconjugation of drug molecules with controlled-release
37 applications. We synthesized cathepsin-cleavable ADCs using a dimeric prodrug
38 system based on a self-immolative dendritic scaffold, resulting in a high drug-antibody
39 ratio (DAR) with the potential to reach 16 payloads due to its dendritic structure,
40 increased stability in the circulation and efficient release profile of a highly cytotoxic
41 payload at the targeted site. Using our novel cleavable linker technologies, we
42 conjugated the anti-human epidermal growth factor receptor 2 (anti-HER2) antibody,
43 trastuzumab, with topoisomerase I inhibitors, exatecan or belotecan. The newly
44 synthesized ADCs were tested *in vitro* on mammary carcinoma cells overexpressing
45 human HER2, demonstrating a substantial inhibitory effect on the proliferation of
46 HER2-positive cells. Importantly, a single dose of our trastuzumab-based ADCs
47 administered *in vivo* to mice bearing HER2-positive tumors, showed a dose-dependent
48 inhibition of tumor growth and survival benefit, with the most potent antitumor
49 effects observed at 10 mg/kg, which resulted in complete tumor regression and
50 survival of 100% of the mice. Overall, our novel dendritic technologies using the
51 protease-cleavable Val-Cit linker present an opportunity for the development of highly
52 selective and potent controlled-released therapeutic payloads. This strategy could
53 potentially lead to the development of novel and effective ADC technologies for
54 patients diagnosed with HER2-positive cancers. Moreover, our proposed ADC linker
55 technology can be implemented in additional medical conditions such as other
56 malignancies as well as autoimmune diseases that overexpress targets, other than
57 HER2.

58

59 Introduction

60 Human epidermal growth factor receptor 2 (HER2) is overexpressed in a broad
61 number of cancer types, including breast, ovarian, colorectal, gastric, head and neck,
62 and liver cancers^{1, 2}. Specifically, in breast cancer, HER2 is overexpressed in ~15-30%
63 of patients, due to amplification or activating somatic mutations of gene encoding
64 HER2 (ERBB2), closely correlating with poor prognosis and poor overall survival rates,
65 thus, presenting an attractive candidate for targeted therapeutic applications^{3, 4}.
66 Trastuzumab (Herceptin[®]) is a clinically approved HER2 humanized monoclonal
67 antibody and a standard-of-care for HER2-positive early and advanced breast cancer
68 and advanced gastric cancer, either as a single agent or in combination with
69 chemotherapy^{1, 5}. Although trastuzumab targeted therapy transformed the treatment
70 of HER2-positive cancers, many patients treated with trastuzumab-based therapy
71 gradually develop resistance, and some experience severe side-effects such as
72 cardiotoxicity, especially when combined with anthracycline-based chemotherapy
73 treatment^{6, 7}.

74 The design of novel treatments with superior anti-tumor efficacy and reduced off-
75 target toxicities continues to be a clinical challenge. Targeted therapy, using small
76 molecules or biologics, has gained significant attention in the last decade^{8, 9}. One
77 special class of targeted therapy is antibody-drug conjugates (ADCs) which are a
78 rapidly growing class of anti-cancer therapeutics, representing a unique opportunity
79 to increase the safety of highly toxic drugs by utilizing the specificity of antibodies to
80 obtain targeted delivery of potent cytotoxic molecules to specific tissues. Currently,
81 there have been 14 ADCs approved for marketing worldwide for the treatment of
82 hematological malignancies and solid tumors¹⁰ and over 100 ADC candidates in
83 different stages of clinical development. Specifically, for HER2-positive tumors,
84 trastuzumab has been effectively exploited to target cytotoxic agents directly to HER2-
85 expressing cancers, as illustrated by the US Food and Drug Administration (FDA)
86 approval of two trastuzumab-based ADCs, Ado-trastuzumab emtansine (Kadcyla[®]; T-
87 DM1) and fam-trastuzumab deruxtecan-nxki (Enhertu[®]; T-DXd).

88 Much of the success of ADCs is attributed to technological advances made in the
89 design of the linker between the antibody and the therapeutic payloads. The linker

90 technology employed has a significant impact on balancing between ADC therapeutic
91 efficacy and off-target toxicity^{17, 18}. Thus, several key linker properties should be
92 addressed when designing efficient and selective ADCs. Ideal ADCs should present
93 high stability in circulation to limit the premature release of toxic agents in the
94 bloodstream and off-target compartments, high water solubility to aid bioconjugation
95 and prevent the formation of ADC aggregates, and should efficiently release highly
96 cytotoxic payloads at desired targeted sites¹⁸. Furthermore, homogenous conjugation
97 of the ADC contributes to its efficacy. Broader DAR distribution produces more
98 heterogenous ADC, which results in product inconsistency and suboptimal
99 efficiency¹⁹. *In vivo* studies have shown improved therapeutic window and tolerability
100 of developed ADCs with DAR2 with high homogeneity (>90%)²⁰.

101 The vast majority of ADCs in clinical development employ cleavable linkers with
102 specific release mechanisms to allow the controlled release of solitary drug molecules
103 at the target site²¹. Considering that each conjugation site between an antibody and a
104 linker has the potential to impact the antibody's binding affinity with its antigen, it would
105 be advantageous to explore the development of linkers with cleavage sites capable of
106 initiating the release of multiple drug molecules upon a single cleavage event. Another
107 key component of ADCs is the cytotoxic payload. Due to the fact that only a low
108 fraction of ADCs administered reach their target, and the drug loading capacity on the
109 antibody is limited, highly potent drugs are required for effective ADCs. Moreover, as
110 ADC technology is highly selective to its target, it allows the use of highly potent drugs
111 that could not have been used in their free unbound form. Currently, the main
112 cytotoxic drugs incorporated in ADCs under clinical development include microtubule
113 inhibitors and DNA targeting agents, some of which could not have been used as free
114 drugs due to their toxicity and/or hydrophobicity²².

115 Here we report an efficient construction of novel trastuzumab-based ADCs using self-
116 immolative dendritic prodrug dimeric-platform for bioconjugation of topoisomerase I
117 inhibitor compounds, exatecan or belotecan, to trastuzumab. Our novel dendritic-
118 based linker system enables the conjugation of two drugs per linking site and utilizes
119 the intrinsic properties of the tumor cells in the context of a protease-mediated linker
120 cleavage. The valine-citrulline (Val-Cit) dipeptide present in the linker is recognized

121 and processed predominantly by lysosomal proteases, such as cathepsins, which are
122 overexpressed in cancer cells, thereby enabling accurate and selective payload release
123 at the tumor site²³. Furthermore, we have incorporated a short solubilizing enhancer
124 moiety in order to increase the aqueous solubility of the hydrophobic structure.
125 Dendritic prodrugs that are activated through a single catalytic reaction by a specific
126 enzyme offer advantage in augmenting tumor growth inhibition, especially if the
127 targeted or secreted enzyme exists at relatively low levels in the malignant tissue. In
128 addition, by attachment of a dimeric-prodrug unit, the number of conjugation sites on
129 the antibody molecule is reduced by a factor of 2-fold to reach the same drug-to-
130 antibody ratio (DAR) and a higher DAR can be achieved.

131 Herein, we evaluated the anti-tumor potential of our ADCs with exatecan or belotecan
132 payloads at DAR4 (*i.e.*, 2 linkers carrying 4 drug molecules) and compared their
133 therapeutic efficacy to T-DXd (DAR8) in HER2-positive breast cancer model. In general,
134 trastuzumab-exatecan dendrimer ADC demonstrated higher anti-tumor activity than
135 belotecan and similar therapeutic potential to T-DXd despite bearing half the number
136 of drug molecules attached to the antibody.

137

138 **Materials and Methods**

139 **Prodrug synthesis**

140 Detailed synthetic schemes, chemical preparation and experimental procedures of the
141 linker-drug used in this study, are provided in the **Supplementary Data** section.

142 **Preparation of antibody-drug conjugates (ADCs)**

143 Exatecan-based prodrug 1 or belotecan-based prodrug 2 were conjugated to
144 trastuzumab (Herceptin®; Roche PZN-10816760) using a two-step chemoenzymatic
145 approach as described by Dickgiesser *et al.*²⁴. In a first step, thiol-bearing cysteamine
146 (2-amino-ethanethiol) was enzymatically conjugated to trastuzumab using a microbial
147 transglutaminase (MTG) specifically addressing glutamine 295 in both antibody heavy
148 chains (EU numbering). The two conjugated cysteamine moieties served as handles
149 for the attachment of thiol-reactive prodrugs 1 (exatecan prodrug) and 2 (belotecan

150 prodrug) in a second step resulting in ADCs trastuzumab-exatecan (Tras-Exa; DAR4):
151 Trastuzumab-Thiol spacer at Q295-Dendron-Cat B-cleavable linker (Val-Cit)-Exatecan;
152 trastuzumab-belotecan (Tras-Bel; DAR4): Trastuzumab-Thiol spacer at Q295-Dendron-
153 Cat B-cleavable linker (Val-Cit)-Belotecan, respectively.

154 Drug-linker Gly-Gly-Phe-Gly(GGFG)-DXd was conjugated to trastuzumab or rituximab
155 (Roche 10179132-02; PZN-08709904) targeting endogenous antibody cysteine
156 residues as described previously²⁵. Briefly, antibodies were incubated with 10 molar
157 equivalents of tris(2-carboxyethyl)phosphine for disulfide reduction. Afterwards, 16-
158 18 molar equivalents of prodrug 3 (DXd prodrug; see Supplementary Data **Figure S26**)
159 were added for attachment to reduced cysteine thiols generating trastuzumab-
160 deruxtecan (T-DXd; DAR8): Trastuzumab-Maleimide-GGFG-DXd or rituximab-
161 deruxtecan (Ritux-DXd; DAR8): Rituximab-Maleimide-GGFG-DXd ADCs.

162 Conjugates were purified by size-exclusion chromatography using HiLoad™ 16/600
163 Superdex™ 200 pg columns (Cytiva) and 10 mM Histidine, 3 % Trehalose, 100 mM
164 NaCl, pH 5.5 as running buffer. ADCs were analyzed using reversed phase
165 chromatography (RP-HPLC; PLRPS column, 4000 Å, 5 µm, 2.1 × 50 mm, Agilent
166 technologies) and analytical size exclusion chromatography (SE-HPLC; TSKgel
167 SuperSW3000 column, 4.6 x 300 mm, Tosoh Bioscience) as described previously²⁴. For
168 liquid chromatography mass spectrometry (LC-MS) DAR determination, ADCs were
169 diluted with 0.1 % formic acid to a final concentration of 0.05 mg/mL. Subsequently,
170 100 µL of this solution were reduced with 1 µL TCEP (500 mM) for 5 min at room
171 temperature. LC-MS analysis was performed using an Exion HPLC system (Buffer A:
172 0.1% formic acid in water (Biosolve, 23244101), Buffer B: 0.1% formic acid in
173 acetonitrile (Biosolve, 01934101) coupled to a Sciex 6600+ mass spectrometer by a
174 Turbo V ESI source. 4 µl protein solution was loaded onto an Phenomenex bioZen 3.6
175 µm Intact C4 column (50 x 2.1 mm) and eluted with a linear gradient from 15 % to 95
176 % Buffer B within 3 min and 0.25 mL/min flow rate. Data were acquired in IDA mode
177 with positive polarity, in a mass range from 400 to 4000 m/z. Other instrument
178 settings were as follows: source voltage 5.5 kV, declustering potential 180 V,
179 accumulation time 1 s, source temperature 450 °C, gas1 50 L/h, and gas2 25 L/h. The
180 mass spectrometer was calibrated with ESI positive calibration solution 5600.

181 Acquired data were processed with Genedata Expressionist 16.5. All the ADCs used in
182 this study were conjugated in house as described above. The serum stability assay was
183 performed using the commercial Enhertu® (please see below).

184 **Serum stability**

185 The serum stability assay was conducted as previously described^{26, 27} applying some
186 minor modifications: Tras-Exa (DAR4), Tras-Bel (DAR4) and trastuzumab-deruxtecan
187 [T-DXd, Enhertu® (Daiichi Sankyo; NDC 65597-406-01; Asaman, MA, USA); DAR8:
188 Trastuzumab-Maleimide-(GGFG)-DXd] conjugates were incubated at a final
189 concentration of 5 µM conjugated payload (considering the DAR of each construct) in
190 human and mouse serum (Biowest). Moreover, serum samples were supplemented
191 with 5 µM deuterated 7-Ethyl-d3-camptothecin (D3-CPT) as an internal standard.

192 **Pharmacokinetics study**

193 For pharmacokinetic (PK) study plan concerning animal care have been reviewed by
194 the RBM Designated Veterinarian. Protection of animals used, housing and welfare
195 are guaranteed according to the Italian D.Lvo. No. 26 of March 4, 2014. Physical
196 facilities for accommodation and care of animals are in accordance with the provisions
197 of the Italian D.Lvo. 2014/26 and of Directive 2010/63/EU. The Institute is fully
198 authorized by Italian Ministry of Health.

199 C57BL/6 wild type mice and hu FcRn transgenic mice were purchased from Charles
200 River Laboratories (Italia Srl, Via Indipendenza, 11 - 23885 Calco). ADCs were
201 administered to the animals (n=6/group) at 3 mg/kg single dose, by intravenous
202 injection. Mice were divided (n=3/time point) and 70 µL of blood samples were
203 collected from mice #1, 2, 3 at 0.08, 24, 72, 240, and 504 h and from mice #4, 5, 6 at
204 4, 48, 168 and 336 h after dosing. Plasma samples were tested by using an
205 immunoassay method for the quantification of total human IgG antibodies in mouse
206 sera for all groups. The lower limit of quantification (LLOQ) of total antibodies was 50
207 ng/mL and values below the LLOQ were considered 0 ng/mL for descriptive statistics
208 and necessary condition analysis (NCA). PK analysis was performed using Phoenix
209 WinNonlin® version 6.3 (Pharsight Corporation, USA) with a non-compartmental
210 (model independent) approach.

211 **Cell lines**

212 HCC-1954, and MDA-MB-468 human mammary carcinoma cells were obtained from
213 the American Type Culture Collection (ATCC, Manassas, VA, USA). JIMT-1 cells were
214 obtained from the German Collection of Microorganisms and Cell Cultures GmbH
215 (DSMZ). HCC-1954 and JIMT-1 cells were cultured in Dulbecco's modified Eagle's
216 medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100
217 IU/mL penicillin, 100 µg/ml streptomycin, 12.5 U/mL nystatin, 2 mM L-glutamine and
218 100 µg/mL sodium pyruvate (Biological Industries, Israel). MDA-MD-468 cells were
219 cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; Gibco) supplemented
220 with 10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 12.5 U/mL nystatin, 2
221 mM L-glutamine. All cells were grown at 37°C in 5% CO₂. All experiments were
222 performed with mycoplasma-free cells. All human cell lines were purchased within
223 the last three years and hence, were authenticated using STR profiling by the ATCC.

224 **HER2 quantification**

225 For each sample, 0.8x10⁶ cells (HCC1954, JIMT-1 or MDA-MB-468) were prepared in
226 50 µL of Dulbecco's phosphate-buffered saline (DPBS)x1 containing 1% bovine serum
227 albumin (BSA; #BAH65; Equitech-Bio). Trastuzumab was added to a final
228 concentration of 100 nM. The sample was incubated on ice for 45 min. A wash step
229 was performed with 2 mL of cold DPBS + 1% BSA, the sample was spun down and
230 supernatant was discarded. The detection antibody (AlexaFluor 488 F(ab')₂ Donkey
231 Anti-human IgG-Fc) (#709-546-098; Jackson ImmunoResearch Laboratories) was
232 added in a volume of 80 µL and incubated for 30 min on ice. Cells were then washed
233 twice in DPBS + 1% BSA, resuspended in 500 µL of DPBSx1 and then taken for flow
234 cytometry analysis (BD FACSAria™ III). Analysis was done using the FCS Express™
235 software (De Novo Software).

236 A calibration curve was prepared for each cell line, according to manufacturer's
237 instructions of the Quantum™ Simply Cellular anti-human IgG kit (Bangs Laboratories
238 Inc.), using the trastuzumab (Herceptin®) antibody and the same detection antibody
239 that was used for the experiments.

240

241 **HER2-ADC binding assay**

242 For each sample 0.8×10^6 cells (HCC1954, JIMT-1 or MDA-MB-468) were prepared in 50
243 μL of DPBSx1 + 1% BSA. The following ADCs and antibodies were added to a final
244 concentration of 100 nM: T-DXd (DAR8), Tras-Exa (DAR4), Tras-Bel (DAR4), Isotype
245 control Ritux-DXd and trastuzumab. The samples were incubated on ice for 45 min. A
246 wash step was performed with 2 mL of cold DPBS + 1% BSA, centrifugation and
247 supernatants were discarded. The detection antibody (AlexaFluor 488 F(ab')₂ Donkey
248 Anti-human IgG-Fc) was added in a volume of 80 μL , and incubated for 30 min on ice.
249 Cells were then washed twice in DPBS + 1% BSA, resuspended in 500 μL of DPBSx1 and
250 then taken for flow cytometry analysis, in a BD FACSAria™ III. Analysis was done using
251 the FCS Express™ software (De Novo Software).

252 **Cell viability assay**

253 HCC-1954, JIMT-1 and MDA-MB-468 cells were plated in 24-well culture plates (5000,
254 5000 and 10,000 cells/well, respectively) and incubated for 24 h. Cells were then
255 exposed to serial dilutions of trastuzumab, free exatecan, or Tras-Exa (DAR4) ADC. Cell
256 viability was evaluated following 6 days incubation using MTT (3 $\mu\text{g}/\text{mL}$, Sigma). MTT
257 absorbance was measured at 570 nm using SpectraMax M5e multi-detection reader.

258 ***In vivo* study**

259 All animals were housed in the Tel Aviv University Specific Pathogen Free (SPF) animal
260 facility. The experiments were approved by the animal care and use committee
261 (IACUC) of Tel Aviv University (approval no. 01-19-088) and conducted in accordance
262 with NIH guidelines.

263 HCC-1954 cells (1×10^6) were injected into the mammary fat pad of 6-weeks old female
264 severe combined immune deficient (SCID) mice. Tumor growth was monitored by
265 caliper and the volume was defined as $0.52 \times \text{width} \times \text{length}^2$. When tumor volume
266 reached approximately 95 mm^3 , mice were randomized into groups ($n=9/\text{group}$) and
267 intravenously injected with a single dose of T-DXd (DAR8), Tras-Exa (DAR4) or Tras-
268 Bel (DAR4), at three different concentrations (1, 3 or 10 mg/kg). As controls, mice
269 were treated either with 10 mg/kg of Ritux-DXd (DAR8) or with a vehicle control (10
270 mM Histidine, 3% Trehalose, 100 mM NaCl, pH 5.5). All treatments were administered

271 at a volume of 200 μ L/20 g mouse. Mice were euthanized when tumor volume
272 reached 1000 mm³, when tumors became necrotic and/or ulcerated, or when mice
273 lost 15% of their body weight.

274 **Statistical analyses**

275 Data is presented as mean \pm standard error of mean (SEM), unless stated otherwise.
276 The statistical significance in overall survival was determined with a log-rank (Mantel-
277 Cox) test using GraphPad Prism 9 software. Statistical significance was defined as $p <$
278 0.05.

279

280 **Results and Discussion**

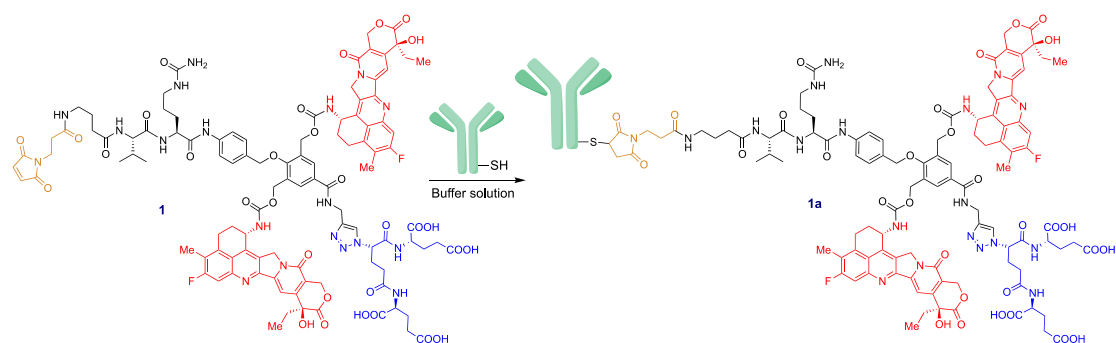
281 **Molecular design, bioconjugation, and characterization of ADCs**

282 Almost 20 years ago, we have demonstrated the ability of *para*- and *ortho*-quinone-
283 methide eliminations to occur consecutively on the same aromatic phenol or aniline
284 derivative, by the introduction of proper leaving groups on such positions²⁸. This
285 double elimination mechanism is initiated by the removal of the head-substrate and
286 formation of a phenol or an aniline intermediate, which then undergoes *p*-quinone-
287 methide elimination to release the first reporter group; this is followed by *o*-quinone-
288 methide elimination to release the second reporter. Incorporation of drug molecules
289 as the tail units and an enzyme substrate as the trigger generated a dimeric-prodrug
290 unit that was activated upon a single enzymatic cleavage. This self-immolative
291 dendritic scaffold enables attachment and then consecutive release of two identical
292 or different chemotherapeutic drugs²⁹⁻³¹.

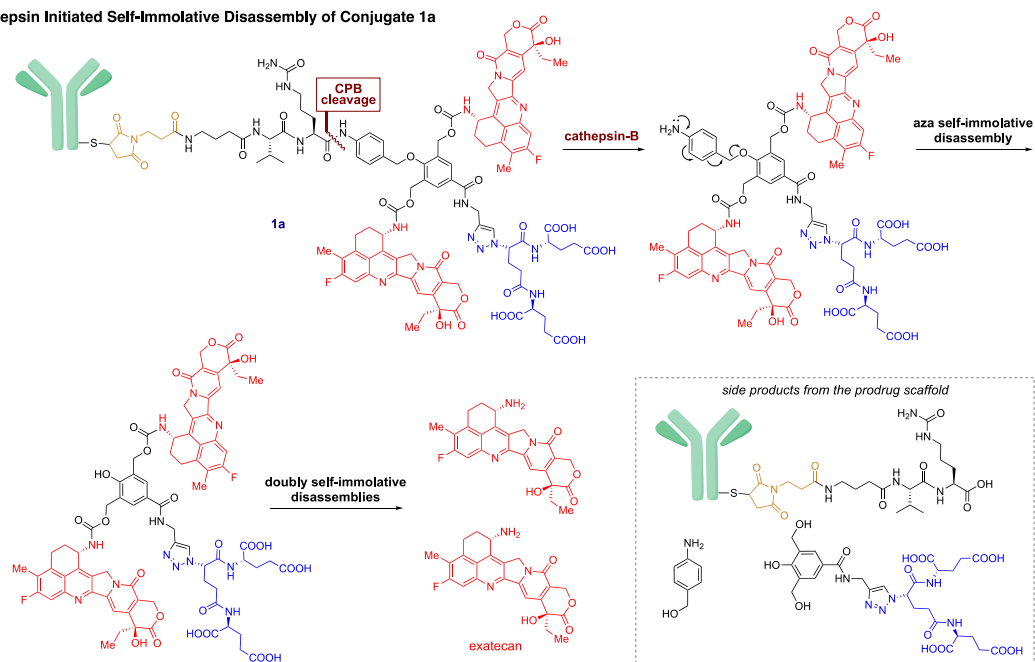
293 In this work, we synthesized a new dimeric prodrug system based on an AB₂ self-
294 immolative dendritic scaffold, where exatecan (red) is the chemotherapeutic drug and
295 Val-Cit dipeptide is used as a substrate for cathepsin B (**Fig. 1A; prodrug 1**, see
296 **Supplementary Data Figures S1-S24** for detailed synthesis). A maleimide moiety
297 (brown) was introduced at the focal site in order to enable conjugation to an antibody
298 molecule *via* reaction with a thiol functionality (thiol functional groups were
299 introduced via glutamine modification using transglutaminase ligation with
300 cysteamine). In addition, a short solubilizing moiety, composed of tetra-carboxylate

301 (blue), was attached to the dendritic prodrug scaffold in order to increase the aqueous
 302 solubility of the hydrophobic structure. The self-immolative dendritic linker, when
 303 paired with two copies of exatecan, creates a molecular structure that is extremely
 304 hydrophobic, rendering it insoluble in water. Consequently, these dendritic molecules
 305 cannot be effectively conjugated with an antibody, as the highly lipophilic system
 306 undergoes complete precipitation in aqueous media. The inclusion of a hydrophilic
 307 unit in the para-position of the AB2 self-immolative dendritic linker dramatically
 308 improved the hydrophilicity of this molecular system and thus enabled sufficient
 309 water solubility to allow conjugation with the antibody. Furthermore, trials using a
 310 solubilizing unit on the dendritic linker, which is less hydrophilic than the current one,
 311 resulted in aggregation of the ADC molecules.

A. Conjugation Reaction of Prodrug 1 with Antibody



B. Cathepsin Initiated Self-Immolative Disassembly of Conjugate 1a



312

313 **Figure 1. (A) Conjugation of prodrug 1 to an antibody.** Cysteamine was attached to
 314 antibodies using microbial transglutaminase (MTG) which was then conjugated with

315 exatecan self-immolative dimeric prodrug *via* thiol-maleimide chemistry (see
316 Methods). The Buffer solution for the antibody conjugation of drug-linker was PBS, 1
317 mM EDTA, pH 7.4. **(B) Cathepsin-B initiated self-immolative disassembly of conjugate**
318 **1a**. The release mechanism of the two exatecan drug molecules from the ADC system
319 by cathepsin B. PBS - Phosphate, buffered saline; CPB - cathepsin B.

320

321 The release mechanism of the two exatecan drug molecules from the ADC system is
322 presented in **Figure 1B**. The prodrug disassembly is initiated by enzymatic cleavage of
323 the specific amide bond between the Val-Cit dipeptide and the aniline linker by
324 cathepsin B. The generated aniline undergoes aza-quinone-methide elimination to
325 release a phenol intermediate, which then undergoes two consecutive quinone-
326 methide eliminations to release the two exatecan chemotherapeutic drug molecules.

327 The belotecan dimeric prodrug 2 was synthesized in an analogue manner to that
328 described for the exatecan prodrug (**Fig. S25**).

329 Prodrugs 1 and 2 were conjugated to trastuzumab using a site-specific,
330 chemoenzymatic two-step conjugation strategy to generate ADCs Tras-Exa and Tras-
331 Bel, respectively, carrying two dimeric prodrugs, hence four drugs per antibody. T-DXd
332 and Ritux-DXd were produced by attaching prodrug 3 (**Fig. S26**) directly to endogenous
333 antibody cysteines to yield ADC with DARs around 8. A slightly higher DAR of 4.3 for
334 Tras-Exa indicated some unspecific conjugation, but overall the anticipated DARs
335 could be reached, and analytical size-exclusion chromatography (SEC) analysis
336 revealed high purity for all four ADCs (**Table 1, Fig. S27**).

337 **Table 1. Generated ADCs and key data.**

Antibody + prodrug	ADC	DAR	Purity (%)
Trastuzumab + Val-Cit-Exatecan dendrimer [prodrug 1]	Tras-Exa	4.3	97.8
Trastuzumab + Val-Cit-Belotecan dendrimer [prodrug 2]	Tras-Bel	3.9	99.7
Trastuzumab + GGFG-DXd [prodrug 3]	T-DXd	7.9	96.3
Rituximab + GGFG-DXd [prodrug 3]	Ritux-DXd	8.0	99.0

338 DAR values were determined by LC-MS and refer to the total number of cytotoxic
339 drugs (each prodrug 1 or 2 accounts for two drugs). Purity values were determined by
340 SE-HPLC and calculated by dividing the peak area of the monomeric species by the
341 overall area including high and low molecular weight species.

342

343

344

345 ***In vitro* serum stability, binding and cytotoxicity of Tras-Exa/Bel ADCs**

346 First, we assessed the linker-drug stability of our trastuzumab-based ADCs in serum.
 347 To this end, Tras-Exa (DAR4), Tras-Bel (DAR4) and T-DXd (DAR8) were monitored
 348 overtime for their payload release in mouse (**Table 2, Fig. S28**) and human (**Table 2,**
 349 **Fig. S28**) serum by the detection of free exatecan, belotecan and DXd *via* LC-MS/MS.
 350 Following 96 h incubation of the ADCs in sera, minimal amounts of the conjugated
 351 payload were released from both Tras-Exa and Tras-Bel ADCs in mouse (0.4% and
 352 0.2%, respectively) and human (0.3% and 0.2%, respectively) sera after. Similarly, we
 353 analyzed control ADC-bearing exatecan at DAR4 *via* Val-Cit linker in mouse, monkey
 354 and human serum and monitored for changes in the DAR. Following incubation of the
 355 ADC in different serum samples at 37°C for 96 h, the mean DAR remained stable over
 356 time at ~4 (**Fig. S29**). Overall, these data indicated that our ADCs are highly stable while
 357 maintaining the desired DAR. Moreover, as many preclinical studies are performed
 358 using murine systems, linker stability is of great importance when evaluating the
 359 therapeutic potential of new drugs, specifically in such models. These results
 360 demonstrated superior stability in mice for our dimeric Val-Cit linker compared to the
 361 classic monomeric Val-Cit linker, previously reported to exhibit a very short half-life of
 362 2.3 h in mouse plasma³².

363 **Table 2. Drug-linker stability.**

ADC	Free payload (% of total payload)			
	Mouse serum		Human serum	
	0 h	96 h	0 h	96 h
T-DXd	0.2	1.9	0	1.3
Tras-Exa	0	0.4	0	0.3
Tras-Bel	0	0.2	0	0.2

364 Percentage of ADC remaining over time in mouse and human sera following
 365 incubation at 37°C for 96 h, calculated from free exatecan, belotecan or DXd that were
 366 measured *via* LC-MS/MS. Numbers show the released fraction (%) relative to initially
 367 conjugated payload.

368

369 Prior to evaluating our trastuzumab-Exa/Bel conjugates for their anti-tumor effects *in*
 370 *vitro* and *in vivo*, we confirmed the expression levels of HER2 in two different HER2-
 371 positive human breast cancer cell lines used in this study. In line with previous reports,
 372 which classified HCC-1954 as HER2 medium/high-expressing cells and JIMT-1 as low-

373 expressing cells^{33, 34}, cell surface quantification of HER2 on HCC-1954 and JIMT-1 cell
374 showed approximately 1,700,000 and 190,000 receptors per cell, respectively (**Fig.**
375 **2A**). In comparison, the HER2-negative breast cancer cell line MDA-MB-468³⁵ showed
376 777 HER2 receptors per cell (which might be below the limit of detection of the kit
377 used) (**Fig. 2A**).

378 Next, while it is not expected that the conjugation of prodrugs to the heavy chains of
379 trastuzumab will affect its ability to bind HER2, we validated the binding of Tras-
380 Exa/Bel ADCs, unconjugated naked trastuzumab (Herceptin®) and T-DXd in an *in vitro*
381 cellular antigen-binding assay using flow cytometry. Considering the potential
382 limitation of such an assay when comparing ADCs with different Fc regions, linkers and
383 drugs, we used the same detection antibody. This analysis showed that at fixed
384 concentration of 100 nM, the three ADCs and naked trastuzumab bound similarly to
385 HER2-positive cells, with no difference in binding ability detected due to the use of
386 different linkers and/or payloads. Binding of all trastuzumab-based antibodies to HCC-
387 1954 cells was higher than to JIMT-1 cells, correlating with their respective HER2
388 expression levels (**Fig. 2B1,2B2**). Moreover, no binding of either antibody was
389 observed in the HER2-negative MDA-MB-468 cells (**Fig. 2B3**).

390 These results suggest that the bioconjugation techniques used to generate Tras-Exa
391 and Tras-Bel ADCs, did not impair the quaternary structure or masked the
392 complementarity-determining regions of trastuzumab, resulting in intact binding of
393 the antibodies to their target on the tumor cells, as compared to the naked
394 trastuzumab.

395 In correlation with the previous HER2 expression experiment, *in vitro* cytotoxicity
396 assay showed that HCC-1954 cells were significantly more sensitive to Tras-Exa
397 compared to MDA-MB-468 (**Fig. 2C1,2C3**). Furthermore, Tras-Exa had a lower IC₅₀
398 compared to free exatecan in HCC-1954 cells (**Fig. 2C1,2C4**). Moreover, Tras-Exa did
399 not show any advantage compared to free exatecan in JIMT-1 cells (**Fig. 2C2**). This may
400 be attributed to lower HER2 expression levels in JIMT-1 compared to HCC-1954,
401 demonstrating the correlation of our trastuzumab-based ADC and HER2 levels. Of
402 note, Tras-Exa showed some cytotoxic effects in MDA-MB-468 cells (**Fig. 2C3,2C4**).
403 While cathepsins are normally associated with intracellular lysosomal activity, it has

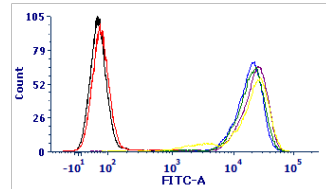
404 been shown that cathepsin B is also secreted into the extracellular matrix by cancer
405 cells, including breast tumor cells³⁶. Thus, in such *in vitro* settings, HER2-negative cells
406 might be affected by exatecan cleaved from the ADC, regardless of the HER2-directed
407 delivery of the therapeutic agent. Moreover, mechanisms of target-independent
408 uptake mediated by non-specific endocytosis may also contribute to such off-target
409 toxicity³⁷.

A. HER2 extracellular quantitation

	HCC-1954	JIMT-1	MDA-MB-468
Amount of HER2/cell	1,707,241	189,082	777

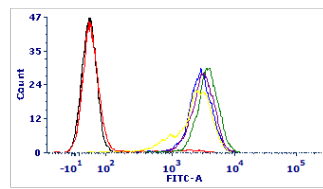
B. HER2-ADC binding

B1. HCC-1954 (HER2+++)



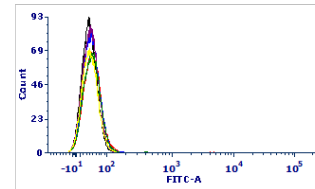
Antibody	MFI
AlexaFluor 488	53.56
Ritux-DXd	62.83
T-DXd	17084.09
Tras-Exa	20823.51
Tras-Bel	17919.94
Trastuzumab	19737.37

B2. JIMT-1 (HER2+)



Antibody	MFI
AlexaFluor 488	33.99
Ritux-DXd	38.11
T-DXd	2509.08
Tras-Exa	2770.70
Trast-Bel	3312.48
Trastuzumab	2182.57

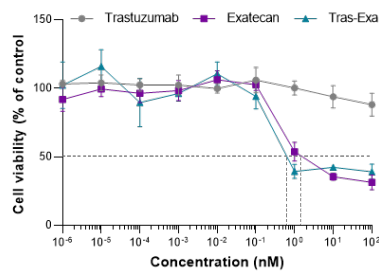
B3. MDA-MB-468 (HER2-)



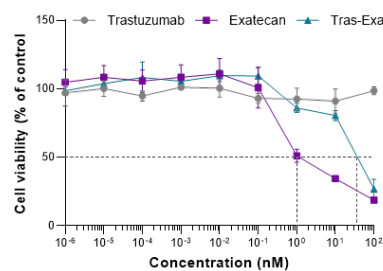
Antibody	MFI
AlexaFluor 488	29.87
Ritux-DXd	39.14
T-DXd	36.05
Tras-Exa	33.99
Tras-Bel	38.11
Trastuzumab	31.93

C. Cytotoxicity

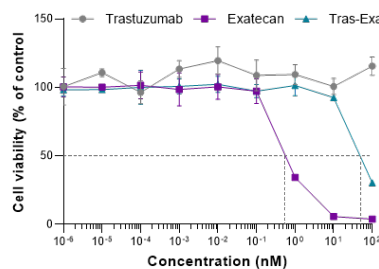
C1. HCC-1954 (HER2+++)



C2. JIMT-1 (HER2+)



C3. MDA-MB-468 (HER2-)



C4. IC₅₀ (nM)

	HCC-1954	JIMT-1	MDA-MB-468
Trastuzumab	N/A	N/A	N/A
Exatecan	1.5	1	0.6
Tras-Exa	0.6	40	50
Tras-Exa/Exa	0.4	40	83

410 **Figure 2. *In vitro* evaluation of Tras-Exa ADC. (A)** HER2 cell surface expression. Data
 411 represent the average values of n=2; **(B)** Trastuzumab and trastuzumab-based ADCs
 412 binding to HER2(+) HCC-1954 cells (B1), JIMT-1 cells (B2) and HER2(-) MDA-MB-468
 413 cells (B3 data is representative of n=2); **(C)** Viability of HCC-1954 (C1), JIMT-1 (C2), and
 414 MDA-MB-468 (C3) cells following 144 h (6 days) treatment with serial dilutions of
 415 trastuzumab, exatecan or Tras-Exa. (C4) Table summarizing the IC₅₀ values of the
 416 different treatments. Data represent mean ± SD. Data is representative of n=3.

417

418 **Pharmacokinetic (PK) parameters and therapeutic efficacy in a HER2-positive breast**
419 **cancer model**

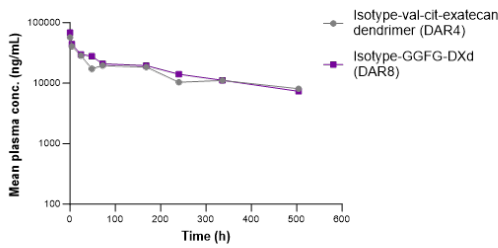
420 The PK parameters of control-ADC [isotype-Val-Cit-exatecan dendrimer (DAR4)] were
421 assessed and compared to isotype-GGFG-DXd (DAR8) following a single intravenous
422 administration at 3 mg/kg. For both ADCs no clear differences were observed and
423 demonstrated similar PK profiles in both hu FcRn and C57BL/6 strains (**Fig. 3A**).

424 Finally, Tras-Exa ADC (DAR4) and Tras-Bel ADC (DAR4) were evaluated for their *in vivo*
425 anti-tumor efficacy. To this end, mice bearing HCC-1954 xenografts were treated once
426 with Tras-Exa or Tras-Bel at three doses (1, 3 and 10 mg/kg) and monitored for tumor
427 growth and survival. The therapeutic efficacy of our ADCs was compared to T-DXd
428 (DAR8), which was also administered at three doses (1, 3 and 10 mg/kg), and to an
429 isotype control of Ritux-Dxd (DAR8), which was given only at the highest dose of 10
430 mg/kg. An additional control group was treated with the vehicle. A dose-dependent
431 inhibition of tumor growth was observed, with the most potent antitumor effect
432 observed at 10 mg/kg for the three trastuzumab-based ADCs (**Fig. 3B**). Interestingly, a
433 single dose of Tras-Exa or T-DXd at the highest concentration (10 mg/kg) resulted in a
434 complete tumor regression. Conversely, 4 out of 9 (44%) of the Tras-Bel-treated mice
435 at 10 mg/kg, relapsed 60 days following treatment (**Fig. 3B1**). Furthermore, a
436 moderate anti-tumor activity was observed at 3 mg/kg for all ADCs, with T-DXd
437 demonstrating slightly stronger tumor growth inhibition among the different ADCs
438 (**Fig. 3B2**). Overall, Tras-Exa and T-DXd showed a superior anti-tumor activity
439 compared to Tras-Bel ADC at 3 and 10 mg/kg, with no statistically significant difference
440 in efficacy between these two ADCs for both concentrations. In contrast, treatment
441 with the lowest dose (1 mg/kg) had no impact on tumor growth compared to the
442 controls in either ADC (**Fig. 3B3**). These results correlate with *in silico* outcomes using
443 our newly-established prediction algorithm for ADC performance based on Shah *et*
444 *al.*³⁸

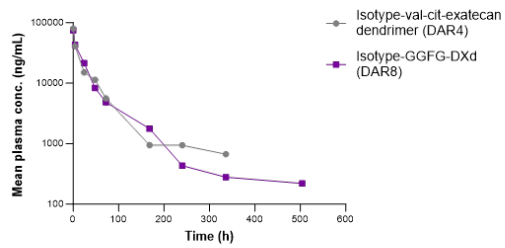
445 Of note, following treatments, mice of all experimental groups, including the vehicle
 446 and isotype control, exhibited transient body weight loss, which was recovered after
 447 5 days (Fig. 3B4). Overall, our trastuzumb-based ADCs were well-tolerated at all doses
 448 evaluated, similarly to T-DXd, vehicle or isotype control, with net positive weight gain
 449 over the course of the study (Fig. 3B4).

A. Pharmacokinetic parameters

A1. C57BL/6



A2. hu FcRn

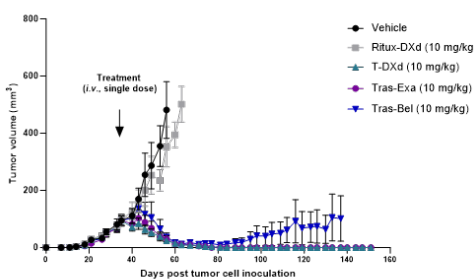


A3.

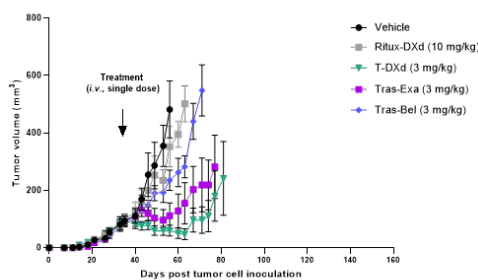
Strain	ADC	C _{last} (mg/mL)	AUC _{last} (h*mg/mL)	Ext (%)	AUC _{INF} (h*mg/mL)	Beta t _{1/2} (days)	Cl (mL/h/kg)		V _{ss} (mL/kg)	
							Estimate	CV%	Estimate	CV%
hu FcRn	Isotype-GGFG-DXd (DAR8)	0.00067	1.67	2	1.7	2.74	1.76	4	112	2
hu FcRn	Isotype-val-cit-exatecan dendrimer (DAR4)	0.000221	1.77	0.8	1.79	3.49	1.68	4	104	2
C57BL/6	Isotype-GGFG-DXd (DAR8)	0.0081	7.94	30	11.1	11.67	0.270	7	107	1.5
C57BL/6	Isotype-val-cit-exatecan dendrimer (DAR4)	0.00737	8.11	30	11.1	11.79	0.270	10.6	103	3.2

B. In vivo antitumor activity

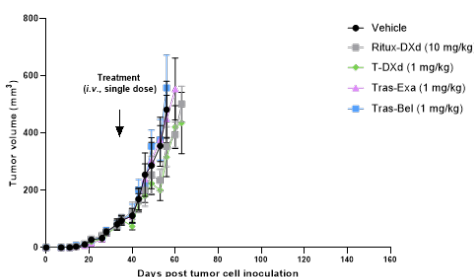
B1. 10 mg/kg



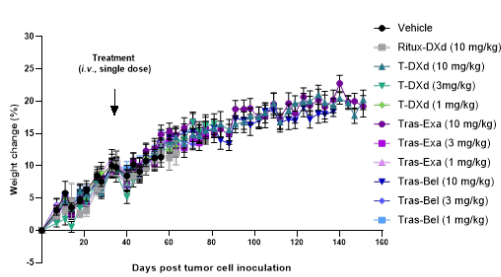
B2. 3 mg/kg



B3. 1 mg/kg



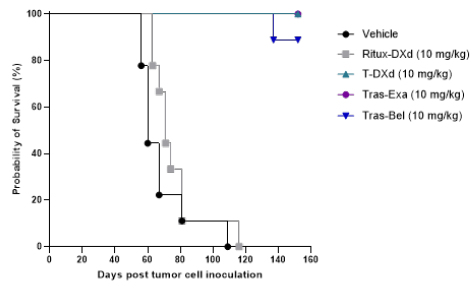
B4. Body weight change



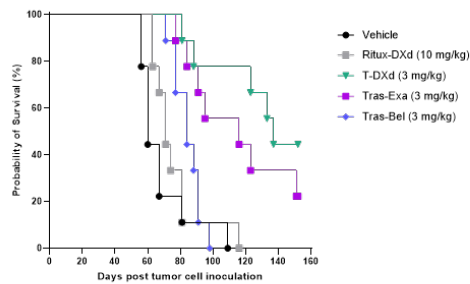
450 **Figure 3. Trastuzumab-based ADCs lead to a dose-dependent inhibition of tumor**
451 **growth. (A)** PK parameters following a single intravenous administration of isotype-
452 GGFG-DXd (DAR8) and isotype-Val-Cit-exatecan dendrimer (DAR4) at 3 mg/kg to two
453 mice strains (hu FcRn and C57BL/6) for 21 days (n=3 mice/time point; in total n=6
454 mice/group). C_{last} – last measurable concentration; AUC_{last} – area under the curve up
455 to the last quantifiable time-point; Ext – extrapolated; AUC_{inf} – area under the curve
456 extrapolated to the infinite time; Cl – clearance; V_{ss} – volume of distribution at steady
457 state; CV – coefficient of variation. PK statistical analyses were done for AUC and Cl.
458 Ext (%) calculated as the percent of the AUC that is extrapolated. **(B)** Growth curves of
459 HCC1954 tumors following single intravenous injection of trastuzumab-based ADCs at
460 three concentrations (n=9 mice/group): 10 mg/kg (B1); 3 mg/kg (B2) and 1 mg/kg (B3).
461 All treatment groups were included in the same experiment; data are presented in
462 separate graphs for clarity. **(B4)** Body weight change, expressed as percent change
463 from the day of tumor cell inoculation. Data are presented as mean \pm s.e.m.
464

465 In line with the data described above, Kaplan-Meier analyses demonstrated a dose-
466 dependent survival benefit of the different ADCs evaluated. At the end of the study
467 (day 152), 100% of mice treated with 10 mg/kg of Tras-Exa or T-DXd and 89% of mice
468 treated with Tras-Bel at the same concentration were alive; while none of the control-
469 treated mice survived past day 109 (vehicle) and 116 (Ritux-Dxd) (**Fig. 4A1,4B**).
470 Moreover, treatment at 3 mg/kg significantly prolonged the median survival of mice
471 treated with Tras-Exa (116 days) or T-DXd (137 days) compared to vehicle control (60
472 days) and Ritux-Dxd (71 days) (**Fig. 4A2,4B**). Tras-Bel (3 mg/kg) had no significant
473 effect on mice survival compared to the controls, with a median survival of 84 days
474 (**Fig. 4A2,4B**). At the lowest dose of 1 mg/kg, neither ADC showed any therapeutic
475 efficacy in comparison to vehicle and isotype controls (**Fig. 4A3,4B**).
476 Overall Tras-Exa showed similar efficacy to T-DXd at 3 and 10 mg/kg despite having
477 lower DAR of 4 compared to DAR8 of T-DXd.

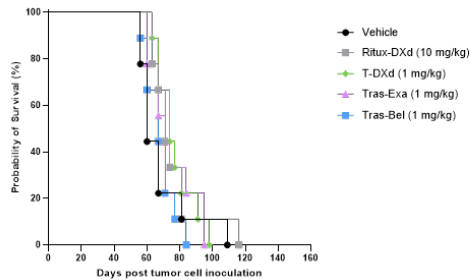
A1. 10 mg/kg



A2. 3 mg/kg



A3. 1 mg/kg



B.

Comparison of survival curves	Log-Rank (Mantel-Cox) test
Vehicle vs. Ritux-DXd 10 mg/kg	ns
Vehicle vs. T-DXd 10 mg/kg	<0.0001
Vehicle vs. T-DXd 3 mg/kg	<0.0001
Vehicle vs. T-DXd 1 mg/kg	ns
Vehicle vs. Tras-Exa 10 mg/kg	<0.0001
Vehicle vs. Tras-Exa 3 mg/kg	0.0007
Vehicle vs. Tras-Exa 1 mg/kg	ns
Vehicle vs. Tras-Bel 10 mg/kg	<0.0001
Vehicle vs. Tras-Bel 3 mg/kg	ns
Vehicle vs. Tras-Bel 1 mg/kg	ns
Ritux-DXd 10 mg/kg vs. T-DXd 10 mg/kg	<0.0001
Ritux-DXd 10 mg/kg vs. T-DXd 3 mg/kg	0.0001
Ritux-DXd 10 mg/kg vs. T-DXd 1 mg/kg	ns
Ritux-DXd 10 mg/kg vs. Tras-Exa 10 mg/kg	<0.0001
Ritux-DXd 10 mg/kg vs. Tras-Exa 3 mg/kg	0.0017
Ritux-DXd 10 mg/kg vs. Tras-Exa 1 mg/kg	ns
Ritux-DXd 10 mg/kg vs. Tras-Bel 10 mg/kg	<0.0001
Ritux-DXd 10 mg/kg vs. Tras-Bel 3 mg/kg	ns
Ritux-DXd 10 mg/kg vs. Tras-Bel 1 mg/kg	ns
T-DXd 10 mg/kg vs. T-DXd 3 mg/kg	0.0101
T-DXd 10 mg/kg vs. T-DXd 1 mg/kg	<0.0001
T-DXd 3 mg/kg vs. T-DXd 1 mg/kg	0.0002
Tras-Exa 10 mg/kg vs. Tras-Exa 3 mg/kg	0.0008
Tras-Exa 10 mg/kg vs. Tras-Exa 1 mg/kg	<0.0001
Tras-Exa 3 mg/kg vs. Tras-Exa 1 mg/kg	0.003
Tras-Bel 10 mg/kg vs. Tras-Bel 3 mg/kg	<0.0001
Tras-Bel 10 mg/kg vs. Tras-Bel 1 mg/kg	<0.0001
Tras-Bel 3 mg/kg vs. Tras-Bel 1 mg/kg	0.0016
T-DXd 10 mg/kg vs. Tras-Exa 10 mg/kg	ns
T-DXd 10 mg/kg vs. Tras-Bel 10 mg/kg	ns
Tras-Exa 10 mg/kg vs. Tras-Bel 10 mg/kg	ns
T-DXd 3 mg/kg vs. Tras-Exa 3 mg/kg	ns
T-DXd 3 mg/kg vs. Tras-Bel 3 mg/kg	0.0012
Tras-Exa 3 mg/kg vs. Tras-Bel 3 mg/kg	0.0076
T-DXd 1 mg/kg vs. Tras-Exa 1 mg/kg	ns
T-DXd 1 mg/kg vs. Tras-Bel 1 mg/kg	ns
Tras-Exa 1 mg/kg vs. Tras-Bel 1 mg/kg	ns

479 **Figure 4. Trastuzumab-based ADCs prolong the survival of mice in a dose-dependent**
 480 **manner. (A)** Kaplan–Meier overall survival curves of HCC-1954 tumor-bearing SCID
 481 mice treated intravenously with a single injection of trastuzumab-based ADCs at three
 482 concentrations (n=9 mice/group): 10 mg/kg (A1); 3 mg/kg (A2) and 1 mg/kg (A3). All
 483 treatment groups were included in the same experiment; data are presented in
 484 separate graphs for clarity. **(B)** Table summarizing the statistical significance of
 485 survival curves between different treatment groups, as analyzed using the log-rank
 486 (Mantel-Cox) test. P < 0.05; ns = not significant.

487 **Conclusions**

488 In this study, we developed a novel and highly effective trastuzumab-based HER2-
489 targeting ADC using a new dimeric prodrug system based on an AB2 self-immolative
490 dendritic scaffold. Our novel trastuzumab-exatecan ADC demonstrated high stability
491 in serum while maintaining intact HER2 binding ability. Furthermore, our linker is more
492 stable compared to conventional Val-Cit linkers, which show instability in mice, *i.e.*
493 half-life of 2.3 h³² *versus* ~70 days for our linker, according to the $t_{1/2}$ of our control
494 ADC in mice (Data not shown). Importantly, trastuzumab-exatecan ADC exhibited a
495 highly potent antitumor activity *in vivo* in HER2-positive breast cancer model,
496 compared to the FDA-approved T-DXd (Enhertu®; DAR8) despite having half the DAR,
497 *i.e.*, DAR4. Overall, our findings highlight the therapeutic potential of this novel
498 trastuzumab-exatecan ADC as an effective option for the treatment of patients
499 overexpressing HER2. Furthermore, our novel linker-drug technology has the potential
500 to serve as a platform for further technological advances. It can potentially assist in
501 the development of trastuzumab-based therapy given either as combination with a
502 different active agent or as monotherapy ADCs using distinct payloads attached to the
503 same antibody. Moreover, this linker technology can be employed in the development
504 of novel ADCs for a wide array of molecular targets beyond HER2. Nevertheless, there
505 is a need for further investigations of trastuzumab-exatecan ADC in a clinical setting.

506

507 **Acknowledgements**

508 The Satchi-Fainaro and Shabat laboratories received partial funding from Merck
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510 Advanced Grant Agreement no. [835227]-3DBrainStrom; The Israel Science
511 Foundation (Grant No. 1969/18); and The Israel Cancer Research Fund (PROF-18-682).

512 **Declaration of competing interests**

513 RS-F is a Board Director at Teva Pharmaceutical Industries Ltd, relevant to work
514 unrelated to this article. RS-F and DS received research funding from Merck.

515

516 **Supplementary Data**

517 **General information**

518 All reactions requiring anhydrous conditions were performed under an Argon
519 atmosphere. All reactions were carried out at room temperature (RT) unless stated
520 otherwise. Chemicals and solvents were either A.R. grade or purified by standard
521 techniques. Thin-layer chromatography (TLC): silica gel plates Merck 60 F254:
522 compounds were visualized by irradiation with UV light. Column chromatography (FC):
523 silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses.
524 Reverse-phase high-pressure liquid chromatography (RP-HPLC): C18 5u, 250x4.6mm,
525 eluent given in parentheses. Preparative RP-HPLC: C18 5u, 250x21mm, eluent given in
526 parentheses. ¹H-NMR spectra were measured using Bruker Avance operated at 400
527 MHz. ¹³C-NMR spectra were measured using Bruker Avance operated at 101 MHz.
528 Chemical shifts were reported in ppm on the δ scale relative to a residual solvent
529 (CDCl₃: δ = 7.26 for ¹H-NMR and 77.16 for ¹³C-NMR, DMSO-d₆: δ = 2.50 for ¹H-NMR
530 and 39.52 for ¹³C-NMR). Mass spectra were measured on Waters Xevo TQD. All
531 general reagents, including salts and solvents, were purchased from Sigma-Aldrich.

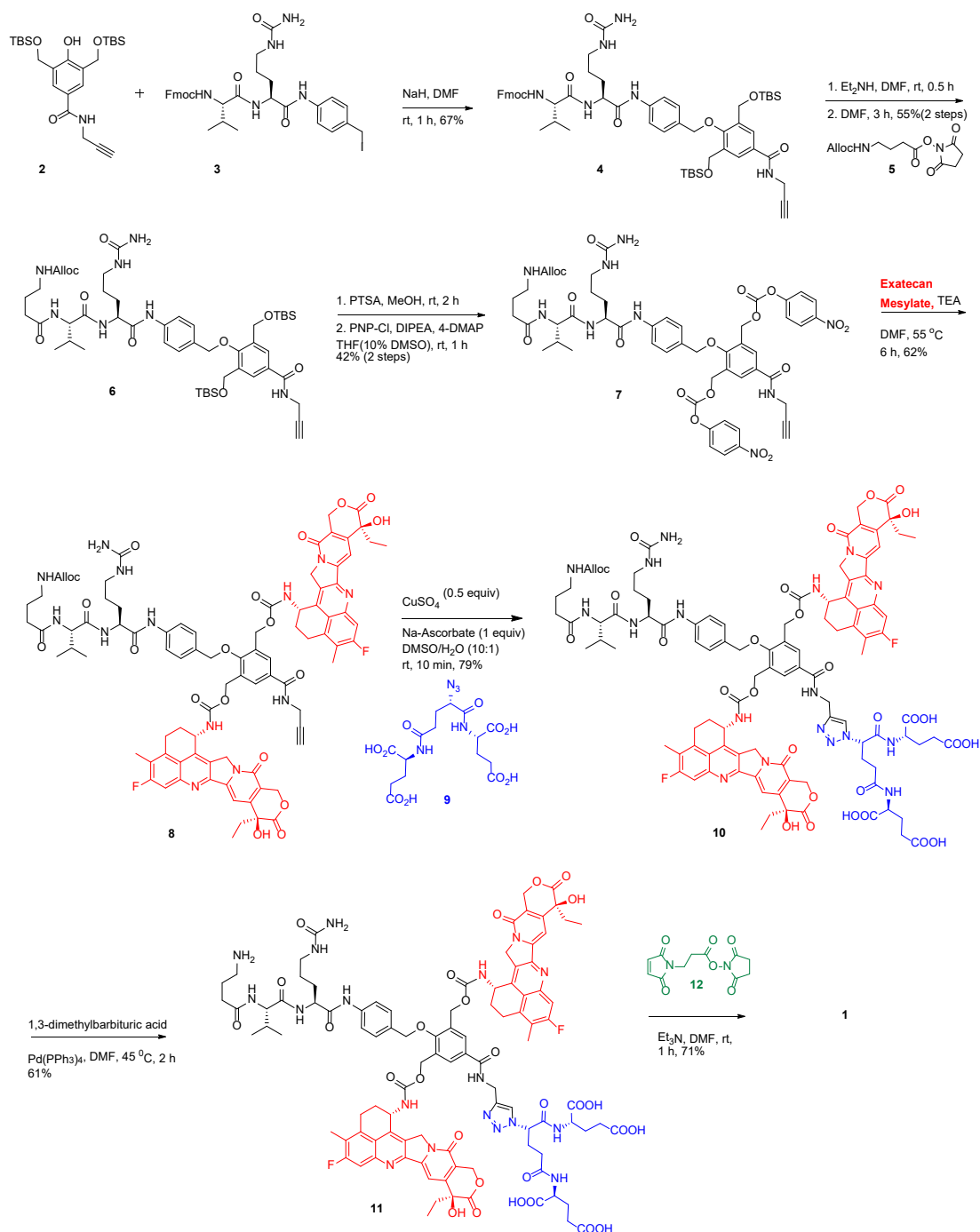
532

533 **Abbreviations**

534 **ACN** - Acetonitrile, **CDI** - 1,1'-Carbonyldiimidazole, **DCC** - *N, N'*-Dicyclohexylcarbo-
535 diimide, **DCM** - dichloromethane, **DIPEA** - *N,N*-Diisopropylethylamine, **DMF** - *N,N'*-
536 Dimethylformamide, **DMBA** - Dimethylbarbituric acid, **DMAP** - 4-(Dimethylamino)-
537 pyridine, **DMSO** - Dimethyl sulfoxide, **EDC** - 1-Ethyl-3-(3-dimethylaminopropyl)-
538 carbodiimide, **EEDQ** - *N*-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, **EtOAc** -
539 Ethylacetate, **HBTU** - 2-(1*H*-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium
540 hexafluorophosphate, **Hex**- Hexane, **TFA** - Trifluoroacetic acid, **TEA** - Triethylamine, **THF**
541 - Tetrahydrofuran, **TMSCl** - Trimethylsilyl chloride, **PBS** - Phosphate-buffered saline.

542 Synthetic schemes and experimental procedures

543



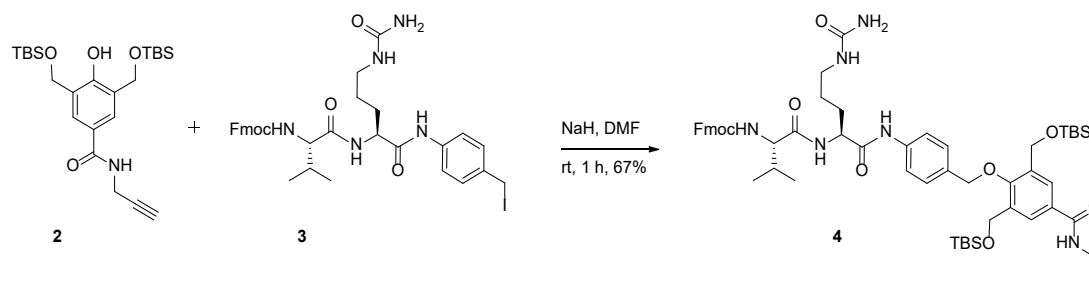
544

545 **Figure S1. Synthesis of prodrug 1.** The synthesis of exatecan self-immolative dimeric
 546 Prodrug 1 was achieved as presented. Phenol 2 was alkylated with iodide 3 to generate
 547 ether 4. The Fmoc protecting group of Compound 4 was then removed to give the
 548 corresponding amine, which was further reacted with NHS ester 5 to produce amide
 549 6. The TBS protecting groups of Compound 6 were removed and the obtained di-
 550 hydroxy-benzylalcohol was activated with 4-nitrophenyl chloroformate to form di-4-
 551 nitrophenyl carbonate 7. The latter was reacted with two equivalents of exatecan to

552 give Compound **8**. The acetylene functionality of Compound **8** was clicked with azide
553 9 to produce triazole 10. Finally, the Alloc protecting group of Compound **10** was
554 removed and the obtained amine was reacted NHS ester of maleimide-carboxylic acid
555 to furnish Prodrug 1.

556

557



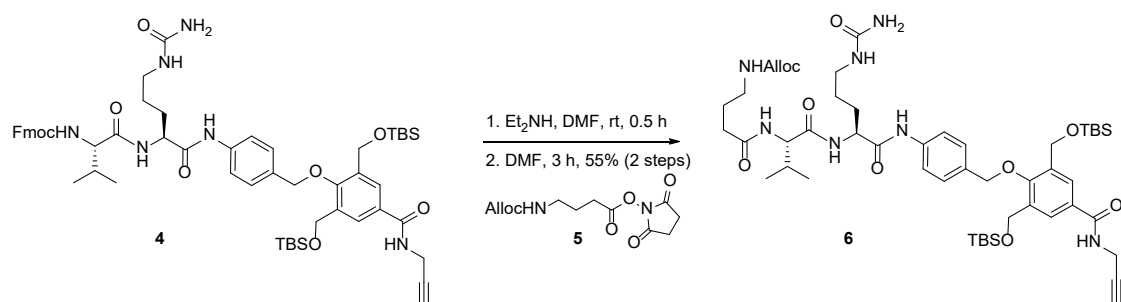
558

559 **Figure S2. Synthesis of Compound 4.** Compound **2**^{31, 39, 40} (1.0 g, 2.16 mmol) was
560 dissolved in dry DMF (5 mL) under argon atmosphere and cooled to 0°C. Sodium
561 hydride (91 mg, 2.27 mmol, 60% dispersion in mineral oil) was added and the reaction
562 was allowed to warm to RT. After stirring for 15 min at RT, Compound **3**⁴¹ (1.54 g, 2.16
563 mmol) was added and the reaction was monitored by TLC (MeOH:EtOAc 10:90). Upon
564 completion, the reaction was diluted with EtOAc (30 mL) and NH₄Cl (10 mL). The
565 biphasic mixture was then extracted with EtOAc (3 x 30 mL), washed with brine (30
566 mL), dried over Na₂SO₄ and evaporated under reduced pressure. The crude product
567 was purified by column chromatography on silica gel to afford the titled Compound **4**
568 as an off-white solid (1.61 g, 67%). ¹H NMR (400 MHz, DMSO) δ 10.09 (s, 1H), 8.85 (s,
569 1H), 8.12 (d, *J* = 6.4 Hz, 1H), 8.01 – 7.54 (m, 6H), 7.37 (m, 6H), 5.97 (s, 1H), 5.40 (s, 2H),
570 4.89 (s, 2H), 4.64 (s, 4H), 4.44 (d, *J* = 4.0 Hz, 1H), 4.40 – 4.15 (m, 4H), 4.04 (s, 2H), 3.94
571 (d, *J* = 6.7 Hz, 2H), 3.09 – 2.91 (m, 3H), 2.87 (s, 2H), 1.99 (d, *J* = 5.5 Hz, 1H), 1.78 – 1.53
572 (m, 2H), 1.49 – 1.28 (m, 2H), 0.86 (s, 28H), 0.04 (s, 12H). MS (ES+) *m/z* calculated for
573 C₅₇H₇₈N₆O₉Si₂: 1046.5, found 1047.9 for [M+H]⁺.

574

575

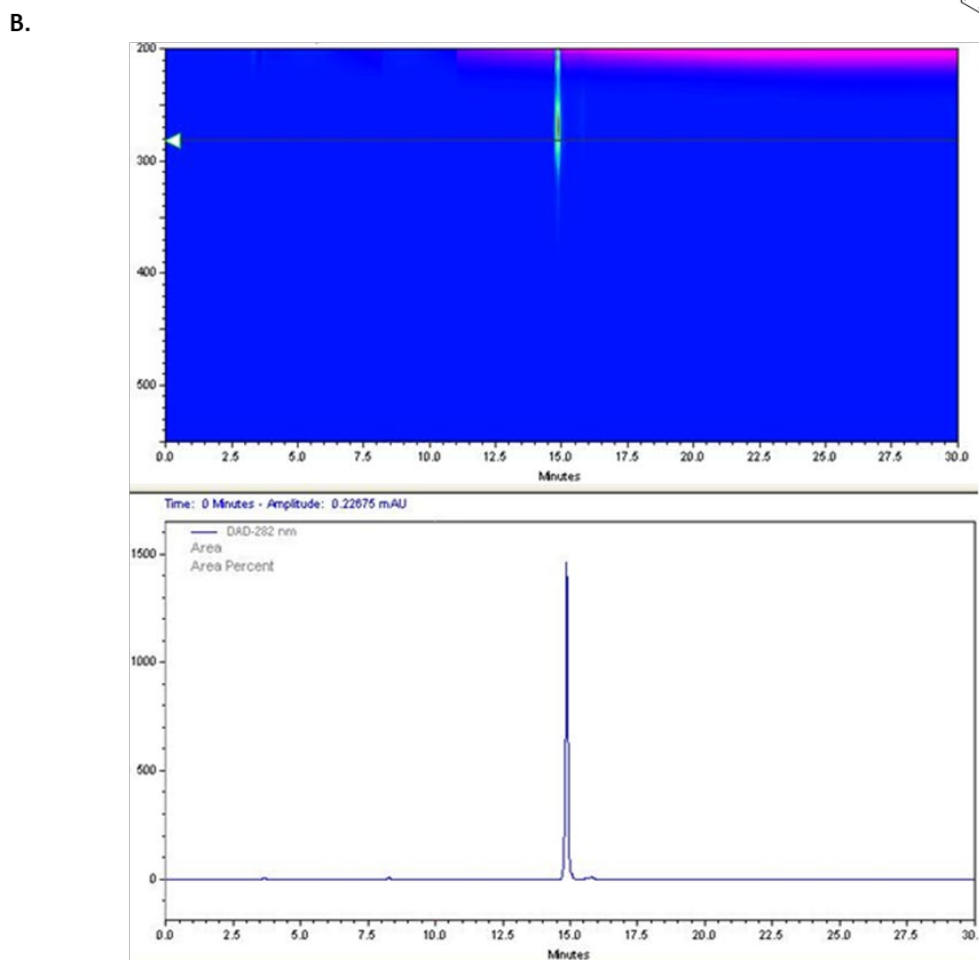
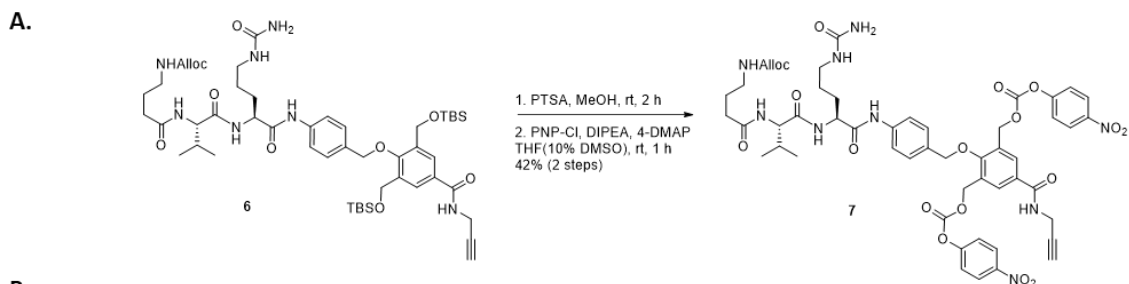
576



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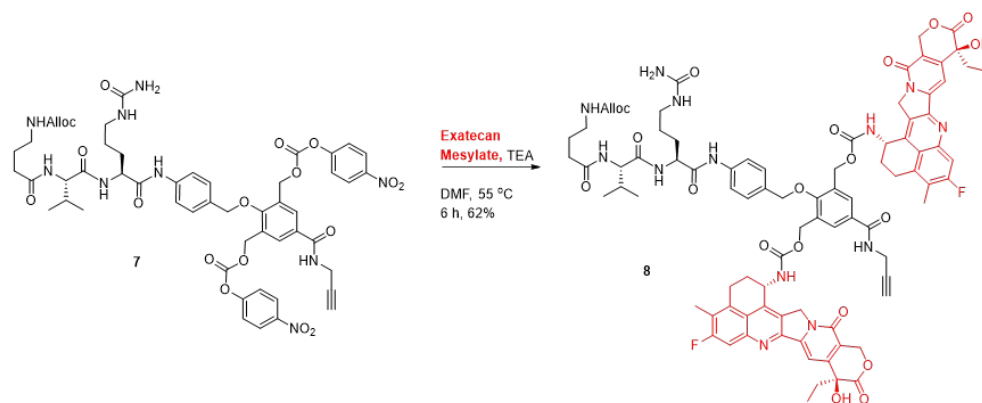
579 **Figure S3. Synthesis of Compound 6.** Compound 4 (1.2 g, 1.15 mmol) was dissolved in
 580 DMF (6 mL) and diethylamine (2 mL) was added. The reaction was monitored by TLC.
 581 Upon disappearance of starting material, the solvent and diethylamine were removed
 582 under reduced pressure. The product was dried under vacuum and directly taken for
 583 the next reaction. The crude material was dissolved in DMF (5 mL), and Compound 5
 584 (327 mg, 1.15 mmol) was added. The mixture was stirred at RT for 2 h and the reaction
 585 was monitored by TLC. Upon completion, the solvent was removed under reduced
 586 pressure. The crude product was purified by column chromatography on silica gel to
 587 afford the titled Compound 6 as an off-white solid (630 mg, 55% over 2 steps). ¹H NMR
 588 (400 MHz, MeOD) δ 7.86 (s, 1H), 7.66 (d, *J* = 8.0 Hz, 2H), 7.48 (d, *J* = 8.0 Hz, 1H), 7.34
 589 (d, *J* = 8.3 Hz, 2H), 7.10 (d, *J* = 8.3 Hz, 1H), 6.00 – 5.85 (m, 1H), 5.29 (d, *J* = 17.2 Hz, 1H),
 590 5.17 (d, *J* = 10.3 Hz, 1H), 4.98 (s, 2H), 4.85 (s, 2H), 4.79 (s, 1H), 4.70 (s, 2H), 4.57 – 4.48
 591 (m, 3H), 4.21 – 4.12 (m, 2H), 3.26 – 3.06 (m, 4H), 2.39 – 2.29 (m, 2H), 2.15 – 2.05 (m,
 592 1H), 1.87 – 1.72 (m, 4H), 1.69 – 1.53 (m, 2H), 1.05 – 0.96 (m, 6H), 0.92 (s, 18H), 0.09
 593 (s, 9H). MS (ES+) *m/z* calculated for C₅₀H₇₉N₇O₁₀Si₂: 993.5, found 1016.9 for [M+Na]⁺.



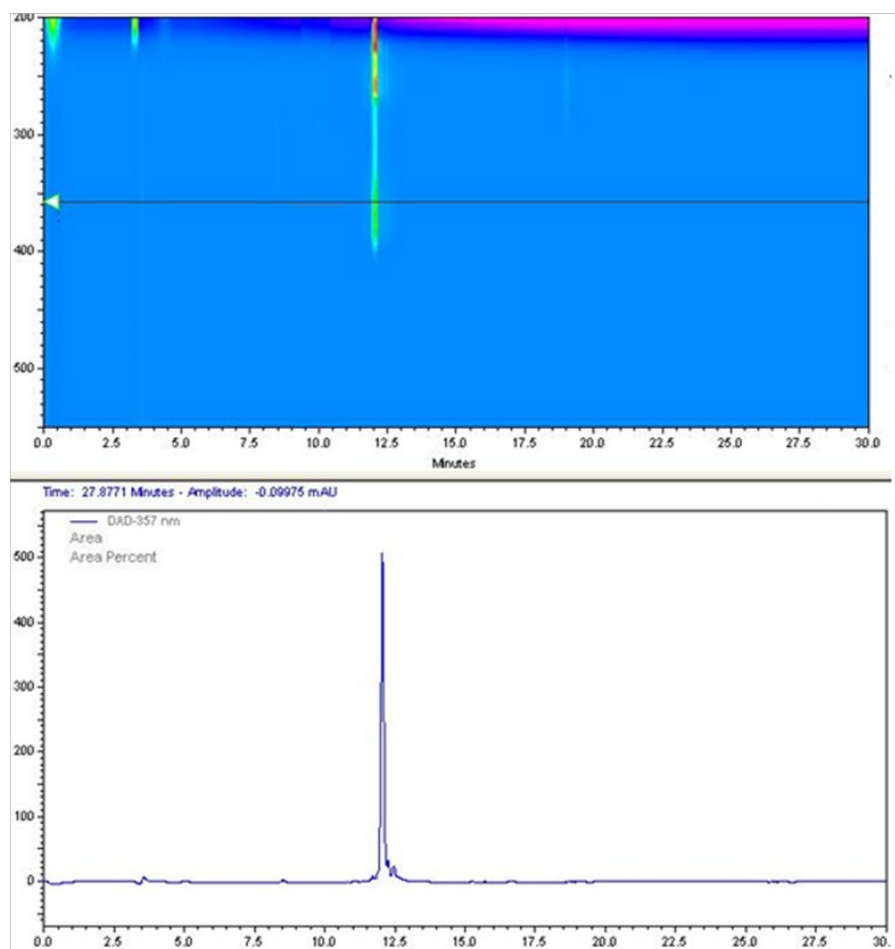
594 **Figure S4. Synthesis and RP-HPLC chromatograms of Compound 7.** (A) Compound 6
595 (625 mg, 0.63 mmol) was dissolved in MeOH (4 mL) and 4-touenesulfonic acid
596 monohydrate (PTSA, 12 mg, 0.063 mmol) was added. The reaction was monitored by
597 TLC. Upon completion the reaction mixture was diluted with EtOAc (20 mL) and
598 saturated NaHCO₃ (5 mL) was added. The biphasic mixture was then extracted with
599 EtOAc (3 x 20 mL), washed with brine (20 mL), dried over Na₂SO₄, and evaporated
600 under reduced pressure. The crude product was dissolved in dry THF/DMSO solvent
601 system (8 mL, THF: DMSO 10:1) before DIPEA (0.45 mL, 2.52 mmol) and 4-DMAP (4
602 mg, 0.03 mmol) were added. The mixture was cooled to 0°C. 4-Nitrophenyl
603 chloroformate (PNP-Cl, 317 mg, 1.58 mmol) was then added portion wise and the
604 reaction was stirred for 1 h at RT. The progress of the reaction was monitored by TLC.

605 Upon completion, the solution was directly loaded on silica gel and the product was
606 purified by column chromatography to afford Compound **7** as off-white solid (289 mg,
607 42% over 2 steps). MS (ES+) m/z calculated for $C_{52}H_{57}N_9O_{18}$: 1095.4, found 1096.7 for
608 $[M+H]^+$. Analytical RP-HPLC: Column C18 5 μ , 250x4.6 mm. Eluent: ACN/H₂O (H₂O with
609 0.1% of TFA). Method: 30-100 % ACN gradient. t_R for Compound **7**: 15.0 min. **(B)** HPLC
610 elution gradient ACN and water with 0.1 TFA (30-100%).

A.

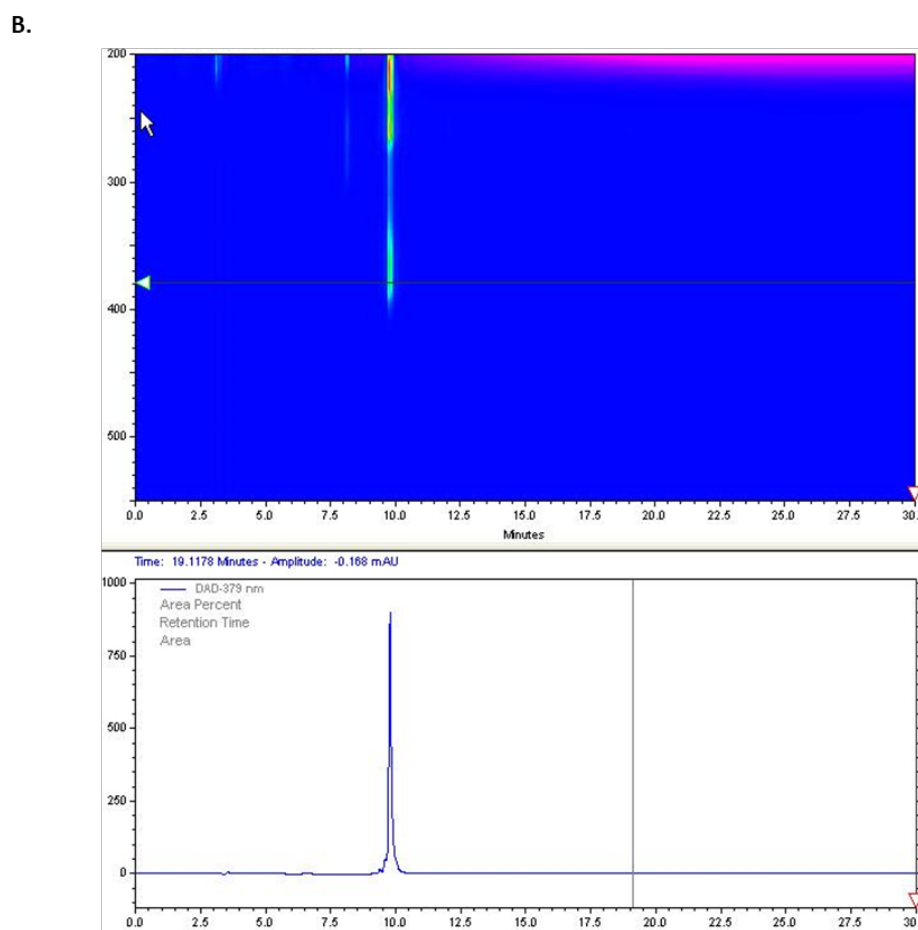
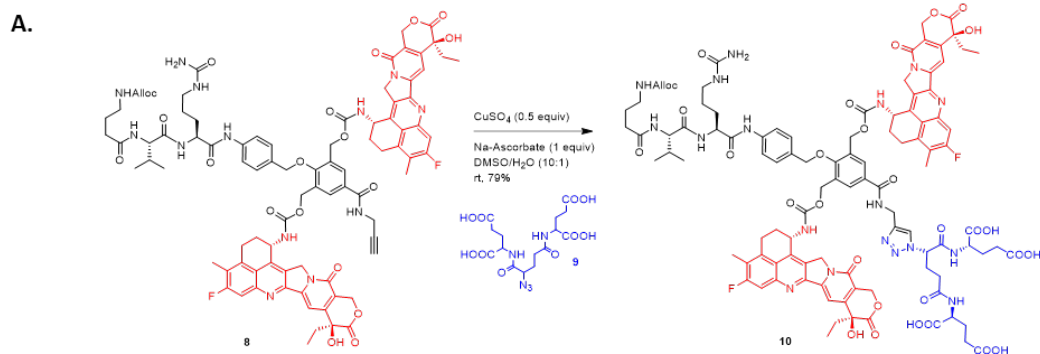


B.



611

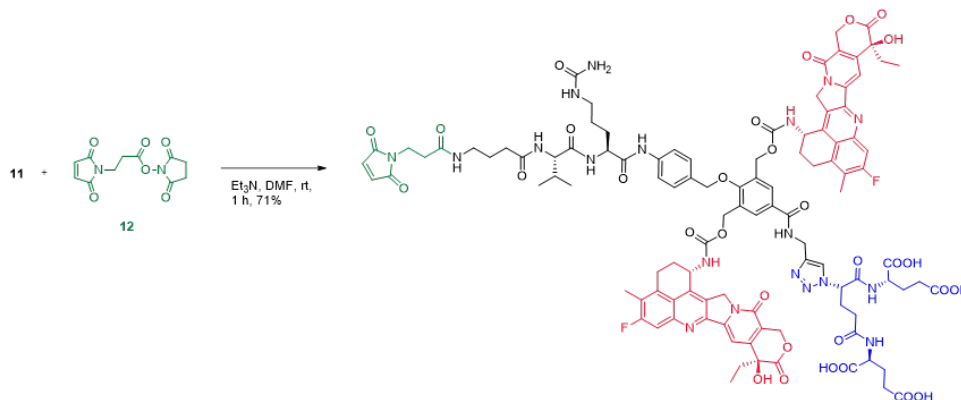
612 **Figure S5. Synthesis and RP-HPLC chromatograms of Compound 8. (A)** Compound 7
613 (80 mg, 0.07 mmol) was dissolved in dry DMF (2 mL) at RT. Exatecan Mesylate (77 mg,
614 0.14 mmol) was added followed by Et₃N (60 μL, 0.44 mmol). The reaction mixture was
615 stirred at 55°C for 6 h and the progress of the reaction was monitored by RP-HPLC.
616 Upon completion the product was isolated by reverse phase preparative HPLC (30-
617 100% ACN in water with 0.1% TFA, 20 min) to afford Compound 8 as yellow solid (76
618 mg, 62%). MS (ES+) *m/z* calculated for C₈₈H₉₁F₂N₁₃O₂₀: 1687.6, found 845.7 for
619 [M/2+2H]⁺. Analytical RP-HPLC: Column C18 5μ, 250x4.6 mm. Eluent: ACN/H₂O (H₂O
620 with 0.1% of TFA). Method: 30-100% ACN gradient. *t_R* for Compound 8: 12.4 min. **(B)**
621 HPLC elution gradient ACN and water with 0.1 TFA (30-100%).



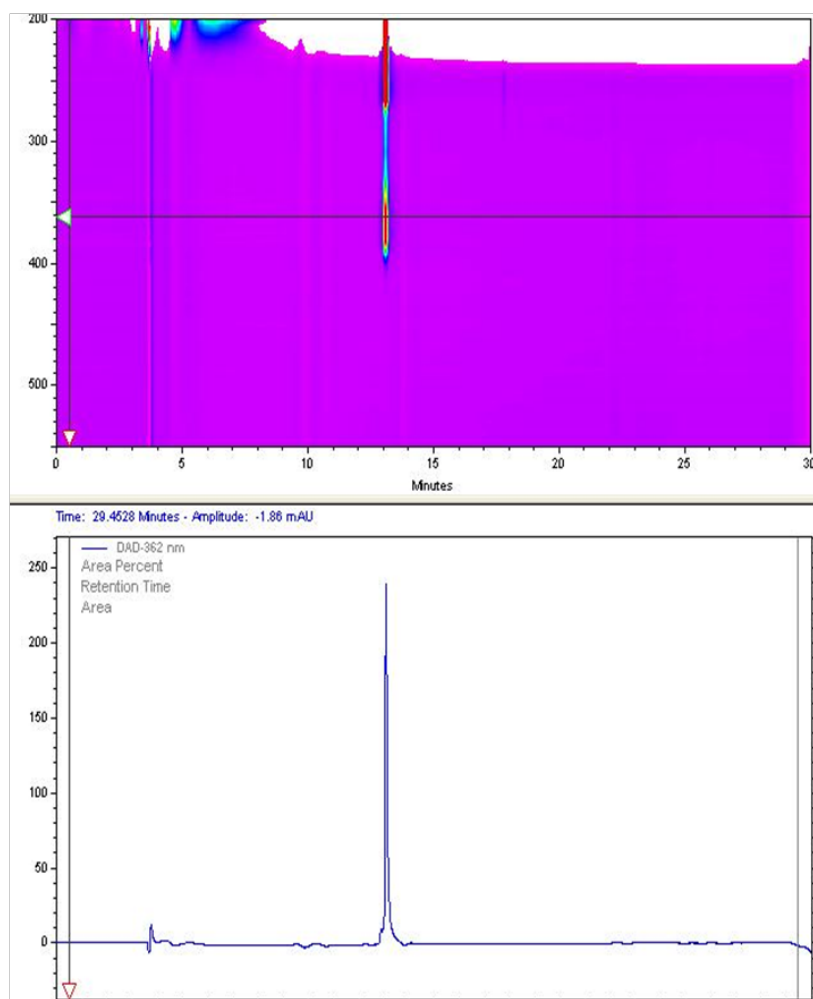
622 **Figure S6. Synthesis and RP-HPLC chromatograms of Compound 10.** (A) Compound 8
 623 (65 mg, 0.04 mmol) and azide 9 (86 mg, 0.2 mmol) was dissolved in a solvent system
 624 DMSO/H₂O (10:1, 2 mL) followed by the addition of CuSO₄·5H₂O (5 mg, 0.02 mmol)
 625 and sodium ascorbate (3.2 mg, 0.03 mmol). The solution was degassed with argon for
 626 30 min and then stirred at RT. After 30 min of stirring the progress of the reaction was
 627 monitored by RP-HPLC. Upon completion the product was isolated by reverse phase
 628 preparative HPLC (30-100% ACN in water with 0.1% TFA, 20 min) to afford Compound
 629 **10** as yellow solid (64 mg, 79%). MS (ES⁺) *m/z* calculated for C₁₀₃H₁₁₂F₂N₁₈O₃₀: 2119.9,
 630 found 1061.4 for [M/2+H]⁺. Analytical RP-HPLC: Column C18 5μ, 250x4.6 mm. Eluent:
 631 ACN/H₂O (H₂O with 0.1% of TFA). Method: 30-100% ACN gradient. *t_R* for Compound
 632 **10**: 10.07 min. (B) HPLC elution gradient ACN and water with 0.1 TFA (30-100%).

642 for $C_{99}H_{108}F_2N_{18}O_{28}$: 2035.7, found 1019.2 for $[M/2+H]^+$. Analytical RP-HPLC: Column
643 C18 5 μ , 250x4.6 mm. Eluent: ACN/H₂O (H₂O with 0.1% of TFA). Method: 30-100 % ACN
644 gradient. t_R for Compound **11**: 6.5 min. **(B)** HPLC elution gradient ACN and water with
645 0.1 TFA (30-100%).

A.



B.

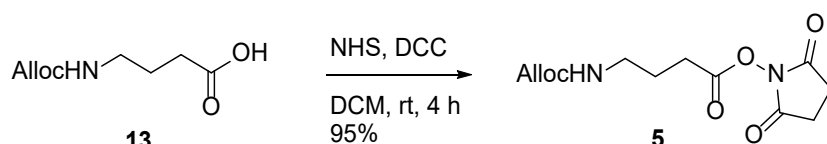


646

647 **Figure S8. Synthesis and RP-HPLC chromatograms of Prodrug 1.** (A) Compound **11** (25
648 mg, 0.012 mmol) and Compound **12** (4 mg, 0.014 mmol) were dissolved in dry DMF
649 (1.5 mL). Et_3N was added and the reaction mixture was stirred at RT for 1 h. Upon

650 completion, as monitored by RP-HPLC, the product was purified by reverse phase
651 preparative HPLC (10-90% ACN in water with 0.1% TFA, 20 min) to afford the final
652 Prodrug **1** as yellow solid (19 mg, 71%). MS (ES+) *m/z* calculated for C₁₀₆H₁₁₃F₂N₁₉O₃₁:
653 2186.79, found 1094.6 for [M/2+H]⁺. Analytical RP-HPLC: Column C18 5μ, 250x4.6 mm.
654 Eluent: ACN/H₂O (H₂O with 0.1% of TFA). Method: 10-90 % ACN gradient. *t_R* for
655 Prodrug **1**: 13.2 min.

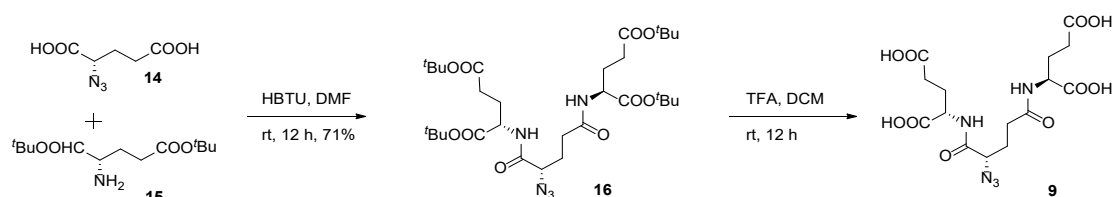
656



658

659 **Figure S9. Synthesis of Compound 5.** Compound **13**⁴² (750 mg, 4.0 mmol) was
660 dissolved in DCM (8 mL) and cooled to 0°C. N-hydroxysuccinimide (692 mg, 6.0 mmol)
661 was added followed by N,N'-Dicyclohexylcarbodiimide (908 mg, 4.4 mmol). The
662 reaction was allowed to warm up to RT and stirring continued for 4 h. Upon
663 completion the turbid mixture was filtered, washed with DCM. The combined organic
664 solution was concentrated, and the product was purified by silica gel column
665 chromatography to afford Compound **4** (1.08 g, 95%) as white solid. ¹H NMR (400 MHz,
666 CDCl₃) δ 5.89 (ddt, *J* = 16.2, 10.7, 5.5 Hz, 1H), 5.28 (dd, *J* = 17.2, 1.2 Hz, 1H), 5.18 (d, *J*
667 = 10.4 Hz, 1H), 4.53 (d, *J* = 4.8 Hz, 2H), 3.27 (q, *J* = 6.4 Hz, 2H), 2.82 (s, 4H), 2.66 (dd, *J*
668 = 13.2, 5.9 Hz, 2H), 1.95 (p, *J* = 6.9 Hz, 2H). ¹³C NMR (101 MHz, CDCl₃) δ 169.33, 168.40,
669 156.51, 132.97, 117.73, 65.66, 39.82, 28.36, 25.67, 25.02. MS (ES+) *m/z* calculated for
670 C₁₂H₁₆N₂O₆:284.1, found 285.3 for [M+H]⁺.

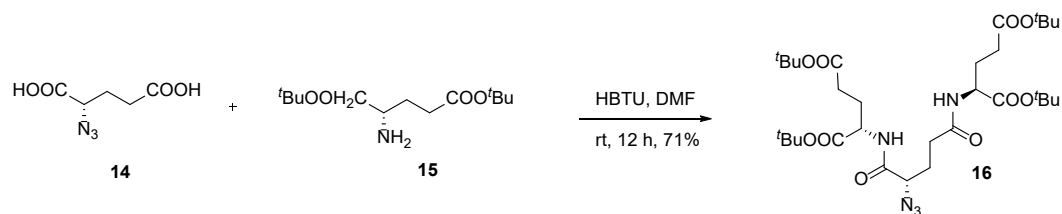
671



673 **Figure S10. Synthesis of Compound 9.**

674

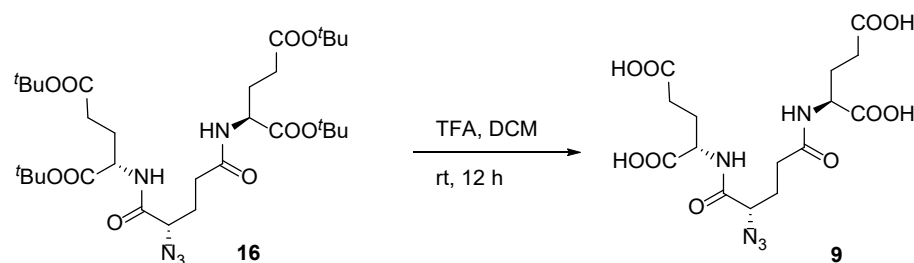
675



676

677 **Figure S11. Synthesis of Compound 16.** Compound **16** is prepared according to the
 678 literature procedure⁴³. The L-azidoglutamic acid **14**⁴⁴ (222 mg, 1.3 mmol) was
 679 dissolved in dry DMF (10 mL) under an argon atmosphere. HBTU (1.23 g, 3.25 mmol)
 680 was added to the solution and the mixture was stirred for 5 min at RT. DIPEA (2.30 mL,
 681 13.0 mmol) was added to the solution in a single portion, followed immediately by L-
 682 glutamic acid di-tert-butyl ester **15** (760 mg, 2.6 mmol). The reaction mixture was
 683 stirred overnight, after which the solvent was removed in vacuum. The crude product
 684 was purified by silica gel column chromatography to afford Compound **16** (597 mg,
 685 71% with respect to **14**) as gummy liquid. ¹H NMR (400 MHz, CDCl₃) δ 8.17 (d, *J* = 8.7
 686 Hz, 1H), 7.83 (d, *J* = 8.7 Hz, 1H), 4.59 (td, *J* = 9.6, 4.3 Hz, 1H), 4.49 (td, *J* = 9.5, 4.3 Hz,
 687 1H), 3.40 (dd, *J* = 11.4, 4.2 Hz, 1H), 2.45 – 2.23 (m, 6H), 2.14 (dt, *J* = 18.2, 6.2 Hz, 4H),
 688 1.91 – 1.72 (m, 2H), 1.44 (m, *J* = 13.5, 3.3 Hz, 36H). ¹³C NMR (101 MHz, CDCl₃) δ 174.36,
 689 173.78, 172.52, 171.60, 170.54, 83.58, 83.53, 80.97, 80.89, 59.98, 52.50, 52.38, 32.11,
 690 31.93, 31.78, 28.18, 28.12, 28.09, 27.79, 26.86, 26.71. MS (ES⁺) *m/z* calculated for
 691 C₃₁H₅₃N₅O₁₀: 655.4, found 656.7 for [M+H]⁺.

692



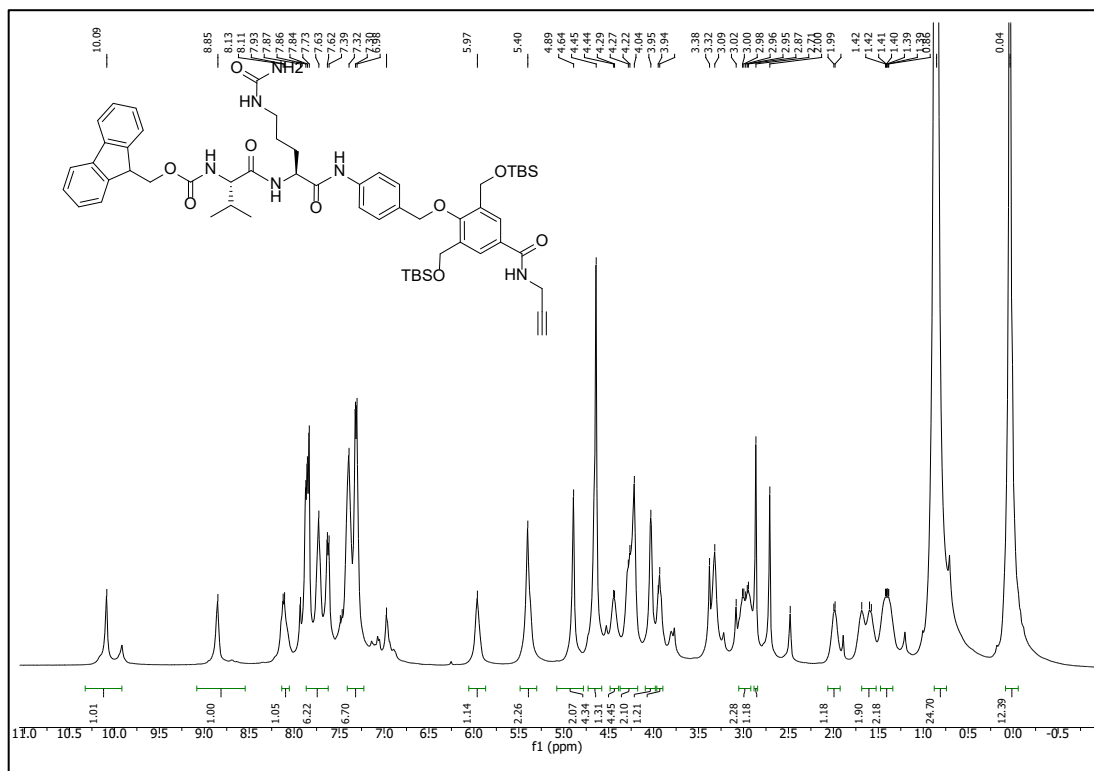
693

694 **Figure S12. Compound 9.** Compound **16** (550 mg, mmol) was dissolved in DCM (3 mL)
 695 and TFA (3 mL) was added. The reaction was stirred overnight. Upon completion, as
 696 monitored by TLC, the solvent and TFA were removed under reduced pressure. The
 697 product formation was confirmed by MS and the crude material was directly taken for
 698 the click reaction. MS (ES⁺) *m/z* calculated for C₁₅H₂₁N₅O₁₀: 431.1, found 432.3 for
 699 [M+H]⁺.

700

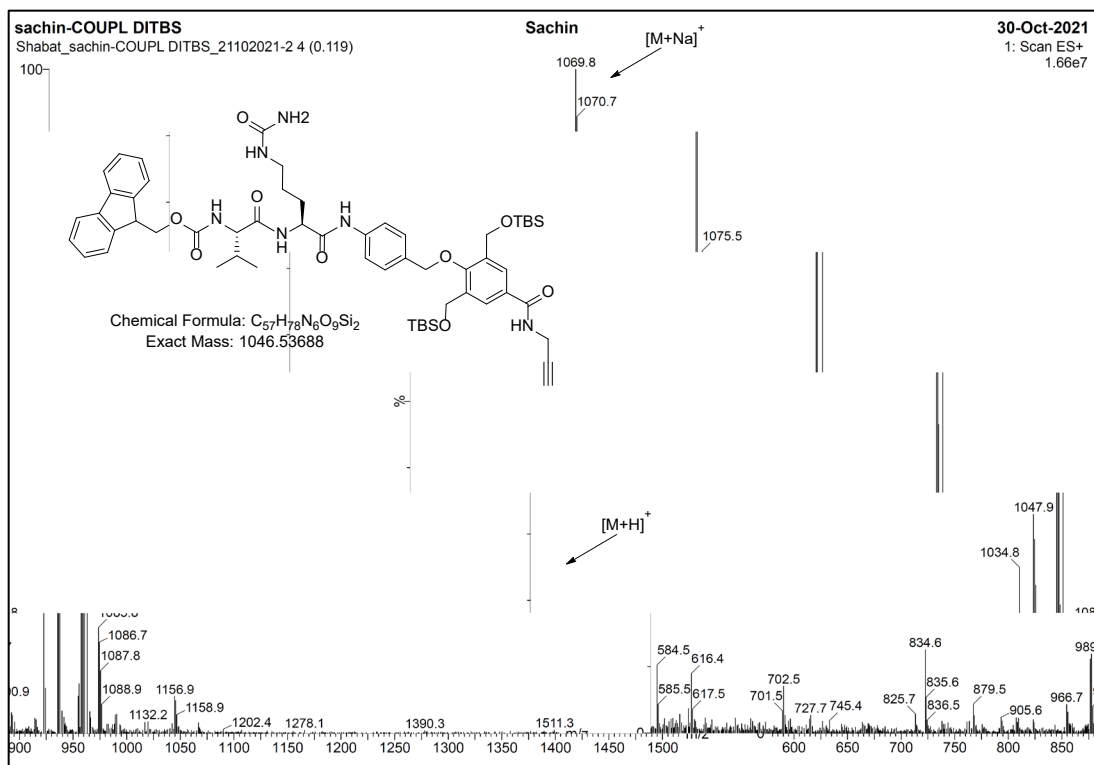
701 NMR and MS Spectra

702 A.



703

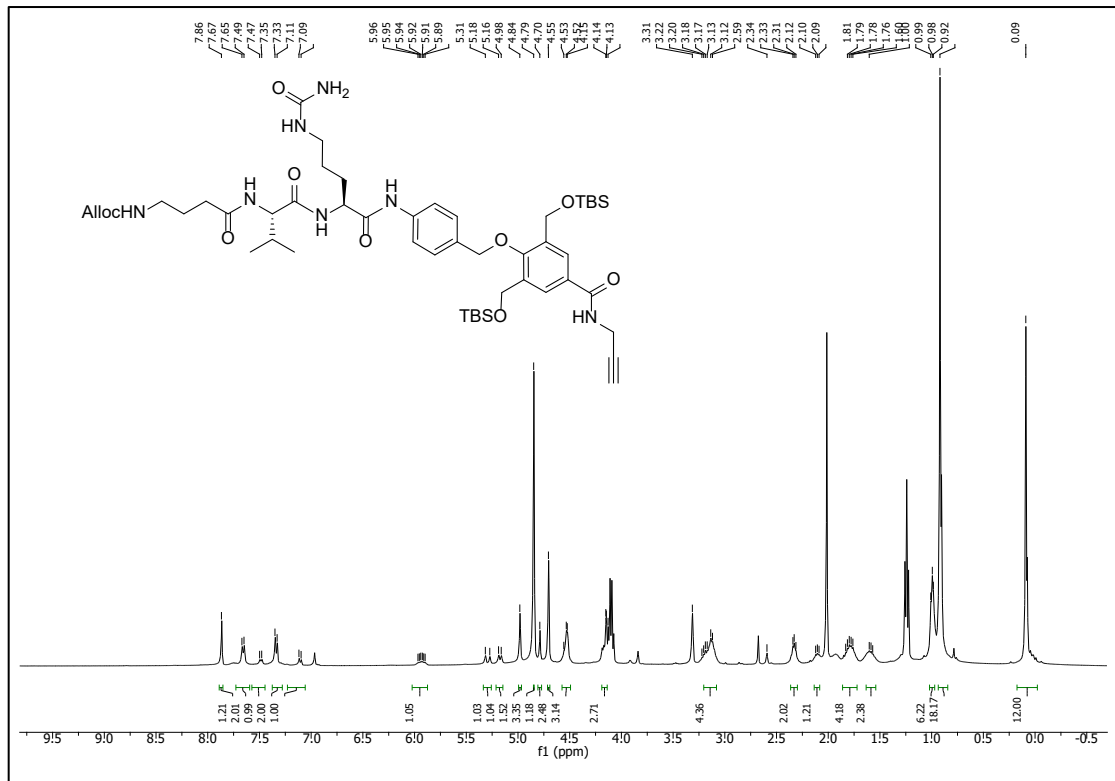
704 B.



705

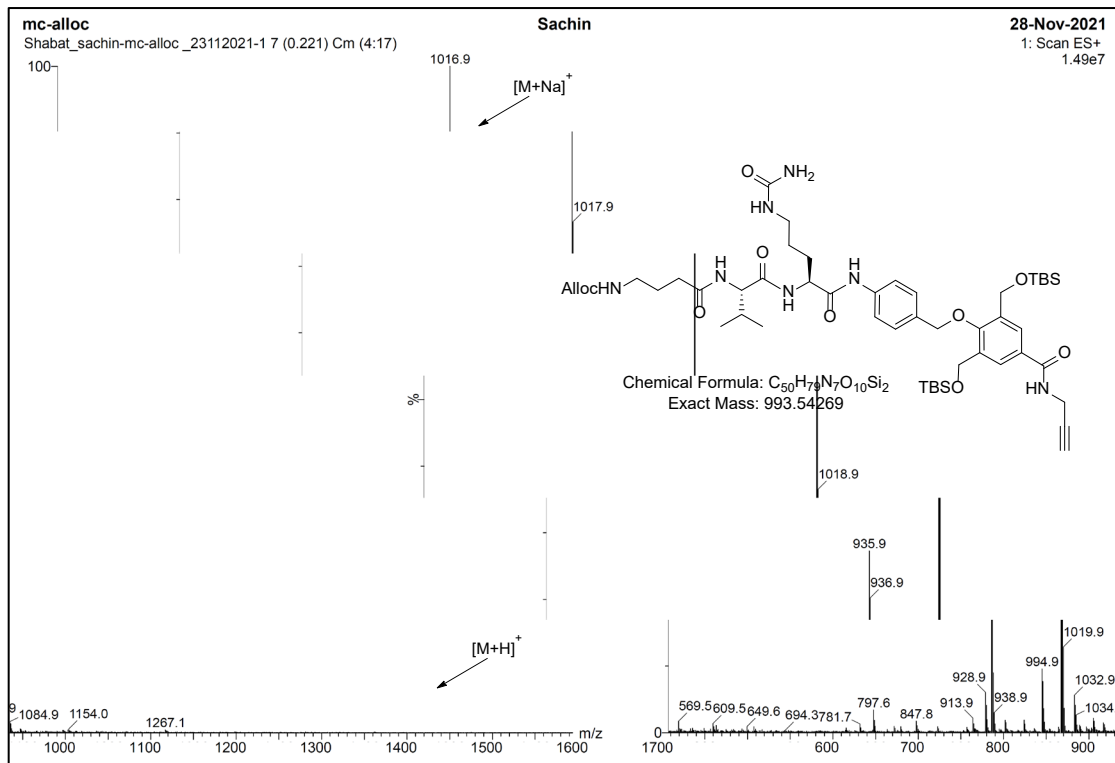
706 Figure S13. (A) 1H -NMR spectra of Compound 4. (B) MS of Compound 4.

707 **A.**



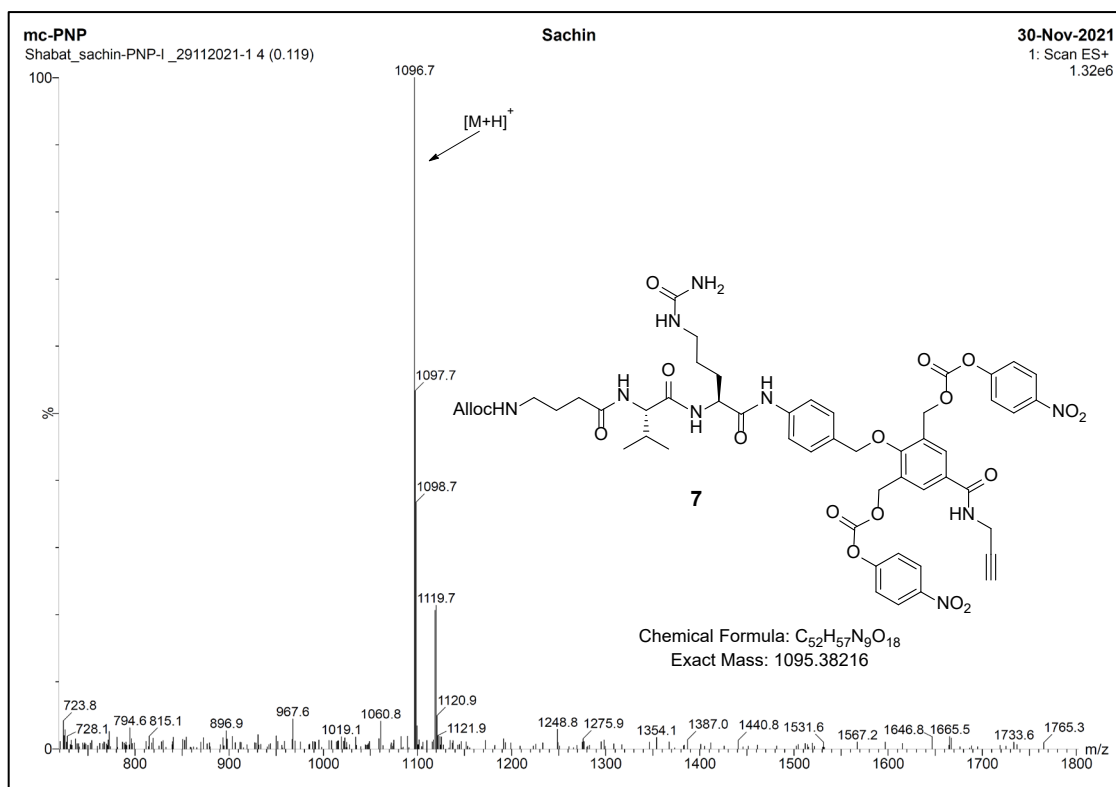
708

709 **B.**



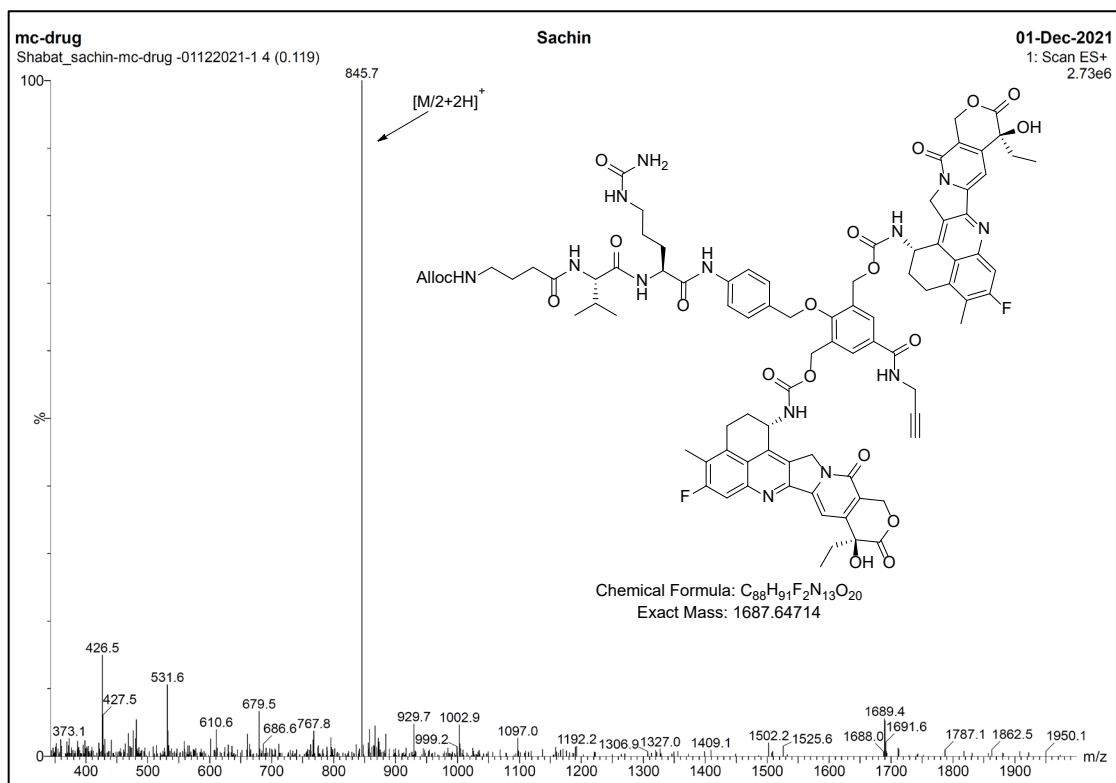
710

711 **Figure S14. (A) ¹H-NMR spectra of Compound 6. (B) MS of Compound 6.**



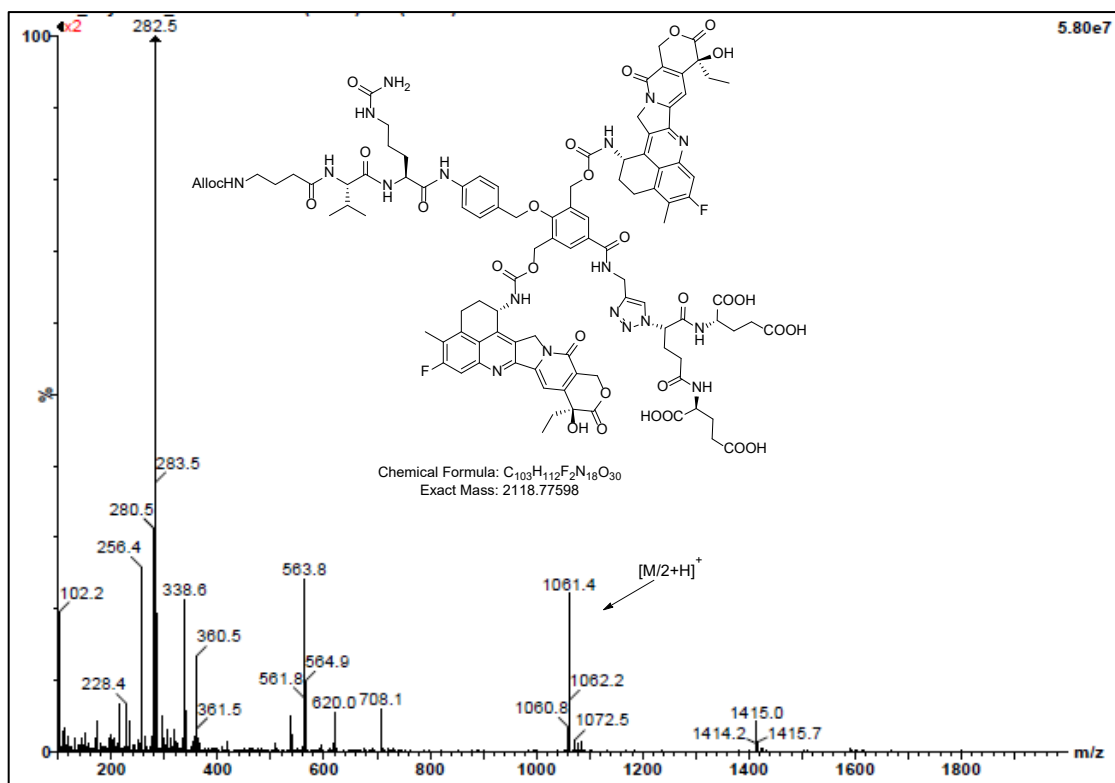
712

713 **Figure S15. MS of Compound 7**



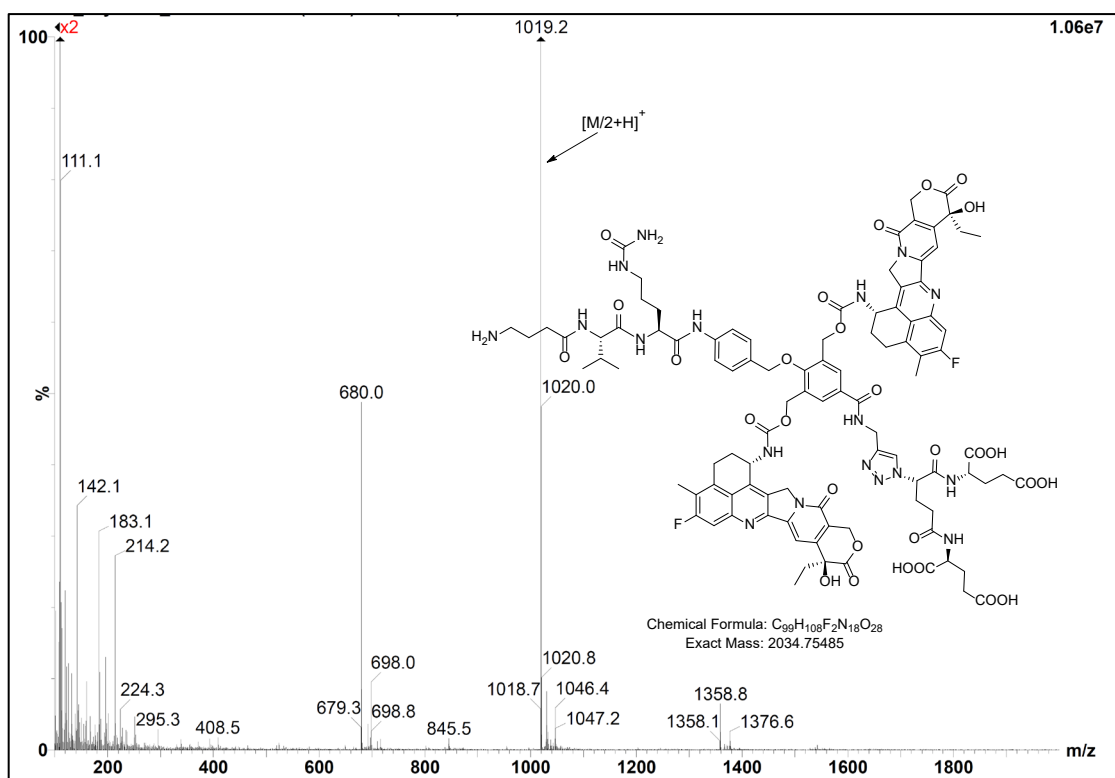
714

715 **Figure S16. MS of Compound 8**



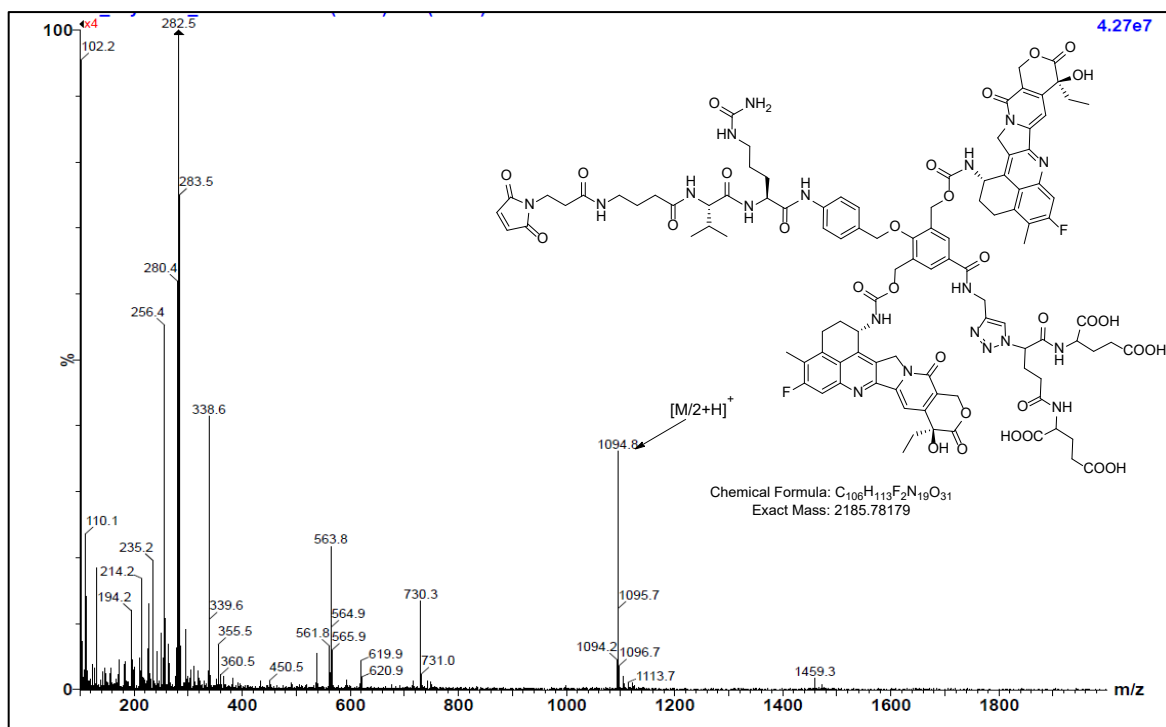
716

717 **Figure S17.** MS of Compound 10



718

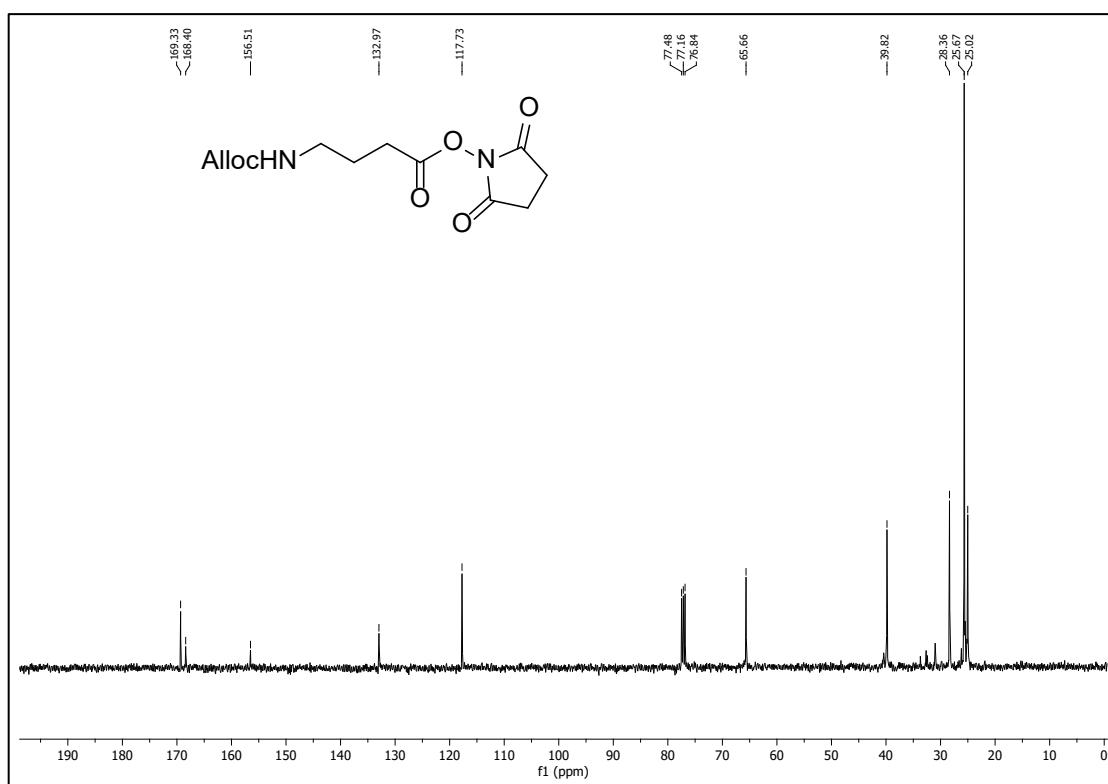
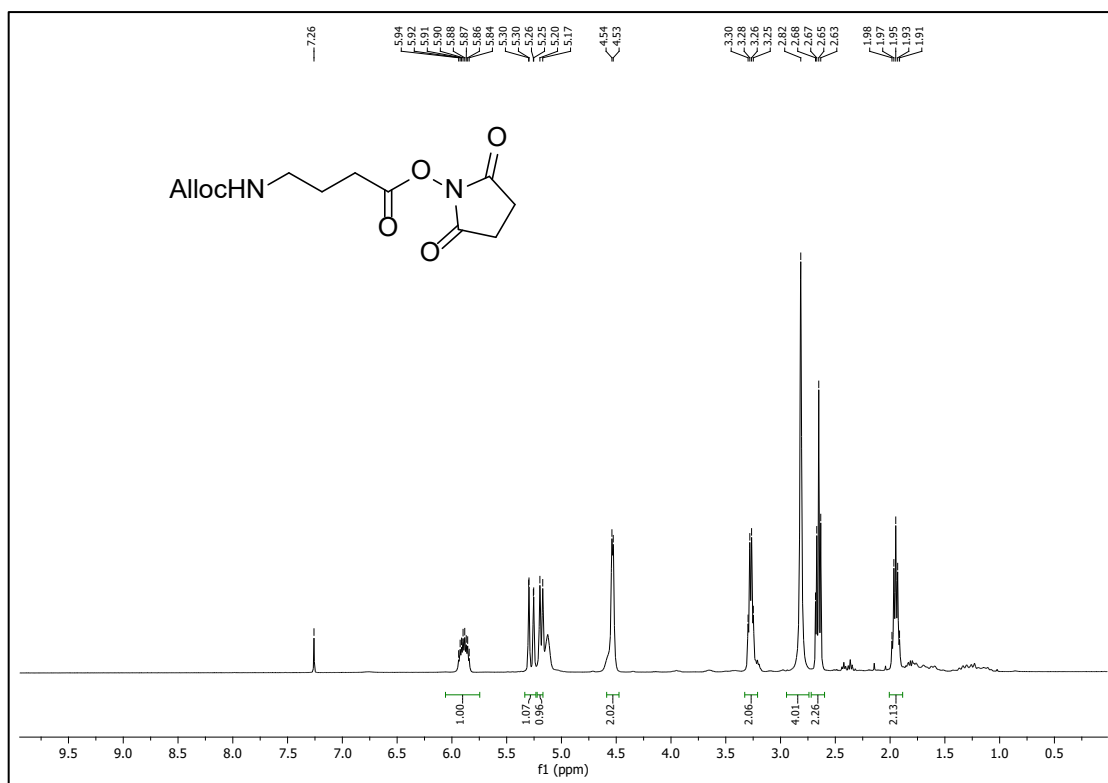
719 **Figure S18.** MS of Compound 11



720

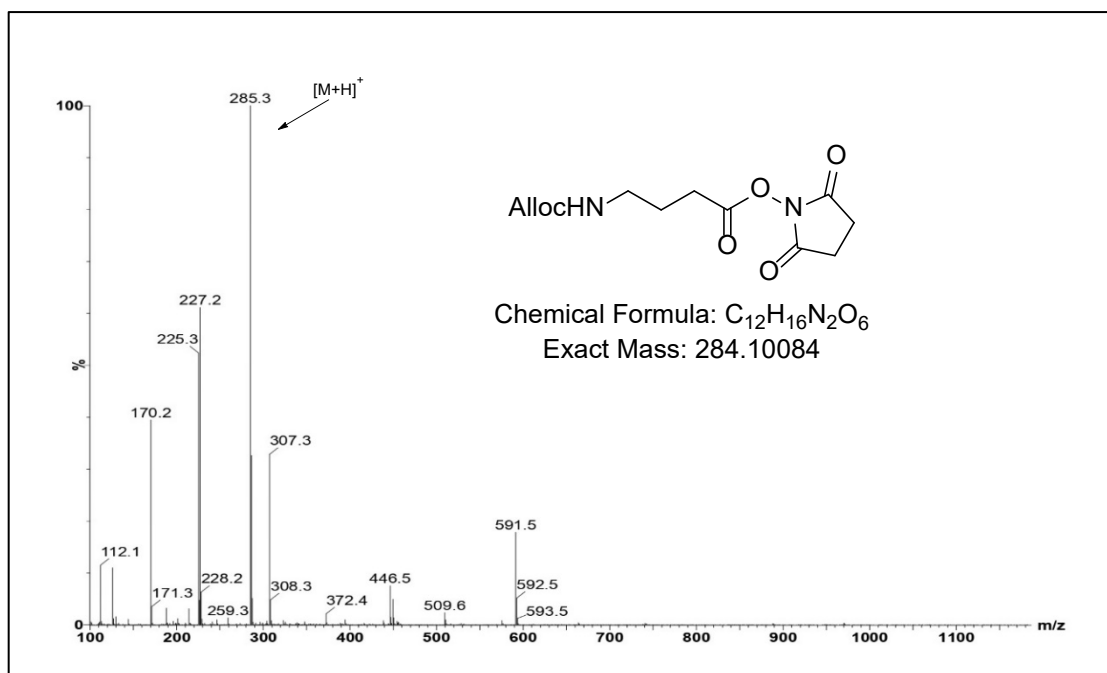
721 **Figure S19. MS of Prodrug 1**

722



725 **Figure S20.** $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra of Compound 5

726



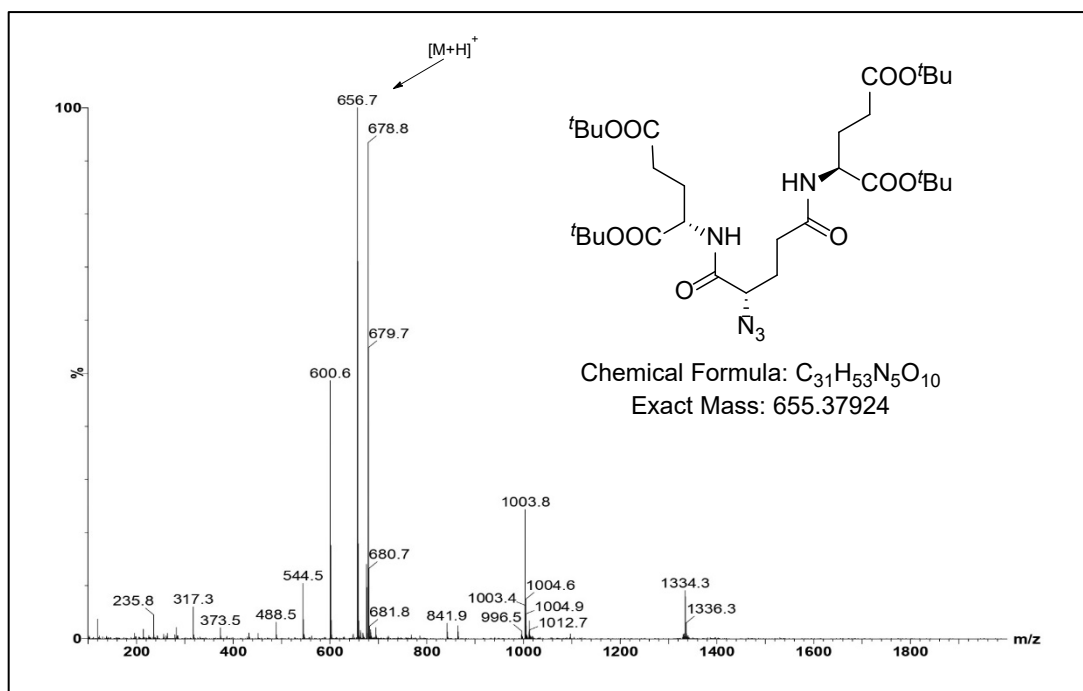
727

728 **Figure S21.** MS of compound 5

729

730

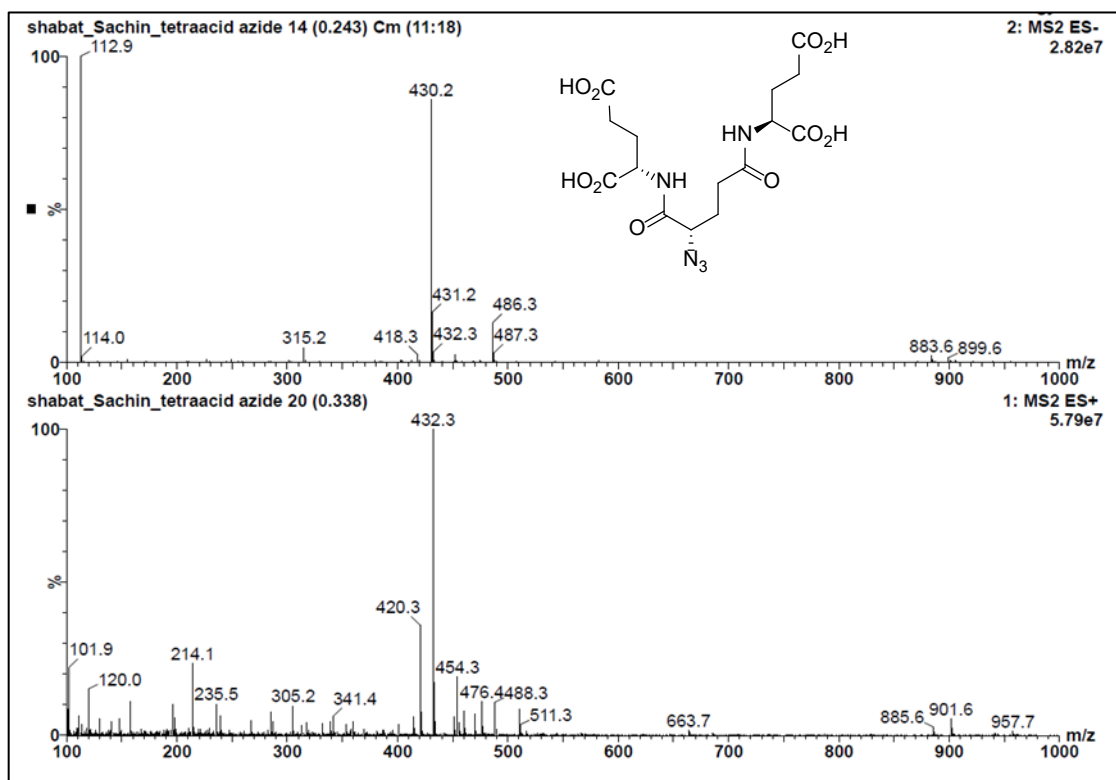
731



736

737 **Figure S23.** MS of Compound 16

738

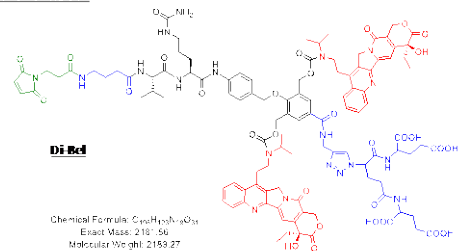


739

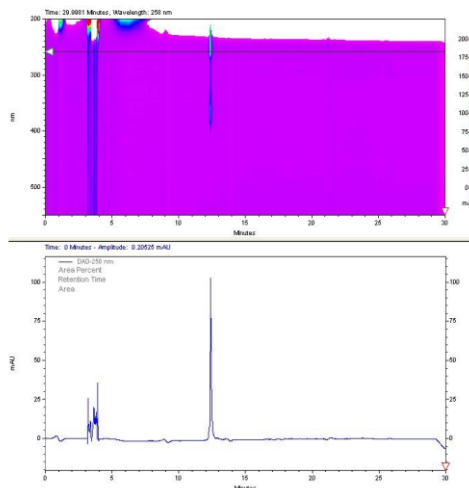
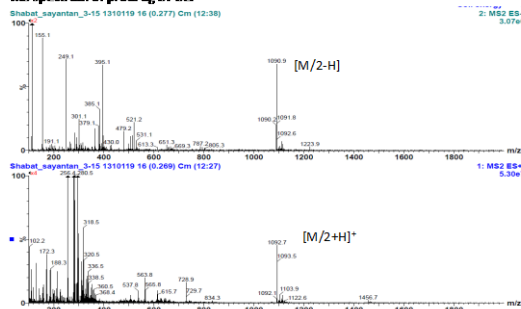
740 **Figure S24.** MS of Compound 9

741

Characterization Data



MS spectrum of prodrug Di Bel



HPLC chromatography: Column C18 5 μ , 250x4.6 mm.
Eluent: ACN/H₂O (H₂O with 0.1% of TFA). Method: 10-90 % ACN gradient.

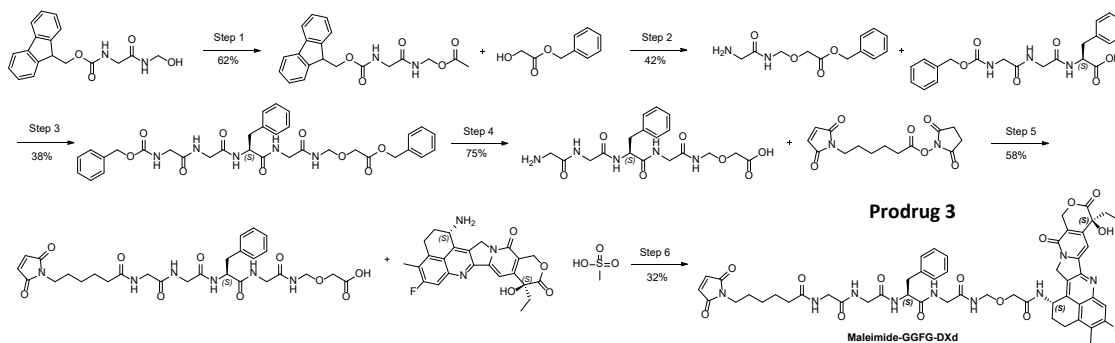
742

743 **Figure S25. Belotecan dimeric prodrug 2 synthesis.** Belotecan dimeric prodrug was
744 synthesized in a similar manner to the procedures described above for the exatecan
745 dimeric prodrug.

746

747

748 DXd prodrug 3 synthesis



749

750 **Figure S26. DXd prodrug 3 synthesis.** Experimental procedures are described below.

751

752 DXd prodrug 3 synthesis – Experimental procedures

753 Step 1:

754 To a stirred solution of [2-(9H-Fluoren-9-ylmethoxycarbonylamino)-acetylamino]-
755 acetic acid (25.00 g; 69.84 mmol; 1.00 eq.) in THF (500.00 ml; 20.00 V) and Toluene

756 (150.00 ml; 6.00 V) (3:1) ratio was added Pyridine (6.83 ml; 83.81 mmol; 1.20 eq.) and
757 followed by triacetoxylumbyl acetate (37.54 g; 83.81 mmol; 1.20 eq.) at RT and
758 heated to reflux for 5 h at 80°C.

759 After that, the reaction solution was cooled to RT, the insoluble material was removed
760 by filtration through Celite bed, and the filtrate was concentrated under reduced
761 pressure. The obtained residue was dissolved in ethyl acetate, washed with water and
762 saturated brine and then the organic layer was dried over anhydrous sodium sulfate.

763 After the solvent was removed under reduced pressure, the obtained residue was
764 purified by column chromatography using 60-120 mesh silica gel eluent at 70 to 85 %
765 ethyl acetate in pet ether to afford Acetic acid [2-(9H-fluoren-9-ylmethoxycarbonylamino)-acetylamino]-methyl ester (16.00 g; 43.35 mmol; 62.1 %;
766 white solid; Purified Product).

768 LC/MS Chromatography: Column: ATLANTIS dC18 (50x4.6mm) 5 µm; Mobile phase:
769 A:0.1% HCOOH in H₂O:ACN(95:5) B:ACN; Flow Rate: 1.5 mL/min: [M+Na]⁺ = 391, RT =
770 2.22 minute

771 ¹H NMR: 400 MHz, DMSO-d₆:8.94 (t, J = Hz, 1H), 7.90 (d, J = 7.20 Hz, 2H), 7.72 (d, J =
772 7.20 Hz, 2H), 7.57 (t, J= Hz, 1H), 7.38 (d, J = 20.00 Hz, 2H), 7.32 (d, J = 0.80 Hz, 2H), 5.11
773 (d, J = Hz, 2H), 4.31-4.24 (m, 3H) 3.35 (d, J = Hz, 2H), 2.00 (s, 3H)

774 **Step 2:**

775 To a stirred solution of Acetic acid [2-(9H-fluoren-9-ylmethoxycarbonylamino)-
776 acetylamino]-methyl ester (16.00 g; 43.35 mmol; 1.00 eq.) in THF (160.00 mL; 10.00
777 V) was added Hydroxy-acetic acid benzyl ester (14.50 g; 86.39 mmol; 1.99 eq.)
778 followed by p-toulenesulfonic acid monohydrate (1.66 mL; 4.76 mmol; 0.11 eq.) at 0°C
779 and stirred at same temperature for 2 h then stirred at RT for 1 h.

780 After completion, the reaction solution was charged with water and extracted with
781 ethyl acetate, washed with 10% NaHCO₃ solution and The obtained organic layer was
782 dried over sodium sulfate and filtered, the filtrate was concentrated to obtain crude
783 product The obtained residue was purified by silica gel (230-400 mesh) column
784 chromatography eluent at 70 to 75 % EA in PE to afford benzyl 2-[[2-((9H-fluoren-9-

785 yl)methoxy]carbonyl}amino)acetamido]methoxy}acetate (9.00 g; 18.35 mmol; 42.3 %;
786 white solid; Purified Product).

787 LC/MS Chromatography: Column: ATLANTIS dC18 (50x4.6 mm) 5 μ m; Mobile phase:
788 A: 0.1% HCOOH in H₂O:ACN(95:5) B: ACN; Flow Rate :1.5 mL/min: [M+Na]⁺ = 497, RT
789 = 2.52 min.

790 ¹H NMR: 400 MHz, DMSO-d₆: 8.75 (t, J = Hz, 1H), 7.90 (d, J = 7.48 Hz, 2H), 7.81 (d, J =
791 65.32 Hz, 2H), 7.61 (t, J = Hz, 1H), 7.58-7.31 (m, 8H), 5.15 (s, 2H), 4.64 (d, J = 6.68 Hz,
792 2H), 4.30-4.21 (m, 3H), 4.18 (d, J = 17.24 Hz, 2H), 3.63 (d, J = 6.04 Hz, 2H)

793 **Step 3:**

794 To a stirred solution of benzyl 2-{{2-(((9H-fluoren-9-
795 yl)methoxy]carbonyl}amino)acetamido] methoxy} acetate (9.00 g; 18.36 mmol; 1.00
796 eq.) in DMF (90.00 mL; 10.00 V) was added 2,3,4,6,7,8,9,10-Octahydropyrimido[1,2-
797 a]azepine (2.85 g; 18.36 mmol; 1.00 eq.) at 0°C and stirred at RT for 30 min, completion
798 of the reaction was confirmed by LC-MS. The reaction mixture was directly used for
799 the next step without further purification.

800 To the solution of the reaction mixture was added (2S)-2-[2-(2-
801 {{{(benzyloxy)carbonyl}amino} acetamido)acetamido]-3-phenylpropanoic acid (7.67 g;
802 18.36 mmol; 1.00 eq.) followed by 1-Hydroxy-pyrrolidine-2,5-dione (2.56 g; 22.03
803 mmol; 1.20 eq.) and (3-Dimethylamino-propyl)-ethyl-carbodiimide hydrochloride
804 (4.27 g; 22.03 mmol; 1.20 eq.) the resulted reaction mixture was stirred at RT for 2h,
805 to that was added 0.1N HCl solution (500 mL) and stirred for 30 min, then the product
806 was extracted with DCM, the collected organic layer was dried over anhydrous sodium
807 sulphate, filtered and concentrated to afford crude product.

808 The crude product was purified by column chromatography using 230-400 mesh
809 silica gel, all Fmoc impurities were removed by eluting 45% to 50% EA in pet ether and
810 the product eluent at 4 to 5 % methanol in DCM to afford impure product which was
811 later agitated with di ethyl ether and filtered, then again agitated with ethyl acetate
812 and filtered, dried to afford benzyl 2-{{2-[(2S)-2-[2-(2-{{{(benzyloxy)carbonyl]

813 amino}acetamido)acetamido]-3-phenylpropanamido]acetamido}methoxy) acetate
814 (5.00 g; 6.97 mmol; 37.9 %; off white solid; Purified Product).

815 LC/MS Chromatography: Column: ATLANTIS dC18 (50x4.6 mm) 5 μ m; Mobile phase:
816 A:0.1% HCOOH in H₂O:ACN(95:5) B: ACN; Flow Rate :1.5 mL/min: [M+Na]⁺ = 670, RT =
817 2.15 min.

818 **Step 4:**

819 To a stirred solution of benzyl benzyl 2-({2-[(2S)-2-[2-(2-
820 {[(benzyloxy)carbonyl]amino}acetamido) acetamido]-3-
821 phenylpropanamido]acetamido}methoxy)acetate (5.00 g; 6.97 mmol; 1.00 eq.) in
822 DMF (50.00 mL; 10.00 V) was added Palladium on carbon (10% w/w) (4.00 g; 3.76
823 mmol; 0.54 eq.) and stirred at RT under hydrogen bladder pressure for 6 h.

824 After completion, reaction mixture was filtered through celite bed and washed the
825 celite bed with mixture of THF and water (100 : 50) and concentrated, the obtained
826 crude product was co-evaporated with toluene twice and dried to afford 2-({2-[(2S)-
827 2-[2-(2-aminoacetamido)acetamido]-3-
828 phenylpropanamido]acetamido}methoxy)acetic acid (2.50 g; 5.20 mmol; 74.7 %; off
829 white solid; Crude Product).

830 LC/MS Chromatography: Column: ATLANTIS dC18 (50x4.6 mm) 5 μ m; Mobile phase:
831 A:0.1% HCOOH in H₂O:ACN(95:5) B: ACN; Flow Rate :1.5 mL/min: [M+H]⁺ = 424, RT =
832 0.87 min.

833 **Step 5:**

834 To a stirred solution of 2-({2-[(2S)-2-[2-(2-aminoacetamido)acetamido]-3-
835 phenylpropanamido] acetamido} methoxy)acetic acid (2.50 g; 5.21 mmol; 1.00 eq.) in
836 DMF (50.00 mL; 20.00 V) was added 6-(2,5-Dioxo-2,5-dihydro-pyrrol-1-yl)-hexanoic
837 acid 2,5-dioxo-pyrrolidin-1-yl ester (2.00 g; 6.42 mmol; 1.23 eq.) and followed by
838 Triethyl-amine (0.82 mL; 5.82 mmol; 1.12 eq.) at 0°C and stirred at RT for 2 h.

839 After completion of the reaction, reaction mass was concentrated to dryness to afford
840 crude product. The crude product was purified by reverse phase column
841 chromatography eluent at 30% of 0.1% HCOOH in water and ACN, the collected

842 fractions were lyophilized to afford pure product 2-({2-[(2S)-2-(2-{2-[6-(2,5-dioxo-2,5-
843 dihydro-1H-pyrrol-1-yl)hexanamido]acetamido}acetamido)-3-
844 phenylpropanamido]acetamido}methoxy)acetic acid (1.90 g; 3.04 mmol; 58.4 %; off
845 white solid; Purified Product).

846 LC/MS Chromatography: Column: ATLANTIS dC18 (50x4.6 mm) 5 μ m; Mobile phase:
847 A:0.1% HCOOH in H₂O:ACN(95:5) B: ACN; Flow Rate :1.5 mL/min: [M-H]⁻ = 615, RT =
848 1.51 min.

849 ¹H NMR: 400 MHz, DMSO-d₆:12.80 (s, 1H), 8.57 (t, J = 13.20 Hz, 1H), 8.31 (t, J = 11.60
850 Hz, 1H), 8.15-8.01 (m, 3H), 7.28-7.16 (m, 5H), 7.01 (s, 2H), 4.61 (d, J = 6.80 Hz, 2H),
851 4.50-4.50 (m, 1H), 3.98 (s, 1H), 3.77-3.66 (m, 6H), 3.57-3.34 (m, 1H), 2.81-2.80 (m, 1H),
852 2.12 (t, J = 7.20 Hz, 2H), 1.51-1.47 (m, 4H), 1.21-1.17 (m, 2H).

853 **Step 6:**

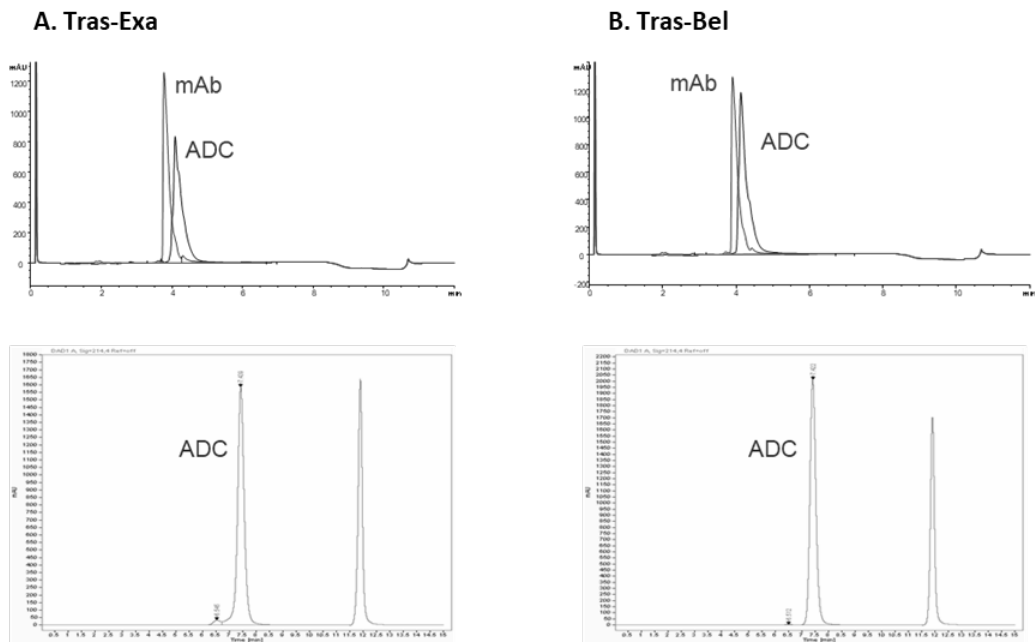
854 2-({2-[(2S)-2-(2-{2-[6-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-
855 yl)hexanamido]acetamido}acetamido)-3-
856 phenylpropanamido]acetamido}methoxy)acetic acid (427.38 mg; 0.66 mmol; 1.00
857 eq.) was dissolved in N,N-Dimethylformamide for spectroscopy Uvasol® (2.00 mL).
858 HATU (185.89 mg; 0.79 mmol; 1.20 eq.) was added and the solution was stirred at RT
859 30 min. Then exatecan methanesulfonic acid (350.00 mg; 0.66 mmol; 1.00 eq.) and 4-
860 Methylmorpholine for synthesis (144.78 μ L; 1,32 mmol; 2.00 eq.) were added and the
861 solution was stirred at RT overnight. The reaction solution was injected directly onto
862 the column of the prep. HPLC and the fractions containing product were combined
863 and lyophilized afford Maleimide-GGFG-DXd (220 mg; 0.21 mmol; 32 %; pure product,
864 yellow solid).

865 LC/MS Chromatography: Column: ATLANTIS dC18 (50x4.6 mm) 5 μ m; Mobile phase:
866 A:0.1% HCOOH in H₂O:ACN(95:5) B: ACN; Flow Rate :1.5 mL/min: [M+H]⁺ = 1034, RT =
867 0.87 min.

868

869

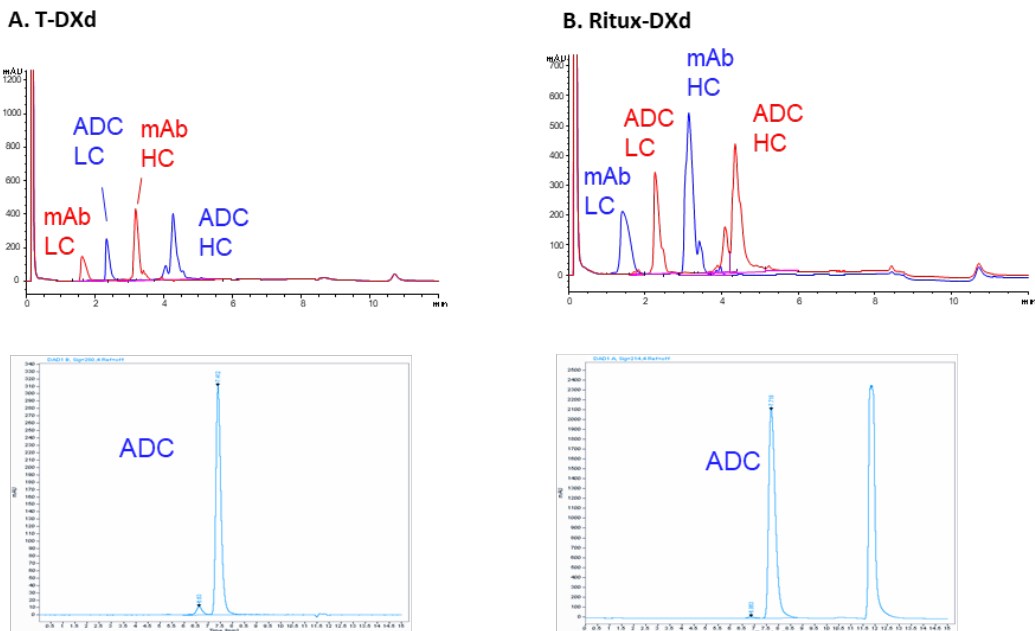
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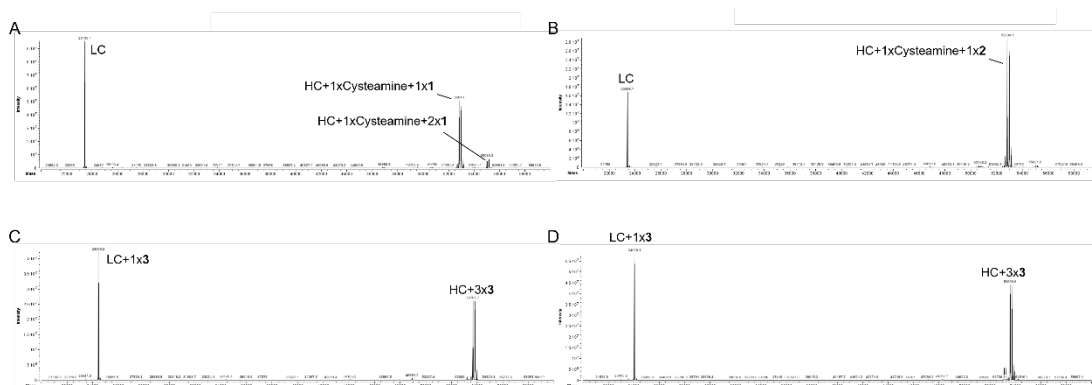
872 **Figure S27a.** RP-HPLC overlays (upper panel) and SE-HPLC (lower panel)
873 chromatograms of non-reduced Tras-Exa (A) and Tras-Bel (B). Signals were recorded
874 at 214 nm.

875



876 **Figure S27b.** RP-HPLC overlays (upper panel) and SE-HPLC (lower panel)
877 chromatograms of T-DXd (A) and Ritux-DXd (B). Since the thiol conjugation process
878 results in depletion of interchain disulfide bonds, all samples were reduced prior to
879 RP-HPLC analysis and therefore separated into heavy chains (HC) and light chains (LC).
880 Signal for SE-HPLC of T-DXd was recorded at 280 nm, all other signals were recorded
881 at 214 nm.

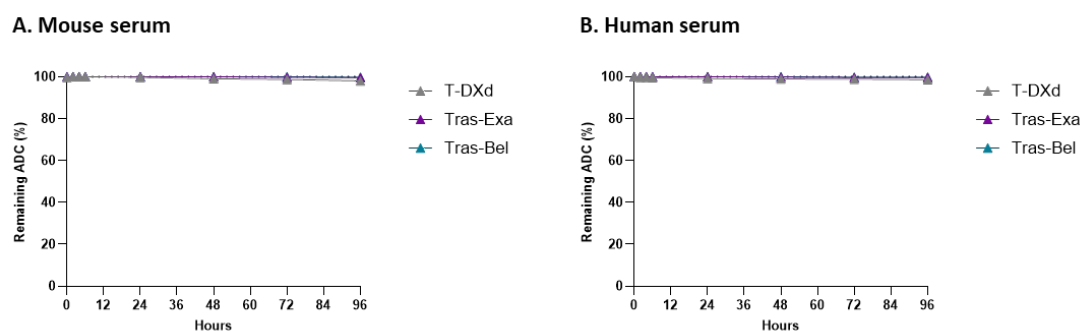
882



883

884 **Figure S27c.** LC-MS spectra of Tras-Exa (A), Tras-Bel (B), T-DXd (C) and Ritux-DXd (D).
885 All samples were reduced prior to LC-MS analysis and therefore separated into heavy
886 chains (HC) and light chains (LC). Multiple peaks were detected for the heavy chains
887 due to different glycosylated species. Heavy chains of Tras-Exa (A) and Tras-Bel (B)
888 were conjugated with cysteamine serving as a handle for the attachment of 1 or 2
889 resulting in DARs 4.3 and 3.9, respectively. For T-DXd (C) and Ritux-DXd (D), 3 was
890 conjugated to the mAb interchain cysteines resulting in attachment of one molecule
891 to each LC and three to each HC (overall DARs of 7.9 and 8.0, respectively).

892



893 **Figure S28. Drug-linker stability.** Percentage of ADC remaining over time in mouse (A)
894 and human (B) sera following incubation at 37°C for 96 h, calculated from free
895 exatecan, belotecan or DXd that were measured via LC-MS/MS. Numbers show the
896 released fraction (%) relative to initially conjugated payload.

897

898

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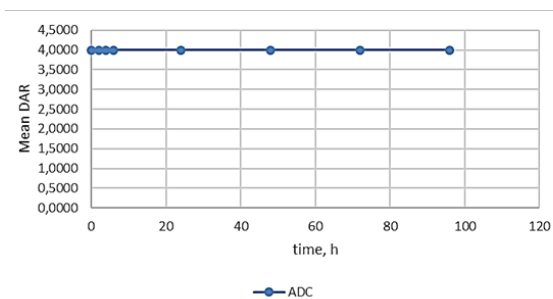
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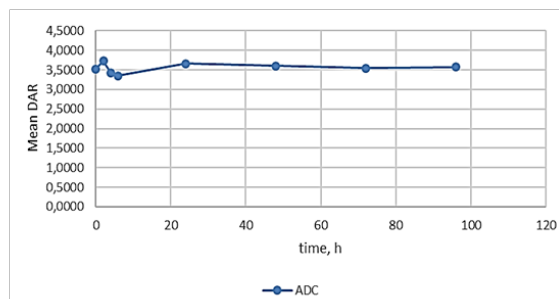
902 Serum Stability

903 Serum samples from mice and humans were purchased from Biowest. Serum was
904 thawed at ambient temperature and supplemented with 2 M HEPES buffer (Merck
905 KGaA, Darmstadt), pH 7.55 in order to obtain a 0.3 M final HEPES concentration.
906 Supplementation was performed in order to maintain serum pH levels close to
907 physiological pH ranging between pH 7.3 and 7.4. Buffered serum was filtered through
908 a 0.22 μm filter (Merck KGaA, Darmstadt). ADC stock solutions were added to the
909 plasma samples at final concentration of 50 μg ADC protein/mL, mixed and transferred
910 to PCR tubes (Thermo Fisher Scientific) with a final volume of 20 μL per aliquot and
911 followed by incubation at 37°C in a CO₂ incubator. Triplicates were prepared for each
912 sample. Sample time series were collected at timepoints 0, 2, 4, 6, 24, 48, 72 and 96 h
913 and stored at -80°C until further analysis. ADC serum samples were transferred to PCR
914 tubes (5 μL) and supplemented with internal standard, followed by extraction with
915 150 μL methanol and analysis of exatecan by UPLC MS/MS. For the UPLC system, the
916 ACQUITY UPLC H-Class System (Waters) was used along with the ACQUITY UPLC BEH
917 C18 1.7 μm 2.1 x 50 mm column (Waters). For the mobile phase, solvent A was used
918 with 0.1% formic acid in H₂O and acetonitrile for solvent B. Mass spectrometer
919 measurements were performed using a Sciex Triple Quad 6500 system (AB Sciex) and
920 ABSciex Analyst 1.7 software for data analysis.

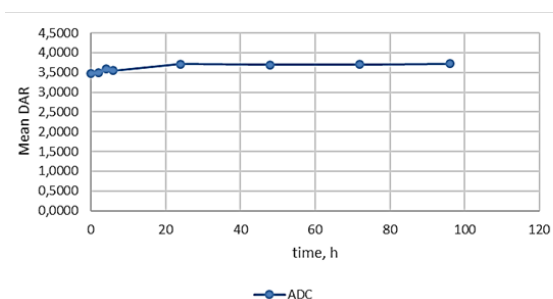
A. Mouse serum



B. Monkey serum



C. Human serum



921 **Figure S29 Drug-linker stability.** Mean DAR of ADC over time in mouse serum (A),
922 monkey serum (B) and human serum (C) was determined by UPLC MS/MS.

923

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976 [information-approved-drugs/fda-grants-accelerated-approval-fam-trastuzumab-](https://www.fda.gov/drugs/resources-information-approved-drugs/fda-grants-accelerated-approval-fam-trastuzumab-deruxtecan-nxki-her2-mutant-non-small-cell-lung)
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