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13. ABSTRACT ***(Maximum 200 Words)***

During the period supported by this award, we accomplished the synthesis of three of the four protected **Linker-Drug** conjugates of doxorubicin and 5-fluorouracil (5-FU) proposed in the original application. We determined the stability of two 5-FU **Linker-Drug** conjugates originally designed and found them to be unstable and not suitable for incorporation into prodrugs. We modified the structure and synthesized two new linkers. The new **Linker-Drug** conjugates of 5-FU were found to be stable under physiological conditions in the masked form and could undergo once unmasked the cyclization activation process as originally proposed to release the drug 5-FU. We also accomplished the synthesis of a **Peptide-Linker-Drug** conjugate albeit with an unstable linker. But, the chemistry developed will be useful for the construction of more promising **Peptide-Linker-Drug** conjugates. Recently, we turned our attention to the synthesis of a **Peptide-Linker-Drug** conjugate with much less bulky linker. Results are encouraging and will be further investigated.

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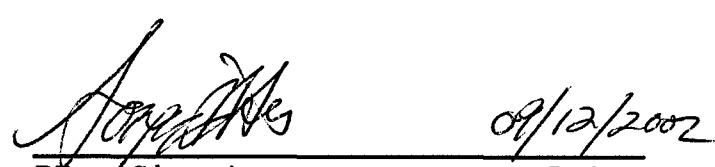

09/12/2002

PI - Signature Date

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Introduction

The goal of this project is to design, synthesize and evaluate peptide-based prodrugs containing an effective clinical anticancer agent such as doxorubicin or 5-fluorouracil for greater tissue-specific activation in order to increase the efficacy and to decrease the systemic toxicity of anticancer drugs used in the treatment of advanced prostate cancer. Peptide-based prodrugs in the form of **Peptide-Linker-Drug** were designed. It was proposed that the peptide portion would be cleaved site-specifically by a prostate tissue-specific enzyme, prostate specific antigen (PSA). After enzymatic cleavage in the targeted prostate cancer tissue, the **Linker-Drug** will undergo a cyclization activation process to release the **Drug**, which will produce the desired cytotoxic effect. We first needed to synthesize the Linker-Drug conjugates to test the cyclization activation process. If the Linker system works, we would continue with the synthesis of **Peptide-Linker-Drug** conjugates and test the activation using enzyme and cell culture assays in vitro.

The grant was first awarded when the PI was at the University of Oklahoma Health Sciences Center and transferred with the PI to Rutgers University in March 2000. There was a gap of about six months during the grant transfer and this past year, this project is under a no-cost extension. Two reports were submitted earlier: first one covered the first year in Oklahoma and the second one covered the 1.5 years at Rutgers just before the no-cost extension. This is the third and final report covering the entire grant period including the one year in Oklahoma and the two and half years at Rutgers. During the initial one year period, we focused on the synthesis of **Linker-Drug** conjugates of doxorubicin and 5-fluorouracil (5-FU) proposed in the original application to test the second cyclization activation step. Quickly, we found that the doxorubicin conjugates were difficult to test due to its inherent easily reduced functional group in doxorubicin. For the 5-FU conjugates, we soon uncovered an unexpected stability problem in our original design. We modified the linker portion and solved the stability problem of the **Linker-Drug** conjugates. Finally, we successfully accomplished the synthesis of two **Peptide-Linker-Drug** conjugates of 5-FU: one incorporating the previously unstable linker and the other incorporating a self-disintegrating *gem*-diamine linker. The latter showed promise as a prodrug activated by our target enzyme PSA.

Final Report Body

Because of the potential facile cyclization of the **Linkers** having a free amino group to form the corresponding cyclic urea or lactam, we used synthetic strategies that mask the amino group as an inert nitro group. Reduction to the corresponding amino group will take place only when needed.

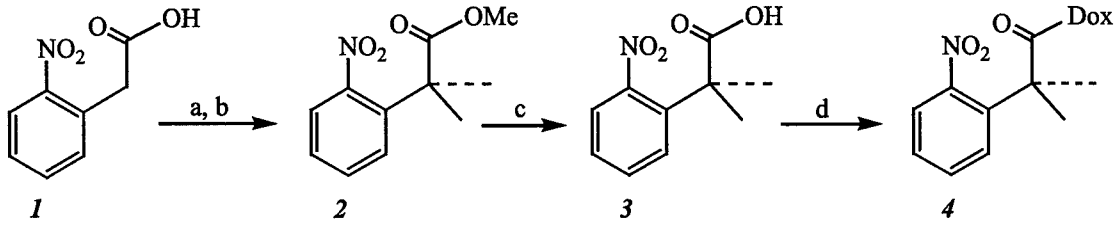
1) Synthesis of protected Linker-Drug conjugates of doxorubicin and 5-FU

Because of the potential facile cyclization of **Linkers** having a free amino group to form the corresponding lactam or cyclic urea, we used synthetic strategies that mask the amino group as an inert nitro or azido group. Reduction to the corresponding amino group will take place only when needed.

Synthesis of 2-(2-nitrophenyl)-2-methyl-propionic acid-Doxorubicin conjugate (4). As shown in Scheme 1, we started the synthesis with *2*-nitrophenylacetic acid (***1***). Esterification of ***1*** using thionyl chloride in methanol followed by α,α-dialkylation using sodium hydride and methyl iodide in the presence of catalytic amount of 18-crown-6 gave the α,α-dimethyl analogue ***2*** in quantitative yield. Sodium hydroxide-mediated hydrolysis converted ester ***2*** to its corresponding acid ***3*** in 93% yield. Coupling of the acid ***3*** to the amino group of doxorubicin was accomplished using its HOBt activated ester to give the protected **Linker-Drug** conjugate ***4*** in 40% yield.

Synthesis of 2-(1-azidoisopropyl)-benzoic acid-Doxorubicin conjugate (***10***). As shown in Scheme 2, Grignard reaction of the commercially available phthalide (***5***) with methyl magnesium bromide gave diol ***6*** in quantitative yield. The primary alcohol in ***6*** was selectively protected by *t*-butyldiphenylsilyl group to form the silyl ether compound ***7*** in 92.4% yield. The secondary hydroxyl group in compound ***7*** was replaced by an azido group using sodium azide and TFA giving the azido

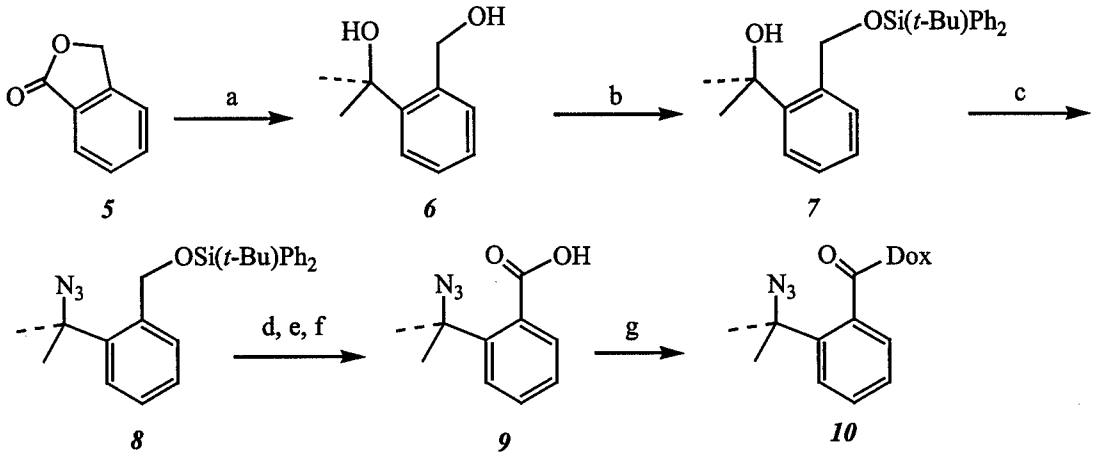
Scheme 1.



1 *2* *3* *4*

a) $SOCl_2$/MeOH, 100%; b) MeI/NaH, 18-crown-6, 100%; c) 2 N NaOH/MeOH, reflux, 6 hr, 93.0%; d) HBTU/DIEA, Dox·HCl, 40.0%

Scheme 2.



5 *6* *7* *8* *9* *10*

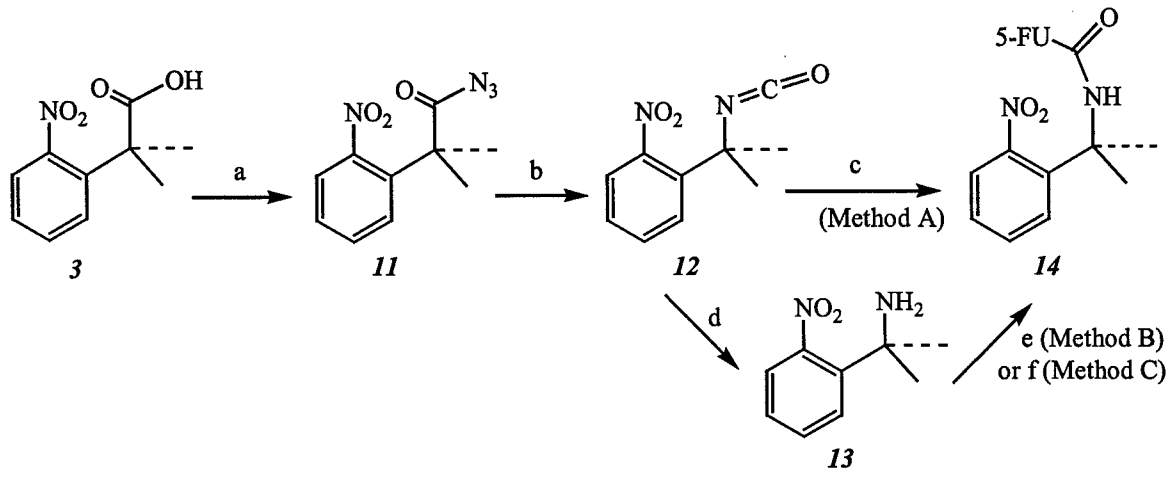
a) MeMgBr followed by H_2O, 100%; b) 1.1 eq. *t*-$BuPh_2SiCl$/imidazole/THF, 92.4%; c) NaN_3/TFA/CH_2Cl_2, 73%; d) TBAF/THF, 73.4%; e) PDC/DMF, 100%; f) $NaClO_2$, NaH_2PO_4, 64%; g) HBTU/DIEA/DMF, Dox·HCl, 62.6%

compound ***8*** in 73% yield. Deprotection of the silyl ether ***8*** by fluoride ion and subsequent two-step oxidation of primary alcohol to the corresponding carboxylic acid afforded compound ***9*** in 47.0% yield. Coupling of the carboxylic acid to the drug doxorubicin led to the protected **Linker-Drug** conjugate ***10*** in 62.6% yield.

The problem with testing the cyclization activation process of doxorubicin conjugates due to the presence of an easily reduced functional group in doxorubicin itself and the consideration of the unfavorable huge molecular size incorporating doxorubicin lead us to focus on the development of 5-FU conjugates for further evaluation.

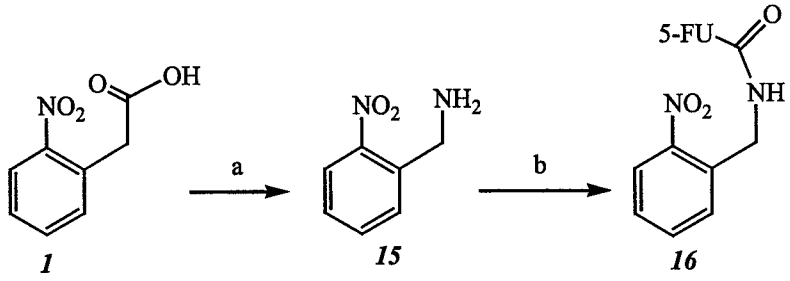
*Synthesis of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (**14**) and N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (**16**).* Scheme 3 shows the synthesis of nitro-**Linker-Drug** conjugate ***14*** of 5-FU starting from compound ***3***. The acid ***3*** was first converted to the acyl azide followed by trapping of the Curtius rearrangement intermediate ***12*** with 5-FU to give N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-FU (***14***) in an overall yield of 3.6% (Method A). The strong refluxing condition and low yield in the last step of this method prompted us to explore an alternative route. Acid hydrolysis of the Curtius rearrangement intermediate ***12*** gave the free primary amine ***13***. Final condensation with 5-FU could then be accomplished by reaction of the amine with diphosgene followed by coupling of the amine-chlorofomate with 5-FU sodium salt (Method B) or reaction of the free amine with N^1-chloroformyl-5-FU (Method C). We found that method C offers a very mild reaction condition as well as a better yield.

Scheme 3.



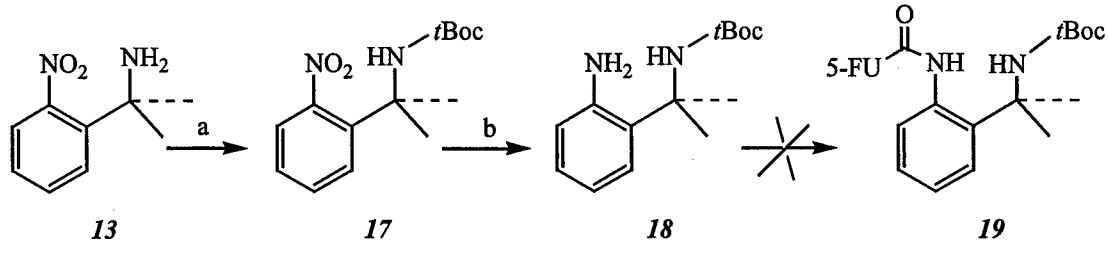
a) $ClCO_2Et/Et_3N$ followed by NaN_3, 31.3%; b) reflux, toluene, 2 hr, 79.5%; c) 5-FU/Et_3N, toluene, reflux, 18 hr, 14.3%; d) HCl, 32.0%; e) diphosgene/C, followed by 5-FUNa 16.7%; f) 5-FU-COCl, rt, 50.0%

Scheme 4.



a) H_2SO_4, NaN_3, $CHCl_3$, 50 °C, 1.5 hr, followed by NaOH, 76.1%; b) 5-FU-COCl, rt, 34.2%

Scheme 5.



a) $(Boc)_2O$, 88.2%; b) H_2, 10% Pd/C, 70.9%;

To study the effect of the two methyl groups on the rate of cyclization activation process, we also synthesized N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***16***), an analogue without the two methyl groups. As shown in Scheme 4, 2-nitrophenylacetic acid was converted to 2-nitrobenzylamine in 76.1% yield by treatment with sodium azide and sulfuric acid in chloroform at 50 °C for 1.5 h followed by neutralization with sodium hydroxide. Coupling with 5-FU by using N^1-chloroformyl-5-FU afforded the desired N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***16***) in 34.2% yield.

Synthesis of N^1-[2-(1-azidoisopropyl)phenylcarbamoyl]-5-fluorouracil. We are having trouble synthesizing this compound as originally proposed because of the inherent difficulty of coupling an aromatic amine with N^1-chloroformyl-5-FU. As shown in Scheme 5, reaction of compound ***18***, where the azido group is replaced by a *t*-Boc-amino group, with N^1-chloroformyl-5-FU failed to give the desired conjugate ***19***. This has been reported by Ozaki and colleagues [1]. Because of this problem, our research was later focused on compound ***14*** and analogues.

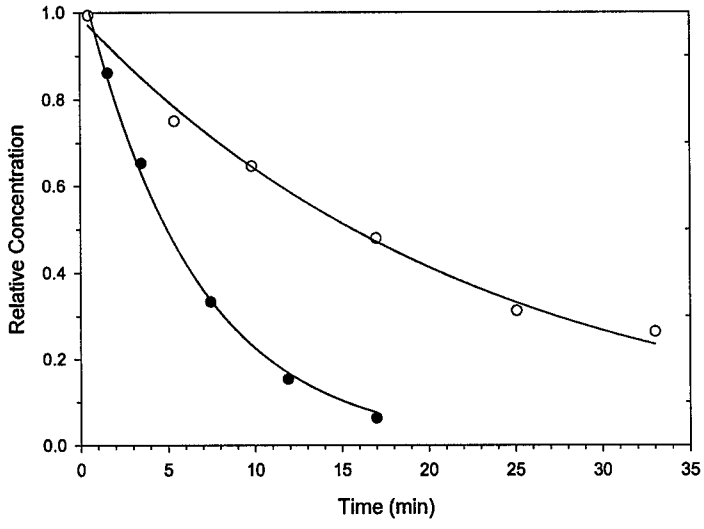
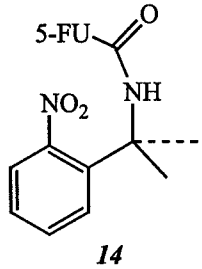


Figure 1. The stability of compounds 1 (●) and 2 (O) in phosphate buffer, pH 7.4 at 37 °C as monitored by HPLC.

2) Stability of protected Linker-Drug conjugates *14* and *16* of 5-FU

In the first year, we synthesized the Linker-Drug conjugates ***14*** and ***16***. During second year, we found that compounds ***14*** and ***16***, were having stability problems. The urea linkage is rather unstable in phosphate buffer at pH 7.4 and 37 °C with half lives of 15.9 and 4.4 min (Figure 1), respectively, making them unstable for incorporation into prodrugs. Thus, our research was then focused on the design and synthesis of new analogues with increased stability.



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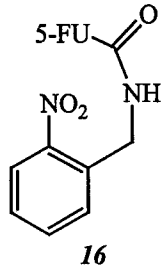

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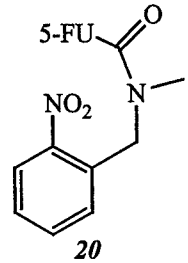

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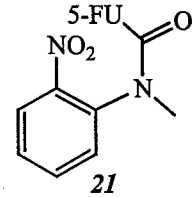

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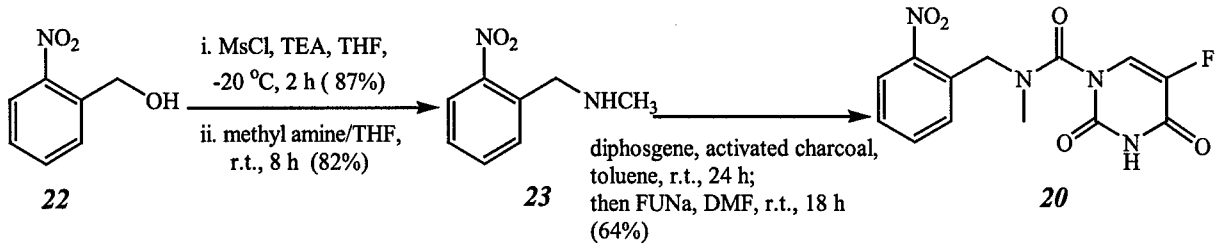
3) Synthesis of protected Linker-Drug conjugates *20* and *21* of 5-FU

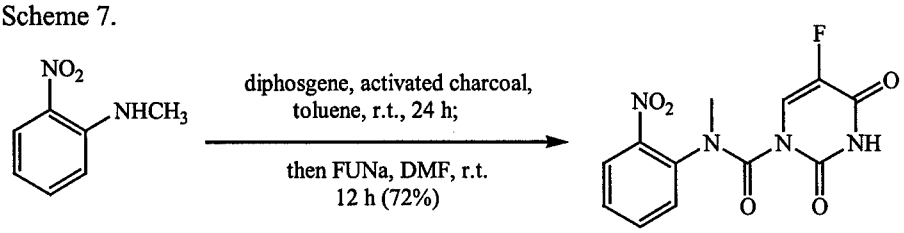
The synthesis of compounds ***20*** and ***21*** are outlined in Schemes 6 and 7, respectively. *N*-Methyl-2-nitro-benzylamine ***23*** was prepared by substituting the hydroxyl group on 2-nitrobenzyl alcohol (***22***) via an activated mesylate in a yield of 72%. The other starting material, *N*-methyl-2-nitroaniline (***24***), is commercially available. Phosgene chemistry was used to link the amines ***23*** and ***24*** with 5-FU. Diphosgene is a much safer substitution for phosgene, as it can generate phosgene *in situ* in the presence of activated charcoal. Therefore, the amine was first mixed with diphosgene and activated charcoal in toluene to form the carbamoyl chloride. Excess phosgene was purged by a smooth flow of nitrogen, then the carbamoyl chloride intermediate was further reacted with 5-FU sodium salt in DMF to afford the desired product. Compounds ***20*** and ***21*** were obtained in yields of 64% and 72%, respectively.

Scheme 6.



22 *23* *20*

Scheme 7.



24 *21*

4) Stability of protected Linker-Drug conjugates *20* and *21* of 5-FU

Compounds ***20*** and ***21*** synthesized above were found to be stable. No significant hydrolysis was observed after three days of incubation in phosphate buffer at pH 7.4 and 37 °C. The introduction of a methyl group on the nitrogen of the urea linkage dramatically increased the stability of 5-FU conjugates.

5) Cyclization of Linker-Drug conjugates *20* and *21* of 5-FU

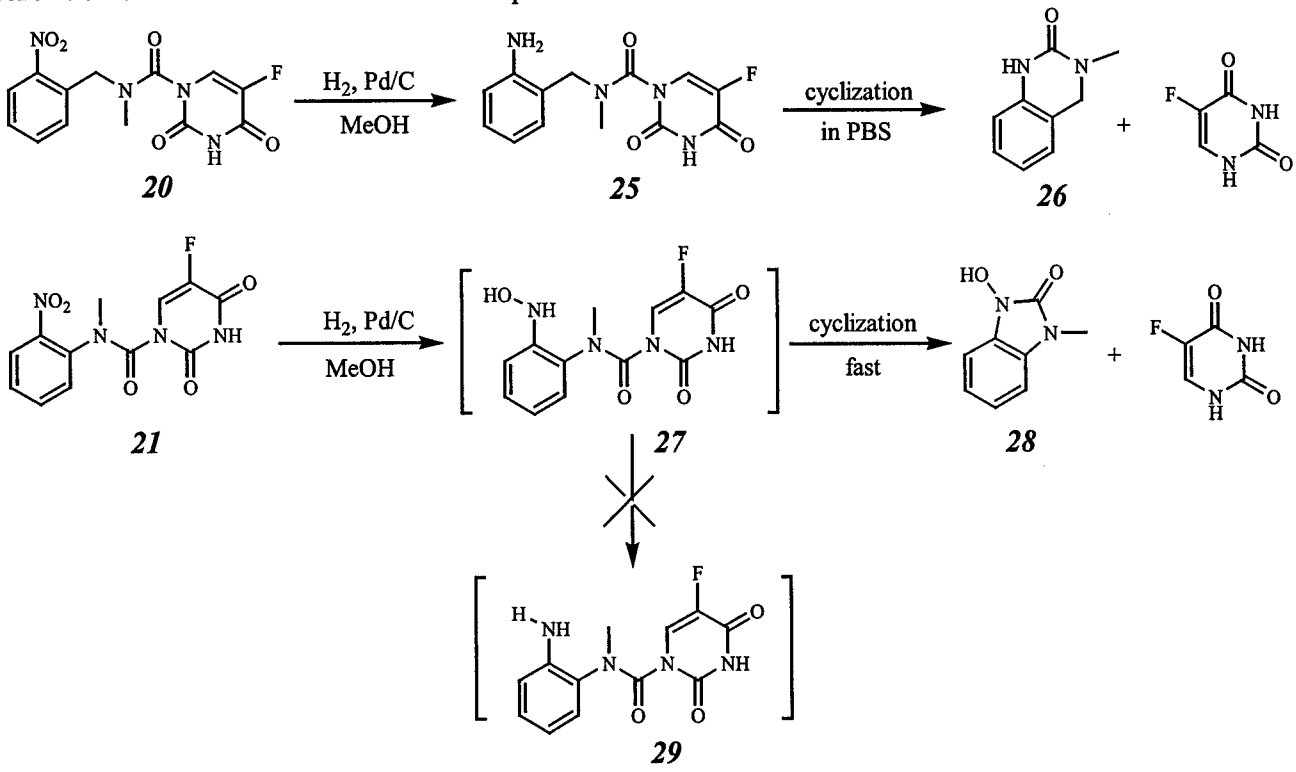
Hydrogenation was employed with the two compounds to test the feasibility of releasing 5-FU (Scheme 8). Amine ***25***, obtained from the reduction of compound ***20***, cyclized in phosphate buffer to free the active drug with a half-life of 2.86 h. Interestingly, hydrogenation of compound ***21*** resulted in the formation of compound ***28***, which apparently was derived from the hydroxylamine intermediate ***27***. The structure of ***28*** was confirmed by ^{1}H NMR spectra and HRMS. It was hypothesized that in this case, the hydroxylamine cyclized much faster than its further reduction to form the amine intermediate.

6) Synthesis of a Peptide-Linker-Drug conjugate of 5-FU

The peptide sequence of L-Serine-L-Alanine-L-Leucine-L-Leucine was chosen as the peptide portion for our prodrugs. It is one of the fastest cleaved sequences *N*-terminal to the cleavage site for PSA. Although not specific for PSA, the simple peptide sequence would still enable us to test our activation mechanism, and future studies might involve the structure or sequence modification to make prodrugs as specific substrates for PSA with respect to other serine proteases.

*Synthesis of protected peptide **36**.* The preparation of protected tetrapeptide ***36*** in solution is summarized in Scheme 9. Treatment of *N-tert*-butoxycarbonyl (*t*-Boc) protected L-leucine ***30*** with thionyl chloride in methanol resulted in the protection of the carboxylic acid methyl ester ***31***. Removal of the *t*-Boc group under acidic condition and coupling of the resulting amine with *t*-Boc-protected L-alanine via the activation by *N*-hydroxybenzotriazole (HOBT) and 1,3-dicyclohexylcarbodiimide (DCC) gave protected dipeptide ***32***. The same method was applied to the coupling of dipeptide **15** with protected L-serine to give protected tripeptide ***33***. Lithium hydroxide mediated hydrolysis converted the tripeptide methyl ester ***33*** to its corresponding carboxylic acid ***34***. Preparation of the protected tetrapeptide ***35*** from the coupling of acid ***34***and leucine methyl ester, followed by lithium hydroxide mediated hydrolysis furnished the desired peptide ***36*** in a total yield of 33%.

Scheme 8. Chemical reductive activation of compounds ***20*** and ***21***.



20 ***25*** ***26*** ***21*** ***27*** ***28*** ***29***

Scheme 9. Synthesis of tetrapeptide ***36***.

N-t-Boc-Leu-OH (***30***) $\xrightarrow[4\text{ h (100\%)}]{SOCl_2,\ MeOH,\ 0\ ^{\circ}C\text{ - r.t.}}$ Leu-OCH_3•HCl (***31***) $\xrightarrow[\text{ii. } N\text{- } t\text{-Boc-Ala-OH, HOBT, DCC, MeCN-THF, -5 °C, 1 h (100\%)}]{\text{i. TEA, MeCN}}$ *N-t*-Boc-Ala-Leu-OCH_3 (***32***)

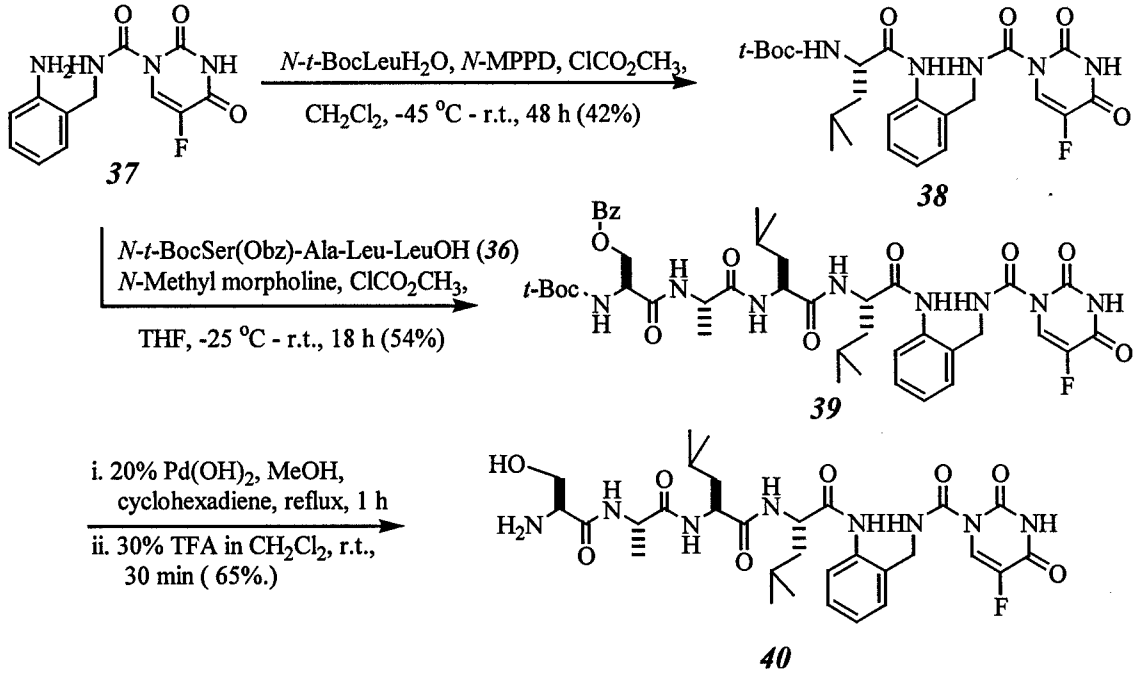
$\xrightarrow[\text{ii.}N\text{-}t\text{-Boc-Ser(OBn)-OH, HOBT, DCC, MeCN-THF, -5 °C-r.t. (89\%)}]{\text{i. TFA, } CH_2Cl_2\text{, r.t., 40 min}}$ *N-t*-Boc-Ser(OBn)-Ala-Leu-OCH_3 (***33***)

$\xrightarrow[13\text{ h (97\%)}]{LiOH,\ MeOH\text{-}H_2O,\ 0\ ^{\circ}C}$ *N-t*-Boc-Ser(OBn)-Ala-Leu-OH (***34***) $\xrightarrow[\text{DCC, MeCN, THF, 0 °C, 4 h (91\%)}]{\text{Leu-OCH}_3\text{.HCl, TEA, HOBT,}}$

N-t-Boc-Ser(OBn)-Ala-Leu-Leu-OCH_3 (***35***) $\xrightarrow[0\ ^{\circ}C,\ 5\text{ h}\ (42\%)]{LiOH,\ MeOH\text{-}H_2O}$ *N-t*-Boc-Ser(OBn)-Ala-Leu-Leu-OH (***36***)

*Synthesis of initial target **40**.* The synthesis of our first peptide-linker-drug ***40*** is outlined in Scheme 10. A model test was first performed to construct the peptide bond between *N-t*-Boc protected leucine and the aromatic amino group in compound ***37***, which was derived from reduction of the nitro compound ***16***. The conditions using HOBT/DCC and N-hydroxysuccinimide (HOSU)/DCC failed to accomplish the coupling, and compound ***38*** could only be produced in 42% yield through an activated anhydride. The difficulty in forming the peptide bond in this case might be due to the weak nucleophilicity of the aromatic amine in ***37***. Subsequently, using these conditions, conjugate ***39*** was

Scheme 10. Synthesis of compound ***40***.



obtained in 54% yield from the coupling of the tetrapeptide ***36*** with the Linker-Drug ***37***. In the last step of deprotecting the hydroxyl group and amino group on the serine residue of the peptide, the sequence of deprotection was found to be very important. If the *N*-*t*-Boc group was removed first, the benzyl ether protecting the hydroxyl would not been cleaved by hydrogenation, which might be explained by the poisoning of the palladium catalyst by free amino groups. Therefore, deprotection of the hydroxyl group was first carried out by heating ***39*** with 20% $Pd(OH)_2$ catalyst and cyclohexadiene in methanol. Further treatment with a solution of trifluoroacetic acid (TFA) in dichloromethane (30%) removed the *t*-Boc protecting group and afforded the final compound ***40*** in 65% yield.

7) Experimental Section

General Methods. Melting points were determined on a Mel–Temp capillary apparatus and were uncorrected. Infrared (IR) spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer and are reported in wave numbers (cm^{-1}) with broad signals denoted by (br). ^1H NMR spectra were recorded in deuterated solvents at 200 or 300 MHz on Varian Gemini 200 or 300 MHz spectrometer as indicated. ^{13}C NMR spectra were recorded at 50 MHz on a Varian Gemini 200 MHz spectrometer. Coupling constants are reported in hertz (Hz). UV spectra were recorded on HP-8451A diode array spectrophotometer. HPLC analysis was performed on Spectra-Physics HPLC system. Mass spectra (MS) were obtained from mass spectrometry laboratories in University of Oklahoma and University of Kansas. Analytical LCMS spectra were obtained from Department of Pharmaceutics, Rutgers, The State University of New Jersey.

All reactions were stirred magnetically. Moisture-sensitive reactions were performed in flame-dried glassware under a positive pressure of nitrogen or argon as indicated. Air and moisture-sensitive liquids and solutions were transferred via syringes and were introduced into reaction vessels through rubber septa. Analytical thin-layer chromatography (TLC) was carried out on Whatman TLC plates precoated with silica gel 60 F_{254} (250-μm layer thickness). Flash column chromatography was performed on EM Science silica gel 60 (230–400 mesh) purchased from Aldrich. Organic solutions were concentrated using a Büchi rotary evaporator at 15~20 mmHg.

Tetrahydrofuran (THF), diethyl ether (Et_2O) were distilled from sodium metal/benzophenone. Pyridine, dichloromethane (CH_2Cl_2) and acetonitrile (CH_3CN) were distilled from calcium hydride. *N,N*-Dimethylformamide (DMF) was distilled under reduced pressure from calcium hydride and stored over 4-Å molecular sieves. Dioxane was distilled from calcium oxide (CaO). Anhydrous toluene was purchased from Aldrich and used directly. 5-Fluorouracil (5-FU) was purchased from ICN Biomedicals Inc. All

amino acids were obtained from Advanced Chem. Tech. Deuterated solvents were purchased from Cambridge Isotope Laboratories Inc. All other commercially available chemicals were purchased from Sigma and Aldrich Chemical Co., and were used without further purification unless otherwise indicated.

Synthesis of 2-(2-nitrophenyl)-2-methylpropionic acid methyl ester (2).

To a solution of 2-nitrophenylacetic acid ***1*** (40 g, 0.22 mol) in 240mL methanol was added with stirring $SOCl_2$ (36 mL) over 30 min while maintaining the temperature at 0-4 °C. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 18 h. After removal of solvent, the residue was dissolved with ethyl acetate, washed with water, and dried over $MgSO_4$. Removal of ethyl acetate under reduced pressure gave the corresponding methyl ester (45 g, 100%). MS (FAB, NBA) *m/z* 196.2 (MH^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 3.72 (s, 3H, OCH_3) 4.04 (s, 2H, CH_2), 7.38-8.14 (m, 4H, Ph).

The methyl ester (19.5 g, 100 mmol), MeI (14.3 mL, 250 mmol, 2.5eq) and 18-crown-6 (6.6 g, 25 mmol, 0.25 eq) was dissolved in 130 mL of DMF and stirred at -4-0 °C. A small amount of sodium hydride (60% in oil) was added slowly until the color suddenly turned to blue; then NaH (9.2g, 230 mmol, 2.3eq,) was added with stirring over 40 min while maintaining the temperature at 0-4 °C. The reaction mixture gradually turned to a green then yellow slurry after standing overnight. The reaction mixture was then diluted with ethyl acetate, washed with 1 N HCl, 1 N $KHCO_3$, and brine, and dried over $MgSO_4$. Removal of ethyl acetate afforded desired 2-(2-nitrophenyl)-2-methylpropionic acid methyl ester ***2*** (26.9 g, 100%). MS (FAB, NBA) *m/z* 224.2 (MH^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.68 (s, 6H, CH_3), 3.65 (s, 3H, CH_3), 7.26-7.89 (m, 4H, Ph).

Synthesis of 2-(2-nitrophenyl)-2-methylpropionic acid (3).

2-(2-Nitrophenyl)-2-methylpropionic acid methyl ester ***2*** (8 g, 36 mmol) was refluxed in 216 mL of 1 N NaOH (6 eq) methanol/water (1 : 1) solution for 6 h. After evaporation of methanol, the aqueous solution was acidified with 1 N HCl to pH 2 and extracted with ethyl acetate. The ethyl acetate phase was then washed with brine, and dried over $MgSO_4$. Removal of ethyl acetate gave 2-(2-nitrophenyl)-2-methylpropionic acid ***3*** (5.5 g, 93%) as a yellow solid in. IR (KBr) cm^{-1} 2900 (br), 2600, 1700; MS (EI) *m/z* 164.3 (M^+-COOH, 3.6%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.71 (s, 6H, CH_3), 7.61-7.99 (m, 4H, Ph).

Synthesis of 2-(1-hydroxylisopropyl)-benzyl alcohol (6).

A solution of phthalide (20 g, 149 mmol) in 100 mL CH_2Cl_2 was added through a dropping funnel into a 100 mL solution of 3 M MeMgBr in Et_2O at 0-5 °C (ice-salt bath) under N_2. The reaction mixture was stirred at 5-10 °C for 1 hour, and then diluted with ethyl acetate. After washing with aqueous NH_4Cl and brine, it was dried over $MgSO_4$. Removal of organic solvents gave the desired product ***6*** (25.2 g, 100%) as an oil. MS (EI) *m/z* 148 (M^+-H_2O, 83.4%). ^1H NMR (300 MHz, $CDCl_3$) δ 1.67 (s, 6H, CH_3), 3.40-3.60 (b, 2H, OH), 4.81 (s, 2H, $HOCH_2$), 7.20-7.29 (m, 4H, Ph).

Synthesis of 2-(1-hydroxylisopropyl)-benzyloxy-t-butyl-diphenylsilane (7).

Flame-dried 3-necked flask was charged with diol ***6*** (10 g, 60.2 mmol), *t*-butyldiphenylsilyl chloride (18.1 g, 66.2 mmol, 1.1 eq) and 60 mL of tetrahydrofuran under N_2. The mixture was then cooled to 0 °C. A solution of DMAP (30 mg, 0.004 eq), imidazole(20.5 g, 300.8 mmol, 5 eq) in 90 mL THF was added slowly. After the reaction mixture was stirred for 1 h, it was diluted with ethyl acetate, washed with 5% $KHSO_4$ solution and brine, dried over $MgSO_4$. Removal of solvent afforded the desired product *7* (22.5 g, 92.4%) as an oil. MS (FAB, NBA) *m/z* 405 (MH^+); ^1H NMR (300 MHz, $CDCl_3$) δ 1.05 (s, 9H, *t*-Bu), 1.63 (s, 6H, CH_3), 5.01 (s, 2H, OCH_2), 7.12-7.68 (m, 14H, Ph).

*Synthesis of 2-(1-azidoisopropyl)-benzyloxy-t-butyl-diphenylsilane (**8**).*

To a solution of trifluoroacetic acid (15mL, 14 eq) in 120 mL of CH_2Cl_2 was added sodium azide (8 g, 8 eq) at 0 °C. After 10 min at 0 °C, a solution of 2-(1-hydroxylisopropyl)-benzyloxy-*t*-butyl-diphenylsilane *7* (5.9 g, 14.6 mmol) in 120 mL of CH_2Cl_2 was added dropwise over 15 min. The reaction mixture was allowed to warm up to room temperature and stirred for 48 h. It was then diluted with CH_2Cl_2, washed with sat. $KHCO_3$ and brine, then dried over Na_2SO_4. After evaporation of solvent under reduced pressure, the residue was separated by flash column chromatography to give the desired product ***8*** (3.4 g, 73% after recovering 1.73 g starting material) as an oil. IR (KBr) cm^{-1} 2100 ($-N_3$); MS (FAB, NBA) *m/z* 386 (M^+-HN_3, 2.8%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.08 (s, 9H, *t*-Bu), 1.53 (s, 6H, CH_3), 5.05 (s, 2H, OCH_2), 7.30-7.90 (m, 14H, Ph).

*Synthesis of 2-(1-azidoisopropyl)-benzoic acid (**9**).*

To a solution of 2-(1-azidoisopropyl)-benzyloxy-t-butyl-diphenylsilane ***8*** (918 mg, 2.1 mmol) in 5 mL of THF was added 3.4 mL of 1 M TBAF (1.6 eq) in THF. The reaction mixture was stirred at room temperature for 2 h before it was diluted with ethyl acetate, washed with 5% $NaHSO_4$ and brine, dried over Na_2SO_4. After removal of solvent, the residue was separated by flash column chromatography to give 2-(1-azidoisopropyl)-benzyl alcohol (300 mg, 73.4%) as an oil. IR (KBr) cm^{-1} 3320 (br, -OH), 2100 ($-N_3$); MS (EI) *m/z* 149.2 (M^+-N_3, 3.2%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.75 (s, 6H, CH_3), 2.4 (br, 1H, OH), 4.92 (s, 2H, OCH_2), 7.26-7.55 (m, 4H, Ph).

A suspension of 2-(1-azidoisopropyl)-benzyl alcohol (900 mg, 4.7 mmol) and PDC (6.7 g, 3.8 eq) in 20 mL DMF was stirred at room temperature for 4.5 h. The reaction mixture was filtered through a pad of celite, washed with water and brine until no color was present in the organic phase, and then dried over $MgSO_4$. Removal of solvent gave 2-(1-azidoisopropyl)-benzaldehyde as an oil in quantitative yield. IR (KBr): cm^{-1} 2100 ($-N_3$), 1670 (-CHO); MS (FAB, NBA) *m/z* 147.2 (M^+-N_3, 4.9%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.80 (s, 6H, CH_3), 7.43-7.96 (m, 4H, Ph), 10.90 (s, 1H, CHO)

To a solution of 2-(1-azidoisopropyl)-benzaldehyde (900 mg, 4.7 mmol) in 50 mL of *t*-butanol and 12 mL of 2-methyl-2-butene was added dropwise with stirring a solution of 2.0 g of $NaClO_2$ (5 eq) and 2.3 g of $NaH_2PO_4 \cdot H_2O$ in 20 mL water. Stirring was continued for 2 h at room temperature before the reaction mixture was diluted with water and washed with ether. The aqueous phase was acidified to pH 2 with 1 N HCl solution and extracted with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4. Removal of solvent under vacuum, the residue was subjected to flash column chromatography to give 2-(1-azidoisopropyl)-benzoic acid ***9*** (620mg, 64%) as a solid. IR (KBr) cm^{-1} 2900(br, -COOH), 2100 ($-N_3$), 1650 (-COOH); MS (FAB, NBA) *m/z* 206.2 (MH^+, 11.2%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.79 (s, 6H, CH_3), 7.32-7.53 (m, 4H, Ph), 8.6 (br, 1H, COOH).

*Synthesis of 2-(1-azidoisopropyl)-benzoic acid-Doxorubicin conjugate (**10**).*

2-(1-Azidoisopropyl)-benzoic acid (12 mg, 0.059 mmol) and HBTU (23 mg, 0.059 mmol) were dried under vacuum for 1 h before dry DMF was introduced under N_2. After the addition of DIEA (15 μL, 0.059 mmol), the reaction mixture was stirred at room temperature for 15 min followed by the addition of a solution of Dox·HCl (34 mg, 0.059 mmol) and DIEA (15 μL, 0.059 mmol) in 0.5 mL DMF. The reaction mixture was stirred at room temperature for 1 h, diluted with ethyl acetate, washed with 1 N HCl, sat. $NaHCO_3$ and brine, and dried over Na_2SO_4. After removal of solvent under vacuum, the residue was subjected to flash column chromatography to obtain the desired doxorubicin conjugate ***10*** (27 mg, 62.6%) as a red solid. TLC ($CH_3OH/CHCl_3$, 1 : 6) R_f 0.4; IR (KBr) cm^{-1} 3400, 2900, 2100; ^1H NMR (300 MHz, $CDCl_3$), δ 1.33 (d, J = 6.6 Hz, 3H), 1.70 (s, 3H, CH_3), 1.74 (s, 3H, CH_3), 1.90-1.96 (dd, J = 4.2, 13.8 Hz, 1H), 1.98-2.04 (dd, J = 5.4, 14.0 Hz, 1H), 2.22 (d, J = 4.2 Hz, 1H), 2.40 (d, J = 14.3 Hz, 1H), 3.01 (s, 1H), 3.08 (s, 1H), 3.85 (s, 1H), 4.09 (s, 3H), 4.24 (q, J = 6.7 Hz, 1H), 4.30-4.40 (m, 1H),

4.79 (d, J = 4.8 Hz, 2H), 5.31 (s, 1H), 5.56 (d, J = 3.3 Hz, 2H), 6.05 (d, J = 8.1 Hz, 1H), 7.24-8.06 (m, 7H, Ph).

*Synthesis of 2-(2-nitrophenyl)-2-methylpropionyl azide (**11**)* and *1-(2-nitrophenyl)-isopropyl isocyanate (**12**).*

2-(2-Nitrophenyl)-2-methylpropionic acid ***3*** (1.2 g, 6 mmol) was dissolved in 15 mL of dry acetone, to which was added slowly triethylamine (0.92 mL, 1.1 eq) under N_2. The solution was then cooled and kept at -5-0 °C when $ClCO_2Et$ (0.51 mL, 1.1 eq) was added slowly. After stirring for an additional 15 min, NaN_3 (780 mg, 2 eq) in 3 mL water was added slowly over 10 min at -5-0 °C. The stirring was continued for another 30 min at 0 °C before the reaction mixture was poured into cold water and extracted with methylene chloride. The organic phase was washed with brine and dried over Na_2SO_4. Removal of methylene chloride gave the desired acyl azide product ***11*** (400 mg, 31.3%) as an oil after recovering 60 mg starting material. IR (KBr) cm^{-1} 2140, 1700; MS (FAB) *m/z* 235.1(MH^+, 22.2%); ^1H NMR (300 MHz, $CDCl_3$) δ1.65 (s, 6H, CH_3), 7.46-7.99(m, 4H, Ph).

The acyl azide ***11*** (100 mg, 0.43 mmol) was dissolved in 2 mL of toluene and refluxed for 2 h. The residue after evaporation of solvent was subjected to flash column chromatography on silica gel to give the corresponding isocyanate ***12*** (70 mg, 79.5%) as an oil. IR (KBr) cm^{-1} 2260 (-N=C=O); MS (FAB) *m/z* 205.1 (M^+-1, 3.5%); ^1H NMR (300 MHz, $CDCl_3$) δ1.87 (s, 6H, CH_3), 7.41-7.52 (m, 4H, Ph).

*One-pot synthesis of 2-nitrophenylisopropylamine (**13**) from 2-(2-nitrophenyl)-2-methylpropionic acid (**3**).*

To a solution of 2-(2-nitrophenyl)-2-methylpropionic acid ***3*** (1.25 g, 6 mmol) in 10 mL of acetone was added slowly with stirring 0.92mL (1.1eq) of Et_3N. The mixture was cooled to -5-0 °C before $ClCO_2Et$ (0.63 mL, 1.1 eq) in 2 mL of acetone was added slowly. The reaction mixture was stirred for an additional 15 min at -5-0 °C before a solution of NaN_3 (780 mg, 2eq) in 3 mL water was added slowly. After stirring was continued for another 30 min at -5-0 °C, the reaction mixture was poured into 25 mL cold water and extracted with toluene (2x25 mL). The organic phase was dried over $MgSO_4$ (150 mg acid was recovered from aqueous phase). The toluene extract was transferred to a one necked round bottomed flask equipped with a reflux condenser; the stirred solution was heated cautiously under reflux for 1 h on an electric bath. Toluene was then removed at 50 °C with a rotavap; the flask containing the residual was again fitted with reflux condenser, the oil was stirred and cooled in an ice bath before 10 mL of 8 N HCl was added. The cooling bath was removed and the stirred mixture was gradually heated under reflux for 10 min. Then evacuated and warmed in a bath at 50 °C for 10 min; 10 mL ice water was added to the flask while cooled in an ice bath, 30 mL of 3 N NaOH solution was added slowly to adjust the pH to 12. Ethyl acetate extraction followed by washing with brine, drying over $MgSO_4$, solvent evaporation gave the desired product 2-nitrophenylisopropylamine ***13*** (300 mg, 32%) after flash column chromatography. IR (KBr) cm^{-1} 3300, 2950, 1500, 1350; MS (FAB) *m/z* 181.1 (MH^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ1.58 (s, 6H, CH_3), 1.65 (s, 1H, NH), 1.70 (s, 1H, NH), 7.28-7.54 (m, 4H, Ph).

*Synthesis of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (**14**).*
Method A

Isocyanate ***12*** (150 mg, 0.7 mmol) and 5-FU (95 mg, 0.7 mmol, 1 eq) were dissolved in 3.5 mL of toluene. After the addition of 0.1 mL of Et_3N, the reaction mixture was refluxed for 18 h. The residue after evaporation of solvent was subjected to flash column chromatography on silica gel to obtain the desired 5-FU conjugate ***14*** (35 mg, 14.3%) as a solid. TLC (EtOAc/PE=1:1) R_f = 0.5; MS (FAB) *m/z* 337.1(MH^+, 12.4%); HRMS (FAB, NBA) $C_{14}H_{14}FN_4O_5$ (MH^+) calcd 337.0948, found 337.0950; ^1H NMR (300 MHz, $CDCl_3$) δ1.88 (s, 6H, CH_3), 7.26-7.54 (m, 4H, Ph), 8.32 (d, J = 7.2 Hz, 1H), 9.47 (s, 1H).

Method B

2-Nitrophenylisopropylamine ***13*** (32 mg, 0.2 mmol) and 5 mg of activated charcoal in 1 mL anhydrous toluene were stirred under N_2 at 0 °C. 50 μL of diphosgene (2 eq) was added and the reaction mixture was stirred at room temperature for 18 h. N_2 was bubbled through the reaction mixture to remove excess phosgene; after filtration and CH_2Cl_2 wash, the solvent was evaporated. The residue and 30 mg of 5-FUNa (1 eq) were dried under vacuum for 1 h before 1 mL of anhydrous DMF was introduced under N_2. Stirring was continued at room temperature for 20 h. The reaction mixture was then diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4. After removal of solvent, the residue was separated using flash column chromatography on silica gel to give the desired 5-FU conjugate ***14*** (10 mg, 16.7%) as a solid.

Method C

5-FU (20 mg, 0.154 mmol) and 10 mg of active charcoal were dried under vacuum for 1 h. 2 mL of pyridine was introduced under argon and mixture was cooled down to 0 °C. Diphosgene (40 μL, 0.308 mmol) was added with stirring. After 2 h, N_2 was bubbled through to remove excess phosgene. After removal of charcoal, the filtrate containing N^1-chloroformyl-5-FU was added directly to 2-nitrophenylisopropylamine ***13*** (20 mg, 0.111 mmol). The reaction mixture was stirred at room temperature for 18 h before solvent was removed by rotavap. The residue was dissolved in acetone and the pyridinium salt was removed by filtration. Flash column chromatography gave the desired 5-FU conjugate ***14*** (23 mg, 50%).

Stability test in phosphate buffer

The masked Linker-5-FU conjugates (1.5 mg) was dissolved in 200 mL of CH3CN and stored at 0 °C. A solution (10 μL) was withdrawn and quickly added to 190 μL of 100 mM phosphate buffer (pH 7.4) pre-warmed at 37 °C to give a final concentration of 1 mM. The resulting solution was incubated at 37 °C while aliquots (25 μL) of sample were removed at intervals and injected directly into the HPLC injection port. HPLC analysis with C-18 reverse-phase column used either a gradient mobile phase from 28% CH_3CN to 52% CH_3CN over 10 min or isocratic elution with a mobile phase of 40% CH_3CN, at a flow rate of 1 mL/min and detection wavelengths of 220 nm and 280 nm.

General procedure for hydrogenation

A solution of nitro compound (0.05 mmol) in 3 mL of methanol underwent atmospheric hydrogenation in the presence of 10% Pd/C. Both HPLC and TLC were used to monitor the progress of reduction. At the end of reaction, the catalyst was removed by filtration, the residue was washed with methanol (3 × 5 mL). The combined organic phase was condensed *in vacuo*, and the residue was purified by flash column chromatograhpy on silica gel eluted with acetone–hexanes to afford the cyclized lactam, parent drug 1, and/or the amine intermediate. HPLC analysis was performed on a C-18 reversed-phase column (150 × 4.6 mm), using first a isocratic elution of 2% CH_3CN for 5 min followed by a gradient elution from 2% CH_3CN to 70% CH_3CN over 15 min and a final isocratic elution of 70% CH_3CN for 5 min, at a flow rate of 1 mL/min and detection wavelengths of 220 nm and 280 nm.

General procedure for cyclization

A solution of nitro compound (0.05 mmol) in 3 mL of methanol underwent hydrogenation according to prodedure A. The product after isolation was the amine intermediate. The amine compound was then incubated in 100 mM phosphate buffer (pH 7.4) at 37 °C for half an hour and monitored by HPLC. At the end of reaction, the solution was extracted with ethyl acetate, and the organic phase was washed with brine, dried over anhydrous Na2SO4 and filtered. Removal of the solvent *in vacuo* afforded the lactam. FUDR in the aqueous phase was detected by HPLC.

Methyl-(2-nitrobenzyl)amine (23)

To a solution of 2-nitrobenzylalcohol **5** (2 g, 13 mmol) and triethylamine (2.72 mL, 19.6 mmol) in 40 mL of THF was added methanesulfonyl chloride (1.06 mL, 13.65 mmol) dropwise at –20 °C under argon atmosphere. After being stirred for 2 h at this temperature, the suspension was added 50 mL of ice water and extracted with *t*-butyl methyl ether (3 × 30 mL). The orgainc phase was washed with 1 N HCl solution (40 mL) and saturated NaHCO3 solution, dried over anhydrous MgSO4, and condensed *in vacuo* to afford 2.61 g (87%) of mesylate as a pale yellow solid. mp 95.5–97 °C; ^{1}H NMR (200 MHz, CDCl3) δ 8.20–7.53 (m, 4 H), 5.67 (s, 2 H), 3.14 (s, 3 H), 1.70 (br s, 1 H); ^{13}C NMR (50 MHz, CDCl3) δ 134.53, 130.32, 129.86, 129.55, 125.48, 68.25, 37.95; IR (KBr) 3098.3, 3028.9, 3015.5, 1613.7, 1578.3, 1525.1, 1443.2, 1383.4, 1342.1, 1270.9, 1198.6, 1174.0, 1149.8, 1050.4, 1010.1, 989.1, 967.1, 870.2, 805.8, 792.4, 736.4 cm^{-1}; MS (ESI) *m/z* (rel intensity): 135.85 [(M–OMs)$^+$, 100].

To a solution of 2 N methylaminé in THF (6.72 mL) was added mesylate (2.596 g, 11.2 mmol) as prepared above. The reaction mixture was stirred at room temperature for 8 h and then diluted with *t*-butyl methyl ether. The organic layer was washed with saturated NaHCO3 solution and brine, dried over anhydrous MgSO4, and condensed *in vacuo*. The residue was subjected to flash column chromatography on silica gel eluted with methanol–dichloromethane–1% triethylamine (1:40 → 1:30 → 1: 10 → 1: 5) to afford 1.52 g (82%) of **23** as a brown oil. ^{1}H NMR (200 MHz, CDCl3) δ 7.96–7.37 (m, 4 H), 3.98 (s, 2 H), 2.46 (s, 3 H), 1.70 (br s, 1 H); ^{13}C NMR (50 MHz, CDCl3) δ 135.86, 133.62, 131.75, 128.44, 125.23, 53.26, 36.63; IR (neat) 3344.0, 2944.0, 2841.4, 2790.2, 1610.3, 1528.4, 1441.2, 1343.8, 1123.3, 851.5, 784.8, 723.3 cm^{-1}; MS (ESI) *m/z* (rel intensity): 167.01 (MH$^+$, 100).

1-(N-Methyl-N-2-nitrobenzylcarbamoyl)-5-fluorouracil (20)

To a suspension of methyl-(2-nitrobenzyl)amine **23** (130 mg, 0.78 mmol) and 20 mg of activated charcoal in 5 mL of anhydrous toluene was added diphosgene (190 μL, 1.57 mmol) dropwise under argon atmosphere. After the reaction mixture was stirred at room temperature for 24 h, argon was bubbled through for 5 min to get rid of the excess phosgene. The activated charcoal was removed by vacuum filtration. After removal of the solvent *in vacuo*, the residue was dissolved in 5 mL of anhydrous DMF, to which was added 5-fluorouracil sodium salt (178 mg, 1.17 mmol) under argon atmosphere. The reaction mixture was stirred for 18 h at room temperature and condensed *in vacuo*. The residue was subjected to column chromatography on silica gel eluted with acetone–hexanes (1:5 → 1:3 → 1: 2 → 1: 1) to afford 161 mg (64%) of **20** as a white solid. mp 218–220 °C; ^{1}H NMR (200 MHz, DMSO-*d6*) δ 12.15 (s, 1 H), 8.21 (d, 1 H, J = 6.2 Hz), 8.17–7.57 (m, 4 H), 5.25–4.86 (m, 2 H), 3.01 (s, 3 H); IR (KBr) 3425.6, 3189.7, 3076.9, 2964.1, 2923.1, 1733.3, 1712.8, 1517.9, 1476.9, 1441.0, 1353.8, 1338.5, 1271.8, 1261.5, 1205.1, 1233.3, 1066.7, 928.2, 882.1, 861.5, 841.0, 794.9, 723.1, 702.6, 605.1 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 323.1 (MH$^+$, 12), 307.1 (85), 154.1 (100); HRMS calcd for C13H12N4O5F (MH$^+$) 323.0792 found 323.0814.

1-(N-Methyl-N-2-nitrophenylcarbamoyl)-5-fluorouracil (21)

To a suspension of commercially available *N*-methyl-2-nitroaniline **24** (304.1 mg, 1.99 mmol) and 40 mg of activated charcoal in 10 mL of anhydrous toluene was added diphosgene (483 μL, 3.98 mmol) dropwise under argon atmosphere. After the reaction mixture was stirred at room temperature for 24 h, argon was bubbled through for 5 min to get rid of the excess phosgene. The activated charcoal was removed by vacuum filtration and toluene was evaporated *in vacuo*. The residue was dissolved in 10 mL of anhydrous DMF, to which was added 5-fluorouracil sodium salt (304 mg, 2.0 mmol) under argon atmosphere. The reaction mixture was stirred for 12 h at room temperature and condensed *in vacuo*. The residue was subjected to column chromatography on silica gel eluted with acetone–hexanes (1:5 → 1:3 → 1: 2 → 1: 1) to afford 443 mg (72%) **21** as a yellow oil. ^{1}H NMR (200 MHz, CDCl3) δ 8.09 (d, 1 H, J =

8.4 Hz), 7.74–7.48 (m, 4 H), 3.48 (s, 3 H); IR (KBr) 3425.6, 3087.2, 1723.1, 1707.7, 1528.2, 1343.6, 1266.7, 1138.5, 784.6, 702.6 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 309.1 (MH^+, 29), 179.1 (9), 154.1 (100); HRMS calcd for C12H10N4O5F (MH^+) 309.0635 found 309.0635.

3-Methyl-3,4-dihydro-1H-quinazolin-2-one (26)

A solution of 1-(*N*-methyl-*N*-2-nitrobenzylcarbamoyl)-5-fluorouracil **3** (20 mg, 0.062 mmol) in 5 mL of methanol underwent atmospheric hydrogenation in the presence of 10 % Pd/C for 40 min. The reaction mixture was filtered and condensed *in vacuo* to afford 1-(*N*-methyl-*N*-2-aminobenzylcarbamoyl)-5-fluorouracil **25** as a colorless oil quantitatively after evaporating the solvent *in vacuo*. ^1H NMR (200 MHz, DMSO) δ 11.13 (br s, 1 H), 8.18 (d, 1 H, J = 6.2 Hz), 7.15–6.54 (m, 4 H), 5.08 (br s, 2 H), 4.51–3.77 (m, 2 H), 2.83 (d, 3 H, J = 11.6 Hz).

1-(*N*-Methyl-*N*-2-aminobenzylcarbamoyl)-5-fluorouracil **25** was incubated in phosphate buffer (pH 7.4, 100 mM) at 37 °C for 5 h and the reaction progress was monitored by HPLC. At the end of the reaction, the mixture was extracted with ethyl acetate. The organic phase was dried over anhydrous Na2SO4 and condensed *in vacuo* to afford cyclized compound **26** as a white solid quantitatively. mp 196–198 °C; ^1H NMR (200 MHz, CDCl3) δ 7.18–6.67 (m, 4 H), 4.47 (s, 2 H), 3.06 (s, 3 H); ^{13}C NMR (50 MHz, CDCl3) δ 154.39, 137.20, 128.40, 125.63, 122.05, 113.67, 51.06, 34.85; IR (KBr) 3435.9, 3210.3, 3128.2, 3066.7, 2923.1, 1671.8, 1610.3, 128.5, 1497.4, 1441.0, 1400.0, 1328.3, 1302.6, 1282.1, 1251.3, 1035.9, 753.8, 717.9 cm^{-1}; MS (ESI) *m/z* (rel intensity): 185.99(MNa^+, 10), 162.90 (MH^+, 100).

1-Hydroxy-3-methyl-1,3-dihydro-benzoimidazol-2-one (28)

A solution of 1-(*N*-methyl-*N*-2-nitrophenylcarbamoyl)-5-fluorouracil **21** (20 mg, 0.065 mmol) in 5 mL of methanol underwent atmospheric hydrogenation in the presence of 10 % Pd/C for 15 min. The catalyst was removed by vacuum filtration and product **28** was obtained as a white solid quantitatively after evaporating the solvent *in vacuo*. mp 180–182 °C; ^1H NMR (200 MHz, CDCl3) δ 10.59 (br s, 0.01 H), 7.38–6.70 (m, 4 H), 3.41 (s, 3 H); ^{13}C NMR (50 MHz, CDCl3) δ 153.21, 128.19, 126.14, 122.39, 121.86, 108.17, 108.06, 27.60; IR (KBr) 3449.3, 3097.4, 2765.4, 1685.5, 1488.0, 1446.2, 1388.1, 1333.4, 1260.8, 1220.5, 1125.7, 984.3, 753.5, 740.5, 715.3 cm^{-1}; MS (ESI) *m/z* (rel intensity): 186.95 (MNa^+, 12), 164.92 (MH^+, 100); HRMS calcd for C8H9N2O2 (MH^+) 165.0664 found 165.0670.

HCl•Leu-OCH3 (31)

A solution of *N-t*-Boc-Leu-OH **30** (3.5 g, 15 mmol) in 20 mL of methanol at 0 °C was added slowly 2.7 mL of thionyl chloride while stirring. After the addition, the reaction mixture was gradually warmed to room temperature and stirred for another 4 h. Removal of the solvent *in vacuo* afforded 2.5 g of **31** as a white foam. ^1H NMR (300 MHz, CDCl3) δ 3.72 (s, 3 H), 3.48 (dd, 1 H, J = 5.7, 5.85 Hz), 1.76 (m, 1 H), 1.57 (ddd, 1 H, J = 5.4, 6.6, 7.8 Hz), 1.47 (br s, 2 H), 1.45 (m, 1 H), 0.95 (d, 3 H, J = 5.1 Hz), 0.93 (d, 3 H, J = 5.4 Hz).

N-t-Boc-Ala-Leu-OCH3 (32)

To a solution of *N-t*-Boc-Ala-OH (1.9 g, 10 mmol) in 175 mL of THF and 75 mL of CH3CN were added HCl•Leu-OCH3 **31** (2.0 g, 11 mmol) and triethylamine (13.4 mL) followed by HOBT•H2O (2.02 g, 15 mmol). After the addition, the reaction mixture was cooled to –5 °C and charged with a solution of DCC (3.1 g, 15 mmol) in 10 mL of THF. After stirred for 1 h, the reaction mixture was filtered and 50 mL of THF was added to wash. After the combined organic phase was concentrated *in vacuo*, the residue was diluted with CH2Cl2, washed with 5% citric acid solution, 5% NaHCO3 solution and brine, dried over anhydrous Na2SO4 and filtered. Removal of the solvent *in vacuo* afforded product **32** as a white foam quantitatively. ^1H NMR (300 MHz, CDCl3) δ 6.60 (br s, 1 H), 5.06 (br s, 1 H), 4.61

(m, 1 H), 4.19 (br s, 1 H), 3.73 (s, 3 H), 1.67–1.55 (m, 3 H), 1.45 (s, 9 H), 1.35 (d, 3 H, J = 7.2 Hz), 0.93 (d, 6 H, J = 5.7 Hz); IR (KBr) 3314.8 (br), 2959.9, 2861.5, 1751.2, 1680.2, 1660.4, 1535.0, 1512.8, 1458.3, 1367.1, 1254.7, 1160.3, 1068.5, 854.2 cm^{-1}; MS (ESI) *m/z* (rel intensity) 317.45 (MH^+, 100).

N-t-Boc-Ser(Obz)-Ala-Leu-OCH3 (33)

N-t-Boc-Ala-Leu-OCH3 **32** (10 mmol) was treated with 50 mL of 50% TFA in CH2Cl2 at room temperature for 40 min. Removal of the solvent *in vacuo* afforded TFA salt of dipeptide, which was used directly in the next step. To a solution of *t*-Boc-Ser(Obz)-OH (3.2 g, 11 mmol) in 70 mL of THF and 30 mL of CH3CN were added the solution of TFA salt of dipeptide in 14 mL of triethylamine and HOBT•H2O (2.02 g, 15 mmol) sequentially with stirring. After the addition, the reaction mixture was cooled to –5 °C and charged with a solution of DCC (3.1 g, 15 mmol) in 10 mL of THF. The reaction mixture was warmed up to room temperature within one hour, and stirred at this temperature for 18 h. DCU was filtered off and 25 mL of THF was used to wash. After removal of the solvent *in vacuo*, the residue was diluted with CH2Cl2. The organic phase was washed with 5% citric acid solution, 5% aqueous NaHCO3 solution, and brine, dried over anhydrous Na2SO4 and filtered. After removal of the solvent *in vacuo*, the residue was subjected to flash column chromatography on silica gel eluted with ethyl acetate–hexanes (1:10 → 1:5 → 1:2) to afford 4.38 g (89%) of **33** as a white foam. ^{1}H NMR (300 MHz, CDCl3) δ 7.36–7.35 (m, 5 H), 7.16 (d, 1 H, J = 7.5 Hz), 7.01 (d, 1 H, J = 8.1 Hz), 5.49 (d, 1 H, J = 7.2 Hz), 4.57 (m, 2 H), 4.54 (s, 2 H), 4.32 (br s, 1 H), 3.88 (dd, 1 H, J = 4.2, 9.3 Hz), 3.72 (s, 3 H), 3.61 (dd, 1 H, J = 6.0, 9.3 Hz), 1.66–1.48 (m, 3 H), 1.42 (s, 9 H), 1.38 (d, 3 H, J = 7.2 Hz), 0.91 (d, 6 H, J = 5.7 Hz); IR (KBr) 3310.0 (br), 2961.1, 2872.0, 1752.0, 1717.9, 1646.0, 1523.1, 1453.1, 1367.2, 1248.5, 1208.9, 1166.0, 1115.3, 1025.3, 738.2, 698.4 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 494.2 (MH^+, 20.3), 438.2 (22.3), 146.0 (100).

N-t-Boc-Ser(Obz)-Ala-Leu-OH (34)

To a solution of *N-t*-Boc-Ser(Obz)-Ala-Leu-OCH3 **33** (100 mg, 0.2 mmol) in 3 mL of methanol and 1 mL of water was added lithium hydroxide (18 mg, 0.75 mmol) at 0 °C with stirring. After the reaction proceeding at this temperature for 13 h, the solvent was removed *in vacuo*. The residue was diluted with water and extracted with ethyl acetate. The aqueous phase was adjusted with 0.2 N HCl solution to pH 3, extracted with ethyl acetate, dried over anhydrous Na2SO4 and filtered. Removal of the solvent *in vacuo* afforded 98.7 mg (97%) of **34** as a white foam. ^{1}H NMR (300 MHz, CDCl3) δ 7.34–7.25 (m, 5 H), 7.04 (d, 1 H, J = 7.5 Hz), 6.95 (d, 1 H, J = 8.1 Hz), 5.50 (br s, 1 H), 4.55 (s, 2 H), 4.52 (m, 2 H), 4.32 (br s, 1 H), 3.85 (dd, 1 H, J = 4.2, 9.3 Hz), 3.62 (dd, 1 H, J = 6.0, 9.3 Hz), 1.75–1.58 (m, 3 H), 1.45 (s, 9 H), 1.39 (d, 3 H, J = 7.2 Hz), 0.91 (d, 6 H, J = 5.7 Hz); IR (KBr) 3306.0 (br), 2961.9, 2872.6, 1711.5, 1641.9, 1535.5, 1454.5, 1393.0, 1367.9, 1251.6, 1170.1, 1109.1, 1026.4, 738.5, 698.6 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 480.3 (MH^+, 10.6), 424.2 (7.5), 154.1 (100); HRMS calcd for C24H38N3O7 (MH^+) 480.2710 found 480.2712.

N-t-Boc-Ser(Obz)-Ala-Leu-Leu-OCH3 (35)

To a suspension of Leu-OCH3 HCl salt(350 mg, 1.92 mmol) in 14 mL of THF and 6 mL of CH3CN were added 2.15 mL of triethylamine, *N-t*-Boc-Ser(Obz)-Ala-Leu-OH **34** (837 mg, 1.745 mmol) and HOBT•H2O (353 mg, 2.62 mmol) sequentially. The reaction mixture was cooled to 0 °C, charged with DCC (500 mg, 2.62 mmol), and stirred for 4 h. After filtration, the reaction mixture was diluted with ethyl acetate. The organic phase was washed by 5% citric acid, 5% NaHCO3 solution, and brine, dried over anhydrous Na2SO4 and filtered. Removal of the solvent *in vacuo* afforded 960 mg (91%) of **35** as a white foam. ^{1}H NMR (200 MHz, CDCl3) δ 7.36–7.24 (m, 4 H), 7.24–6.97 (m, 3 H), 5.54 (d, 1 H, J = 6.2 Hz), 4.58–4.48 (m, 5 H, singlet at 4.51), 4.30 (d, 1 H, J = 5.6 Hz), 3.80 (dd, 1 H, J = 4.72, 9.6 Hz), 3.69 (s, 3 H), 3.64 (dd, 1 H, J = 5.27, 9.7 Hz), 1.79–1.26 (m, 19 H, singlet at 1.43, doublet at 1.35, J = 7.0 Hz), 0.90–0.84 (m, 12 H); ^{13}C NMR (50 MHz, CDCl3) δ 173.54, 172.43, 172.33, 170.90, 156.38, 137.80,

128.99, 128.69, 128.45, 128.17, 81.05, 73.89, 70.22, 55.50, 52.64, 52.13, 51.24, 49.98, 41.37, 41.11, 32.05, 28.73, 25.24, 23.36, 23.31, 23.12, 22.34, 22.21, 18.73, 14.59; IR (KBr) 3290.7 (br), 2958.8, 2872.0, 1749.6, 1719.5, 1641.1, 1542.4, 1452.4, 1367.4, 1252.0, 1209.4, 1166.7, 1114.4, 736.8, 698.4 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 607.4 (MH^+, 100), 551.3 (32); HRMS calcd for C31H51N4O8 (MH^+) 607.3707 found 607.3689.

N-t-Boc-Ser(Obz)-Ala-Leu-Leu-OH (36)

To a solution of *N-t*-Boc-Ser(Obz)-Ala-Leu-Leu-OCH3 **35** (3.03 g, 5 mmol) in 60 mL of methanol and 30 mL of water was added lithium hydroxide (1.06 g, 44 mmol) at 0 °C with stirring. After the reaction proceeding at this temperature for 5 h, methanol was removed *in vacuo*. The residue was partitioned between water and ethyl acetate. The aqueous phase was adjusted with 1 N HCl solution to pH 3, and extracted with *t*-butyl methyl ether (3 × 100 mL). The organic phase was dried over anhydrous MgSO4, filtered, and condensed *in vacuo* to afford 1.25 g (42%) of **36** as a white foam. ^{1}H NMR (200 MHz, CDCl3) δ7.46–7.27 (m, 6 H), 7.13 (d, 2 H, J = 6.58 Hz), 5.51 (m, 1 H), 4.54–4.40 (m, 5 H, singlet at 4.54), 4.27 (m, 1 H), 3.83 (dd, 1 H, J = 4.44, 9.54 Hz), 3.67 (dd, 1 H, J = 4.76, 9.76 Hz), 1.73–1.53 (m, 6 H), 1.45 (s, 9 H), 1.39 (d, 3 H, J = 6.96 Hz), 0.96–0.87 (m, 12 H); ^{13}C NMR (50 MHz, CDCl3) δ 174.60, 173.62, 172.76, 171.32, 156.60, 137.68, 129.05, 128.56, 128.46, 128.39, 128.27, 81.54, 73.98, 73.90, 69.86, 55.75, 52.55, 52.36, 50.46, 41.31, 40.52, 28.73, 25.25, 23.36, 22.18, 18.86; IR (KBr) 3303.8 (br), 2960.3, 2872.3, 1717.9, 1646.9, 1534.3, 1453.2, 1368.1, 1251.7, 1166.5, 736.5, 698.3 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 593.4 (MH^+, 100), 537.3 (50); HRMS calcd for C30H49N4O8 (MH^+) 593.3550 found 593.3552.

1-(2-Aminobenzylcarbamoyl)-5-fluorouracil (37)

A solution of 1-(2-nitrobenzylcarbamoyl)-5-fluorouracil **16** (11 mg, 0.036 mmol) in 3 mL of methanol underwent atmospheric hydrogenation in the presence of 10 % Pd/C for 25 min. The catalyst was removed by vacuum filtration and the filtrate was condensed *in vacuo* to afford **37** as a white solid quantitatively. mp 242–244 °C (acetone/hexanes); ^{1}H NMR (300 MHz, acetone-d_6) δ9.65 (br s, 1 H), 8.43 (d, 1 H, J = 7.8 Hz), 7.18–6.61 (m, 4 H), 4.51 (d, 2 H, J = 5.7 Hz); IR (KBr) 3424.0, 3251.3, 3087.2, 1736.0, 1686.3, 1651.3, 1523.2, 1498.0, 1458.7, 1339.7, 1266.2, 1246.2, 1210.3, 815.4, 755.6 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 279.1 (MH^+, 17.0), 176.0 (44.5), 154.0 (100), 106.0 (48.3); HRMS calcd for $C_{12}H_{12}FN_4O_3$ (MH^+) 279.0893 found 279.0855.

N-t-Boc-Leu-linker-5-FU conjugate (38)

To a solution of *N-t*-Boc-Leu•H2O (30 mg, 0.13 mmol) in 1 mL of CH2Cl2 cooled at –45 °C under argon atmosphere, were added *N*-methylpiperidine (15 μL, 0.121 mmol) followed by methyl chloroformate (10 μL, 0.121 mmol). After being stirred at this temperature for 10 min, the reaction mixture was added to 1-(2-aminobenzylcarbamoyl)-5-fluorouracil **37** (33 mg, 0.12 mmol), warmed up to room temperature and stirred for 48 h. After removal of the solvent *in vacuo*, the residue was subjected to flash column chromatography on silica gel eluted with ethyl acetate–petroleum ether (1:10 → 1:5 → 1:2) to afford 25 mg (42%) of **38** as a white foam. ^{1}H NMR (300 MHz, CDCl3) δ9.06 (br s, 0.5 H), 8.47 (d, 1 H, J = 7.2 Hz), 7.85 (d, 1 H, J = 8.1 Hz), 7.36–7.05 (m, 4 H), 5.05 (m, 1 H), 4.47 (m, 1 H), 4.34 (d, 2 H, J = 6.3 Hz), 1.80–1.70 (m, 3 H), 1.44 (s, 9 H), 1.02 (d, 6 H, J = 6.0 Hz); IR (KBr) 3424.4 (br), 2964.1, 1720.0, 1702.6, 1525.6, 1456.7, 1369.2, 1338.5, 1249.8, 1164.1, 1112.8, 1066.7, 762.1 cm^{-1}; MS (FAB, *m*-NBA) *m/z* (rel intensity): 492.3 (MH^+, 8), 262.2 (13.3), 154.1 (100); HRMS calcd for C23H31N5O6F (MH^+) 492.2258, found 492.2255.

N-t-Boc-Ser(Obz)-Ala-Leu-Leu-linker-5-FU conjugate (39)

To a solution of *N-t*-Boc-Ser(Obz)-Ala-Leu-Leu-OH **36** (300 mg, 0.5 mmol) in 15 mL of THF cooled at –25 °C under argon atmosphere, was added *N*-methylmorpholine (66 μL, 0.6 mmol) followed by methyl chloroformate (46 μL, 0.6 mmol). After being stirred at this temperature for 10 min, the reaction mixture was charged with a solution of 1-(2-aminobenzylcarbamoyl)-5-fluorouracil 44 (130 mg, 0.5 mmol) in 6 mL of THF, warmed up to room temperature and stirred for 18 h. After removal of the solvent *in vacuo*, the residue was subjected to flash column chromatography on silica gel eluted with acetone–hexanes (1:10 → 1:5 → 1:4 → 1:2) to afford 116 mg (54%) of **39** as a colorless oil. ^{1}H NMR (200 MHz, CDCl3) δ 10.0 (m, 1 H), 9.51 (m, 1 H), 8,80 (s, 1 H), 8.40 (d, 1 H, J = 6.88 Hz), 7.72 (m, 1 H), 7.51–7.11 (m, 10 H), 6.83 (m, 1 H), 5.50 (m, 1 H), 4.61–4.23 (m, 7 H, singlet at 4.53), 4.13 (m, 1 H), 3.85–3.68 (m, 2 H), 1.90–1.67 (m, 6 H), 1.50–1.30 (m, 12 H), 0.98–0.82 (m, 12 H); MS (FAB, *m*-NBA) *m/z* (rel intensity): 875.42 (MNa^+, 98), 853.44 (MH^+, 15), 629.36 (100); HRMS calcd for C42H58N8O10F (MH^+) 853.4260, found 853.4252.

H-Ser-Ala-Leu-Leu-linker-5-FU conjugate (40)

A solution of *N-t*-Boc-Ser(Obz)-Ala-Leu-Leu-linker-5-FU conjugate **39** (20 mg, 0.0235mmol), 5 mg of 20% Pd(OH)2 and cyclohexadiene (22 μL, 0.235 mmol) in 1 mL of methanol was heated under refluxing for 1.0 h with stirring. The catalyst was removed by filtration. Removal of the solvent *in vacuo* afforded the crude intermediate containing free hydroxyl group. The residue was treated with a solution of 30% TFA in CH2Cl2 (0.5 mL) at room temperature for 30 min. Removal of the solvent *in vacuo* afforded **40** as a yellow oil. ^{1}H NMR (200 MHz, CD3OD) δ 8.45 (d, 1 H, J = 6.24 Hz), 8.20 (m, 1 H), 7.75–7.20 (m, 9 H), 4.65–4.40 (m, 5 H, singlet at 4.61), 4.10 (m, 1 H), 3.90–3.70 (m, 3 H), 1.78–1.59 (m, 5 H), 1.39–1.24 (m, 6 H), 1.03–0.85 (m, 12 H); IR (KBr) 3424.1 (br), 2959.6, 2871.8, 1717.9, 1655.6, 1538.5, 1527.2, 1455.7, 1410.3, 1214.7, 1092.3, 753.8, 697.4 cm^{-1}; MS (ESI) *m/z* (rel intensity): 645.09 (M^+–OH, 55), 623.20 (100).

Key Research Accomplishments

1) **Synthesis of protected Linker-Drug conjugates of doxorubicin and 5-FU**

- Synthesis of 2-(2-nitrophenyl)-2-methyl-propionic acid-Doxorubicin conjugate (***4***).
- Synthesis of 2-(1-azidoisopropyl)-benzoic acid-Doxorubicin conjugate (***10***).
- Synthesis of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (***14***)
- Synthesis of N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***16***)

2) **Synthesis of protected Linker-Drug conjugates of 5-FU**

- Synthesis of N^1-[methyl (2-nitrobenzyl)carbamoyl]-5-fluorouracil (***20***)
- Synthesis of N^1-[methyl (2-nitrophenyl)carbamoyl]-5-fluorouracil (***21***)

3) **Synthesis of Peptide-Linker-Drug conjugate of 5-FU**

- Synthesis of N^1-[2-(H-Ser-Ala-Leu-Leu-amino)benzylcarbamoyl]-5-fluorouracil (***40***)

4) **Selective reduction and the kinetic analysis of the cyclization-activation process**

- Selective reduction of N^1-[methyl (2-nitrobenzyl)carbamoyl]-5-fluorouracil (***20***)
- Selective reduction of N^1-[methyl (2-nitrophenyl)carbamoyl]-5-fluorouracil (***21***)
- Kinetic analysis of the cyclization-activation process of N^1-[methyl (2-aminobenzyl)carbamoyl]-5-fluorouracil (***25***)

Reportable Outcomes

Bin Liu received her Master's degree in Pharmaceutical Sciences in December 2000. She was in part supported by this grant.

Some of this work and work done in the first year of support was published. The following is a list of publications as a result of research funded in part by this grant.

1. Chengzhi Yu, Bin Liu, and **Longqin Hu** A Simple One-Pot Procedure for the Direct Conversion of Alcohols to Azides via Phosphate Activation. *Org. Lett.* **2000**, *2*(13), 1959-1961.
2. Chengzhi Yu, Bin Liu, and **Longqin Hu** A convenient biphasic process for the monosilylation of symmetrical 1,n-primary diols. *Tetrahedron Lett.* **2000**, *41*(22), 4281-4285.
3. **Longqin Hu**, Bin Liu, and Douglas Hacking 5′-[2-(2-Nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine as a potential bioreductively activated prodrug of FUDR: synthesis, stability, and reductive activation. *Bioorg. Med. Chem. Lett.* **2000**, *10*(8), 197-800.
4. Chengzhi Yu, Bin Liu, and **Longqin Hu** A modified procedure for the deprotection of methoxylmethyl ether. *Tetrahedron Lett.* **2000**, *41*(6), 819-822.
5. **Longqin Hu**, Bin Liu, Zhiyong Cui, and Chengzhi Yu 2-Nitrophenylalkanoic Acid Esters of FUDR: Synthesis, Stability and Kinetics of Reductive Activation. *Abstracts: 27th National Medicinal Chemistry Symposium, Kansas City, Missouri*, June 13-17, **2000**, A21.
6. **Longqin Hu**, Bin Liu, and Douglas R. Hacking Synthesis and Biomimetic Reductive Activation of Potential Prodrugs of FUDR and 5-FU. *Abstracts: 217th ACS national meeting* **1999**, MEDI 0218.

Conclusions

We accomplished the synthesis of three of the four protected **Linker-Drug** conjugates of doxorubicin and 5-fluorouracil (5-FU) proposed in the original application. The doxorubicin conjugates were difficult to test due to the presence of an easily reduced functional group in doxorubicin itself. The two 5-FU Linker-Drug conjugates (***14*** and ***16***) were found to be unexpectedly unstable under physiological conditions. Thus, two new conjugates were designed and synthesized with modified urea linkers and were found to be stable under the same conditions tested. The new conjugates were synthesized in a protected form (NO2) and chemically reduced to test the cyclization activation process. It was found that both new linker-drug conjugates of 5-FU could release the drug 5-FU upon conversion to the nucleophilic amino or hydroxylamino group. These two new linker systems will provide the basis for further conjugation with peptide and test the PSA-activation process. We also synthesized a **Peptide-Linker-Drug** conjugate of 5-FU using the unstable linker in ***16***. The chemistry developed would be useful in synthesizing the more stable conjugate of 5-FU using the new linkers developed. Recently, we turned our attention to the synthesis of a **Peptide-Linker-Drug** conjugate with much less bulky linker. Results are encouraging and will be further investigated. The support by this award enable us to test the cyclization activation process, a necessary second step in our **Peptide-Linker-Drug** conjugates designed for activation by PSA. While this work was in progress, reports have been published about using PSA to activate peptide-doxorubicin conjugates without the linker. The results are encouraging. The incorporation of our linker to the prodrugs should further improve the efficiency of PSA activation and release of the original cytotoxic drug making this therapy a reality. Research will continue in our laboratory towards this goal.

Bibliography

Publications.

1. Chengzhi Yu, Bin Liu, and **Longqin Hu** A Simple One-Pot Procedure for the Direct Conversion of Alcohols to Azides via Phosphate Activation. *Org. Lett.* **2000**, *2*(13), 1959-1961.
2. Chengzhi Yu, Bin Liu, and **Longqin Hu** A convenient biphasic process for the monosilylation of symmetrical 1,n-primary diols. *Tetrahedron Lett.* **2000**, *41*(22), 4281-4285.
3. **Longqin Hu**, Bin Liu, and Douglas Hacking 5′-[2-(2-Nitrophenyl)-2-methylpropionyl]-2′-deoxy-5-fluorouridine as a potential bioreductively activated prodrug of FUDR: synthesis, stability, and reductive activation. *Bioorg. Med. Chem. Lett.* **2000**, *10*(8), 197-800.
4. Chengzhi Yu, Bin Liu, and **Longqin Hu** A modified procedure for the deprotection of methoxylmethyl ether. *Tetrahedron Lett.* **2000**, *41*(6), 819-822.

Meeting abstracts

1. **Longqin Hu**, Bin Liu, Zhiyong Cui, and Chengzhi Yu 2-Nitrophenylalkanoic Acid Esters of FUDR: Synthesis, Stability and Kinetics of Reductive Activation. *Abstracts: 27th National Medicinal Chemistry Symposium, Kansas City, Missouri*, June 13-17, **2000**, A21.
2. **Longqin Hu**, Bin Liu, and Douglas R. Hacking Synthesis and Biomimetic Reductive Activation of Potential Prodrugs of FUDR and 5-FU. *Abstracts: 217th ACS national meeting* **1999**, MEDI 0218.

List of Personnel Receiving Pay from this Project

Longqin Hu, Ph.D., PI
Wayne Yu, Ph.D., Postdoctoral Fellow
Jun Zhao, Ph.D., Postdoctoral Fellow
Bin Liu, Graduate Student
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
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