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Conjugates of Modified Cryptophycins and RGD-Peptides Enter Target Cells by Endocytosis

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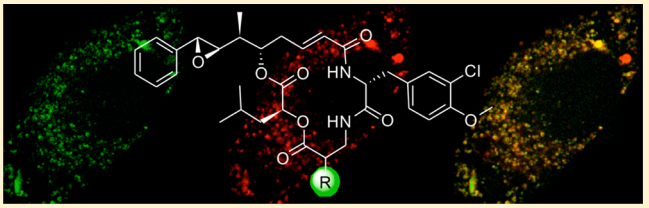
Supporting Information

ABSTRACT: Tumor targeting anticancer drug conjugates that contain a tumor recognition motif (homing device) are of high current relevance. Cryptophycins, naturally occurring cytotoxic *cyclo*-depsipeptides, have been modified by total synthesis to provide analogues suitable for conjugation to peptide-based homing devices. An array of functionalized β^2-amino acids was synthesized and incorporated into cryptophycins. All analogues proved to be highly active in the cytotoxicity assay using the human cervix carcinoma cell line KB-3-1 and its multidrug-resistant subclone KB-V1. Conformational analysis of cryptophycin-52 and two synthetic analogues was performed by NMR and MD methods to obtain information on the influence of the unit C configuration on the overall conformation. An azide-functionalized cryptophycin was connected by CuAAC to an alkyne-containing fluorescently labeled cyclic RGD-peptide as the homing device for internalization studies. Confocal fluorescence microscopy proved integrin-mediated internalization by endocytosis and final lysosomal localization of the cryptophycin prodrug.



INTRODUCTION

Cryptophycins, cyclic depsipeptides of cyanobacterial origin, interact with the protein tubulin and display very high cytotoxicity, sometimes even against multidrug-resistant cancer cells. Different naturally occurring cryptophycins were found to induce apoptosis while inhibiting microtubule dynamics.[1−8] Cryptophycin-1 (**1a**) showed the highest biological activity of all subtypes (Figure 1). Cryptophycin-52 (**1b**), a highly bioactive synthetic analogue of **1a**, had been chosen by Eli Lilly for clinical trials. However, neurotoxicity prevented administration of **1b** at an effectively high dosage in phase I and II studies.[9−12] In addition, there was no complete remission observed in any patient, but 42% of them obtained a clinical benefit.

Conventional antitumor chemotherapy relies on the assumption that cancer cells divide more rapidly than healthy cells and, consequently, are more susceptible toward cytotoxic agents. This is restricted by the large number of quiescent tumor cells and due to the fact that solid tumors divide very slowly. In fact, antitumor chemotherapeutics have no or only poor specificity, resulting in systemic toxicity and undesired side effects, for instance, hair loss, as well as kidney, liver, and bone marrow damage. In the last three decades, progress in cancer therapy has been made with the design of tumor targeting drug delivery systems. Such conjugates usually consist of a tumor targeting group (homing device) connected directly or by a suitable linker to the cytotoxic drug. Tumor targeting systems can be classified based on the type of cancer cell recognition moiety including monoclonal antibodies, hyaluronic acid, polyunsaturated fatty acids, folic acid, and peptides.[13] While antibody−drug conjugates (ADC) show promise for curative cancer treatments, only very few have been approved

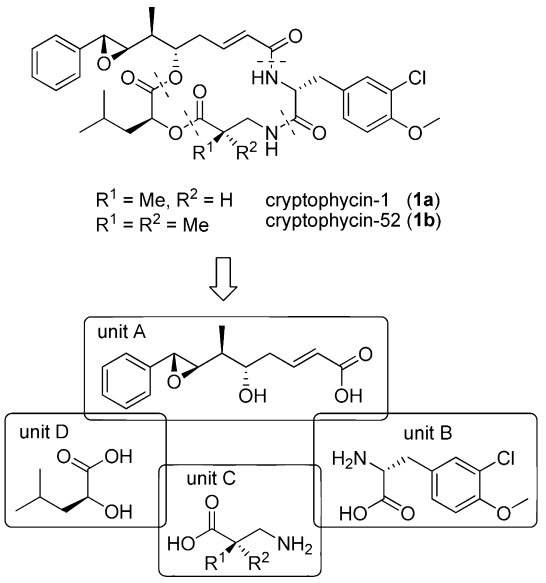


Figure 1. Retrosynthetic disconnection of cryptophycin-1 (**1a**) and cryptophycin-52 (**1b**).

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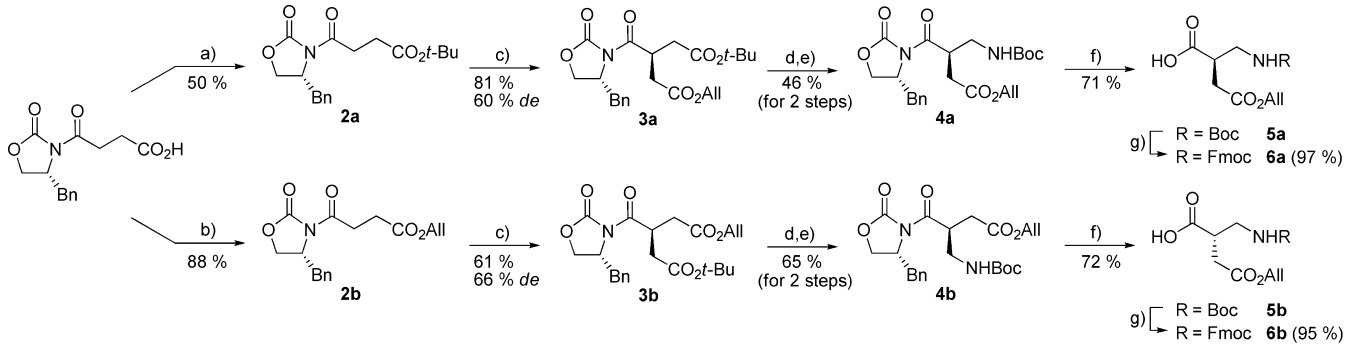
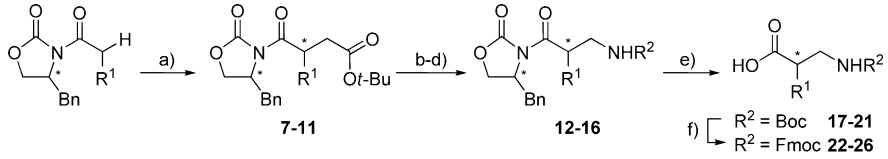
Scheme 1. Synthesis of Orthogonally Protected (*R*)- and (*S*)-β^2-Homoaspartate[a]

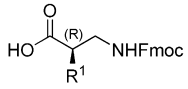
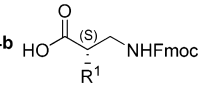


[a]Reagents and conditions: (a) P_2O_5, *t*-BuOH, $CHCl_3$, rt, 60 h; (b) AllBr, $KHCO_3$, DMF, rt, 64 h; (c) (1) NaHDMS, THF, −78 °C, 30 min, (2) **3a**, $ClCH_2CO_2All$, NaI, 16 h, **3b** $BrCH_2CO_2t$-Bu, NaI, 4 h, −100 °C → −78 °C; (d) TFA, CH_2Cl_2, rt, 16 h; (e) (1) ethyl chloroformate, NEt_3, THF, 0 °C → rt, 1 h, (2) NaN_3, THF/H_2O, 0 °C, 1 h, (3) *t*-BuOH, toluene, reflux, 16 h; (f) H_2O_2, LiOH·H_2O, THF, 0 °C, 40 min; (g) (1) TFA, CH_2Cl_2, rt, 16 h, (2) Fmoc-OSu, $NaHCO_3$, acetone, H_2O, rt, 4 h.

Scheme 2. Stereoselective Synthesis of Diverse Functionalized Unit C Building Blocks 22−26[a]



R^1	a)			b-d)			e)		f)	
	product	yield	*de*	R^2	product	yield	product	yield	product	yield
CO_2Allyl	**7**	50 %	82 %	Boc	**12**	53 %	**17**	85 %	**22**	88 %
OAllyl	**8a**	90 %	85 %	Boc	**13a**	55 %	**18a**	87 %	**23a**	80 %
OAllyl	**8b**	80 %	89 %	Boc	**13b**	55 %	**18b**	70 %	**23b**	70 %
N_3	**9a**	37 %	n.d.	Boc	**14a**	73 %	**19a**	88 %	**24a**	81 %
N_3	**9b**	46 %	n.d.	Boc	**14b**	78 %	**19b**	85 %	**24b**	94 %
N_3	**10a**	98 %	78 %	Boc	**15a**	68 %	**20a**	99 %	**25a**	90 %
N_3	**11**	50 %	n.d.	Boc	**16**	55 %	**21**	91 %	**26**	92 %

22,23a-25a,26  **23b, 24b** 

[a]Reagents and conditions: (a) NaHDMS, $BrCH_2CO_2t$-Bu, NaI, THF, −78 °C, 3−4 h; (b) (1) TFA, CH_2Cl_2, rt, 16 h, (2) ethyl chloroformate, NEt_3, rt, 1 h; (c) NaN_3, H_2O, 0 °C, 1 h; (d) *t*-BuOH, toluene, reflux, 16 h; (e) H_2O_2, LiOH·H_2O, THF, 0 °C, 4 h; (f) (1) TFA, CH_2Cl_2, rt, 16 h, (2) Fmoc-OSu, $NaHCO_3$, acetone, H_2O, rt, 4 h.

for treatment or are close to approval.[14] If the conjugate is systemically nontoxic and the active drug is exclusively released upon internalization into the cancer cell, the conjugate can be considered as a prodrug. Among selective receptor-targeting small peptides, integrin-binding RGD-peptides appear to be attractive candidates as homing devices. Recently, a variety of RGD-peptide containing drug conjugates was developed and shows promising activities in vitro and in vivo.[15−18] In comparison to antibodies as homing devices, peptides are easy to synthesize, have a well-defined, homogeneous molecular composition, do not compromise the immune system, and conjugation to cytotoxic drugs usually is straightforward.

Application of cryptophycins in tumor targeting conjugates might be a potential solution to overcome their high systemic cytotoxicity and side effects. However, cryptophycin-1 (**1a**) and -52 (**1b**) both lack functional groups to be used for conjugation. Therefore, functionalized cryptophycin derivatives are required. Such a structural analogue would ideally be an inactive prodrug and activated only upon delivery to the target cancer cell. In this context, glycinate esters of the chlorohydrin derivative cryptophycin-55 have been investigated by Eli Lilly.[19] The role of the epoxide moiety in unit A of the most active cryptophycins is still under debate, but its integrity was shown to be crucial for biological activity.[20−22] Consequently, the cryptophycin framework should be modified at a different position, which has been proven amenable with some analogues functionalized in unit D.[23]

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Each of the four cryptophycin units A, B, C,[24,25] and D[3,26−28] (Figure 1), as well as connections between these subunits,[25,28−32] have been modified in structure−activity relationship studies. Modifications at unit C were shown to be tolerated with respect to cytotoxicity. Several α,α-disubstituted β^2-amino acids, β^3-amino acids, and $\beta^{2,3}$-amino acids have been introduced into the macrocycle.[24,25] Cytotoxicity decreased only moderately in most cases. Even the combination of α-amino acids as unit C and the β^3-homologue of tyrosine as unit B provided biologically active analogues, albeit with lower EC_{50} values.[33] Otherwise, combination of α-amino acids as unit C with the native unit B caused a significant decrease of bioactivity.[25] The unit B/C region seems to confer flexibility on the macrocycle.

Cryptophycin-1 (**1a**) contains the α-monosubstituted β^2-amino acid (*R*)-β^2-homoalanine as unit C. However, other monosubstituted β^2-amino acids, especially with functional groups in the side chain, have not yet been investigated as building blocks of cryptophycins so far. Therefore, we embarked on a project devoted to the total synthesis of cryptophycin analogues with functional groups in the unit C side chain with the goal to explore whether conjugation of cryptophycins across unit C provides bioactive derivatives.

RESULTS AND DISCUSSION

Synthesis of β^2-Amino Acids. Unit C building blocks appropriate for further conjugation require a functional group in the side chain which can be derivatized after the synthesis of the macrocycle. This moiety should be addressed orthogonally to the benzylic epoxide, the α,β-unsaturated amide, and the two ester linkages. Three functional groups were envisaged: (1) an allyl protected ester for amide or ester formation, (2) an allyl ether moiety, and (3) an azido function for copper catalyzed azide−alkyne cycloaddition (CuAAC). All three of them were considered to be connected across different linker lengths to the cryptophycin macrocycle to assess the influence of linker length and functional groups on bioactivity.

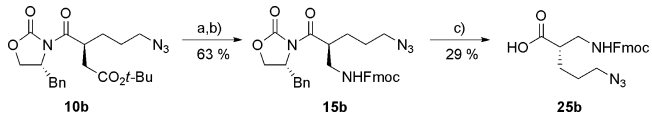
However, the required side chain functionalities were not compatible with most synthetic approaches toward β^2-amino acids.[34,35] Therefore, all unit C building blocks were synthesized employing a combined Evans' auxiliary/Curtius rearrangement approach (see Schemes 1 and 2 and Supporting Information).[36−38]

In the case of the allylester-functionalized unit C building blocks **6a** and **6b**, both enantiomers were obtained in a stereodivergent manner, starting from the succinic acid derivatized Evans' auxiliary (Scheme 1). This carboxylic acid was protected either as *tert*-butylester **2a** or as allylester **2b.** The stereogenic center of the β^2-amino acids was introduced by deprotonation with NaHMDS, followed by alkylation using either allyl or *tert*-butyl iodoacetate. Subsequently, the *tert*-butylesters **3a** and **3b** were deprotected using TFA, and the free acids were converted to the corresponding *tert*-butyl carbamates **4a** and **4b** by Curtius rearrangement. Finally, the β^2-amino acids were cleaved from the auxiliary by treatment with lithium hydroperoxide. The Fmoc-protecting group was installed in a deprotection/reprotection reaction sequence, and the unit C building blocks **6a** and **6b** were obtained in high yields.

In a more general procedure, the enantiomeric unit C building block pairs were obtained using the appropriately configured Evans' auxiliary. The corresponding starting materials modified with various R^1 residues were diastereoselectively functionalized in α-position with an Fmoc-protected aminomethylene moiety in a four-step sequence (Scheme 2). Deprotonation of the modified auxiliaries with NaHMDS followed by alkylation with *tert*-butyl bromoacetate/NaI at low temperature gave **7**−**11** in satisfactory to good diastereoselectivity (Scheme 2, step a). The crude products were purified by recrystallization of the alkylated product or after cleaving the *tert*-butylesters (Scheme 2, step b). In all cases, the intermediates were obtained diastereomerically pure. The precursors were then converted to *tert*-butyl carbamates **12**−**16** via Curtius rearrangement in the presence of *tert*-butanol (Scheme 2, steps c−d), and subsequently the Boc-protected β^2-amino acids **17**−**21** were obtained upon cleavage from the auxiliary by treatment with lithium hydroperoxide in 77−99% yield (Scheme 2, step e). The Boc group was then replaced by the Fmoc group in a high yielding final deprotection/reprotection step (Scheme 2, step f).

Additionally, the unit C building block **25b** was synthesized according to a slight modification of the method as depicted in Scheme 3. In this case, *tert*-butyl ester **10b** was cleaved to give

Scheme 3. Synthesis of Azidopropyl-Functionalized β^2-Amino Acid 25b[a]



[a]Reagents and conditions: (a) TFA, CH_2Cl_2, rt, 16 h; (b) (1) ethyl chloroformate, NEt_3, THF, 0 °C → rt, 1 h, (2) NaN_3, THF/H_2O, 0 °C, 1 h, (3) 9-fluorenylmethanol, toluene, reflux, 18 h; (c) (1) H_2O_2, LiOH·H_2O, THF, 0 °C, 75 min, (2) Na_2SO_3, H_2O, 0 °C → rt.

the free carboxylic acid. Next, the intermediate isocyanate obtained by Curtius rearrangement of the acyl azide was directly transformed into the corresponding *N*-Fmoc derivative **15b** by treatment with 9-fluorenylmethanol. Cleavage of the Fmoc-β^2-amino acid **25b** from the auxiliary using lithium hydroperoxide was accompanied by cleavage of the base-sensitive Fmoc group under the reaction conditions. However, **25b** was obtained in a yield of 29%.

Synthesis of the Cryptophycin Macrocycle. The array of different β^2-amino acid units C called for a common precursor comprising units D−A−B with a free hydroxy group at unit D and trichloroethyl protection at unit B to allow a straightforward incorporation into the macrocycle. Hence, unit A−B building block **27**[23] and unit D building block **28**[29] were coupled in a DMAP-induced esterification to obtain the D−A−B precursor **29** (Scheme 4). Selective cleavage of the TBS-ether **29** to alcohol **30** turned out to be an unexpected challenge. A fluoride-mediated cleavage of the silyl ether was not possible due to the base sensitivity of the trichloroethyl ester. This particular ester serves as a protective group but is at the same time susceptible toward attack by nucleophiles. Buffering a TBAF solution with acetic acid or benzoic acid prevented the ester cleavage. However, the hydrolysis reaction became very slow and stayed incomplete even after two days. An alternative desilylation protocol[39] led to better results: Compound **30** was obtained after deprotection at 0 °C with a methanolic solution of ceric ammonium nitrate (CAN). Decomposition products were observed only after overnight reaction, when the reaction was quenched and **30** was obtained in 57% yield. Unreacted starting material **29** could be reisolated by column chromato-

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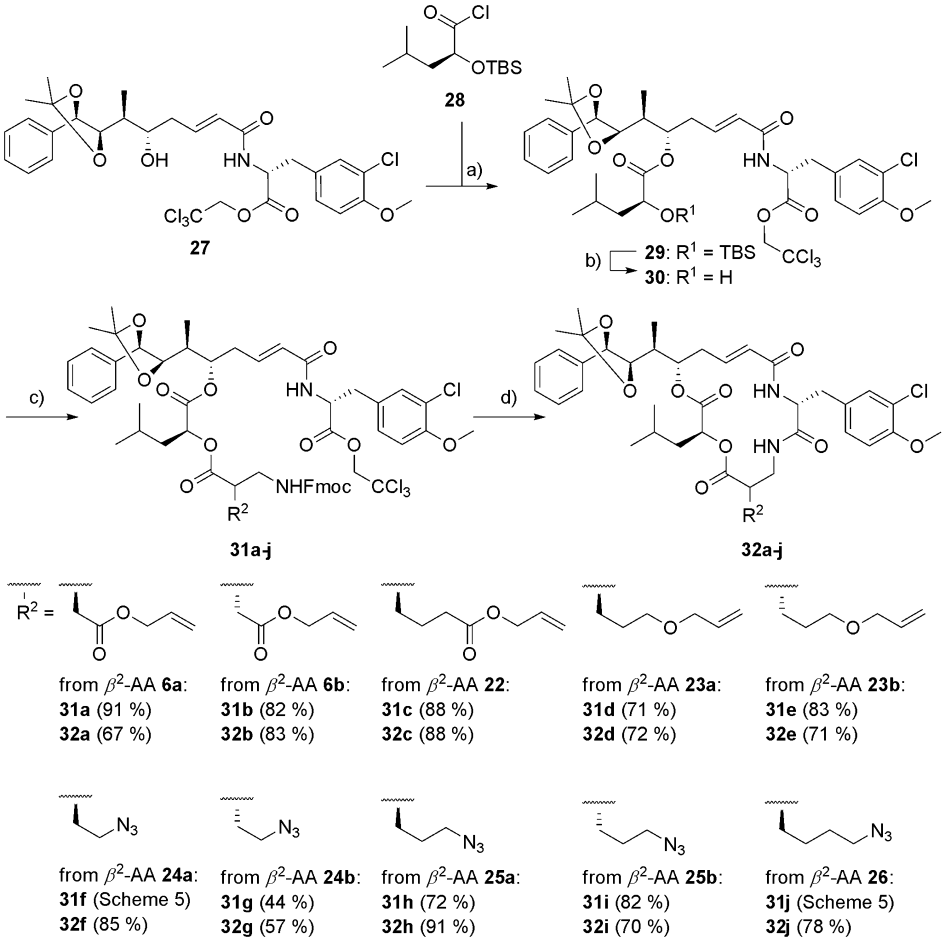
Scheme 4. Synthesis of the Macrocycles 32a–j: The Acyclic ABCD Precursors Were Obtained by a Unit AB + D + C Coupling Approach for 31a–e and 31g–i[a]



[a]Reagents and conditions: (a) DMAP, NEt_3, CH_2Cl_2, 0 °C, 1 h, 75%; (b) CAN, abs. MeOH, 0 °C, 17 h, 57%; (c) unit C, DCC, DMAP, CH_2Cl_2, rt, 16 h; (d) piperidine, DMF, rt, 16 h.

graphy in 23% yield. The array of functionalized β^2-amino acids was then incorporated in a two-step reaction sequence. In the first step, the particular β^2-amino acids were esterified by a Steglich condensation with the D–A–B fragment **30** to give the *seco*-depsipeptides **31a–e** and **31g–i**.

The acyclic compounds **31f/j** were synthesized by a more convergent approach, which also avoided any desilylation step (Scheme 5). Here, a suitable unit C–D precursor was synthesized by coupling β^2-amino acids **24a** and **26a** to unit D building block **33**.[40,41] The *tert*-butyl ester of **34a/b** was then cleaved, and the obtained unit C–D fragments **35a/b** were esterified with the unit A–B precursor **27**.

The obtained *seco*-compounds (Schemes 4 and 5) were cyclized according to a procedure by Moher et al., which consists of a base-induced cleavage of the Fmoc group and subsequent intramolecular aminolysis of the trichloroethyl ester.[41] The cyclization products **32a–j** were obtained in good yields without formation of oligomers. For completion of the cryptophycin synthesis, the acetonide function of **32a–j** had to be converted into the corresponding benzylic epoxide (Scheme 6). First, the acetonides were cleaved to give the diols **36a–j** within 3 h. The free diols **36a–j** were obtained in high purity and subjected to the final diol-epoxide transformation without further purification.[23,29,42] In the first step, **36a–j** were converted with trimethylorthoformate to the cyclic orthoesters **37a–j**. These cyclic intermediates were ring-opened by acetyl bromide to the bromohydrin formates **38a–j**. Finally, the epoxides **39a–j** were generated by saponification of the formates with K_2CO_3 in DME/ethylene glycol. No purification was needed throughout this reaction sequence. Only the final cryptophycins **39a–j** were purified by column chromatography and subsequently lyophilized. The overall yields for this four-step acetonide to epoxide transformation varied between 35 and 80%.

Cytotoxicity Assay. The biological activities of all synthesized unit C cryptophycin analogues were evaluated using a cell-based resazurin cytotoxicity assay with fluorescence read-out (Table 1). The human cervix carcinoma cell lines KB-3-1 and KB-V1 were employed for IC_{50} determination. KB-V1 is a subclone of KB-3-1 that expresses P-glycoprotein, an ATP-driven efflux pump conferring multidrug resistance by removing xenobiotics from inside the cell. Hence, cell lines expressing P-glycoprotein withstand higher drug concentrations compared to cell lines not expressing P-glycoprotein.

Some of the newly synthesized analogues show very high cytotoxicity. In particular, **39b** is even more potent than cryptophycin-52 (**1b**) against KB-3-1 cells. Interestingly, there is no general trend whether one unit C configuration confers more potency against the KB-3-1 cell line than the epimer. However, the (*R*)-configured unit C epimer is more active

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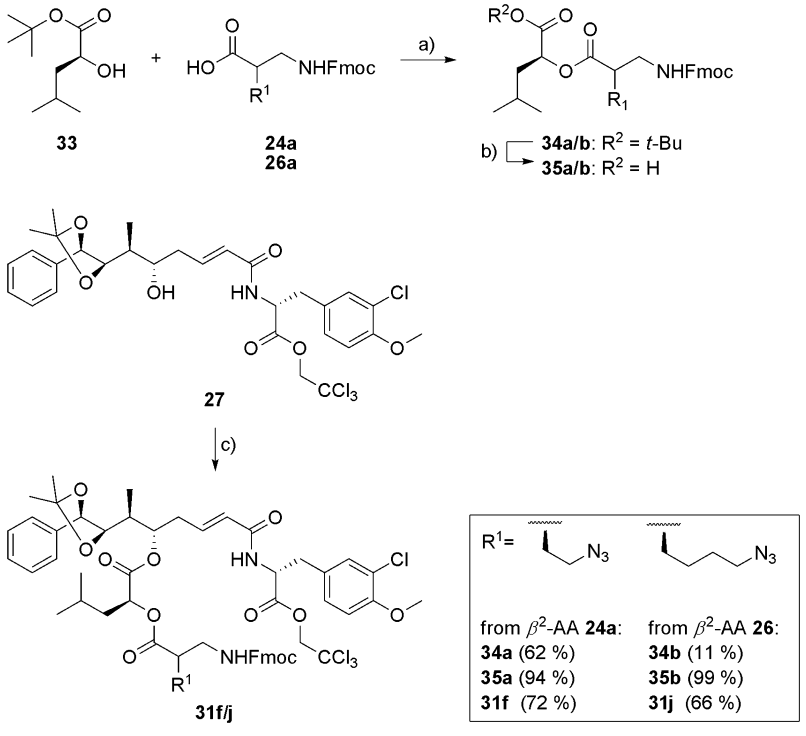
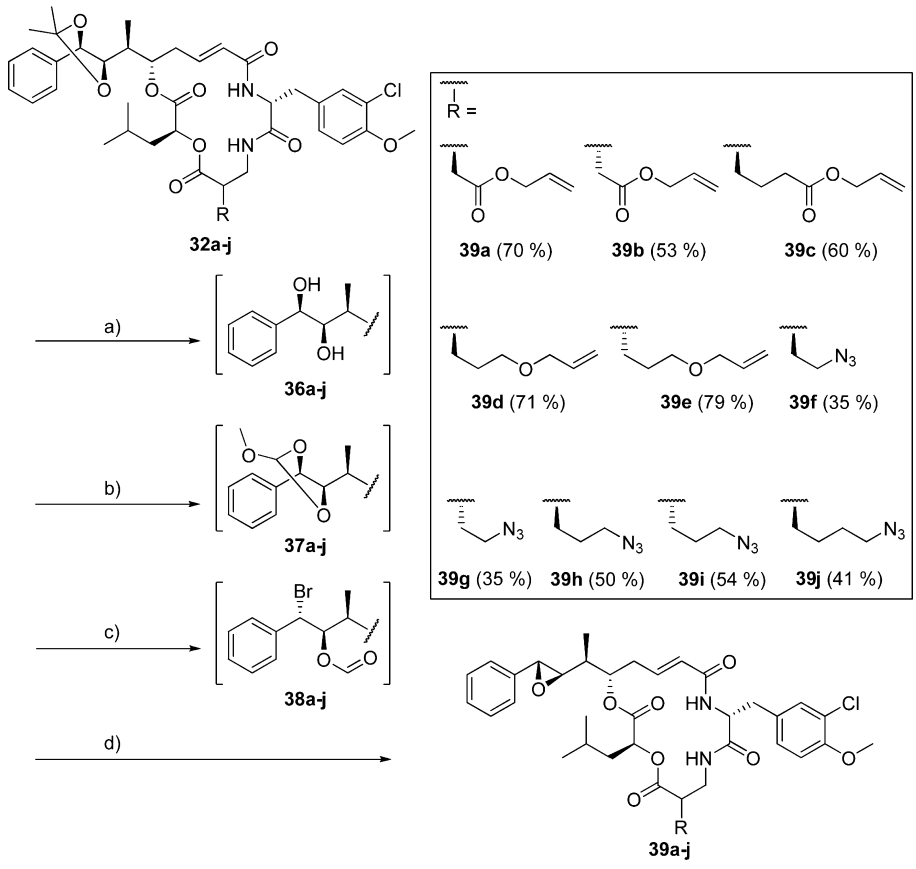
Scheme 5. Alternative Synthesis of an ABCD Precursor by a Unit AB + DC Coupling Approach[a]



[a]Reagents and conditions: (a) DCC, DMAP, CH_2Cl_2, rt, 16 h; (b) TFA, 0 °C, 3.5 h; (c) **35a**/**b**, DCC, DMAP, CH_2Cl_2, rt, 16 h.

Scheme 6. Acetonide to Epoxide Conversion[a]



[a]Reagents and conditions: (a) TFA, CH_2Cl_2, H_2O, 0 °C, 3 h; (b) $(CH_3O)_3CH$, PPTS, CH_2Cl_2, rt, 2 h; (c) AcBr, CH_2Cl_2, rt, 4 h; (d) K_2CO_3, DME/ethylene glycol (2:1 v/v), rt, 3 min (reported yields for four steps).

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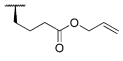
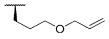
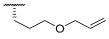
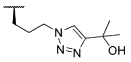
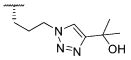
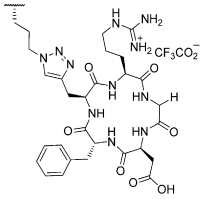
Table 1. IC_{50} values of Cryptophycin Analogues

		IC_{50} [pM] KB-3-1	IC_{50} [nM] KB-V1
1b		15.5	0.26
39a		96.1	2.7
39b		8.3	6.0
39c		432.8	45.2
39d		148.8	26.6
39e		52.7	38.0
39f		21.6	2.1
39g		54.6	14.1
39h		111.9	18.4
39i		11450	114.5
39j		249.3	23.5
40h		100.6	410.6
40i		312.4	3021
41		55800	1800

against the multidrug-resistant KB-V1 cells in all cases. It is noteworthy that all those cryptophycins isolated by Moore et al. from natural sources containing a chiral unit C building block are also (*R*)-configured at C^{α} in unit C.[3] Analogue **41** exhibits only a very low cytotoxicity compared to the parent drug and hence can be considered as a model for a prodrug. However, because the triazole ring is stable under physiological conditions, cryptophycin cannot be released from this conjugate. Linker moieties cleavable after endocytosis would liberate the cytotoxic cryptophycin derivative from the prodrug.

Conjugation of Modified Cryptophycins by CuAAC. The selective delivery of drug conjugates to tumor cells represents a promising strategy to overcome the undesired side effects of cryptophycins. For this purpose, cryptophycin has to be conjugated to a homing device. Moreover, linking cryptophycin conjugates to reporter molecules like fluorescent dyes enables further experiments to clarify the biological mode of action, e.g., by confocal laser microscopy.

Preliminary click experiments were carried out with the alkylazide-functionalized cryptophycins **39h/i** (see Table 1 and Supporting Information for further details).[43] Both compounds were reacted in a CuAAC with 2-methyl-propyne-2-ol. This particular reaction partner was chosen for the model reactions because its tertiary hydroxyl group would most unlikely lead to alcoholysis of the epoxide.

In the presence of an excess of CuI and DIPEA in toluene, both azido-derivatized cryptophycins **39h/i** formed the corresponding triazole derivates **40h/i** within 20 h. The newly introduced alcohol function would allow the conjugation to a targeting moiety via ester bonds. Such ester linkages are known to be cleaved at pH 4 with a half-life of 20 h. Diastereomer **39h** is less soluble in toluene in comparison to **39i**, which resulted in a lower turnover and a reduced yield of the CuAAC. Similar differences in polarity were found for all pairs of cryptophycin unit C diastereomers and could be quantified by significantly different retention times in RP-HPLC.

The diastereomer **39i** was chosen for a conjugation experiment with the alkyne-functionalized cyclic RGD-peptide c-(-Arg-Gly-Asp-D-Phe-Xaa-)[44] with Xaa = propargylglycine to give a first prototype of tumor targeting drug conjugates (see Supporting Information). Cyclic RGD-pentapeptides with D-Phe located after the RGD sequence have been shown to bind with high affinity to integrin $\alpha_V\beta_3$, a membrane protein highly abundant, e.g., on several cancer cells and activated endothelial cells.[45−47] The CuAAC reaction was carried out with copper powder in *tert*-BuOH/H_2O because of the hydrophilic nature of the peptide. The low copper concentration in the solution simplified the removal of most of the copper by filtration. A complete conversion to the corresponding conjugate **41** was achieved within 8−12 h, and after preparative HPLC, the coupling product was obtained in 68% yield.

An alkyne modified carboxyfluorescein derivative was chosen to connect a cyclic RGD-peptide and cryptophycin **39i** (Scheme 7). The RGD-peptide Arg-Gly-Asp-D-Phe-Lys was synthesized on 2-chlorotrityl chloride resin using the Fmoc/*tert*-Bu protecting scheme. After deprotection of the Alloc group, the carboxyfluorescein analogue was coupled using DIC/HOBt, and then the terminal Fmoc protecting group was cleaved. Afterward the conjugate was cleaved from the resin, cyclized, and fully deprotected. Cryptophycin **39i** was coupled via CuAAC to the fluorescent peptide using the reaction conditions described above. The fluorescent cyclic RGD-peptide−cryptophycin conjugate **43** was obtained within 8 h in a yield of 43%. As the envisaged conjugate was planned for internalization studies only, cytotoxicity was not a prerequisite.

Cell Adhesion Assay. The affinity of conjugate **43** to RGD-binding integrins was investigated in a competitive cell adhesion assay. The human epithelial cancer cell line WM-115 is considered a model cell line mainly expressing integrins $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$.[48] For cell adhesion measurements, vitronectin (Vn), a prominent interaction partner of RGD-binding integrins, and especially of α_v integrins, was immobilized on a Nunc Maxisorp surface.[49] WM-115 cells were prestained with fluorescein, preincubated with varying concentrations of conjugate **43**, and dispensed to immobilized Vn. An IC_{50} value of 7.9 μM was observed for conjugate **43**, which indicates that the cyclic RGD-peptide c-(-Arg-Gly-Asp-D-Phe-Lys-) conjugated to a cryptophycin across the fluorescent linker is capable to bind to integrins. For comparison, one of the most affine integrin ligand, c-(-Arg-Gly-Asp-D-Phe-Val-), has an IC_{50} value of 0.5 μM in the same assay. Consequently, this strategy has potential for tumor targeting of cancer cells highly expressing integrins. A detailed description of the experimental procedure is given in the Supporting Information.

Confocal Microscopy. Human melanoma cells WM-115 were incubated with the fluorescent cryptophycin−RGD-peptide conjugate **43** and LysoTrackerRed for colocalization experiments. After incubation for different time intervals, the cells were fixed with paraformaldehyde and investigated by

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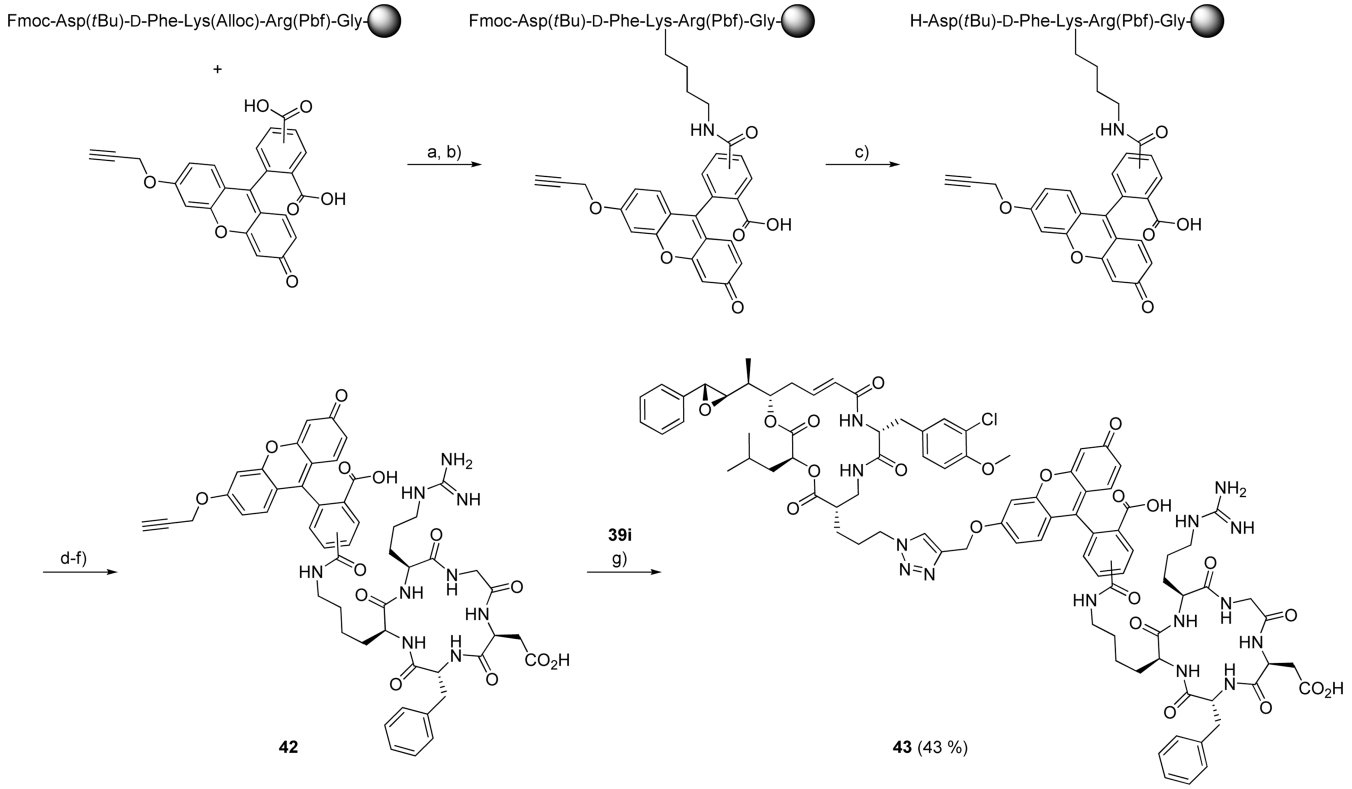
Scheme 7. Synthesis of the Cyclic RGD-Peptide−Fluorescein Conjugate 42 and Coupling to Cryptophycin 39i to Give Cryptophycin Conjugate 43[a]



[a]Reagents and conditions: (a) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, 10 min, rt; (b) DIC, HOBt, DMF, 18 h, rt; (c) piperidine, DMF, 50 min, rt; (d) HFIP, CH_2Cl_2, 45 min, rt; (e) HATU, HOAt, DIPEA, DMF, 2 h, rt; (f) TFA/H_2O/TIS (95:2.5:2.5), 3 h, rt; (g) copper powder, *t*-BuOH/H_2O (2:1), 8 h, rt.

confocal microscopy (Figure 2). Endocytosis of the fluorescent cryptophycin−RGD-peptide conjugate was observed already after 15 min incubation.

The overlay of the intracellular green fluorescence of **43** and lysosomes stained in red with LysoTrackerRed showed a high degree of colocalization. In the initial phase some structures were stained in green, presumably endosomes, and lysosomes were stained in red. The congruence of these signals was increasing with time, which means that the colocalization was enhanced. After incubation for 4 h, the degree of colocalization was almost 100%. Endocytosis could not be detected when the cells were incubated with a conjugate consisting of the cryptophycin and the fluorescent linker only (data not shown). Images were taken using a 63× glycerol objective lens (NA 1.3) with 4× frames averaging. Sequential mode was used for image acquisition to minimize cross-talk and bleed-through. Images were corrected for chromatic shift using Hyugens Essential version 4. This setup secures best confidence in concluding true physical colocalization.

Conformational Analysis. The biological activity of drugs depends on their three-dimensional shape. Therefore, conformational analysis by NMR and molecular dynamics calculation was performed for cryptophycin-52 (**1b**) and its analogues **39d** and **39e**. These particular analogues were chosen because of their relatively high biological activities against both cell lines. In the KB-3-1 cells, the (*R*)-configured epimer was three times more potent. To correlate this difference in cytotoxicity with a different three-dimensional shape of the epimers, the solution conformations were obtained on the basis of NOE distance information. Starting structures were generated in a distance geometry/simulated annealing step followed by restrained molecular dynamics calculations and, finally, by unrestrained molecular dynamics calculations. After each step, torsion angle clustering was done, and the resulting clusters were then used in the next step.

The ester and amide bonds were found to be *s-trans*-configured in all clusters. Major differences between the clusters of the cryptophycins were found for the α,β-unsaturated amide of unit A, which can be *s-trans*- or *s-cis*-configured within the enecarbonyl moiety and in the peptide bond between unit B and unit C (Figure 3). A clear preference for enecarbonyl *s-cis* planarity is supported by the NOE cross peak intensities of uB H^N/uA H^α and uB H^N/uA H^β. The calculated distances were 1.90−2.01 Å and 2.50−3.94 Å, respectively, which fits well with the measured distances in the final conformations. This observation is in accordance with the crystal structure and NMR studies of cryptophycin-3.[3]

A detailed description of each cluster conformation found for the three cryptophycins is given in the Supporting Information. The comparison of the main conformations of each cryptophycin shows a high similarity between cryptophycin-52 (**1b**) and analogue **39d** (Figure 4). The conformation of analogue **39e** differs in a flipped peptide bond between units B and C and a flipped ester bond between units D and A. However, this does not significantly change the overall shape of the backbone compared to the two other cryptophycins. The flip of the peptide bond between units B and C leads to two favored conformations, where the amide proton of unit C is

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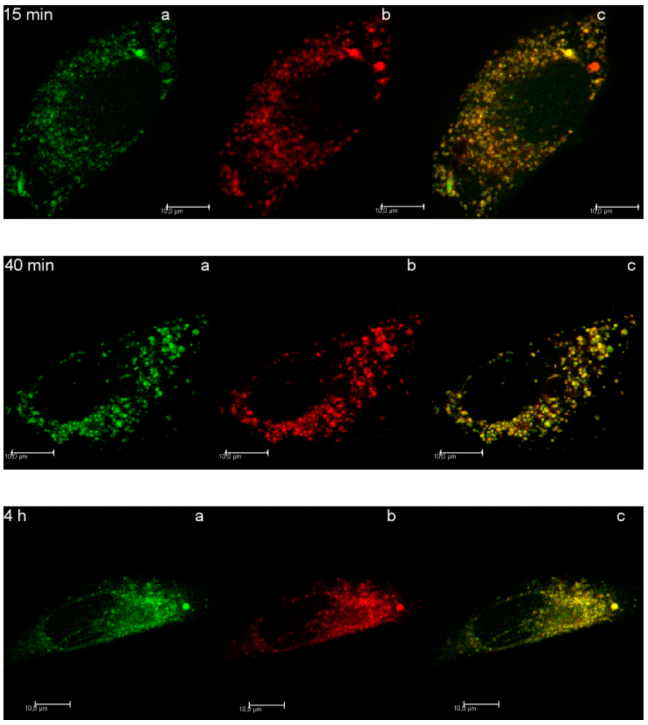

Figure 2. Confocal microscopy. Incubation of WM-115 melanoma cells with conjugate **43** for (I) 15 min, (II) 40 min, (III) 4 h. (a) The fluorescent conjugate **43** (λ_{ex} = 458 nm) is localized intracellularly. (b) Lysosomes are stained with LysoTrackerRed (λ_{ex} = 561 nm). (c) The overlay clearly proves colocalization.

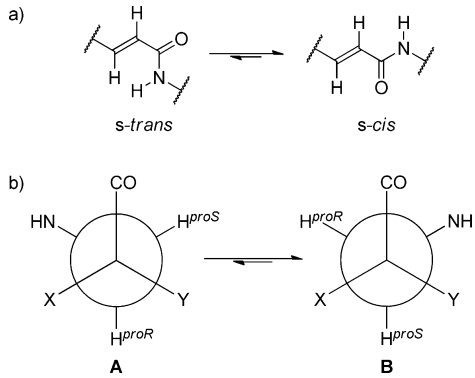

Figure 3. (a) *s-trans* and *s-cis* planar conformation of the enecarbonyl system in unit A. (b) Newman projections of the $C^{\alpha}-C^{\beta}$ bond in unit C. (±)-Synclinal: 60° ± 30° or −60° ± 30°, antiperiplanar: 180° ± 30°. Cryptophycin-52: X,Y = CH_3. **39d**: X = H, Y = $(CH_2)_3$OAll. **39e**: X = $(CH_2)_3$OAll, Y = H.

involved in a hydrogen bond with the carbonyl group of unit A or with the carbonyl group of unit C. The temperature gradients of the amide proton of unit C hint to a conformation where the proton is either involved in a hydrogen bond or otherwise shielded from the solvent (see Supporting Information, Table 1).

Unit C is a β^2-amino acid with an additional torsion angle μ ($N-C^{\beta}-C^{\alpha}-CO$) that preferred the amide group in a (+)-*synclinal* position relative to the carbonyl moiety irrespective of the unit C configuration.[50] Additionally, the unit C side chains of the analogues **39d/e** have almost the same C^{α} → side chain vectors as the corresponding methyl groups in cryptophycin-52 (**1b**). This might give an explanation for the

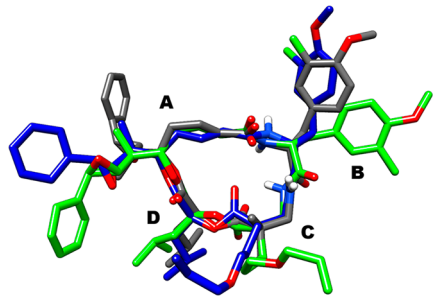

Figure 4. Superimposition of the main conformations found for cryptophycin-52 (**1b**) (gray), analogue **39d** (blue), and analogue **39e** (green).

minimal influence of the stereochemistry in unit C on the biological activity. We assume that the differences in cytotoxicity are due to the steric demand of the unit C side chains.

CONCLUSIONS

Several cryptophycins with different functional groups in the side chain of unit C were synthesized. An array of β^2-amino acids was obtained according to Evans' auxiliary chemistry. The newly synthesized cryptophycin analogues display high biological activity as determined in a cell-based cytotoxicity assay using the human cervix carcinoma cell line KB-3-1 and its multidrug-resistant subclone KB-V1. While the (*R*)-configured analogues are consistently more efficient against the multidrug-resistant cell line KB-V1, no clear-cut structure−activity relationship could be observed for the KB-3-1 cells with respect to the absolute configuration of unit C.

Comparison of the solution structures of the highly active cryptophycin-52 (**1b**) with the analogues **39d** and **39e** by conformational analysis using NMR/MD methods revealed only minor differences in conformation, e.g., in the enecarbonyl system of unit A, in the rotation of the $C^{\alpha}-C^{\beta}$ bond in unit C and in the peptide bond between unit B and C. For the enecarbonyl system a clear preference for *s-cis* planarity was found. Most importantly, the different unit C configurations in **39d** and **39e** do not induce significant changes in overall conformation.

The functional groups present in unit C of the analogues enable a conjugation of the antimitotic agents, e.g., to homing devices. Azide-functionalized cryptophycins **39h/i** were coupled to different alkynes, among them the propargylglycine-containing RGD-peptide c-(-Arg-Gly-Asp-D-Phe-Xaa-), by CuAAC. Cyclic RGD-peptides address integrins like $\alpha_v\beta_3$ that are highly expressed on the surface of many tumor cells and, consequently, show promise as homing devices in tumor targeting with peptide−drug conjugates.

The cryptophycin−fluorescein−RGD-peptide conjugate **43** was designed to investigate the internalization and final localization of a cryptophycin prodrug. We were able to show by confocal fluorescence microscopy that the majority of the conjugate had undergone endocytosis even within 15 min and was found in the lysosomes of human tumor cells. To our knowledge, this is the first time that lysosomal localization of a RGD-peptide−drug conjugate after endocytosis was visualized inside the cell by colocalization. The work presented here highlights the potential of peptide−drug conjugates containing cryptophycin derivatives and provides first principles for the further modification of such analogues.

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EXPERIMENTAL SECTION

All reactions were performed under inert conditions (argon) unless otherwise stated. Dichloromethane, DMF, and triethylamine were dried over calcium hydride. Toluene and ethylene glycol were distilled from sodium, and THF and DME were distilled from sodium/benzophenone. All other chemicals, reagents, and solvents were used as purchased. Macherey Nagel silica gel "Kieselgel 60" was used as stationary phase for column chromatography (70−230 mesh) and flash chromatography (230−400 mesh).

^{1}H NMR spectra were recorded using the Bruker instruments ARX 250 MHz, DRX 500 MHz, or AVANCE 600 MHz. Chemical shifts are reported in parts per million (ppm) relative to an internal solvent reference. Significant peaks are tabulated in the order multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants, and number of protons. The acronyms uA, uB, uC, and uD describe signals pertaining to cryptophycin units A−D. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonik APEX III FT-ICR-mass spectrometer equipped with a (nano-)ESI ion source. Mass spectrometry results are reported as the ratio of mass over charge. The purity of all final compounds was investigated by analytical RP-HPLC. Analytical RP-HPLC was performed using the Thermo Scientific HPLC unit Accela (controller SN 4000, pump Accela 600, autosampler Accela, detector Accela PDA, UV-absorption measured at λ = 254 nm), equipped with a Thermo Scientific Hypersil Gold column (dimensions 2.1 mm (ID) × 150 mm, grain size 3 μm) at a 1.0 mL/min flow rate. Eluent A was 5% acetonitrile in water with 0.1% TFA, and eluent B was 5% water in acetonitrile with 0.1% TFA. Analytical RP-HPLC was performed using the following methods. Method M1: 90% eluent A and 10% eluent B hold for 3 min, then 10−100% eluent B from 3 to 35 min, hold at 100% eluent B for 10 min, followed by washing with 90% eluent A for 5 min. Otherwise the Thermo Scientific Spectra System (controller SN 4000, pump P 4000, autosampler AS 100, detector UV 6000 LP, UV absorption measured at λ = 254 nm), equipped with a Phenomenex Jupiter C18 column (dimensions 4.6 mm (ID) × 250 mm) at a 0.7 mL/min flow rate was used for analytical RP-HPLC. Using the same eluents as above method M2 was applied: 0−100% eluent B from 0 to 5 min, then 0−100% eluent A from 5 to 6 min, followed by washing with 100% eluent A from 6 to 6.5 min.

Cryptophycins **39a−j** were synthesized starting from the corresponding acetonides **32a−j** via a four-step synthesis according to the general procedures (GP) 1−4.

GP-1: Acetonide Cleavage. The corresponding macrocyclic acetonide **32a−j** (0.10 mmol) was dissolved in dry CH_2Cl_2 (1.0 mL). TFA (1.0 mL) was added at 0 °C, followed by H_2O (5 drops). The reaction solution was stirred for 3 h at 0 °C, followed by evaporation to dryness in vacuo (bath ≤25 °C). The residue was partitioned between EtOAc (50 mL) and saturated $NaHCO_3$ solution (75 mL). The aqueous phase was additionally extracted with EtOAc (3 × 25 mL). The combined organic phases were dried over $MgSO_4$ and evaporated to dryness. The resulting crude diol **36a−j** was dried in high vacuum and was used without further purification in the following step (i.e., GP-2).

GP-2: Synthesis of Cyclic Orthoformates. A mixture of the crude diol **36a−j** (1.0 equiv, 0.05−0.25 mmol) and PPTS (2.5 equiv) was dried in vacuo. The dry solid mixture was taken up in CH_2Cl_2 (30 mL/mmol) and trimethyl orthoformate (10 mL/mmol), and the reaction mixture was stirred for 2 h at rt. The reaction mixture was filtered through silica gel (5 × 2 cm, 40−63 mm), which was washed with EtOAc/CH_2Cl_2 (1:1 v/v, 300 mL). The combined organic filtrates were evaporated to dryness. The resulting crude orthoformates **37a−j** were dried in vacuo and used without further purification in the following step (i.e., GP-3).

GP-3: Conversion of the Cyclic Orthoformates to Bromohydrin Formates. The corresponding orthoformate **37a−j** (0.05−0.25 mmol) was dissolved in dry CH_2Cl_2 (15 mL/mmol), and a solution of acetylbromide (2.5 equiv) in dry CH_2Cl_2 (0.5 M) was added. The reaction solution was stirred for 4 h at rt. Subsequently, the solution was diluted with dry CH_2Cl_2 (10 mL) and poured at 0 °C into a stirred 1:1-mixture of saturated $NaHCO_3$ solution and H_2O (50 mL). The aqueous phase was separated and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phases were dried over $MgSO_4$ and evaporated to dryness. The resulting bromohydrin formates **38a−j** were used without further purification in the following step (i.e., GP-4).

GP-4: Epoxide Synthesis. The corresponding bromohydrin formate **38a−j** (1.0 equiv, 0.05−0.25 mmol) was taken up in a freshly shaken 0.2 M K_2CO_3/ethylene glycol/DME emulsion (25 mL/mmol, 5 equiv K_2CO_3). The reaction mixture was stirred for 3 min at rt and was subsequently diluted with dry CH_2Cl_2 (20 mL). The resulting mixture was immediately poured into a separatory funnel containing ice cooled 0.5% aq $KHSO_4$ solution (20 mL). The reaction flask was washed with additional dry CH_2Cl_2 (20 mL). After mixing and phase separation, the aqueous phase was extracted with CH_2Cl_2 (2 × 20 mL). The extraction was performed as quickly as possible to prevent hydrolysis of the epoxide **39a−j** in the acidic aqueous medium. For that reason, the formation of two clear phases was not awaited. The combined organic phases were dried over $MgSO_4$ and evaporated to dryness. The residue was chromatographically purified and subsequently lyophilized.

*uC[(R)-CH_2CO_2All]-cryptophycin-1 (**39a**).* The acetonide **32a** (106 mg, 0.133 mmol) was converted to the corresponding epoxide **39a** according to GP-1−GP-4. Purification by flash chromatography (eluent: EtOAc/hexane 3:1 v/v) afforded the cryptophycin-1 analogue **39a** (69 mg, 70%, four steps) as a colorless solid. HPLC: t_R = 30.6 min (method M1). HRMS calcd for $[C_{39}H_{47}ClN_2O_{10} + Na]^+$ 761.2811; found 761.2820. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.82 (d, 3H), 0.84 (d, 3H), 1.14 (d, 3H), 1.33 (ddd, 1H), 1.61 (m, 1H), 1.69 (ddd, 1H), 1.84 (m, 1H), 2.45 (ddd, 1H), 2.56 (dd, 1H), 2.57 (m, 1H), 2.78 (dd, 1H), 2.92 (dd, 1H), 2.98 (dd, 1H), 3.16 (m, 1H), 3.16 (dd, 1H), 3.28 (ddd, 1H), 3.58 (ddd, 1H), 3.69 (d, 1H), 3.86 (s, 3H), 4.54−4.61 (m, 2H), 4.83 (dd, 1H), 4.83 (m, 1H), 5.10 (ddd, 1H), 5.24 (dm, 1H), 5.32 (dm, 1H), 5.75 (dm, 1H), 5.78 (m, 1H), 5.89 (dddd, 1H), 6.64 (ddd, 1H), 6.71 (m, 1H), 6.83 (d, 1H), 7.08 (dd, 1H), 7.21 (d, 1H), 7.23−7.39 (m, 5H).

*uC[(S)-CH_2CO_2All]-cryptophycin-1 (**39b**).* The acetonide **32b** (125 mg, 0.157 mmol) was converted to the corresponding epoxide **39b** according to GP-1−GP-4. Purification by flash chromatography (eluent: EtOAc/hexane 3:1 v/v) afforded the cryptophycin-1 analogue **39b** (62 mg, 53%, four steps) as a colorless solid. HPLC: t_R = 30.0 min (method M1). HRMS calcd for $[C_{39}H_{47}ClN_2O_{10} + Na]^+$ 761.2811; found 761.2800. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.825 (d, 3H), 0.834 (d, 3H), 1.15 (d, 3H), 1.31 (ddd, 1H), 1.65 (m, 1H), 1.70 (ddd, 1H), 1.81 (dqm, 1H), 2.46 (ddd, 1H), 2.51−2.57 (m, 2H), 2.74 (dd, 1H), 2.93 (dd, 1H), 2.93 (m, 1H), 3.07−3.14 (m, 2H), 3.44 (ddd, 1H), 3.66 (ddd, 1H), 3.69 (d, 1H), 3.86 (s, 3H), 4.51 (ddm, 1H), 4.57 (ddm, 1H), 4.79 (ddd, 1H), 4.86 (dd, 1H), 5.11 (ddm, 1H), 5.23 (dm, 1H), 5.29 (dm, 1H), 5.66 (m, 1H), 5.78 (dm, 1H), 5.87 (dddd, 1H), 6.62 (ddd, 1H), 6.79 (m, 1H), 6.84 (d, 1H), 7.08 (dd, 1H), 7.20 (d, 1H), 7.24−7.38 (m, 5H).

*uC[(R)-$(CH_2)_3CO_2$All]-cryptophycin-1 (**39c**).* The acetonide **32c** (125 mg, 0.151 mmol) was converted to the corresponding epoxide **39c** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/hexane 2:1 v/v) afforded the cryptophycin-1 analogue **39c** (70 mg, 60%, four steps) as a colorless solid. HPLC: t_R = 31.1 min (method M1). HRMS calcd for $[C_{41}H_{51}ClN_2O_{10} + Na]^+$ 789.3122; found 789.3124. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.85 (d, 3H), 0.87 (d, 3H), 1.14 (d, 3H), 1.36 (ddd, 1H), 1.46−1.83 (m, 7H), 2.36−2.39 (m, 2H), 2.45 (ddd, 1H), 2.53−2.59 (m, 2H), 2.92 (dd, 1H), 3.04 (dd, 1H), 3.12 (dd, 1H), 3.36 (ddd, 1H), 3.49 (ddd, 1H), 3.69 (d, 1H), 3.86 (s, 3H), 4.57−4.59 (m, 2H), 4.79−4.84 (m, 2H), 5.14 (ddd, 1H), 5.22 (dm, 1H), 5.31 (dm, 1H), 5.64 (m, 1H), 5.74 (dm, 1H), 5.92 (dddd, 1H), 6.63 (ddd, 1H), 6.83 (d, 1H), 6.89 (m, 1H), 7.06 (dd, 1H), 7.21 (d, 1H), 7.24−7.26 (m, 2H), 7.32−7.39 (m, 3H).

*uC[(R)-$(CH_2)_3$OAll]-cryptophycin-1 (**39d**).* The acetonide **32d** (85 mg, 0.107 mmol) was converted to the corresponding epoxide **39d** according to GP-1−GP-4. Purification by flash chromatography (eluent: EtOAc/hexane 2:1 v/v) afforded the cryptophycin-1 analogue

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39d (56 mg, 71%, four steps) as a colorless solid. HPLC: t_R = 31.1 min (method M1). HRMS calcd for $[C_{40}H_{51}ClN_2O_9 + Na]^+$ 761.3175; found 761.3175. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.85 (d, 3H), 0.87 (d, 3H), 1.14 (d, 3H), 1.36 (ddd, 1H), 1.54 (ddd, 1H), 1.59−1.84 (m, 6H), 2.45 (ddd, 1H), 2.54 (ddm, 1H), 2.60 (m, 1H), 2.92 (dd, 1H), 3.05 (dd, 1H), 3.12 (dd, 1H), 3.34 (ddd, 1H), 3.42−3.46 (m, 2H), 3.51 (ddd, 1H), 3.69 (d, 1H), 3.84 (s, 3H), 3.94−3.97 (m, 2H), 4.80−4.84 (m, 2H), 5.13 (ddd, 1H), 5.16 (dm, 1H), 5.27 (dm, 1H), 5.63 (m, 1H), 5.75 (dm, 1H), 5.90 (dddd, 1H), 6.62 (ddd, 1H), 6.83 (d, 1H), 6.85 (m, 1H), 7.06 (dd, 1H), 7.21 (d, 1H), 7.24−7.26 (m, 2H), 7.32−7.39 (m, 3H).

*uC[(S)-(CH$_2$)$_3$OAll]-cryptophycin-1 (**39e**).* The acetonide **32e** (103 mg, 0.129 mmol) was converted to the corresponding epoxide **39e** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/hexane 3:1 v/v) afforded the cryptophycin-1 analogue **39e** (75 mg, 79%, four steps) as a colorless solid. HPLC: t_R = 30.9 min (method M1). HRMS calcd for $[C_{40}H_{51}ClN_2O_9 + Na]^+$ 761.3175; found 761.3178. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.84 (d, 3H), 0.85 (d, 3H), 1.15 (d, 3H), 1.33 (m, 1H), 1.53−1.84 (m, 7H), 2.45 (ddd, 1H), 2.56 (ddm, 1H), 2.67 (m, 1H), 2.93 (dd, 1H), 3.06 (dd, 1H), 3.12 (dd, 1H), 3.37−3.40 (m, 2H), 3.42 (ddd, 1H), 3.49 (ddd, 1H), 3.69 (d, 1H), 3.86 (s, 3H), 3.92−3.95 (m, 2H), 4.76 (dd, 1H), 4.90 (ddm, 1H), 5.12−5.18 (m, 2H), 5.25 (dm, 1H), 5.65 (m, 1H), 5.75 (dm, 1H), 5.88 (dddd, 1H), 6.66 (ddd, 1H), 6.71 (m, 1H), 6.83 (d, 1H), 7.06 (dd, 1H), 7.20 (d, 1H), 7.23−7.26 (m, 2H), 7.32−7.38 (m, 3H).

*uC[(R)-(CH$_2$)$_2$N$_3$]-cryptophycin-1 (**39f**).* The acetonide **32f** (112 mg, 0.146 mmol) was converted to the corresponding epoxide **39f** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/petroleum ether 2:1 v/v) afforded the cryptophycin-1 analogue **39f** (36.4 mg, 35%, four steps) as a colorless solid. HPLC: t_R = 5.3 min (method M2). HRMS calcd for $[C_{36}H_{44}ClN_5O_8 + Na]^+$ 732.2770; found 732.2788. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.83 (d, 3H), 0.85 (d, 3H), 1.14 (d, 3H), 1.32 (ddd, 1H), 1.65−1.76 (m, 3H), 1.80 (m, 1H), 2.01 (m, 1H), 2.45 (ddd, 1H), 2.57 (ddd, 1H), 2.76 (m, 1H), 2.92 (dd, 1H), 2.97 (dd, 1H), 3.11 (dd, 1H), 3.26 (ddd, 1H), 3.48 (ddd, 1H), 3.55- 3.67 (m, 2H), 3.68 (d, 1H), 3.86 (s, 3H), 4.69 (ddd, 1H), 4.83 (dd, 1H), 5.19 (ddd, 1H), 5.68−5.76 (m, 2H), 6.71 (ddd, 1H), 6.83 (d, 1H), 7.03 (dd, 1H), 7.12 (d, 1H), 7.19−7.25 (m, 3H), 7.31−7.40 (m, 3H).

*uC[(S)-(CH$_2$)$_2$N$_3$]-cryptophycin-1 (**39g**).* The acetonide **32g** (27.5 mg, 0.036 mmol) was converted to the corresponding epoxide **39g** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/hexane 3:1 v/v) afforded the cryptophycin-1 analogue **39g** (9 mg, 35%, four steps) as a colorless solid. HPLC: t_R = 28.4 min (method M1). HRMS calcd for $[C_{36}H_{44}ClN_5O_8 + Na]^+$ 732.2770; found 732.2769. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.86 (d, 3H), 0.88 (d, 3H), 1.15 (d, 3H), 1.39 (m, 1H), 1.64−1.77 (m, 4H), 1.82 (m, 1H), 2.47 (ddd, 1H), 2.54 (dd, 1H), 2.73 (m, 1H), 2.93 (dd, 1H), 3.04 (dd, 1H), 3.21 (dd, 1H), 3.28−3.35 (m, 2H), 3.38 (m, 1H), 3.69 (d, 1H), 3.74 (ddd, 1H), 3.87 (s, 3H), 4.84 (ddd, 1H), 4.87 (dd, 1H), 5.08 (m, 1H), 5.61(d, 1H), 5.80 (dm, 1H), 6.43 (m, 1H), 6.58 (ddd, 1H), 6.84 (d, 1H), 7.05 (dd, 1H), 7.21 (d, 1H), 7.22−7.25 (m, 2H), 7.33−7.39 (m, 3H).

*uC[(R)-(CH$_2$)$_3$N$_3$]-cryptophycin-1 (**39h**).* The acetonide **32h** (184 mg, 0.235 mmol) was converted to the corresponding epoxide **39h** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/hexane 2:1 v/v) afforded the cryptophycin-1 analogue **39h** (75 mg, 50%, four steps) as a colorless solid. HPLC: t_R = 31.0 min (method M1). HRMS calcd for $[C_{37}H_{46}ClN_5O_8 + Na]^+$ 746.2927; found 746.2937. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.85 (d, 3H), 0.87 (d, 3H), 1.15 (d, 3H), 1.36 (ddd, 1H), 1.54 (ddd, 1H), 1.62−1.84 (m, 6H), 2.46 (ddd, 1H), 2.54−2.59 (m, 2H), 2.92 (dd, 1H), 3.03 (dd, 1H), 3.11 (dd, 1H), 3.28 (ddd, 1H), 3.33 (ddd, 1H), 3.41 (ddd, 1H), 3.47 (ddd, 1H), 3.69 (d, 1H), 3.87 (s, 3H), 4.79 (ddd, 1H), 4.83 (dd, 1H), 5.16 (ddd, 1H), 5.66 (m, 1H), 5.74 (dm, 1H), 6.66 (ddd, 1H), 6.83 (d, 1H), 7.00 (m, 1H), 7.05 (dd, 1H), 7.20 (d, 1H), 7.24−7.26 (m, 2H), 7.32−7.39 (m, 3H).

*uC[(S)-(CH$_2$)$_3$N$_3$]-cryptophycin-1 (**39i**).* The acetonide **32i** (129 mg, 0.165 mmol) was converted to the corresponding epoxide **39i** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/hexane 3:1 v/v) afforded the cryptophycin-1 analogue **39i** (65 mg, 54%, four steps) as a colorless solid. HPLC: t_R = 28.5 min (method M1). HRMS calcd for $[C_{37}H_{46}ClN_5O_8 + Na]^+$ 746.2927; found 746.2937. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.85 (d, 3H), 0.86 (d, 3H), 1.15 (d, 3H), 1.35 (ddd, 1H), 1.52−1.70 (m, 5H), 1.72 (ddd, 1H), 1.81 (m, 1H), 2.45 (ddd, 1H), 2.56 (dm, 1H), 2.65 (m, 1H), 2.93 (dd, 1H), 3.08 (dd, 1H), 3.12 (dd, 1H), 3.25−3.28 (m, 2H), 3.40 (ddd, 1H), 3.53 (ddd, 1H), 3.69 (d, 1H), 3.87 (s, 3H), 4.77 (ddd, 1H), 4.91 (dd, 1H), 5.14 (ddd, 1H), 5.67 (m, 1H), 5.76 (dm, 1H), 6.65 (ddd, 1H), 6.68 (m, 1H), 6.84 (d, 1H), 7.06 (dd, 1H), 7.21 (d, 1H), 7.23−7.26 (m, 2H), 7.32−7.39 (m, 3H).

*uC[(R)-(CH$_2$)$_4$N$_3$]-cryptophycin-1 (**39j**).* The acetonide **32j** (30 mg, 0.038 mmol) was converted to the corresponding epoxide **39j** according to GP-1−GP-4. Purification by column chromatography (eluent: EtOAc/petroleum ether 2:1 v/v) afforded the cryptophycin-1 analogue **39j** (11.5 mg, 41%, four steps) as a colorless solid. HPLC: t_R = 5.3 min (method M2). HRMS calcd for $[C_{38}H_{48}ClN_5O_8 + Na]^+$ 760.3098; found 760.3083. ^{1}H NMR (500 MHz, $CDCl_3$, TMS): δ (ppm) = 0.85 (d, 3H), 0.87 (d, 3H), 1.14 (d, 3H), 1.35 (m, 1H), 1.45−1.55 (m, 3H), 1.59−1.64 (m, 2H), 1.68−1.76 (m, 3H), 1.80 (m, 1H), 2.46 (ddd, 1H), 2.51−2.59 (m, 2H), 2.92 (dd, 1H), 3.04 (dd, 1H), 3.11 (dd, 1H), 3.24−3.35 (m, 2H), 3.39 (m, 1H), 3.46 (m, 1H), 3.69 (d, 1H), 3.86 (s, 3H), 4.78 (ddd, 1H), 4.82 (dd, 1H), 5.16 (ddd, 1H), 5.60 (m, 1H), 5.74 (dm, 1H), 6.66 (ddd, 1H), 6.83 (d, 1H), 7.00 (m, 1H), 7.05 (dd, 1H), 7.20 (d, 1H), 7.22−7.26 (m, 2H), 7.33−7.39 (m, 3H).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

All details of the syntheses, molecular modeling coordinates, and details on the conformational analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

AcBr, acetyl bromide; ADC, antibody−drug conjugate; CuAAC, copper-catalyzed azide−alkyne cycloaddition; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; FmocOSu, *N*-(9-fluorenylmethoxycarbonyloxy)-succinimide; HATU, 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HFIP, hexafluoroisopropanol; HOAt, 1-hydroxy-7-azabenzotriazole; HOBt, 1-hydroxybenzotriazole; MeOH, methanol; NaHMDS, sodium hexamethyldisilazide; nd, not determined; R, residue; RP, reversed phase; *t*-BuOH, *tert*-butanol; TIS, triisopropylsilane; u (as in uB), unit; Vn, vitronectin

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