ドラッグ研究においても，水溶性補助基を導入するという従来の考え方から一歩踏み出して，補助基を持たない水溶性プロドラッグ"イソタキソイド"を開発することができた。創薬という観点では，このプロドラッグはまだ開発の入り口にあるが，今後さらに実用化に向けた検討を重ねて行きたい。そして今後も，機能性有機分子としてのα-ヒドロキシ-β-アミノ酸の新たな特性に注目して，有意義な応用研究を展開したいと考えている。

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文　献

1) (a) H. Umezawa, T. Aoyagi, H. Suda, M. Hamada, T. Takeuchi, *J. Antibiot.*, 29, 97 (1976); (b) M. Ishizuka, T. Masuda, N. Kanbayashi, S. Fukasawa, T. Takeuchi, T. Aoyagi, H. Umezawa, *ibid.*, 33, 642 (1980)
2) T. Aoyagi, H. Tobe, F. Kojima, M. Hamada, T. Takeuchi, H. Umezawa, *J. Antibiot.*, 31, 636 (1978)
3) K. Iizuka, T. Kamijo, H. Harada, K. Akahane, T. Kubota, H. Umeyama, T. Ishida, Y. Kiso, *J. Med. Chem.*, 33, 2707 (1990)
4) (a) T. Mimoto, N. Hattori, H. Takaku, S. Kisanuki, T. Fukazawa, K. Terashima, R. Kato, S. Nojima, S. Misawa, T. Ueno, J. Imai, H. Enomoto, S. Tanaka, H. Sakikawa, M. Shintani, H. Hayashi, Y. Kiso, *Chem. Pharm. Bull.*, 48, 1310 (2000); (b) T. Mimoto, R. Kato, H. Takaku, S. Nojima, K. Terashima, S. Misawa, T. Fukazawa, T. Ueno, H. Sato, M. Shintani, Y. Kiso, H. Hayashi, *J. Med. Chem.*, 42, 1789 (1999); (c) Y. Kiso, H. Matsumoto, S. Mizumoto, T. Kimura, Y. Fujiwara, K. Akaji, *Biopolymers*, 51, 59 (1999); (d) Y. Kiso, *ibid.*, 40, 235 (1996) and other references cited therein; (e) E. Takashiro, I. Hayakawa, T. Nitta, A. Kasuya, S. Miyamoto, Y. Ozawa, R. Yagi, I. Yamamoto, T. Shibayama, A. Nakagawa, Y. Yabe, *Bioorg. Med. Chem.*, 7, 2063 (1999); (f) T. Komai, S. Higashida, M. Sakurai, T. Nitta, A. Kasuya, S. Miyamoto, R. Yagi, Y. Ozawa, H. Handa, H. Mohri, A. Yasuoka, S. Oka, T. Nishigaki, S. Kimura, K. Shimada, Y. Yabe, *ibid.*, 4, 1365 (1996)
5) (a) H. M. Abdel-Rahman, T. Kimura, K. Hidaka, A. Kiso, A. Nezami, E. Freire, Y. Hayashi, Y. Kiso, *Biol. Chem.*, 385, 1035 (2004); (b) A. Kiso, K. Hidaka, T. Kimura, Y. Hayashi, A. Nezami, E. Freire, Y. Kiso, *J. Peptide Sci.*, 10, 641 (2004); (c) A. Nezami, T. Kimura, K. Hidaka, A. Kiso, J. Liu, Y. Kiso, D. E. Goldberg, E. Freire, *Biochemistry*, 42, 8459 (2003); (d) A. Nezami, I. Luque, T. Kimura, Y. Kiso, E. Freire, *ibid.*, 41, 2273 (2002).
6) (a) T. Kimura, D. Shuto, Y. Hamada, N. Igawa, S. Kasai, P. Liu, K. Hidaka, T. Hamada, Y. Hayashi, Y. Kiso, *Bioorg. Med. Chem. Lett.*, 15, 211 (2005); (b) T. Kimura, D. Shuto, S. Kasai, P. Liu, K. Hidaka, T. Hamada, Y. Hayashi, C. Hattori, M. Asai, S. Kitazume, T. C. Saido, S. Ishiura, Y. Kiso, *ibid.*, 14, 1527 (2004); (c) D. Shuto, S. Kasai, T. Kimura, P. Liu, K. Hidaka, T. Hamada, S. Shibakawa, Y. Hayashi, C. Hattori, B. Szabo, S. Ishiura, Y. Kiso, *ibid.*, 13, 4273 (2003)
7) (a) T. M. Mekhail, M. Markman, *Expert Opin. Pharmacotherapy*, 3, 755 (2002); (b) C. Ferlini, I. Ojima, M. Distefano, D. Gallo, A. Riva, P. Morazzoni, E. Bombardelli, S. Mancuso, G. Scambia, *Curr. Med. Chem. Anti-Cancer Agents*, 3, 133 (2003)
8) Y. Hayashi, Y. Kinoshita, K. Hidaka, A. Kiso, H. Uchibori, T. Kimura, Y. Kiso, *J. Org. Chem.*, 66, 5537 (2001)
9) (a) T. Kotake, S. Rajesh, Y. Hayashi, Y. Mukai, M. Ueda, T. Kimura, Y. Kiso, *Tetrahedron Lett.*, 45, 3651 (2004); (b) T. Kotake, Y. Hayashi, S. Rajesh, Y. Mukai, Y. Takiguchi, T. Kimura, Y. Kiso, *Tetrahedron*, 61, 3819 (2005)
10) (a) Y. Sohma, Y. Hayashi, M. Skwarczynski, Y. Hamada, M. Sasaki, T. Kimura, Y. Kiso, *Biopolymers*, 76, 344 (2004); (b) Y. Sohma, M. Sasaki, Y. Hayashi, T. Kimura, Y. Kiso, *Tetrahedron Lett.*, 45, 5965 (2004); (c) *idem*, *Chem. Commun.*, 2004, 124
11) (a) M. Skwarczynski, Y. Sohma, M. Kimura, Y. Hayashi, T. Kimura, Y. Kiso, *Bioorg. Med. Chem. Lett.*, 13, 4441 (2003); (b) Y. Hayashi, M. Skwarczynski, Y. Hamada, Y. Sohma, T. Kimura, Y. Kiso, *J. Med. Chem.*, 46, 3782 (2003); (c) Y. Hamada, H. Matsumoto, S. Yamaguchi, T. Kimura, Y. Hayashi, Y. Kiso, *Bioorg. Med. Chem.*, 12, 159 (2004); (d) Y. Hamada, H. Matsumoto, T. Kimura, Y. Hayashi, Y. Kiso, *Bioorg. Med. Chem. Lett.*, 13, 2727 (2003); (e) Y. Hamada, J. Ohtake, Y. Sohma, T. Kimura, Y. Hayashi, Y. Kiso, *ibid.*, 10, 4155 (2002); (f) Y. Kiso, S. Yamaguchi, H. Matsumoto, T. Kimura, K. Akaji, "*Peptides, Frontiers of Peptide Science, Proc. 15th American Peptide Symposium*" eds. J. P. Tam, P. T. P. Kaumaya, Kluwer Academic, Netherlands, 1999, p 678; (g) T. Kimura, J. Ohtake, S. Nakata, H. Enomoto, H. Moriwaki, K. Akaji, Y. Kiso, "*Peptide Chemistry 1994*" ed. M. Ohno, Protein Research Foundation, Osaka, 1995, p 157; (h) M. Skwarczynski, Y. Sohma, M. Noguchi, M. Kimura, Y. Hayashi, Y. Hamada, T. Kimura, Y. Kiso, *J. Med. Chem.*, 48, 2655 (2005)
12) (a) M. T. Reetz, M. W. Drewes, K. Harms, W. Reif, *Tetrahedron Lett.*, 29, 3295 (1988); (b) R. Herranz, J. Castro-Pichel, S. Vinuesa, M. T. Garcia-Lopez, *J. Org. Chem.*, 55, 2232 (1990); (c) W. H. Pearson, J. V. Hines, *ibid.*, 54, 4235 (1989); (d) Y. Kobayashi, Y. Takemoto, T. Kamijo, H. Harada, Y. Ito, S. Terashima, *Tetrahedron*, 48, 1853 (1992); (e) T. Kawabata, Y. Kiryu, Y. Sugiura, K. Fuji, *Tetrahedron Lett.*, 34, 5127 (1993); (f) A. Dondoni, D. Perrone, T. Semola, *Synthesis* 1995, 181; (g) J. Kearns, M. M. Kayser, *Tetrahedron Lett.*, 35 2845 (1994); (h) H. Sasaki, W-S. Kim, T. Suzuki, M. Shibasaki, *ibid.*, 35, 6123 (1994); (i) Y. Hamashima, D. Sawada, M Kanai, M. Shibasaki, *J. Am. Chem. Soc.*, 121, 2641 (1999); (j I. Ojima, F. Delaloge, *Chem. Soc. Rev.*, 26, 377 (1997); (k) M Seki, K. Matsumoto, *Synthesis*, 1999, 924
13) L. A. Carpino, *J. Am. Chem. Soc.*, 115, 4397 (1993)
14) (a) W. König, R. Geiger, *Chem. Ber.*, 103, 2034 (1970); (b) E Acerton, L. Cameron, M. Meldal, R. J. Sheppard, *J. Chem Soc., Chem. Commun.*, 1986, 1763
15) (a) W. König, R. Geiger, *Chem. Ber.*, 103, 788 (1970); (b) *iden ibid.*, 103, 2024 (1970); (c) *idem*, *ibid.*, 106, 3626 (1973)
16) (a) D. A. Evans, *Aldrichim. Acta*, 15, 23 (1982); (b) D. J. Age I. Prakash, D. R. Schaad, *ibid.*, 30, 3 (1997)
17) (a) A. V. Purandare, S. Natarajan, *Tetrahedron Lett.*, 38, 877 (1997); (b) C. W. Phoon, C. Abell, *ibid.*, 39, 2655 (1998); (c) D. Winkler, W. McCoull, *ibid.*, 39, 4935 (1998); (d) G. Faita, Paio, P. Quadrelli, F. Rancati, P. Seneci, *ibid.*, 41, 1265 (2000 (e) *idem.*, *Tetrahedron*, 57, 8313 (2001); (f) G. Desimoni, Faita, A. Galbiati, D. Pasini, P. Quadrelli, F. Rancati, *Tetrah dron:Asymmetry*, 13, 333 (2002)
18) K. Burgess, D, Lim, *Chem. Commun.*, 1997, 785
19) (a) S. P. Bew, S. D. Bull, S. G. Davies, *Tetrahedron Lett.*, 4 7577 (2000); (b) S. P. Bew, S. D. Bull, S. G. Davies, E.

Savory, D. J. Watkin, *Tetrahedron*, 58, 9387 (2002)
20) (a) D. A. Evans, T. C. Britton, J. A. Ellman, *Tetrahedron Lett.*, 28, 6141 (1987); (b) D. A. Evans, J. A. Ellman, *J. Am. Chem. Soc.*, 111, 1063 (1989)
21) E. Atherton, N. L. Benoiton, E. Brown, R. C. Sheppard, B. J. Williams, *J. Chem. Soc., Chem. Commun.*, 1981, 336
22) (a) J. M. Stewart, "The Peptides", Vol. 3, eds. E. Gross, J. Meienhofer, Academic Press, New York, 1981, p 170; (b) H. Tamamura, T. Kato, A. Otaka, N. Fujii, *Org. Biomol. Chem.*, 1, 2468 (2003); (c) L. Mouls, G. Subra, C. Enjalbal, J. Martinez, J-L. Aubagnac, *Terahedron Lett.*, 45, 1173 (2004)
23) (a) E. Atherton, V. Woolley, R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1980, 970; (b) V. Pillai, M. Mutter, *Acc. Chem. Res.*, 14, 122 (1981); (c) A. G. Ludwick, L. W. Jelinski, D. Live, A. Kintaner, J. J. Dumais, *J. Am. Chem. Soc.*, 108, 6493 (1986); (d) S. B. H. Kent, *Annu. Rev. Biochem.*, 232, 341 (1988); (e) R. C. d. L. Milton, S. C. F. Milton, P. A. Adams, *J. Am. Chem. Soc.*, 112, 6039 (1990); (f) J. P. Tam, Y. A. Lu, *ibid.*, 117, 12058 (1995); (g) C. Dhalluin, C. Boutillon, A. Tartar, G. Lippens, *ibid.*, 119, 10494 (1997)
24) (a) T. Haack, M. Mutter, *Tetrahedron Lett.*, 33, 1589 (1992); (b) A. Nefzi, K. Schenk, M. Mutter, *Protein Pept. Lett.*, 1, 66 (1994); (c) T. Wöhr, F. Wahl, A. Nefzi, B. Rohwedder, T. Sato, X. Sun, M. Mutter, *J. Am. Chem. Soc.*, 118, 9218 (1996); (d) M. Keller, C. Sager, P. Dumy, M. Schutkowski, G. S. Fischer, M. Mutter, *ibid.*, 120, 2714 (1998); (e) A. Wittelsberger, M, Keller, L. Scarpellino, L. Patiny, H. Acha-Orbea, M. Mutter, *Angew. Chem. Int. Ed.*, 39, 1111 (2000); (f) J-F. Guichou, L. Patiny, M. Mutter, *Tetrahedron Lett.*, 43, 4389 (2002)
25) (a) T. Johnson, M. Quibell, D. Owen, R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, 1993, 369; (b) T. Johnson, M. Quibell, R. C. Sheppard, *J. Peptide Sci.*, 1, 11 (1995); (c) M. Quibell, L. C. Packman, T. Johnson, *J. Am. Chem. Soc.*, 117, 11656 (1995); (d) *idem*, *J. Chem. Soc., Perkin Trans. 1*, 1996, 1227; (e) J. Howe, M. Quibell, T. Johnson, *Tetrahedron Lett.*, 41, 3997 (2000); (f) L. P. Miranda, W. D. F. Meutermans, M. L. Smythe, P. F. Alewood, *J. Org. Chem.*, 65, 5460 (2000)
26) For examples: (a) D. Burdick, B. Soreghan, M. Kwon, J. Kosmoski, M. Knauer, A. Henschen, J. Yates, C. Cotman, C. Glabe, *J. Biol. Chem.*, 267, 546 (1992); (b) M. Quibell, W. G. Turnell, T. Johnson, *J. Chem. Soc., Perkin Trans. I*, 1995, 2019; (c) C-L. Shen, R. M. Murphy, *Biophys. J.*, 69, 640 (1995); (d) H. Fukuda, T. Shimizu, M. Nakajima, H. Mori, T. Shirasawa, *Bioorg. Med. Chem. Lett.*, 9, 953 (1999); (e) S. C. F. Milton, R. C. D. Milton, S. A. Kates, C. Glabe, *Lett. Peptide Sci.*, 6, 151 (1999); (f) K. Murakami, K. Irie, A. Morimoto, H. Ohigashi, M. Shindo, M. Nagano, T. Shimizu, T. Shirasawa, *Biochem. Biophys. Res. Commun.*, 294, 5 (2002)
27) (a) D. J. Selkoe, *Nature*, 399, A 23 (1999); (b) C. Geula, C. K. Wu, D. Saroff, A. Lorenzo, M. L. Yuan, B. A. Yankner, *Nature Med.*, 4, 827 (1998); (c) S. Sinha, I. Lieberburg, *Proc. Natl. Acad. Sci. U.S.A.*, 96, 11049 (1999)
28) W. B., Jr. Stine, K. N.Dahlgren, G. A. Krafft, M. J. Ladu, *J. Biol. Chem.*, 278, 11612 (2003)
29) T. M. Mekhail, M. Markman, *Expert Opin. Pharmacotherapy*, 3, 755 (2002); (b) C. Ferlini, I. Ojima, M. Distefano, D. Gallo, A. Riva, P. Morazzoni, E. Bombardelli, S. Mancuso, G. Scambia, *Curr. Med. Chem. Anti-Cancer Agents*, 3, 133 (2003)
30) A. Gennari, B. Salvadori, A. Tognoni, P. F. Conte, *Ann. Oncol.*, 7, 978 (1996)
31) (a) J. W. Singer, B. Baker, P. De Vries, A. Kumar, S. Shaffer, E. Vawter, M. Bolton, P. Garzone, *Adv. Exp. Med. Biol.*, 519, 81 (2003); (b) J. M. Meerum Terwogt, W. W.ten Bokkel Huinink, J. H. M. Schellens, M. Schot, I. A. M. Mandjes, M. G. Zurlo, M. Rocchetti, H. Rosing, F. J. Koopman, J. H. Beijnen, *Anti-Cancer Drugs*, 12, 315 (2001); (c) A. Sparreboom, A. C. Wolff, J. Verweij, Y. Zabelina, D. M. van Zomeren, G. L. McIntire, C. S. Swindell, R. C. Donehower, S. D. Baker, *Clin. Cancer Res.*, 9, 151 (2003); (d) W. Wrasidlo, A. Niethammer, S. Deger, J. Sehouli, A. Kulozik, W. Geilen, G. Henze, G. Gaedicke, H. N. Lode, *Curr. Therapeutic Res.*, 63, 247 (2002)
32) (a) A. L. Seligson, R. C. Terry, J. C. Bressi, J. G. Douglass III, M. Sovak, *Anti-cancer Drugs*, 12, 305 (2001); (b) A. K. Singla, A. Garg, D. Aggarwal, *Int. J. Pharmaceutics*, 235, 179 (2002)

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No Auxiliary, No Byproduct Strategy for Water-Soluble Prodrugs of Taxoids: Scope and Limitation of O−N Intramolecular Acyl and Acyloxy Migration Reactions†

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Since numerous new taxoids active against multidrug resistant (MDR) tumors have been developed and their poor water-solubility is a very real problem in intravenous administration, we have designed and synthesized a series of novel water-soluble taxoid prodrugs (isotaxoids). These prodrugs, a 2′-*O*-isoform of taxoids, showed promising results with higher water solubility (0.8−1.1 mg/mL) and proper kinetics for parent drug release by a simple pH-dependent chemical mechanism via O−N intramolecular acyl migration. No additional functional auxiliaries are released during the conversion to parent drugs, which would be an advantage in toxicology and general pharmacology, and the cost for the evaluations of auxiliary units in these fields could be saved in prodrug development. In addition, we demonstrate for the first time the successful application of the O−N intramolecular acyloxy migration reaction in the prodrug design, with the exception of the *tert*-butyloxycarbonyl group, and that this reaction can be provided with no organic solvent and no side products.

Introduction

The introduction of the anticancer agents paclitaxel (Taxol, **1**)[1] and docetaxel (Taxotere, **2**)[2] has revolutionized the treatment of cancer and markedly improved the survival time of patients.[3−5] However, despite the hope and promise that taxoids such as paclitaxel and docetaxel have engendered, their lack of activity against multidrug resistant (MDR) tumors as well as dose-limiting toxicity corresponding to the side toxicity of taxoids are significant drawbacks. Additionally, their poor water solubility, based on their hydrophobic common taxane ring structure, is a very real problem in intravenous administration. Since paclitaxel can be dissolved with a detergent, Cremophor EL, at low concentrations, a prolonged intravenous administration time is required. Significant side effects associated with hypersensitivity to Cremophor EL have also been observed and a premedication schedule that includes a corticosteroid and antihistamine is required.[6] Moreover, it was recently reported that both Cremophor EL and Tween 80 used in the formulation of paclitaxel and docetaxel reduce the antitumor efficacy of these drugs by reducing their antiangiogenic activity.[7] To overcome these problems, two main strategies can be distinguished: the design of new paclitaxel derivatives and the temporary modification of paclitaxel for a prodrug form. Thus, numerous new taxoids have been designed and synthesized with improved properties,[8−15] and some of them are in clinical trials.[11−15] Also, water-soluble paclitaxel prodrugs and targeted prodrugs with reduced systemic toxicity through the introduction of hydrophilic or targeting moieties to C-2′or/and C-7 positions have been developed,[16−31] some also currently undergoing clinical evaluation[27−31] (Chart 1). It would therefore be more attractive to connect these two strategies, namely, the utilization of a prodrug strategy to novel taxoids.

In all standard prodrug strategies, auxiliary moieties are employed and their release may have negative effects in vivo.[32] Hence, prodrug designs that avoid the use of additional moieties are very promising. Since the O−N intramolecular acyl migration reaction proceeded under mild basic or neutral condition with no byproduct formation,[33−40] we anticipate that this reaction would be ideal. Previously, we demonstrated the utilization of the O−N intramolecular acyl migration strategy in paclitaxel and canadensol prodrugs, isotaxoids **4** and **6**, respectively (Figure 1).[41−43] These prodrugs, 2′-*O*-acyl isoforms of **1** and **3**, showed (1) higher water solubility than that of parent drugs and (2) complete and prompt conversion to parent drugs under physiological conditions (pH = 7.4), while being stable in stock solution and in storage conditions as solid HCl salts as well as during synthesis. Therefore, this strategy was demonstrated to be effective.

In this present paper, based on these promising results, we successfully expanded the O−N intramolecular acyl migration strategy to a number of taxoids that are active against MDR tumors, since they all possess the α-hydroxy-β-amino acid moiety necessary for migration. Herein, we also demonstrate the first successful application of the O−N intramolecular acyloxy migration reaction in a prodrug design. We designed water-soluble prodrugs of 3′-*N*-carbamate taxoids and considered the possibility of utilizing this strategy in a docetaxel prodrug, **5**. Despite problems in the application of this strategy to **2**, the O−N intramolecular

† This paper is dedicated to Prof. Iwao Ojima on the occasion of his 60th birthday.

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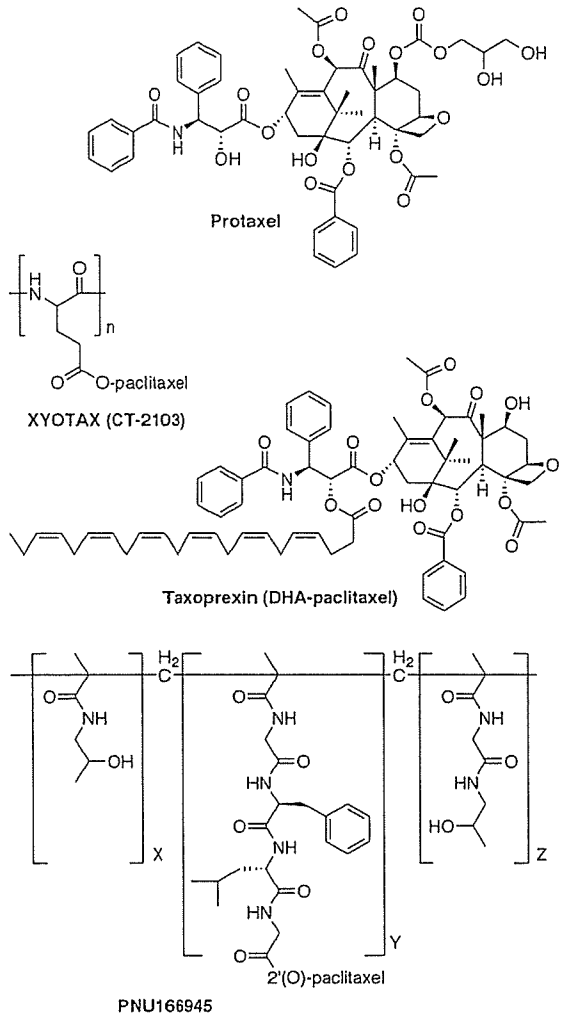
Chart 1



acyloxy migration reaction for all other prodrugs of 3′-*N*-carbamate taxoids proceeded promptly and with no side product formation under physiological conditions. Thus, we demonstrated the successful application of the O−N intramolecular acyloxy migration reaction in prodrug design for the first time. This is also the first report that showed that this reaction can be provided with no organic solvent and with no side products. In addition, both O−N acyl and acyloxy migration reactions were pH-dependent, and the migration rate was correlated to the structure of acyl/acyloxy groups. In contrast, no relationship was observed between the water solubility of the prodrug and the structure of the acyl/acyloxy moieties. However, all prodrugs showed significantly increased water solubility compared to the parent drug.

Chemistry

The synthesis of the prodrugs **8**−**13** is shown in Scheme 1. Compound **7**, which was synthesized by a previously described method,[41] was coupled with a corresponding carboxylic acid by the EDC−DMAP method (EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DMAP, 4-(dimethylamino)pyridine) or chloroformate in pyridine/CH_2Cl_2 solution, and subsequent deprotection of both 2,2,2-trichloroethyloxycarbonyl (Troc) groups using Zn−AcOH and the following purification with ion exchange by HPLC gave prodrugs **8**−**13** as a HCl salt. Additionally, parent drugs were synthesized by the deprotection of both Troc groups of **7** to afford compound **14** (Scheme 1), then the product of this reaction was coupled with the desired acid by the EDC−HOBt method (HOBt, 1-hydroxybenzotriazole) in DMF or chloroformates in CH_2Cl_2/saturated $NaHCO_3$ solution, resulting in the formation of the parent drugs **15**−**20**.

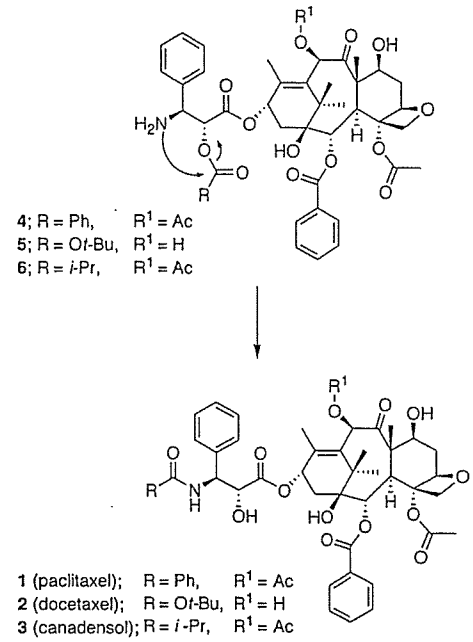
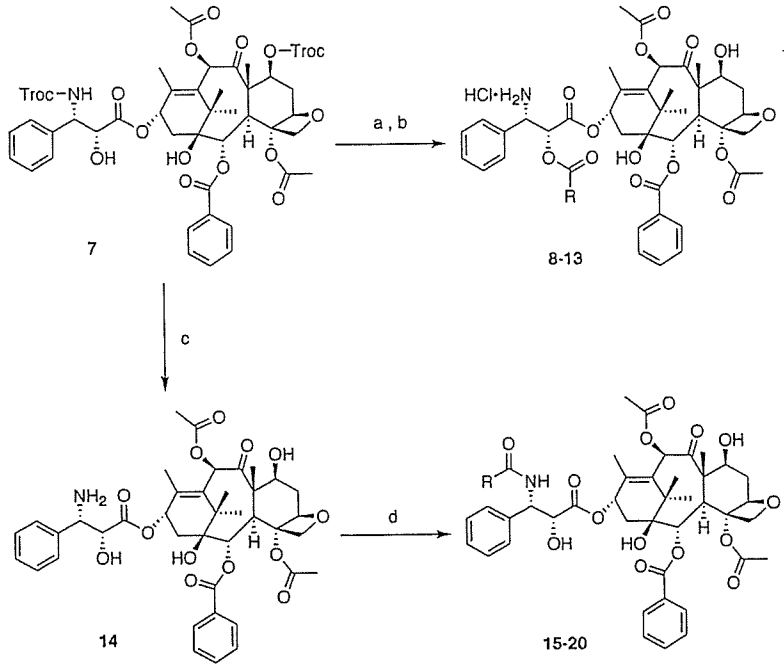


Figure 1. The O−N acyl/acyloxy migration reaction of taxoid prodrugs to their corresponding parent drugs under physiological conditions.

The synthesis of docetaxel prodrug **5** (Scheme 2) was carried out using a similar strategy to that described previously for isotaxel **4**.[41] Commercially available (2*R*,3*S*)-phenylisoserine·HCl **21** after protection of an amine moiety by the benzyloxycarbonyl group (Z) and formation of the methyl ester was allowed to react with 4-methoxybenzaldehyde dimethyl acetal in the presence of a catalytic amount of pyridinium *p*-toluenesulfonate (PPTS) under kinetic control to afford 1,3-oxazolidine derivative **22** as a diastereomeric mixture with 14% of the minor and undesired diastereomer. The formation of the minor, *S*-diastereomer of 1,3-oxazolidine, as expected according to the literature,[44] was also observed in the previously described synthesis of isotaxel **4** (data not showed), although the desired *R*-diastereomer was isolated after careful chromatographic purification.[41] As the minor diastereomer of **22** was difficult to separate by column chromatography, **22** was used for the next step as a mixture. Compound **22**, after hydrolysis to the coresponding carboxylic acid without further purification (due to high lability in an acidic medium, even to silica gel), was coupled with 10-deacetyl-7,10-dibenzyloxycarbonylbaccatin III[45] in the presence of DCC to give the corresponding crude ester **23**, which was immedi-

Scheme 1[a]

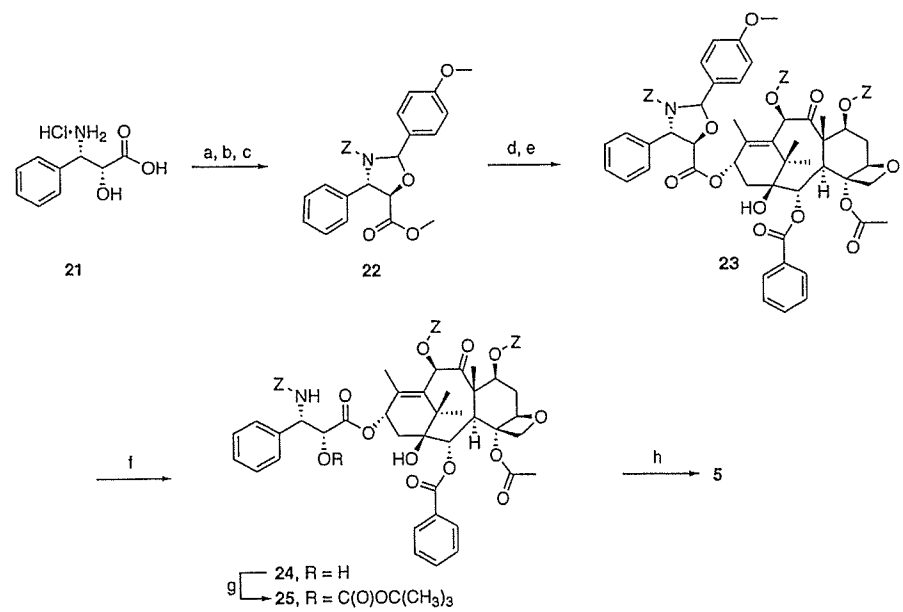


compd	RC(O)·
8	1-cyclohexenecarbonyl
9	*trans*-2-hexenoyl
10	*n*-hexanoyl
11	isopropyloxycarbonyl
12	*n*-butyloxycarbonyl
13	benzyloxycarbonyl (Z-)

compd	RC(O)·
15	1-cyclohexenecarbonyl
16	*trans*-2-hexenoyl
17	*n*-hexanoyl
18	isopropyloxycarbonyl
19	*n*-butyloxycarbonyl
20	benzyloxycarbonyl (Z-)

[a] Reagents and conditions: (a) RCOOH, EDC·HCl, DMAP, CH_2Cl_2, rt (for **8**–**10**), or RCOCl, CH_2Cl_2, pyridine, rt (for **11**–**13**); (b) Zn, AcOH, MeOH, rt, then ion exchange HPLC with 12 mM HCl, (**8**, 76%; **9**, 47%; **10**, 62%; **11**, 65%; **12**, 71%; **13**, 53%) over two steps; (c) Zn, AcOH, AcOEt, rt, 60%; (d) RCOOH, EDC·HCl, HOBt, DMF, rt, for **15**–**17**, or RCOCl, CH_2Cl_2, sat. $NaHCO_3$, rt, for **18**–**20**, (**15**, 77%; **16**, 56%; **17**, 56%; **18**, 87%; **19**, 93%; **20**, 95%).

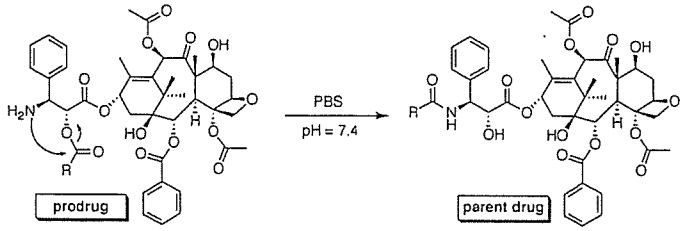
Scheme 2[a]

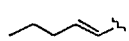
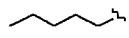
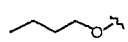
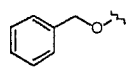


[a] Reagents and conditions: (a) $SOCl_2$, MeOH, 0 °C to rt, overnight; (b) benzyloxycarbonyl chloride, CH_2Cl_2, sat. $NaHCO_3$, 0 °C to rt, 3 h; (c) 4-methoxybenzaldehyde dimethyl acetal, PPTS, toluene, distillation, 40 min, 96% over three steps; (d) KOH, MeOH, rt, 2 h.; (e) 10-deacetyl-7,10-dibenzyloxycarbonylbaccatin III, DCC, DMAP, CH_2Cl_2, rt, 2 h; (f) PTS, MeOH, rt, 5 h, 80% over two steps; (g) Boc_2O, DMAP, CH_2Cl_2:pyridine 1:1, rt, overnight, 84%; (h) Pd/C, H_2, AcOEt, rt, 8 h, then 0.1 M HCl in methanol, 99%.

ately allowed to react with PTS to afford **24**. Although the oxazolidine ring of **23** was easily cleaved with the major diastereomer, the minor diastereomer was stable even with excess PTS and a long reaction time. Despite this, the yield of **24** formation was good (80%). The Boc group was then introduced to **24** to afford compound **25**. Finally, three Z groups were deprotected under standard conditions, and HCl was added to afford the final

Table 1. Water Solubility and Conversion Time of Prodrugs



prodrug	parent drug	R	water solubility: prodrug (mg/mL)	water solubility: parent drug (mg/mL)	water solubility: ratio	$t_{1/2}$ (min)[c]
4[a]	1		0.45	0.00025	1800	15.1
6[b]	3		2.26	0.22	10	4.3
8	15		0.8	0.021	38	60.0
9	16		1.1	0.0013	850	29.3
10	17		0.56	0.008	70	6.0
11	18		1.7	0.018	90	19.2
12	19		0.8	0.004	200	3.8
13	20		1.0	<0.00025	>4000	< 1

[a] Data from ref 41. [b] Data from ref 42. [c] $t_{1/2}$ is the time required for 50% release of the parent drug from prodrug at 37 °C in phosphate-buffered saline (pH = 7.4).

prodrug **5** as a HCl salt quantitatively with a good total yield (67% calculated from baccatin III derivative coupling). It is noteworthy that prodrug **5** is unstable, even under aqueous mildly acidic conditions, due to the hydrolysis of the Boc group; even reverse-phase HPLC purification could not be employed. However, the deprotection of three Z groups and hydrochloride salt formation required no purification, as described in the Experimental Section.

Results

In our previous study, we synthesized water-soluble prodrug **4**[41] and **6**[42] on the basis of an O−N intramolecular acyl migration reaction, which is well-known in peptide chemistry.[46] To diversify this strategy and to understand the effect of acyl structures on the O−N acyl migration reaction, three reported taxoids were chosen. Taxoids **15** and **16**, which have 1-cyclohexenecarbonyl and *trans*-2-hexenoyl moieties, respectively, instead of 3′-*N*-benzoyl in **1**, show high activity against MDR tumors,[47,48] and **17**, which has a hexanoyl moiety at the same position, is also more potent than paclitaxel.[49] The prodrugs, isotaxoids **8**−**10**, *O*-acyl isoforms of parent taxoids **15**−**17**, were synthesized, and the water solubility and kinetic profile of the prodrugs were evaluated. As shown in Table 1, prodrugs **8**−**10** showed practical water solubility with values from 0.56 to 1.1 mg/mL, 38−850-fold higher than of parent drugs **15**−**17** (0.021−0.0013 mg/mL). To study the kinetics of O−N intramolecular acyl migration, prodrugs were dissolved in phosphate-buffered saline (PBS, pH = 7.4) and incubated at 37 °C, and the migration profile was monitored periodically by HPLC. The migration rate was significantly different among the three isotaxoids **8**−**10**. Sterically hindered prodrug **8** with the cyclohexene moiety showed the longest migration time ($t_{1/2}$ = 60.0 min), four times longer than that of isotaxel **4** ($t_{1/2}$ = 15.1 min), while prodrug **9** with the *trans*-2-*n*-hexenoyl

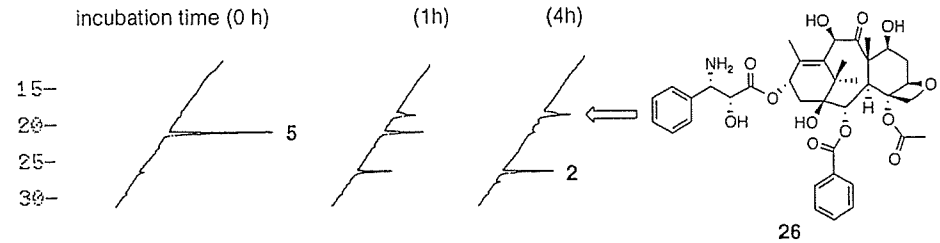


Figure 2. HPLC profiles of O–N intramolecular acyloxy migration in prodrug **5** in PBS (pH 7.4, 37 °C).

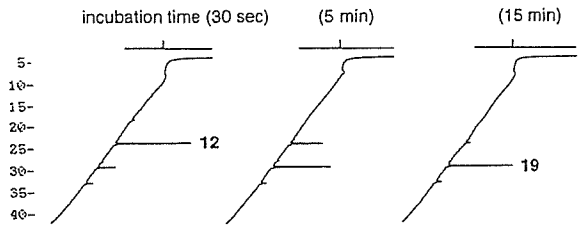


Figure 3. HPLC profiles of O–N intramolecular acyloxy migration of prodrug **12** in PBS (pH 7.4, 37 °C).

group migrated for twice as long as **4**. Prodrugs **6** and **10** with simple aliphatic acyl groups, isobutanoyl and hexanoyl, respectively, released the parent drugs fastest ($t_{1/2}$ = 4.3 and 6.0 min, respectively). Importantly, in each case, the migration reaction proceeded smoothly with no side reactions, such as the simple hydrolysis of the newly introduced ester bond in the prodrugs.

In addition, on the basis of these promising results in prodrugs **8**–**10**, we considered that O–N intramolecular migration could be applied to important and clinically contributing docetaxel **2**, which possesses a *tert*-butyloxycarbonyl (Boc) group in place of the benzoyl group in paclitaxel. Namely, the O–N intramolecular acyloxy migration in docetaxel prodrug **5** was expected. Hence, prodrug **5**, which is the 2′-*O*-acyloxy isoform of docetaxel **2**, was designed and synthesized. This prodrug **5** showed a high water solubility of more than 6 mg/mL. However, in the conversion profile in PBS (pH 7.4, 37 °C), undesired hydrolysis of the Boc group, resulting in the production of compound **26**, was detected, while the desired O–N acyloxy migration from **5** to **2** was also observed (Figure 2).

On the basis of these observations, we assumed that this strategy should be considered for other carbonates more stable than Boc. Therefore, for further examination of the O–N acyloxy migration strategy, three reported taxoids, i.e., **18**,[50] **19**,[51] and **20**,[52] with structurally different acyloxy groups (isopropyloxycarbonyl, *n*-butyloxycarbonyl, and benzyloxycarbonyl, respectively) were chosen. Prodrugs **11**–**13**, including newly formed carbonate groups, were synthesized as HCl salts, and their water solubility was determined in the range from 0.8–1.7 mg/mL, 90–4000-fold higher than those of the parent drugs **18**–**20** (Table 1). Interestingly, complete migration was observed in all cases with no byproduct formation in PBS (pH 7.4, 37 °C) (Figure 3), and in contrast to compound **5**, no hydrolysis was detected. Prodrug **13** had the fastest migration in this group ($t_{1/2}$ < 1 min). Prodrug **11**, having the bulky isopropyloxy group, showed a slower migration ($t_{1/2}$ = 19.2 min) than isotaxoid **12** with the *n*-butyloxy group ($t_{1/2}$ = 3.8 min). On the other hand, the release of the

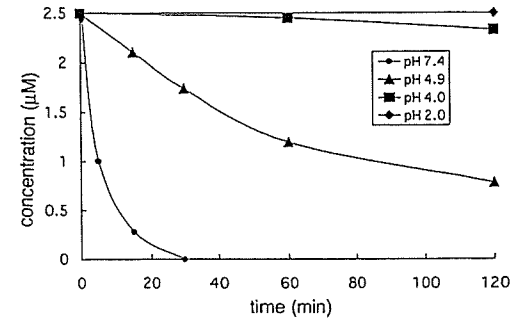


Figure 4. Migration of prodrug **12** at different pH values at 37 °C: pH 7.4 and 4.9 (PBS), pH 4.0 (0.035% citric acid saline), and pH 2.0 (glycine-HCl buffered saline).

parent drug from prodrug **12** slowed at pH 4.9 to $t_{1/2}$ = 55.5 min, and no O–N acyloxy intramolecular migration was observed at pH 2 (Figure 4). Moreover, compound **12** was almost stable in 0.035% citric acid saline (pH 4.0) at room temperature (<2% of the parent drug was released after 1 h of incubation), suggesting a possible condition for injection.[20] These results indicate that the kinetics of O–N acyloxy migration are clearly pH-dependent, and a faster migration could be obtained under physiological conditions (pH 7.4) than under acidic conditions. This result is comparable to that observed previously in O–N intramolecular acyl migration.[38–40]

Discussion

Prodrugs are bioreversible derivatives of the drug molecule used to overcome some barriers in the parent drug utilities, such as solubility, permeability, oral absorption, stability, toxicity, and targeting.[32] In 2001 and 2002, 14% of all new approved chemical drugs were prodrugs.[53] This trend caused a dramatic increase in the number of submitted prodrug patents (>2000% increase in 2002 compared to 1993), with claims for cancer treatment comprising 37%,[53] which clearly shows the importance of this field in the development of anticancer agents. However, auxiliary units are used in all standard prodrug strategies, and the release of these moieties may cause some undesired side effects.[32] Therefore, the potential side effects of the auxiliary unit should be evaluated; otherwise, safe and nontoxic auxiliary units already accepted by the registration authorities should be used. However, even commonly used pivalic acid showed some toxicity connected with changes in carnitine homeostasis,[54] as well as the phosphate group (popularly used in prodrug design), which was reported to cause hypocalcemic effects, and special caution should be implemented, for example, for

patients with renal impairment.[55] Also, the formaldehyde released upon hydrolysis of some linker can be a toxicological concern.[32] In consideration, prodrug strategies that avoid any additional auxiliary moieties are very promising.

Along these lines, we previously developed isotaxel **4**, a 2′-*O*-acyl isoform of paclitaxel **1**. This prodrug **4** showed 1800-fold higher water solubility (0.45 mg/mL) than **1** and released parent drug **1** via an O−N intramolecular acyl migration reaction with no side reaction under physiological conditions (pH 7.4).[41] In a similar manner, we designed and synthesized the first water-soluble prodrug of canadensol **3**,[42] a promising candidate as a new taxoid drug. In both cases, the production of parent drugs was pH-dependent, while prodrugs were stable in the solid form and under acidic conditions, which suggested a possible injection condition for practical clinical use. Thus, the O−N acyl migration reaction could be one of the leading strategies in prodrug design, since isotaxoids **4** and **6** showed higher water solubility and there was no byproduct formation during parent drug formation.

On the other hand, it was demonstrated that paclitaxel prodrug 7-(2″,3″-dihydroxypropylcarbonoxy)-paclitaxel (protaxel),[20] which had improved water solubility (0.05 mg/mL) and released **1** in a similar pH-dependent manner (pH 7.4, $t_{1/2}$ = 10 min,) via intramolecular cyclization, showed lower systemic toxicity than paclitaxel. Therefore, it can be administered in higher doses. Protaxel is currently in clinical trials.[28] In relation to those results, we also expected the water solubility and kinetics of our prodrugs to be promising in practice.

In this paper, prodrugs **8**−**10** of important taxoids, which have different acyl groups and can be converted to parent drugs by the O−N intramolecular acyl migration reaction, were synthesized and evaluated. All showed significant increases in water solubility (38−800-fold) compared to the parent drugs. The water-soluble prodrug **8** with the cyclohexene carbonyl group showed the slowest migration rate ($t_{1/2}$ = 60 min), which is thought to result from its steric hindrance. Indeed, the less sterically hindered prodrug **4** with the flat benzoyl group showed faster migration ($t_{1/2}$ = 15.1 min), and prodrug **10** with linear hexanoyl moiety showed even faster migration ($t_{1/2}$ = 6.0 min). However, in contrast to these results, the significantly longer migration time of prodrug **9**, with the hexenoyl moiety ($t_{1/2}$ = 29.3 min), than **10** ($t_{1/2}$ = 6.0 min) was rather unexpected. The decelerating influence of the double bond on the migration rate seems difficult to explain by the steric or electrical effects of only this bond. Moreover, the faster migration rate of prodrug **6** with the isopropyl group ($t_{1/2}$ = 4.3 min) similar to **10** with the hindered hexanoyl group is surprising. These complicated results suggest the difficulty of precise theoretical prediction of O−N intramolecular acyl migration kinetics among various acyl moieties. However, the important conclusions are that (1) the obtained $t_{1/2}$ values of a series of synthetic taxoids prodrugs (4−60 min) are though to be in not too short time for systemic distribution and not too long for metabolism, although the optimal migration rate should be estimated from in vivo evaluation, and (2) no byproduct formation based on like acyl group hydrolysis was observed, which is a very important factor for practical use of the prodrugs. Additionally, these prodrugs, *O*-acyl isoforms of taxoids, are not expected to be active, based on previous SAR studies on paclitaxel derivatives.[56,57] Namely, it is known that both *N*-acyl and free 2′-OH groups are required for taxoid activity.[57]

Taxoids can be divided into two groups, 3′-*N*-acyl derivatives such as paclitaxel (**1**) and 3′-*N*-acyloxy derivatives such as docetaxel (**2**). Therefore, our prodrug strategy was also applied to an O−N intramolecular "acyl-like" (acyloxy) migration to docetaxel (**2**). A model prodrug of **2** was designed in which the baccatin III moiety was replaced by a bulky cyclohexyl ester. However, a simple hydrolysis of the Boc group, besides the desired O−N acyloxy migration for the production of the *N*-acyl parent model compound, was observed under physiological conditions.[42] On the other hand, this strategy was recently used for the prodrug design of HIV-1 protease inhibitors and no hydrolysis of carbonate moieties (3-tetrahydrofuranyloxycarbonyl) was observed.[58] Considering this feature, we decided to reconsider this strategy for the synthesis of 3′-*N*-acyloxy taxoid prodrugs, including **2**, a docetaxel prodrug **5**. However, the undesired hydrolysis of the Boc group in PBS (pH 7.4, 37 °C) was replicated in this real taxoid prodrug (Figure 2). We suggested that replacement of the *tert*-butyl group in the primary or secondary aliphatic groups could avoid undesired hydrolysis. Hence, the application of new acyloxy groups in our prodrug strategy has been demonstrated. Three isotaxoids, **11**−**13**, prodrugs of the reported 3′-*N*-carbamate taxoids **18**−**20**,[50−52] based on the O−N acyloxy migration strategy, were synthesized and showed practical water solubility, ranging from 0.8 to 1.7 mg/mL, than isotaxel **4** (Table 1). The prodrugs **11** and **12** showed promising kinetic data. Prodrug **11**, having the bulky isopropyloxy group, due to steric hindrance, clearly showed a slower migration ($t_{1/2}$ = 19.2 min) than prodrug **12** ($t_{1/2}$ = 3.8 min). Prodrug **13** showed the fastest migration in this group ($t_{1/2}$ < 1 min), due to the electron-withdrawing effect of the phenyl group. This suggests that the steric effect of the phenyl ring on acyl migration appears only in cases when the phenyl ring is directly connected to the carbonyl carbon (**4**, $t_{1/2}$ = 15.1 min). As expected, no byproduct formation, such as hydrolysis, was observed. Interestingly, in the previously mentioned prodrugs of HIV-1 protease inhibitors using the hydroxyethylamine core structure,[58] a byproduct of oxazolidinone was produced during the parent drug formation (Figure 5A). The lack of oxazolidinone formation in our case was probably due to the electron-withdrawing influence of a neighboring ester group in the phenylisoserine moiety (Pis), and thus the hydroxyl group of Pis is a good leaving group, as shown in Figure 5B. Thus, herein, we reported the first successful atom economical O−N intramolecular acyloxy migration reaction under aqueous conditions without the formation of side products, as we reported previously (hydrolysis),[42] as did GlaxoSmithKline (oxazolidone formation).[58] In addition, it was shown that there is no major difference in kinetics between the previously reported model compounds with the cyclohexanol moiety instead of baccatin III and real prodrugs.[41−43] This suggests that the time of parent

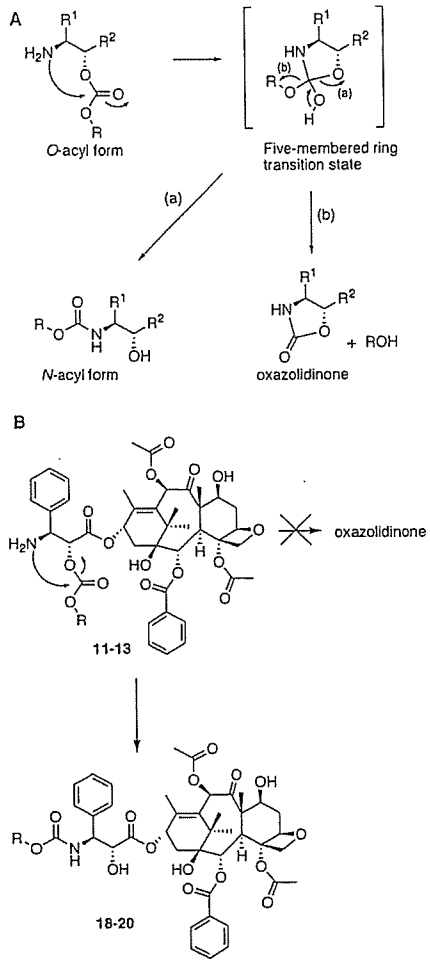


Figure 5. Possible products of amine group nucleophilic attack on carbonate moiety (A) and pure O−N intramolecular acyloxy migration reaction observed in prodrugs **11**−**13** (B).

drug formation does not necessarily depend on the modification of the baccatin III structure, namely the kinetics of migration observed for prodrugs **4**, **6**, **8**−**13** can be expected to replicate without significant differences among all prodrugs that have the same phenylisoserine derivative moiety. Thus, for example, similar kinetic behavior can be expected for other taxoids with isopropyloxycarbonyl and *n*-butyloxycarbonyl groups, which showed very promising anticancer activity,[51,59,60] different from drugs **18** and **19** only in baccatin III moiety. Additionally, differences in migration rates between prodrug **10** ($t_{1/2}$ = 6.0 min) and its isoster prodrug **12** ($t_{1/2}$ = 3.8 min) demonstrated that O−N acyloxy migration is kinetically similar to O−N acyl migration. There is no major difference in $t_{1/2}$ values between these two compounds (Table 1).

In addition, pH-dependent kinetics observed in both types of migration reactions suggest a design for the injectable solution. We demonstrated previously that isotaxel **4** was almost stable for several hours at pH 4.0,[41] which is an acceptable value for injection in practical clinical use.[20] The prodrug of canadensol **6** was also stable at pH 4.0 for at least 1 h.[42] In this study, prodrug **12**, which also had a very small $t_{1/2}$ value at pH 7.4 (3.8 min), showed only slight migration (<2%) after 1 h of incubation in 0.035% citric acid saline (pH 4.0). Naturally, prodrugs with higher $t_{1/2}$ values under physiological conditions can remain stable for a longer time under these injectable pH 4.0 conditions. Therefore, it should be possible to prepare a stable prodrug solution just before injection. One exception is prodrug **13**, which has a very small $t_{1/2}$ value for parent drug release, and it would be difficult to adopt this pH 4.0 and even lower pH conditions because the precipitate would be formed just after injection in the body without complete distribution.

Interestingly, the water-solubility ratios of prodrugs compared to parent drugs exist over a wide range (10−4000-fold), but the resulting water solubility of the prodrugs is very similar over the range of 0.45−2.3 mg/mL. This suggests that the solubility of prodrugs is related much more to the whole structure of isotaxoids and the ionized amine group than to the R acyl/acyloxy groups, in contrast to the solubility of parent drugs, which is highly dependent on the nature of acyl/acyloxy groups. However, in all cases, the improved water solubility of prodrugs suggests that the utility of the O−N acyl/acyloxy intramolecular migration strategy for water-soluble taxoid produgs can be generalized.

Conclusion

We developed water-soluble prodrugs of taxoids based on the O−N acyl migration reaction. All prodrugs showed a significant increase in water solubility and promising kinetic data. These prodrugs, a 2′-*O*-isoform of taxoids, have no additional functional auxiliaries released during conversion to the parent drugs. This would be an advantage in toxicology and general pharmacology, since the detergent for solubilization, which has some side effects, can be omitted and the potential side effects caused by reported auxiliaries can be avoided. In addition, no costly toxicological and general pharmacological evaluation of released auxiliary units is required. Therefore, we can recommend this strategy as a first choice for water-soluble prodrug design for other drugs if O−N acyl intramolecular migration is possible. Additionally, this study demonstrates for the first time the pure O−N intramolecular acyloxy migration reaction, which proceeds under aqueous conditions with no side product formation. The first successful application of this reaction in a prodrug strategy was demonstrated with the same promising data as for acyl migration. Thus, our prodrug strategy can be extended to carbamate derivatives of taxoids, except for Boc. Moreover, our success with O−N intramolecular acyloxy migration will open the door for the application of this atom economical reaction in water-soluble prodrug design of other drugs which have a carbamate moiety and the neighboring hydroxy group.

Experimental Section

Chemistry. Reagents and solvents were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), and Aldrich Chemical Co. Inc. (Milwaukee, WI) and used without further purification. Column chromatography was performed on Merck 107734 silica gel 60 (70−230 mesh). TLC was performed using Merck silica gel $60F_{254}$ precoated plates. Analytical HPLC was performed using a C18 reverse phase column (4.6 × 150 mm; YMC Pack ODS

AM302) with a binary solvent system: a linear gradient of CH_3CN in 0.1% aqueous TFA at a flow rate of 0.9 mL min^{-1}, detected at 230 nm. Preparative HPLC was carried out on a C18 reverse phase column (20 × 250 mm; YMC Pack ODS SH343−5) with a binary solvent system: a linear gradient of CH_3CN in 12 mM aqueous HCl at a flow rate of 5.0 mL min^{-1}, detected at 230 nm. Solvents used for HPLC were of HPLC grade. All other chemicals were of analytical grade or better. ^1H NMR spectra were obtained using a 400 MHz Varian UNITY INOVA 400NB spectrometer with TMS as an internal standard at 25 °C. FAB-MS was performed on a JEOL JMS−SX102A spectrometer equipped with the JMA-DA7000 data system. ESI-MASS was performed on a Finnigan SSQ-7000 spectrometer.

2′-*O*-(1-Cyclohexenecarbonyl)-3′-*N*-debenzoylpaclitaxel Hydrochloride (8). A solution of **7** (21 mg, 0.019 mmol), 1-cyclohexenecarboxylic acid (2.9 mg, 0.023 mmol), DMAP (0.7 mg, 0.006 mmol), and EDC·HCl (4.4 mg, 0.023 mmol) in dry CH_2Cl_2 (1 mL) was stirred under an argon atmosphere at room temperature for 5 h. Then the reaction mixture was diluted with AcOEt and successively washed with 10% citric acid three times and water and saturated $NaHCO_3$ twice. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was dissolved in a mixture (2 mL) of MeOH:AcOH (1:1), zinc dust was added in three portions (a total of 38 mg, 0.57 mmol) within 30 min, and the reaction mixture was stirred for an additional 15 min at room temperature. The reaction mixture was directly applied to preparative HPLC, which was eluted with a linear gradient of 10−60% CH_3CN in 12 mM aqueous HCl over 100 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give a white powder of **8** as an HCl salt (12.9 mg, 14.4 μmol, 76%). ^1H NMR (CD_3OD, 400 MHz): δ = 8.08−8.06 (m, 2 H, CH), 7.75−7.71 (m, 1 H, CH), 7.65−7.49 (m, 6 H, CH), 7.39−7.35 (m, 2 H, CH), 6.41 (s, 1 H, CH), 5.93 (bt, ^3J(H,H) = 9.3 Hz, 1 H, CH), 5.60 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.40 (d, ^3J(H,H) = 9.0 Hz, 1H, CH), 4.97 (bd, ^3J(H,H) = 7.7 Hz, 1H, CH), 4.85 (d, 1 H, CH, partially overlapping with signal from CD_3OD), 4.30 (dd, ^3J(H,H) = 6.6, 11.0 Hz, 1 H, CH), 4.17, 4.14 (2d, ^2J(H,H) = 8.2 Hz, 2 H, CH_2), 3.72 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.48−2.41 (m, 1 H, CH_2), 2.34−2.31 (m, 4H, CH_2), 2.27 (s, 3 H, CH_3), 2.16 (s, 3 H, CH_3), 1.92 (dd, ^3J(H,H) = 9.3 Hz, ^2J(H,H) = 15.4 Hz, 1 H, CH_2), 1.85 (d, ^4J(H,H) = 1.1 Hz, 3 H, CH_3), 1.82−1.68 (m, 5 H, CH_2), 1.62 (s, 3 H, CH_3), 1.47 (dd, ^3J(H,H) = 8.8 Hz, ^2J(H,H) = 15.5 Hz, 1 H, CH_2), 1.12 (s, 3 H, CH_3), 1.10 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{47}H_{56}NO_{14}$ [M^+ + H] 858.3701, found 858.3705. Anal. ($C_{47}H_{56}ClNO_{14}$·3.5H_2O) C, N, H: calcd, 6.63; found, 6.05.

2′-*O*-(*trans*-2-Hexenoyl)-3′-*N*-debenzoylpaclitaxel Hydrochloride (9). The above procedure with *trans*-2-hexenoic acid afforded **9** (47%): ^1H NMR (CD_3OD, 400 MHz): δ = 8.08−8.05 (m, 2 H, CH), 7.75−7.71 (m, 1 H, CH), 7.65−7.48 (m, 6 H, CH), 7.37−7.30 (m, 1 H, CH), 7.26(dt, ^3J(H,H) = 7.0, 15.6 Hz, 1H, CH), 6.41 (s, 1 H, CH), 6.09 (dt, ^4J(H,H) = 1.5 Hz, ^3J(H,H) = 15.6 Hz, 1 H, CH), 5.93 (bt, ^3J(H,H) = 8.9 Hz, 1 H, CH), 5.59 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.33 (d, ^3J(H,H) = 9.0 Hz, 1H, CH), 4.96 (bd, ^3J(H,H) = 9.5 Hz, 1H, CH), 4.80 (d, ^3J(H,H) = 8.6 Hz, 1H, CH), 4.30 (dd, ^3J(H,H) = 6.5, 10.9 Hz, 1 H, CH), 4.16, 4.13 (2d, ^2J(H,H) = 8.9 Hz, 2 H, CH_2), 3.72 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.48−2.40 (m, 1 H, CH_2), 2.34−2.28 (m, 2H, CH_2), 2.26 (s, 3 H, CH_3), 2.15 (s, 3 H, CH_3), 1.90 (dd, ^3J(H,H) = 6.0 Hz, ^2J(H,H) = 15.6 Hz, 1 H, CH_2), 1.85 (d, ^4J(H,H) = 1.1 Hz, 3 H, CH_3), 1.82−1.74 (m, 1 H, CH_2), 1.62 (s, 3 H, CH_3), 1.62−1.53 (m, 2 H, CH_2), 1.44 (dd, ^3J(H,H) = 8.7 Hz, ^2J(H,H) = 15.5 Hz, 1 H, CH_2), 1.12 (s, 3 H, CH_3), 1.09 (s, 3 H, CH_3), 1.00 (t, ^3J(H,H) = 7.4 Hz, 3 H, CH). HRMS (FAB+): calcd for $C_{46}H_{56}NO_{14}$ [M^+ + H] 846.3701, found 846.3698. Anal. ($C_{46}H_{56}ClNO_{14}$·2.5H_2O) C, H, N.

2′-*O*-Hexanoyl-3′-*N*-debenzoylpaclitaxel Hydrochloride (10). The above procedure with hexanoic acid afforded **10** (62%): ^1H NMR (CD_3OD, 400 MHz): δ = 8.08−8.06 (m, 2 H, CH), 7.76−7.71 (m, 1 H, CH), 7.66−7.48 (m, 6 H, CH), 7.37−7.33 (m, 1 H, CH), 6.41 (s, 1 H, CH), 5.93 (bt, ^3J(H,H) = 8.5 Hz, 1 H, CH), 5.59 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.31 (d, ^3J(H,H) = 9.0 Hz, 1H, CH), 4.97 (dd, ^3J(H,H) = 2.0, 9.7 Hz, 1H, CH), 4.78 (bd, ^3J(H,H) = 8.4 Hz, 1H, CH), 4.30 (dd, ^3J(H,H) = 6.5, 11.1 Hz, 1 H, CH), 4.16, 4.14 (2d, ^2J(H,H) = 8.4 Hz, 2 H, CH_2), 3.72 (d, ^3J(H,H) = 7.2 Hz, 1 H, CH), 2.57 (t, ^3J(H,H) = 7.2 Hz, 1 H, CH), 2.48−2.40 (m, 1 H, CH_2), 2.26 (s, 3 H, CH_3), 2.16 (s, 3 H, CH_3), 1.93−1.88 (m, 1H, CH_2), 1.86 (d, ^4J(H,H) = 1.1 Hz, 3 H, CH_3), 1.81−1.74 (m, 1 H, CH_2), 1.73−1.66 (m, 2 H, CH_2), 1.62 (s, 3 H, CH_3), 1.44 (dd, ^3J(H,H) = 9.0 Hz, ^2J(H,H) = 15.4 Hz, 1 H, CH_2), 1.39−1.31 (m, 4 H, CH_2), 1.12 (s, 3 H, CH_3), 1.10 (s, 3 H, CH_3), 0.93 (t, ^3J(H,H) = 7.0 Hz, 3 H, CH). HRMS (FAB+): calcd for $C_{46}H_{58}NO_{14}$ [M^+ + H] 848.3857, found 848.3854. Anal. ($C_{46}H_{58}ClNO_{14}$·2.5H_2O) C, H, N.

2′-*O*-Isopropyloxycarbonyl-3′-*N*-debenzoylpaclitaxel Hydrochloride (11). Isopropyloxycarbonyl chloride (5 μL, 43.6 μmol) was added to the stirring solution of **7** (24.0 mg, 21.8 μmol) in dry CH_2Cl_2 (1 mL) and dry pyridine (1 mL), and the mixture was stirred under an argon atmosphere at room temperature for 30 min. The reaction mixture was diluted with AcOEt and then washed with water, 1 N hydrochloric acid twice, and then brine. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was dissolved in a mixture (2 mL) of MeOH:AcOH (1:1), zinc dust was added in three portions (a total of 42 mg, 0.65 mmol) within 30 min, and the reaction mixture was stirred for an additional 15 min at room temperature. The reaction mixture was directly applied to preparative HPLC, which was eluted with a linear gradient of 30−70% CH_3CN in 12 mM aqueous HCl over 40 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give a white powder of **11** as an HCl salt (12.3 mg, 14.1 μmol, 65%). ^1H NMR (CD_3OD, 400 MHz): δ = 8.08−8.06 (m, 2 H, CH), 7.74−7.70 (m, 1 H, CH), 7.64−7.50 (m, 6 H, CH), 7.40−7.36 (m, 1 H, CH), 6.41 (s, 1 H, CH), 5.99−5.95 (m, 1 H, CH), 5.60 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.34 (d, ^3J(H,H) = 8.8 Hz, 1H, CH), 4.99−4.80 (m, 3 H, CH, partially overlapping with signal from CD_3OD), 4.30 (dd, ^3J(H,H) = 6.6, 11.0 Hz, 1 H, CH), 4.17, 4.16 (2d, ^2J(H,H) = 8.4 Hz, 2 H, CH_2), 3.72 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.49−2.41 (m, 1 H, CH_2), 2.29 (s, 3 H, CH_3), 2.16 (s, 3 H, CH_3), 1.94 (dd, ^3J(H,H) = 9.6 Hz, ^2J(H,H) = 15.3 Hz, 1 H, CH_2), 1.86 (d, ^4J(H,H) = 1.3 Hz, 3 H, CH_3), 1.82−1.75 (m, 1 H, CH_2), 1.63 (s, 3 H, CH_3), 1.53 (dd, ^3J(H,H) = 8.9 Hz, ^2J(H,H) = 15.3 Hz, 1 H, CH_2), 1.36 (d, ^3J(H,H) = 6.2 Hz, 3 H, CH_3), 1.35 (d, ^3J(H,H) = 6.2 Hz, 3 H, CH_3), 1.13 (s, 3 H, CH_3), 1.11 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{44}H_{54}NO_{15}$ [M^+ + H] 836.3493, found 836.3502. Anal. ($C_{44}H_{54}ClNO_{15}$·4.5H_2O) C, H, N.

2′-*O*-*n*-Butyloxycarbonyl-3′-*N*-debenzoylpaclitaxel Hydrochloride (12). The above procedure with *n*-butyloxycarbonyl chloride (6 equiv) afforded **12** (71%): ^1H NMR (CD_3OD, 400 MHz): δ = 8.08−8.06 (m, 2 H, CH), 7.75−7.70 (m, 1 H, CH), 7.64−7.49 (m, 6 H, CH), 7.39−7.36 (m, 1 H, CH), 6.42 (s, 1 H, CH), 5.96 (bt, ^3J(H,H) = 9.5 Hz, 1 H, CH), 5.60 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.31 (d, ^3J(H,H) = 8.8 Hz, 1H, CH), 4.96 (dd, ^3J(H,H) = 1.8, 9.7 Hz, 1H, CH), 4.82 (d, 1 H, CH, partially overlapping with signal from CD_3OD), 4.34−4.22 (m, 3 H, CH, CH_2), 4.17, 4.14 (2d, ^2J(H,H) = 8.4 Hz, 2 H, CH_2), 3.73 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 2.48−2.41 (m, 1 H, CH_2), 2.27 (s, 3 H, CH_3), 2.16 (s, 3 H, CH_3), 1.92 (dd, ^3J(H,H) = 9.4 Hz, ^2J(H,H) = 15.5 Hz, 1 H, CH_2), 1.87 (d, ^4J(H,H) = 1.1 Hz, 3 H, CH_3), 1.82−1.75 (m, 1 H, CH_2), 1.75−1.67 (m, 2H, CH_2), 1.63 (s, 3 H, CH_3), 1.53−1.40 (m, 3 H, CH, CH_2), 1.13 (s, 3 H, CH_3), 1.11 (s, 3 H, CH_3), 0.97 (t, ^3J(H,H) = 7.3 Hz, 3 H, CH_3). HRMS (FAB+): calcd for $C_{45}H_{56}NO_{15}$ [M^+ + H] 850.3650, found 850.3655. Anal. ($C_{45}H_{56}ClNO_{15}$·3.5H_2O) C, H, N.

2′-*O*-Benzyloxycarbonyl-3′*N*-debenzoylpaclitaxel Hydrochloride (13). The above procedure with benzyloxycarbonyl chloride (20 equiv) afforded **13** (53%): ^1H NMR (CD_3OD, 400 MHz): δ = 8.09−8.05 (m, 2 H, CH), 7.74−7.70 (m, 1 H, CH), 7.63−7.59 (m, 2 H, CH), 7.57−7.34 (m, 10 H, CH), 6.41 (s, 1 H, CH), 5.94 (dt, ^4J(H,H) = 1.3 Hz, ^3J(H,H) = 9.2 Hz, 1 H, CH), 5.60 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.35 (d, ^3J(H,H) = 8.6 Hz, 1H, CH), 5.31, 5.27 (2d, ^2J(H,H) = 12.0 Hz, 2 H,

CH_2), 4.97 (dd, ^3J(H,H) = 1.9, 9.6 Hz, 1H, CH), 4.81 (d, ^3J(H,H) = 8.8 Hz, 1 H, CH), 4.30 (dd, ^3J(H,H) = 6.6, 11.0 Hz, 1 H, CH), 4.17, 4.14 (2d, ^2J(H,H) = 9.0 Hz, 2 H, CH_2), 3.72 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 2.50–2.42 (m, 1 H, CH_2), 2.27 (s, 3 H, CH_3), 2.17 (s, 3 H, CH_3), 1.91 (dd, ^3J(H,H) = 9.3 Hz, ^2J(H,H) = 15.4 Hz, 1 H, CH_2), 1.81 (d, ^4J(H,H) = 1.3 Hz, 3 H, CH_3), 1.82–1.76 (m, 1 H, CH_2), 1.63 (s, 3 H, CH_3), 1.49 (dd, ^3J(H,H) = 9.0 Hz, ^2J(H,H) = 15.4 Hz, 1 H, CH_2), 1.13 (s, 3 H, CH_3), 1.10 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{48}H_{54}NO_{15}$ [M^+ + H] 884.3493, found 884.3486. Anal. ($C_{48}H_{55}ClNO_{15}$·1.5H_2O) C, H, N.

3′-*N*-Debenzoylpaclitaxel (14). Zinc dust was added in three portions (a total of 0.71 g, 10.9 mmol) to the stirring solution (9 mL) of **7** (400 mg, 0.363 mmol) in AcOEt:AcOH (8:1) within 5 h and the reaction mixture was stirred for an additional 3 h at room temperature. The zinc dust was filtered, and the resulting mixture was diluted with AcOEt and washed with saturated $NaHCO_3$ three times. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was purified by silica gel column chromatography (MeOH:$CHCl_3$ 10:1) to yield a white powder **14** after lyophilization (164 mg, 0.219 mmol, 60%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.07–8.05 (m, 2 H, CH), 7.67–7.63 (m, 1 H, CH), 7.54–7.50 (m, 2H, CH), 7.38–7.36 (m, 4 H, CH), 7.28–7.21 (m, 1 H, CH), 6.27 (s, 1 H, CH), 6.14 (dt, ^4J(H,H) = 1.3 Hz, ^3J(H,H) = 8.9 Hz, 1 H, CH), 5.63 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 4.93 (dd, ^3J(H,H) = 2.0, 9.5 Hz, 1H, CH), 4.40 (dd, ^3J(H,H) = 6.7, 10.9 Hz, 1 H, CH), 4.31–4.26 (m, 3H, CH_2, CH), 4.14(d, ^3J(H,H) = 8.2 Hz, 1H, CH), 3.75 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 2.57–2.49 (m, 1 H, CH_2), 2.24 (s, 3 H, CH_3), 2.13 (s, 3 H, CH_3), 2.19–1.82 (m, 3 H, CH_2), 1.87 (d, ^4J(H,H) = 1.3 Hz, 3 H, CH_3), 1.65 (s, 3 H, CH_3), 1.24 (s, 3 H, CH_3), 1.13 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{40}H_{48}NO_{13}$ [M^+ + H] 750.3126, found 750.3120. Anal. ($C_{40}H_{47}NO_{13}$·H_2O) C, H, N.

3′-*N*-(1-Cyclohexenylcarbonyl)-3′-*N*-debenzoylpaclitaxel (15). A solution of **14** (10.4 mg, 0.014 mmol), 1-cyclohexenecarboxylic acid (2.1 mg, 0.017 mmol), HOBt (2.5 mg, 0.017 mmol), and EDC·HCl (3.2 mg, 0.017 mmol) in DMF (1 mL) was stirred at room temperature for 3 h. Then DMF was removed under reduced pressure and the reaction mixture was dissolved with AcOEt. After washing with 10% citric acid three times and water and saturated $NaHCO_3$ twice, the organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was applied to preparative HPLC, which was eluted with a linear gradient of 40%–80% CH_3CN in 12 mM aqueous HCl over 80 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give a white powder of **15** (9.2 mg, 10.7 μmol, 77%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.13–8.10 (m, 2 H, CH), 7.64–7.60 (m, 1 H, CH), 7.53–7.49 (m, 2H, CH), 7.41–7.38 (m, 4 H, CH), 7.37–7.31 (m, 1 H, CH), 6.65–6.63 (m, 1H, CH), 6.47 (d, ^3J(H,H) = 8.8 Hz, 1 H, NH) 6.27 (s, 1 H, CH), 6.20 (bt, ^3J(H,H) = 8.2 Hz, 1 H, CH), 5.67 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 5.61 (dd, ^3J(H,H) = 2.8, 8.8 Hz, 1H, CH), 4.95–4.93 (m, 1H, CH), 4.71 (dd, ^3J(H,H) = 2.8, 5.4 Hz, 1 H, CH), 4.43–4.37 (m, 1 H, CH), 4.30, 4.19 (2d, ^2J(H,H) = 8.3 Hz, 2 H, CH_2), 3.78 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 3.57 (d, ^3J(H,H) = 5.5 Hz, 1 H, OH) 2.59–2.51 (m, 1 H, CH_2), 2.47 (d, ^3J(H,H) = 4.0 Hz, 1 H, OH), 2.35 (s, 3 H, CH_3), 2.29 (dd, ^3J(H,H) = 5.2 Hz, ^2J(H,H) = 8.9 Hz, 1 H, CH_2), 2.25 (s, 3 H, CH_3), 2.22–2.11 (m, 4H, CH_2), 1.91–1.85 (m, 1 H, CH_2), 1.85 (s, 1H, OH), 1.79 (d, ^4J(H,H) = 1.1 Hz, 3 H, CH_3), 1.68 (s, 3 H, CH_3), 1.66–1.63 (m, 5 H, CH_2, partially overlapping with signal from H_2O), 1.26 (s, 3 H, CH_3), 1.15 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{47}H_{56}NO_{14}$ [M^+ + H] 858.3701, found 858.3696.

3′-*N*-(*trans*-2-Hexenoyl)-3′-*N*-debenzoylpaclitaxel (16). The above procedure with *trans*-2-hexenoic acid afforded **16** (56%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.14–8.12 (m, 2 H, CH), 7.64–7.60 (m, 1 H, CH), 7.55–7.31 (m, 7 H, CH), 6.79 (dt, ^3J(H,H) = 7.0, 15.4 Hz, 1H, CH), 6.27 (s, 1 H, CH), 6.25–6.17 (m, 2 H, CH, NH), 5.79 (bd, ^3J(H,H) = 15.6 Hz, 1 H, CH), 5.67 (d, ^3J(H,H) = 7.0 Hz, 1H, CH), 5.62 (dd, ^3J(H,H) = 2.6, 8.6 Hz, 1H, CH), 4.94 (dd, ^3J(H,H) = 2.0, 7.3 Hz, 1 H, CH), 4.71 (bs, 1 H, CH), 4.43–4.38 (m, 1H, CH), 4.30, 4.19 (2d, ^2J(H,H) = 8.8 Hz, 2 H, CH_2), 3.79 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 3.58 (d, ^3J(H,H) = 5.3 Hz, 1 H, OH) 2.59–2.51 (m, 1 H, CH_2), 2.46 (bs, 1 H, OH), 2.37 (s, 3 H, CH_3), 2.33–2.27 (m, 1 H, CH_2), 2.25 (s, 3 H, CH_3), 2.13–2.08 (m, 2H, CH_2), 1.91–1.84 (m, 1 H, CH_2), 1.81 (d, ^4J(H,H) = 1.1 Hz, 3 H, CH_3), 1.68 (s, 3 H, CH_3), 1.64–1.59 (m, 1 H, CH_2, partially overlapping with signal from H_2O), 1.44–1.35 (m, 2H, CH_2), 1.26 (s, 3 H, CH_3), 1.15 (s, 3 H, CH_3), 0.87 (t, ^3J(H,H) = 7.3 Hz, 3 H, CH_3). HRMS (FAB+): calcd for $C_{46}H_{56}NO_{14}$ [M^+ + H] 846.3701, found 846.3707.

3′-*N*-(Hexanoyl)-3′-*N*-debenzoylpaclitaxel (17). The above procedure with hexanoic acid afforded **17** (56%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.12–8.10 (m, 2 H, CH), 7.64–7.60 (m, 1 H, CH), 7.53–7.32 (m, 7 H, CH), 6.28 (s, 1 H, CH), 6.23–6.20 (m, 2 H, CH, NH), 5.68 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.58 (dd, ^3J(H,H) = 2.3, 8.9 Hz, 1H, CH), 4.94 (dd, ^3J(H,H) = 1.8, 9.5 Hz, 1 H, CH), 4.68 (bs, 1 H, CH), 4.40 (dd, ^3J(H,H) = 6.4, 11.0 Hz, 1 H, CH), 4.30, 4.19 (2d, ^2J(H,H) = 8.3 Hz, 2 H, CH_2), 3.79 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 3.47 (bs, 1 H, OH) 2.58–2.50 (m, 1 H, CH_2), 2.47 (bs, 1 H, OH), 2.34 (s, 3 H, CH_3), 2.32–2.25 (m, 1 H, CH_2), 2.25 (s, 3 H, CH_3), 2.19 (t, ^3J(H,H) = 7.6 Hz, 2H, CH_2), 1.93–1.84 (m, 1 H, CH_2), 1.82 (d, ^4J(H,H) = 1.13 Hz, 3 H, CH_3), 1.68 (s, 3 H, CH_3), 1.65–1.53 (m, 3 H, CH, CH_2), 1.28–1.22 (m, 4H, CH_2), 1.27 (s, 3 H, CH_3), 1.15 (s, 3 H, CH_3), 0.83 (t, ^3J(H,H) = 7.0 Hz, 3 H, CH_3). HRMS (FAB+): calcd for $C_{46}H_{58}NO_{14}$ [M^+ + H] 848.3857, found 848.3864.

3′-*N*-Debenzoyl-3′-*N*-isopropyloxycarbonylpaclitaxel (18). 3′-*N*-Debenzoylpaclitaxel **14** (10.0 mg, 13.3 μmol) was dissolved in CH_2Cl_2 (0.5 mL) and a saturated solution of $NaHCO_3$ (0.5 mL) was added. Then isopropyloxycarbonyl chloride (1.7 μL, 17 μmol) was added with vigorous stirring. After 1 h, the next portion of isopropyloxycarbonyl chloride (1.7 μL, 17 μmol) was added, and after an hour, the reaction mixture was diluted with EtOAc and washed with water. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The crude product was applied to preparative HPLC, which was eluted with a linear gradient of 30%–70% CH_3CN in 12 mM aqueous HCl over 80 min at a flow rate of 5 mL/min. The desired fraction was collected and lyophilized to give a white powder of **18** (9.7 mg, 11.6 μmol, 87%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.10 (d, ^3J(H,H) = 7.3 Hz, 2 H, CH), 7.68–7.64 (m, 1 H, CH), 7.58–7.54 (m, 2 H, CH), 7.39 (d, ^3J(H,H) = 4.6 Hz, 4 H, CH), 7.28–7.24 (m, 1 H), 6.46 (s, 1 H, CH), 6.15 (t, ^3J(H,H) = 10.0 Hz, 1 H, CH), 5.64 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.15 (bs, 1H, CH), 5.00–4.97 (m, 1 H, CH), 4.81–4.75 (m, 1 H, CH), 4.52 (d, ^3J(H,H) = 4.4 Hz, 1 H, CH), 4.32 (dd, ^3J(H,H) = 6.6, 11.0 Hz, 1 H, CH), 4.18 (bs, 2 H, CH_2), 3.82 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.50–2.42 (m, 1 H, CH_2), 2.33 (s, 3 H, CH_3), 2.28–2.19 (m, 1 H, CH_2), 2.17 (s, 3 H, CH_3), 2.00–1.95 (m, 1 H, CH_2), 1.92 (s, 3 H, CH_3), 1.84–1.77 (m, 1 H, CH_2), 1.66 (s, 3 H, CH_3), 1.19 (d, ^3J(H,H) = 6.4 Hz, 6 H, CH_3), 1.17 (s, 3 H, CH_3), 1.16 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{44}H_{54}NO_{15}$ [M^+ + H] 836.3493, found 836.3502.

3′-*N*-*n*-Butyloxycarbonyl-3′-*N*-debenzoylpaclitaxel (19). The above procedure with *n*-butyloxycarbonyl chloride afforded **19** (93%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.10 (d, ^3J(H,H) = 7.5 Hz, 2 H, CH) 7.68–7.64 (m, 1 H, CH), 7.58–7.54 (m, 2 H, CH), 7.39 (d, ^3J(H,H) = 4.6 Hz, 4 H, CH), 7.28–7.24 (m, 1 H), 6.46 (s, 1 H, CH), 6.16 (bt, ^3J(H,H) = 8.5 Hz, 1 H, CH), 5.64 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 5.17 (d, ^3J(H,H) = 4.6 Hz, 1 H, CH), 4.99 (dd, ^3J(H,H) = 1.8, 9.5 Hz, 1H, CH), 4.54 (d, ^3J(H,H) = 4.0 Hz, 1H, CH), 4.33 (dd, ^3J(H,H) = 6.6, 11.0 Hz, 1 H, CH), 4.19, 4.17 (2d, ^2J(H,H) = 8.8 Hz, 2 H, CH_2), 4.05–3.94 (m, 2 H, CH, CH_2), 3.82 (d, ^3J(H,H) = 7.3 Hz, 1 H, CH), 2.50–2.42 (m, 1 H, CH_2), 2.34 (s, 3 H, CH_3), 2.29–2.18 (m, 1 H, CH_2), 2.17 (s, 3 H, CH_3), 2.03–1.97 (m, 1H, CH_2), 1.92 (s, 3 H, CH_3), 1.84–1.77 (m, 1 H, CH_2), 1.66 (s, 3 H, CH_3), 1.61–1.55 (m, 2 H, CH_2), 1.38–1.29 (m, 2H, CH_2), 1.18 (s, 3 H, CH_3), 1.16 (s, 3 H, CH_3), 0.90 (t, ^3J(H,H) = 7.2 Hz, 3 H, CH_3). HRMS (FAB+): calcd for $C_{45}H_{55}NO_{15}$ [M^+ + H] 872.3469, found 884.3477.

3′-*N*-Benzyloxycarbonyl-3′-*N*-debenzoylpaclitaxel (20). The above procedure with benzyloxycarbonyl chloride afforded

20 (95%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.10 (d, ^3J(H,H) = 9.5 Hz, 2 H, CH), 7.67−7.63 (m, 1 H, CH), 7.57−7.52 (m, 2 H, CH), 7.45−7.24 (m, 10 H), 6.44 (s, 1 H, CH), 6.15 (t, ^3J(H,H) = 8.9 Hz, 1 H, CH), 5.64 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.20 (d, ^3J(H,H) = 4.6 Hz, 1H, CH), 5.08, 5.05 (2d, ^2J(H,H) = 12.6 Hz, 2 H, CH_2), 4.98 (dd, ^3J(H,H) = 1.9, 9.6 Hz, 1H, CH), 4.55 (d, ^3J(H,H) = 4.7 Hz, 1 H, CH), 4.30 (dd, ^3J(H,H) = 6.6, 11.0 Hz, 1 H, CH), 4.19, 4.17 (2d, ^2J(H,H) = 9.2 Hz, 2 H, CH_2), 3.81 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.50−2.42 (m, 1 H, CH_2), 2.34 (s, 3 H, CH_3), 2.34−2.18 (m, 1 H, CH_2), 2.18 (s, 3 H, CH_3), 1.99−1.90 (m, 1 H, CH_2), 1.90 (s, 3 H, CH_3), 1.83−1.76 (m, 1 H, CH_2), 1.66 (s, 3 H, CH_3), 1.16 (s, 3 H, CH_3), 1.15 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{48}H_{54}NO_{15}$ [M^+ + H] 884.3493, found 884.3498.

(4*S*,5*R*)-5-Methoxycarbonyl-2-(4-methoxyphenyl)-4-phenyl-3-benzoxycarbonyl-1,3-oxazolidine (22). (2*R*,3*S*)-3-Phenylisoserine hydrochloride **21** (1.00 g, 4.59 mmol) was dissolved in anhydrous MeOH (25 mL), and $SOCl_2$ (0.50 mL, 6.89 mmol) was added dropwise at 0 °C under an argon atmosphere. The reaction mixture was stirred overnight at room temperature and then quenched with $NaHCO_3$, evaporated under reduced pressure, and diluted with H_2O. The water phase was extracted with AcOEt three times, the combined organic solution was dried over $MgSO_4$, and the solvent was removed under reduced pressure. Benzyloxycarbonyl chloride (1.3 mL, 9.18 mmol) was added dropwise at 0 °C to the resulting oil dissolved in CH_2Cl_2 (30 mL) and saturated $NaHCO_3$ (30 mL). The whole mixture was stirred vigorously at room temperature for 3 h. The desired compound was extracted with AcOEt. The organic solution was dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to yield 1.42 g of white solid. A portion of the resulting solid (360 mg) was dissolved in anhydrous toluene (30 mL) and pyridinium *p*-toluenesulfonate (PPTS) (2.7 mg, 0.011 mmol) was added. Toluene distillation was then started and 4-methoxybenzaldehyde dimethyl acetal (0.22 mL, 1.31 mmol) was added dropwise under an argon atmosphere. During 40 min of azeotropic distillation, approximately half of the solvent was removed and then the reaction mixture was allowed to cool to ambient temperature and diluted with Et_2O. The organic layer was washed with water, saturated $NaHCO_3$, water, and brine and dried over $MgSO_4$, and the solvent was removed under reduced pressure. The resulting oil was purified by silica gel column chromatography (AcOEt:hexane 1:4) to yield a product **22** (469 mg, 1.05 mmol, 96%) as a mixture of diastereomers in the ratio 17:3 (detected by ^{1}H NMR and HPLC analysis). ^{1}H NMR ($CDCl_3$, 400 MHz), major diastereomer: δ = 7.42−7.32 (m, 7 H, CH), 7.22−7.06 (m, 3 H, CH), 6.92−6.84 (m, 2 H, CH), 6.75 (d, ^3J(H,H) = 7.3 Hz, 2 H, CH), 6.42 (bs, 1 H, CH), 5.50 (bs, 1 H, CH), 4.92, 4.77 (2d, ^2J(H,H) = 12.3 Hz, 2 H, CH_2), 4.59 (d, ^3J(H,H) = 4.0 Hz, 1 H, CH), 3.82 (s, 3 H, CH_3), 3.59 (s, 3 H, CH_3); minor diastereomer: δ = 7.42−7.32 (m, 7 H, CH), 7.22−7.06 (m, 3 H, CH), 6.92−6.84 (m, 2 H, CH), 6.75 (d, ^3J(H,H) = 7.3 Hz, 2 H, CH), 6.50 (bs, 1 H, CH), 5.34 (bs, 1 H, CH), 5.09, 5.05 (2d, ^2J(H,H) = 12.4 Hz, 2 H, CH_2), 4.88 (d, ^3J(H,H) = 3.5 Hz, 1 H, CH), 3.83 (s, 3 H, CH_3), 3.59 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{26}H_{26}NO_6$ [M^+ + H] 448.1760, found 448.1763.

13-(3′-*N*-Benzyloxycarbonylphenylisoserine)-10-deacetyl-7,10-dibenzyloxycarbonylbaccatin III (24). A solution of KOH (66 mg, 1.18 mmol) in water (10 mL) was added slowly at room temperature to a stirring solution of **22** (440 mg, 0.983 mmol) in MeOH (30 mL). The reaction mixture was stirred for 2 h and then MeOH was evaporated under reduced pressure. The residual mixture was diluted with water, washed with Et_2O, acidified with 1 N HCl, and extracted with AcOEt. This organic phase was dried over $MgSO_4$ and evaporated under reduced pressure. The resulting oil, 10-deacetyl-7,10-dibenzyloxycarbonylbaccatin III (399 mg, 0.491 mmol), and DMAP (12 mg, 0.098 mmol) were dissolved in anhydrous CH_2Cl_2 (20 mL), DCC was added, and the reaction mixture was stirred for 2 h at room temperature under an argon atmosphere. The reaction mixture was diluted with AcOEt and washed with saturated NH_4Cl, water, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was dissolved in MeOH (25 mL) and a solution of *p*-toluenesulfonic acid (112 mg, 0.589 mmol) in MeOH (10 mL) was added. After stirring for 5 h at room temperature, the reaction mixture was diluted with AcOEt and washed three times with saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was purified by silica gel column chromatography (AcOEt:hexane 2:3), and unreacted minor diastereomer was discarded. The desired product was dissolved in a few milliliters of CH_3CN, the remaining dicyclohexylurea was filtered off, and solution was lyophilized to give a white powder of **24** (438 mg, 0.395 mmol, 80%). ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.10 (d, ^3J(H,H) = 7.7 Hz, 2 H, CH), 7.67−62 (m, 1 H, CH), 7.57−7.53 (m, 2 H, CH), 7.42−7.22 (m, 20 H, CH), 6.24 (s, 1 H, CH), 6.17 (t, ^3J(H,H) = 8.7 Hz, 1H, CH), 5.65 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.52 (dd, ^3J(H,H) = 7.2, 10.5 Hz, 1 H, CH), 5.22 (d, ^3J(H,H) = 4.6 Hz, 1 H, CH), 5.20−4.99 (m, 7 H, CH, CH_2), 4.57 (d, ^3J(H,H) = 4.8 Hz, 1 H, CH), 4.20, 4.18 (2d, ^2J(H,H) = 9.2 Hz, 2 H, CH_2), 3.90 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 2.60−2.52 (m, 1 H, CH_2), 2.36 (s, 3 H, CH_3), 2.27−2.21 (m, 1 H, CH_2), 2.05−1.99 (m, 1 H, CH_2), 1.93 (s, 3 H, CH_3), 1.90−1.83 (m, 1 H, CH_2), 1.79 (s, 3 H, CH_3), 1.82 (s, 3 H, CH_3), 1.16 (s, 3 H, CH_3), 1.11 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{62}H_{64}NO_{18}$ [M^+ + H] 1111.4123, found 1111.4127.

13-(3′-*N*-Benzyloxycarbonyl-2′-*tert*-butoxycarbonylphenylisoserine)-10-deacetyl-7,10-dibenzyloxycarbonylbaccatin III (25). To a solution of **24** (200 mg, 0.180 mmol) were added dry CH_2Cl_2 (1 mL) and pyridine (3 mL), DMAP (4.4 mg, 0.036 mmol), and then Boc_2O (59 mg, 0.27 mmol) in dry CH_2Cl_2 (2 mL) at 0 °C under an argon atmosphere. The mixture was stirred at room temperature overnight. The reaction mixture was diluted with AcOEt and successively washed with 10% citric acid three times, water, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was purified by silica gel column chromatography (AcOEt:hexane 1:2) to yield a white solid **25** (182 mg, 0.150 mmol, 84%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 8.12−8.10 (m, 2 H, CH), 7.64−7.60 (m, 1 H, CH), 7.53−7.50 (m, 2 H, CH), 7.43−7.20 (m, 20 H, CH), 6.27 (s, 1 H, CH), 6.23 (bt, ^3J(H,H) = 8.9 Hz, 1H, CH), 5.71 (bd, 1 H, CH, partially overlapping with next signal), 5.68 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.56 (dd, ^3J(H,H) = 7.0, 10.7 Hz, 1 H, CH), 5.47 (bd, ^3J(H,H) = 9.0 Hz, 1 H, CH), 5.28 (bs, 1 H, NH), 5.21, 5.16 (2d, ^2J(H,H) = 11.9 Hz, 2 H, CH_2), 5.14 (s, 2 H, CH_2), 5.07, 5.00 (2d, ^2J(H,H) = 12.5 Hz, 2 H, CH_2), 4.95 (d, ^3J(H,H) = 7.9 Hz, 1H, CH), 4.32, 4.18 (2d, ^2J(H,H) = 8.5 Hz, 2 H, CH_2), 3.93 (d, ^3J(H,H) = 7.0 Hz, 1 H, CH), 2.64−2.56 (m, 1 H, CH_2), 2.43 (s, 3 H, CH_3), 2.36−2.28 (m, 1 H, CH_2), 2.05 (s, 3 H, CH_3), 2.01−1.94 (m, 2 H, CH_2), 1.83 (s, 3 H, CH_3), 1.44 (s, 9 H, CH_3), 1.21 (s, 3 H, CH_3), 1.17 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{67}H_{72}NO_{20}$ [M^+ + H] 1210.4648, found 1210.4642.

2′-*O*-*tert*-Butoxycarbonyl-3′-*N*-de(*tert*-butoxycarbonyl)docetaxel Hydrochloride (5). Pd/C was added (16 mg) to the stirring solution of **25** (31 mg, 0.0256 mmol) in EtOAc (1 mL), and the reaction mixture was vigorously stirred for 8 h at room temperature under a hydrogen atmosphere. The catalyst was filtered off and 1 equiv of 0.1 M HCl in methanol (260 μL, 0.026 mmol) was added. The solvent was removed under reduced pressure to give a white powder of **5** as a HCl salt (21.4 mg, 0.0253 mmol, 99%). The product was pure, required no purification, and remained stable under refrigeration in solid form for at least 2 weeks. ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.08−8.06 (m, 2 H, CH), 7.74−7.70 (m, 1 H, CH), 7.64−7.60 (m, 2 H, CH), 7.57−7.50 (m, 4 H), 7.39−7.34 (m, 1 H, CH), 5.98 (dt, ^4J(H,H) = 1.1 Hz, ^3J(H,H) = 9.2 Hz, 1 H, CH), 5.59 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.28 (d, ^3J(H,H) = 8.8 Hz, 1H, CH), 5.22 (s, 1 H, CH), 4.97 (dd, ^3J(H,H) = 2.0, 9.7 Hz, 1H, CH), 4.76 (d, ^3J(H,H) = 8.8 Hz, 1 H, CH), 4.22−4.14 (m, 3 H, CH, CH_2), 3.78 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.47−2.39 (m, 1 H, CH_2), 2.29 (s, 3 H, CH_3), 1.93 (dd, ^3J(H,H)

= 9.5 Hz, ^2J(H,H) = 15.4 Hz, 1 H, CH_2), 1.88−1.77 (m, 1 H, CH_2), 1.84 (d, ^4J(H,H) = 1.3 Hz, 3 H, CH_3), 1.66 (s, 3 H, CH_3), 1.55−1.47 (m, 1 H, CH_2), 1.52 (s, 9 H, CH_3), 1.11 (s, 3 H, CH_3), 1.09 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{43}H_{54}NO_{14}$ [M^+ + H] 808.3544, found 808.3550. Anal. ($C_{43}H_{54}ClNO_{14}\cdot 3H_2O$) C, H, N.

3′-*N*-De(*tert*-butoxycarbonyl)docetaxel Hydrochloride (26). Pd/C was added (16 mg) to the stirring solution of **24** (31 mg, 0.0279 mmol) in EtOAc (1 mL), and the reaction mixture was vigorously stirred for 8 h at room temperature under a hydrogen atmosphere. The catalyst was filtered off and 0.1 M HCl in methanol (280 μL, 0.028 mmol) was added. The solvent was removed under reduced pressure to give **26** as the HCl salt, a white powder (20.6 mg, 0.0277 mmol, 99%). The product was pure and required no purification. ^{1}H NMR (CD_3OD, 400 MHz): δ = 8.05−8.02 (m, 2 H, CH), 7.73−7.68 (m, 1 H, CH), 7.62−7.50 (m, 6 H, CH), 7.44−7.39 (m, 1 H, CH), 6.07 (dt, ^4J(H,H) = 1.3 Hz, ^3J(H,H) = 9.0 Hz, 1 H, CH), 5.59 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 5.23 (s, 1 H, CH), 4.97 (dd, ^3J(H,H) = 2.1, 9.6 Hz, 1H, CH), 4.58 (d, ^3J(H,H) = 8.6 Hz, 1H, CH), 4.55 (d, ^3J(H,H) = 8.4 Hz, 1 H, CH), 4.22−4.14 (m, 3 H, CH, CH_2), 3.80 (d, ^3J(H,H) = 7.1 Hz, 1 H, CH), 2.46−2.39 (m, 1 H, CH_2), 2.21 (s, 3 H, CH_3), 1.96 (dd, ^3J(H,H) = 9.2 Hz, ^2J(H,H) = 15.2 Hz, 1 H, CH_2), 1.87 (d, ^4J(H,H) = 1.5 Hz, 3 H, CH_3), 1.85−1.78 (m, 1 H, CH_2), 1.72 (dd, ^3J(H,H) = 9.0 Hz, ^2J(H,H) = 15.2 Hz, 1 H, CH_2), 1.67 (s, 3 H, CH_3), 1.12 (s, 3 H, CH_3), 1.09 (s, 3 H, CH_3). HRMS (FAB+): calcd for $C_{38}H_{46}NO_{12}$ [M^+ + H] 708.3020, found 708.3024 Anal. ($C_{38}H_{46}ClNO_{12}\cdot 2H_2O$) C, H, N.

Water Solubility. The parent drugs (**2**, **15**−**20**) and the prodrugs (**5**, **8**−**13**) were saturated in distilled water and shaken vigorously. The saturated solutions were sonicated for 15 min at 25 °C and passed through a centrifugal filter (0.45 μm filter unit, Ultrafree-MC, Millipore). The filtrate was analyzed using RP-HPLC.

Stability Studies of 8−13 in PBS Buffer. The conversion profiles of **5**, **8**−**13** were determined in phosphate-buffered saline (PBS, pH 7.4). To 990 μL of PBS (pH 7.4) was added 5 μL of DMSO and 5 μL of solution including **5**, **8**−**13** (1 mM in DMSO), and the mixture was incubated at 37 °C. At the desired time points, 1 mL of MeOH was added to the samples to dissolve parent drugs completely, and 1 mL of the mixture was directly analyzed by RP-HPLC. In the case of **12**, PBS (pH 4.9) and HCl/glycine-buffered saline (pH 2.0) were also employed to evaluate the conversion profiles. HPLC was performed using a C18 (4.6 × 150 mm; YMC Pack ODS AM302) reverse phase column with a binary solvent system: linear gradient of CH_3CN (0−100%, 40 min) in 0.1% aqueous TFA at a flow rate of 0.9 mL/min, UV detection at 230 nm.

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References

(1) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J. Am. Chem. Soc.* **1971**, *93*, 2325−2327.

(2) Gueritte-Voegelein, F.; Guenard, D.; Lavelle, F.; Le Goff, M. T.; Mangatal, L.; Potier, P. Relationships between the structure of taxol analogs and their antimitotic activity. *J. Med. Chem.* **1991**, *34*, 992−998.

(3) Mekhail, T. M.; Markman, M. Paclitaxel in cancer therapy. *Expert Opin. Pharmacother.* **2002**, *3*, 755−766.

(4) Ferlini, C.; Ojima, I.; Distefano, M.; Gallo, D.; Riva, A.; Morazzoni, P.; Bombardelli, E.; Mancuso, S.; Scambia, G. Second generation taxanes: From the natural framework to the challenge of drug resistance. *Curr. Med. Chem. Anti-Cancer Agents* **2003**, *3*, 133−138.

(5) Nabholtz, J.-M.; Vannetzel, J.-M.; Llory, J.-F.; Bouffette, P. Advances in the use of taxanes in the adjuvant therapy of breast cancer. *Clin. Breast Cancer* **2003**, *4*, 187−192.

(6) Singla, A. K.; Garg, A.; Aggarwal, D. Paclitaxel and its formulations. *Int. J. Pharmaceutics* **2002**, *235*, 179−192.

(7) Ng, S. S. W.; Figg, W. D.; Sparreboom A. Taxane-mediated antiangiogenesis in vitro: Influence of formulation vehicles and binding proteins. *Cancer Res.* **2004**, *64*, 821−824.

(8) Ter Haar, E. Taxanes and other microtubule stabilizing agents. *Exp. Opin. Ther. Patents* **1998**, *8*, 571−586.

(9) Lin, S.; Ojima, I. Recent strategies in the development of taxane anticancer drugs. *Exp. Opin. Ther. Patents* **2000**, *10*, 869−889.

(10) Dubois, J.; Guenard, D.; Gueritte, F. Recent developments in antitumour taxoids. *Exp. Opin. Ther. Patents* **2003**, *13*, 1809−1823.

(11) Cassinelli, G.; Lanzi, C.; Supino, R.; Pratesi, G. Zuco, V.; Laccabue, D.; Cuccuru, G.; Bombardelli, E.; Zunino, F. Cellular bases of the antitumor activity of the novel taxane IDN 5109 (BAY59−8862) on hormone-refractory prostate cancer. *Clin. Cancer Res.* **2002**, *8*, 2647−2654.

(12) Shionoya, M.; Jimbo, T.; Kitagawa, M.; Soga, T.; Tohgo, A. DJ-927, a novel oral taxane, overcomes P-glycoprotein-mediated multidrug resistance in vitro and in vivo. *Cancer Sci.* **2003**, *94*, 459−466.

(13) Sun, W.; Stevenson, J. P.; Gallagher, M. L.; Vaughn, D.; Hahn, S. M.; Haller, D. G.; Cohen, M.; Kopit, J.; Gallant, G.; O'Dwyer, P. J. Phase I and pharmacokinetic trial of the novel taxane BMS-184476 administered as a 1-hour intravenous infusion in combination with cisplatin every 21 days. *Clin. Cancer Res.* **2003**, *9*, 5221−5227.

(14) Advani, R.; Fisher, G. A.; Lum, B. L.; Jambalos, C.; Cho, C. D.; Cohen, M.; Gollerkeri, A.; Sikic, B. I. Phase I and pharmacokinetic study of BMS-188797, a new taxane analog, administered on a weekly schedule in patients with advanced malignancies. *Clin. Cancer Res.* **2003**, *9*, 5187−5194.

(15) Ojima, I.; Geney, R. 109881(Aventis). *Curr. Opin. Investig. Drugs* **2003**, *4*, 737−740.

(16) Nicolau, K. C.; Riemer, C.; Kerr, M. A.; Rideout, D.; Wrasidlo, W. Design, synthesis and biological activity of protaxols. *Nature* **1993**, *364*, 464−466.

(17) Greenwald, R. B.; Gilbert, C. W.; Pendri, A.; Conover, C. D.; Xia, J.; Martinez, A. Drug delivery systems: Water soluble Taxol 2′-poly(ethylene glycol) ester prodrugs-design and in vivo effectiveness. *J. Med. Chem.* **1996**, *39*, 424−31.

(18) Khmelnitsky, Y. L.; Budde, C.; Arnold, M. J.; Usyatinsky, A.; Clark, D. S.; Dordick, J. S. Synthesis of water-soluble paclitaxel derivatives by enzymatic acylation. *J. Am. Chem. Soc.* **1997**, *119*, 11554−11555.

(19) de Groot, F. M. H.; van Berkom, L. W. A.; Scheeren, H. W. Synthesis and biological evaluation of 2′-carbamate-linked and 2′-carbonate-linked prodrugs of paclitaxel. Selective activation by the tumor-associated protease plasmin. *J. Med. Chem.* **2000**, *43*, 3093−3102.

(20) Seligson, A. L.; Terry, R. C.; Bressi, J. C.; Douglass III, J. G.; Sovak, M. A new prodrug of paclitaxel: Synthesis of Protaxel. *Anti-cancer Drugs* **2001**, *12*, 305−313.

(21) Niethammer, A.; Gaedicke, G.; Lode, H. N.; Wrasidlo, W. Synthesis and preclinical characterization of a paclitaxel prodrug with improved antitumor activity and water solubility. *Bioconjugate Chem.* **2001**, *12*, 414−420.

(22) Wrasidlo, W.; Gaedicke, G.; Guy, R. K. Renaud, J.; Pitsinos, E.; Nicolaou, K. C.; Reisfeld, R. A.; Lode, H. N. A Novel 2′-(*N*-methylpyridinium acetate) prodrug of paclitaxel induces superior antitumor responses in preclinical cancer models. *Bioconjugate Chem.* **2002**, *13*, 1093−1099.

(23) Kirschberg, T. A.; VanDeusen, C. L.; Rothbard, J. B.; Yang, M.l; Wender, P. A. Arginine-based molecular transporters: The synthesis and chemical evaluation of releasable taxol-transporter conjugates. *Org. Lett.* **2003**, *5*, 3459−3462.

(24) Rodrigues, P. C. A.; Scheuermann, K.; Stockmar, C.; Maier, G.; Fiebig, H. H.; Unger, C.; Muelhaupt, R. Kratz, F. Synthesis and in vitro efficacy of acid-sensitive poly(ethylene glycol) paclitaxel conjugates. *Bioorg. Med. Chem.* **2003**, *13*, 355−360.

(25) de Groot, F. M. H.; Albrecht, C.; Koekkoek, R.; Beusker, P. H.; Scheeren, H. W. "Cascade-release dendrimers" liberate all end groups upon a single triggering event in the dendritic core. *Angew. Chem., Int. Ed. Engl.* **2003**, *42*, 4490−4494.

(26) Liu, C.; Strobl, J. S.; Bane, S.; Schilling, J. K.; McCracken, M.; Chatterjee, S. K.; Rahim-Bata, R.; Kingston, D. G. I. Design, synthesis, and bioactivities of steroid-linked Taxol analogues as potential targeted drugs for prostate and breast cancer. *J. Nat. Prod.* **2004**, *67*, 152−159.

(27) Meerum Terwogt, J. M.; ten Bokkel Huinink, W. W.; Schellens, J. H. M.; Schot, M.; Mandjes, I. A. M.; Zurlo, M. G.; Rocchetti, M.; Rosing, H.; Koopman, F. J.; Beijnen, J. H. Phase I clinical and pharmocokinetic study of PNU166945, a novel water soluble polymer-conjugated prodrug of paclitaxel. *Anti-Cancer Drugs* 2001, *12*, 315–323.
(28) Wrasidlo, W.; Niethammer, A.; Deger, S.; Sehouli, J.; Kulozik, A.; Geilen, W.; Henze, G.; Gaedicke, G.; Lode, H. N. Pilot study of hydrolytically activated paclitaxel prodrug therapy in patients with progressive malignancies. *Curr. Ther. Res.* 2002, *63*, 247–262.
(29) Singer, J. W.; Baker B.; De Vries P.; Kumar A.; Shaffer S.; Vawter E.; Bolton M.; Garzone P. Poly-(L)-glutamic acid-paclitaxel (CT-2103) [XYOTAX], a biodegradable polymeric drug conjugate: Characterization, preclinical pharmacology, and preliminary clinical data. *Adv. Exp. Med. Biol.* 2003, *519*, 81–99.
(30) Sparreboom, A.; Wolff, A. C.; Verweij, J.; Zabelina, Y.; van Zomeren, D. M.; McIntire, G. L.; Swindell, C. S.; Donehower, R. C.; Baker, S. D. Disposition of docosahexaenoic acid-paclitaxel, a novel taxane, in blood: In vitro and clinical pharmacokinetic studies. *Clin. Cancer Res.* 2003, *9*, 151–159.
(31) Wolff, A. C.; Donehower, R. C.; Carducci, M. K.; Carducci, M. A.; Brahmer, J. R.; Zabelina, Y.; Bradley, M. O.; Anthony, F. H.; Swindell, C. S.; Witman, P. A.; Webb, N. L.; Baker, S. D. Phase I study of docosahexaenoic acid-paclitaxel: A taxane-fatty acid conjugate with a unique pharmacology and toxicity profile. *Clin. Cancer Res.* 2003, *9*, 3589–3597.
(32) Ettmayer, P.; Amidon, G. L.; Clement, B.; Testa, B. Lessons learned from marketed and investigational prodrugs. *J. Med. Chem.* 2004, *47*, 2393–2404.
(33) Kimura, T.; Ohtake, J.; Nakata, S.; Enomoto, H.; Moriwaki, H.; Akaji, K.; Kiso, Y. Synthesis of prodrug of HIV protease inhibitors. In *Peptide Chemistry 1994*, Ohno, M., Ed.; Protein Research Foundation: Osaka, 1995; Vol. 32, pp 157–160.
(34) Kiso, Y.; Kimura, T.; Ohtake, J.; Nakata, S.; Enomoto, H.; Moriwaki, H.; Nakatani, M.; Akaji, K. "*O*→*N* Intramolecular acyl migration"-type prodrugs of tripeptide inhibitors of HIV protease. *Peptides: Chemistry, Structure and Biology (Proceedings of the Fourteenth American Peptide Symposium)*; Mayflower Scientific: England, 1996; pp 157–159.
(35) Yamaguchi, S.; Mitoguchi, T.; Nakata, S.; Kimura, T.; Akaji, K.; Nojima, S.; Takaku, H.; Mimoto, T.; Kiso, Y. Synthesis of HIV protease dipeptide inhibitors and prodrugs. In *Peptide Chemistry 1996*; Kitada, C., Ed.; Protein Research Foundation: Osaka, 1997; Vol. 34, pp 297–300.
(36) Kiso, Y., Matsumoto, H.; Yamaguchi, S.; Kimura, T. Design of small peptidomimetic HIV-1 protease inhibitors and prodrug form. *Lett. Pept. Sci.* 1999, *6*, 275–281.
(37) Kiso, Y.; Yamaguchi, S.; Matsumoto, H.; Kimura, T.; Akaji, K. "*O,N*-Acyl migration"-type prodrug of dipeptide HIV protease inhibitors. *Peptides: Frontiers of peptide science;* Kluwer Academic Publishers: The Netherlands, 1999; pp 678–679.
(38) Hamada, Y.; Ohtake, J.; Sohma, Y.; Kimura, T.; Hayashi, Y.; Kiso, Y. New water-soluble prodrugs of HIV protease inhibitors based on *O*→*N* intramolecular acyl migration. *Bioorg. Med. Chem.* 2002, *10*, 4155–4167.
(39) Hamada, Y.; Matsumoto, H.; Kimura, T.; Hayashi, Y.; Kiso, Y. Effect of the acyl groups on *O*→*N* acyl migration in the water-soluble prodrugs of HIV-1 protease inhibitor. *Bioorg. Med. Chem. Lett.* 2003, *13*, 2727–2730.
(40) Hamada, Y.; Matsumoto, H.; Yamaguchi, S.; Kimura, T.; Hayashi, Y.; Kiso, Y. Water-soluble prodrugs of dipeptide HIV protease inhibitors based on *O*→*N* intramolecular acyl migration: Design, synthesis and kinetic study. *Bioorg. Med. Chem.* 2004, *12*, 159–170.
(41) Hayashi, Y.; Skwarczynski, M.; Hamada, Y.; Sohma, Y.; Kimura, T.; Kiso, Y. A Novel approach of water-soluble paclitaxel prodrug with no auxiliary and no byproduct: Design and synthesis of isotaxel. *J. Med. Chem.* 2003, *46*, 3782–3784.
(42) Skwarczynski, M.; Sohma, Y.; Kimura, M.; Hayashi, Y.; Kimura, T.; Kiso, Y. O–N Intramolecular acyl migration strategy in water-soluble prodrugs of taxoids. *Bioorg. Med. Chem. Lett.* 2003, *13*, 4441–4444.
(43) Sohma, Y.; Hayashi, Y.; Skwarczynski, M.; Hamada, Y.; Sasaki, M.; Kimura, T.; Kiso, Y. O–N Intramolecular acyl migration reaction in the development of prodrugs and the synthesis of difficult sequence-containing bioactive peptides. *Biopolymers* 2004, *76*, 344–356.
(44) Didier, E.; Fouque, E.; Taillepied, I.; Commerçon, A. 2-Monosubstituted-1,3-oxazolidines as improved protective groups of *N*-Boc-phenylisoserine in docetaxel preparation. *Tetrahedron Lett.* 1994, *35*, 2349–2352.
(45) Sisti, N. J.; Brinkman, H. R.; McChesney, J. D.; Chander, M. C.; Liang, X.; Zygmunt, J. Methods and useful intermediates for paclitaxel synthesis from C-7, C-10 di-Cbz 10-deacetylbaccatin III. US patent, 6,448,417, 2002.
(46) Stewart J. M. Protection of the hydroxyl group in peptide synthesis. in *The Peptides*; Gross, E., Meienhofer, J., Eds.; Academic Press: New York, 1981; Vol. 3, pp 170–201.
(47) Roh, E. J.; Kim, D.; Lee, C. O.; Choi, S. U.; Song, C. E. Structure–activity relationship study at the 3′-*N*-position of paclitaxel: Synthesis and biological evaluation of 3′-*N*-acyl-paclitaxel analogues. *Bioorg. Med. Chem.* 2002, *10*, 3145–3151.
(48) Roh, E. J.; Kim, D.; Choi, J. Y.; Lee, B.-S.; Lee, C. O.; Song, C. E. Synthesis, biological activity and receptor-based 3-D QSAR study of 3′-*N*-substituted-3′-*N*-debenzoylpaclitaxel analogues. *Bioorg. Med. Chem.* 2002, *10*, 3135–3143.
(49) Kobayashi, J. I.; Hosoyama, H. Wang, X.; Shigemori, H.; Koiso, Y.; Iwasaki, S.; Sasaki, T.; Naito, M.; Tsuruo, T. Effects of taxoids from Taxus cuspidata on microtubule depolymerization and vincristine accumulation in MDR cells. *Bioorg. Med. Chem. Lett.* 1997, *7*, 393–398.
(50) Zamir, L.; Caron, G.; Zheng, Y. F. US patent, 6,410,756, 1997; *Chem. Abstr.* 1998, *128*, 321780.
(51) Baloglu, E.; Hoch, J. M.; Chatterjee, S. K.; Ravindra, R.; Bane, S. Kingston, D. G. I. Synthesis and biological evaluation of C-3′NH/C-10 and C-2/C-10 modified paclitaxel analogues. *Bioorg. Med. Chem.* 2003, *11*, 1557–1568.
(52) Swindell, C. S.; Heerding, J. M.; Krauss, N. E.; Horwitz, S. B.; Ringel, I. Characterization of the Taxol structure–activity profile for the locus of the A-ring side chain. *Bioorg. Med. Chem. Lett.* 1994, *4*, 1531–1536.
(53) Stella, V. J. Prodrug as therapeutics. *Exp. Opin. Ther. Patents* 2004, *14*, 277–280.
(54) Brass, E. P. Pivalate-generating prodrugs and carnitine homeostasis in man. *Pharmacol. Rev.* 2002, *54*, 589–598.
(55) Luer, M. S. Fosphentoin. *Neurol. Res.* 1998, *20*, 178–182.
(56) Zhu, Q.; Guo, Z.; Huang, N.; Wang, M.; Chu, F. Comparative molecular field analysis of a series of paclitaxel analogues. *J. Med. Chem.* 1997, *40*, 4319–4328.
(57) Kingston, D. G. I. Recent advances in the chemistry of Taxol. *J. Nat. Prod.* 2000, *63*, 726–734.
(58) Kazmierski, W. M.; Bevans, P.; Furfine, E.; Spaltenstein, A.; Yang, H. Novel prodrug approach to Amprenavir-based HIV-1 protease inhibitors via *O*→*N* acyloxy migration of P1 moiety. *Bioorg. Med. Chem. Lett.* 2003, *13*, 2523–2526.
(59) Maring, C. J.; Grampovnik, D. J.; Yeung, C. M.; Klein, L. L.; Li, L.; Thomas, S. A.; Plattner, J. J. C-3′-*N*-acyl analogs of 9(R)-dihydrotaxol: Synthesis and structure–activity relationships. *Bioorg. Med. Chem. Lett.* 1994, *4*, 1429–32.
(60) Mastalerz, H.; Cook, D.; Fairchild, C. R.; Hansel, S.; Johnson, W.; Kadow, J. F.; Long, B. H.; Rose, W. C.; Tarrant, J.; Wu, M.-J.; Xue, M. Q. Zhang, G.; Zoeckler, M.; Vyas, D. M. The discovery of BMS-275183: An orally efficacious novel taxane. *Bioorg. Med. Chem.* 2003, *11*, 4315–4323.

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Original article

Establishment of a biological assay system for human retroviral protease activity

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Abstract

In order to obtain indicator cell lines that are exquisitely susceptible to human T-lymphotropic virus type 1 (HTLV-1), luciferase gene driven by HTLV-1 long terminal repeat (LTR) was transfected into lymphocytic H9 cells with *neo* gene, and cell lines were selected by G418. A cell line (H9/K30*luc*) was found to produce an extremely high level of luciferase only when co-cultured with HTLV-1 producer MT-2 cells. Both in the absence and presence of a reverse transcriptase (RT) inhibitor azidothymidine, H9/K30*luc* cells generated similarly high luciferase activity upon co-cultivation with MT-2 cells. To develop an equivalent system for human immunodeficiency virus type 1 (HIV-1), H9/NL432 cells, which are stably infected with HIV-1 and producing a low level of the virus-like MT-2 cells for HTLV-1, were generated. Together with the indicator cell line H9/H1*luc* for HIV-1 already reported, antiviral effects of some agents on HTLV-1 and HIV-1 could be readily and sensitively evaluated by similar methods. In fact, by using our system, an HIV-1 protease inhibitor, saquinavir, was demonstrated to be highly effective against HIV-1 but not against HTLV-1.
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Keywords: HTLV-1; HIV-1; Luciferase; Retroviral protease

1. Introduction

Human T-lymphotropic virus type 1 (HTLV-1), the first well-characterized human retrovirus, causes adult T cell leukemia/lymphoma (ATL) and is associated with several lymphocyte-mediated disorders such as HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1–4]. ATL is the result of a clonal outgrowth of a CD4-positve T cell that contains integrated, and frequently defective HTLV-1 DNA. HAM/TSP is linked with the immune system-mediated destruction of cells in the spinal cord. Although there is no evidence that expression of the HTLV-1 genome is a significant feature of symptomatic ATL, many studies have strongly suggested that HAM/TSP involves enhanced virus replication as shown by increases in the blood and cerebral spinal fluid of anti-HTLV-1-antibodies, of activated T cells, and of proviral DNA [5–13]. HAM/TSP, therefore, can be treated effectively by reducing the level of replicating HTLV-1 in infected individuals.

Quantitative monitoring of virus infectivity is prerequisite for various basic and clinical studies on viruses. The growth property of HTLV-1 is now difficult to follow in contrast to that of human immunodeficiency virus type 1 (HIV-1), for which various useful assay systems are available [14]. The lack of an eminent quantitative method for HTLV-1 infectivity would be mainly because it grows in cells much more poorly than HIV-1 [15,16], and hampers the systematic analytical study on HTLV-1. We have recently established new indicator cell lines for HIV-1, and have successfully used them to characterize various clones of HIV and simian immunodeficiency virus (SIV) ([14] and unpublished results). By the same strategy, we have established an indicator lymphocytic cell line for HTLV-1 replication carrying luciferase gene as reporter in this study. Our results described here indicated that simply by co-culturing the indicator cells and virus-

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producing cells and by monitoring luciferase activity in the co-cultures, some potential antiviral agents against HTLV-1 and HIV-1 can be examined for their effects.

2. Materials and methods

2.1. Cell cultures, cell viability, and transfection

Cell lines designated 293T [17], H9 [18], H9/H1*luc* [14], MT-2 [19], MT-4 [19], and M8166 [20] were cultured as previously described [21]. An indicator cell line for HTLV-1 designated H9/K30*luc* was established and maintained as reported for the HIV-1 indicator cell line H9/H1*luc* [14]. H9/NL432 cells, which are persistently infected with HIV-1 and producing stably a low level of the virus, were generated by electroporation [21] of an infectious HIV-1 DNA clone pNL432 [21] into H9 cells and culturing them for several months. Cell viability was monitored by the Cell Counting Kit-8 (Wako Pure Chemical Industries Ltd., Osaka, Japan). For transfection of 293T cells, the calcium-phosphate coprecipitation method was used as previously described [21].

2.2. Luciferase and reverse transcriptase (RT) assays

Luciferase activity was determined by the Luciferase Assay System (Promega Co., Madison, WI, USA). RT activity was measured as previously described [22].

2.3. DNA constructs

Expression vectors for the *neo* gene designated pRVSV-neo [23] and HTLV-1 Tax designated pCG-Tax [24] have been previously described. A full-length molecular clone of HTLV-1 designated pK30 was obtained through NIH AIDS Research and Reference Reagent Program (catalog no. 2817). A luciferase reporter vector designated pK30*luc* was constructed by insertion of polymerase chain reaction-amplified entire long terminal repeat (LTR) of pK30 into the *Xho*I and *Hin*dIII sites of pGL3-Basic Vector (Promega Co.).

3. Results

3.1. Establishment and characterization of luciferase system for HTLV-1 infection

Sensitive and quantitative methods to determine HTLV-1 infectivity were currently unavailable. In order to establish indicator cell lines to monitor HTLV-1 infection easily and rapidly, we constructed a reporter clone carrying luciferase gene under the control of HTLV-1 LTR. The resultant construct pK30*luc* was co-transfected with an HTLV-1 Tax expression vector pCG-Tax into 293T cells, and the production level of luciferase was determined. As shown in Fig. 1A, pK30*luc* directed the synthesis of luciferase at a highly enhanced level in response to HTLV-1 Tax. Stable indicator H9 cell lines were selected by co-electroporation of pK30*luc* and pRVSVneo (approximately 10:1 molar ratio) followed by culturing in the presence of G418 (1 mg/ml). As shown in Fig. 1B, out of six clones obtained, clone no.1 generated a high level of luciferase upon co-cultivation with HTLV-1 producer MT-2 cells [19], and was designated H9/K30*luc*. The indicator cell line for HIV-1 designated H9/H1*luc* [14] responded poorly to co-cultivation with MT-2 cells (Fig. 2), which was consistent with the results previously reported [25,26].

We determined whether the observed activation of H9/K30*luc* cells by MT-2 cells can be caused by cell-free HTLV-1 and by newly synthesized HTLV-1 Tax after co-culture. Cell-free virus samples were prepared from various cell cultures including HTLV-1-positive (MT-2), HTLV-1 DNA-positive (MT-4 and M8166), and HTLV-1-negative (H9) cell lines, and inoculated into H9/K30*luc* to monitor luciferase production. As shown in Fig. 3, no evidence for cell-free HTLV-1 infection was obtained. We then examined the effect of azidothymidine (AZT) on the production of luciferase upon co-cultivation of H9/K30*luc* and MT-2 cells. The two cell lines were co-cultured for 48 h in the presence of AZT at various concentrations, and the luciferase activity expressed in the cultures was assayed. As shown in Fig. 4, no

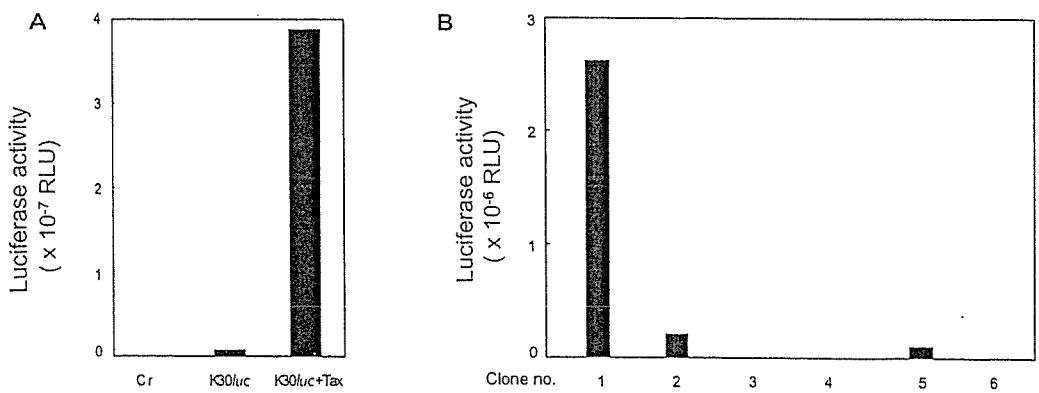

Fig. 1. Activation of HTLV-1 (K30) LTR. (A) Response of the K30*luc* reporter construct to HTLV-1 Tax. 293T cells were co-transfected with pK30*luc* (10 μg) and pUC19 (10 μg) or with pK30*luc* (10 μg) and pCG-Tax (10 μg) as indicated, and 2 days later, cell lysates were prepared for luciferase assay. Control (Cr) 293T cells were singly transfected with 20 μg of pUC19. RLU, relative light unit. (B) Luciferase production in co-cultures of HTLV-1 producer MT-2 cells and H9 cell clones harboring pK30*luc*. G418-resistant H9 cell clones (5×10^5), which had been obtained as described in the text, were co-cultured with MT-2 cells (5×10^5), and on the next day, cell lysates were prepared for luciferase assay.

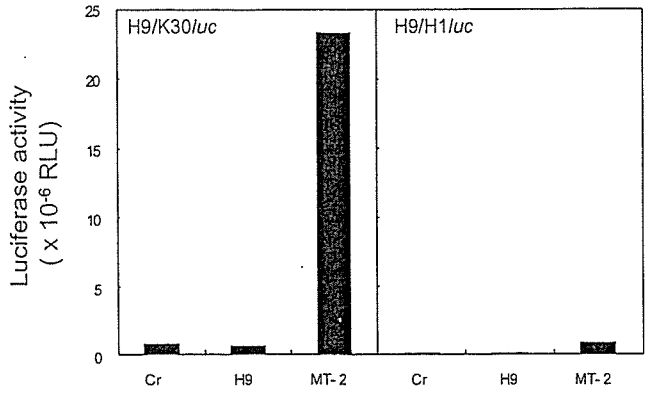

Fig. 2. Enhancement of luciferase production in co-cultures of indicator and MT-2 cells. Indicator cell lines H9/K30*luc* for HTLV-1 (this paper) and H9/H1*luc* for HIV-1 [14] (10^6) were co-cultured with H9 or HTLV-1 producer MT-2 cells (10^6), and 2 days later, cell lysates were prepared for luciferase assay. Cultures of indicator cells only served as Cr.

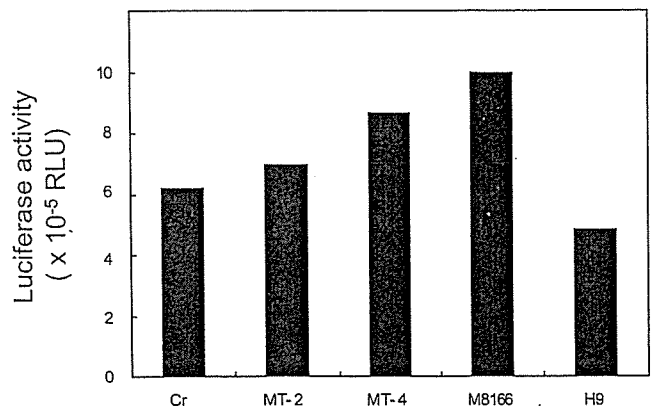

Fig. 3. Potential of cell-free virus from MT-2 to augment luciferase production in H9/K30*luc* cells. Cell-free culture supernatants were prepared from various cultures (MT-2, MT-4, M8166, and H9) maintained at growing phase for 2 days, and inoculated into the indicator cell line H9/K30*luc*. On the next day, cell lysates were prepared for luciferase assay. Culture of indicator cells only served as Cr. Cell lines MT-2 [19] and H9 [18] are HTLV-1-positive and -negative, respectively. Cell lines MT-4 [19] and M8166 [20] are HTLV-1 DNA-positive but negative for HTLV-1.

significant difference was observed among co-cultures of H9/K30*luc* and MT-2 cells.

3.2. Effects of saquinavir (SQV) on HTLV-1 and HIV-1 as determined by our luciferase system

Based on the results described above, we assumed that Tax transported from MT-2 to H9/K30*luc* cells by Env-mediated membrane fusion enhances the luciferase production, and that, if this process is suppressed, luciferase production is significantly reduced. It has been reported for HIV-1 recently that interactions between unprocessed Gag and the cytoplasmic tail of Env-gp41 suppress cell fusion [27]. We, therefore, checked by our system the effects of a protease inhibitor SQV on HIV-1 and HTLV-1. SQV has been reported to be very effective against HIV-1 protease but fails to inhibit HTLV-I Gag processing in infected cells [28]. To obtain appropriate HIV-1 producer cells, which are stably infected with HIV-

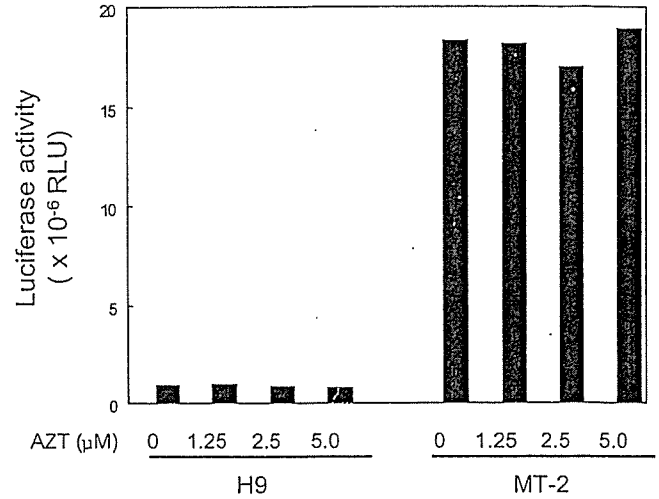

Fig. 4. Effect of AZT on luciferase production in the co-cultures of H9/K30*luc* and MT-2 cells. Indicator H9/K30*luc* cells (10^6) were co-cultured with H9 or HTLV-1 producer MT-2 cells (10^6) in the absence or presence of AZT as indicated for 48 h, and cell lysates were prepared for luciferase assay. H9 and MT-2 cells had been pre-cultured for 24 h in the absence or presence of AZT as above before co-culture started. No cytotoxic effects were observed in these conditions.

1 and producing a low level of the virus-like MT-2 cells for HTLV-1, H9 cells were electroporated with pNL432 and cultured for months. The resultant H9/NL432 cells were easily maintained, and produced a low level of HIV-1 as monitored by RT assay (data not shown).

By the use of H9/H1*luc*, H9/K30*luc*, H9/NL432, and MT-2 cells as indicator and virus producer cells, we determined the inhibitory effects of SQV on HIV-1 and HTLV-1 by monitoring luciferase activity. The effects of SQV on viability of cells were also determined to confirm that there would be no experimental error caused by cytotoxicity. As shown in Fig. 5, while luciferase production in the HIV-1 co-culture was severely inhibited by SQV, no appreciable effects were observed for the HTLV-1 co-culture. These data were in good agreement with our assumption and the results previously reported [27,28] as mentioned above.

4. Discussion

In this report, we have established an indicator cell line for HTLV-1 infection based on luciferase assay (Figs. 1 and 2). Although the cell line H9/K30*luc* was highly susceptible to infection by the co-culture method (Fig. 1), it was insensitive to infection with cell-free HTLV-1 (Fig. 3) as expected [15,16]. Enhanced production of luciferase observed in the co-cultures of H9/K30*luc* and MT-2 cells was probably due to the Tax already present in MT-2 cells before co-cultivation (Fig. 4). Therefore, we have concluded that our HTLV-1 system described here monitors the efficiency of Env-mediated membrane fusion, and that it is useful for evaluating the ability of various factors or agents affecting the process. Indeed, SQV was demonstrated to be a powerful inhibitor for HIV-1 by affecting cell fusion indirectly (Fig. 5).

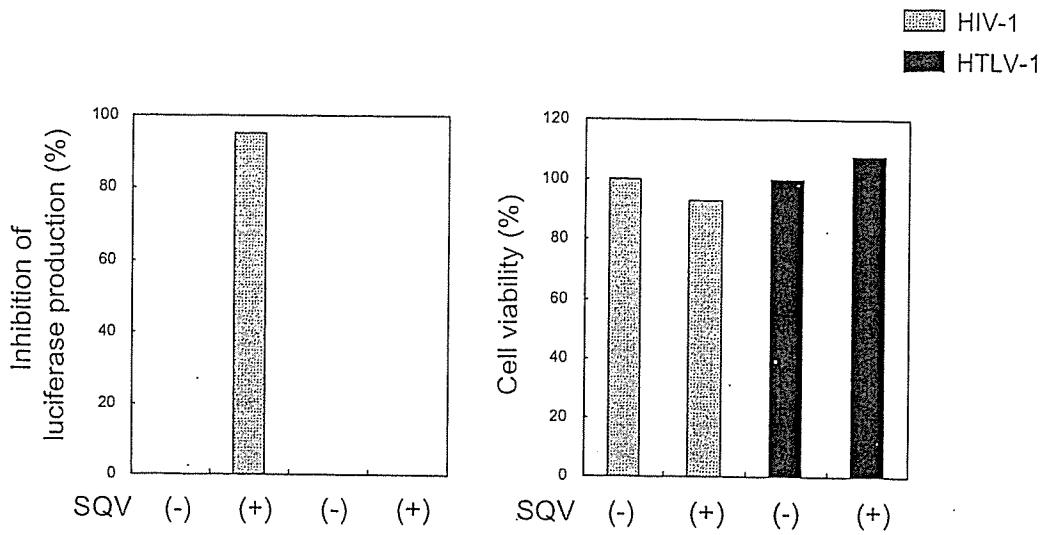

Fig. 5. Effects of SQV on the co-cultures of indicator and virus-producing cells. Indicator (H9/H1*luc* for HIV-1 and H9/K30*luc* for HTLV-1) and virus-producing cells (H9/NL432 for HIV-1 and MT-2 for HTLV-1) (10^6 for each) were co-cultured for 48 h in the absence (–) or presence of SQV (+, 2 μM), and cell lysates were prepared for luciferase assay. Data presented are relative to those of cultures without SQV. H9/NL432 and MT-2 cells had been pre-cultured for 24 h in the absence or presence of SQV as above before co-culture started. Cell viability was determined by the Cell Counting Kit-8, and relative values are presented.

Our results described here strongly suggest that our biological assay system can be used for screening of inhibitors against HTLV-1 and HIV-1 proteases. In particular, because HIV-1 protease inhibitors cannot be effective against HTLV-1 protease (Fig. 5) [28], and because no other good assay methods are available for HTLV-1, the screening by the H9/K30*luc*-MT-2 system would be important. Furthermore, pathogenesis of HAM/TSP can be controlled by reducing the level of HTLV-1 in infected individuals [5–13].

Our protocol for monitoring the inhibitory effects of potential protease inhibitors on HTLV-1 and HIV-1 is summarized as shown in Fig. 6. By using appropriate producer and indicator cells, a large number of antiviral agents can be checked readily for their ability to inhibit the replication of HTLV-1 and HIV-1 within days. Screening of various candidate protease inhibitors by the protocol in Fig. 6 is now in progress in our laboratory.

Virus-producer cells
↓
Culture in the presence of protease inhibitors for 24hrs
↓
Co-cultivation with indicator cells in the presence of protease inhibitors for 48hrs
↓
Assays for luciferase and cell viability

Virus	Producer cells	Indicator cells
HIV-1	H9/NL432	H9/H1*luc*
HTLV-1	MT-2	H9/K30*luc*

Fig. 6. Evaluation system for the effects of human retroviral protease inhibitors. Based on the results in this report, validity of human retroviral protease inhibitors can be readily evaluated as shown in this figure.

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References

[1] M. Yoshida, I. Miyoshi, Y. Hinuma, Isolation and characterization of retrovirus from cell lines of human adult T-cell leukemia and its implication in the disease, Proc. Natl. Acad. Sci. USA 79 (1982) 2031–2035.

[2] A. Gessain, F. Barin, J.C. Vernant, O. Gout, L. Maurs, A. Calender, G. De The, Antibodies to human T-lymphotropic virus type I in patients with tropical spastic paraparesis, Lancet ii (1985) 407–410.

[3] M. Osame, K. Usuku, S. Izumo, N. Ijichi, H. Amitani, A. Igata, M. Matsumoto, M. Tara, HTLV-I associated myelopathy, a new clinical entity, Lancet i (1986) 1031–1032.

[4] K. Barmak, E. Harhaj, C. Grant, T. Alefantis, B. Wigdahl, Human T-cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration, Virology 308 (2003) 1–12.

[5] M. Osame, M. Matsumoto, K. Usuku, S. Izumo, N. Ijichi, H. Amitani, M. Tara, A. Igata, Chronic progressive myelopathy associated with

elevated antibodies to human T-lymphotropic virus type I and adult T-cell leukemialike cells, Ann. Neurol. 21 (1987) 117–122.

[6] M. Mori, K. Kinoshita, N. Ban, Y. Yamada, H. Shiku, Activated T-lymphocytes with polyclonal gammopathy in patients with human T-lymphotropic virus type I-associated myelopathy, Ann. Neurol. 24 (1988) 280–282.

[7] M. Yoshida, M. Osame, H. Kawai, M. Toita, N. Kuwasaki, Y. Nishida, Y. Hiraki, K. Takahashi, K. Nomura, S. Sonoda, N. Eiraku, S. Ijichi, K. Usuku, Increased replication of HTLV-I in HTLV-I-associated myelopathy, Ann. Neurol. 26 (1989) 331–335.

[8] S.J. Greenberg, S. Jacobson, T.A. Waldmann, D.E. McFarlin, Molecular analysis of HTLV-I proviral integration and T-cell receptor arrangement indicates that T cells in tropical spastic paraparesis are polyclonal, J. Infect. Dis. 159 (1989) 741–744.

[9] M. Nishimura, A. Adachi, I. Akiguchi, N. Shirahata, M. Maeda, A. Ishimoto, T. Mezaki, J. Kimura, High ratio of HTLV-1-infected cells in HTLV-1 associated myelopathy (HAM), Acta Neurol. Scand. 81 (1990) 209–214.

[10] K. Nagasato, T. Nakamura, K. Ohishi, K. Shibayama, M. Motomura, K. Ichinose, et al., Active production of anti-human T-lymphotropic virus type I (HTLV-I) IgM antibody in HTLV-I-associated myelopathy, J. Neuroimmunol. 32 (1991) 105–109.

[11] J. Kira, Y. Koyanagi, T. Yamada, Y. Itoyama, I. Goto, N. Yamamoto, H. Sasaki, Y. Sakaki, Increased HTLV-I proviral DNA in HTLV-I-associated myelopathy: a quantitative polymerase chain reaction study, Ann. Neurol. 29 (1991) 194–201.

[12] R. Kubota, T. Fujiyoshi, S. Izumo, S. Yashiki, I. Maruyama, M. Osame, H. Sasaki, Y. Sakaki, Fluctuation of HTLV-I proviral DNA in peripheral blood mononuclear cells of HTLV-I-associated myelopathy, J. Neuroimmunol. 42 (1993) 147–154.

[13] R. Kubota, F. Umehara, S. Izumo, S. Ijichi, K. Matsumoto, S. Yashiki, T. Fujiyoshi, S. Sonoda, M. Osame, HTLV-I proviral DNA amounts correlates with infiltrating CD4+ lymphocytes in the spinal cord from patients with HTLV-I-associated myelopathy, J. Neuroimmunol. 53 (1994) 23–29.

[14] T. Nagao, A. Yoshida, A. Sakurai, A. Piroozmand, A. Jere, M. Fujita, T. Uchiyama, A. Adachi, Determination of HIV-1 infectivity by lymphocytic cell lines with integrated luciferase gene, Int. J. Mol. Med. 14 (2004) 1073–1076.

[15] N. Fan, J. Gavalchin, B. Paul, K.H. Wells, M.J. Lane, B.J. Poiesz, Infection of peripheral blood mononuclear cells and cell lines by cell-free human T-cell lymphoma/leukemia virus type I, J. Clin. Microbiol. 30 (1992) 905–910.

[16] D. Derse, S.A. Hill, P.A. Lloyd, H.K. Chung, B.A. Morse, Examining human T-lymphotropic virus type 1 infection and replication by cell-free infection with recombinant virus vectors, J. Virol. 75 (2001) 8461–8468.

[17] J.S. Lebkowski, S. Clancy, M.P. Calos, Simian virus 40 replication in adenovirus-transformed human cells antagonizes gene expression, Nature 317 (1985) 169–171.

[18] D.L. Mann, S.J. O'Brien, D.A. Gilbert, Y. Reid, M. Popovic, E. Read-Connole, R.C. Gallo, A.F. Gazdar, Origin of the HIV-susceptible human CD4+ cell line H9, AIDS Res. Hum. Retroviruses 5 (1989) 253–255.

[19] S. Harada, Y. Koyanagi, N. Yamamoto, Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay, Science 229 (1985) 563–566.

[20] R. Shibata, M. Kawamura, H. Sakai, M. Hayami, A. Ishimoto, A. Adachi, Generation of a chimeric human and simian immunodeficiency virus infectious to monkey peripheral blood mononuclear cells, J. Virol. 65 (1991) 3514–3520.

[21] A. Adachi, H.E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, M.A. Martin, Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone, J. Virol. 59 (1986) 284–291.

[22] R.L. Willey, D.H. Smith, L.A. Lasky, T.S. Theodore, P.L. Earl, B. Moss, D.J. Capon, M.A. Martin, In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity, J. Virol. 62 (1988) 139–147.

[23] H. Sakai, R. Shibata, J. Sakuragi, T. Kiyomasu, M. Kawamura, M. Hayami, A. Ishimoto, A. Adachi, Compatibility of rev gene activity in the four groups of primate lentiviruses, Virology 184 (1991) 513–520.

[24] R.A. Furuta, K. Sugiura, S. Kawakita, T. Inada, S. Ikehara, T. Matsuda, J. Fujisawa, Mouse model for the equilibration interaction between the host immune system and human T-cell leukemia virus type 1 gene expression, J. Virol. 76 (2002) 2703–2713.

[25] E. Bohnlein, M. Siekevitz, D.W. Ballard, J.W. Lowenthal, L. Rimsky, H. Bogerd, J. Hoffman, Y. Wano, B.R. Franza, W.C. Greene, Stimulation of the human immunodeficiency virus type 1 enhancer by the human T-cell leukemia virus type I tax gene product involves the action of inducible cellular proteins, J. Virol. 63 (1989) 1578–1586.

[26] H. Cheng, J. Tarnok, W.P. Parks, Human immunodeficiency virus type 1 genome activation induced by human T-cell leukemia virus type 1 Tax protein is through cooperation of NF-kappaB and Tat, J. Virol. 72 (1998) 6911–6916.

[27] T. Murakami, S. Ablan, E.O. Freed, Y. Tanaka, Regulation of human immunodeficiency virus type 1 Env-mediated membrane fusion by viral protease activity, J. Virol. 78 (2004) 1026–1031.

[28] S.C. Pettit, R. Sanchez, T. Smith, R. Wehbie, D. Derse, R. Swanstrom, HIV type 1 protease inhibitors fail to inhibit HTLV-I Gag processing in infected cells, AIDS Res. Hum. Retroviruses 14 (1998) 1007–1014.

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The '*O*-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides: application to the synthesis of Alzheimer's disease-related amyloid β peptide (Aβ) 1–42

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Abstract: An efficient '*O*-acyl isopeptide method' for the synthesis of difficult sequence-containing peptides was applied successfully to the synthesis of amyloid β peptide (Aβ) 1–42 via a water-soluble *O*-acyl isopeptide of Aβ1–42, i.e. '26-*O*-acyl isoAβ1–42' (**6**). This paper describes the detailed synthesis of Aβ1–42 focusing on the importance of resin selection and the analysis of side reactions in the *O*-acyl isopeptide method. Protected '26-*O*-acyl isoAβ1–42' peptide resin was synthesized using 2-chlorotrityl chloride resin with minimum side reactions in comparison with other resins and deprotected crude 26-*O*-acyl isoAβ1–42 was easily purified by HPLC due to its relatively good purity and narrow elution with reasonable water solubility. This suggests that only one insertion of the isopeptide structure into the sequence of the 42-residue peptide can suppress the unfavourable nature of its difficult sequence. The migration of *O*-acyl isopeptide to intact Aβ1–42 under physiological conditions (pH 7.4) via *O–N* intramolecular acyl migration reaction was very rapid and no other by-product formation was observed while **6** was stable under storage conditions. These results concluded that our strategy not only overcomes the solubility problem in the synthesis of Aβ1–42 and can provide intact Aβ1–42 efficiently, but is also applicable in the synthesis of large difficult sequence-containing peptides at least up to 50 amino acids. This synthesis method would provide a biological evaluation system in Alzheimer's disease research, in which 26-*O*-acyl isoAβ1–42 can be stored in a solubilized form before use and then rapidly produces intact Aβ1–42 *in situ* during biological experiments. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Aβ1–42; *O*-acyl isopeptide method; Alzheimer's disease; Alzheimer's disease research tool; difficult sequence-containing peptide; *O–N* intramolecular acyl migration reaction

INTRODUCTION

The synthesis of 'difficult sequence'-containing peptides is one of the most problematic areas in peptide chemistry, and such peptides are often obtained with low yield and purity in solid-phase peptide synthesis (SPPS) [1–6]. These difficult sequences are generally hydrophobic and promote aggregation in solvents during synthesis and purification. This aggregation is attributed to intermolecular hydrophobic interaction and hydrogen bond network among resin-bound peptide chains, resulting in the formation of extended secondary structures such as β-sheets [1,2]. The tendency for aggregation depends on the nature of the peptide and side chain protecting groups. In particular, it is known that peptides with sequences containing Ala, Val, Ile, Asn and Gln residues in high frequency show a strong propensity for difficult sequences.

To solve the synthetic problem of difficult sequence-containing peptides, Sheppard and Johnson *et al.* reported a building block, 2-hydroxy-4-methoxybenzyl (Hmb), a protecting group for the backbone amide nitrogen [3,4]. Mutter *et al.* also introduced building blocks, so-called pseudo-prolines, which are dipeptide derivatives, consisting of Ser/Thr-derived oxazolidines or Cys-derived thiazolidine [5,6]. These special building blocks were designed to disrupt the secondary structure formed by interchain hydrogen bonding. However, to prepare building blocks, prior modifications of Fmoc-amino acids by 2–6 steps of additional solution phase synthesis are required in these approaches, and a strong acid treatment is also required to remove the building blocks. Therefore, the development of novel methods using conventional amino acid derivatives are of great significance in the synthesis of difficult sequence-containing peptides.

Hence, a novel and efficient '*O*-acyl isopeptide method' was developed for the synthesis of difficult sequence-containing peptides based on the synthesis of hydrophilic '*O*-acyl isopeptides' followed by *O–N* intramolecular acyl migration reaction (Figure 1A)

Abbreviations: PBS, phosphate buffered saline; Pmc, 2, 2, 5, 7, 8-pentamethylchroman-6-sulfonyl; Pns, phenylnorstatine = (2*R*, 3*S*)-3-amino-2-hydroxy-4-phenylbutyric acid; otherwise as in *J. Peptide Sci* **9**: 1–8 (2003).

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