Synthesis of Water-Soluble Paclitaxel Derivatives by Enzymatic Acylation

Yuri L. Khmelnitsky,[†] Cheryl Budde,[†] J. Michael Arnold,[†]
Alexander Usyatinsky,[†] Douglas S. Clark,[*,‡] and
Jonathan S. Dordick[*,§]

*EnzyMed, Inc., 2501 Crosspark Road
Oakdale Research Park, Iowa City, Iowa 52242
Department of Chemical Engineering
University of California
Berkeley, California 94720
Department of Chemical and Biochemical Engineering
University of Iowa, Iowa City, Iowa 52242*

Received September 3, 1997

Paclitaxel,[1] a diterpenoid originally isolated from the bark of the Pacific yew, *Taxus brevifolia*, is a powerful antimitotic agent[2] that acts by promoting tubulin assembly into stable aggregated structures. Although paclitaxel has shown tremendous potential as an anticancer compound,[3] its use as an anticancer drug is compromised by its poor aqueous solubility. For this reason, a number of water-soluble paclitaxel prodrugs have been synthesized that contain hydrophilic or charged functionalities attached to specific sites on the paclitaxel molecule.[4]

Acylation at the 2′ position (for the structure of paclitaxel, see Figure 1) can be a very effective strategy for improving the water solubility of paclitaxel.[4] Interestingly, acylation of the C-2′ hydroxyl eliminates microtubule stabilization but not cytotoxicity, which is consistent with the hydrolytic regeneration of paclitaxel from pro-paclitaxel within the cell.[5] Water soluble pro-paclitaxels modified at the 2′ position include arylsulfonyl ethoxycarbonates and thiodiglycolic esters synthesized by Nicolaou et al.,[4a] the most soluble of which were 100−1000 times more soluble than paclitaxel.

In the present work, we establish for the first time that paclitaxel can be enzymatically derivatized[6] in an organic solvent[7] to generate new potential prodrugs possessing high solubility in water. Our approach is based on a unique strategy

† EnzyMed, Inc.
‡ University of California.
§ University of Iowa.
(1) For reviews on paclitaxel see: (a) Kingston, D. G. I. *Trends Biotechnol.* **1994,** *12,* 222. (b) Rowinsky, E. K.; Onerto, N.; Canetta, R. M.; Arbuck, S. G.; *Sem. Oncol.* **1992,** *19,* 646. (c) Nicolaou, K. C.; Dai, W. M.; Guy, R. K. *Angew. Chem., Int. Ed. Eng.* **1994,** *33,* 15. (d) Kingston, D. G. I. *Pharmacol. Ther.* **1991,** *52,* 1.
(2) Schiff, P. B.; Fant, J.; Horwitz, S. B. *Nature* **1979,** *277,* 665.
(3) (a) Rowinsky, E. K.; Cazenave, L. A.; Donehower, R. C. *J. Nat. Cancer Inst.* **1990,** *82,* 1247. (b) McGuire, W. P.; Rowinsky, E. K.; Rosenshein, N. B.; Grumbine, F. C.; Ettinger, D. S.; Armstrong, D. K.; Donehower, R. C. *Ann. Int. Med.* **1989,** *111,* 273.
(4) (a) Nicolaou, K. C.; Riemer, C.; Kerr, M. A.; Rideout, D.; Wrasidlo, W. *Nature* **1993,** *364,* 464. (b) Deutsch, H. M.; Glinski, J. A.; Hernandez, M.; Haugwitz, R. D.; Narayanan, V. L.; Suffness, M.; Zalkow, L. H. *J. Med. Chem.* **1989,** *32,* 788. (c) Mathew, A. E.; Mejillano, M. R.; Nath, J. P.; Himes, R. H.; Stella, V. J. *J. Med. Chem.* **1992,** *35,* 145. (d) Ueda, Y.; Wong, H.; Matiskella, J. D.; Mikkilineni, A. B.; Farina, V.; Fairchild, C.; Rose, W. C.; Mamber, S. W.; Long, B. H.; Kerns, E. H.; Casazza, A. M.; Vyas, D. M. *Bioorg. Med. Chem. Lett.* **1994,** *4,* 1861. (e) Zhou, Z.; Kingston, D. G. I.; Crosswell, A. R. *J. Nat. Prod.* **1991,** *54,* 1607.
(5) (a) Kingston, D. G. I.; Samaranayake, G.; Ivey, C. A. *J. Nat. Prod.* **1990,** *53,* 1. (b) Potier, P. *Chem. Soc. Rev.* **1992,** 113.
(6) Recently, two enzymes from *Nocardioides* strains (isolated from soil) were shown to catalyze the regioselective hydrolysis of the 10-acetyl and 13-side chain esters. Since these enzymatic reactions were carried out in aqueous solutions, they resulted in low productivities (due to the insolubility of paclitaxel) and hydrolytic (and hence degradative) reactions. Hanson, R. L.; Wasylyk, J. M.; Nanduri, V. B.; Cazzulino, D. L.; Patel, R. N.; Szarka, L. J. *J. Biol. Chem.* **1994,** *269,* 22145.
(7) For reviews on enzymatic catalysis in organic solvents, see: (a) Dordick, J. S. In *Applied Biocatalysis*; Blanch, H. W. Clark, D. S., Eds.; Marcel Dekker: New York, 1991; Vol. 1, pp 1−51. (b) Klibanov, A. M. *Acc. Chem. Res.* **1990,** *23,* 114. (c) Khmelnitsky, Yu. L.; Levashov, A. V.; Klyachko, N. L.; Martinek, K. *Enzyme Microb. Technol.* **1988,** *10,* 710.
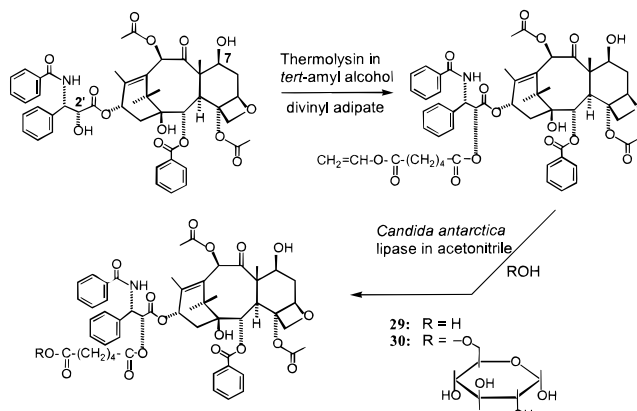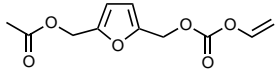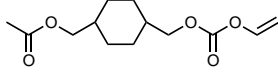

Figure 1. Two-step enzymatic modification of paclitaxel resulting in paclitaxel 2′-adipic acid (**29**) and paclitaxel 2′-adipoylglucose (**30**). Reaction conditions are described in the text.

of two-step enzymatic acylation depicted in Figure 1. In the first step, the starting compound is reacted with a bifunctional acylating agent to give an activated acyl derivative, which is then used as a complex acyl donor in the second step of the derivatization procedure. In accordance with this strategy, the starting point of the present work was to identify an appropriate enzyme catalyst suitable for acylation of paclitaxel in the first step. After a wide range of enzymes and solvents[8] were tested, thermolysin (an extracellular protease from *Bacillus thermoproteolyticus rokko*) suspended in anhydrous *tert*-amyl alcohol was identified to be the most effective catalyst for paclitaxel acylation.[9] In particular, this enzyme-catalyzed acylation of paclitaxel with a bifunctional acyl donor, divinyl adipate, as determined by TLC and HPLC. The reactivity of thermolysin toward paclitaxel was enhanced ca. 20-fold by lyophilizing the enzyme in the presence of KCl prior to use.[10] Using the salt-activated enzyme preparation (5.7 mg/mL protein), ca. 90% conversion of paclitaxel (14 mM solution) was obtained in 96 h in the presence of 45 mM divinyl adipate. Following termination of the reaction,[11] two products were isolated from the reaction mixture via preparative HPLC. The identities of these products were determined by mass and ^{1}H NMR spectroscopies to be paclitaxel 2′-vinyl adipate (**7**, major) and 7-epipaclitaxel 2′-vinyl adipate (**14**, minor) (Table 1). Isolated yields of the products (based on the starting amount of paclitaxel) were 60 and 18%, respectively. Thus, thermolysin is an extremely regioselective enzyme toward the 2′-hydroxyl moiety of paclitaxel, as no other hydroxyl groups on the paclitaxel molecule were esterified in the enzymatic reaction. In addition to divinyl adipate, several other straight-chain vinyl esters were suitable for the thermolysin-catalyzed acylation of paclitaxel under conditions described above for divinyl adipate.

(8) Over 50 different commercially available lipases and proteases were screened for paclitaxel acylation using vinyl butyrate as the acyl donor. The following enzymes were found to possess paclitaxel acylation activity: α-chymotrypsin, subtilisin Carlsberg, and thermolysin. Among these enzymes thermolysin showed the highest activity (ca. 3- and 40-fold higher than α-chymotrypsin and subtilisin, respectively) and, therefore, was used as a catalyst in all subsequent reactions.
(9) The ability of thermolysin to catalyze a transesterification reaction, such as that used in paclitaxel acylation, has never been observed before. This unusual finding reveals an interesting new feature of the zinc-containing protease which has been used as a catalyst for synthesis of peptides: Miyanaga, M.; Tanaka, T.; Sakiyama, T.; Nakanishi, K. *Biotechnol. Bioeng.* **1995,** *46,* 631.
(10) Salt-activated thermolysin was prepared following the published procedure (Khmelnitsky, Yu. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994,** *116,* 2647).
(11) The workup of the reaction mixture included removal of the suspended enzyme by centrifugation and evaporation of the solvent under vacuum.

Table 1. Enzymatically Synthesized 2′-Acyl Paclitaxel Derivatives[a]

acyl R group in paclitaxel-OR	conversion (%)[b]			
	cmpd	paclitaxel deriv	cmpd	7-epipaclitaxel deriv[c]
Esters				
-C(O)CH$_3$	**1**	57	**8**	30
-C(O)CH$_2$Cl	**2**	70	**9**	3
-C(O)CH=CH$_2$	**3**	80	**10**	10
-C(O)CH$_2$CH$_3$	**4**	78	**11**	12
-C(O)(CH$_2$)$_2$CH$_3$	**5**	67	**12**	11
-C(O)(CH$_2$)$_4$CH$_3$	**6**	50	**13**	7
-C(O)CH$_2$(CH$_2$)$_4$C(O)-OCH=CH$_2$	**7**	69	**14**	31
Carbonates				
-C(O)O(CH$_2$)$_3$CH$_3$	**15**	80	**22**	20
-C(O)ON=C(CH$_3$)$_2$	**16**	89	**23**	11
-C(O)OCH$_2$CH=CHCH$_2$-OC(O)OCH=CH$_2$	**17**	81	**24**	10
-C(O)OCH$_2$CH$_2$CH(CH$_3$)-OC(O)OCH=CH$_2$	**18**	60	**25**	9
-C(O)OCH$_2$CH$_2$CH$_2$OC-(O)OCH=CH$_2$	**19**	69	**26**	9
	20	83	**27**	13
	21	25	**28**	4

[a] Formation of 2′-substituted paclitaxel derivatives was confirmed on the basis of the characteristic downfield shift of the C2′ proton signal from 4.7 to 5.6 ppm, representing an unequivocal proof of 2′-substitution.[18] Signals from other paclitaxel ring protons were essentially identical to those previously reported for 2′-acyl paclitaxels.[18] [b] Determined from relative peak areas on HPLC chromatograms. For structural confirmation, part of the reaction mixture was subjected to preparative HPLC to isolate a small amount of the product needed for mass spectral and NMR analyses. [c] Formation of 7-epipaclitaxel was found to be a spontaneous process not related to enzyme action. Epimerization occurred during prolonged incubation of paclitaxel in *tert*-amyl alcohol at the increased temperature required for enzymatic acylation (35 °C). Spontaneous epimerization of paclitaxel in mildly basic aqueous solutions has been observed previously.[19] Epimerization at the 7 position was established on the basis of the characteristic merging of signals from protons at C20 into a singlet at 4.3 ppm and the shift of the C7 proton signal from 4.4 ppm to 3.7 ppm, which unambiguously indicate the formation of 7-epimer.[20]

In all cases, acylation was specific to the 2′-hydroxyl group of paclitaxel with 96 h conversions of at least 50% (Table 1).[12,13]

In the second step of the two-step acylation procedure, following purification of the paclitaxel 2′-vinyl adipate by preparative HPLC, hydrolysis of the terminal vinyl ester group was performed in acetonitrile (containing 1% water) catalyzed

by the lipase from *Candida antarctica* (Novozym 435, Novo Nordisk) (75 mg/ml) to give paclitaxel 2′-adipic acid (**29**) with 75% isolated yield. Paclitaxel 2′-vinyl adipate was also used as the acyl donor for transesterification in dry acetonitrile containing glucose (0.36 M) as the acyl acceptor resulting in the formation of paclitaxel 2′-adipoylglucose (**30**) with 85% isolated yield (presumably linked selectively to the 6-hydroxyl moiety of the sugar[16]). Using a similar procedure, we also succeeded in synthesizing paclitaxel 2′-adipoylmannose and paclitaxel 2′-adipoylfructose starting from paclitaxel 2′-vinyl adipate and the corresponding sugar. This two-step process demonstrates the unique advantage of enzymatic catalysis, namely the high regioselectivity of hydrolysis/transesterification to generate paclitaxel derivatives.[17]

Both the free adipic acid and sugar-containing paclitaxel derivatives were more soluble in water than paclitaxel itself. Specifically, the solubility of paclitaxel ($<4 \mu g/mL$) is increased 58 and 1625-fold for the paclitaxel 2′-adipoylglucose and paclitaxel 2′-adipic acid, respectively. Thus, the enzymatic addition of polar functionalities onto the 2′-position of paclitaxel results in dramatic improvement in paclitaxel's water solubility.

In summary, paclitaxel is a substrate for thermolysin-catalyzed transacylation reactions in *tert*-amyl alcohol, being transformed into water-soluble potential prodrugs with hydrophilic functionalities off of the 2′-hydroxyl group.

Supporting Information Available: ^{1}H NMR spectral data for compounds **1**, **3**−**8**, **10**−**12**, **14**, **15**, **29**, and **30** and mass spectral data for compounds **1**, **3**−**6**, **15**, **29**, and **30** (8 pages). See any current masthead page for ordering and Internet access instructions.

(13) To verify their potential as prodrugs, two paclitaxel esters, paclitaxel 2′-chloroacetate (**2**) and paclitaxel 2′-acrylate (**3**), were tested for cytotoxicity against HL-60 cells, a promyelocytic leukemia cell line, and MOLT-4 cells, a lymphoblastic leukemia cell line.[15] Both derivatives had IC$_{50}$ values about 10 times higher than that of paclitaxel for each cell line.

(14) Vinyl carbonates were synthesized from vinyl chloroformate and the corresponding alcohols using procedures similar to those published previously: Pozo, M.; Pulido, R.; Gotor, V. *Tetrahedron* **1992**, *48*, 6477.

(15) Cells were seeded in 96-well plates at densities of 30 000 cells/well and grown in RPMI-1640 medium containing 10% bovine calf serum at 37 °C for 24 h. The medium was then replaced with fresh medium containing the paclitaxel esters (excluding paclitaxel, which had been removed by preparative TLC) dissolved in DMSO at final concentrations ranging from 100 to 0.1 nM. The final concentration of DMSO in the cell medium was 0.5%. After 72 h, samples were removed for cell counts. Total cell number and viability were determined by trypan blue exclusion and manual cell counting on a hemacytometer.

(16) A great deal of evidence exists that lipases are highly selective for the primary hydroxyls of monosaccharides in transesterification reactions: (a) Martin, B. D.; Ampofo, S. A.; Linhardt, R. J.; Dordick, J. S. *Macromolecules* **1992**, *25*, 7081. (b) Therisod, M.; Klibanov, A. M. *J. Am. Chem. Soc.* **1986**, *108*, 5638.

(17) Although the chemical acylation of paclitaxel at the 2′ position is facile,[4e] the subsequent modification of such an ester group may be complicated by the presence of native esters on the paclitaxel nucleus which may be labile to nonselective hydrolysis/transesterification. This is in stark contrast to the high regioselectivity of enzymatic catalysis as demonstrated in this work.

(18) Mellado, W.; Magri, N. F.; Kingston, D. G. I.; Garcia-Arenas, R.; Orr, G. A.; Horwitz, S. B. *Biochem. Biophys. Res. Commun.* **1984**, *124*, 329.

(19) Ringel, I.; Horwitz, S. B. *J. Pharmac. Exp. Ther.* **1987**, *242*, 692.

(20) Chmurny, G. N.; Hilton, B. D.; Brobst, S.; Look, S. A.; Witherup, K. M.; Beutler, J. A. *J. Nat. Prod.* **1992**, *55*, 414.

(12) Thermolysin was also capable of catalyzing the regioselective synthesis of paclitaxel carbonates. Specifically, butyl vinyl carbonate[14] was an excellent carbonate donor for salt-activated thermolysin. The reaction was performed in *tert*-amyl alcohol containing 5 mM paclitaxel, 75 mM butyl vinyl carbonate, and the salt-activated enzyme preparation (5 mg/mL protein). After 48 h of reaction at 45 °C, essentially all of the paclitaxel was converted to two products, paclitaxel 2′-butyl carbonate (**15**, major) and 7-epipaclitaxel 2′-butyl carbonate (**22**, minor) (Table 1). Thus, as was the case with paclitaxel ester synthesis, the thermolysin-catalyzed paclitaxel carbonate synthesis was specific for the 2′-hydroxyl moiety. In addition to butyl vinyl carbonate, a number of divinyl dicarbonates and acetone oxime vinyl carbonate were used as carbonate donors. In all cases, conversions of paclitaxel ranged from 30 to 100% (Table 1).