Elongated Multiple Electronic Cascade and Cyclization Spacer Systems in Activatible Anticancer Prodrugs for Enhanced Drug Release

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The design and synthesis of several novel elongated self-elimination spacer systems for application in prodrugs is described. These elongated spacer systems can be incorporated between a cleavable specifier and the parent drug. Naphthalene- and biphenyl-containing spacers were synthesized but did not eliminate. Prodrugs of the anticancer agents doxorubicin and paclitaxel are reported that contain two or three electronic cascade spacers. A novel catalytic application of HOBt was found for the synthesis of *N*-aryl carbamates through reacting a 4-nitrophenyl carbonate with an aniline derivative, to connect the 1,6-elimination spacers via a carbamate linkage. In addition, a double spacer-containing paclitaxel prodrug was synthesized, comprising a 1,6-elimination spacer and a bis-amine linker connected to paclitaxel via a 2′-carbamate linkage. Prodrugs in which the novel spacer systems were incorporated between a specific tripeptide specifier and the parent drug doxorubicin or paclitaxel proved to be significantly faster activated by plasmin in comparison with prodrugs containing conventional spacer systems. It is expected that the generally applicable novel spacer systems reported herein will contribute to future development of improved enzymatically activated prodrugs.

Introduction

Drug delivery of therapeutic parent drugs via prodrugs can offer multiple advantages over administration of the free parent drug. One advantage is that an inactive prodrug can be site-specifically activated to release the active drug, which then exerts its biological function at the target site. Target-selective delivery of biologically active compounds is expected to become increasingly important for application in diseases, like cancer, where selectivity of existing drugs for the diseased area is desired.[1,2] Most chemotherapeutic agents used for treatment of cancer have a small therapeutic window and cause severe, sometimes life-threatening side effects, while little therapeutic effect is seen.

Many reported prodrugs consist of three components: a specifier, a spacer, and a parent drug. The specifier, which is a substrate for a site-specific enzyme, is often connected to the parent drug via a self-elimination spacer. The spacer is incorporated to facilitate enzymatic cleavage of the specifier. After specifier removal, the spacer must spontaneously eliminate to release free drug. The existence of tumor-associated enzymes can be exploited to cleave the specifier of the prodrug (prodrug monotherapy[3]) or the enzyme can be targeted to tumor tissue in an additional step preceding prodrug administration (for example via antibody-directed enzyme prodrug therapy, ADEPT[4]).

One very important criterion for the development of a successful in vivo selective prodrug is that the prodrug must be efficiently activated by the site-specific enzyme under physiological conditions.[5] In this paper, several elongated self-elimination spacer systems are presented which enable fast prodrug activation.

Several structurally different enzymes have been shown to cleave anticancer prodrugs containing a self-elimination spacer much more readily than the corresponding prodrugs lacking a spacer. In some cases with prodrugs lacking a spacer, specifier removal did not occur at all. This clearly demonstrates the beneficial effect of spacer incorporation on prodrug activation characteristics. Evidence for this recurring theme was obtained for example with prodrug substrates for enzymes such as β-glucuronidase[6−9] and cathepsin.[10] In our laboratory it was found that if a specific tripeptide is coupled to an anthracycline via a self-elimination spacer, the resulting

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(1) (a) Dubowchik, G. M.; Walker, M. A. *Pharmacol. Ther.* **1999**, *83*, 67−123. (b) Huang, P. S.; Oliff, A. *Curr. Opin. Gen. Dev.* **2001**, *11*, 104−110.

(2) Damen, E. W. P.; de Groot, F. M. H.; Scheeren, H. W. *Exp. Opin. Ther. Patents* **2001**, *11*, 651−666.

(3) de Groot, F. M. H.; Damen, E. W. P.; Scheeren, H. W. *Curr. Med. Chem.* **2001**, *8*, 1093−1122.

(4) Senter, P. D. *Faseb J.* **1990**, 188−193.

(5) (a) Connors, T. A. *Biochemie* **1978**, *60*, 979−987. (b) Carl, P. L. *Development of Target-Oriented Anticancer Drugs*; Raven Press: New York, 1983; pp 143−155.

(6) Haisma, H. J.; van Muijen, M.; Pinedo, H. M.; Boven, E. *Cell Biophys.* **1994**, *24/25*, 185−192.

(7) Leenders, R. G. G.; Scheeren, H. W.; Houba, P. H. J.; Boven, E.; Haisma, H. J. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2975−2980.

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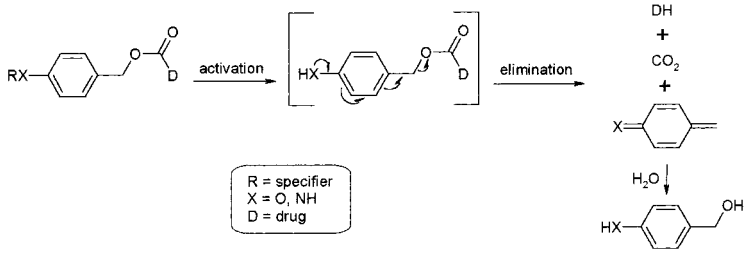
Scheme 1



prodrug is efficiently hydrolyzed by plasmin,[11,12] whereas a plasmin substrate directly coupled to an anthracycline amino group is not activated by the enzyme.[13,14]

Two types of self-elimination spacers can be distinguished: (i) cyclization spacers, and (ii) electronic cascade spacers. The most prominent example of an electronic cascade spacer is the 1,6-elimination spacer developed by Carl et al. After unmasking the aromatic amine or hydroxyl function of these spacers, the amine or hydroxyl group becomes electron-donating and initiates an electronic cascade that leads to the expulsion of the leaving group, which releases the free drug after elimination of carbon dioxide (Scheme 1).[16]

In general, electronic cascade spacers eliminate faster upon unmasking than most cyclization spacers, for which elimination is often characterized by long half-lives. In our laboratory, *p*-aminobenzyl oxycarbonyl (PABC) electronic cascade spacer systems are used because they eliminate virtually instantaneously upon unmasking of the amine.[16,17] If the spacer rapidly eliminates after prodrug activation, it is the enzymatic prodrug activation itself that determines the efficiency of drug release.

Most of the research (including that in our own laboratory) to enhance drug release from prodrugs has been conducted to increase the rate of enzymatic activation by placing electron-withdrawing substituents at aromatic positions of self-immolative electronic cascade linkers. However, the beneficial effect on prodrug activation of spacers containing electron-withdrawing substituents over unsubstituted spacers in most cases proved to be only marginal.[18] In addition, electron-withdrawing substituents on the phenyl ring may affect spacer fragmentation rates.[19] It can be questioned whether major improvement of drug release from prodrugs containing electronic cascade spacers can be achieved by means of introducing spacer substituents.

We hypothesized that elongated spacer systems could decrease steric hindrance to a larger extent than conventional spacers by virtue of the enlarged spacing between the parent drug and the specifier. Indeed, in several electronic cascade spacer-containing prodrugs differences in enzymatic activation rates can still be observed when different parent drugs are connected with the same promoiety (= part of prodrug that is coupled to parent drug) or when a parent drug is connected to the same promoiety via a different site of the drug. For example, β-glucuronidase cleaves the glucuronide from a β-glucuronide-cyclization spacer promoiety much slower when paclitaxel is the parent drug[20] in comparison with the prodrug containing doxorubicin as the parent drug.[8a] In another example, a dipeptide derivative of paclitaxel, linked via a PABC spacer was more readily cleaved by cathepsin B when paclitaxel was linked via its 7-position than via its 2′-position.[10b] In addition, half-lives of cathepsin B cleavage of electronic cascade spacer-containing prodrugs of doxorubicin or mitomycin C were much shorter than the half-life of the corresponding prodrugs with paclitaxel as the parent drug.[10b] Finally, plasmin cleaves the tripeptide from an electronic cascade spacer-containing doxorubicin prodrug much more readily than the tripeptide from the corresponding paclitaxel prodrug.[13,21]

Thus, in several prodrug systems the parent drug still exerts a significant effect on the rate of enzymatic activation, even though the prodrugs mentioned contained a spacer.

In the synthetic studies presented herein, the design, synthesis, and kinetic properties of novel improved linker

(8) (a) Leenders, R. G. G.; Gerrits, K. A. A.; Ruijtenbeek, R.; Scheeren, H. W.; Haisma, H. J.; Boven, E. *Tetrahedron Lett.* **1995**, *26*, 1701−1704. (b) Leenders, R. G. G.; Damen, E. W. P.; Bijsterveld, E. J. A.; Scheeren, J. W.; Houba, P. H. J.; van der Meulen-Muileman, I. H.; Boven, E.; Haisma, H. J. *Bioorg. Med. Chem.* **1999**, *7*, 1597−1610.

(9) Madec-Lougerstay, R.; Florent, J.-C.; Monneret, C. *J. Chem. Soc., Perkin Trans. 1* **1999**, 1369−1375.

(10) (a) Dubowchik, G. M.; Firestone, R. A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3341−3346. (b) Dubowchik, G. M.; Mosure, K.; Knipe, J. O.; Firestone, R. A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3347−3352.

(11) Yamashita, Y.-I.; Ogawa, M. *Int. J. Oncol.* **1997**, *10*, 807−813.

(12) Irigoyen, J. P.; Muñoz-Cánoves, P.; Montero, L.; Koziczak, M.; Nagamine, Y. *Cell. Mol. Life Sci.* **1999**, *56*, 104−132.

(13) de Groot, F. M. H.; de Bart, A. C. W.; Verheijen, J. H.; Scheeren, H. W. *J. Med. Chem.* **1999**, *42*, 5277−5283.

(14) Chakravarty, P. K.; Carl, P. L.; Weber, M. J.; Katzenellenbogen, J. A. *J. Med. Chem.* **1983**, *26*, 638−644.

(15) (a) Nicolaou, M. G.; Yuan, C.-S.; Borchardt, R. T.; *J. Org. Chem.* **1996**, *61*, 8636−8641. (b) Wang, B.; Gangwar, S.; Pauletti, G. M.; Siahaan, T. J.; Borchardt, R. T. *J. Org. Chem.* **1997**, *62*, 1363−1367.

(16) Carl, P. L.; Chakravarty, P. K.; Katzenellenbogen, J. A. *J. Med. Chem.* **1981**, *24*, 479−480.

(17) In contrast to aminobenzyl spacer systems, hydroxybenzyl electronic cascade spacers need electron-withdrawing substituents on the phenyl part of the spacer in order to let spacer elimination take place: (a) Monneret, C.; Florent, J.-C.; Gesson, J.-P.; Jacquesy, J.-C.; Tillequin, F.; Koch, M. *ACS Symp. Ser.* **1995**, *574*, 78−99. (b) Florent, J.-C.; Dong, X.; Gaudel, G.; Mitaku, S.; Monneret, C.; Gesson, J.-P.; Jacquesy, J.-C.; Mondon, M.; Renoux, B.; Andrianomenjanahary, S.; Michel, S.; Koch, M.; Tillequin, F.; Gerken, M.; Czech, J.; Straub, R.; Bosslet, K. *J. Med. Chem.* **1998**, *41*, 3572−3581. (c) Platel, D.; Bonoron-Adèle, S.; Dix, R. K.; Robert, J. *Br. J. Cancer* **1999**, *81*, 24−27.

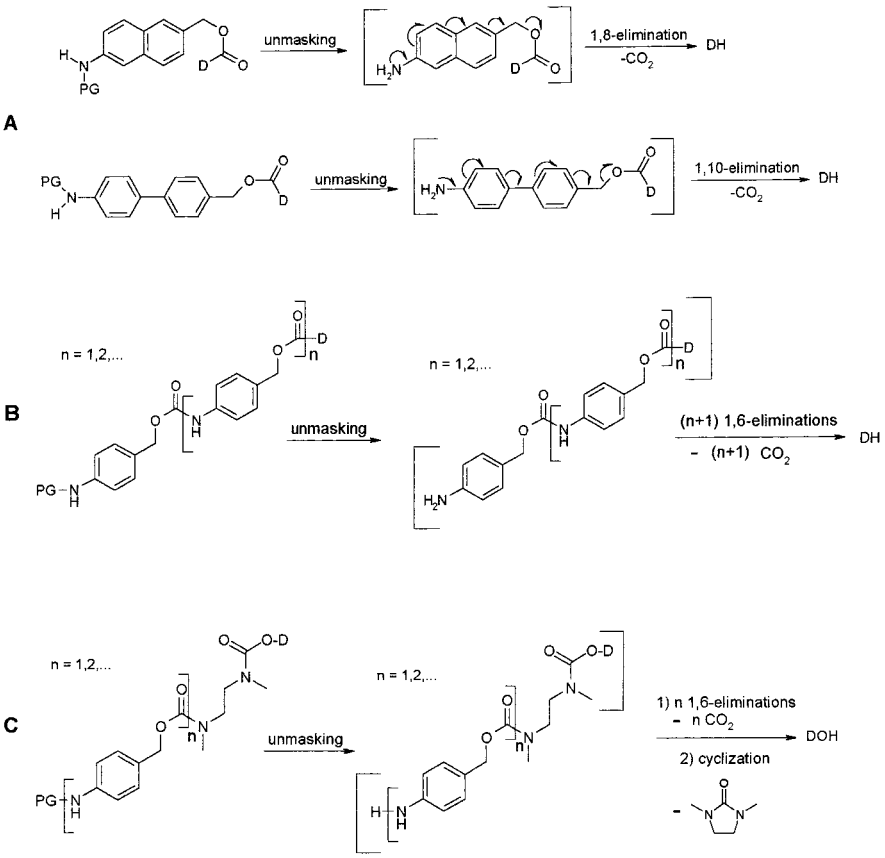
(18) A chloro substituent on an aminobenzyl spacer only marginally enhanced the rate of enzymatic prodrug activation by plasmin.[13] In the case of aminobenzyl spacer-containing anthracycline prodrugs for activation by β-glucuronidase, a variety of substituents showed no beneficial or only a marginal effect[8b] and anthracycline prodrugs containing a spacer without substituents were used for further in vivo evaluation: (a) Houba, P. H. J.; Boven, E.; van der Meulen-Muileman, I. H.; Leenders, R. G. G.; Scheeren, J. W.; Pinedo, H. M.; Haisma, H. J. *Br. J. Cancer* **2000**, *84*, 1−8. (b) Houba, P. H. J.; Boven, E.; van der Meulen-Muileman, I. H.; Leenders, R. G. G.; Scheeren, J. W.; Pinedo, H. M.; Haisma, H. J. *Int. J. Cancer* **2001**, *91*, 550−554.

(19) Electron-donating substituents on the phenyl ring may accelerate fragmentation because of stabilization of the developing positive charge on the benzylic carbon: Hay, M. P.; Sykes, B. M.; Denny, W. A.; O'Connor, C. J. *J. Chem. Soc., Perkin Trans. 1* **1999**, 2759−2770.

(20) de Bont, D. B. A.; Leenders, R. G. G.; Haisma, H. J.; van der Meulen-Muileman, I.; Scheeren, H. W. *Bioorg. Med. Chem.* **1997**, *5*, 405−414.

(21) de Groot, F. M. H.; van Berkom, L. W. A.; Scheeren, H. W. *J. Med. Chem.* **2000**, *43*, 3093−3102.

Scheme 2



systems, which are generally applicable in prodrugs to facilitate release of free parent compound, are reported. The chemotherapeutic agents doxorubicin and paclitaxel serve as parent drugs.

Synthesis

Three possibilities that have been explored for creating elongated electronic cascade spacer systems are depicted in Scheme 2. First, additional aromatic groups can be incorporated in conjugation with the phenyl group present in existing 1,6-elimination spacers (type A). Second, two or more electronic cascade spacers can be incorporated between the specifier and the parent drug (type B). To our knowledge, no papers describing either of these possibilities have been published before. Finally, prodrugs containing a cyclization spacer can be improved by combination of the cyclization spacer in one spacer system with (an) additional electronic cascade spacer(s) (type C). Type C spacer systems may be particularly suitable for application in prodrugs of parent drugs that contain a hydroxyl group.

The incorporation of additional aromatic groups in conjugation with the phenyl group present in existing electronic cascade spacers (type A, Scheme 2) was explored by synthesizing spacer systems that consist of N-capped naphthyl or biphenyl derivatives that contain functionalized hydroxymethylene groups. These elongated aromatic spacers are designed to eliminate according to the mechanism depicted in Scheme 2. After unmasking the primary amine, a 1,8-elimination or a 1,10-elimination electronic cascade should lead to expulsion of the leaving group and subsequent drug release.

More than one electronic cascade spacer can be incorporated between a specifier and the parent drug. In that case the parent drug is released after specifier removal and two or more subsequent spacer eliminations (type B, Scheme 2). Especially in the case of aminobenzyl oxycarbonyl spacers this could also be a feasible approach under physiological conditions, because (i) these spacers eliminate rapidly upon unmasking,[16] and (ii) PABC spacers can be connected to one another via a carbamate linkage, which is expected to be relatively stable against ubiquitous enzymes.

Prodrugs of parent drugs that contain a hydroxyl group (such as paclitaxel) can be improved by incorporation of an elongated spacer system that contains one or more electronic cascade spacers between the specifier and a bis-amine cyclization spacer (type C, Scheme 2). A major advantage of the use of type C elongated spacer systems is that these promoieties can be linked to the OH-drug via a relatively stable carbamate linkage.

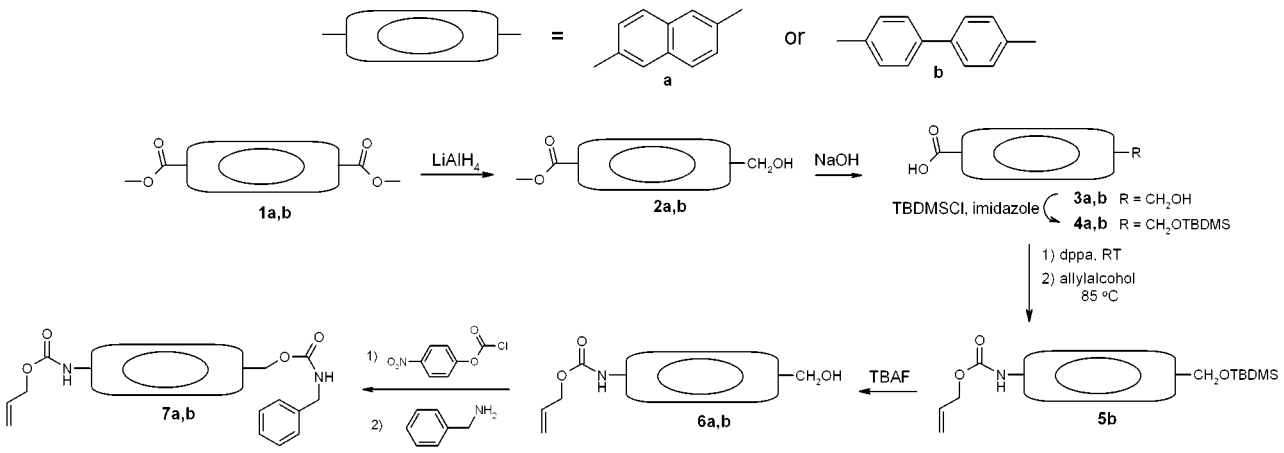
To deliver proof of principle for the proposed 1,8- and 1,10-elimination processes (type A, Scheme 2), allyl oxycarbonyl (Aloc) protected naphthalene and biphenyl spacer systems were synthesized that contained benzylamine as a model compound[22] for an amine-containing drug (Scheme 3).

The commercially available dimethyl ester analogues **1a**,**b** of the desired naphthalene and biphenyl compounds were reduced to the mono-alcohols **2a**,**b** using $LiAlH_4$.

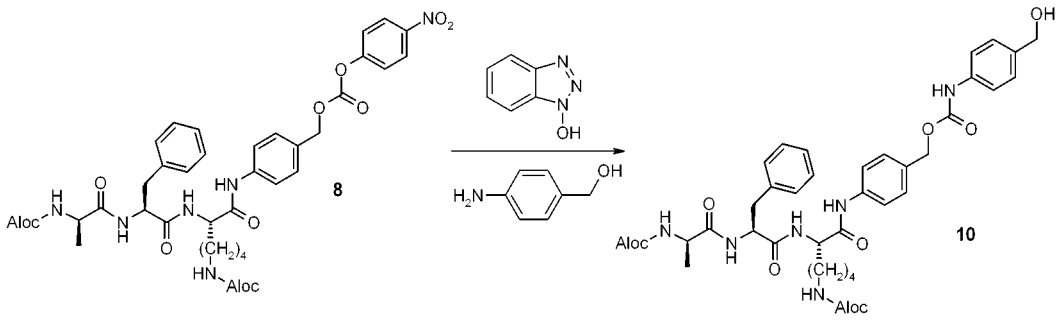
Subsequent saponification of the esters to carboxylic acids **3a**,**b**, followed by protection using *tert*-butyl dimethylsilyl chloride yielded silyl ethers **4a**,**b**. The key step of the synthesis of the model compounds was the conversion of carboxylic acids **4a**,**b** into the corresponding

(22) Griffin, R. J.; Evers, E.; Davison, R.; Gibson, A. E.; Layton, D.; Irwin, W. J. *J. Chem. Soc., Perkin Trans. 1* **1996**, 1205−1211.

Scheme 3



Scheme 4



Aloc protected amines in a one-pot procedure. First, the carboxylic acid was reacted with diphenyl phosphoryl azide at room temperature. This resulted in the formation of the corresponding acyl azide. Second, allyl alcohol was added, and the reaction mixture was heated to a temperature of 85 °C. The acyl azide rearranged into the isocyanate that was immediately trapped by allyl alcohol to lead to the Aloc protected amines. In case of naphthalene spacer **4a**, the Aloc was introduced and the silyl protecting group was removed in one pot, affording **6a**. In case of biphenyl spacer **4b**, the silyl group was not removed yielding **5b**. After deprotection of the silyl group of **5b**, activation of the resulting alcohols **6a**,**b** to the corresponding 4-nitrophenyl carbonates, and subsequent substitution with benzylamine, the naphthalene and biphenyl model compounds **7a**,**b** were obtained.

Exploration of type B elongated spacer systems (Scheme 2) was performed by synthesizing plasmin-activated prodrugs that contained more than one 1,6-elimination spacer. As a plasmin substrate, the specific tripeptide D-Ala-Phe-Lys was employed as a specifier. The synthesis of the doubly Aloc protected tripeptide Aloc-D-Ala-Phe-Lys(Aloc)-OH and the corresponding activated tripeptide−spacer conjugate **8** (Scheme 4) was described in a previous paper.[13]

The key step in the synthesis of these derivatives was coupling a second spacer to the benzylic alcohol function of a tripeptide−spacer conjugate. Several attempts were unsuccessful. The activated 4-nitrophenyl carbonate derivative **8** of the tripeptide−spacer conjugate did not react with 4-aminobenzyl alcohol at room temperature, whereas at 60 °C the starting material was degraded. In the literature an example of formation of an *N*-aryl carbamate is reported, in which an aniline was reacted with a 4-nitrophenyl carbonate at 60 °C for 10 h.[23]

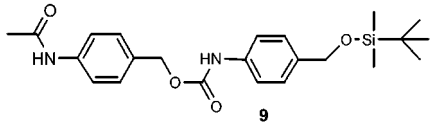


Figure 1. Double spacer-containing model compound **9**.

An attempt was then made to couple a model compound, *N*-acetyl-4-aminobenzyl alcohol, with a carbonyl diimidazole (CDI) activated O-TBDMS protected 4-aminobenzyl alcohol spacer. This reaction succeeded, and the desired double spacer-containing model compound (**9**, Figure 1) could be isolated. Disappointingly, when the same CDI activated spacer was reacted with the tripeptide−spacer conjugate, no coupling reaction was observed.

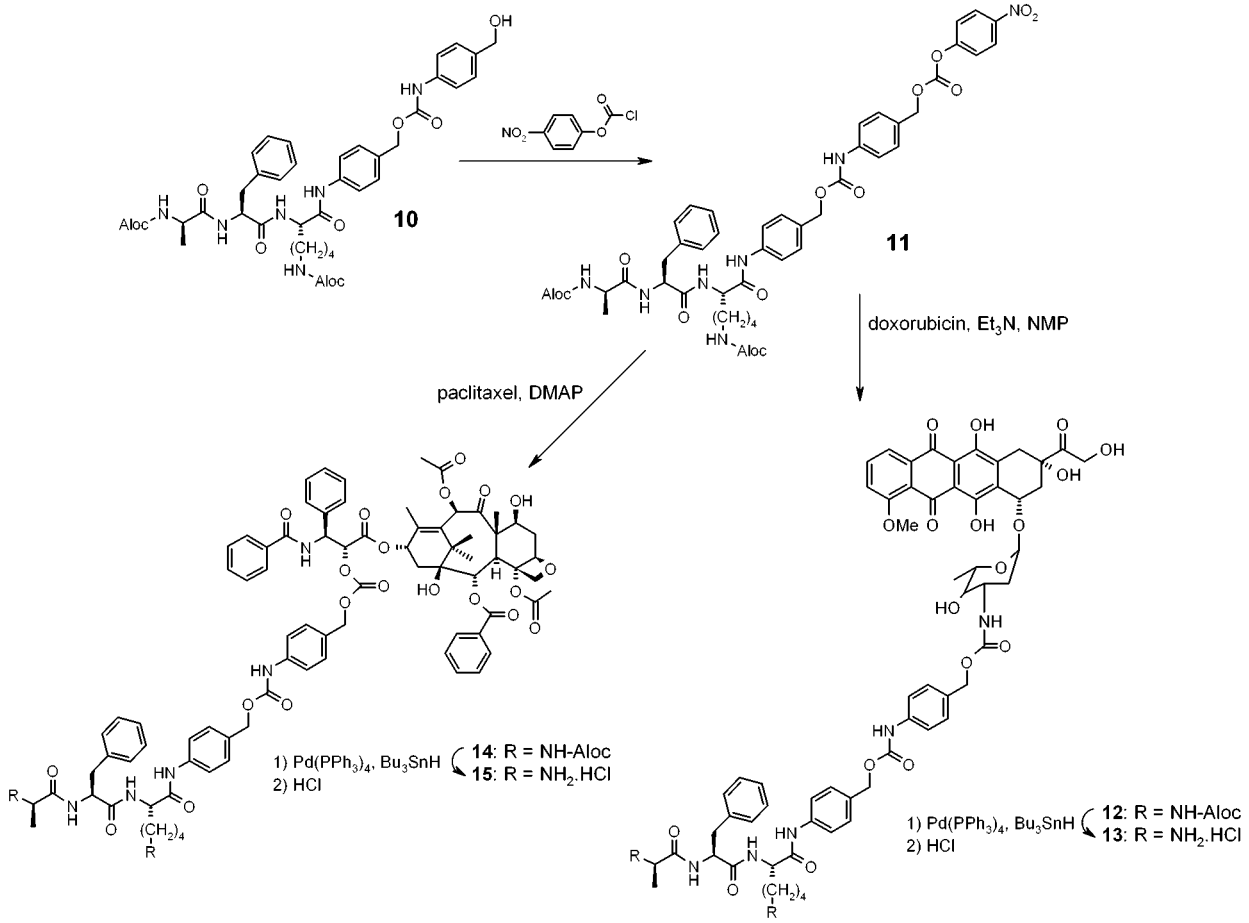
In another attempt to couple both spacers, the tripeptide−spacer conjugate was added to an isocyanate derivative of the spacer,[22] formed via a modified Curtius rearrangement. Even under high-pressure conditions at 15 kbar, formation of the desired compound was not observed.

The possibility of synthesizing the *N*-aryl carbamate via the benzyl chloroformate derivative of the tripeptide−spacer conjugate was not investigated, as it appears both from the literature[24] and our own experience, that it is difficult to obtain the benzylic chloroformate without decomposition to the benzyl chloride.

(23) Obi, K.; Ito, Y.; Terashima, S. *Chem. Pharm. Bull.* **1990**, *38*, 917−924.

(24) Niculescu-Duvaz, D.; Niculescu-Duvaz, U.; Friedlos, F.; Martin, J.; Spooner, R.; Davies, L.; Marais, R.; Springer, C. J. *J. Med. Chem.* **1998**, *41*, 5297−5309.

Scheme 5



At this point we started to look for a catalyst that could catalyze the attack of an aromatic amine on an activated nitrophenyl carbonate (Scheme 4). When the 4-nitrophenyl carbonate activated tripeptide−spacer conjugate **8** was reacted with 4-aminobenzyl alcohol in the presence of diphenyl phosphinic acid,[25] at 40 °C, and the reaction mixture was worked up after 6 days, the desired product **10** was isolated in a yield of 16%.

Then, hydroxy benzotriazole (HOBt) was tested for its catalytic properties. It was previously reported that this alcohol was able to catalyze the coupling reaction of an aziridine nitrogen with a 4-nitrophenyl carbonate.[26] We hypothesized that it might also be an effective catalyst for the synthesis of *N*-aryl carbamates. In the presence of 0.2−0.3 equiv of HOBt, the desired *N*-aryl carbamate **10** was obtained at room temperature in a high yield (97%) (Scheme 4). To our knowledge, this is the first report describing the use of HOBt as a catalyst for coupling an aromatic amine to an activated carbonate.

Both doxorubicin and paclitaxel prodrugs containing two aminobenzyl oxycarbonyl spacers were synthesized starting from the double spacer-containing conjugate **10** (Scheme 5). Activation of benzylic alcohol **10** to carbonate **11** and subsequent coupling with the amine of doxorubicin or the 2′-hydroxyl group of paclitaxel led to protected prodrugs **12** and **14**. Deprotection in the presence of palladium catalyst and tributyltin hydride[27] afforded prodrugs **13** and **15**.

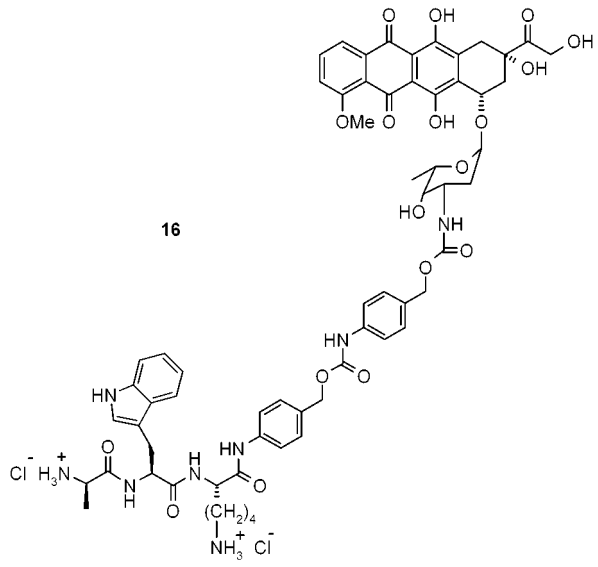


Figure 2. Double spacer-containing doxorubicin prodrug **16** with a tryptophan residue in the tripeptide sequence.

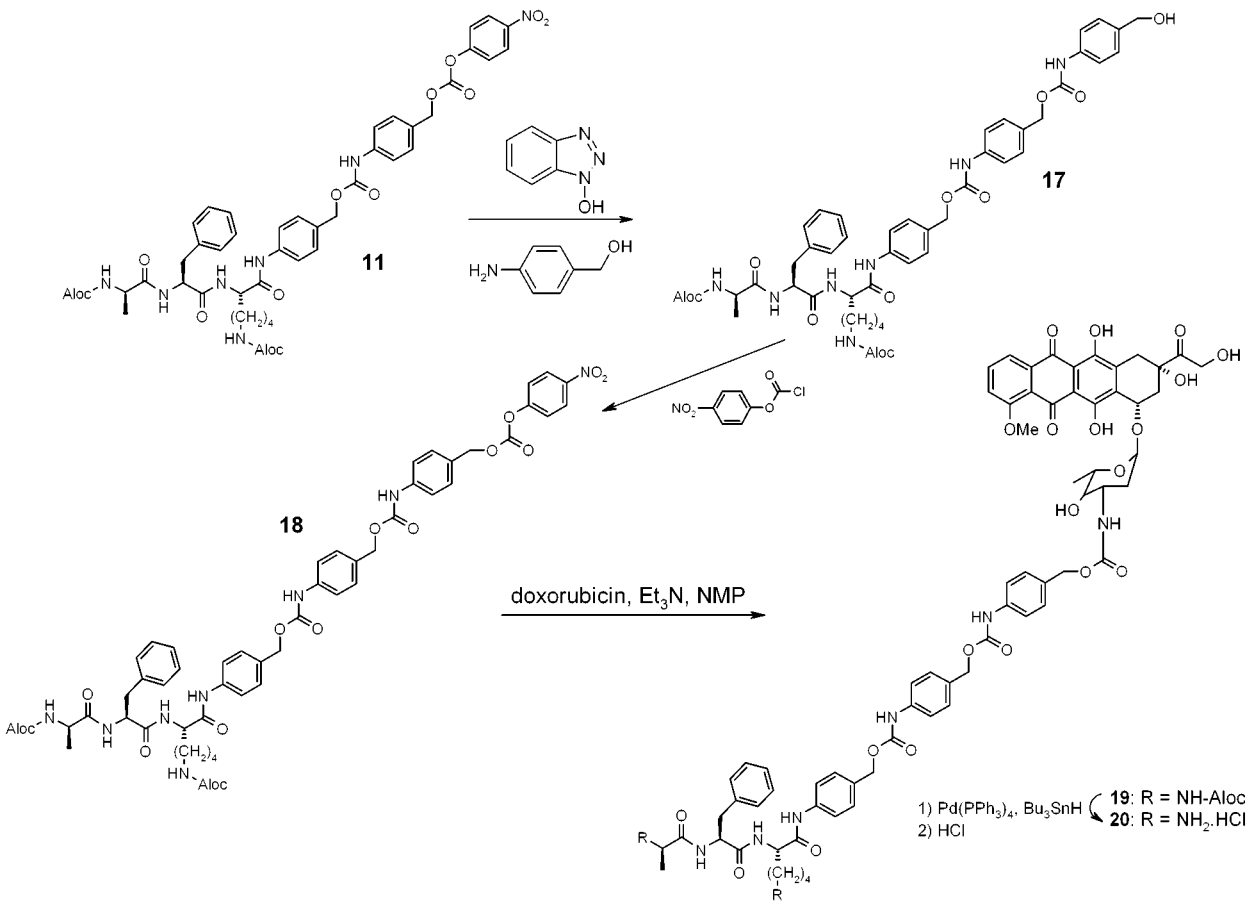
A double 1,6-elimination spacer-containing doxorubicin prodrug with a tryptophan residue at the P2 position instead of a Phe (H-D-Ala-Trp-Lys-PABC-PABC-Doxorubicin, **16**, Figure 2) was also synthesized, because tryptophan is presumed to improve plasmin substrate properties.[28]

(25) Aresta, M.; Berloco, C.; Quaranta, E. *Tetrahedron* **1995**, *51*, 8073−8088.

(26) Dubowchik, G. M.; King, H. D.; Pham-Kaplita, K. *Tetrahedron Lett.* **1997**, *38*, 5261−5264.

(27) Dangles, O.; Guibé, F.; Balavoine, G.; Lavielle, S.; Marquet, A. *J. Org. Chem.* **1987**, *52*, 4984−4993.

Scheme 6



To extend the scope of elongated spacer systems and to reveal information on the optimal number of spacers incorporated, a doxorubicin prodrug was prepared that contained three 1,6-elimination spacers. Starting from carbonate **11**, a third spacer molecule was linked to the peptide double spacer conjugate employing again HOBt as a catalyst, smoothly affording *N*-aryl carbamate **17** (Scheme 6). Activation to carbonate **18**, coupling with doxorubicin to yield **19** and deprotection afforded the triple spacer-containing prodrug **20**.

Paclitaxel served as OH-drug for investigation of type C elongated spacer systems (Scheme 2). A route to synthesize paclitaxel-2′-carbamate prodrugs was previously reported.[21] In the literature, there exists one example of a prodrug of a phenol derivative that contains a combined electronic cascade spacer/cyclization spacer system, although elongation of the spacer system was not mentioned as a purpose.[29]

A paclitaxel prodrug containing both a 1,6-elimination PABC spacer and an *N*,*N*′-dimethyl ethylenediamine spacer may possess desirable properties such as the elongated nature of the spacer system and the nature of the linkage between promoiety and drug being a carbamate.[21,30] After proteolysis, paclitaxel would be released following 1,6-elimination and subsequent intramolecular cyclization (type C, Scheme 2).

The paclitaxel prodrug **26** was prepared in a reasonable yield through a convergent synthetic approach (Scheme 7). A deprotected spacer−drug conjugate **24** was synthesized by coupling a benzyl oxycarbonyl (Z) monoprotected spacer **22** to the previously reported 2′-[4-nitrophenyl carbonate]-paclitaxel (**21**)[21] to afford **23**, followed by hydrogenolysis using H_2, Pd/C in the presence of acetic acid to yield the protonated amine **24**. This amine was coupled with the activated specifier-spacer conjugate **8** to afford protected prodrug **25**, by adding base to a concentrated solution of **24** and **8** in order to favor the intermolecular coupling reaction over premature intramolecular spacer cyclization of **24**. Deprotection of **25** yielded the desired paclitaxel prodrug **26**. Another synthetic route, in which a Boc-protected cyclization spacer was coupled to the activated tripeptide−spacer conjugate **8**, failed, because after deprotection of the Boc group using 0.5 M hydrochloric acid/ethyl acetate, an undesired product was isolated.[31] Coupling of two separate fragments according to the strategy depicted in Scheme 7, in which the chemical link between the two spacers was established in the final stage,

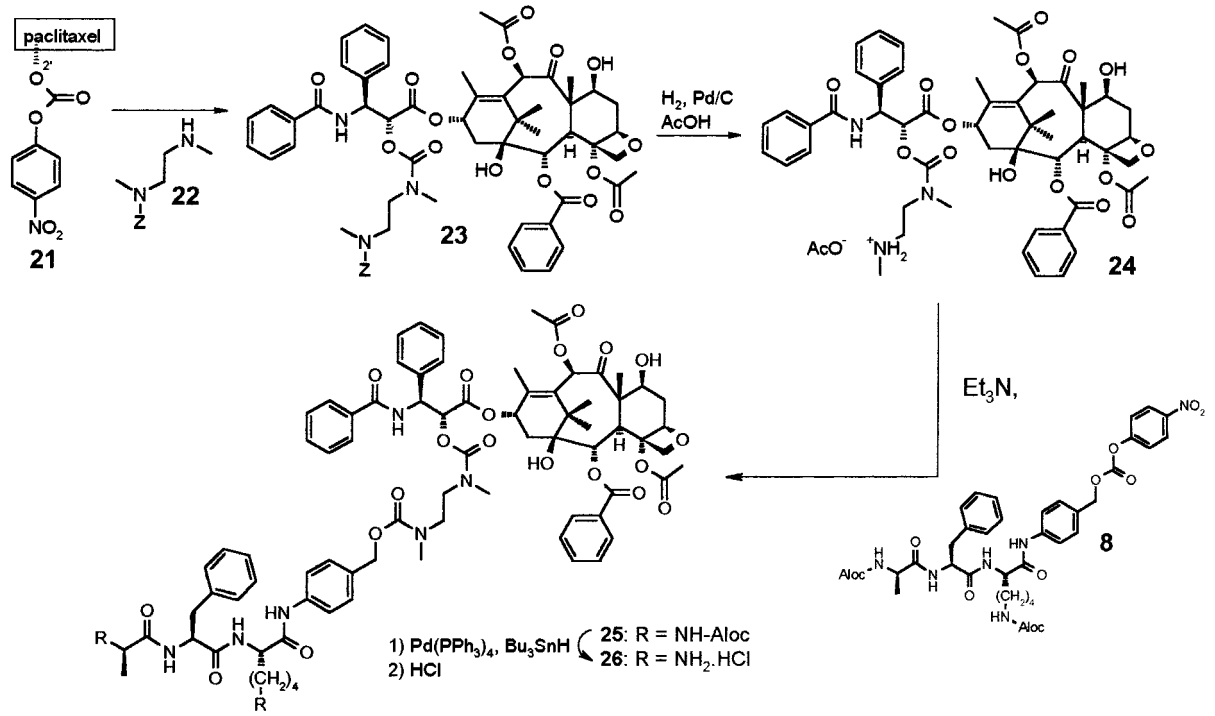
(28) Tryptophan instead of phenylalanine may fit better into the S2 pocket of plasmin, a pocket specific for aromatic amino acids. In a study where a library of inhibitors was screened, Trp provided up to a 80-fold increase in affinity in comparison with Phe. (a) Backes, B. J.; Harris, J. L.; Leonetti, F.; Craik, C. S.; Ellman, J. A. *Nature Biotech.* **2000**, *18*, 187−193. (b) Abato, P.; Conroy, J. L.; Seto, C. T. *J. Med. Chem.* **1999**, *42*, 4001−4009.

(29) Lougerstay-Madec, R.; Florent, J.-C.; Monneret, C.; Nemati, F.; Poupon M. F. *Anti-Cancer Drug Des.* **1998**, *13*, 995−1007.

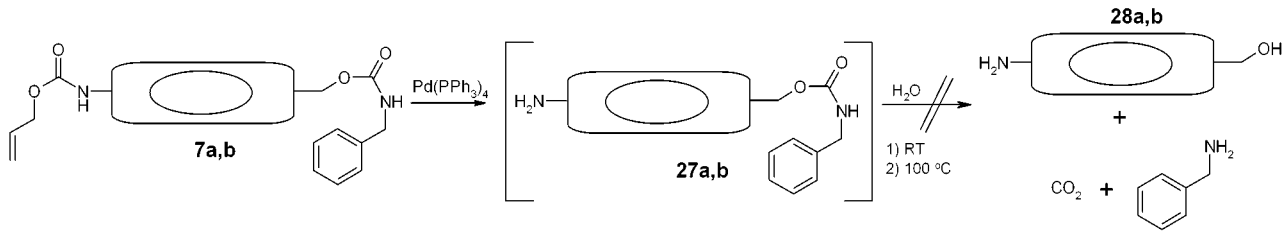
(30) Saari, W. S.; Schwering, J. E.; Lyle, P. A.; Smith, S. J.; Engelhardt, E. L. *J. Med. Chem.* **1990**, *33*, 97−101.

(31) When the Boc group was deprotected from the tripeptide double spacer conjugate, the corresponding tripeptide aminobenzyl chloride conjugate (MS (FAB) *m/e* 656 $(M + H)^+$, 678 $(M + Na)^+$) was obtained, presumably formed upon acidic cleavage of the benzyl carbamate. In the literature a low yield (30%) was reported for a similar Boc deprotection.[29] The strategy depicted in Scheme 7 may provide a more efficient synthesis of type C elongated spacer system-containing prodrugs.

Scheme 7



Scheme 8



provided the most efficient route to the paclitaxel prodrug.

Proof of Principle and Kinetic Studies. The Aloc functions of the naphthalene- and biphenyl-containing spacers **7a**,**b** were deprotected, to generate free amines **27a**,**b**. The deprotected products should subsequently undergo spontaneous spacer elimination to release free benzylamine and regenerate amino-naphthyl methanol (**28a**) or amino-biphenyl methanol (**28b**), as depicted in Scheme 8.

Formation of a new and more polar product was observed; however, no benzylamine or amino alcohols **28a**,**b** were detected at room temperature. Even heating of the reaction mixtures up to 100 °C under basic conditions did not lead to the desired elimination reaction. Both amines **27a**,**b** were isolated. To verify whether lack of elimination could be partly ascribed to the leaving group ability of the *N*-benzyl carbamic acid, the *N*-phenyl leaving group analogue **29** was synthesized, because of its assumed improved leaving group ability, again using HOBt as a catalyst (Figure 3).

Even with this improved leaving group, the desired 1,10-elimination of the biphenyl part of **29** occurred neither at room temperature nor at 100 °C.

The synthesized elongated spacer system-containing prodrugs (types B and C) were subjected to incubation

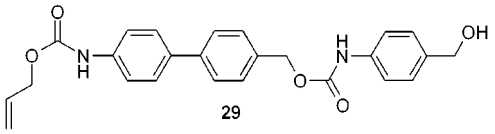


Figure 3. Biphenyl spacer-containing model compound **29** with an aromatic amine in the leaving group.

Table 1. Plasmin Activation Rates and Spacer Cyclization Rate of Single and Double Spacer-Containing Paclitaxel Prodrugs

	spacer system	$T_{1/2}$ act. (min)	$T_{1/2}$ cycl (min)
30[21]	1 × PABC	42	
26	1 × PABC, 1 × cyclization	4	47
15	2 × PABC	7.5	

experiments in the presence of the protease plasmin, to determine the rate of enzymatic prodrug activation.

In the case of the paclitaxel prodrugs, incubation was performed at 37 °C in 0.1 M Tris/hydrochloric acid buffer (pH 7.3), at a prodrug concentration of 200 μM and 0.03 U/mL human plasmin. Using capillary electrophoresis, half-lives of plasmin activation and in case of paclitaxel prodrug **26** also the half-life of spacer cyclization were determined (Table 1). Both double spacer-containing paclitaxel prodrugs were converted to yield the corresponding parent drug. A plasmin activated paclitaxel

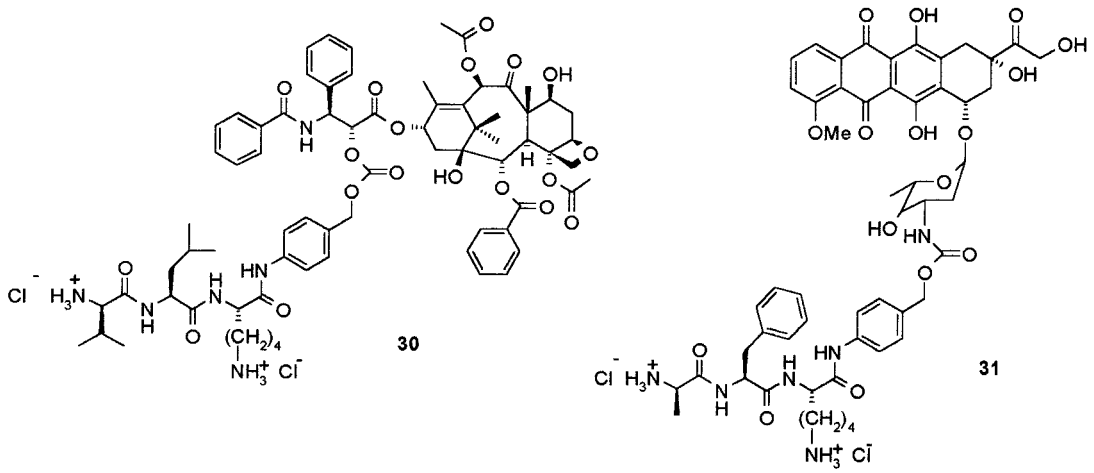


Figure 4. Reference prodrugs **30** and **31** that contain a single spacer.

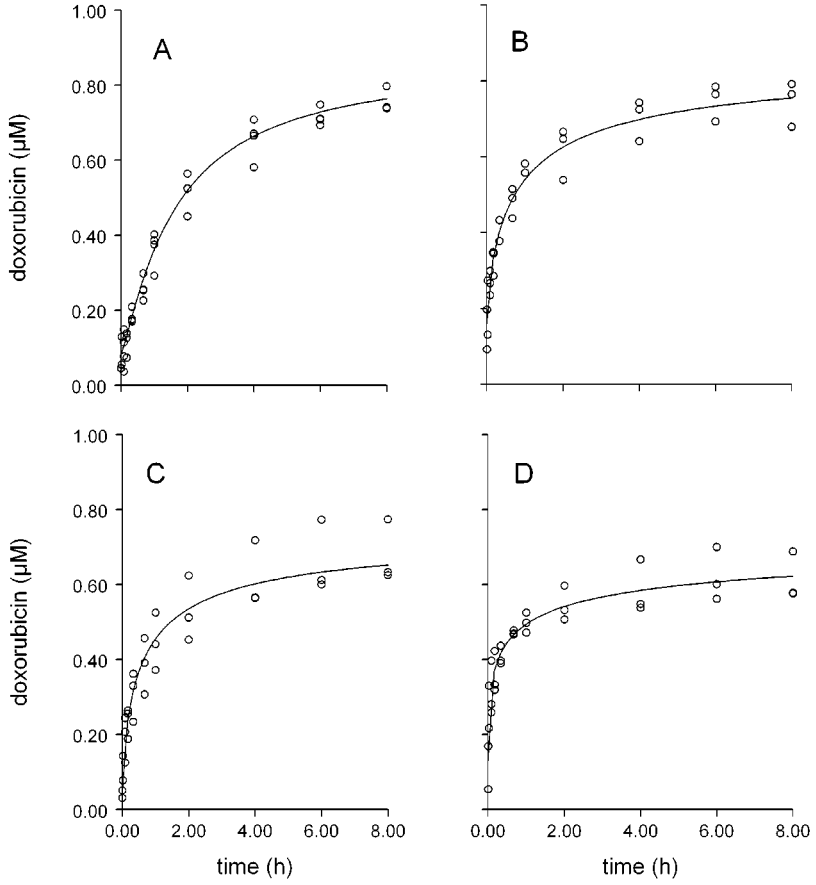


Figure 5. Release of free parent drug doxorubicin from elongated spacer system-containing prodrugs **13** (B), **16** (C), and **20** (D) in comparison with single spacer-containing prodrug **31** (A).

prodrug containing one 1,6-elimination spacer (H-D-Val-Leu-Lys-PABC-Paclitaxel, **30**, Figure 4) was reported previously.[21] This prodrug contains a D-Val-Leu-Lys sequence, which shows plasmin substrate properties comparable to D-Ala-Phe-Lys.[32]

The doxorubicin prodrugs containing multiple spacer systems were incubated at 37 °C in 0.1 M Tris/hydrochloric acid buffer (pH 7.3), at an expected maximally approachable doxorubicin concentration of approximately 1 μM in the presence of 0.0025 U/mL human plasmin, on three or four separate occasions. Using a fully validated HPLC method for detection of doxorubicin,[33] the rates of enzymatic activation by plasmin of the multiple electronic cascade spacer system-containing doxorubicin prodrugs **13**, **16**, and **20** were determined. The plasmin-activated doxorubicin prodrug containing one 1,6-elimination spacer (H-D-Ala-Phe-Lys-PABC-Doxorubicin, **31**, Figure 4) was reported previously.[13] In Figure 5 the doxorubicin concentration after addition of plasmin (at

(32) Eisenbrand, G.; Lauck-Birkel, S.; Tang, W. C. *Synthesis* **1996**, 1246−1258.

(33) de Bruijn, P.; Verweij, J.; Loos, W. J.; Kolker, H. J.; Planting, A. S. T.; Nooter, K.; Stoter, G.; Sparreboom, A. *Anal. Biochem*. **1999**, *266*, 216−221.

Table 2. Summary of Constants of the Hill Equation for the Different Doxorubicin Prodrugs

	spacer system	T_{50} (h)	γ	r	p
31[13]	1 × PABC	1.66 ± 0.23	1.18 ± 0.085	0.991	<0.001
13	2 × PABC	0.82 ± 0.28	0.72 ± 0.093	0.978	<0.001
16[a]	2 × PABC	0.64 ± 0.062	0.72 ± 0.039	0.958	<0.001
20	3 × PABC	0.57 ± 0.11	0.43 ± 0.031	0.949	<0.001

[a] Contains a D-Ala-Trp-Lys specifier.

T = 0) is depicted for doxorubicin prodrugs **31**, **13**, **16**, and **20**.

The constants of the Hill equation[34] that follow from the data depicted in Figure 5 are outlined in Table 2. The time needed to reach a 50% conversion to doxorubicin is T_{50}, whereas γ (the Hill constant) represents the sigmoidicity of the curve. Since the hydrolysis of the prodrugs started directly after the addition of plasmin, the Hill constant is representative for the initial prodrug conversion rate. A lower γ means a steeper curve, and a higher initial prodrug conversion rate. An r-value close to 1 and a p-value close to 0 indicate that all data fit significantly in the Hill curve.

As shown in Figure 5 and Table 2, all curves could be significantly fitted using the Hill equation. The time needed to reach a 50% conversion to doxorubicin was in the following order: **31** > **13** > **16** > **20**.

All prodrugs were incubated in 0.1 M Tris/HCl buffer (pH 7.3) for 3 days at 37 °C and showed no formation of degradation products. Because a markedly decreased cytotoxicity of a prodrug in comparison with the parent drug is a requirement of a successful prodrug, all prodrugs were tested for their cytotoxicity in a panel of seven human tumor cell lines. Paclitaxel prodrugs **15** and **26** showed an average decrease of in vitro cytotoxicity of respectively 3.7-fold and 73-fold in comparison with parent paclitaxel upon incubation for 5 days. Doxorubicin prodrugs **13**, **16**, and **20** showed an average decrease of in vitro cytotoxicity of 30-fold, 24-fold, and 22-fold, respectively, when compared with parent doxorubicin under similar conditions.

Discussion and Conclusions

Incorporation of elongated spacer systems in plasmin-activated prodrugs resulted in increased enzymatic activation rates when compared to prodrugs containing a conventional spacer. At the prodrug and enzyme concentrations used, elongated spacer-containing doxorubicin prodrugs were activated with a 2- to 3-fold higher rate, whereas elongated spacer-containing paclitaxel prodrugs showed a 6- to 10-fold higher activation rate. Application of elongated spacer systems resulted in major improvements of enzymatic activation rates, particularly when a bulky drug, such as paclitaxel, was the parent drug. It is feasible to assume that there exists an optimum for the length of spacer systems required for ensuring a maximal enzymatic prodrug activation rate. This optimum will be determined by several parameters, including the enzyme and the parent drug that is used.

Naphthalene and Biphenyl Spacer Systems. Although several examples in the literature suggest that the (nonaromatic) structures that were hypothesized to be generated are not inconceivable,[35] the naphthalene and biphenyl spacer systems (type A, Scheme 2) unfortunately were resistant to the proposed 1,8- and 1,10-elimination reactions, respectively. This observation may be explained by the fact that in both cases aromaticity of two ring systems must be sacrificed and possibly this energy barrier for dearomatization of the bicyclic spacers is too high. An additional drawback for elimination of the biphenyl system in comparison with the naphthalene spacer is the repulsion of the biphenyl ortho hydrogens when both phenyl groups leave their tilted orientation to reach a flat structure to make the electronic cascade possible.

Multiple PABC Spacer Systems. The synthesized doxorubicin and paclitaxel prodrugs contain linker systems comprising two (**13**, **15**, and **16**) or three (**20**) electronic cascade PABC spacers (type B, Scheme 2). To the best of our knowledge, linker systems comprising two or more connected electronic cascade spacers have not been published up till now. Release of the leaving group (the drug) occurred after two or three subsequent electronic cascade spacer eliminations. The half-lives for enzymatic prodrug activation were significantly lower when elongated spacer systems were incorporated.

The synthesis of multiple PABC spacer systems was enabled through a novel synthetic application of HOBt as a catalyst. The HOBt-catalyzed coupling appears to be a fruitful reaction mainly when an alcohol that must be converted to an *N*-aryl-carbamate is sterically hindered. It has been shown that HOBt can be employed to covalently link aminobenzyl oxycarbonyl electronic cascade spacers via carbamate linkages.

Plasmin hydrolysis of the double and triple 1,6-elimination spacer-containing doxorubicin prodrugs is, at the plasmin and prodrug concentrations used, at least two times faster than plasmin hydrolysis of the corresponding single spacer-containing prodrug,[13] reflected both in T_{50} and γ values. The triple spacer-containing prodrug **20** shows the highest enzymatic activation rate. Additional incubation experiments would be necessary to enable more significant discrimination between the double and triple spacer systems. The Hill constants γ indicate that the initial plasmin activation rate is higher for the double spacer-containing prodrugs **13** and **16** than for single spacer-containing prodrug **31**, whereas the prodrug containing the longest spacer system (triple spacer-containing prodrug **20**) shows the highest initial rate of doxorubicin release.

In case of prodrugs with paclitaxel as the parent drug, the double electronic cascade spacer-containing prodrug **15** released the parent drug with a 6-fold increased rate when compared to single spacer-containing prodrug **30**, upon incubation with plasmin.

Combination of PABC Spacer(s) with a Bisamine Cyclization Spacer. Prodrugs of parent drugs that are coupled via a hydroxyl group may be improved by attaching the specifier to the drug via both one or more electronic cascade spacers and a bisamine cyclization spacer, which is connected to the OH-drug via a carbamate function (type C, Scheme 2). Applying this approach

(34) Holford, N. H. G.; Sheiner, L. B. *Clin. Pharmacokin.* **1981**, *6*, 429−453.

(35) (a) Biewer, M. C.; Biehn, C. R.; Platz, M. S.; Després, A.; Migirdicyan, E. *J. Am. Chem. Soc.* **1991**, *113*, 616−620. (b) Fukushima, T.; Okazeri, N.; Miyashi, T.; Suzuki, K.; Yamashita, Y.; Suzuki, T. *Tetrahedron Lett.* **1999**, *40*, 1175−1178. (c) Minabe, M.; Mochizuki, H.; Yoshida, M.; Toda, T. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 68−72. (d) Szunerits, S.; Utley, J. H. P.; Nielsen, M. F. *J. Chem. Soc., Perkin Trans. 2* **2000**, 669−675.

to paclitaxel yielded prodrug **26**, which showed a markedly decreased cytotoxicity, and a 10-fold increased plasmin hydrolysis rate when compared to the prodrug containing a single spacer (**30**).

Prodrug Stability. Several conclusions can be drawn from the cytotoxicity data. Single-, double-, and triple-spacer-containing doxorubicin prodrugs **31**, **13**, **16**, and **20**, show, respectively, 17-fold,[13] 30-fold, 24-fold, and 22-fold reduced cytotoxicity in comparison with doxorubicin. The fact that the number of spacers does not show a distinct effect on prodrug cytotoxicity may imply that the carbamate bond connecting two PABC spacers is relatively stable, at least in vitro, against ubiquitous enzymes.

When considering that double spacer-containing paclitaxel-2′-carbonate prodrug **15** shows much higher in vitro cytotoxicity than single spacer-containing paclitaxel-2′-carbonate prodrug **30**,[21] one can imagine that the enlarged spacing between specifier and drug not only facilitates the desired prodrug activation, but may also facilitate ubiquitous cleavage of the promoiety−drug bond, depending on the chemical nature of this linkage.

The 2′-carbonate linked paclitaxel prodrug **15** shows 20-fold higher in vitro cytotoxicity than the 2′-carbamate linked paclitaxel prodrug **26**. The markedly lower cytotoxicity of the double spacer-containing 2′-carbamate paclitaxel prodrug **26** shows that linkage of a promoiety to paclitaxel via a 2′-carbamate function may increase prodrug stability against ubiquitous enzymes. Thus, combination of electronic cascade spacer(s) with a bisamine cyclization spacer (type C) seems to yield a spacer system that is particularly useful for incorporation in prodrugs that are coupled via a hydroxyl group of the parent drug.

In vivo studies with prodrugs that contain the elongated spacer systems described are currently in progress.

It is anticipated that the elongated spacer systems reported herein can be employed in prodrugs or bioconjugates that are designed for enzymatic activation. Use of these spacer systems is not limited to anticancer prodrugs, as they can serve also for targeting to other diseases or diseased areas where a specific disease-associated or targeted enzyme is present.

Experimental Section

Mass spectra were recorded with a MAT 9005, using FAB and EI modes. For other analytical instruments used, see ref 21. All solvents were, if necessary, distilled and dried prior to use, following standard procedures. Thin-layer chromatography was performed using Merck precoated silica gel ($60F_{254}$) plates and compounds were detected with UV light, ammonium molybdate solution, or with a Chloro-TDM test. Column chromatography was performed using Baker silica gel in the solvents indicated. Purchased compounds were used without further purification.

Reduction of 1a to 2a. To a solution of 6.04 g (24.7 mmol) of dimethyl 2,6-naphthalenedicarboxylate **1a** in dry THF under an argon atmosphere was added $LiAlH_4$ (1.17 g, 1.25 equiv). The reaction mixture was stirred at room temperature and after 1 and 2 h 0.5 equiv of $LiAlH_4$ were added. After 3 h the organic layer was evaporated and the product was purified by means of column chromatography (EtOAc−hexane 1:1) yielding 1.65 g (31%) of the desired product **2a**. Mp 113 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.08 (s, 1H), 3.98 (s, 3H), 4.88 (d, 2H, J = 3.6 Hz), 7.50−7.54 (m, 1H), 7.82−7.85 (m, 2H), 7.92 (d, 1H, J = 8.5 Hz), 8.02−8.06 (m, 1H), 8.57 (s, 1H); MS (EI) *m/e* 216 $(M)^+$. Anal. ($C_{13}H_{12}O_3$) calculated C 72.21%, H 5.59%, measured C 72.08%, H 5.59%.

Saponification of 2a to 3a. To a solution of 10 equiv of NaOH (3.05 g) in 50 mL of water was added 1.64 g (7.6 mmol) of **2a**. After 40 min, 2 mL of MeOH was added to dissolve the ester. MeOH was evaporated after 3 h, and 1 M HCl solution was added until pH 3 was reached. The mixture was filtered, and the white solid was dissolved in a mixture of CH_2Cl_2 and MeOH. After drying over anhydrous Na_2SO_4, the organic layer was evaporated to dryness, yielding 1.44 g (94%) of the desired product **3a**. Mp 234 °C; ^{1}H NMR (300 MHz, DMSO) δ 4.69 (s, 2H), 7.54 (d, 1H, J = 8.5 Hz), 7.90 (s, 1H), 7.96 (s, 2H), 8.05 (d, 1H, J = 8.5 Hz), 8.56 (s, 1H); MS (EI) *m/e* 202 $(M)^+$. Anal. ($C_{12}H_{10}O_3$) calculated C 71.28%, H 4.98%, measured C 71.00%, H 5.04%.

Silyl Protection of 3a to 4a. A solution of 2.5 equiv (1.04 g) of *tert*-butyl dimethylsilyl chloride and 3.0 equiv (0.466 g) of imidazole in dry THF was stirred under an argon atmosphere. After 15 min, 1.40 g (6.9 mmol) **3a** in dry THF was added dropwise. The reaction mixture was stirred overnight, THF was evaporated, and CH_2Cl_2 was added. The organic layer was washed with 0.5 M $KHSO_4$ and brine and evaporated to yield a solid compound, which was dissolved in THF. To this mixture was added saturated $NaHCO_3$ to hydrolyze the silyl ester. After stirring for 1 h, THF was evaporated and the solution was neutralized using 0.5 M $KHSO_4$. The product was extracted with ether and washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness yielding 0.82 g (37%) of the desired product **4a**, which was used without further purification. Mp 168 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.14 (s, 6H), 0.97 (s, 9H), 4.92 (d, 2H, J = 4.8 Hz), 7.51 (d, 1H, J = 8.5 Hz), 7.84−7.96 (m, 3H), 8.10−8.13 (m, 1H), 8.70 (s, 1H).

Curtius Rearrangement and Aloc Protection To Give 6a. To a solution of 242 mg (0.765 mmol) of **4a** in dry toluene under an argon atmosphere were added diphenylphosphoryl azide (198 μL, 1.2 equiv) and Et_3N (129 μL, 1.2 equiv). After stirring overnight at room temperature, allyl alcohol (1.5 mL) was added and the mixture was stirred at 85 °C for 5 h, and then $CHCl_3$ was added. The organic layer was washed with saturated $NaHCO_3$, 0.5 M $KHSO_4$, 10% citric acid and brine, dried over anhydrous Na_2SO_4 and evaporated. The residual crude product was purified by means of column chromatography (EtOAc−hexane 2:5) to afford 96 mg (49%) of the Aloc-protected desilylated product **6a**. Mp 112 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.70−4.72 (m, 2H), 4.83 (s, 2H), 5.27−5.43 (m, 2H), 5.94−6.07 (m, 1H), 6.85 (s, 1H), 7.37−7.47 (m, 2H), 7.74 (s, 2H), 7.77 (s, 1H), 7.99 (s, 1H). MS (EI) *m/e* 257 $(M)^+$. Anal. ($C_{15}H_{15}O_3N$) calculated C 70.02%, H 5.88%, measured C 69.82%, H 5.95%.

Activation of 6a and Coupling of Benzylamine To Give 7a. To a solution of 88 mg of **6a** (0.34 mmol) in dry THF under an argon atmosphere were added 4-nitrophenyl chloroformate (138 mg, 2.0 equiv) and dry pyridine (83 μL, 3.0 equiv). The reaction mixture was stirred at room temperature and after 4, 19, and 21 h were added, respectively, 3 equiv (112 mL), 3 equiv (112 mL), and 1.5 equiv (56 mL) of benzylamine. After 23 h, THF was evaporated and EtOAc was added. The organic layer was washed with 0.1 M NaOH, 10% citric acid, and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness, yielding 135 mg (100%) of the desired product **7a**. Mp 163 °C; ^{1}H NMR (300 MHz, DMSO) δ 4.22 (d, 2H, J = 6.2 Hz), 4.66 (d, 2H, J = 5.4 Hz), 5.18 (s, 2H), 5.24−5.37 (m, 2H), 5.95−6.08 (m, 1H), 7.21−7.34 (m, 4H), 7.44 (d, 1H, J = 8.7 Hz), 7.55−7.58 (m, 1H), 7.77−7.87 (m, 4H), 8.08 (s, 1H), 9.97 (s, 1H); MS (EI) *m/e* 390 $(M)^+$. Anal. ($C_{23}H_{22}O_4N_2(\cdot^1/_2H_2O)$) calculated C 69.16%, H 5.80%, N 7.01%, measured C 69.20%, H 5.86%, N 6.75%.

Reduction of 1b to 2b. To a solution of 9.99 g (37.0 mmol) of dimethyl 4,4′-biphenyldicarboxylate **1b** in dry CH_2Cl_2 (75 mL) under an argon atmosphere was added $LiAlH_4$ (1.053 g, 0.75 equiv). The reaction mixture was stirred at room temperature. Subsequently were added, after 2 h, dry THF (7 mL), dry CH_2Cl_2 (40 mL), and 0.5 equiv of $LiAlH_4$ (708 mg), after 24 h, CH_2Cl_2 (40 mL) and 0.3 equiv of $LiAlH_4$ (420 mg), and after 25, 26, and 27 h, respectively, 0.3 equiv (429 mg), 0.5 equiv (710 mg), and 0.25 equiv (355 mg) of $LiAlH_4$. After 28 h,

CH_2Cl_2 and 10% citric acid were added, and the mixture was filtered over Hyflo. The organic layer was washed with 10% citric acid and brine, dried over anhydrous Na_2SO_4, and evaporated yielding the crude product. The product was purified by means of column chromatography ($CHCl_3$, $CHCl_3$−MeOH 9:1; respectively) to afford 2.00 g (25%, conversion 88%) of the desired product **2b**. Mp 170 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.94 (s, 3H), 4.77 (d, 2H, J = 3.3 Hz), 7.47 (d, 2H, J = 8.1 Hz), 7.63 (d, 2H, J = 8.5 Hz), 7.66 (d, 2H, J = 8.6 Hz), 8.11 (d, 2H, J = 8.3 Hz); MS (EI) *m/e* 242 $(M)^+$. Anal. ($C_{15}H_{14}O_3$) calculated C 74.36%, H 5.82%, measured C 74.01%, H 6.06%.

Saponification of 2b to 3b. To a solution of 10 equiv NaOH (325 mg) in a water/THF (50 mL/40 mL) mixture was added 1.97 g (32.9 mmol) of **2b.** After 2 h, 30 mL of MeOH was added. MeOH was evaporated after 4.5 h, and 1 M HCl solution was added until a pH of 3 was reached. After filtration of the mixture, the residue was washed with 10% citric acid and water and dissolved in MeOH. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness yielding 1.855 g (100%) of the desired product **3b**. Mp 247 °C; ^{1}H NMR (300 MHz, DMSO) δ 4.56 (d, 2H, J = 4.8 Hz), 7.44 (d, 2H, J = 8.0 Hz), 7.70 (d, 2H, J = 8.1 Hz), 7.80 (d, 2H, J = 8.3 Hz), 8.02 (d, 2H, J = 8.2 Hz); MS (EI) *m/e* 228 $(M)^+$. Anal. ($C_{14}H_{12}O_3 \cdot {}^1/_4$ MeOH) calculated C 72.44%, H 5.55%, measured C 72.20%, H 5.30%.

Silyl Protection of 3b to 4b. A solution of 2.5 equiv (1.65 g) of *tert*-butyl dimethylsilyl chloride and 3.0 equiv (0.895 g) of imidazole in dry THF was stirred under argon atmosphere. After 15 min, 1.01 g (4.43 mmol) **3b** in dry THF was added. After stirring overnight the THF was evaporated and CH_2Cl_2 was added. The organic layer was washed with 0.5 M $KHSO_4$ and brine and evaporated to yield a solid compound, which was dissolved in THF. To this mixture was added saturated $NaHCO_3$, to saponify the silyl ester. After stirring for 1 h, THF was evaporated, and the solution was neutralized with 0.5 M $KHSO_4$. The product was extracted with CH_2Cl_2 and washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness yielding 1.44 g (95%) of the desired product **4b**. Mp 227 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 0.14 (s, 6H), 0.97 (s, 9H), 4.81 (s, 2H), 7.43 (d, 2H, J = 8.0 Hz), 7.62 (d, 2H, J = 8.0 Hz), 7.68 (d, 2H, J = 8.2 Hz), 8.11 (d, 2H, J = 8.2 Hz); MS (EI) *m/e* 342 $(M)^+$. Anal. ($C_{20}H_{26}O_3Si$) calculated C 70.14%, H 7.65%, measured C 70.47%, H 7.69%.

Curtius Rearrangement and Aloc Protection To Give 5b. To a solution of 306 mg (0.893 mmol) of **4b** in dry toluene under an argon atmosphere were added diphenylphosphoryl azide (227 μL, 1.2 equiv) and Et_3N (148 μL, 1.2 equiv). The reaction mixture was stirred at room temperature, and after 15 and 21 h, respectively, 0.4 equiv (76 μL) and 0.5 equiv (95 μL) of diphenylphosphoryl azide were added. After 39 h, allyl alcohol (3 mL) was added and the mixture was stirred at 85 °C for 3 h, then $CHCl_3$ was added. The organic layer was washed with saturated $NaHCO_3$, 10% citric acid, and brine, dried over anhydrous Na_2SO_4, and evaporated. The residual crude product was purified by means of column chromatography (EtOAc−hexane 1:9) to afford 292 mg (82%) of the desired product **5b**. Mp 78 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.12 (s, 6H), 0.96 (s, 9H), 4.68−4.70 (m, 2H), 4.78 (s, 2H), 5.26−5.41 (m, 2H), 5.92−6.08 (m, 1H), 6.68 (s, 1H), 7.25−7.28 (m, 1H), 7.38 (d, 2H, J = 8.1 Hz), 7.45 (d, 2H, J = 8.6 Hz), 7.51−7.56 (m, 3H); MS (EI) *m/e* 397 $(M)^+$.

Deprotection of 5b to 6b. To a solution of 274 mg (0.689 mmol) of **5b** in dry THF under an argon atmosphere was added tetrabutyl ammoniumfluoride (1.03 mL, 1.5 equiv). After stirring for 1 h, EtOAc (50 mL) was added and THF was evaporated. The mixture was washed with 10% citric acid, saturated $NaHCO_3$, and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness yielding 158 mg (81%) of the desired product **6b**. Mp 146 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) 4.67−4.68 (2 × s, 4H), 5.25−5.41 (m, 2H), 5.93−6.06 (m, 1H), 7.41 (d, 2H, J = 7.6 Hz), 7.48−7.57 (m, 4H); MS (EI) *m/e* 283 $(M)^+$. Anal. ($C_{17}H_{17}O_3N$) calculated C 72.07%, H 6.05%, N 4.94%, measured C 71.53%, H 6.06%, N 4.88%.

Activation of 6b and Coupling of Benzylamine To Give 7b. To a solution of 41 mg of **6b** (0.14 mmol) in dry THF under an argon atmosphere were added 4-nitrophenyl chloroformate (31 mg, 1.0 equiv) and dry Et_3N (30 μL, 1.5 equiv). The reaction mixture was stirred at room temperature and after 1 and 2 h were added, respectively, 1.0 equiv (20 μL) and 3.0 equiv (60 μL) of Et_3N. After 3 and 4 h was added 2 equiv (56 mg) of 4-nitrophenyl chloroformate. After 19, 21, and 23 h were added, respectively, 1.2 equiv (19 μL), 2.4 equiv (38 μL), and 2.4 equiv (38 μL) of benzylamine. After 40 h, THF was evaporated and EtOAc was added. The organic layer was washed with 0.1 M NaOH, 10% citric acid, and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness, yielding the desired product **7b** quantitatively. Mp 154 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 4.35 (s, 2H), 4.65 (m, 2H), 5.15 (s, 2H), 5.24−5.41 (m, 2H), 5.94−6.03 (m, 1H), 7.21−7.33 (m, 8H), 7.40−7.46 (m, 1H), 7.52−7.57 (m, 4H). MS (EI) *m/e* 416 $(M)^+$. Anal. ($C_{25}H_{24}O_4N_2$) calculated C 72.10%, H 5.81%, measured C 72.24%, H 6.09%.

Activation of 6b and Coupling of 4-Aminobenzyl Alcohol To Give 29. To a solution of 40 mg of **6b** (0.14 mmol) in dry THF under an argon atmosphere were subsequently added in a period of 64 h: 180 mg of 4-nitrophenyl chloroformate (6.0 equiv), Et_3N (10 drops), 4-aminobenzyl alcohol (63 mg, 3.6 equiv), HOBt (12 mg, 0.6 equiv), DIPEA (5 drops). After 64 h, dry DMF was added and THF was, under an argon atmosphere, evaporated. Subsequently, 8 mg of HOBt (0.4 equiv) and 5 drops of DIPEA were added. After 88 h DMF was evaporated, and EtOAc (50 mL) was added. The organic layer was washed with 0.1 M NaOH, 10% citric acid, and brine, dried over anhydrous Na_2SO_4, and evaporated. The residual crude product was purified by means of column chromatography ($CHCl_3$−MeOH 9:1) to afford 32 mg (52%) of the desired product **29**. Mp 182 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 4.58 (s, 2H), 4.68 (d, 2H, J = 5.6 Hz), 5.22 (s, 2H), 5.25−5.41 (m, 2H), 5.92−6.03 (m, 1H), 7.28 (d, 2H, J = 8.5 Hz), 7.36−7.58 (m, 10H); MS (EI) *m/e* 432 $(M)^+$, 283 $(M_{Aloc-spacer})^+$. Anal. ($C_{25}H_{24}O_5N_2$) calculated C 69.43%, H 5.59%, N 6.48%, measured C 68.95%, H 5.58%, N 6.37%.

Aloc Deprotection To Give 27a. To a solution of 10 mg (0.026 mmol) of **7a** in dry THF under an argon atmosphere were added AcOH (7.0 μL, 5.0 equiv), tributyltin hydride (21.0 μL, 3.0 equiv) and a catalytic amount of palladium tetrakis(triphenylphosphine). After 18 h, the THF was evaporated and the crude product was purified by means of column chromatography (EtOAc/heptane 2/5) followed by preparative TLC (EtOAc/heptane 1.5/1) to afford 8 mg (100%) of the spacer-leaving group conjugate **27a**. Mp 130 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.40 (d, 2H, J = 5.9 Hz), 5.23 (s, 2H), 6.93−6.97 (m, 2H), 7.24−7.38 (m, 6H), 7.57−7.68 (m, 3H); MS (EI) *m/e* 306 $(M)^+$.

Aloc Deprotection To Give 27b. To a solution of 8.0 mg (0.019 mmol) of **7b** in dry THF under an argon atmosphere were added AcOH (5.5 μL, 5.0 equiv), tributyltin hydride (16.0 μL, 3.0 equiv), and a catalytic amount of palladium tetrakis(triphenylphosphine). After 1 h, another catalytic amount of palladium tetrakis(triphenylphosphine) was added. After 2 h, THF was evaporated and the crude product was purified by means of preparative TLC (EtOAc/heptane 1.5/1) to afford 4 mg (63%) of the spacer-leaving group conjugate **27b**. Mp 128 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.40 (d, 2H, J = 5.8 Hz), 5.16 (s, 2H), 7.26−7.43 (m, 11H), 7.52 (d, 2H, J = 8.1 Hz); MS (EI) *m/e* 332 $(M)^+$.

Aloc Deprotection of 29 To Give the Corresponding Biphenyl Spacer Aminobenzyl Alcohol Conjugate. To a solution of 15 mg (0.035 mmol) of **29** in dry THF under an argon atmosphere were added AcOH (10.0 μL, 5.0 equiv), tributyltin hydride (28.0 μL, 3.0 equiv), and a catalytic amount of palladium tetrakis(triphenylphosphine). After 1 h, the THF was evaporated and the crude product was purified by means of preparative TLC (EtOAc/heptane 1.5/1) to afford 8 mg (66%) of the spacer-leaving group conjugate. Mp 121 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.59 (s, 2H), 5.21 (s, 2H), 7.28−7.64 (m, 12H); MS (EI) *m/e* 348 $(M)^+$.

Synthesis of the Double Spacer-Containing Model Compound 9. TBDMS protected spacer was synthesized by adding imidazole (1.34 g, 3.1 equiv) to a solution of *tert*-butyl dimethylsilyl chloride (2.48 g, 2.6 equiv) in dry THF. After 30 min, 4-aminobenzyl alcohol (780 mg, 6.33 mmol) was added and the reaction mixture was stirred for 2 h. The solution was concentrated to dryness, and the residual product was dissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$, and the organic layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo. The crude product was subjected to column chromatography (SiO_2, $CHCl_3$/MeOH 9/1) and freeze-dried twice from dioxane to obtain 1.35 g (90%) of the desired silyl protected spacer. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.07 (s, 6H), 0.92 (s, 9H), 3.60 (bs, 2H), 4.62 (s, 2H), 6.64 (d, 2H, J = 8.4 Hz), 7.11 (d, 2H, J = 8.3 Hz) ppm.

N−Acetyl protected spacer was synthesized by adding $NaHCO_3$ (2.75 g, 1.1 equiv) and acetic anhydride (2.96 mL, 1.05 equiv) to a solution of 3.67 g of 4-aminobenzyl alcohol (29.8 mmol). After 2 h, the solution was filtered and the filtrate was evaporated to dryness. The crude product was subjected to column chromatography (SiO_2, $CHCl_3$/MeOH 9/1) to obtain 4.30 g (87%) of the desired *N*-acetyl protected spacer as a white solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.18 (s, 3H), 4.66 (s, 2H), 7.32 (d, 2H, J = 8.4 Hz), 7.49 (d, 2H, J = 8.4 Hz) ppm.

A solution of O-silyl protected spacer (218 mg, 0.918 mmol) in dry THF was added dropwise to a solution of carbonyl diimidazole (149 mg, 1.0 equiv) in dry THF, and the mixture was stirred for 24 h. A solution of *N*-acetyl protected spacer (152 mg, 1.0 equiv) and Et_3N (127 μL, 1.0 equiv) in THF was added dropwise, and the reaction mixture was allowed to stir for 96 h. The solution was concentrated to dryness, and the residual product was dissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$, a 10% citric acid solution, and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo. The crude product was subjected to column chromatography (SiO_2, $CHCl_3$/MeOH 20/1) to obtain 85 mg (22%) of the desired model compound **9**. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.08 (s, 6H), 0.92 (s, 9H), 2.11 (s, 3H), 4.67 (s, 2H), 5.10 (s, 2H), 7.08 (bs, 1H), 7.25 (m, 4H), 7.35 (d, 2H, J = 8.2 Hz), 7.45 (d, 2H, J = 8.3 Hz), 7.80 (bs, 1H) ppm.

Coupling of spacer to 8 to give Aloc-D-Ala-Phe-Lys-(Aloc)-PABC-PABA (10). To a solution of 156 mg (0.194 mmol) of compound **8** and 26.3 mg (1.1 equiv) 4-aminobenzyl alcohol in dry DMF under an argon atmosphere were added DIPEA (34 μL, 1.0 equiv) and a catalytic amount of *N*-hydroxy benzotriazole (7.9 mg, 0.3 equiv). The reaction solution was stirred for 24 h after which it was diluted with 10% propanol-2/EtOAc. The organic layer was washed with saturated $NaHCO_3$, 0.5 M $KHSO_4$, and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The yellow residual film was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 9/1) to yield 148 mg (97%) of the desired product **10**. Mp 196−197 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (d, 3H, J = 6.4 Hz), 1.27−2.05 (m, 6H), 2.99−3.27 (m, 4H), 4.00−4.64 (m, 7H), 4.57 (s, 2H), 5.14 (s, 2H), 5.06−5.37 (m, 4H), 5.72 (m, 1H), 5.88 (m, 1H), 7.10−7.46 (m, 11H), 7.64 (d, 2H, J = 8.3 Hz) ppm; MS (FAB) m/e 809 $(M + Na)^+$. Anal. $C_{41}H_{50}N_6O_{10}$(·$^1/_2H_2O$) calculated C 61.87%, H 6.46%, N 10.56%, measured C 61.84%, H 6.38%, N 10.38%.

Activation of 10 to Aloc-D-Ala-Phe-Lys(Aloc)-PABC-PABC-PNP (11). A solution of 80.2 mg (0.102 mmol) of compound **10**, pyridine (25 μL, 3.0 equiv), and 4-nitrophenyl chloroformate (44.3 mg, 0.220 mmol) was stirred in dry THF/CH_2Cl_2 under an argon atmosphere at 0 °C for 2 h and overnight at room temperature. The solution was evaporated in vacuo, and the residual product was dissolved in CH_2Cl_2. After washing the organic layer with brine and 0.5 M $KHSO_4$, the organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. The resulting crude product was subjected to column chromatography (SiO_2, $CHCl_3$/MeOH 20/1) to obtain 61.9 mg (84%) of compound **11**. Mp 69−70 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.23 (d, 3H, J = 7.0 Hz), 1.10−2.08 (m, 6H), 3.04−3.27 (m, 4H), 4.06 (m, 1H), 4.26 (m, 1H), 4.35−4.70 (m, 5H), 5.04−5.47 (m, 4H), 5.14 (s, 2H), 5.24 (s, 2H), 5.72 (m, 1H), 5.90 (m, 1H), 7.10−7.46 (m, 13H), 7.65 (d, 2H, J = 8.3 Hz), 8.27 (d, 2H, J = 9.1 Hz) ppm; MS (FAB) m/e 952 $(M + H)^+$, 974 $(M + Na)^+$. Anal. $C_{40}H_{46}N_6O_{12}$(·$^1/_4H_2O$) calculated C 59.51%, H 5.81%, N 10.41%, measured C 59.52%, H 5.54%, N 10.12%.

Doxorubicin coupling To Give Aloc-D-Ala-Phe-Lys-(Aloc)-PABC-PABC-Doxorubicin (12). The double spacer-containing 4-nitrophenyl carbonate **11** (140 mg, 0.147 mmol) and doxorubicin·HCl (94.1 mg, 1.1 equiv) in *N*-methylpyrrolidinone were treated at room temperature with Et_3N (22.5 μL, 1.1 equiv). The reaction mixture was stirred in the dark for 72 h, again Et_3N (1.1 equiv) was added and after an additional 24 h the reaction mixture was diluted with 10% 2-propanol/EtOAc. The organic layer was washed with water and brine and was dried (Na_2SO_4). After evaporation of the solvents, the crude product was purified by means of column chromatography ($CHCl_3$−MeOH; 9:1) followed by circular chromatography using a Chromatotron supplied with a 2 mm silica plate ($CHCl_3$−MeOH; 9:1), to yield 72 mg (36%) of protected prodrug **12**. Mp 129 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.22 (d, 3H, J = 7.1 Hz), 1.27 (d, 3H, J = 6.7 Hz), 1.25−2.00 (m, 8H), 2.15 (dd, 1H), 2.36 (bd, 1H), 3.04 (bd, 1H), 2.90−3.50 (m, 5H), 3.37 (bs, 1H), 3.58 (m, 1H), 3.85 (m, 1H), 4.08 (s, 3H), 4.14 (m, 1H), 4.29 (dd, 1H), 4.37−4.68 (m, 5H), 4.76 (s, 2H), 4.96 (s, 2H), 5.11 (s, 2H), 5.02−5.40 (m, 4H), 5.48 (bs, 1H), 5.61−6.00 (m, 3H), 7.08−7.39 (m, 9H), 7.33 (d, 2H, J = 8.3 Hz), 7.42 (d, 1H, J = 8.4 Hz), 7.62 (d, 2H, J = 8.0 Hz), 7.80 (t, 1H, J = 8.1 Hz), 8.03 (d, 1H, J = 7.5 Hz) ppm; MS (FAB) m/e 1378 $(M + Na)^+$. Anal. $C_{69}H_{77}N_7O_{22}$(·$2H_2O$) calculated C 59.52%, H 5.86%, N 7.04%, measured C 59.34%, H 5.71%, N 6.66%.

Deprotection To Give prodrug H-D-Ala-Phe-Lys-PABC-PABC-Doxorubicin (13). To a solution of 48 mg (0.035 mmol) of protected prodrug **12** in dry THF/CH_2Cl_2 under an argon atmosphere was added morpholine (31 μL, 10 equiv) together with a catalytic amount of $Pd(PPh_3)_4$. The reaction mixture was stirred for 1 h in the dark. The red precipitate was collected by means of centrifugation. EtOAc was added, and the mixture was acidified using 1.0 mL of 0.5 M hydrochloric acid/EtOAc. The precipitate was collected by means of centrifugation and washed several times with EtOAc. *tert*-Butyl alcohol was added and evaporated, and the resulting red film was freeze-dried in water, yielding 37 mg (83%) of doxorubicin prodrug **13**. Mp > 300 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.20 (d, 3H, J = 7.0 Hz), 1.27 (d, 3H, J = 6.5 Hz), 1.38−2.05 (m, 8H), 2.18 (dd, 1H), 2.36 (bd, 1H), 2.82−3.41 (m, 6H), 3.37 (s, 1H), 3.60 (bs, 1H), 4.02 (m, 1H), 4.08 (s, 3H), 4.18 (m, 1H), 4.53 (dd, 1H), 4.66 (m, 1H), 4.77 (s, 2H), 4.95 (bs, 2H), 5.14 (s, 2H), 5.27 (bs, 1H), 5.48 (bs, 1H), 7.09−7.50 (m, 11H), 7.58 (d, 2H, J = 8.4 Hz), 7.82 (t, 1H, J = 8.0 Hz), 8.03 (d, 1H, J = 7.6 Hz) ppm; MS (FAB) m/e 1188 $(M + H)^+$, m/e 1210 $(M + Na)^+$. Anal. (duplo) $C_{61}H_{69}N_7O_{18}$(·5.7HCl) calculated C 52.42%, H 5.39%, N 7.01%, measured C 52.38%, H 5.71%, N 7.14%.

Paclitaxel Coupling To Give 2′-[Aloc-D-Ala-Phe-Lys-(Aloc)-PABC-PABC]-Paclitaxel (14). 4-Nitrophenyl carbonate **11** (47.4 mg, 0.0498 mmol) and paclitaxel (42.3 mg, 1.0 equiv) in dry THF/CH_2Cl_2 under an argon atmosphere were treated at room temperature with *N*,*N*-dimethyl-4-aminopyridine (6.7 mg, 1.1 equiv). The reaction mixture was stirred in the dark for 48 h and was then concentrated to dryness. The product was dissolved in CH_2Cl_2, and the organic layer was washed with saturated $NaHCO_3$, 0.5 M $KHSO_4$ and brine and dried over anhydrous Na_2SO_4. After evaporation of the solvents the residual yellow film was purified by means of column chromatography (SiO_2, EtOAc/Hex/MeOH 5/5/1), to yield 67.5 mg (82%) of protected paclitaxel prodrug **14**. Mp 137−138 °C;^{1}H NMR (300 MHz, $CDCl_3$) δ 1.14 (s, 3H), 1.23 (s, 3H), 1.27 (d, 3H, J = 7.1 Hz), 1.05−2.10 (m, 6H), 1.67 (s, 3H), 1.89 (s, 3H), 2.22 (s, 3H), 2.44 (s, 3H), 2.97−3.21 (m, 4H), 3.81 (d, 1H, J = 7.0 Hz), 4.03 (m, 1H), 4.20 (d, 1H, J = 8.4 Hz), 4.31 (d, 1H, J = 8.4 Hz), 4.43 (m, 1H), 4.34−4.74 (m, 6H), 4.90−5.37 (m, 11H), 5.44 (d, 1H, J = 2.9 Hz), 5.63 (m, 1H), 5.69 (d, 1H, J = 7.1 Hz), 5.87 (m, 1H), 5.97 (bd, 1H, J = 2.9 Hz, J = 9.2 Hz), 6.26 (m, 1H), 6.29 (m, 1H), 7.05−7.80 (m, 26H), 8.14 (d, 2H, J = 7.2 Hz) ppm; MS (FAB) m/e 1668 (M +

H)$^+$, 1689 (M + Na)$^+$. Anal. $C_{89}H_{99}N_7O_{25}(\cdot 2H_2O)$ calculated C 62.78%, H 6.10%, N 5.76%, measured C 62.55%, H 5.82%, N 5.57%.

Deprotection To Give Prodrug 2′-[H-D-Ala-Phe-Lys-PABC-PABC]-Paclitaxel (15). To a solution of 51.4 mg (0.0308 mmol) protected prodrug **14** in dry THF under an argon atmosphere was added glacial AcOH (8.9 μL, 5 equiv) together with tributyltin hydride (24.6 μL, 3 equiv) and a catalytic amount of $Pd(PPh_3)_4$. After 30 min, 1 mL of 0.5 M HCl/EtOAc was carefully added to the reaction solution. The product was precipitated by addition of diethyl ether, and the white precipitate was collected by means of centrifugation and washed several times with ether. *tert*-Butyl alcohol was added and evaporated again to remove an excess of HCl, and the resulting product was dissolved in water and freeze-dried, yielding 46.9 mg (100%) of prodrug **15**. Mp > 192 °C (dec);^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$): δ 1.15 (s, 3H), 1.21 (s, 3H), 1.10−2.00 (m, 9H), 1.67 (s, 3H), 1.90 (s, 3H), 2.20 (s, 3H), 2.43 (s, 3H), 2.85 (m, 4H), 3.80 (d, 1H, *J* = 6.9 Hz), 4.24 (d, 1H, *J* = 8.4 Hz), 4.31 (d, 1H, *J* = 8.4 Hz), 4.39 (dd, 1H), 4.56 (m, 1H), 5.68 (m, 1H), 4.98 (d, 1H), 5.08 (m, 4H), 5.43 (d, 1H, *J* = 2.7 Hz), 5.70 (d, 1H, *J* = 7.0 Hz), 5.97 (m, 1H), 6.22 (m, 1H), 6.32 (m, 1H), 7.05−7.68 (m, 24H), 7.71 (d, 1H, *J* = 7.2 Hz), 8.14 (d, 2H, *J* = 7.3 Hz) ppm; MS (FAB) *m/e* 1499 (M + H)$^+$, 1521 (M + Na)$^+$. Anal. $C_{81}H_{91}N_7O_{21}(\cdot 3.7HCl)$ calculated C 59.60%, H 5.85%, N 6.01%, measured C 59.60%, H 5.88%, N 5.98%.

Coupling Reaction To Give Fmoc-Trp-Lys(Boc)-OBu^t. To a solution of 3.00 g (5.73 mmol) Fmoc-Trp-ONSu (ONSu = *N*-hydroxysuccinimide) in dry CH_2Cl_2 under an argon atmosphere were added at 0 °C 0.791 mL (1.00 equiv) Et_3N and 2.12 g (1.10 equiv) of H-Lys(Boc)-OBu^t·HCl. The mixture was stirred at room temperature for 5 h, CH_2Cl_2 was added, and the organic layer was washed with 10% citric acid, saturated $NaHCO_3$, and water, dried over anhydrous Na_2SO_4, and evaporated. The white solid (3.52 g, 86%) was used without further purification. Mp 77 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.10−1.92 (m, 24H), 2.80−3.20 (m, 3H), 3.52 (d, 1H), 4.19 (t, 1H), 4.29−4.82 (m, 5H), 6.54 (d, 1H), 7.06−7.76 (m, 12H) ppm; MS (FAB) *m/e* 734 (M+ Na)$^+$,1444 (2M + Na)$^+$. Anal. $C_{41}H_{50}N_4O_7(\cdot 4H_2O)$ calculated C 62.90%, H 6.30%, N 7.15%, measured C 63.22%, H 6.49%, N 7.13%.

Deprotection and Coupling To Give Boc-D-Ala-Trp-Lys(Boc)-OBu^t. 3.52 g (4.95 mmol) of Fmoc-Trp-Lys(Boc)-OBu^t was dissolved in 100 mL of dioxane/MeOH/2 M NaOH (70/25/5) and stirred at room temperature for 1 h. The mixture was neutralized with AcOH (0.570 mL), and organic solvents were evaporated. Water and dioxane were added, and the solution was freeze-dried. Diisopropyl ether was added, and after filtration the filtrate was evaporated. The product was dissolved in dry CH_2Cl_2 and added at 0 °C to a solution of 1.41 g (4.93 mmol) of Boc-D-Ala-ONSu and 0.756 mL (1.10 equiv) of Et_3N in dry CH_2Cl_2. The mixture was stirred for 16 h after which CH_2Cl_2 was added. The organic layer was washed with 10% citric acid, saturated $NaHCO_3$, and water, dried over Na_2SO_4, and evaporated. The product was purified by means of column chromatography ((SiO_2, first EtOAc/heptane 1/1 and then $CHCl_3$/MeOH 9/1) to afford 2.26 g (3.42 mmol, 69%) of the tripeptide as a white foam. Mp 117 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.99−1.90 (m, 36 H), 2.80−3.50 (m, 4H), 3.99 (m, 1H), 4.33 (m, 1H), 4.77 (bd, 1H), 6.90−7.72 (m, 5H) ppm; MS (FAB) *m/e* 660 (M + H)$^+$, 682 (M + Na)$^+$. Anal. $C_{34}H_{53}N_5O_8(\cdot H_2O)$ calculated C 60.25%, H 8.17%, N 10.33%, measured C 60.47%, H 8.08%, N 9.73%.

Deprotection and Aloc Protection To Give Aloc-D-Ala-Trp-Lys(Aloc)-OH. A 2.56 g (4.13 mmol) amount of Boc-D-Ala-Trp-Lys(−Boc)-OBu^t was stirred in a solution of hydrochloric acid in EtOAc (3 M). After 5 h, the solvent was evaporated, and *tert*-butyl alcohol was added and evaporated twice to remove remaining hydrochloric acid. The product was freeze-dried in dioxane/water to yield a brown colored powder.

To a solution of 1.71 g (3.62 mmol) D-Ala-Phe-Lys-OH in water/CH_3CN was added Et_3N until a pH of 8 was reached. Then a solution of 1.58 g (2.20 equiv) Aloc-ONSu in CH_3CN was added, and the mixture was kept basic by adding Et_3N. After the pH of the mixture did not alter any more, a 0.5 M solution of hydrochloric acid in EtOAc was added until a pH of 3 was reached. The mixture was thoroughly extracted with CH_2Cl_2. The organic layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated. The product was purified by means of column chromatography (SiO_2, $CHCl_3$/AcOH/MeOH 85/10/5) to afford 302 mg (15%) of product as a cream colored powder. Mp 116 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00−1.80 (m, 9H), 2.80−3.35 (m, 4H), 4.13 (m, 1H), 4.30−4.95 (m, 6H), 5.01−5.40 (m, 4H), 5.70−6.30 (m, 3H), 6.90−7.70 (m, 5H) ppm; MS (FAB) *m/e* 572 (M + H)$^+$, 594 (M + Na)$^+$, 1143 (2M + Na)$^+$. Anal. $C_{28}H_{37}N_5O_8(\cdot 1^1/_2H_2O)$ calculated C 56.18%, H 6.44%, N 11.70%, measured C 56.07%, H 6.22%, N 11.21%.

Coupling of Spacer To Give Aloc-D-Ala-Trp-Lys(Aloc)-PABA. A solution of 239 mg (0.419 mmol) of Aloc-D-Ala-Trp-Lys(Aloc)-OH was dissolved in dry THF under an argon atmosphere and cooled to −40 °C. *N*-Methylmorpholine (48.3 μL, 1.05 equiv) and isobutyl chloroformate (57.0 μL, 1.05 equiv) were added. The reaction mixture was stirred for 2 h at a temperature below −30 °C. A solution of 4-aminobenzyl alcohol (51.5 mg, 1.00 equiv) and *N*-methylmorpholine (50.6 μL, 1.1 equiv) in dry THF was added dropwise to the reaction mixture. After 2 h, THF was evaporated and CH_2Cl_2 was added. The organic layer was washed with saturated $NaHCO_3$, a 0.5 M $KHSO_4$ solution and brine, dried over anhydrous Na_2SO_4, and evaporated to afford 265 mg (94%) of the desired product as a cream-colored powder, which was used without further purification. Mp 132 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.00−1.62 (m, 9H), 2.90−3.70 (m, 4H), 4.10 (m, 1H), 4.48−4.92 (m, 6H), 4.72 (s, 2H), 5.00−5.50 (m, 4H), 5.35−6.05 (m, 2H), 6.80−7.83 (m, 9H) ppm; MS (FAB) *m/e* 677 (M + H)$^+$, 699 (M + Na)$^+$.

Activation To Give Aloc-D-Ala-Trp-Lys(Aloc)-PABC-PNP. To a solution of 384 mg (0.602 mmol) of Aloc-D-Ala-Trp-Lys(Aloc)-PABA in dry THF/CH_2Cl_2 under an argon atmosphere, 4-nitrophenyl chloroformate (182 mg, 1.50 equiv) and dry pyridine (73 μL, 1.50 equiv) were added. The mixture was stirred at room temperature for 48 h, and then EtOAc was added. The organic layer was washed with 10% citric acid, brine, and water, dried over anhydrous Na_2SO_4, and evaporated yielding a yellow solid. The product was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 30/1) to afford 324 mg (67%) of the product as a cream-colored powder. Mp 100 °C;^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00−2.10 (m, 9H), 2.90−3.70 (m, 4H), 3.81 (m, 1H), 4.10 (m, 1H), 4.38−4.81 (m, 5H), 5.10−5.35 (m, 4H), 5.21 (s, 2H), 5.40−6.00 (m, 2H), 7.00−7.85 (m, 11H), 8.25 (d, 2H, *J* = 8.1 Hz); MS (FAB) *m/e* 842 (M + H)$^+$, 864 (M + Na)$^+$.

Coupling of Spacer To Give Aloc-D-Ala-Trp-Lys(Aloc)-PABC-PABA. To a solution of 219 mg (0.260 mmol) of Aloc-D-Ala-Trp-Lys(Aloc)-PABC−PNP and 35.2 mg (1.1 equiv) of 4-aminobenzyl alcohol in dry DMF under an argon atmosphere were added DIPEA (45.3 μL, 1.00 equiv) and a catalytic amount of *N*-hydroxybenzotriazole (10.5 mg, 0.30 equiv). The reaction solution was stirred for 48 h after which it was diluted with 10% propanol-2/EtOAc. The organic layer was washed with saturated $NaHCO_3$, 0.5 M $KHSO_4$, and brine, dried over anhydrous Na_2SO_4, and evaporated to dryness. The pale yellow residual film was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 15/1) to yield 192 mg (89%) of the desired product. Mp 287 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90−2.10 (m, 9H), 2.90−3.70 (m, 4H), 3.82 (m, H), 4.08 (m, 1H), 4.40−4.86 (m, 5H), 4.59 (s, 2H), 4.90−5.40 (m, 4H), 5.08 (s, 2H), 5.50 (m, 1H), 5.92 (m, 1H), 6.72−7.82 (m, 13H) ppm; MS (FAB) *m/e* 848 (M + Na)$^+$. Anal. $C_{43}H_{51}N_7O_{10}(\cdot 2^3/_4H_2O)$ calculated C 58.99%, H 6.50%, N 11.20%, measured C 59.15%, H 6.25%, N 11.15%.

Activation To Give Aloc-D-Ala-Trp-Lys(Aloc)-PABC-PABC-PNP. A solution of 70 mg (0.085 mmol) of Aloc-D-Ala-Trp-Lys(Aloc)-PABC−PABA, pyridine (17 μL, 2.5 equiv), and 4-nitrophenyl chloroformate (34 mg, 2.0 equiv) was stirred under an argon atmosphere at 0 °C for 2 h and for 24 h at room temperature. The solution was evaporated in vacuo, and the residual product was dissolved in $CHCl_3$. After washing the organic layer with brine and 0.5 M $KHSO_4$, the organic

layer was dried over anhydrous Na_2SO_4 and concentrated to dryness. The resulting crude product was subjected to column chromatography (SiO_2, $CHCl_3$/MeOH 20/1) to obtain 54 mg (64%) of the desired product as a pale yellow solid. ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.90−2.10 (m, 9H), 2.90−3.27 (m, 4H), 4.20 (m, 1H), 4.35−4.78 (m, 6H), 4.90−5.52 (m, 4H), 5.13 (s, 2H), 5.34 (s, 2H), 5.60 (m, 1H), 5.94 (m, 1H), 7.10−7.46 (m, 15H), 8.36 (d, 2H) ppm; MS (FAB) *m/e* 991 $(M + H)^+$, 1013 $(M + Na)^+$. Anal. $C_{50}H_{54}N_8O_{14}(\cdot^3/_4H_2O)$ calculated C 59.78%, H 5.57%, N 11.15%, measured C 60.12%, H 5.89%, N 10.76%.

Doxorubicin Coupling To Give Aloc-D-Ala-Trp-Lys-(Aloc)-PABC-PABC-Doxorubicin. Aloc-D-Ala-Trp-Lys(Aloc)-PABC-PABC-PNP (41 mg, 0.041 mmol) and doxorubicin·HCl (26 mg, 1.1 equiv) in *N*-methylpyrrolidinone were treated at room temperature with Et_3N (6.3 μL, 1.1 equiv). The reaction mixture was stirred in the dark for 48 h, again Et_3N (1.1 equiv) was added, and after an additional 24 h the reaction mixture was diluted with 10% 2-propanol/EtOAc. The organic layer was washed with water and brine and was dried over anhydrous Na_2SO_4. After evaporation of the solvents, the crude product was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 9/1) followed by circular chromatography using a Chromatotron supplied with a 2 mm silica plate ($CHCl_3$−MeOH 9/1), to yield 45 mg (78%) of the protected prodrug. Mp 130 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.92−1.52 (m, 14H), 2.15 (dd, 1H), 2.36 (bd, 1H), 3.18 (bd, 1H), 2.90−3.10 (m, 6H), 3.59 (bs, 1H), 3.82 (m, 1H), 3.85 (m, 1H), 4.11 (s, 3H), 4.21 (m, 1H), 4.45 (dd, 1H), 4.30−4.62 (m, 5H), 4.76 (s, 2H), 4.96 (s, 2H), 5.11 (s, 2H), 5.13−5.40 (m, 4H), 5.48 (bs, 1H), 5.58 (m, 2H), 5.91(m, 1H), 6.70−7.39 (m, 11H), 7.41 (d, 1H, *J* = 8.4 Hz), 7.63 (d, 2H), 7.78 (t, 1H), 8.03 (d, 1H, *J* = 7.6 Hz) ppm; MS (FAB) *m/e* 1417 $(M + Na)^+$.

Deprotection To Give Prodrug H−D-Ala-Trp-Lys-PABC-PABC-Doxorubicin (16). To a solution of 36 mg (0.026 mmol) protected prodrug Aloc-D-Ala-Trp-Lys(Aloc)-PABC−PABC-Doxorubicin in dry THF/CH_2Cl_2 under an argon atmosphere was added morpholine (22 μL, 10 equiv) together with a catalytic amount of $Pd(PPh_3)_4$. The reaction mixture was stirred for 1 h in the dark. The red precipitate was collected by means of centrifugation. EtOAc was added, and the mixture was acidified using 0.5 mL of 1 M hydrochloric acid/EtOAc. The precipitate was collected by means of centrifugation and washed several times with EtOAc. *tert*-Butyl alcohol was added and evaporated, and the resulting red film was freeze-dried in water yielding 28 mg (72%) of the doxorubicin prodrug **16**. Mp 95 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.10−1.96 (m, 14H), 2.09 (m, 1H), 2.35 (bd, 1H), 2.79−3.39 (m, 6H), 3.60 (s, 1H), 4.00 (bs, 1H), 4.09 (s, 3H), 3.95−4.15 (m, 2H), 4.29 (m, 1H), 4.51 (m, 1H), 4.77 (s, 2H), 4.86 (2 × d, 2H), 5.02 (s, CH_2), 5.38 (bs, 1H), 5.48 (bs, 1H), 6.99−7.72 (m, 11H), 7.55 (d, 2H, *J* = 8.2 Hz), 7.62 (d, 1H, *J* = 7.6 Hz), 7.83 (t, 1H), 8.05 (d, 1H, *J* = 7.7 Hz) ppm; MS (FAB) *m/e* 1228 $(M + H)^+$. Anal. $C_{63}H_{70}N_8O_{18}(\cdot 7\ ^1/_2HCl)$ calculated C 50.42%, H 5.21%, N 7.47%, measured C 50.56%, H 5.48%, N 7.35%.

Coupling of a Third Spacer to 11 To Give Aloc-D-Ala-Phe-Lys(Aloc)-PABC-PABC-PABA (17). A 100 mg (0.105 mmol) amount of Aloc-D-Ala-Phe-Lys(Aloc)-PABC-PABC-PNP **11** was dissolved in dry DMF under an argon atmosphere and cooled to −8 °C. 4-Aminobenzyl alcohol (14.2 mg, 1.1 equiv), DIPEA (18.3 μL, 1.0 equiv), and 1-hydroxybenzotriazole (HOBt) (4 mg, 0.3 equiv) were added. The reaction mixture was stirred for 48 h at room temperature and diluted with 10% 2-propanol/EtOAc. The organic layer was washed with water, saturated $NaHCO_3$, 0.5 M $KHSO_4$, and brine, dried over anhydrous Na_2SO_4, and evaporated to yield the desired product **17** as a cream-colored powder 86 mg (88%), which was used without further purification. Mp 126 °C; ^{1}H NMR (300 MHz $CDCl_3$) δ 0.95−2.05 (m, 9H), 2.88−3.11 (m, 4H), 3.95−4.62 (m, 7H), 4.75 (s, 2H), 5.12−5.21 (m, 6H), 5.09 (s, 2H), 5.65−6.00 (m, 2H), 6.79−7.41 (m, 15H), 7.62 (d, 2H) ppm; MS (FAB) *m/e* 959 $(M + Na)^+$.

Activation of 17 to Aloc-D-Ala-Phe-Lys(Aloc)-PABC-PABC-PABC-PNP (18). To a solution of 59 mg (0.063 mmol) of **17** in dry THF and CH_2Cl_2 under an argon atmosphere were added at −40 °C, respectively, pyridine (13 μL, 2.5 equiv) and 4-nitrophenyl chloroformate (25 mg, 2.0 equiv). After stirring for 4.5 h at −40 °C and overnight at 6 °C, pyridine (10 μL, 2.0 equiv) and 4-nitrophenyl chloroformate (25 mg, 2.0 equiv) were added again. This was repeated after 48 h stirring at 6 °C. After another 48 h, the solution was evaporated in vacuo and the residual product was dissolved in $CHCl_3$. The organic layer was washed with 10% citric acid, brine, and water, dried over anhydrous Na_2SO_4, and evaporated, yielding a yellow solid. The crude product was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 15/1) to give the desired product **18** quantitatively. ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.12−1.89 (m, 9H), 3.04 (m, 1H), 3.14 (m, 2H), 3.27 (bd, 1H), 4.09 (m, 1H), 4.28 (m, 1H), 4.34−4.68 (m, 5H), 5.02−5.40 (m, 4H), 5.14 (s, 2H), 5.21 (s, 2H), 5.31 (s, 2H), 5.72 (m, 1H), 5.90 (m, 1H), 7.10−7.52 (m, 17H), 7.63 (d, 2H, *J* = 8.3 Hz), 8.27 (d, 2H, *J* = 9.1 Hz) ppm; MS (FAB) *m/e* 1102 $(M + H)^+$, 1124 $(M + Na)^+$.

Doxorubicin Coupling To Give Aloc-D-Ala-Phe-Lys-(Aloc)-PABC-PABC-PABC-Doxorubicin (19). The 4-nitrophenyl carbonate **18** (69 mg, 0.063 mmol) and doxorubicin·HCl (40 mg, 1.1 equiv) in *N*-methylpyrrolidinone were treated at room temperature with Et_3N (9.7 μL, 1.1 equiv). The reaction mixture was stirred in the dark for 24 h, and the reaction mixture was diluted with 10% 2-propanol/EtOAc. The organic layer was washed with water and brine and was dried over anhydrous Na_2SO_4. After evaporation of the solvents, the crude product was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 9/1) followed by circular chromatography using a Chromatotron supplied with a 2 mm silica plate ($CHCl_3$/MeOH; 9/1), to yield 65 mg (71%) of the protected prodrug **19.** ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.10−1.80 (m, 14H), 2.14 (m, 1H), 2.36 (m, 1H), 2.82−3.41 (m, 6H), 3.37 (s, 1H), 3.60 (bs, 1H), 4.03 (m, 1H), 4.29 (m, 1H), 4.07 (s, 3H), 4.29 (dd, 1H), 4.37−4.68 (m, 5H), 4.76 (s, 2H), 4.95 (bs, 2H), 5.10 (s, 2H), 5.14 (s, 2H), 5.02−5.35 (m, 4H), 5.27 (bs, 1H), 5.47 (bs, 1H), 5.70 (m, 1H), 5.89 (m, 1H), 7.09−7.50 (m, 16H), 7.64 (d, 2H, *J* = 8.4 Hz), 7.79 (t, 1H, *J* = 8.1 Hz), 8.06 (d, 1H, *J* = 7.5 Hz) ppm; MS (FAB) *m/e* 1506 $(M + H)^+$, 1528 $(M + Na)^+$.

Deprotection To Give Prodrug H-D-Ala-Phe-Lys-PABC-PABC-PABC-Doxorubicin (20). To a solution of 40 mg protected prodrug **19** (0.027 mmol) in dry THF/CH_2Cl_2 under an argon atmosphere were added morpholine (24 μL, 10 equiv) and a catalytic amount of $Pd(PPh_3)_4$. The reaction mixture was stirred for 1 h in the dark. The red precipitate was collected by means of centrifugation and washed several times with EtOAc. Water and dioxane were added and the mixture was acidified using 4.3 mL of 0.125 mM hydrochloric acid. After freeze-drying 26 mg (69%) of doxorubicin prodrug **20** was obtained. Mp 66 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.19 (d, 3H, *J* = 6.9 Hz), 1.27 (d, 3H, *J* = 6.6 Hz), 1.25−2.00 (m, 8H), 2.18 (dd, 1H), 2.33 (bd, 1H), 2.89−3.38 (m, 6H), 3.60 (s, 1H), 3.72 (m, 1H), 4.08 (s, 3H), 4.03−4.20 (m, 2H), 4.53 (dd, 1H), 4.66 (m, 1H), 4.77 (s, 2H), 4.96 (s, 2H), 5.11 (s, 2H), 5.17 (s, 2H), 5.27 (bs, 1H), 5.48 (bs, 1H), 7.05−7.35 (m, 16H), 7.52 (d, 2H, *J* = 8.5 Hz), 7.84 (t, 1H), 8.01 (d, 1H, *J* = 7.7 Hz) ppm.; MS (FAB) *m/e* 1337 $(M + H)^+$, 1359 $(M + Na)^+$. Anal. $C_{69}H_{76}N_8O_{20}(\cdot 6^1/_2HCl)$ calculated C 52.64%, H 5.28%, N 7.12%, measured C 52.66%, H 5.36%, N 6.78%.

Synthesis of Z-Protected Spacer 22. To a solution of 1.21 g (13.7 mmol) *N*,*N*′-dimethylethylenediamine in dry CH_2Cl_2 under an argon atmosphere at room temperature was added dropwise a solution of Z-ONSu (338 mg, 1.36 mmol) in dry CH_2Cl_2. After stirring for 120 min, the solution was concentrated in vacuo. The residual product was dissolved in EtOAc, and the organic layer was washed with brine. The organic solvent was dried over anhydrous Na_2SO_4 and evaporated to dryness. The oily product was purified by means of column chromatography (SiO_2, $CHCl_3$/MeOH 1/1) to obtain 249 mg (83%) of the product **22** as an oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.42 (bd, 3H), 2.73 (m, 2H), 2.95 (s, 3H), 3.41 (bs, 2H), 5.13 (s, 2H), 7.25−7.40 (m, 5H) ppm.

Coupling of 22 To Give 2′-[Z-N(Me)-$(CH_2)_2$-N(Me)CO]-Paclitaxel (23). To a solution of 114 mg (0.112 mmol) of 2′-activated paclitaxel and 25 mg of Z-protected *N*,*N*′-dimethyl-

ethylenediamine **22** in dry CH_2Cl_2 under an argon atmosphere at −50 °C was added Et_3N (20.0 μL, 0.144 mmol). The reaction solution was stirred 7 h at −40 °C and then overnight at room temperature. The solution was diluted with CH_2Cl_2 and washed with saturated $NaHCO_3$, brine, and 0.5 M $KHSO_4$. The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo to obtain a yellow film. The product was purified by column chromatography (SiO_2, EtOAc/Hex 2/1) to obtain 113 mg (92%) of the desired product **23**. Mp 130−131 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.12 (s, 3H), 1.21 (s, 3H), 1.70 (s, 3H), 2.00 (s, 3H), 2.26 (s, 3H), 2.60 (s, 3H), 2.90 (s, 3H), 2.94 (s, 3H), 2.97 (m, 1H), 3.06 (m, 1H), 3.54 (m, 1H), 3.78 (m, 1H), 3.84 (d, 1H, *J* = 7.2 Hz), 4.23 (d, 1H, *J* = 8.4 Hz), 4.32 (d, 1H, *J* = 8.4 Hz), 4.47 (m, 1H), 4.69 (d, 1H, *J* = 12.4 Hz), 4.85 (d, 1H, *J* = 12.4 Hz), 5.01 (m, 1H), 5.47 (d, 1H, *J* = 2.9 Hz), 5.68 (d, 1H, *J* = 7.0 Hz), 6.19 (dd, 1H, *J* = 9.8 Hz, *J* = 2.9 Hz), 6.28 (s, 1H), 6.33 (m, 1H), 6.94−7.70 (m, 16H), 7.83 (d, 2H, *J* = 7.3 Hz), 8.16 (d, 2H, *J* = 7.1 Hz), 8.57 (d, 1H, *J* = 9.8 Hz) ppm; MS (FAB) *m/e* 1102 $(M + H)^+$, 1124 $(M + Na)^+$. Anal. $C_{60}H_{67}N_3O_{17}(\cdot H_2O)$ calculated C 64.33%, H 6.21%, N 3.73%, measured C 64.65%, H 6.11%, N 3.76%.

Deprotection of 23 to 2′-[HN(Me)−$(CH_2)_2$-N(Me)CO]-Paclitaxel (24). To a solution of 61.8 mg (0.0561 mmol) **23** in 5% AcOH/MeOH was added a catalytic amount of 10% Pd/C. The mixture was stirred for 1 h under a H_2 atmosphere. The Pd−C was removed by means of centrifugation. EtOAc was added, and MeOH was evaporated in vacuo. The organic layer was extracted with water. The water layer was freeze-dried yielding 78.0 mg (100%) of the desired protonated product **24**.

Coupling of Fragments 8 and 24 to 2′-[Aloc-D-Ala-Phe-Lys(Aloc)-PABC-N(Me)-$(CH_2)_2$-N(Me)CO]-Paclitaxel (25). To a solution of 152 mg (0.0958 mmol) of paclitaxel-spacer **24** and 80.7 mg (0.101 mmol) of compound **8** in dry THF under an argon atmosphere was added Et_3N (200 μL, 1.44 mmol). After 24 h the solution was concentrated to dryness and the residual product was dissolved in CH_2Cl_2 and washed with saturated $NaHCO_3$ and brine. The organic layer was dried over anhydrous Na_2SO_4 and evaporated in vacuo. The crude product was subjected to column chromatography (SiO_2, EtOAc/Hex/MeOH 5/5/1) to obtain 113 mg (72%) of the protected prodrug **25**. Mp 127−128 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13 (s, 3H), 1.22 (s, 3H), 1.27 (d, 3H, *J* = 5.6 Hz), 1.04−2.00 (m, 6H), 1.69 (s, 3H), 2.00 (s, 3H), 2.22 (s, 3H), 2.59 (s, 3H), 2.90 (s, 3H), 2.91 (s, 3H), 2.76−3.46 (m, 6H), 3.54 (m, 1H), 3.74 (m, 1H), 3.84 (d, 1H, *J* = 7.0 Hz), 4.00−5.00 (m, 3H), 4.23 (d, 1H, *J* = 8.4 Hz), 4.32 (d, 1H, *J* = 8.4 Hz), 4.48 (m, 1H), 4.62 (d, 1H, *J* = 12.3 Hz), 4.83 (d, 1H, *J* = 12.4 Hz), 4.30−4.73 (m, 4H), 4.93−5.39 (m, 5H), 5.48 (d, 1H, *J* = 2.9 Hz), 5.69 (d, 1H, *J* = 7.0 Hz), 5.54−5.78 (m, 1H), 5.88 (m, 1H), 6.18 (bd, 1H), 6.30 (s, 1H), 6.33 (m, 1H), 7.05−7.78 (m, 20H), 7.82 (d, 2H, *J* = 7.4 Hz), 8.16 (d, 2H, *J* = 7.2 Hz) ppm; MS (FAB) *m/e* 1653 $(M + Na)^+$. Anal. $C_{86}H_{102}N_8O_{24}$ calculated C 62.61%, H 6.35%, N 6.79%, measured C 62.40%, H 6.31%, N 6.36%.

Deprotection To Give Prodrug 2′-[H-D-Ala-Phe-Lys-PABC-N(Me)-$(CH_2)_2$-N(Me)CO]-Paclitaxel (26). To a solution of 83.0 mg (0.0509 mmol) protected prodrug **25** in dry THF under an argon atmosphere was added glacial AcOH (12 μL, 4.0 equiv) together with tributyltin hydride (41 μL, 3.0 equiv) and a catalytic amount of $Pd(PPh_3)_4$. After 30 min the product was precipitated by addition of diethyl ether. The white precipitate was collected by means of centrifugation and washed several times with diethyl ether. *tert*-Butyl alcohol was added and evaporated again to remove an excess of HCl, and the resulting product was dissolved in water/dioxane and freeze-dried, yielding 56 mg (70%) of prodrug **26**. Mp 142 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.13 (s, 3H), 1.21 (s, 3H), 1.26 (d, 3H, *J* = 6.6 Hz), 1.05−2.00 (m, 6H), 1.69 (s, 3H), 2.00 (s, 3H), 2.22 (s, 3H), 2.58 (s, 3H), 2.89 (s, 3H), 2.91 (s, 3H), 2.67−3.64 (m, 3H), 2.95 (m, 1H), 3.07 (m, 2H), 3.15 (m, 1H), 3.78 (m, 1H), 3.83 (d, 1H, *J* = 7.1 Hz), 4.10−5.05 (m, 2H), 4.22 (d, 1H, *J* = 8.4 Hz), 4.32 (d, 1H, *J* = 8.4 Hz), 4.46 (m, 1H), 4.60 (m, 1H), 4.65 (d, 1H, *J* = 12.3 Hz), 4.80 (d, 1H, *J* = 12.4 Hz), 4.99 (bd, 5H, *J* = 7.4 Hz), 5.47 (d, 1H, *J* = 2.9 Hz), 5.68 (d, 1H, *J* = 6.9 Hz), 6.17 (bd, 1H, *J* = 2.9 Hz, *J* = 9.6 Hz), 6.30 (s, 1H), 6.31 (m, 1H), 7.05−7.70 (m, 20H), 7.82 (d, 2H, *J* = 7.5 Hz), 8.16 (d, 2H, *J* = 7.2 Hz), 8.54 (d, 1H, *J* = 9.6 Hz) ppm; MS (FAB) *m/e* 1463 $(M + H)^+$, 1485 $(M + Na)^+$. Anal. $C_{85}H_{97}N_7O_{22}(\cdot 3AcOH)$ calculated C 61.04%, H 6.50%, N 6.71%, measured C 60.91%, H 6.45%, N 7.10%.

Stability of the Double Spacer-Containing Paclitaxel Prodrugs 15 and 26 in Buffer. The prodrugs were incubated at a concentration of 150 μM in 0.1 M Tris/HCl buffer (pH 7.3) for 72 h at 37 °C and showed no formation of degradation products (TLC, RP_{18}; CH_3CN/H_2O/AcOH 19/19/2).

Stability of the Double and Triple Spacer-Containing Doxorubicin Prodrugs 13, 16, and 20 in Buffer. The prodrugs were incubated at a concentration of 100−135 μM in 0.1 M Tris/HCl buffer (pH 7.3) for 72 h at 37 °C and showed no formation of degradation products (TLC, RP_{18}; CH_3CN/H_2O/AcOH 19/19/2).

Cytotoxicity. The antiproliferative effect of prodrugs and parent drugs was determined in vitro applying seven well-characterized human tumor cell lines and the microculture sulforhodamine B (SRB) test. The antiproliferative effects were determined and expressed as ID_{50} values (ng/mL), which are the (pro)drug concentrations that gave 50% inhibition when compared to control cell growth after 5 days of incubation. Cell lines: MCF-7; breast cancer. EVSA-T; breast cancer. WIDR; colon cancer. IGROV; ovarian cancer. M19; melanoma. A498; renal cancer. H226; nonsmall cell lung cancer.

cell line	MCF-7	EVSA-T	WIDR	IGROV	M19	A498	H226
prodrug **13**	242	546	627	896	302	2303	503
prodrug **15**	11	5	5	22	7	25	7
prodrug **16**	466	448	445	234	162	631	857
prodrug **20**	481	244	568	115	198	354	907
prodrug **26**	60	119	117	499	96	681	62
paclitaxel	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2
doxorubicin	10	8	11	60	16	90	199

Enzymatic Hydrolysis of the Elongated Spacer System-Containing Paclitaxel Prodrugs by Plasmin. Enzymatic hydrolysis of the paclitaxel prodrugs was investigated by incubation at a prodrug concentration of 200 μM in 0.1 M Tris/hydrochloric acid buffer (pH 7.3) in the presence of 100 μg/mL human plasmin (Fluka) (0.3 U/mg) at 37 °C. Both double spacer-containing paclitaxel prodrugs were converted to yield the corresponding parent drug. Capillary electrophoresis was carried out with a CE Ext. Light Path Capillary (80.5 cm, 50 μm), with 1:1 MeOH/0.05 M sodium phosphate buffer (pH 7.0) as eluent. Detection was performed at 200 and 254 nm.

Enzymatic Hydrolysis of the Elongated Spacer System-Containing Doxorubicin Prodrugs by Plasmin. Enzymatic hydrolysis of the doxorubicin prodrugs was investigated by incubation in a 0.1 M Tris/HCl buffer (pH = 7.3) in the presence of 0.0025 U/mL human plasmin at 37 °C, on three or four separate occasions. The prodrugs were incubated at concentrations, yielding a maximal doxorubicin concentration of approximately 1 μM in a total volume of 2 mL. At 1, 5, 10, 20, and 40 min and 1, 2, 4, and 8 h after the start of the incubation an aliquot of 100 μL was withdrawn and added to 900 μL of ice-cold human plasma in a glass tube, to which a volume of 0.5 mL of acetone was added. After vigorous mixing, the samples were stored at −80 °C until analysis. A preincubation sample was taken in the absence of human plasmin.

Doxorubicin Analysis. The concentration of doxorubicin, released from the prodrugs, was determined by a validated reversed-phase high-performance liquid chromatographic assay published by de Bruijn et al.[33] In brief, after addition of 100 μL of the internal standard daunorubicin in acetone and 100 μL of aqueous zinc sulfate (70%), the samples were vigorously mixed in a vortex for 10 min. Subsequently, the samples were centrifuged for 15 min at 4000*g*. The clear supernatant was transferred to a clean glass tube and evaporated under a gentle stream of nitrogen for 45 min at 60 °C. Aliquots of 100 μL of the residue were injected onto a HPLC. Doxorubicin and daunorubicin were separated isocratically at

50 °C on a stainless steel column of 150 × 4.6 mm, packed with Inertsil ODS 80A material (5 μm particle size). The mobile phase was composed of water (pH = 2.0)/CH_3CN/THF (76:24:0.5, v/v/v). The mobile phase was delivered at a flow rate of 1.25 mL/min, and the column effluent was monitored fluorimetrically at excitation and emission wavelengths of 480 and 560 nm, respectively.

Data Analysis of Doxorubicin Incubation Experiments. The data were fitted to a sigmoidal maximum effect model based on the modified Hill equation, as follows: $C = C_{min} + C_{max} * [(T^{\gamma})/(T^{\gamma} + T_{50}\gamma)]$, in which C_{min} is the concentration of doxorubicin in the sample in the absence of plasmin, C_{max} is the maximum reachable doxorubicin concentration, T is the time in hours, T_{50} is the time in hours needed to reach 50% of the maximum reachable doxorubicin concentration and γ is the Hill constant describing the sigmoidicity of the curve.

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