Synthesis and Biological Evaluation of 2′-Carbamate-Linked and 2′-Carbonate-Linked Prodrugs of Paclitaxel: Selective Activation by the Tumor-Associated Protease Plasmin

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Received January 14, 2000

The nontoxic paclitaxel-2′-carbamate prodrugs **2**−**5** and paclitaxel-2′-carbonate prodrug **6** were synthesized and tested for activation by the tumor-associated enzyme plasmin. A generally applicable method for the synthesis of paclitaxel-2′-carbamates was developed. In buffer solution, prodrug **2**, which contained an unsubstituted ethylenediamine spacer, was not stable, whereas prodrugs **3**−**6** were highly stable. Prodrugs **3**−**6** showed on average a decrease in cytotoxicity of more than 8000-fold in comparison with the parent drug in seven human tumor cell lines. Prodrugs **5** and **6** are the most nontoxic prodrugs of paclitaxel that yield the free parent drug upon selective activation currently reported. Enzyme hydrolysis and spacer elimination rates were determined by incubation of prodrugs **5** and **6** in the presence of human plasmin. From these results, prodrug **6** was selected as the promising prodrug for further in vivo studies.

Introduction

The lack of selectivity of chemotherapeutic anticancer agents such as paclitaxel (**1**) (Chart 1) is still a serious drawback in conventional cancer chemotherapy. To render anticancer drugs more selective for tumor cells, the existence of tumor-associated enzymes can be exploited to convert a nontoxic prodrug into the biologically active parent compound in a site-specific manner. A high level of a cytotoxic anticancer drug in tumor tissue can be generated via this concept.

The serine protease plasmin plays a key role in tumor invasion and metastasis.[1,2] The proteolytically active form of plasmin is localized at the tumor level because it is formed from its inactive proenzyme form plasminogen by urokinase-type plasminogen activator, produced by cancer and/or stroma cells.[3] Active plasmin is very rapidly inhibited by inhibitors such as α_2-antiplasmin in the blood circulation. Thus, plasmin is a very promising enzyme for exploitation in a tumor-specific prodrug approach and can serve as a suitable target for prodrug monotherapy.[4−6] In a preceding paper,[6] we reported the first anthracycline prodrugs that were efficiently activated by plasmin. The first paclitaxel prodrugs designed for activation by plasmin are now described.

Paclitaxel (Taxol)[7] is a chemotherapeutic agent with promising antitumor activity,[8] especially against ovarian, breast, and lung cancers.[9] Although paclitaxel, like other cytotoxic agents, shows severe side effects, relatively few efforts have been made to develop selective antitumor prodrugs of paclitaxel. An additional problem for clinical application of this compound is its low water solubility, and for administration it must be dissolved in Chremophor EL, which is believed to cause hypersensitivity reactions.[10] The low therapeutic index of paclitaxel begs development of an improved delivery system.[11] The conversion of paclitaxel into prodrugs that are hydrolyzed by the tumor-associated protease plasmin might yield more selective derivatives that, in addition, show improved water solubility.

A majority of the efforts for derivatization of paclitaxel with peptides were performed to obtain derivatives with improved water solubility,[12−16] and only a few examples of the targeting of paclitaxel have been reported.[17−19] Recently, peptide conjugates of paclitaxel[18,19] have been reported which were claimed to be extracellularly stable and that contained a monoclonal antibody which was needed for tumor specificity. After internalization by the cell, the dipeptide needed to be cleaved intracellularly by lysosomal enzymes (cathepsin B).

It was reasoned that 2′-carbamate prodrugs of paclitaxel would best fulfill the requirements of an ideal prodrug, i.e., low cytotoxicity for healthy tissue, high stability against unspecific enzymes, and fast hydrolysis by the tumor-associated enzyme. By modification of the 2′-hydroxyl functionality the prodrug could be expected to lose its cytotoxic activity.[20−22] It is important to note that linkage of the promoiety to the 2′-position of paclitaxel is only feasible if the resulting bond is biologically stable and does not lead to premature drug release. In literature several paclitaxel-2′-esters and paclitaxel-2′-carbonates have been reported that were hydrolyzed at the 2′-position by nonspecific enzymes.[23,24] Very recently, two papers appeared in which paclitaxel prodrugs were reported containing a labile 2′-ester linkage.[25,26] In the first report,[25] a peptide derivative of paclitaxel was described which was designed for binding to a cell surface receptor. Although the paclitaxel conjugate was shown to retain receptor binding properties, the conjugate might be unspecifically cleaved at the paclitaxel-2′-succinate linkage by ubiquitous extracellular esterases. In the second paper,[26] a tetrasaccharide conjugate of paclitaxel was presented in which the

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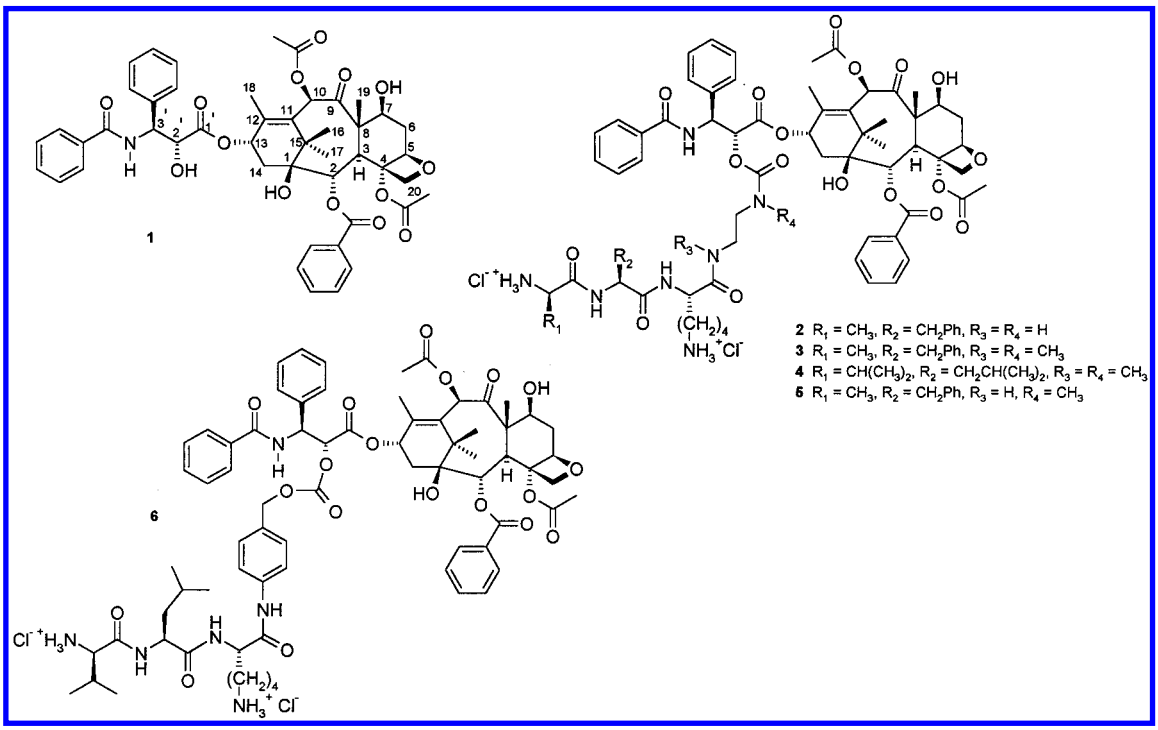
10.1021/jm0009078 CCC: $19.00
Published on Web 07/19/2000

Chart 1



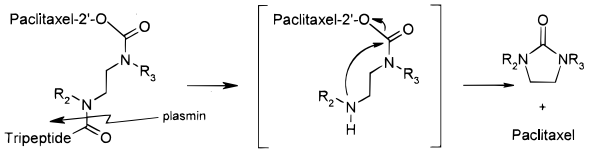
promoiety was linked to the 2′-position of paclitaxel via a labile 2′-ester linkage. This paclitaxel conjugate was designed for binding to hyaluronic acid receptors.

A high priority in the design of our paclitaxel prodrugs is that they should show a marked decrease in cytotoxicity, relative to paclitaxel. By linking the specifier to the drug via a 2′-carbamate, the resulting bond is expected to be stable against unspecific hydrolysis by esterases, proteases, and other enzymes distributed ubiquitously throughout the body. None of the paclitaxel derivatives reported until now, designed for exerting increased tumor selectivity, contain a paclitaxel-2′-carbamate linkage.

On the basis of the above rationale, paclitaxel carbamate prodrugs **2**−**5** (Chart 1) were synthesized. After plasmin hydrolysis the spacer should spontaneously cyclize to yield a cyclic urea derivative, thereby releasing free paclitaxel, as depicted in Scheme 1.[27] The diamine spacers were selected because they are appropriate for attachment to the tripeptide via an amide bond and attachment to the drug via a carbamate bond. In addition, the incorporation of a spacer might enhance the rate of plasmin hydrolysis. Since it has been reported that a *N*,*N*′-dimethyl-substituted ethylenediamine spacer gives a faster cyclization reaction than the unsubstituted spacer,[27] this entity was also incorporated in paclitaxel tripartate prodrugs.

Although a carbonate linkage between drug and promoiety would be expected to be less stable against unspecific enzymes than a carbamate linkage, it would probably be much less susceptible to esterase cleavage than an ester linkage. The paclitaxel-2′-carbonate prodrug **6** which contains a 1,6-elimination spacer was then synthesized (Chart 1). This aromatic spacer proved to be a versatile self-eliminating connector in our plasmin prodrugs of anthracyclines.[6] If cleavage of the tripeptide by plasmin should occur, then spacer elimination is assured.[28]

Scheme 1



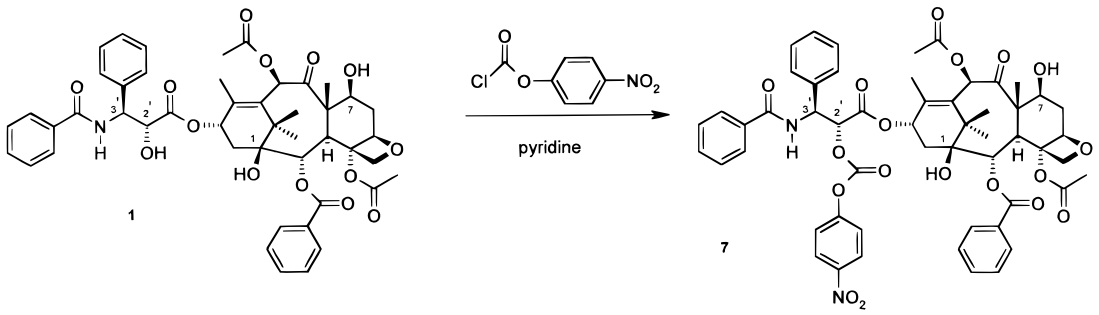
The synthesized prodrugs were characterized biologically by determination of (i) stability during 3 days of incubation in buffer solution, (ii) kinetics of enzymatic activation and spacer cyclization during incubation in the presence of plasmin, and (iii) cytotoxicity in seven human tumor cell lines compared with the cytotoxicity of the parent drug, paclitaxel.

Chemistry

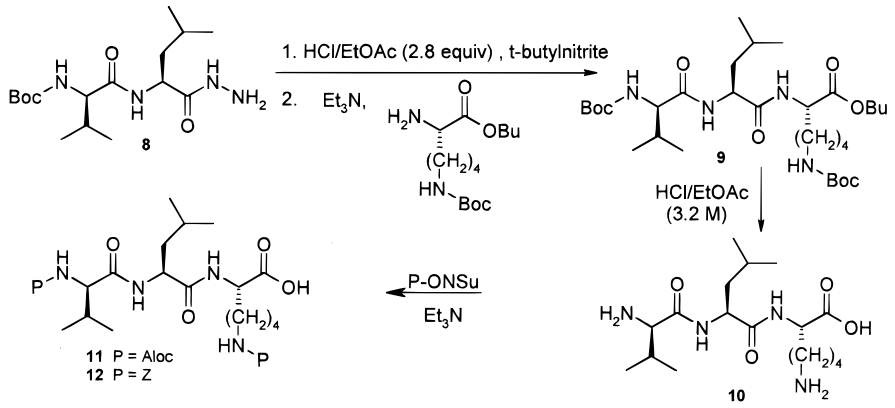
The prodrugs in the present paper were prepared by the coupling reactions between the drug and the spacer end of the tripeptide spacer moieties. D-Ala-Phe-Lys and D-Val-Leu-Lys were selected as tripeptides, since they can serve as suitable plasmin substrates.[29]

Since 2′-carbamate prodrugs of paclitaxel have not been reported, we developed a route to selectively convert the 2′-hydroxyl group into the corresponding carbamate. It was thought that the most feasible route for the preparation of the 2′-carbamate was to react the amino group of the tripeptide spacer moiety with a 2′-activated paclitaxel derivative. During its synthesis however, the 2′-activated paclitaxel derivative should not react with the 3′-nitrogen in a cyclization reaction to give the corresponding oxazolone which is formed easily.[12,24] When the activation of the 2′-hydroxyl function was attempted with carbonyldiimidazole (CDI), the undesired ring-closed product was obtained. Reaction of paclitaxel with 4-nitrophenyl chloroformate at 0 °C also led to the undesired 2′,7-disubstituted product.

Scheme 2



Scheme 3



When paclitaxel was treated with 10 equiv of 4-nitrophenyl chloroformate at −50 °C in the presence of pyridine, the desired activated 2′-carbonate **7** was formed in a reasonable yield (Scheme 2). No oxazolone was detected, and the 7-hydroxyl group remained unaffected.

The synthesis of the D-Ala-Phe-Lys tripeptide was described in a preceding paper;[6] D-Val-Leu-Lys was prepared via well-established peptide chemistry (Scheme 3). Allyloxycarbonyl (Aloc) or benzyloxycarbonyl (Z) was chosen as the protecting group. Doubly protected tripeptides **11** and **12** were synthesized starting with the coupling of H-Lys(Boc)-OBu to the hydrazide of Boc-protected D-Val-Leu via in situ formation of the corresponding azide (Scheme 3). Removal of the Boc groups and the *tert*-butyl ester protection under acidic conditions yielded deprotected tripeptide **10**, and following protection of the amines using Aloc-ONSu or Z-ONSu under basic conditions yielded the doubly protected tripeptides **11** and **12**. The protection of D-Ala-Phe-Lys[6] with Z groups yielding Z-D-Ala-Phe-Lys(Z)-OH (**13**) was performed in a similar manner.

Ethylenediamine was then coupled to the Z-protected tripeptide **13** using isobutyl chloroformate, and the obtained tripeptide spacer was isolated as its acetic acid salt (**14**, Scheme 4). This salt was added to paclitaxel-2′-carbonate (**7**) in the presence of a base to yield protected prodrug **15**. The Z groups were removed by hydrogenolysis resulting in the formation of prodrug **2**. The presence of 5% acetic acid in the solvent was essential to prevent formation of baccatin III.

The attachment of the *N*,*N*′-dimethyl-substituted ethylenediamine spacer to the tripeptide was attempted in a manner similar to that for the unsubstituted spacer. However, under basic conditions the amino group of the spacer was substituted with a Z group, probably via an intermolecular reaction, to give a fully protected tripeptide spacer moiety. The spacer was monoprotected by reaction with excess of spacer with di-*tert*-butyldicarbonate prior to coupling to prevent this. Compound **17** was then connected to both tripeptides **12** and **13** to yield protected promoieties **18** and **19**, and the Boc group was removed using hydrochloric acid/ethyl acetate resulting in the formation of ammonium salts **21** and **22**. The final coupling step was performed by addition of **21** or **22** to a solution of 2′-activated paclitaxel **7** and base to yield both protected prodrugs **24** and **25**. Hydrogenolysis in the presence of acetic acid yielded paclitaxel prodrugs **3** and **4**. The synthesis of prodrug **5** containing the monomethyl spacer was achieved in a similar manner. Protected tripeptide **13** was reacted with *N*-Boc-*N*-methylethylenediamine (**16**) to yield compound **20**. The Boc-substituted amine was then deprotected and reacted with activated paclitaxel derivative **7**. Removal of both Z groups in the usual manner yielded paclitaxel prodrug **5**.

For the construction of paclitaxel prodrug **6**, 4-aminobenzyl alcohol was first coupled to the tripeptide Aloc-D-Val-Leu-Lys(Aloc)-OH (**11**) (Scheme 5) using isobutyl chloroformate, and the resultant benzylic alcohol **27** was activated by treatment with 4-nitrophenyl chloroformate, to give carbonate **28**. Reaction of the 2′-hydroxyl of paclitaxel with this activated carbonate derivative in the presence of DMAP led to the protected tripartate prodrug **29**. The Aloc protecting groups were removed using tributyltin hydride in the presence of a palladium catalyst.[30] Final treatment with hydrochloric acid in ethyl acetate provided the corresponding paclitaxel prodrug **6** as its double ammonium salt.

Scheme 4

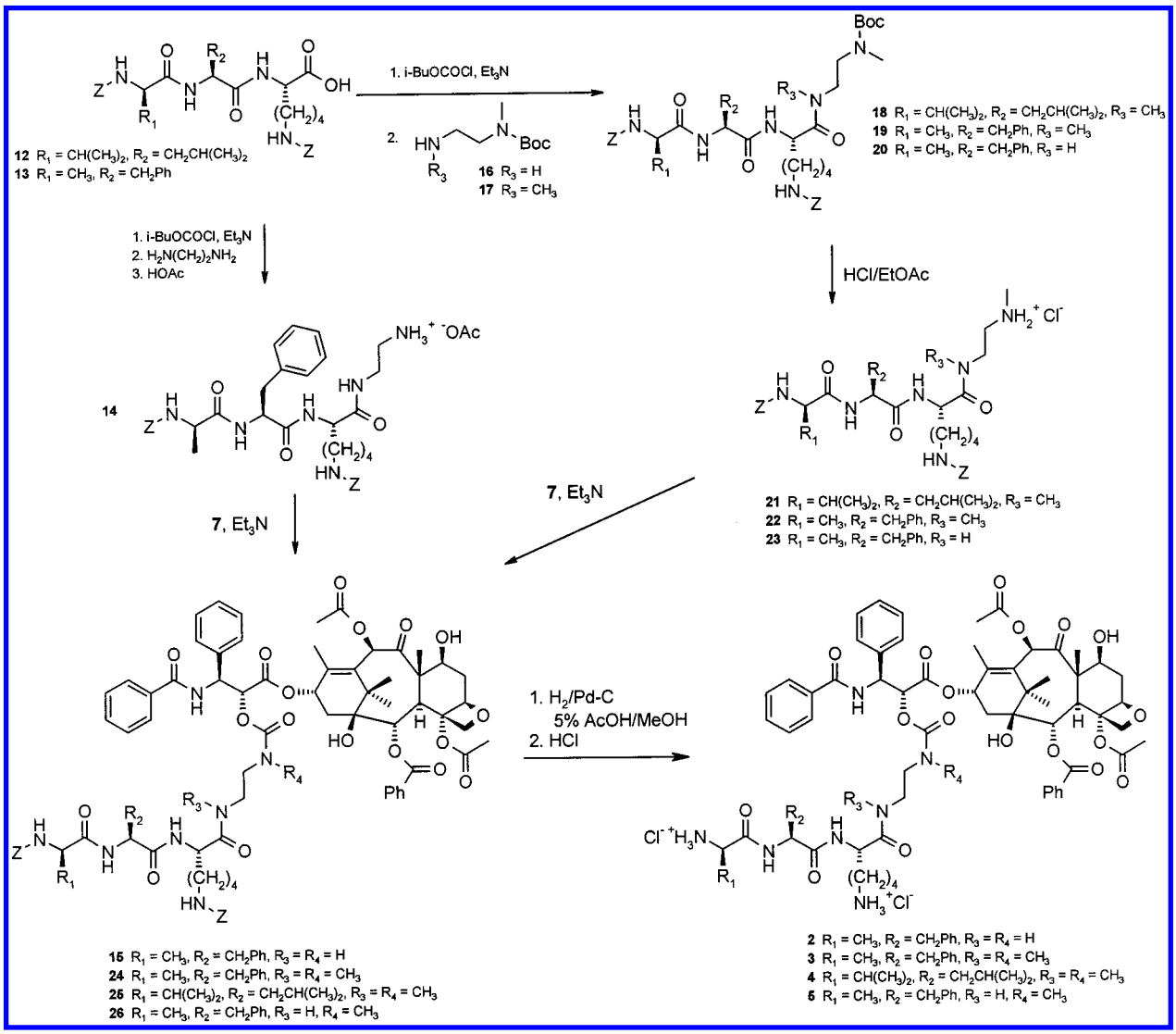


Table 1. Cytotoxicity (ID_{50} values,[a,b] ng/mL) of Paclitaxel (**1**) and the 2′-Modified Paclitaxel Prodrugs **2**−**6** in Various Tumor Cell Lines[32]

compd	MCF-7	EVSA-T	WIDR	IGROV	M19	A498	H226
paclitaxel (**1**)	<3	<3	<3	10	<3	<3	<3
prodrug **2**[c]	16	11	13	19	45	40	41
prodrug **3**[c]	6.6	7.2	10	11	17	38	21
prodrug **4**[c]	6.9	5.2	11	26	24	45	25
prodrug **5**[c]	>63	>63	47	45	47	>63	48
prodrug **6**[c]	9.2	9.3	11	27	20	>60	16

[a] Drug dose that inhibited cell growth by 50% compared to untreated control cultures. [b] SRB cell viability test. [c] $\times 10^3$.

Biological Data

The prodrugs **3**−**6** were highly stable in a Tris buffer solution (pH 7.3). After 3 days of incubation no degradation products were detected. However, prodrug **2** decomposed completely within 24 h, baccatin III being one of the degradation products.

The prodrugs **3**−**6** were incubated with human plasmin in a Tris buffer solution (pH 7.3) in order to determine whether the enzyme was able to hydrolyze them to yield free paclitaxel. Prodrugs **3** and **4** were not converted into paclitaxel; they remained stable in the presence of plasmin for at least 3 days. Prodrugs **5** and **6** produced paclitaxel under these circumstances. The half-lives of enzymatic hydrolysis of prodrugs **5** and **6** were determined by capillary electrophoresis.[31] The prodrugs were incubated at a concentration of 200 μM with 100 μg/mL human plasmin in a Tris buffer solution (pH 7.3). The half-life of enzymatic hydrolysis for prodrug **6** was 42 min, while prodrug **5** was hydrolyzed with a half-life of 3.5 min. As expected the 1,6-elimination of the spacer of prodrug **6** was instantaneous, whereas the half-life of spacer cyclization of prodrug **5**, after peptide cleavage, was 23 h.

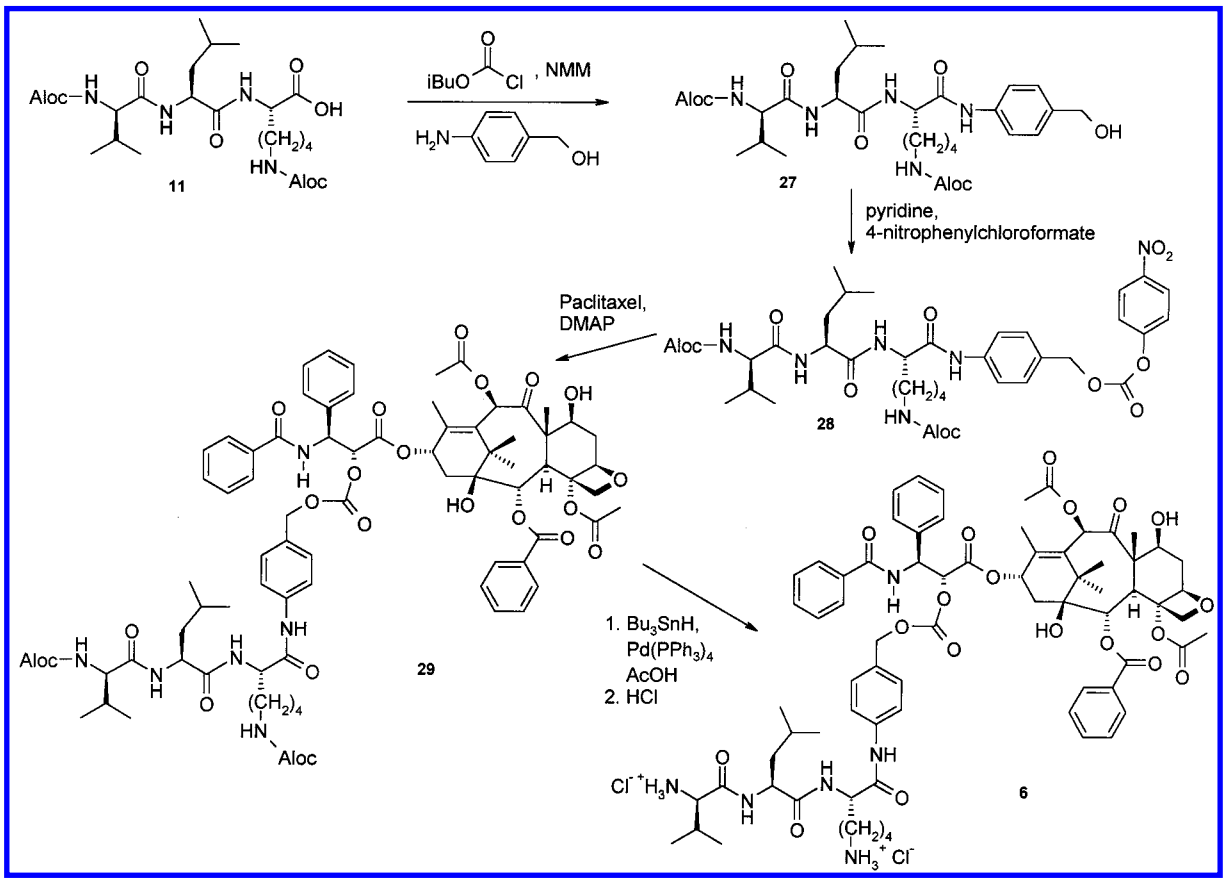
All prodrugs **2**−**6** showed a strongly reduced cytotoxicity in seven well-characterized human tumor cell lines compared with paclitaxel (Table 1).

An average 8000-fold reduction in cytotoxicity was observed, ranging from 1100−20000 times. Even paclitaxel-2′-carbonate prodrug **6** proved to be highly stable in these cell assays. From these results it can be concluded that prodrugs **5** and **6** are, to our knowledge, the most nontoxic prodrugs of paclitaxel reported until now, which do yield the free parent drug upon selective activation.

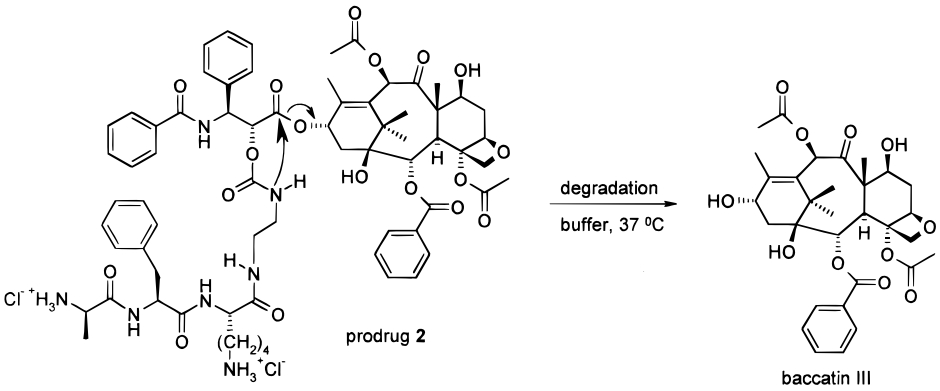
Discussion

It is interesting to note that paclitaxel-2′-carbamate prodrug **2** is not stable in buffer solution at pH 7.3,

Scheme 5



Scheme 6



whereas prodrugs **3** and **4** both proved to be stable in this buffer for 3 days. It was rationalized that the carbamate nitrogen of prodrug **2** could account for the formation of baccatin III by assisting in a kind of addition−elimination sequence, as depicted in Scheme 6. The methyl substituent on the carbamate nitrogen in prodrugs **3** and **4** would hinder this undesired reaction. Unfortunately, the *N*,*N*′-dimethyl-substituted prodrugs are not hydrolyzed by plasmin, probably because of the steric hindrance imposed by the methyl-substituted amide bond on the enzyme active site. Therefore, the prodrug **5** was synthesized. The methyl substituent on the carbamate nitrogen should prevent fragmentation, and the absence of the methyl group on the amide nitrogen might make enzymatic cleavage by plasmin possible. Both of these hypotheses proved to be valid, since prodrug **5** was stable in buffer solution and was hydrolyzed by plasmin, to yield paclitaxel.

The enzymatic activation rates of the paclitaxel prodrugs are comparable to the rates of the anthracycline prodrugs reported in the previous paper.[6] Proteolysis of the anthracycline prodrugs proceeded with half-lives of 7−19 min at a plasmin concentration of 50 μg/mL, while the paclitaxel prodrugs of the current paper are hydrolyzed with half-lives of 3.5 and 42 min at a plasmin concentration of 100 μg/mL. This enzyme concentration is not necessarily the in vivo concentration needed for the concept to work, although from a physiological point of view it can be considered rather high. However, it was demonstrated that the above-mentioned doxorubicin prodrugs were equally cytotoxic to free doxorubicin in u-PA-transfected cells. Apparently, these cells produced sufficient plasmin for complete activation of the anthracycline prodrugs.

When the plasmin hydrolysis rates of prodrugs **5** and **6** are compared to one another, it is expected that the

nature of the tripeptide is not responsible for the different enzyme activation rates. Both D-Ala-Phe-Lys and D-Val-Leu-Lys have shown similar rates of proteolysis by plasmin.[5] Therefore, it is tempting to suggest that plasmin prefers a substrate in which a tripeptide is linked to the more flexible ethylenediamine spacer compared with the more rigid aromatic spacer of prodrug **6**.

The rate of spacer cyclization of 2′-carbamate prodrug **5**, however, is not ideal for application under physiological conditions, as the drug−linker intermediate might diffuse from the site of cleavage before the parent drug is released. As expected, the elimination of the spacer in prodrug **5** is slower than the half-life of 35 min, which is reported for a *N*,*N*′-dimethyl-substituted ethylenediamine spacer which releases 4-hydroxyanisole.[27]

From Table 1 it is clear that the cytotoxicity of the carbamate prodrugs shows a marked decrease in comparison with paclitaxel. This is probably caused by the stable 2′-carbamate linkage of prodrugs **3**−**5**. Decomposition of prodrug **2** according to the mechanism depicted in Scheme 6 leads to nontoxic degradation products such as baccatin III. It is remarkable that prodrug **6** also shows an unambiguous reduction in cytotoxicity, whereas several paclitaxel-2′-carbonates reported in the literature were converted to the corresponding parent drug.[24] In the case of prodrug **6** the presence of the tripeptide possibly causes shielding of the 2′-position toward esterases.

In conclusion, the first paclitaxel prodrugs designed for activation by the tumor-associated enzyme plasmin are reported. The in vitro data for prodrug **6** show that this paclitaxel derivative is very promising for further in vivo studies, since it meets all criteria required for a successful prodrug. The prodrug shows high stability in buffer, is not toxic in seven human tumor cell lines, and shows a reasonable rate of enzymatic activation by plasmin. Furthermore, paclitaxel derivatives **2**−**5** are the first prodrugs of paclitaxel that are connected via a 2′-carbamate functionality. Our studies have shown that in the design of new generations of paclitaxel prodrugs, incorporation of a 2′-carbamate bond can contribute to stability against undesired enzymatic cleavage and thus to low cytotoxicity. As a consequence of these results, we are currently working on 2′-carbamate prodrugs of paclitaxel with enhanced enzyme activation and spacer elimination characteristics.

Experimental Section

Chemistry. ^{1}H NMR spectra were determined using a Bruker AM-300 (300 MHz, FT) spectrometer in the given solvent. Chemical shift values are reported as δ-values in parts per million relative to TMS as an internal standard. Mass spectra were determined using a double-focusing VG 7070E spectrometer. Elemental analyses were performed in triplicate on a Carlo Erba Instruments CHNSO EA 1108 element analyzer and were within 0.4% of the theoretical values calculated for C, H, and N. Melting points were measured on a Reichert Thermopan microscope and are uncorrected. Thin-layer chromatography was performed using precoated silica gel ($60F_{254}$) plates, and compounds were detected with UV light, ammonium molybdate solution, or a Chloro-TDM test. Column chromatography was performed using Baker silica gel in the solvents indicated.

2′-[4-Nitrophenyl-carbonate]paclitaxel (7). To a solution of 194 mg (0.227 mmol) paclitaxel (**1**) in dry dichloromethane under an argon atmosphere was added pyridine (4 drops). At −50 °C, 275 mg (6.0 equiv) of 4-nitrophenyl chloroformate dissolved in dry dichloromethane was added. The reaction mixture was stirred at −50 °C and after 4 h 4-nitrophenyl chloroformate (4.2 equiv) was added. After 1 h the mixture was diluted with dichloromethane and washed with 0.5 N potassium bisulfate and brine and dried over anhydrous sodium sulfate. After evaporation of the solvents the residual yellow film was purified by means of column chromatography (ethyl acetate−hexane, 1:1), to yield 133 mg of activated paclitaxel **7** (78%, 73% conversion): mp 161 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (s, 3H, 17), 1.26 (s, 3H, 16), 1.69 (s, 3H, 19), 1.92 (s, 3H, 18), 2.22 (s, 3H, 10-OAc), 2.49 (s, 3H, 4-OAc), 2.55 (m, 1H, 6a), 3.82 (d, 1H, J = 7.0 Hz, 3), 4.21 (d, 1H, J = 8.4 Hz, 20b), 4.33 (d, 1H, J = 8.4 Hz, 20a), 4.42 (m, 1H, 7), 4.96 (bd, 1H, J = 7.7 Hz, 5), 5.53 (d, 1H, J = 2.7 Hz, 2′), 5.70 (d, 1H, J = 7.1 Hz, 2), 6.10 (dd, 1H, J = 2.6 Hz, J = 9.4 Hz, 3′), 6.29 (s, 1H, 10), 6.34 (m, 1H, 13), 6.90 (d, 1H, J = 9.4 Hz, N−H), 7.34 (d, 2H, J = 9.1 Hz, nitrophenyl), 7.37−7.65 (m, 11H, aromatic), 7.75 (d, 2H, J = 7.2 Hz, aromatic), 8.15 (d, 2H, J = 7.2 Hz, aromatic), 8.26 (d, 2H, J = 9.1 Hz, nitrophenyl); MS (FAB) *m/e* 1020 $(M + H)^+$, 1042 $(M + Na)^+$. Anal. ($C_{54}H_{54}N_2O_{18}$·1.5H_2O) C, H, N.

Boc-D-Val-Leu-Lys(Boc)-OBu (9). To a solution of 2.92 g (8.50 mmol) of Boc-D-Val-Leu-N_2H_3 (**8**) in ethyl acetate under an argon atmosphere at a temperature of −30 °C was added dropwise 7.5 mL of a 3.2 M hydrochloric acid−ethyl acetate solution. The reaction mixture was kept at −30 °C and *tert*-butylnitrite (1.2 mL, 1.2 equiv) was added. After 60 min the solution was neutralized by addition of Et_3N (3.4 mL, 24.5 mmol), then H-Lys(Boc)-OBu·HCl (3.06 g, 1.06 equiv) was added. Again Et_3N (1.4 mL, 10 mmol) was added dropwise to the reaction mixture. The reaction mixture was stirred at 4 °C for 20 h. The mixture was washed with 10% citric acid and sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate and evaporated in vacuo. The product was purified by means of column chromatography (ethyl acetate−hexane, 1:1), obtaining 3.11 g (60%) of the desired product **9**: mp 175−176 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90−1.00 (m, 12H, 2 CH_3−Val and 2 CH_3-Leu), 1.20−1.75 (m, 6H, 3 CH_2-Lys), 1.44 (m, 27H, 6 CH_3 -Boc and 3 CH_3 -OBu), 1.80 (m, 1H, CH-Leu), 2.15 (m, 1H, CH−Val), 3.09 (m, 2H, N−CH_2-Lys), 3.94 (m, 1H, Hα), 4.45 (m, 2H, 2 Hα); MS (FAB) *m/e* 1616 $(M + H)^+$, 637 $(M + Na)^+$. Anal. ($C_{31}H_{58}N_4O_8$) C, H, N.

H-D-Val-Leu-Lys-OH (10). To a solution of 3.71 g (6.03 mmol) of compound **9** in dichloromethane (20 mL) was added 3.2 M hydrochloric acid−ethyl acetate (100 mL). After 60 min the solution was concentrated to dryness in vacuo. *tert*-Butyl alcohol was added and evaporated and the resulting solid (2.60 g, 100%) was freeze-dried in dioxane−water and used without further purification: ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 0.92 (d, 3H, J = 5.9 Hz, CH_3-Leu), 0.98 (d, 3H, J = 5.9 Hz, CH_3-Leu), 1.08 (d, 3H, J = 6.8 Hz, CH_3−Val), 1.09 (d, 3H, J = 6.8 Hz, CH_3−Val), 1.40−2.00 (m, 9H, 3 CH_2-Lys, CH-Leu and CH_2-Leu), 2.26 (m, 1H, CH−Val), 3.00 (m, 2H, N−CH_2-Lys), 3.98 (m, 1H, Hα), 4.30−4.50 (m, 2H, 2 Hα), 8.09 (d, 1H, J = 7.8 Hz, N−H), 8.81 (d, 1H, J = 7.4 Hz, N−H); MS (FAB) *m/e* 359 $(M + H)^+$, 381 $(M + Na)^+$.

General Procedure for Z-Protection of Unprotected Tripeptides. Z-D-Val-Leu-Lys(Z)-OH (12). To solution of 977 mg (2.27 mmol) of H-D-Val-Leu-Lys-OH (**10**) in water−acetonitrile was added triethylamine until a pH of 9.5 was reached. Then a solution of 1.15 g (2.04 equiv) of Z-ONSu in acetonitrile was added and the reaction mixture was kept basic at a pH of 9.0 by adding triethylamine. After the pH of the mixture did not alter anymore, the solution was stirred for 0.5 h. The solution was evaporated in vacuo to remove acetonitrile and the resulting solution was acidified with 1.0 M hydrochloric acid until a pH of 1.5 was reached. A small amount of methanol was added and the water layer was extracted with chloroform. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness. The product was purified by crystallization from methanol−diisopropyl ether to obtain 826

mg (58%) of the protected tripeptide **12**: mp 147 °C; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.80−1.00 (m, 12H, CH_3-Leu and CH_3−Val), 1.25−1.75 (m, 8H, 3 CH_2-Lys and CH_2-Leu), 1.87 (m, 1H, CH-Leu), 2.04 (m, 1H, CH−Val), 3.11 (m, 2H, N−CH_2-Lys), 3.95 (m, 1H, Hα), 4.42 (m, 2H, Hα), 5.06 (m, 4H, CH_2-Z), 7.20−7.40 (m, 10H, aromatic); MS (FAB) *m/e* 627 $(M + H)^+$, 649 $(M + Na)^+$. Anal. ($C_{33}H_{46}N_4O_8$) C, H, N.

Aloc-D-Val-Leu-Lys(Aloc)-OH (11). To solution of 955 mg (2.21 mmol) of D-Val-Leu-Lys-OH (**10**) in water−acetonitrile was added triethylamine until a pH of 9−9.5 was reached. Then a solution of 965 mg (2.2 equiv) of Aloc-ONSu in acetonitrile was added and the reaction mixture was kept basic by adding triethylamine. After the pH of the mixture did not alter anymore, a 0.5 M solution of hydrochloric acid was added until a pH of 3 was reached. The mixture was thoroughly extracted with dichloromethane. The organic layer was washed with water and the water layer was extracted again with dichloromethane. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness to result in the desired product **11** as a white solid (1.123 g, 96%): ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 0.80−1.05 (m, 12H, 4 CH_3 Val and Leu), 1.20−2.15 (m, 10H, CH_2-Lys and CH Val and CH Leu and CH_2 Leu), 3.13 (m, 2H, N−CH_2-Lys), 3.94 (t, 1H, Hα), 4.41 (m, 2H, Hα), 4.54 (m, 4H, Aloc), 5.15−5.35 (m, 4H, Aloc), 5.75−6.10 (m, 2H, Aloc); MS (FAB) *m/e* 527 $(M + H)^+$, 549 $(M + Na)^+$. Anal. ($C_{25}H_{42}N_4O_8 \cdot 0.25H_2O$) calcd C, H, N 10.55; found C, H, N 10.12.

Z-D-Ala-Phe-Lys(Z)-OH (13): yield 51%; mp 180 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.93 (d, 3H, J = 7.1 Hz, CH_3−Ala), 1.07−1.83 (m, 6H, 3 CH_2-Lys), 2.73 (dd, 1H, benzylic), 2.98 (m, 2H, N−CH_2-Lys), 3.08 (dd, 1H, benzylic), 4.00 (m, 1H, Hα), 4.15 (m, 1H, Hα), 4.58 (m, H, Hα), 4.90−5.00 (m, 4H, CH_2-Z), 7.10−7.35 (m, 15H, aromatic), 8.09 (d, 1H, J = 8.6 Hz, N−H), 8.19 (d, 1H, J = 7.5 Hz, N−H); MS (FAB) *m/e* 633 $(M + H)^+$, 655 $(M + Na)^+$. Anal. ($C_{34}H_{40}N_4O_8 \cdot 0.5H_2O$) C, H, N.

General Procedure for the Coupling of Ethylenediamine Spacers to Protected Tripeptides. Z-D-Ala-Phe-Lys(Z)-NH-$(CH_2)_2$-NH_2 (·AcOH) (14). To a solution of 303 mg (0.479 mmol) of Z-D-Ala-Phe-Lys(Z)-OH (**13**) dissolved in dry tetrahydrofuran under an argon atmosphere at −60 °C were added Et_3N (73 μL, 1.1 equiv) and isobutyl chloroformate (68 μL, 1.1 equiv). After 180 min the reaction mixture was added dropwise to a solution of ethylenediamine (20 equiv) in dry dichloromethane at −60 °C. After 90 min the solvent was evaporated and to the resulting solid were added water and dichloromethane. The product was obtained by filtration and purified by means of column chromatography (chloroform−methanol−acetic acid, 85:10:5) yielding 183 mg (52%) of the desired product **14**: mp 155−156 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.91 (d, 3H, J = 6.8 Hz, CH_3−Ala), 1.10−1.70 (m, 6H, 3 CH_2-Lys), 2.52 (t, 2H, CH_2-spacer), 2.73 (dd, 1H, benzylic), 2.80−3.10 (m, 5H, and N−CH_2-Lys, benzylic and CH_2-spacer), 3.95 (m, 1H, Hα), 4.10 (m, 1H, Hα), 4.45 (m, 1H, Hα), 4.94 (m, 4H, 2 CH_2-Z), 7.05−7.48 (m, 15H, aromatic); MS (FAB) *m/e* 675 $(M + H)^+$, 697 $(M + Na)^+$.

General Procedure for Coupling Promoieties 14 and 21−23 to Activated Paclitaxel Derivative 7. 2′-[Z-D-Ala-Phe-Lys(Z)-NH-$(CH_2)_2$-NHCO]paclitaxel (15). To a solution of 26.0 mg (0.0255 mmol) of activated paclitaxel **7** in dry DMF under an argon atmosphere was added Et_3N (10.6 μL, 3 equiv). At −30 °C a solution of **14** (18.7 mg, 1.0 equiv) and Et_3N (170 μL) dissolved in dry DMF was added dropwise. The reaction mixture was stirred at −20 °C for 2.5 h. The solution was diluted with 10% 2-propanol−ethyl acetate and washed with sodium bicarbonate, 0.5 N potassium bisulfate, brine and water. The organic layer was dried (sodium sulfate) and concentrated in vacuo. The residue was subjected to column chromatography (ethyl acetate−hexane, 2:1) to give 23.5 mg (59%) of compound **15**: mp 134−137 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.10−2.00 (m, 15H, CH_3−Ala, CH_2-Lys, 16 and 17), 1.60 (s, 3H, 19), 1.88 (s, 3H, 18), 2.18 (s, 3H, 10-OAc), 2.34 (s, 3H, 4-OAc), 2.54 (m, 1H, 6a), 3.00−3.40 (m, 8H, 2 CH_2-spacer, N−CH_2-Lys and benzylic), 3.67 (d, 1H, J = 6.9 Hz, 3), 4.15 (d, 1H, J = 8.4 Hz, 20b), 4.28 (d, 1H, J = 8.4 Hz, 20a), 4.28−4.60 (m, 3H, 7 and 2 Hα), 4.71 (d, 1H, Hα), 4.90−5.20 (m, 6H, 2 CH_2-Z, 2′ and 5), 5.62 (d, 1H, J = 6.9 Hz, 2), 5.75 (bd, 1H, 3′), 6.05 (bt, 1H, 13), 6.13 (s, 1H, 10), 7.00−7.60 (m, 28H, aromatic), 8.10 (d, 2H, J = 7.5 Hz, aromatic); MS (FAB) *m/e* 1555 $(M + H)^+$, 1577 $(M + Na)^+$. Anal. ($C_{84}H_{95}N_7O_{22} \cdot 2H_2O$) calcd C, H, N 6.16; found C, H, N 5.59.

General Procedure for Removal of Z Protecting Groups from Doubly Protected Prodrugs 15 and 24−26. 2′-[H-D-Ala-Phe-Lys-NH-$(CH_2)_2$-NHCO]paclitaxel (·2HCl) (2). To a solution of 33.2 mg (0.0213 mmol) of protected prodrug **15** in 5% acetic acid−methanol was added a catalytic amount of 10% Pd−C. The mixture was stirred for 90 min under an H_2 atmosphere. The Pd−C was removed by means of centrifugation. Ethyl acetate was added and methanol was evaporated in vacuo. The product was extracted with water and after addition of 1 M hydrochloric acid (85 μL) the solution was freeze-dried. *tert*-Butyl alcohol was added and removed by evaporation in vacuo and the residue was freeze-dried to obtain 24 mg (83%) of the desired product **2**: mp >195 °C dec; ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.07 (d, 3H, J = 6.5 Hz, CH_3−Ala), 1.15 (s, 3H, 17), 1.19 (s, 3H, 16), 1.00−2.00 (m, 6H, 3 CH_2-Lys), 1.68 (s, 3H, 19), 1.91 (s, 3H, 18), 2.07 (m, 1H, 6b), 2.21 (s, 3H, 10-OAc), 2.40 (s, 3H, 4-OAc), 2.48 (m, 1H, 6a), 2.85−3.50 (m, 8H, 2 CH_2-spacer, N−CH_2-Lys and benzylic), 3.78 (d, 1H, J = 7.0 Hz, 3), 4.20−4.45 (m, 3H, 3 Hα), 4.23 (d, 1H, J = 8.2 Hz, benzylic), 4.31 (d, 1H, J = 8.5 Hz, benzylic), 4.60 (dd, 1H, J = 10.4 Hz, J = 4.2 Hz, 7), 4.98 (bd, 1H, J = 9.0 Hz, 5), 5.49 (d, 1H, J = 4.4 Hz, 2′), 5.68 (d, 1H, J = 6.8 Hz, 2), 5.86 (m, 1H, 3′), 6.13 (bt, 1H, 13), 6.32 (s, 1H, 10), 7.10−7.68 (m, 16H, aromatic), 7.73 (d, 2H, J = 7.6 Hz, aromatic), 8.12 (d, 2H, J = 7.7 Hz, aromatic); MS (FAB) *m/e* 1286 $(M + H)^+$, 1309 $(M + Na)^+$. Anal. ($C_{68}H_{83}N_7O_{18} \cdot 9HCl$) C, H, N.[33]

General Procedure for the Boc Protection of Substituted Ethylenediamine Spacers. NH_2-$(CH_2)_2$-N(Me)-Boc (16). To a solution of 4.21 g (56.8 mmol, 10.8 equiv) of *N*-methylethylenediamine in dry tetrahydrofuran under an argon atmosphere at a temperature of 0 °C was added dropwise Boc-O-Boc (1.12 mL, 5.23 mmol) in dry tetrahydrofuran. After 6 h, tetrahydrofuran was evaporated in vacuo and the resulting mixture was dissolved in ethyl acetate and washed with brine. The organic layer was dried (Na_2SO_4) and concentrated in vacuo to dryness. The product was purified by column chromatography (chloroform−methanol, 1:1), obtaining 467 mg (51%) of monoprotected *N*-methylethylenediamine **16** as an oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (s, 2H, NH_2), 1.46 (s, 9H, CH_3−Boc), 2.83 (t, 2H, J = 6.4 Hz, CH_2−NH_2), 2.88 (s, 3H, CH_3−N), 3.27 (t, 2H, J = 6.3 Hz, CH_2−N).

NH(Me)-$(CH_2)_2$-N(Me)-Boc (17): yield 72%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.46 (s, 9H, CH_3−Boc), 2.45 (s, 3H, CH_3−N−Boc), 2.73 (t, 2H, CH_2−N−Boc), 2.88 (s, 3H, CH_3−N), 3.33 (t, 2H, CH_2−N).

Z-D-Ala-Phe-Lys(Z)-N(Me)-$(CH_2)_2$-N(Me)-Boc (19): yield 51%; mp 42 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.16 (d, 3H, J = 7.0 Hz, CH_3−Ala), 1.10−1.75 (m, 6H, 3 CH_2-Lys), 1.43 (s, 9H, CH_3−Boc), 2.70−3.70 (m, 14H, N−CH_2-Lys, benzylic, 2 CH_3-spacer and 2 CH_2-spacer), 4.15 (m, 1H, Hα), 4.60−4.90 (m, 2H, 2 Hα), 5.06 (m, 4H, CH_2-Z), 7.00−7.40 (m, 15H, aromatic); MS (FAB) *m/e* 825 $(M + Na)^+$. Anal. ($C_{43}H_{58}N_6O_9 \cdot 1.5H_2O$) calcd C, H, N 10.13; found C, H, N 9.65.

General Procedure for the Removal of Boc Protection from Protected Promoieties 18−20. Z-D-Ala-Phe-Lys(Z)-N(Me)-$(CH_2)_2$-N(Me) (·HCl) (22). Compound **19** (58.7 mg, 0.0730 mmol) was dissolved in ethyl acetate (4 mL) and 8 mL of 4 M hydrochloric acid was added. After 4 h the mixture was concentrated in vacuo and to the residual product *tert*-butyl alcohol was added and evaporated. The product was freeze-dried in dioxane to obtain 58.0 mg (100%) of the desired product **22** that was used without further purification: ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 1.14 (d, 3H, CH_3−Ala), 1.20−1.80 (m, 6H, CH_2-Lys), 2.64 (m, 3H, CH_3-spacer), 2.90−3.30 (m, 8H, N−CH_2-Lys, benzylic, CH_2−N−Boc and CH_2−N), 4.13 (m, 1H, Hα), 4.59 (m, 2H, Hα), 5.06 (m, 4H, CH_2-Z), 7.10−7.40 (m, 15H, aromatic); MS (FAB) *m/e* 703 $(M + H)^+$, 725 $(M + Na)^+$.

2′-[Z-D-Ala-Phe-Lys(Z)-N(Me)-(CH_2)$_2$-N(Me)CO]paclitaxel (24): the coupling reaction was performed in dichloromethane; yield 52%; mp 116−117 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.75−2.10 (m, 12H, CH_3−Ala, 3 CH_2-Lys and 17), 1.25 (s, 3H, 16), 1.68 (s, 3H, 19), 1.98 (s, 3H, 18), 2.20 (s, 3H, 10-OAc), 2.55 (s, 3H, 4-OAc), 2.70−3.30 (m, 12H, 2 CH_3-spacer, CH_2-spacer, N−CH_2-Lys and benzylic), 3.45 (m, 2H, CH_2-spacer), 4.05−4.90 (m, 6H, 3 Hα, 20a, 20b and 7,), 4.95−5.15 (m, 5H, CH_2-Z and 2′), 5.46 (d, 1H, 2), 5.67 (m, 1H, 3′), 6.15 (m, 1H, 13), 6.30 (s, 1H, 10), 7.05−7.95 (m, 23H, aromatic), 8.15 (m, 2H, aromatic); MS (FAB) *m/e* 1583 (M + H)$^+$, 1606 (M + Na)$^+$. Anal. ($C_{86}H_{99}N_7O_{22}$·2.5H_2O) C, H, N.

2′-[H-D-Ala-Phe-Lys-N(Me)-(CH_2)$_2$-N(Me)CO]paclitaxel (·2HCl) (3): yield 88%; mp >187 °C dec; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.88 (d, 3H, CH_3−Ala), 1.00−2.00 (m, 12H, CH_2-Lys, 16 and 17), 1.52 (s, 3H, 19), 1.68 (s, 3H, 18), 1.95 (s, 3H, 10-OAc), 2.20 (s, 3H, 4-OAc), 2.80−3.35 (m, 11H, CH_3-spacer, N−CH_2-Lys, benzylic and 2CH_2-spacer), 2.88 (s, 3H, CH_3-spacer), 3.45−4.90 (m, 7H, 3Hα, 20a, 20b, 7 and 2′), 5.00 (m, 5H, 5), 5.38 (m, 1H, 2), 5.65 (m, 1H, 3′), 6.10 (m, 1H, 13), 6.40 (s, 1H, 10), 7.05−8.20 (m, 20H, aromatic); MS (FAB) *m/e* 1314 (M + H)$^+$. Anal. ($C_{70}H_{87}N_7O_{18}$·15HCl) calcd C 45.17, H 5.52, N; found C 44.71, H 5.96, N.[33]

Z-D-Val-Leu-Lys(Z)-N(Me)-(CH_2)$_2$-N(Me)-Boc (18): yield 80%; mp 82 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.90−1.05 (m, 12H, 2 CH_3-Leu and 2 CH_3−Val), 1.20−1.80 (m, 8H, 3 CH_2-Lys and CH_2-Leu), 1.44 (s, 9H, CH_3−Boc), 2.25 (m, 2H, CH−Val and CH-Lys), 2.75−4.00 (m, 9H, N−CH_2-Lys, CH_3-spacer, 2 CH_2-spacer), 3.05 (m, 3H, CH_3-spacer), 3.96 (m, 1H, Hα), 4.43 (m, 1H, Hα), 4.84 (m, 1H, Hα), 5.08 (m, 4H, CH_2-Z), 7.25−7.40 (m, 10H, aromatic); MS (FAB) *m/e* 797 (M + H)$^+$, 819 (M + Na)$^+$. Anal. ($C_{42}H_{64}N_6O_9$·0.5H_2O) C, H, N.

Z-D-Val-Leu-Lys(Z)-N(Me)-(CH_2)$_2$-N(Me) (·HCl) (21): yield 100%; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 0.80−1.05 (m, 12H, 2CH_3-Leu and 2 CH_3−Val), 1.20−1.80 (m, 6H, 3 CH_2-Lys and CH_2-Leu), 2.03 (m, 2H, CH−Val and CH-Lys), 2.60−3.30 (m, 9H, N−CH_2-Lys, CH_3-spacer and 2 CH_2-spacer), 3.08 (s, 3H, CH_3-spacer), 3.85−4.65 (m, 3H, 3Hα), 5.09 (m, 4H, CH_2-Z), 7.25−7.40 (m, 10H, aromatic); MS (FAB) *m/e* 697 (M + H)$^+$, 719 (M + Na)$^+$.

2′-[Z-D-Val-Leu-Lys(Z)-N(Me)-(CH_2)$_2$-N(Me)CO]paclitaxel (25): the coupling reaction was performed in dichloromethane; yield 33%; mp 118−120 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.70−1.05 (m, 12H, 2 CH_3-Leu and 2 CH_3−Val), 1.05−2.25 (m, 10H, CH_2-Lys, CH_2-Leu, CH−Val and CH-Leu), 1.13 (s, 3H, 17), 1.23 (s, 3H, 16), 1.69 (s, 3H, 19), 1.88 (s, 3H, 18), 2.22 (s, 3H, 10-OAc), 2.51 (s, 3H, 4-OAc), 2.70−3.60 (m, 12H, N−CH_2-Lys, 2 CH_3-spacer and 2 CH_2-spacer), 3.82 (s, 1H, 3), 3.85−4.55 (m, 5H, 2 Hα, 20a, 20b, 7), 4.80 (m, 1H, Hα), 4.98 (m, 1H, 5), 5.06 (m, 4H, CH_2-Z), 5.45 (bs, 1H, 2′), 5.67 (d, 1H, 2), 6.14 (dd, 1H, 3′), 6.26 (bs, 1H, 13), 6.30 (s, 1H, 10), 7.20−7.65 (m, 21H, aromatic), 7.78 (d, 2H, aromatic), 8.13 (m, 2H, aromatic); MS (FAB) *m/e* 1577 (M + H)$^+$, 1599 (M + Na)$^+$. Anal. ($C_{85}H_{105}N_7O_{22}$·5H_2O) C, H, N.

2′-[H-D-Val-Leu-Lys-N(Me)-(CH_2)$_2$-N(Me)CO]paclitaxel (·2HCl) (4): yield 100%; mp >200 °C dec; ^{1}H NMR (300 MHz, DMSO-d_6) δ 0.80−1.10 (m, 12H, 2 CH_3-Leu and 2 CH_3−Val), 1.10−2.00 (m, 10H, 3 CH_2-Lys, CH_2-Leu), 1.23 (m, 6H, 16 and 17), 1.49 (s, 3H, 19), 1.81 (s, 3H, 18), 2.10 (s, 3H, 10-OAc), 2.15−2.40 (m, 2H, CH−Val and CH-Leu), 2.50 (s, 3H, 4-OAc), 2.60−3.50 (m, 12H, N−CH_2-Lys, 2 CH_3-spacer and 2 CH_2-spacer), 3.75 (bs, 1H, 3), 3.90−4.80 (m, 4H, 2 Hα, 20a and 20b), 4.55 (m, 1H, 7), 4.92 (m, 2H, 5 and Hα), 5.10−5.90 (m, 3H, 2, 2′ and 3′), 5.83 (m, 1H, 13), 6.28 (s, 1H, 10), 7.35−8.40 (m, 15H, aromatic); MS (FAB) *m/e* 1308 (M + H)$^+$.

Z-D-Ala-Phe-Lys(Z)-NH-(CH_2)$_2$-N(Me)-Boc (20): yield 84%; mp 52 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (d, 3H, J = 6.9 Hz, CH_3−Ala), 1.10−1.95 (m, 6H, 3 CH_2-Lys), 1.42 (s, 9H, CH_3−Boc), 2.84 (s, 3H, CH_3-spacer), 3.00−3.50 (m, 8H, N−CH_2-Lys, benzylic, 2 CH_2-spacer), 4.00−4.45 (m, 2H, 2 Hα), 4.62 (m, 1H, Hα), 5.05 (m, 4H, CH_2-Z), 7.10−7.40 (m, 15H, aromatic). Anal. ($C_{42}H_{56}N_6O_9$) C, H, N.

Z-D-Ala-Phe-Lys(Z)-NH-(CH_2)$_2$-N(Me) (·HCl) (23): yield 94%; mp 121 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.19 (d, 3H, J = 7.00 Hz, CH_3−Ala), 1.25−2.00 (m, 6H, CH_2-Lys), 2.68 (s, 3H, CH_3-spacer), 2.90−3.58 (m, 8H, N−CH_2-Lys, 2 CH_2-spacer and benzylic), 4.15 (m, 1H, Hα), 4.24 (m, 1H, Hα), 4.52 (m, 1H, Hα), 5.06 (m, 4H, CH_2-Z), 5.62 (m, 1H, NH), 7.10−7.40 (m, 15H, aromatic). Anal. ($C_{37}H_{48}N_6O_7$·1.5HCl) C, H, N.

2′-[Z-D-Ala-Phe-Lys(Z)-NH-(CH_2)$_2$-N(Me)CO]paclitaxel (26): the coupling reaction was performed in dichloromethane−tetrahydrofuran−*N*-methylpyrrolidinone; yield 80%; mp 132−133 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.00−2.00 (m, 9H, CH_3−Ala, 3 CH_2-Lys), 1.12 (s, 3H, 17), 1.20 (s, 3H, 16), 1.68 (s, 3H, 19), 1.76 (s, 3H, 18), 2.21 (s, 3H, 10-OAc), 2.58 (s, 3H, 4-OAc), 2.79 (s, 3H, CH_3-spacer), 2.80−3.30 (m, 8H, 2 CH_2-spacer, N−CH_2-Lys and benzylic), 3.83 (d, 1H, J = 6.9 Hz, 3), 4.23 (d, 1H, J = 8.3 Hz, 20a), 4.31 (d, 1H, J = 8.4 Hz, 20b), 4.40−4.60 (m, 2H, Hα and 7), 4.80 (m, 1H, Hα), 4.90−5.10 (m, 5H, 5 and CH_2-Z), 5.45 (d, 1H, 2′), 5.69 (d, 1H, J = 7.1 Hz, 2), 6.17 (m, 1H, 3′), 6.26 (bs, 1H, 13), 6.30 (s, 1H, 10), 7.10−7.65 (m, 26H, aromatic), 7.79 (d, 2H, J = 7.7 Hz, N-benzoyl), 8.18 (d, 2H, J = 7.3 Hz, O-benzoyl); MS (FAB) *m/e* 1568 (M + H)$^+$, 1590 (M + Na)$^+$. Anal. ($C_{85}H_{97}N_7O_{22}$·0.75H_2O) C, H, N.

2′-[H-D-Ala-Phe-Lys-NH-(CH_2)$_2$-N(Me)CO]paclitaxel (·2HCl) (5): yield 100%; mp >202 °C dec; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 1.05−1.20 (m, 9H, CH_2−Ala, 16 and 17), 1.10−2.00 (m, 6H, 3 CH_2-Lys), 1.69 (s, 3H, 19), 1.98 (s, 3H, 18), 2.21 (s, 3H, 10-OAc), 2.53 (s, 3H, 4-OAc), 2.91 (s, 3H, CH_3-spacer), 2.92−3.60 (m, 8H, 2 CH_2-spacer, N−CH_2-Lys and benzylic), 3.82 (d, 1H, J = 6.7 Hz, 3), 3.90−4.55 (m, 5H, 7, 20a, 20b, 2 Hα), 4.70 (m, 1H, Hα), 5.02 (m, 5H, 5), 5.39 (m, 1H, 2′), 5.71 (m, 1H, ^3J = 6.9, 2), 6.05 (m, 1H, 3′), 6.19 (m, 1H, 13), 6.36 (s, 1H, 10), 7.15−7.70 (m, 16H, aromatic), 7.80 (d, 2H, J = 7.4 Hz, N-benzoyl), 8.15 (d, 2H, J = 7.4 Hz, O-benzoyl); MS (FAB) *m/e* 1301 (M + H). Anal. ($C_{69}H_{85}N_7O_{18}$·8HCl) C, H, N.[33]

Aloc-D-Val-Leu-Lys(Aloc)-PABA (27). A solution of 450 mg of protected tripeptide **11** (0.854 mmol) was dissolved in dry tetrahydrofuran under an argon atmosphere and cooled to −20 °C. *N*-Methylmorpholine (104 μL, 1.1 equiv) and isobutyl chloroformate (122 μL, 1.1 equiv) were added. The reaction mixture was stirred for 3 h at a temperature below −20 °C. A solution of 4-aminobenzyl alcohol (132 mg, 1.25 equiv) and *N*-methylmorpholine (118 μL, 1.25 equiv) in dry tetrahydrofuran was added dropwise to the reaction mixture. The reaction mixture was allowed to come to room temperature and was stirred for 16 h. Tetrahydrofuran was evaporated and dichloromethane was added. The organic layer was washed with saturated sodium bicarbonate, a 0.5 N potassium bisulfate solution and brine, dried over anhydrous sodium sulfate, and evaporated. The residual crude product was purified by means of column chromatography (chloroform−methanol, 9:1) to afford 376 mg (70%) of the desired product **27** as a white solid: mp 152 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 0.75−1.10 (m, 12H, 4 CH_3 Val and Leu), 1.20−2.15 (m, 10H, CH_2-Lys and CH Val and CH Leu and CH_2 Leu), 3.14 (m, 2H, N−CH_2-Lys), 3.81 (d, 1H, Hα), 4.22 (m, 1H, Hα), 4.25−4.70 (m, 5H, Hα and Aloc), 4.58 (s, 2H, benzylic), 5.00−5.35 (m, 4H, Aloc), 5.55−6.10 (m, 2H, Aloc), 7.27 (d, 2H, aromatic), 7.61 (d, 2H, aromatic); MS (FAB) *m/e* 632 (M + H)$^+$, 654 (M + Na)$^+$. Anal. ($C_{32}H_{49}N_5O_8$·0.25H_2O) calcd C, H, N 11.01; found C, H, N 10.53.

Aloc-D-Val-Leu-Lys(Aloc)-PABC-PNP (28). To a solution of 117 mg (0.185 mmol) of **27** in dry tetrahydrofuran−dichloromethane under an argon atmosphere were added 4-nitrophenyl chloroformate (56 mg, 1.5 equiv) and dry pyridine (23 μL, 1.5 equiv). The reaction mixture was stirred at room temperature and after 24, 48 and 72 h were added respectively 1, 1.5 and 1.5 equiv of both 4-nitrophenyl chloroformate and pyridine. After 96 h ethyl acetate was added. The organic layer was washed with 10% citric acid, brine and water, dried over anhydrous sodium sulfate and evaporated yielding a yellow oil. The product was purified by means of column chromatography (ethyl acetate−hexane, 1:1; chloroform−methanol, 30:1; respectively) to afford 112 mg (76%) of the desired carbonate **28**: mp 171 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85−1.15 (m, 12H, 4 CH_3 Val and Leu), 1.20−2.20

(m, 10H, CH_2-Lys and CH Val and CH Leu and CH_2 Leu), 2.90−3.40 (m, 2H, N−CH_2-Lys), 3.82 (m, 1H, Hα), 4.12 (m, 1H, Hα), 4.30−4.65 (m, 5H, Hα and Aloc), 5.23 (s, 2H, benzylic), 5.00−5.45 (m, 4H, Aloc), 5.58 (m, 1H, Aloc), 5.87 (m, 1H, Aloc), 7.25−7.40 (m, 4H, aromatic), 7.75 (d, 2H, aromatic), 8.26 (d, 2H, aromatic); MS (FAB) *m/e* 797 $(M + H)^+$, 819 $(M + Na)^+$. Anal. ($C_{39}H_{52}N_6O_{12}$) C, H.

2′-[Aloc-D-Val-Leu-Lys(Aloc)-PABC]paclitaxel (29). *p*-Nitrophenyl carbonate **28** (81 mg, 0.102 mmol) and paclitaxel (87 mg, 1 equiv) in dry dichloromethane were treated at room temperature with DMAP (14 mg, 1.1 equiv). The reaction mixture was stirred in the dark for 48 h and was then diluted with dichloromethane. The organic layer was washed with saturated sodium bicarbonate, 0.5 N potassium bisulfate and brine and dried over anhydrous sodium sulfate. After evaporation of the solvents the residual white film was purified by means of column chromatography (chloroform−methanol, 20:1), to yield 151 mg (99%) of protected paclitaxel prodrug **29**: mp 143 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85−1.15 (m, 12H, 4 CH_3 Val and Leu), 1.14 (s, 3H, 17), 1.24 (s, 3H, 16), 1.30−2.30 (m, 11H, CH_2-Lys and CH Val and CH Leu and CH_2 Leu and 6b), 1.69 (s, 3H, 19), 1.91 (s, 3H, 18), 2.23 (s, 3H, 10-OAc), 2.44 (s, 3H, 4-OAc), 2.60 (m, 1H, 6a), 3.00−3.45 (m, 2H, N−CH_2-Lys), 3.72 (m, 2H, Hα), 3.81 (d, 1H, 3), 4.06 (m, 1H, Hα), 4.20 (d, 1H, 20b), 4.32 (d, 1H, 20a), 4.23−4.65 (m, 5H, Aloc and 7), 4.90−5.35 (m, 7H, benzylic spacer and 4 Aloc and 5), 5.44 (d, 1H, 2′), 5.52 (m, 1H, Aloc), 5.69 (d, 1H, 2), 5.88 (m, 1H, Aloc), 5.98 (dd, 1H, 3′), 6.27 (bt, 1H, 13), 6.30 (s, 1H, 10), 6.93 (d, 1H, NH), 7.25−7.55 (m, 12H, aromatic), 7.60 (d, 1H, aromatic), 7.65−7.80 (m, 4H, aromatic), 8.14 (d, 2H, aromatic), 8.39 (bs, 1H, NH); MS (FAB) *m/e* 1512 $(M + H)^+$, 1535 $(M + Na)^+$. Anal. ($C_{80}H_{98}N_6O_{23}$·1.75H_2O) calcd C 62.27, H 6.63, N; found C 61.83, H 6.13, N.

2′-[H-D-Val-Leu-Lys-PABC]paclitaxel (·2HCl) (6). To a solution of 56.4 mg (0.0373 mmol) of protected prodrug **29** in dry tetrahydrofuran under an argon atmosphere was added glacial acetic acid (11 μL, 5 equiv) together with tributyltinhydride (30 μL, 3 equiv) and a catalytic amount of $Pd(PPh_3)_4$. After 45 min tributyltinhydride was added again (30 μL). After 90 min the reaction mixture carefully quenched with 1 mL 0.5 M hydrochloric acid−ethyl acetate. 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 hydrochloric acid and the resulting product was dissolved in water and freeze-dried yielding 36.6 mg (69%) of prodrug **6**: mp 168 °C; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD) δ 0.92 (d, 3H, CH_3-Leu), 0.98 (d, 3H, CH_3-Leu), 1.00−1.13 (m, 6H, 2 CH_3 Val), 1.17 (s, 3H, 17), 1.22 (s, 3H, 16), 1.35−2.32 (m, 11H, CH_2-Lys and CH Val and CH Leu and CH_2 Leu and 6b), 1.70 (s, 3H, 19), 1.85 (s, 3H, 18), 2.23 (s, 3H, 10-OAc), 2.44 (s, 3H, 4-OAc), 2.55 (m, 1H, 6a), 2.99 (bt, 2H, N−CH_2-Lys), 3.81 (d, 1H, 3), 4.07 (m, 1H, Hα), 4.26 (d, 1H, 20b), 4.33 (d, 1H, 20a), 4.36 (m, 2H, Hα), 4.54 (m, 1H, 7), 5.00 (bd, 1H, 5), 5.13 (dd, 2H, benzylic spacer), 5.44 (d, 1H, 2′), 5.71 (d, 1H, 2), 5.97 (bd, 1H, 3′), 6.22 (bt, 1H, 13), 6.35 (s, 1H, 10), 7.20−7.70 (m, 15H, aromatic), 7.74 (d, 2H, aromatic), 8.14 (d, 2H, aromatic); MS (FAB) *m/e* 1343 $(M + H)^+$, 1365 $(M + Na)^+$. Anal. ($C_{72}H_{90}N_6O_{19}$·3.4HCl) C, H, N.[33]

Biological Characterization. Stability of the Prodrug in Buffer Solution. Prodrugs **3**−**6** were incubated at concentrations of 100−270 μM in 0.1 M Tris/hydrochloric acid buffer (pH 7.3) for 3 days and showed no parent drug formation (TLC). Under the same conditions prodrug **2** degraded completely within 24 h, yielding baccatin III.

Kinetics of Enzymatic Hydrolysis. Hydrolysis of prodrugs **3**−**6** was investigated by incubation at a 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). Prodrugs **3** and **4** were not converted to paclitaxel by plasmin and remained intact for 3 days. Prodrug **6** was converted to yield paclitaxel immediately following enzymatic cleavage ($t_{1/2}$ = 42 min), while prodrug **5** showed formation of the intermediate drug−linker moiety before paclitaxel was released ($t_{1/2}$ activation = 3.5 min; $t_{1/2}$ cyclization = 23 h). Capillary electrophoresis was carried out with a CE Ext light path capillary (80.5 cm, 50 μm), with 1:1 methanol/0.05 M sodium phosphate buffer (pH 7.0) as eluent. Detection was performed at 200 and 254 nm.

In Vitro Cytotoxicity in Seven Human Tumor Cell Lines. The antiproliferative effect of prodrugs **2**−**6** and paclitaxel was determined in vitro applying seven well-characterized human tumor cell lines and the microculture sulforhodamine B (SRB) test.[34] The antiproliferative 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.

Acknowledgment. We are grateful to H. Adams and Prof. Dr. G. I. Tesser for their contributions to the peptide synthesis.

Supporting Information Available: Elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM0009078