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Novel 20-Carbonate Linked Prodrugs of Camptothecin and 9-Aminocamptothecin Designed for Activation by Tumour-Associated Plasmin

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Abstract—The first prodrugs of camptothecin and 9-aminocamptothecin that are activated by the tumour-associated protease plasmin are reported. The tripartate prodrugs consist of a tripeptide sequence recognised by plasmin, which is linked to the 20-hydroxyl group of the camptothecins via a 1,6-elimination spacer. After selective *N*-protection of 9-aminocamptothecin with an Aloc group, the promoiety (tripeptide–spacer conjugate) was linked to camptothecin or 9-Aloc-9-aminocamptothecin via a 20-carbonate linkage by reacting parent drugs with the *p*-nitrophenyl carbonate activated promoiety in the presence of DMAP. Both prodrugs showed to be stable in buffer solution and both parent drugs were released upon incubation in the presence of plasmin. Furthermore, the prodrugs showed an average 10-fold decreased cytotoxicity with respect to their parent drugs upon incubation in seven human tumour cell lines.

The camptothecins (CPTs) belong to a relatively new class of promising anticancer agents (Fig. 1).[1] Two CPT derivatives, topotecan (**3**) and irinotecan (**4**; CPT-11) have been clinically approved for treatment of ovarian and colon cancer, respectively.[2] Several other CPT derivatives, such as 9-nitrocamptothecin (9-NCPT) and 9-aminocamptothecin (**2**; 9-ACPT) are currently clinically tested. Both CPT (**1**) and 9-ACPT (**2**) have shown outstanding preclinical effectiveness.[3,4] CPTs inhibit topoisomerase I, an enzyme that breaks and reseals single strand DNA during the DNA replication process in the nucleus.[5–7] An advantage of topoisomerase I as a target is that it is expressed continuously during the cell cycle and in quiescent cells in slowly growing and drug resistant tumours.[5] Therefore, CPTs represent one of the most important classes of anticancer drugs introduced into clinical practice.[6]

Both poor water-solubility[8] and high toxicity of CPTs have restricted their clinical development and therefore these compounds are suitable for incorporation as parent drugs in a prodrug approach.[9,10] Via the prodrug concept, CPT derivatives may be obtained that show both improved tumour-selectivity and water-solubility.

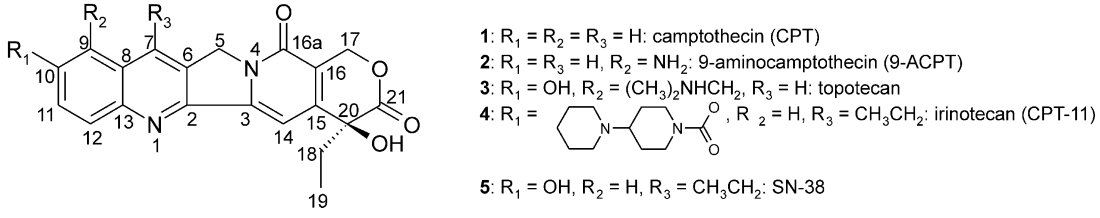

Figure 1. CPT and derivatives.

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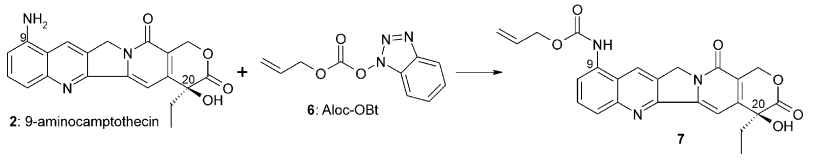


Scheme 1. Selective Aloc-protection of the 9-amino function of 9-ACPT.

The clinically approved CPT-11 (**4**) is in fact a relatively inactive and water-soluble prodrug: after carboxyl esterase cleavage of the dipiperidino side chain at the 10-position, the active metabolite SN-38 (**5**) is formed. Also 9-nitrocamptothecin is a prodrug; it is believed to be reduced in vivo to generate the active 9-ACPT **2**.[11] 9-ACPT has reached phase II clinical trials for several cancer types, such as non-small cell lung cancer, head and neck cancer, breast cancer, and colorectal carcinoma.[12]

In this paper, we report the design and synthesis of prodrugs of CPT and 9-ACPT that are activated by the tumour-associated protease plasmin.[13] Plasmin is a serine protease that is formed upon cleavage of plasminogen by urokinase-type plasminogen activator (u-PA), a protein associated with tumour invasion and metastasis.[14] The first plasmin-activated prodrugs were reported in the 1980s,[15] whereas spacer-containing prodrugs of doxorubicin and paclitaxel that are activated by plasmin have been recently reported by us.[16,17] By derivatisation with a peptide containing two free amino functions, the water-solubility of the plasmin-activated CPT prodrug is expected to be greatly improved, as was observed for peptide prodrugs of paclitaxel.[17]

Both CPT and 9-ACPT possess a hydroxyl group suitable for derivatisation, whereas 9-ACPT contains an additional 9-amino function that can be used for attachment of the promoiety (=part of prodrug coupled to the parent drug). A prodrug of 9-ACPT in which a β-glucuronide–spacer conjugate was connected to the 9-amino group via a carbamate linkage has been reported.[18] Recently, polymeric 9-amide linked 9-ACPT conjugates have been reported that were triggered by consecutive azoreductase and peptidase activation.[19]

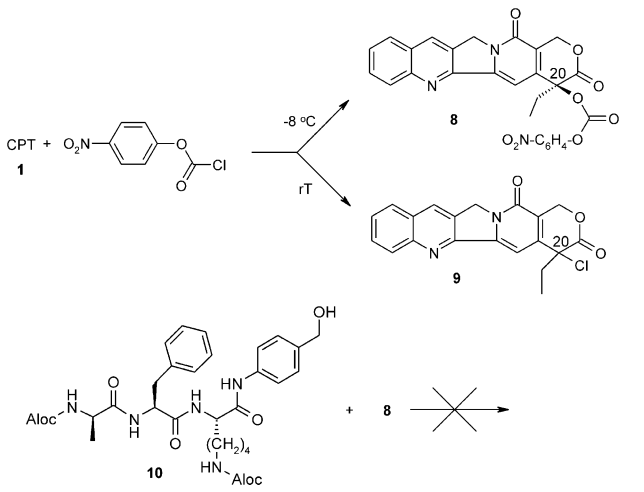
The 20*S*-hydroxyl and 21-lactone moieties in the E-ring of CPTs are essential for biological activity.[20,21] Both groups have been suggested to interact with topoisomerase I.[22] The free 20-hydroxyl group is presumed to hydrogen bond with Asp^{533} from topoisomerase I.[22] For this reason, CPT prodrugs coupled at the 20-position are expected to exert decreased cytotoxicity. It should be considered that under physiological conditions a pH-dependent equilibrium exists between the E-ring lactone and the opened lactone derivative, the carboxylate form.[2] The carboxylate possesses only one tenth of the activity of the closed lactone analogue. The lactone ring of 9-ACPT is even more labile in comparison with the CPT lactone and will readily open to the inactive carboxylate form.[11,23] Rapid opening of the lactone ring is regarded as a crucial obstacle to improving the effectiveness of CPTs.[24] It has been hypothesised that a free 20-hydroxyl group may facilitate the opening reaction of the E-ring lactone by stabilisation of its transition state through intramolecular hydrogen bonding.[25] For this reason, acylation of the 20-hydroxyl group will render the lactone more stable towards ring opening.[26] This may increase the availability of active drug that contains the intact lactone. Therefore, 20-acyl prodrugs of CPTs are not only expected to demonstrate decreased cytotoxicity, but they are also expected to contain a stabilised E-ring lactone functionality. A 9-ACPT prodrug linked via its 20-position may have as an additional advantage that the free 9-amino function can contribute to the water-solubility of the prodrug.

Most CPT prodrugs functionalised at the 20-hydroxyl group that have been published until now contain a 20-ester linkage between promoiety and drug.[27–29] Although the promoieties in these prodrugs are connected to a hindered tertiary alcohol, an ester may be hydrolytically unstable[30] or it may be susceptible in vivo to ubiquitous esterase cleavage, depending on the specific nature of the ester.[28,31] Therefore, a carbonate or carbamate linkage would be more desirable for a targeted prodrug. It was reasoned that incorporation of a self-elimination spacer would enhance enzymatic activation and thus the efficiency of drug release,[32] and it was considered that a 1,6-elimination spacer[33] could be coupled to the 20-hydroxyl function via a carbonate linkage.

We now report the design, synthesis and preliminary biological evaluation of CPT-20-carbonate and 9-ACPT-20-carbonate prodrugs that are activated by tumour-associated plasmin. The prodrugs have been biologically characterised by (i) incubation in a buffer solution to determine their stability, (ii) incubation in the presence of plasmin to confirm prodrug activation by plasmin, and (iii) determination of their cytotoxicity in seven human tumour cell lines.

In order to synthesize a 9-ACPT-20-carbonate prodrug, it is necessary to protect the 9-amino function, as this nucleophilic position is more reactive than the 20-hydroxyl group.[18] Initial attempts to protect the 9-amino group with a fluorenyl methoxycarbonyl group (Fmoc) using Fmoc-carbonate derivatives of hydroxysuccinimide, pentafluorophenol, and hydroxybenzotriazole, were unsuccessful. Also a reaction of 9-ACPT with Fmoc-chloride did not yield 9-Fmoc-9-ACPT. Protection of 9-ACPT with a *t*-butyl oxycarbonyl group (Boc) employing di-*t*-butyl pyrocarbonate failed as well.

It was subsequently tried to selectively protect the amino function of 9-ACPT with an allyl oxycarbonyl group (Aloc). Reacting 9-ACPT with Aloc-carbonate derivatives of *p*-nitrophenol or hydroxysuccinimide did not afford any products. When 9-ACPT was reacted with diallyl pyrocarbonate ($Aloc_2O$) in *N*,*N*-dimethylformamide



Scheme 2. Attempt to synthesize a CPT prodrug by employing an activated CPT-20-nitrophenyl carbonate.

(DMF), the desired product was formed in a low yield. When the reaction was carried out under reflux conditions, the doubly protected 9,20-bis-aloc-9-ACPT was obtained. A higher yield was obtained using allyl-1-benzotriazolyl carbonate (**6**; Aloc-OBt) employing pyridine as a base at 6 °C (Scheme 1), affording the desired product **7** in 8 days in a yield of 87%. This protection proceeded much faster (within 1 day) when it was performed at 80 °C, affording the desired product **7** in a slightly lower yield (80%).[34]

With the protected 9-ACPT derivative in hand, two strategies could be followed to establish the linkage between the promoiety and the parent drug: (i) activation of the drug, followed by nucleophilic attack from the alcohol function of the specifier–spacer conjugate, or (ii) a nucleophilic attack from the sterically hindered and deactivated tertiary 20-hydroxyl group of the drug at an activated specifier–spacer conjugate.

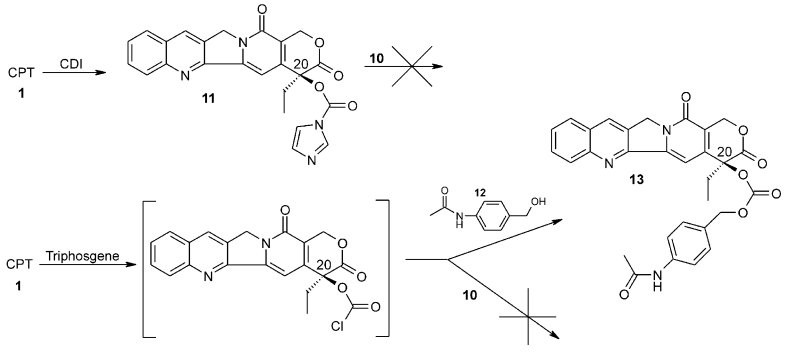
To explore the first strategy, CPT was activated with *p*-nitrophenyl chloroformate to yield the *p*-nitrophenyl carbonate derivative of CPT (**8**, Scheme 2). However, at room temperature this reaction led to formation of 20-deoxy-20-chloro-CPT (**9**, Scheme 2), a CPT derivative previously reported to lack biological activity.[1,35] When the activation was performed at −8 °C, the desired CPT-20-nitrophenyl carbonate **8** was formed. Carbonate **8** was reluctant to react with the alcohol function of the tripeptide–spacer conjugate (Aloc-D-Ala-Phe-Lys(Aloc)-PABA, **10**; PABA = *p*-aminobenzyl alcohol; Scheme 2).[16]

CPT was also activated with carbonyldiimidazole (CDI) to afford the CDI activated CPT derivative **11** (Scheme 3). The alcohol function of tripeptide–spacer promoiety **10** did not react with the activated CPT **11**.

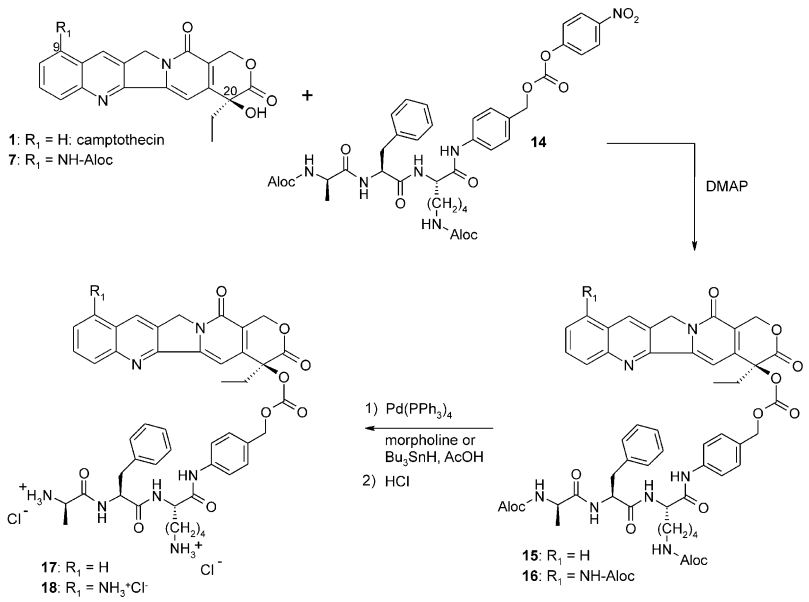
In another attempt to explore the first strategy, CPT was activated with triphosgene in the presence of base to generate in situ the highly reactive intermediate chloroformate.[26] Polyethylene glycol functionalised with a hydroxyl or amino function for example has been reported to react readily with this CPT chloroformate.[26] Although a model alcohol (Ac-PABA, **12**, Scheme 3) was able to react with the CPT-20-chloroformate to yield Ac-PABC-CPT **13** (Scheme 3; PABC = *p*-aminobenzyl oxycarbonyl), the tripeptide–spacer conjugate **10** was reluctant to form the desired carbonate linkage under similar conditions.

Then the second strategy was explored by reacting the parent drug with an activated specifier–spacer conjugate (Scheme 4). CPT and 9-Aloc-9-ACPT were reacted with the *p*-nitrophenyl carbonate activated tripeptide–spacer conjugate **14**.[16] The coupling reaction proceeded only in the presence of the acylation catalyst 4-(dimethylamino)pyridine (DMAP) to give the desired protected prodrugs **15** and **16** in a yield of approximately 15% (non-optimised).[36] Deprotection of the two Aloc groups using tributyltin hydride,[37] acetic acid and catalytic $Pd(PPh_3)_4$ in the case of **15**, followed by addition of hydrochloric acid afforded the desired CPT prodrug **17**.[38] Use of the palladium catalyst with morpholine for deprotection of the three Aloc groups of **16** resulted in the formation of 9-ACPT prodrug **18**.[38]

Both prodrugs were incubated at concentrations of 100 μM in 0.1 M Tris–hydrochloric acid buffer (pH 7.3) for 24 h and showed no parent drug formation (TLC; RP_{18}; acetonitrile–water–acetic acid: 19:19:2). Enzymatic hydrolysis of prodrugs **17** and **18** was investigated by incubation for 24 h at a concentration of 100 μM in 0.1 M Tris–hydrochloric acid buffer (pH 7.3) in the presence of 100 μg/mL human plasmin (Fluka), and both prodrugs were converted to the corresponding parent drugs by plasmin (TLC; RP_{18}; acetonitrile–water–acetic acid: 19:19:2). Furthermore, the in vitro cytotoxicity of



Scheme 3. Attempt to synthesize a CPT prodrug by employing an imidazolyl activated CPT or a CPT-20-chloroformate.



Scheme 4. Synthesis of 20-carbonate prodrugs of CPT and 9-ACPT.

Table 1. Cytotoxicity [ID_{50} values[a],[b] (ng/mL)] of CPTs **1** and **2**, and CPT- and 9-ACPT-20-carbonate prodrugs **17** and **18** in seven human tumour cell lines[40]

	MCF-7	EVSA-T	WIDR	IGROV	M19	A498	H226
CPT **1**	<3.2	<3.2	<3.2	<3.2	13	<3.2	5
9-ACPT **2**	<3.2	<3.2	<3.2	<3.2	24	10	7
Prodrug **17**	10	49	22	20	219	22	111
Prodrug **18**	19	137	7	8	10	39	21

[a]Drug dose that inhibited cell growth by 50% compared to untreated control cultures.
[b]SRB cell viability test.

prodrugs **17** and **18** was determined in seven human tumour cell lines (Table 1).[39]

CPT prodrug **17** showed on the average 11-fold decreased cytotoxicity upon incubation for 5 days in comparison with the parent drug CPT, whereas 9-ACPT prodrug **18** showed an average 9-fold decreased toxicity when compared with 9-ACPT.

Discussion and Conclusions

Plasmin-activated 20-carbonate prodrugs of CPT and 9-ACPT were synthesised and subjected to primary biological characterisation. A new route to synthesise prodrugs of CPTs linked via a carbonate function to their 20-hydroxyl group was found. The coupling of the promoiety to the 20-hydroxyl functionality via a carbonate linkage appeared to be the key step in the synthesis of the prodrugs. The promoiety was reluctant to react with *p*-nitrophenyl carbonate-, chloroformate-, or CDI-activated CPT derivatives. The alcohol function of the tripeptide–spacer conjugate Aloc-D-Ala-Phe-Lys(Aloc)-PABA (**10**) appears to possess only modest reactivity. We have previously experienced similar problems,[32] possibly indicating steric hindrance imposed by the peptide portion of the molecule. This hypothesis is supported by the observation that model alcohol **12** did couple to the chloroformate-activated CPT to yield model prodrug **13**.

The second approach, in which CPT or its protected amino derivative was reacted with the activated promoiety in the presence of DMAP, yielded the desired protected carbonate prodrugs **15** and **16**.

The selectively protected 9-Aloc-9-ACPT derivative **7** was synthesised. We previously reported the catalytic properties of HOBt for the construction of *N*-aryl carbamates through reacting an aromatic amine with a *p*-nitrophenyl carbonate in the presence of HOBt.[32] A HOBt-carbonate is presumably the reactive intermediate.[41] HOBt-carbonates appear to be useful reagents or intermediates for establishing *N*-aryl carbamate linkages.

Prodrugs **17** and **18** showed an average 11-fold and 9-fold decreased cytotoxicity, respectively, when compared with the corresponding free parent drugs in seven human tumour cell lines. Surprisingly, in one cell line (M19) the 9-ACPT prodrug shows a higher cytotoxicity than the corresponding parent drug, an observation which deserves further investigation. Overall, both prodrugs fulfil to the requirement of decreased prodrug toxicity. When it is considered that both CPT and 9-ACPT prodrugs show in vitro stability, release parent drug upon incubation with plasmin, and exhibit decreased in vitro cytotoxicity with respect to the parent compounds CPT and 9-ACPT, it can be concluded that the 20-carbonate linked prodrugs presented herein are promising candidates for further evaluation.

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34. Procedure for the synthesis of 9-Aloc-9-ACPT (**7**): To a heated solution (80 °C) of 250 mg (0.69 mmol) of 9-ACPT **2** and pyridine (61 μL, 1.1 equiv) in dry *N*,*N*-dimethylformamide under an argon atmosphere was added drop wise a solution of Aloc-OBt **6** (1206 mg, 8 equiv) in dry *N*,*N*-dimethylformamide. The mixture was stirred at 80 °C for 6 h after which it was diluted with 10% propanol-2/EtOAc. The organic layer was washed with water, 0.1 M hydrochloric acid and 0.1 M sodium bicarbonate, dried over anhydrous sodium sulphate and evaporated to dryness to yield the desired product **7** as a yellow coloured powder (247 mg, 80%) which was used without further purification. Mp 250 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) 1.04 (t, 3H, $J=7.3$ Hz, 19), 1.91 (m, 2H, 18), 4.74 (m, 2H, Aloc), 5.29 (s, 2H, 5), 5.29–5.54 (m, 3H, 2H Aloc and 17), 5.71 (d, 1H, $J=16.4$ Hz, 17), 6.03 (m, 1H, Aloc), 7.68–7.96 (m, 3H, aromatic), 8.04 (d, 1H, $J=8.4$ Hz, aromatic), 8.63 (s, 1H, 7) ppm; MS (FAB) *m*/*e* 448 $(M+H)^+$. Anal. $(C_{24}H_{21}N_3O_6\cdot 0.3H_2O)$ calcd C 63.66%, H 4.81%; measured C 63.72%, H 4.41%.
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36. Procedure for the coupling reaction to yield protected prodrug 20-[Aloc-D-Ala-Phe-Lys(Aloc)-PABC]-CPT (**15**). To a solution of 100 mg (0.125 mmol) Aloc-D-Ala-Phe-Lys(Aloc)-PABC-PNP (**14**) in dry dichloromethane were added CPT **1** (87 mg, 1 equiv) and DMAP (33 mg, 1.1 equiv). To increase solubility, tetrahydrofuran and *N*,*N*-dimethylformamide were added, respectively. The mixture was subsequently stirred for 96 h after which it was diluted with 10% propanol-2/EtOAc. The organic layer was washed with 10% aqueous citric acid, potassium bisulphate, and brine, and it was dried over anhydrous sodium sulphate. The solvents were evaporated yielding a yellow solid. The product was purified by means of column chromatography (SiO_2–EtOAc/heptane 10/1, EtOAc, and $CHCl_3$/MeOH 9/1, resp.) to afford 41 mg (16%, non-optimised) of the protected prodrug **15**. Mp 95 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.87 (d, 3H, $J=6.1$ Hz, CH_3–Ala), 1.01 (t, 3H, $J=7.4$ Hz, 19), 1.11–2.05 (m, 6H, CH_2–Lys), 2.19 (m, 2H, 18), 2.89–3.32 (m, 4H, benzylic Phe and N–CH_2 Lys), 4.08

(m, 1H, Hα), 4.28 (m, 1H, Hα), 4.35–4.72 (m, 5H, Hα and 4 Aloc), 4.91–5.38 (m, 6H, benzylic spacer, 4 Aloc), 5.32 (s, 2H, 5), 5.41 (d, 1H, $J = 17.2$ Hz, 17), 5.69 (d, 1H, $J = 17.2$ Hz, 17), 5.90 (m, 2H, Aloc), 7.10–7.46 (m, 8H, aromatic), 7.50–7.78 (m, 3H, aromatic), 7.87 (t, 1H, aromatic), 7.99 (d, 1H, $J = 7.3$ Hz, aromatic), 8.23 (d, 1H, $J = 8.6$ Hz, aromatic), 8.48 (s, 1H, 7) ppm; MS (FAB) *m/e* 1013 $(M + H)^+$, 1034 $(M + Na)^+$. Anal. ($C_{54}H_{57}N_7O_{13}\cdot 4H_2O$) calcd C 59.80%, H 6.04%; measured C 59.94%, H 6.04%.

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38. Prodrug **17**: mp 160 °C; ^{1}H NMR (300 MHz, DMSO-D_6) δ 0.78–1.93 (m, 12H, CH_3–Ala, 19 and CH_2–Lys), 2.17 (m, 2H, 18), 2.57–3.12 (m, 4H, benzylic Phe and N–CH_2 Lys), 3.77 (m, 1H, Hα), 4.26–4.80 (m, 2H, Hα), 5.10 (m, 2H, benzylic spacer), 5.32 (s, 2H, 5), 5.52 (s, 2H, 17), 6.95–8.23 (m, 14H, aromatic), 8.70 (s, 1H, 7) ppm; MS (FAB) *m/e* 844 $(M + H)^+$, 884 $(M + K)^+$. Anal. ($C_{46}H_{49}N_7O_9\cdot 15HCl$) calcd C 39.72%, H 4.64%; measured C 39.72%, H 4.76%. Prodrug **18**: ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.95–2.08 (m, 12H, CH_3–Ala, CH_2-Lys and 19), 2.18–2.47 (m, 2H, 18), 2.85–3.03 and 3.22–3.40 (2×m, 4H, benzylic Phe and N-CH_2 Lys), 3.98 (m, 1H, Hα), 4.39–4.61 (m, 2H, Hα), 4.95–5.38 (m, 4H, benzylic spacer and 5), 5.42–5.73 (m, 2H, 17), 7.10–7.94 (m, 13H, aromatic), 8.88 (s, 1H, 7) ppm; MS (FAB) *m/e* 859 $(M + H)^+$. Anal. ($C_{46}H_{50}N_8O_9\cdot 7HCl$) calcd C 49.59%, H 5.16%; measured C 49.88%, H 5.12%.

39. The anti-proliferative effect of CPT, 9-ACPT, and prodrugs **17** and **18** was determined in vitro applying seven well-characterized human tumor cell lines and the microculture sulphorhodamine B (SRB) test. The anti-proliferative effects were determined and expressed as ID_{50} values, which are the (pro)drug concentrations that gave 50% inhibition when compared to control cell growth after 5 days of incubation. Results were averaged from experiments that were performed in quadruplicate. See also: Kepers, Y. P.; Peters, G. J.; ; van Ark-Otte, J.; Winograd, B.; Pinedo, H. M. *Eur. J. Cancer* **1991**, *27*, 897.

40. Cell lines: MCF-7; breast cancer. EVSA-T; breast cancer. WIDR; colon cancer. IGROV; ovarian cancer. M19; melanoma. A498; renal cancer. H226; non-small cell lung cancer.

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