Synthesis of 20-*O*-linked 20(*S*)-Camptothecin Glycoconjugates: Impact of the Side Chain of the Ester-linked Amino Acid on Epimerization During the Acylation Reaction and on Hydrolytic Stability of the Final Glycoconjugates [1])

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Dedicated to Professor Dr. Horst Kunz, University of Mainz, on the Occasion of his 60th Birthday

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Abstract. To improve solubility and tumor selectivity of 20(*S*)-camptothecin the synthesis of 20-*O*-linked glycoconjugates **11A**–**G** is described. Particular focus of the paper is the utilization of *N*-*tert*-butoxycarbonyl protected amino acid *N*-carboxy anhydrides (UNCAs) **2a**–**f** for an efficient acylation of the sterically hindered and deactivated tertiary 20-hydroxy group of 20(*S*)-camptothecin **1**. Depending on the solvent and on the side chain of the amino acid different extents of epimerization of the amino acids during the coupling reaction are observed; however, the epimers can easily be separated after removal of the *tert*-butoxycarbonyl protecting group and camptothecin amino acid conjugates **4B**–**E** with *L*- and *D*- configured amino acids, respectively, are obtained. Particularly, bulky and β-branched amino acids can be attached to camptothecin in high yields and with low epimerization. Starting from the camptothecin amino acid conjugates **4B**–**E** the synthesis of the glycoconjugates **11A**–**G** is straightforward following standard procedures. The glycoconjugate hydrochlorides **11A**–**G** show good water solubility (> 5mg/ml) and hydrolytic stability of the ester bond which again depends on the side chain of the amino acid residue linked to camptothecin. Particularly, glycoconjugates **11B**–**E** with a bulky and β-branched amino acid at the ester linkage show high hydrolytic stability in aqueous solutions with less than 2.5% of 20(*S*)-camptothecin cleaved within 24 h. These results provide a basis for the selection of appropriate spacer groups for delivery systems of 20(*S*)-camptothecin for therapeutic use.

20(*S*)-Camptothecin **1** is a potent cytotoxic agent acting by inhibition of topoisomerase I which has been isolated first by Wall *et al*. from the wood and bark of the Chinese tree *Camptotheca acuminata* [1]. Despite some early evidence for activity, clinical trials were suspended because of unfavourable properties of the compound such as toxicity, poor solubility and a species dependent opening of the lactone-E-ring to give the inactive carboxylate form [2]. To overcome these disadvantages a number of camptothecin analogues, prodrugs and delivery systems have been developed and have recently entered the clinics [3]. The tertiary 20-hydroxy group of camptothecin is an attractive functionality in the molecule for the attachment of carrier residues. However, the chemistry for modifying this particular tertiary hydroxy group is challenging because of the steric hindrance and the deactivation due to the neighboured lactone ring.

20-*O*-Alkyl esters of camptothecin have been prepared by acylation with anhydrides of organic acids to provide prodrugs with increased lactone ring stability [4]. However, the coupling yields decreased dramatically when α-branched organic acids were employed.

To achieve increased circulatory retention as well as a continuous therapeutic release of camptothecin, Greenwald *et al*. utilized the tertiary 20-hydroxy group for esterification with non-immunogenic polyethylene glycol (PEG) 40 kDa dicarboxylic acid and derivatives *e.g*. in the presence of diisopropyl carbodiimide [5], a method previously described for the modification of paclitaxel [6].

To increase water solubility several simple amino acid conjugates of camptothecin and its analogues have been synthesized: Vishnuvajjala et al. functionalized the 20-hydroxy group of camptothecin by chloroacetic acid in pyridine/4-dimethylamino pyridine followed by conversion into the corresponding iodoacetate and amidation with various secondary amines to obtain glycinates [7]. Furthermore, Wani *et al*. and Wadkins *et al*. synthesized 20-*O*-glycinates *via* coupling of *N*-*tert*-butoxycarbonyl-glycine and subsequent deprotection to obtain water soluble prodrugs [8].

Due to the steric hindrance and the deactivation of the tertiary 20-hydroxy group the acylation of camptothecin with chiral amino acid derivatives such as *N*-*tert*-butoxycarbonyl-alanine or *N*-*tert*-butoxycarbonyl-*D*-alanine was accompanied by substantial epimerization of the amino acid [9]. This inconvenience could be overcome by using of the readily available Lewis acid catalyst, scandium triflate [10, 11].

[1]) Abbreviations. *DMAP* – 4-(N,N-dimethylamino)-pyridine; *DMF* – dimethyl formamide; *MTBE* – methyl *tert*-butyl ether; *CPT* – 20(*S*)-Camptothecin; *NCA* – *N*-carboxy anhydride

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Results and Discussion

Our goal is to improve the tumor selectivity of cytotoxic agents by the attachment of glycopeptide derivatives. In previous studies we have optimized modified fucoside residues for preferential receptor-mediated uptake into tumor cells using neoglycoconjugates of bovine serum albumine [12]. Here, we describe the attachment of these optimized fucoside derivatives to the 20-hydroxy group of the camptothecin molecule *via* ester linked dipeptide spacers.

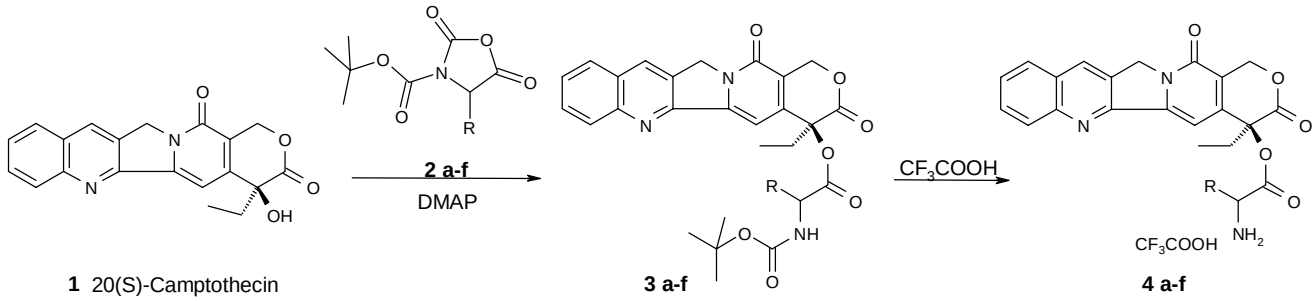
As shown below, to obtain glycoconjugates with sufficient hydrolytic stability of the ester bond a bulky amino acid residue linked to the hydroxy group of camptothecin is of particular importance. Looking for efficient methods convenient also for scale-up for acylating the sterically hindered and deactivated 20-hydroxy group of camptothecin particularly with bulky amino acid residues, we here describe our findings that urethane protected amino acid *N*-carboxy anhydrides (UNCAs) [13] were highly appropriate activated amino acid derivatives for this challenge. The side chain of the amino acid linked to camptothecin has a strong influence on both, the epimerization during the esterification of camptothecin with UNCAs and on hydrolytic stability of the ester bond in the final glycoconjugates.

The acylation of 20(*S*)-camptothecin **1** with *N*-*tert*-butoxycarbonyl-glycine runs smoothly in dichloromethane in the presence of dicyclohexyl carbodiimide and DMAP [7b, 8]. Starting with *N*-*tert*-butoxycarbonyl-glycine *N*-carboxy anhydride (Boc-Gly-NCA) **2a** as an activated amino acid derivative, the acylation of 20(*S*) camptothecin **1** and the subsequent deprotection with trifluoro-acetic acid also runs quickly and in excellent yields (scheme 1).

However, the acylation of the 20-hydroxy group with bulky and β-branched *N*-protected amino gives no or very poor yields when using carbodiimides and DMAP for coupling. In contrast, when the urethane protected amino acid *N*-carboxy anhydrides (UNCAs) **2b**–**f** were used as activated amino acid starting materials, the esterification reaction proceeds highly efficient but slowly (scheme 1, table 1). The inhomogenicity of the reaction mixtures due to the low solubility of camptothecin might be the major factor determining the long reaction times between two and four days rather than kinetic parameters. The esterification reactions are performed under reflux in case of dichloromethane and under sonification in case of DMF. The Boc-protected intermediates **3a**–**f** are formed in high yields and are purified by precipitation or by flash chromatography on silica gel.

Interestingly, depending on the solvent and on the side chain of the amino acid different and in some cases unexpected high extents of epimerization of the amino acids are observed during the coupling reaction. A detection and separation of the epimers is easily possible after removal of the *tert*-butoxycarbonyl protecting group on the step of the free amino acid conjugates **4b**–**f** (scheme 1, table 1). Therefore, the ratio of the epimers of *D*- and *L*-amino acid conjugates formed during the coupling reaction is determined after removal of the protecting group with trifluoroacetic acid in the crude materials **4b**–**f**. The epimeric ratio is determined either by RP-HPLC or by ^{1}H NMR. In table 1 the most important reaction parameters, the yields of the coupling and the deprotection steps as well as the epimeric ratios are summarized for the synthesis of a number of camptothecin amino acid conjugates. In the last column of table 1 the overall yield of pure isolated epimers with respect to coupling, deprotection and purification is indicated.

20(*S*)-camptothecin **1** is efficiently acylated with an excess of 2 equivalents Boc-Val-NCA **2b** in dichloromethane at reflux temperature (table 1, entry 2). Only very low epimerization is observed and after deprotection and purification 20(*S*)-20-*O*-valinyl-camptothecin trifluoroacetate **4B**(***L***) is isolated in an overall yield of 78%. In our hands this was by far the most efficient method to attach valine to camptothecin. If the same reaction is performed under sonification in DMF instead of dichloromethane, the yields are also high, however, about 40% of the *D*-valine epimer is formed (table 1, entry 3). Starting with Boc-D-Val-NCA **2c** and performing the reaction in DMF, the configuration of the amino



Scheme 1 Acylation of 20(*S*)-camptothecin with urethane-protected amino acid *N*-carboxy anhydrides and deprotection

acid is retained with formation of less than 5% of the *L*-epimer (entry 5). In contrast, when the same reaction is performed in dichloromethane, substantial formation of 33% of the *L*-epimer is observed (entry 4). Thus, in the valine series the non polar dichloromethane solvent obviously favours the formation of the *L*-epimer whereas the acylation in DMF predominantly gives the *D*-epimer.

Boc-Ile-NCA **2d** has a β-branch as well and behaves pretty much the same as Boc-Val-NCA **2b** with respect to epimerization during the coupling reaction with 20(*S*)-camptothecin **1** in both, dichloromethane and DMF (table 1, entry 6 and 7).

In contrast, Boc-Leu-NCA **2e**, which has no β-branch, cannot be attached to camptothecin **1** without substantial epimerization (table 1, entry 8, 9). Even when dichloromethane is used as a solvent, about 33% of the *D*-epimer are formed. Remarkably, when performing the acylation with the *L*-configurated Boc-Leu-NCA **2e** in DMF, the *D*-epimer of the amino acid conjugate is formed predominantly with an epimeric ratio of the crude product of *D*:*L* = 3:1. Only if the coupling reaction is performed in dioxane, which is not a very convenient solvent because of poor solubility of camptothecin **1**, the *L*-epimer is formed predominantly but in very low yields (table 1, entry 10). The assignment of the *D*- and *L*-configuration to the polar and non polar epimers in the leucine series is based on an X-ray structure of the acetylated product derived from the polar **4E**(***D***) fraction indicating the *D*-configuration of leucine [14]. Unexpectedly, when Boc-D-Leu-NCA is employed in the coupling reaction, also no configurative stability is observed (table 1, entry 11, 12).

From the epimeric mixtures of the camptothecin amino acid conjugates **4b**–**f** it is easily possible to separate the epimers by crystallization from dichloromethane/methanol with diethyl ether. Repeated crystallization provides pure compounds with epimeric ratios exceeding 30:1. Due to the efficiency of this separation process also minor components can be isolated in pure form (table 1, entry 7 and 8). The overall yield of isolated epimers of high purity (coupling and deprotection, epimeric ratios >15:1) is given in the last column of table 1 with **4B**(***L***), **4D**(***L***) and **4E**(***L***) representing the *L*-amino acid epimers of 20-*O*-valyl-camptothecin, 20-*O*-isoleucyl-camptothecin and 20-*O*-leucyl-camptothecin, respectively, and **4C**(***D***), **4D**(***D***) and **4E**(***D***) representing the *D*-amino acid epimers of 20-*O*-*D*-valyl-camptothecin, 20-*O*-*D*-isoleucyl-camptothecin and 20-*O*-*D*-leucyl-camptothecin, respectively.

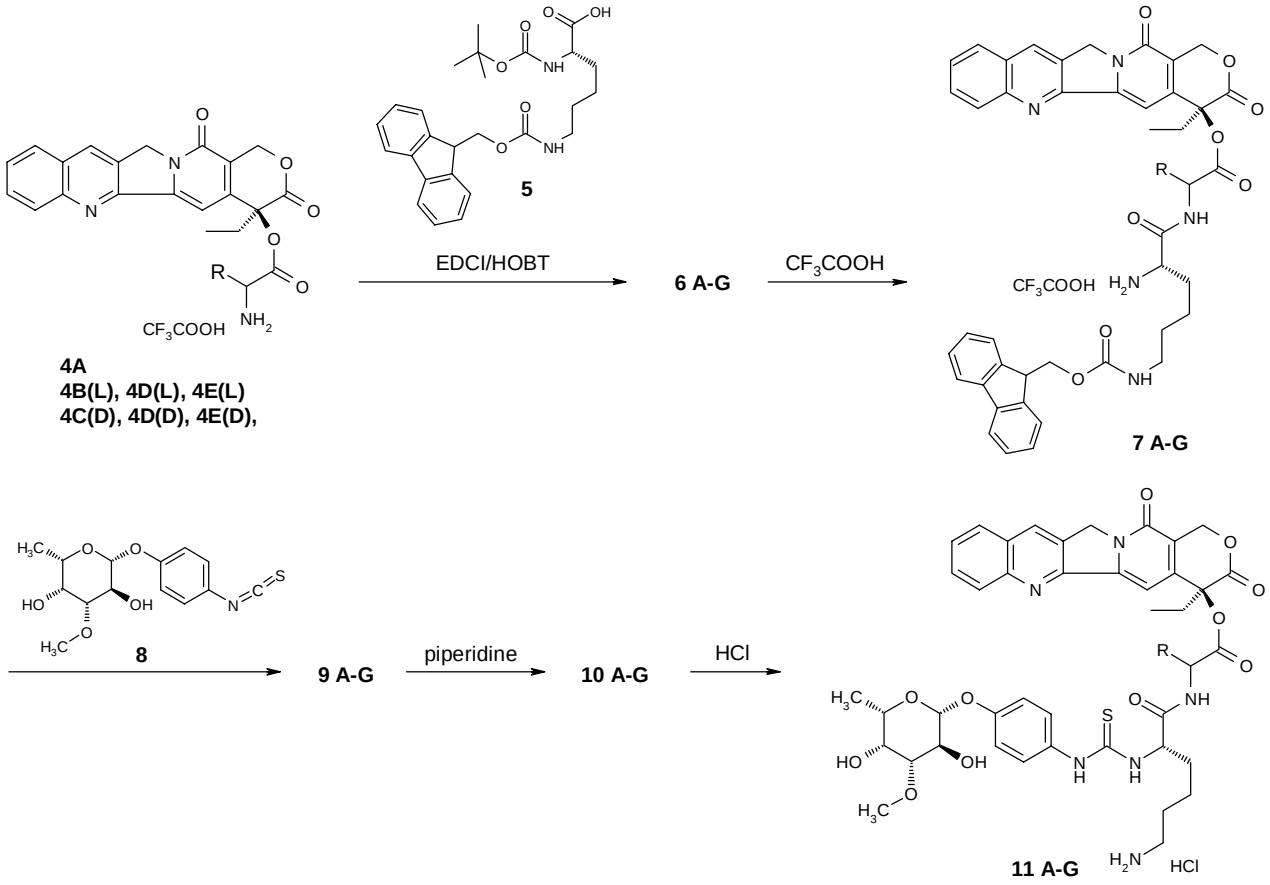
Thus, in each particular case appropriate reaction conditions have been identified to attach bulky and β-branched Boc-amino acid NCAs to the 20-hydroxy group of 20(*S*)-camptothecin **1** in high yields and with low epimerization. Acylation with Boc-Leu-NCAs lacking this β-branch is accompanied with higher epimerization. In all cases amino acid conjugates with high optical purity can easily be obtained by crystallization.

The next goal was to synthesize water soluble glycoconjugates of 20(*S*)-camptothecin carrying *p*-aminophenyl 3-*O*-methyl-fucopyranosyl residues which should be hydrolytically and proteolytically stable. Therefore, a second amino acid with a polar side chain was introduced into the spacer group. To get stability of the final conjugate against proteases the α-amino group should be modified with the carbohydrate moiety and the side chain functional group, after deprotection, should contribute to water solubility.

N^{α}-*tert*-Butoxycarbonyl-N^{ε}-fluorenyl-9-methoxycarbonyl-lysine **5** was attached to the terminal amino group of the purified epimers of the amino acid conjugates **4** using standard conditions (scheme 2). In each case the

Table 1 Acylation of 20(*S*)-camptothecin with urethane-protected amino acid *N*-carboxy anhydrides and deprotection

entry	coupling step				deprotection step		
	educt	solvent	time	product and yield (%)	product and yield (%)	epimeric ratio (**4b**–**f**, *D*:*L*)	Isolated yield of pure epimer **4A**–**4E** (over 2 steps)
1	Boc-Gly-NCA **2a** (1.5eq)	CH_2Cl_2	8 h	**3a** quant.	**4a** 85		**4A** 85%
2	Boc-L-Val-NCA **2b** (2eq)	CH_2Cl_2	4 d	**3b** quant.	**4b** 83	~1:20	**4B**(***L***) 78% (>1:30)
3	Boc-L-Val-NCA **2b** (4.5eq)	DMF	2 d	**3b** 97	**4b** 93	1:1.5	not purified
4	Boc-D-Val-NCA **2c** (3eq)	CH_2Cl_2	7 d	**3c** 90	**4c** 90	2:1	**4C**(***D***) 19% (>40:1)
5	Boc-D-Val-NCA **2c** (2.5eq)	DMF	4 d	**3c** 49	**4c** 37	20:1	**4C**(***D***) 18% (~20:1)
6	Boc-L-Ile-NCA **2d** (2eq)	CH_2Cl_2	2 d	**3d** 92	**4d** 98	1:25	**4D**(***L***) 90% (1:25)
7	Boc-L-Ile-NCA **2d** (3eq)	DMF	4 d	**3d** 68	**4d** 97	1:1.5	**4D**(***L***) 33% (1:16) + **4D**(***D***) 26% (13:1)
8	Boc-L-Leu-NCA **2e** (2eq)	CH_2Cl_2	4 d	**3e** 97	**4e** 94	1:2	**4E**(***L***) 32% (1:13) + **4E**(***D***) 27% (40:1)
9	Boc-L-Leu-NCA **2e** (2eq)	DMF	1 d	**3e** 98	**4e** 89	3:1	**4E**(***D***) 57% (15:1)
10	Boc-L-Leu-NCA **2e** (2eq)	Dioxan	>4 d	**3e** 12	**4e** 56	1:16	**4E**(***L***) 7% (1:16)
11	Boc-D-Leu-NCA **2f** (2eq)	CH_2Cl_2	2.5 d	**3f** 91	**4f** 81	1:1	not purified
12	Boc-D-Leu-NCA **2f** (2.5eq)	DMF	4 d	**3f** 81	**4f** 74	6:1	not further purified



Scheme 2 Synthesis of 20-*O*-linked camptothecin glycoconjugates

coupling reactions run in good yields and without any epimerization. Subsequent removal of the *tert*-butoxycarbonyl-protecting groups give the peptide conjugates **7A**–**G** ready for linkage to the carbohydrate moiety (table 2). The synthesis of *p*-aminophenyl 3-*O*-methyl-β-*L*-fucopyranoside has been described previously [12]. After transformation into the isothiocyanate **8** with thiophosgene the carbohydrate building block is linked to the appropriately protected peptide conjugates of camptothecin **7A**–**G**. Subsequent removal of the fluorenyl-9-methoxycarbonyl protecting group with piperidine, work up and treatment of the compounds **10A**–**G** with 1 equivalent of an aqueous hydrochloride solution gives the glycoconjugates **11A**–**G**. The hydrochlorides **11A**–**G** show good water solubility (>5mg/ml) and hydrolytic stability of the ester bond which depends on the side chain of the amino acid residue linked to camptothecin (table 2). Particularly, glycoconjugates **11B**–**E** with a bulky and β-branched amino acid linked to the 20-hydroxy group of 20(*S*)-camptothecin show high hydrolytic stability in aqueous solutions (c = 1mg/10ml; pH = 4–5) with less than 2.5% of 20(*S*)-camptothecin

Table 2 Synthesis of 20-*O*-linked camptothecin glycoconjugates

entry	educt	dipeptide conjugate: overall yield (%) (coupling and deprotection)			glycoconjugate: overall yield (%) (coupling, deprotection, HCl-salt transformation)			hydrolytic stability (over 24 h) [a]) ratio of peak areas
1	**4A**	H-L-Lys(Fmoc)-Gly-CPT	**7A**	73	3MeFuc-L-Lys(H)-Gly-CPT × HCl	**11A**	42	21/78
2	**4B**(***L***)	H-L-Lys(Fmoc)-L-Val-CPT	**7B**	87	3MeFuc-L-Lys(H)-L-Val-CPT × HCl	**11B**	68	2/94
3	**4C**(***D***)	H-L-Lys(Fmoc)-D-Val-CPT	**7C**	55	3MeFuc-L-Lys(H)-D-Val-CPT × HCl	**11C**	53	1/89
4	**4D**(***L***)	H-L-Lys(Fmoc)-L-Ile-CPT	**7D**	70	3MeFuc-L-Lys(H)-L-Ile-CPT × HCl	**11D**	48	2/81
5	**4D**(***D***)	H-L-Lys(Fmoc)-D-Ile-CPT	**7E**	70	3MeFuc-L-Lys(H)-D-Ile-CPT × HCl	**11E**	37	2/85
6	**4E**(***L***)	H-L-Lys(Fmoc)-L-Leu-CPT	**7F**	87	3MeFuc-L-Lys(H)-L-Leu-CPT × HCl	**11F**	60	6/84
7	**4E**(***D***)	H-L-Lys(Fmoc)-D-Leu-CPT	**7G**	93	3MeFuc-L-Lys(H)-D-Leu-CPT × HCl	**11G**	26	5/88

[a]) glycoconjugate hydrochlorides are dissolved in water (c = 1 mg/10 ml), pH 4–5, hydrolytic stability is assessed by HPLC analysis of the solution after 24 h, comparing the peak areas of cleaved camptothecin **1** and glycoconjugate **11**

cleaved within 24 h. In contrast, glycoconjugate **11A** with ester linked glycine is cleaved readily by hydrolysis.

These results provide a basis for the selection of appropriate spacer groups for delivery systems of 20(*S*)-camptothecin for therapeutic use.

We would like to thank Anna-Maria DiBetta, Andrea Felder and Dirk Wolter for their skilful technical assistance.

Experimental

HPLC-Method for Detection of Camptothecin-conjugates: For HPLC detection Waters Alliance 2690 equipment was used with a LiChrosphere 100 RP-18 (5µM) 250×4mm column (UV detection at 365nm). For eluation of the camptothecin amino acid conjugates a gradient was used with eluent A = 0.01 KH_2PO_4 in water and eluent B = 80% acetonitrile + 20% eluent A.

Preparation of 20-O-Amino Acid Derivatives of 20(S)-Camptothecin 4A–E

20-O-Glycyl-camptothecin trifluoroacetate (**4A**)

A suspension of 2 g (5.75 mmol) of 20(*S*)-camptothecin **1** in 100 ml of absolute dichloromethane is treated under stirring with 1.7 g (1.5 eq.) of *N*-(*tert*-butoxycarbonyl)-glycine-*N*-carboxyanhydride (**2a**) and 200 mg of DMAP. After heating under argon to reflux for 8 h, the mixture is concentrated *in vacuo* to a small volume. 100 ml of MTBE and 100 ml of petroleum ether are then added and the precipitate is filtered. The Boc-protected intermediate compound **3a** is obtained in quantitative yield. This Boc-protected intermediate **3a** is then stirred for 1 h in a mixture of 50 ml of dichloromethane and 3 ml of anhydrous trifluoroacetic acid at 5 °C. Stirring is continued at room temperature until the reaction is complete. After concentrating *in vacuo* to a small volume, the product is precipitated with 100 ml diethylether and filtered. The remaining residue is dissolved in dichloromethane/methanol 1:1 and again precipitated using diethylether. The precipitate is filtered off and 2206 mg (85%) of 20-*O*-Glycyl-camptothecin trifluoroacetate **4a** are obtained after drying. – TLC (acetonitrile/water/glacial acetic acid 5:1:0.2): R_f = 0.44. – ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.05 and 4.15 (2*d*, *J* = 18 Hz, 2×1H, CH_2 Gly), 7,4 (*s*, 1H, CPT D-ring), 7.74(*dd*, *J* = 8 Hz, 1H, CPT A-ring), 7.90 (*dd*, *J* = 8 Hz, 1H, CPT A-ring), 8.08 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.18 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.63 (*s*, 1H, CPT B-ring).

20-O-Valyl-camptothecin trifluoroacetate (**4B(L)**) (coupling reaction in dichloromethane)

A suspension of 8 g (23 mmol) of 20(*S*)-camptothecin **1** in 400 ml of absolute dichloromethane is treated under stirring with 11.2 g (2 eq.) of *N*-(*tert*-butoxycarbonyl)-*L*-valine-*N*-carboxyanhydride (**2b**) and 800 mg of DMAP. After heating under argon to reflux for 4 days, the mixture is concentrated *in vacuo* to a small volume. 150 ml of MTBE are added and the mixture is stirred for 20 min. 300 ml of petroleum ether are then added and the mixture is filtered. The Boc-protected intermediate compound **3b** is obtained in quantitative yield. It contains small amounts of the *D*-valine epimer which, however, can be removed without problems after removal of the protecting group. 12.6 g of this Boc-protected intermediate compound **3b** are then stirred for 1 h in a mixture of 300 ml of dichloromethane and 18 ml of anhydrous trifluoroacetic acid at 5 °C. Stirring is continued overnight at room temperature. After concentrating *in vacuo* to a small volume, the product is precipitated with 300 ml MTBE and filtered. The epimeric ratio *L*-valine : *D*-valine epimer in the crude product is approximately 20:1 as assessed from ^{1}H NMR. The NMR spectra of both epimers differ by slight low field chemical shifts of some CPT protons and particularly of the α-CH of valine in case of the *D*-epimer compared to the proton shifts of the respective *L*-epimer. Convenient NMR-signals to estimate the epimeric ratio from the peak areas are the signals of the α-CH proton and the two singlets in the CPT system.
For further purification of the *L*-valine epimer **4B(*L*)** the crude product is again precipitated from 160 ml methanol using 600 ml MTBE. The precipitate is filtered off and 10.06 g (78%) of 20-*O*-Valyl-camptothecin trifluoroacetate **4B(*L*)** are obtained after drying. – TLC (acetonitrile/water 10:1): R_f = 0.31. – Epimeric ratio: *L*:*D* > 30:1 (according to HPLC and ^{1}H NMR). – ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.06 (*d*, *J* = 4 Hz, 1H, α-CH Val), 7,38 (*s*, 1H, CPT D-ring), 7.73 (*dd*, *J* = 7Hz, 1H, CPT A-ring), 7.89 (*dd*, *J* = 7Hz, 1H, CPT A-ring), 8.06 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.18 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.61 (*s*, 1H, CPT B-ring).

20-O-(D-Valyl)-camptothecin trifluoroacetate (**4C(D)**) (coupling reaction in dichloromethane)

If the coupling reaction is performed analogously in dichloromethane starting with 3 equivalents of Boc-D-Val-NCA **2c**, an epimeric mixture is obtained after deprotection and precipitation with MTBE, which can be detected in TLC, ^{1}H NMR and in RP-HPLC. The overall yield (coupling and deprotection) of the epimeric mixture is 81% in this case. The ratio of *D*-valine : *L*-valine epimer is 2:1 as assessed from NMR. – TLC (acetonitrile/water 20:1): R_f (*L*-epimer): = 0.29; R_f (*D*-epimer): = 0.24. The epimeric mixture is separated by precipitation from 300 ml dichloromethane/methanol 10:1 with 150 ml diethylether. Repeating this process twice leads to the isolation of the *D*-epimer **4C(*D*)** in a yield of 19% and in excellent purity (*D*:*L* > 40:1). – ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.21 (*d*, *J* = 4 Hz, 1H, α-CH D-Val), 7,40 (*s*, 1H, CPT D-ring), 7.73(*dd*, *J* = 7 Hz, 1H, CPT A-ring), 7.89 (*dd*, *J* = 7 Hz, 1H, CPT A-ring), 8.06 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.18 (*d*, *J* = 8Hz, 1H, CPT A-ring), 8.63 (*s*, 1H, CPT B-ring).

20-O-(D-Valyl)-camptothecin trifluoroacetate (**4C(D)**) (coupling reaction in DMF)

A suspension of 1 g (2.87 mmol) of 20(*S*)-camptothecin **1** in 50 ml of DMF is treated under sonification with 1.4 mg (2 eq.) of *N*-(*tert*-butoxycarbonyl)-*D*-valine-*N*-carboxyanhydride **2c** and 100 mg of DMAP for 2.5 days. Additional 350 mg (0.5 eq) of **2c** and 50 mg of DMAP are added and sonification is continued for 1.5 days. Despite the fact that the reaction is still incomplete it is stopped at this time. The mixture

is concentrated *in vacuo* and the residue is purified by flash chromatography on silica gel using ethylacetate/petroleum ether 3:1 as eluent. Relevant fractions are concentrated and precipitated using MTBE. 769 mg (49%) of the protected intermediate are obtained, however, they are still contaminated with Boc-Valine which can be removed after deprotection. The *tert*-butoxycarbonyl protecting group is removed as described above. The product is precipitated with diethyl ether. The precipitate is filtered off and 290 mg (37%) of 20-*O*-(*D*-Valyl)-camptothecin trifluoroacetate **4C**(***D***) are obtained after drying. – TLC (acetonitrile/water 20:1): R_f = 0.24. – Epimeric ratio: $D:L \sim 20:1$ (based on ^{1}H NMR).

20-O-Isoleucyl-camptothecin trifluoroacetate (**4D**(**L**)) (coupling reaction in dichloromethane)

A suspension of 1 g (2.9 mmol) of 20(*S*)-camptothecin **1** in 50 ml of absolute dichloromethane is treated under stirring with 1.55 g (2 eq.) of *N*-(*tert*-butoxycarbonyl)-isoleucine-*N*-carboxyanhydride (**2d**) and 100 mg of DMAP. After heating under argon to reflux for 2 days, the mixture is concentrated *in vacuo* to a small volume. 50 ml of diethyl ether and 50 ml petroleum ether are added, the mixture is stirred for 20 min and then filtered. The residue is purified by flash chromatography on silica gel using acetonitrile as eluent. The Boc-protected intermediate compound **3d** is obtained in a yield of 1.5 g (92%). The *tert*-butoxycarbonyl protecting group is removed as described above. The product is precipitated with diethyl ether and filtered. 1.48 g (98%) of 20-*O*-*L*-isoleucyl-camptothecin trifluoroacetate **4D**(***L***) are obtained after drying. – TLC (acetonitrile/water 20:1): R_f = 0.36. – Epimeric ratio: $L:D$ = 25:1 (according to ^{1}H NMR). – ^{1}H NMR (400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.16 (*d*, *J* = 3.5 Hz, 1H, α-CH L-Ile), 7.33 (*s*, 1H, CPT D-ring), 7.73(*dd*, *J* = 8 Hz, 1H, CPT A-ring), 7.89 (*dd*, *J* = 7 Hz, 1H, CPT A-ring), 8.07 (*d*, *J* = 8 Hz, 1H, CPT A-ring),), 8.18 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.61 (*s*, 1H, CPT B-ring).

20-O-(D-Isoleucyl)-camptothecin trifluoroacetate (**4D**(**D**)) (coupling reaction in DMF)

A suspension of 1 g (2.87 mmol) of 20(*S*)-camptothecin **1** in 50 ml of DMF is treated under sonification with 1.55 g (2 eq.) of *N*-(*tert*-butoxycarbonyl)-isoleucine-*N*-carboxyanhydride (**2d**) and 100 mg of DMAP for 2 days. Additional 773 mg (1 eq) of **2d** and 100 mg of DMAP are added and sonification is continued for 2 days. The mixture is concentrated *in vacuo* and the residue is purified by flash chromatography at silica gel using acetonitrile as eluent. Relevant fractions are concentrated and precipitated using diethyl ether. 1112 mg (68%) of the protected intermediate are obtained. The *tert*-butoxycarbonyl protecting group is removed as described above. The product is precipitated with diethyl ether and filtered. 1028 mg (97%) of an epimeric mixture of 20-*O*-isoleucyl-camptothecin trifluoroacetate and of 20-*O*-(*D*-isoleucyl)-camptothecin trifluoroacetate (**4d**) are obtained after drying. – TLC (acetonitrile/water 20:1): R_f = 0.36 and 0.33 resp. – Epimeric ratio: $D:L \sim 1:1.5$ (based on ^{1}H NMR).
For separation of the epimers the epimeric mixture is stirred with 70 ml of dichloromethane for 4 h and filtered. The polar epimer is enriched in the precipitate whereas the non polar epimer is dissolved in the organic phase. Subsequently, the filter residue is again dissolved in dichloromethane/methanol and precipitated with diethyl ether. The precipitating polar *D*-epimer **4D**(***D***) is isolated in a yield of 397 mg (26% over 2 steps; epimeric ratio $D:L$ = 13:1) and from the mother liquids the non polar L-epimer **4D**(***L***) is isolated in a yield of 517 mg (33% over 2 steps; epimeric ratio $D:L$ = 1:16). The assignment of the epimers is based on the comparison with product **4D**(***L***) isolated after the coupling in dichloromethane and subsequent deprotection. – ^{1}H NMR (polar *D*-epimer **4D**(***D***); 400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.29 (*d*, *J* = 4 Hz, 1H, α-CH D-Ile), 7,40 (*s*, 1H, CPT D-ring), 7.74(*dd*, *J* = 7 Hz, 1H, CPT A-ring), 7.90 (*dd*, *J* = 7 Hz, 1H, CPT A-ring), 8.08 (*d*, *J* = 8 Hz, 1H, CPT A-ring),), 8.18 (*d*, *J* = 8 Hz, 1H, CPT A-ring), 8.62 (*s*, 1H, CPT B-ring).

20-O-Leucyl-camptothecin trifluoroacetate (**4E**(**L**)) and *20-O-(D-Leucyl)-camptothecin trifluoroacetate* (**4E**(**D**)) (coupling reaction in dichloromethane starting from Boc-Leu-NCA **2e**)

A suspension of 20 g (57.4 mmol) of 20(*S*)-camptothecin **1** in 1 ltr. of absolute dichloromethane is treated under stirring with 29.5 g (2 eq.) of *N*-(*tert*-butoxycarbonyl)-leucine-*N*-carboxyanhydride (**2e**) and 2 g of DMAP. After heating under argon to reflux for 4 days, the mixture is concentrated *in vacuo* to a small volume. The crude product is purified by flash chromatography at silica gel using petroleum ether/ethylacetate 1:1 – > 1:3. Relevant fractions are collected, concentrated and dried *in vacuo*. 31.1 g of the Boc-protected intermediate compound **3e** are obtained (97%). The *tert*-butoxycarbonyl protecting group is removed as described above. The crude product is precipitated from dichloromethane/methanol using diethyl ether and isolated by filtration. It contains the *D*-leucine epimer – TLC (acetonitrile/water 20:1): R_f = 0.43 and 0.37 resp. The epimeric ratio *L*-leucine : *D*-leucine epimer is approximately 2:1 as assessed from ^{1}H NMR.
For separation of the *L*-leucine epimer **4E**(***L***) and the *D*-leucine epimer **4E**(***D***) the crude product is again precipitated from dichloromethane/methanol 1:1 using diethylether. The non polar epimer is enriched in the mother liquid whereas the precipitate is a mixture with predominantly polar epimer. The process is repeated until the separation is complete. Relevant fractions of each epimer are collected and finally 20-*O*-leucyl-camptothecin trifluoroacetate **4E**(***L***) is obtained from the mother liquids in an overall yield of 32% and an epimeric ratio $L:D$ = 13:1. The polar *D*-epimer 20-*O*-(*D*-leucyl)-camptothecin trifluoroacetate **4E**(***D***) is obtained from the precipitate in an overall yield of 27%; epimeric ratio $D:L$ > 40:1. The assignment of the *D*- and *L*-configuration to the polar and non polar epimers is based on an X-ray structure of the acetylated product derived from the polar **4E**(***D***) fraction indicating the *D*-configuration of leucine [14]. – ^{1}H NMR of **4D**(***L***) (400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.19 (d*d*, *J* = 8 Hz, *J* = 7 Hz, 1H, α-CH Leu), 7,32 (*s*, 1H, CPT D-ring), 7.73(*dd*, *J* = 7 Hz, 1H, CPT A-ring), 7.89 (*dd*, *J* = 7 Hz, 1H, CPT A-ring), 8.07 (*d*, *J* = 8 Hz, 1H, CPT A-ring),), 8.18 (*d*, *J* = 7 Hz, 1H, CPT A-ring), 8.61 (*s*, 1H, CPT B-ring). – ^{1}H-NMR of **4D**(***D***) (400 MHz; CD_3OD/CD_2Cl_2 1:1): δ/ppm = 4.26 (d*d*, *J* = 7 Hz, *J* = 7 Hz, 1H, α-CH Leu), 7,4 (*s*, 1H, CPT D-ring), 7.73(*dd*, *J* = 7 Hz, 1H, CPT A-ring), 7.89 (*dd*, *J* = 7 Hz, 1H, CPT A-ring), 8.07 (*d*, *J* = 8 Hz, 1H, CPT A-

ring), 8.18 (*d*, *J* = 8.5 Hz, 1H, CPT A-ring), 8.62 (*s*, 1H, CPT B-ring).

20-O-(D-Leucyl)-camptothecin trifluoroacetate (**4E**(**D**)) (coupling reaction in DMF starting from Boc-Leu-NCA **2e**)

The coupling of 20 g Boc-Leu-NCA **2e** to 20(*S*)-camptothecin in DMF runs smoothly within 1 day in the same way as described above and the protected intermediate **3e** is isolated in 98% yield. Subsequent deprotection is performed in the same way as described above. After the first precipitation from the reaction mixture an epimeric mixture with predominant formation of the *D*-epimer (yield crude product: 89%; epimeric ratio *L*:*D* = 1:3) is obtained. Straightforward purification of the predominantly formed *D*-epimer **4E**(***D***) is achieved by precipitation from dichloromethane/methanol using diethyl ether. After two precipitations **4E**(***D***) is isolated in an overall yield of 57% and an epimeric ratio of *D*:*L* = 15:1.

20-O-Leucyl-camptothecin trifluoroacetate (**4E**(**L**)) (coupling reaction in dioxane starting from Boc-Leu-NCA **2e**)

If the coupling reaction is performed in dioxane at 40 °C the reaction mixture remains inhomogeneous and proceeds very slowly when starting with 2 equivalents of Boc-Leu-NCA **2e**. After 5 days the reaction mixture is filtered and the product is purified by flash chromatography as described above. The protected intermediate is obtained in a yield of 12%. Subsequent deprotection gives predominantly the *L*-epimer **4E**(***L***) in an overall yield of 7% (epimeric ratio *L*:*D* = 16:1).

20-O-Leucyl-camptothecin trifluoroacetate (**4E**(**L**)) and *20-O-(D-leucyl)-camptothecin trifluoroacetate* (**4E(D)**) (coupling reaction in dichloromethane starting from Boc-D-Leu-NCA **2f**)

The coupling in dichloromethane and the subsequent deprotection reaction is performed in the same way as described for the coupling of Boc-Leu-NCA **2e**. Both the coupling and deprotection reactions run in good yields with 91% and 81% resp., however, after the first precipitation an epimeric mixture of 1:1 is obtained which has not been purified further.

20-O-Leucyl-camptothecin trifluoroacetate (**4E**(**L**)) and *20-O-(D-leucyl)-camptothecin trifluoroacetate* (**4E**(**D**)) (coupling reaction in DMF starting from Boc-D-Leu-NCA **2f**)

Again both the coupling reaction in DMF and the deprotection run in good yields with 81% and 74% resp. In this case, after the first precipitation an epimeric mixture of 6:1 with predominant retain of the *D*-configuration is obtained which has not been purified further.

Preparation of Fmoc-protected Dipeptide Conjugates of 20(S)-Camptothecin 7A–G (General Procedure)

0.047 mol of N^{α}-(*tert*-butoxycarbonyl)-N^{ε}-(fluorenyl-9-methoxycarbonyl)-lysine (**5**) [Boc-Lys(Fmoc)-OH] are dissolved in 800 ml of anhydrous DMF and cooled down to 0 °C. 0.07 mol of 1-hydroxybenzotriazole (HOBT) and 0.056 mol of *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide, hydrochloride (EDCI) are added and the mixture is stirred for 30 min at 0 °C. Subsequently, 0.039 mol of the trifluoroacetate of a 20-hydroxy-linked amino acid conjugate of camptothecin **4** and finally 24,3 ml *N*-ethyl diisopropylamine are added. The mixture is stirred for 16 h at ambient temperature. The solvent is evaporated at 25 °C. After addition of 1.5 ltr. of water the residue is stirred for 15 min and solidifies. The precipitate is filtered and washed with water. It is dissolved in 800 ml of dichloromethane and remaining water is removed in a separation funnel. The organic layer is concentrated to 150 ml. This solution is dropped to 2 ltr. of MTBE at 0 °C during stirring. The precipitate is filtered, washed with MTBE and dried *in vacuo*. If required, purification by flashchromatography using acetonitrile as eluent is also possible. The orthogonally protected intermediate compound from series **6A**–**G** is dissolved in 600 ml of dichloromethane and the solution is cooled down to 5 °C. 200 ml trifluoroacetic acid are added during stirring. The reaction mixture is allowed to warm up to ambient temperature and is stirred for additional 2 h. The solvent and trifluoroacetic acid are evaporated *in vacuo* at 25 °C bath temperature. MTBE is added to the residue and the mixture is stirred for 10 min. The precipitate is filtered, washed with MTBE and dried *in vacuo* over night. The side chain protected dipeptide conjugates **7A**–**G** are obtained in high purity. The yields for each particular conjugate are listed in table 2.

p-Isothiocyanatophenyl 3-O-methyl-β-L-fucopyranoside (**8**)

0.069 mol of *p*-aminophenyl 3-*O*-methyl-*β*-*L*-fucopyranoside [12] are dissolved in 1.8 ltr. dioxane/water 1:1 (v/v). 7.4 ml (0.096 mol) thiophosgene are added and the mixture is stirred for 10 min at ambient temperature. 71 ml (0.4 mol) *N*-ethyl diisopropylamine are added and the mixture is stirred for additional 15 min. The dioxane is evaporated in vacuo at a bath temperature of max. 25 °C. The aqueous layer is extracted twice with 500 ml dichloromethane. The collected dichloromethane layers are washed twice with 500 ml of water, dried upon sodium sulfate and evaporated. The residue is stirred with 250 ml MTBE. After 10 min 500 ml petrolether are added and the mixture is stirred for additional 10 min. The precipitate is filtered, washed with petrolether dried *in vacuo*. 17.65 g (83%) of **8** are obtained. – TLC (acetonitrile/water 10:1): R_f = 0.7.

Preparation of Glycoconjugate Hydrochlorides of 20(S)-Camptothecin 11A–G (General Procedure)

0.0275 mol of a side chain protected camptothecin dipeptide conjugate **7A**–**G** are dissolved in 1 ltr. anhydrous DMF and cooled down to 0 °C. 18,9 ml (0.11 mol) *N*-ethyl diisopropylamine and 9.4g (0.03 mol) *p*-isothiocyanatophenyl 3-*O*-methyl-*β*-*L*-fucopyranoside (**8**) are added. After stirring for 2 h the reaction mixture is allowed to warm up to room temperature and stirring is continued for 16 h. After evaporating the solvent *in vacuo* at 25 °C, 1.5 ltr. water are added to the residue and the mixture is stirred for 30 min. During this procedure the product solidifies. The precipitate is filtered, washed with water and dried *in vacuo*. If required, the crude product is purified by flash chromatography at silicagel with acetonitrile/water 30:1. The corresponding fractions are collected, concentrated and dissolved in 300 ml dichloromethane. Methanol is added dropwise until complete solution occurs. This solution is added to 1.5 ltr. MTBE and stirred for

30 min. The Fmoc-protected glycoconjugates **9A–G** precipitate and are filtered, washed with MTBE and dried *in vacuo*. For removal of the Fmoc-group 0.021 mol of the Fmoc-protected intermediates **9A–G** are dissolved in 700 ml DMF, the solution is cooled down to 0 °C and 36 ml piperidine are added. The reaction mixture is allowed to warm up to room temperature and stirring is continued for 1 h. The solvent is evaporated and the residue is dissolved in 200 ml dichloromethane and precipitated again with 600 ml MTBE. The precipitate is collected by filtration, dissolved in 400 ml methanol and stirred for 1 h. 400 ml MTBE are added and stirring is continued for 30 min. The precipitating glycoconjugates **10A–G** are filtered, washed with MTBE and then dried *in vacuo*. For transformation into the corresponding hydrochlorides 0.0164 mol of compounds **10A–G** are suspended in 1.4 ltr. distilled water. 0.0164 mol of a 1 M hydrochloric acid are added in 1-ml-portions. The mixture is sonificated for 30 min. The obtained solution is lyophilized in portions of 200 ml and the glycoconjugate hydrochlorides **11 A–G** are obtained as white to yellowish powders. The overall yields of carbohydrate attachment, deprotection and transformation into hydrochlorides are given in table 2.

Investigation of Hydrolytic Stability of the Camptothecin Glycoconjugates 11A–G in Aqueous Solutions

The glycoconjugate hydrochlorides **11A–G** are dissolved in water in a concentration of 1 mg/10 ml water (pH 4–5). The hydrolytic release of 20(*S*)-camptothecin is detected by HPLC analysis of the solutions after 24 h using an RP18 (5 µm) column with 70% $HClO_4$/water (0.4% v/v) as eluent A and acetonitrile as eluent B (UV detection at 365nm). The relative peak areas of cleaved camptothecin versus glycoconjugate are assessed. The results are summarized in table 2.

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