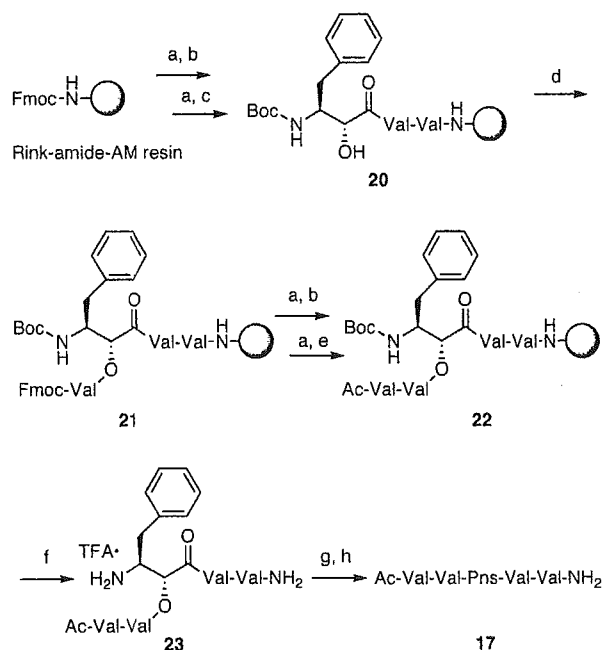


Ac-Val-Val-Pns-Val-Val-NH₂ (17) を選択し、通常の Fmoc 固相合成法および“O-アシルイソペプチド法”の比較検討を実施した。通常の Fmoc 固相合成法では、目的物と同等量の Fmoc-Val-Val-Pns-Val-Val-NH₂ 18 および H-Val-Val-Pns-Val-Val-NH₂ 19 が副生した(図 13A)。これは、Fmoc 基の除去およびカップリングが不完全であったために生成したものと考えられる。また表 3 に示すように、ペプチド 17 の HPLC 精製は、低い溶解性のため困難であり、収率は 7% であった。一方、“O-アシルイソペプチド法”(スキーム 4) では、Pns ヒドロキシ基への Fmoc-Val-OH の導入時に 3.2% のラセミ化が観察されたが、以降の固相合成過程において、本エステル結合の開裂は観察されなかった。そして O-アシルイソペプチド 23 が主生成物として効率良く得られた(図 13B)。この結果は、ペプチド中に挿入された分岐エステル構造が、樹脂上での difficult sequence に起因する悪影響を排除し、カップリング・脱保護効率改善をもたらしたことを意味している。さらに、23 は水およびメタノールに対し良好な溶解性を示したことから、HPLC による精製が容易であり、高い収率(58%)で得られた(表 3)。そして最後に、精製した O-アシルイソペプチド 23 を pH 7.4 リン酸緩衝液(PBS)に溶解させると、半減期 < 1 min という早い O-N 分子内アシル基転位反応により、目的のペプチドアミド 17 が沈殿物として効率良く(総収率 54%)得られた(図 14)^{10c)}。



a) 20% Piperidine/DMF, 20 min; b) Fmoc-Val-OH, DIPCDI, HOBt, DMF, 2 h; c) Boc-Pns-OH, DIPCDI, HOBt, DMF, 2 h; d) Fmoc-Val-OH, DIPCDI, DMAP, CH₂Cl₂, 16 h × 2; e) Ac₂O, TEA, DMF, 2 h; f) TFA-*m*-cresol-thioanisole-H₂O (92.5 : 2.5 : 2.5 : 2.5), 90 min; g) preparative HPLC (a linear gradient of CH₃CN in 0.1% aqueous TFA); h) PBS, pH 7.4, 25 °C.

Scheme 4

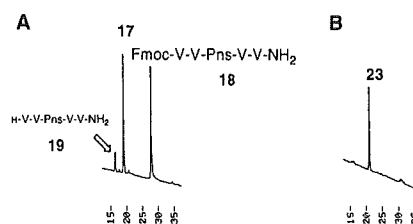


Fig. 13 HPLC profiles of crude deprotected peptides; (A) conventional solid phase peptide synthesis, (B) O-acyl isopeptide method.

Table 3 Solubility and yield of 17 and 23.

Compd	H ₂ O-solubility		MeOH-solubility		Yield (%)
	mg / mL ^a	Ratio ^b	mg / mL ^a	Ratio ^b	
17	0.008 ± 0.001	1	0.065 ± 0.019	1	7
23	59.4 ± 13.6	7500	277 ± 84	4300	58

^a Values are means ± SD of three experiments. ^b Ratio = solubility of O-acyl isopeptide / solubility of parent peptide.

このように、“O-アシルイソペプチド法”は 5 残基の アミノ酸からなる difficult sequence 含有モデルペプチドの合成において、顕著な収率改善をもたらしたことから、difficult sequence 含有ペプチドの新規効率的合成手法になりうることが示唆された。

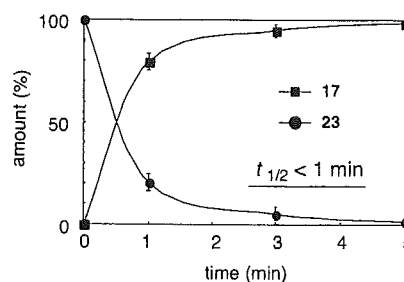


Fig. 14 Migration of O-acyl isopeptide 23 to 17 in PBS (pH 7.4, 25 °C).

3.2 O-N 分子内アシル基転位反応によるアミロイドβペプチド(Aβ)1-42の合成

“O-アシルイソペプチド法”により低分子の α-ヒドロキシβ-アミノ酸を含む、difficult sequence 含有ペプチドの合成が成功したことから、次に高分子 difficult sequence 含有ペプチドの合成に挑戦した。標的 difficult sequence 含有ペプチドとして、その合成の難しさが指摘されているアミロイドβペプチド(Aβ)1-42²⁶⁾に注目した。本ペプチドはアミロイド形成において重要な役割を演じ、アルツハイマー病の病因と考えられており²⁷⁾、化学合成 Aβ1-42 の効率的供給は、Aβ1-42 と発病の因果関係を詳細に解明する上で、重要なポイントである。しかしながら、本ペプチドは非常に疎水性の高いペプチドであり、種々の溶媒中で不溶性の凝集体を形成するため、一般的な固相合成法では収率・純度は極めて低く、さらに HPLC による精製は溶媒への低溶解性と

ていない³¹⁾。これらはすべて、C-2'位またはC-7位水酸基に高極性補助基を付加することで水溶性を改善しているが、親薬物への変換に際して補助基部分が副生成物となることから、常に補助基に対する副作用を懸念する必要がある。

筆者らは、有機化学に基づいて薬剤学的付加価値の高い分子の創製を目指す“chemical pharmaceuticals”の研究展開として、前節で述べた、O-N分子内アシル基転位反応に注目して、水溶性補助基を利用しない新しいタイプの水溶性プロドラッグを考案している。図17に示すように、すでに、 α -ヒドロキシ- β -アミノ酸構造を有するHIV-1プロテアーゼ阻害剤の“O-N分子内アシル基転位型”水溶性プロドラッグを報告している^{11c-g)}。これらは、親化合物のO-アシルイソ体であり、塩形成可能なアミノ基が存在するために水溶性を改善できる。例えば、HIV-1プロテアーゼ阻害剤KNI-727(図17)の分子中央部Apsn残基で、相当するO-アシル型プロドラッグ26にすると、水溶性はKNI-727より8,600倍上昇するとともに、生理的条件下では数分で副反応なく完全にKNI-727に変換された^{11d)}。一方、酸性条件下では安定で、塩酸塩(固体)として長期保存可能であった。

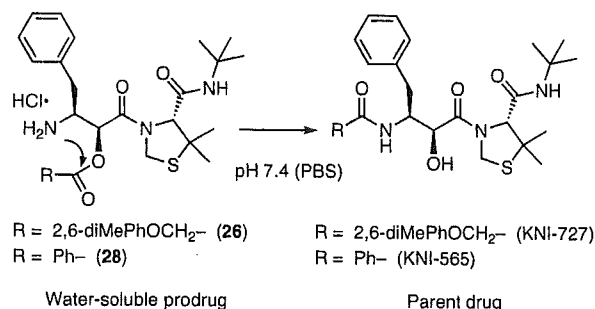


Fig. 17 Water soluble prodrug of HIV-1 protease inhibitors based on O-N intramolecular acyl migration.

筆者らは、このプロドラッグ戦略が、類似した α -ヒドロキシ- β -アミノ酸を例外なく分子内に有する一連のtaxoid類でも、難水溶性の問題を網羅的に解決できるのではないかと考えた。すなわちtaxoidの2'-O-acyl isoformを合成すれば、水溶性プロドラッグとして効果的に機能するのではないかと考え、paclitaxel 25の2'-O-benzoyl isoformであるisotaxel 27をデザインした(図18)。しかし、同様なベンゾイル構造を有するKNI-565(図17)のプロドラッグ28が比較的長い半減期(30分)を示したことから^{11d)}、isotaxel 27の合成に先立ち、タキサン環部分をシクロヘキサン環に変更した27のモデル化合物を合成し、生理的条件下での半減期を測定したところ、12分であった。これは、O-Nアシル基転位反応における5員環遷移状態で、置換基同士の立体配置が立体障害の大きなcisとなる28に比べ、27のモ

デル化合物では、立体障害が小さいtrans配置になるため、転位反応がより早く進行したものと考えられる。得られた半減期は、静脈点滴後、比較的速やかに親化合物を放出できる長さであったことから、実際にスキーム5に示すルートで、27の合成を行った。

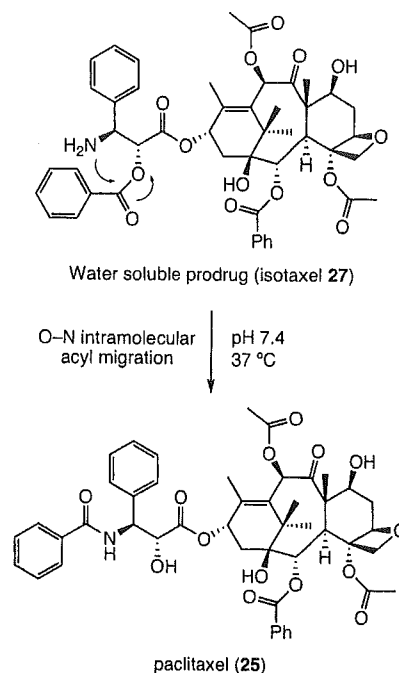
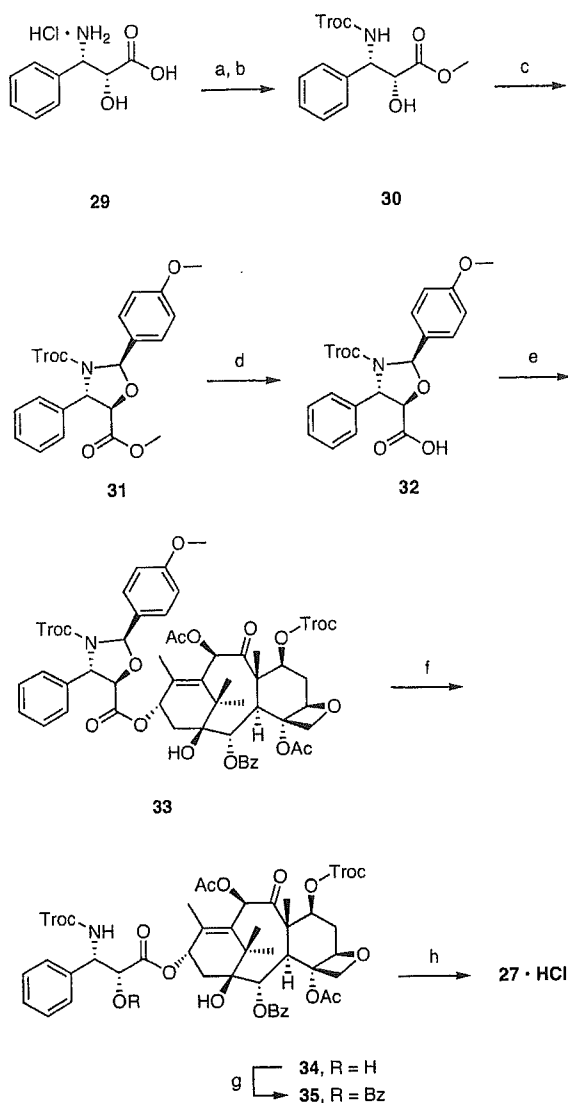


Fig. 18 New water soluble prodrug of paclitaxel.

すなわち、市販の(2R, 3S)-phenylisoserine·HCl 29から調製したN ^{β} -Troc-phenylisoserine methyl ester 30をPPTS触媒下に4-methoxybenzaldehyde dimethyl acetalと反応させ1,3-oxazolidine誘導体31とし、次いでメチルエステルを加水分解後、得られたカルボン酸誘導体32をDCC-DMAP法で7-Troc-baccatin IIIと縮合させた。このエステル化はほぼ定量的に進み、またC-2'位のラセミ化も観察されなかった。得られた化合物33のoxazolidine環をPTSで分解後に、2'-ヒドロキシ基に安息香酸をEDC-DMAP法で導入し、さらに、2つのTroc基をZn-AcOHで除去し、12 mM HClを溶出液とするHPLC精製にて、目的のisotaxel塩酸塩27を総収率58%で得た。

27は親化合物25に比べ水溶性が1,800倍(0.45 mg mL⁻¹)上昇するとともに、生理的条件下(pH 7.4, 37 °C)において副反応を伴うことなく、完全に親薬物に変換された(図19)。また、この半減期はpH 7.4においては15分であり、先に述べたモデル実験に近い値で、静脈投与後の全身への薬物の送達には適度な値と思われる。一方、半減期はpH依存性であり、pH 4.9では252分、pH 2.0では転位は起こらなかった(図20)。また、塩酸塩として固体状態では、冷蔵での長期保存が可能である。さらに、静脈投与可能な0.035%クエン酸生理食塩



a) Succinimidyl-2, 2, 2-trichloroethylcarbonate, NaOH, NaHCO₃, dioxane, rt, 1 h; b) SOCl₂, MeOH, 0 °C to rt, 14 h, 97% over two steps; c) 4-methoxybenzaldehyde dimethyl acetal, PPTS, toluene, distillation 30 min., 92%; d) KOH, MeOH, rt, 30 min. 99%; e) 7-Troc-baccatin III, DCC, DMAP, toluene : CH₂Cl₂ 2 : 1, rt, 3 h, 98%; f) PTS, MeOH, rt, 24 h, 94%; g) benzoic acid, EDC · HCl, DMAP, CH₂Cl₂, rt, 2 h, 92%; h) Zn (dust), MeOH : AcOH 1 : 1, rt, 4 h, then HCl, 77%.

Scheme 5

水(pH 4.0)³²⁾では、室温3時間のインキュベーションでも、親化合物 25 の生成は3%以下であったことから、isotaxel 27 には、実用性があると思われる。一方、副作用の原因ともなる水溶性補助基を有しないことから、新しいタイプのタキソイドプロドラッグ(イソタキソイド)として開発される可能性がある。

筆者らは、さらに本水溶性プロドラッグ手法の一般性を検証するために、最近、他のタキソイド誘導体 canadensol の O-N 分子内アシル基転位型水溶性プロドラッグの合成も行い、良好な結果を得ている^{11a)}。また、taxoid 類では、docetaxel のように、アミノ基の修飾基として、カルバメートを有するものがある。これら

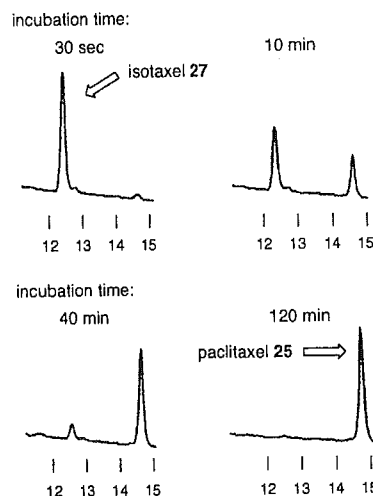


Fig. 19 HPLC profile of O-N benzoyl migration of isotaxel 27 in PBS (pH 7.4, 37 °C).

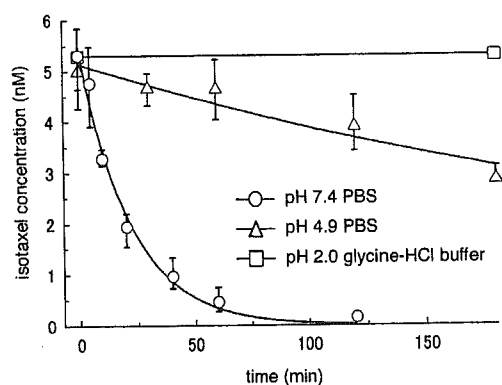


Fig. 20 Migration of isotaxel 27 in different pH conditions (PBS, pH 7.4, 37 °C).

の誘導体の水溶性プロドラッグ体は、炭酸エステル構造を有し、O-N 分子内アシロキシ基転位反応で親化合物に変換される必要がある。最近の検討では Boc 基を有する docetaxel では、一部アシロキシ基の加水分解が起こるが、それ以外のカルバメート型誘導体では、O-N 分子内アシロキシ基転位反応により、適度な半減期で、副生物なしに親化合物に変換されることを見出している^{11h)}。これらの誘導体の中には、次世代の抗腫瘍剤として期待される paclitaxel 耐性腫瘍に対して有効な化合物もあり、今後、本水溶性プロドラッグ(イソタキソイド)の実用的な応用例となる可能性がある。

おわりに

筆者らは、 α -ヒドロキシ- β -アミノ酸の分子機能に注目することで、有機化学・創薬化学での新規な応用研究を展開してきた。特に、 α -ヒドロキシ- β -アミノ酸の特性の1つである O-N 分子内アシル基転位反応に注目することで、difficult sequence 含有ペプチドの新しい合成手法までたどり着くことができた。また、水溶性プロ

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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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-isomer 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.

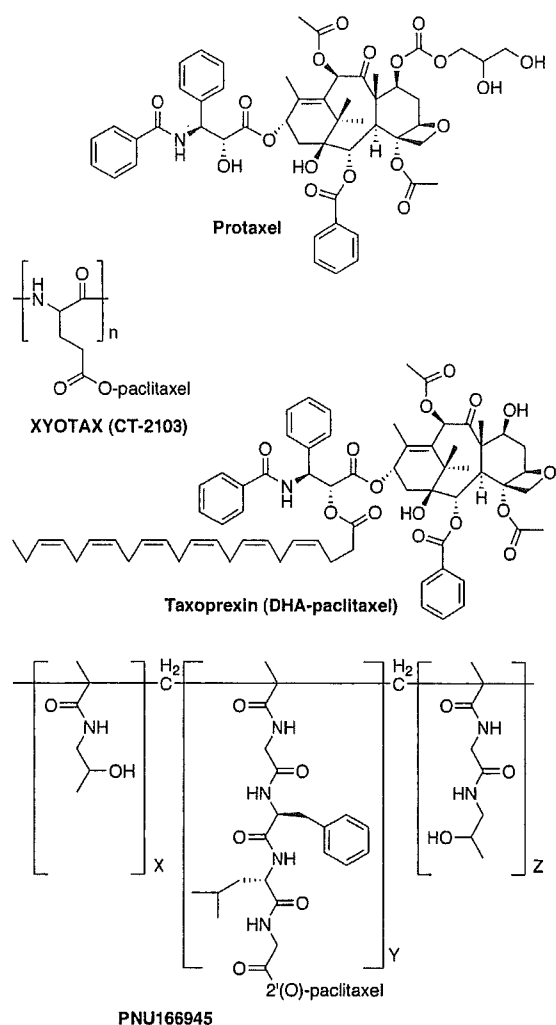
In all standard prodrug strategies, auxiliary moieties are employed and their release may have negative effects *in vivo*.³² Hence, prodrug designs that avoid the use of additional moieties are very promising. Since the O–N intramolecular acyl migration reaction proceeded under mild basic or neutral 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 chloro-

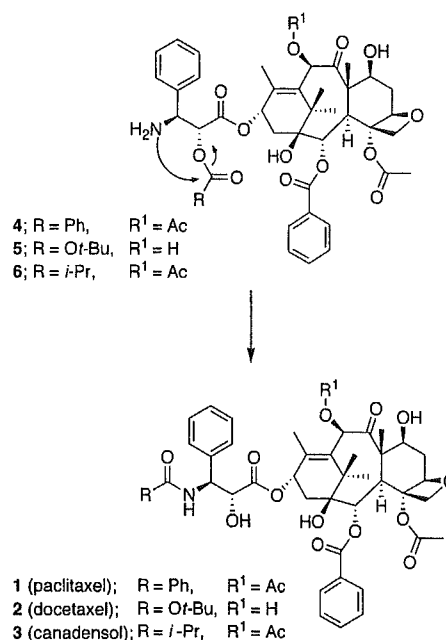
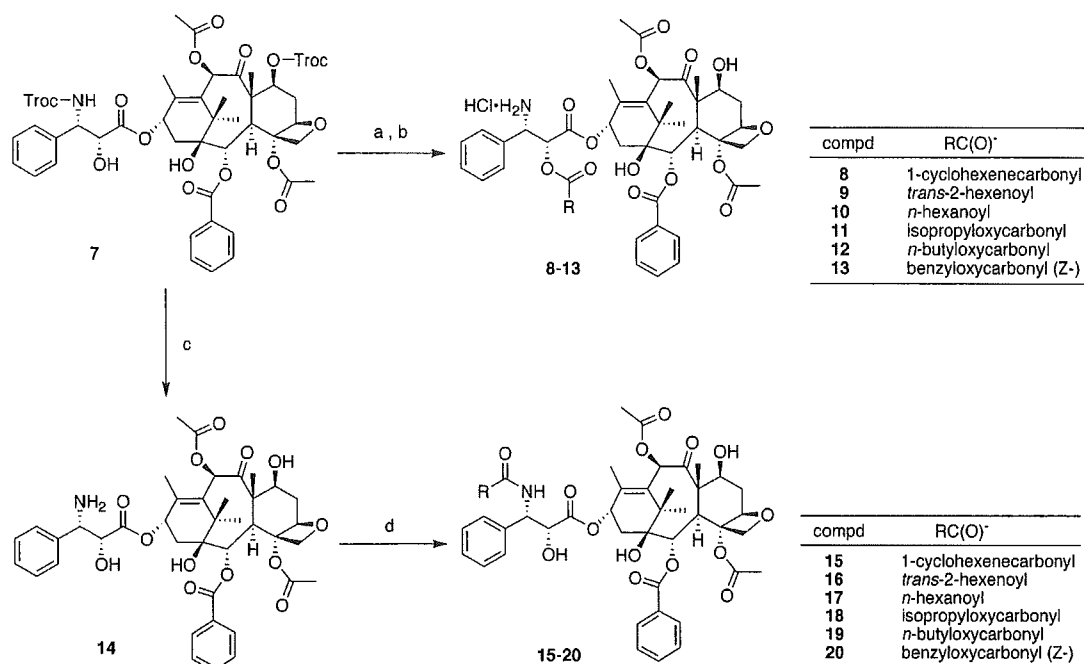


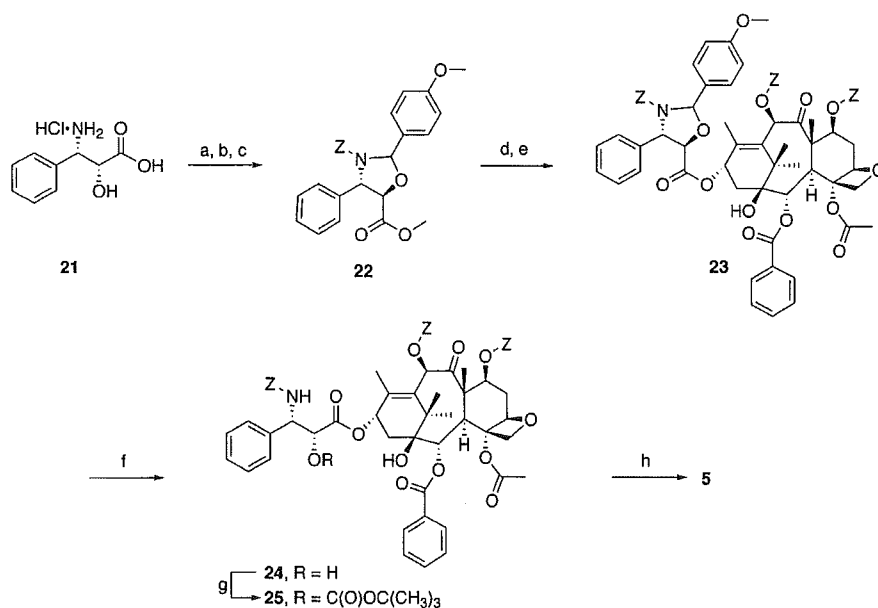
Figure 1. The O–N acyl/acyloxy migration reaction of taxoid prodrugs to their corresponding parent drugs under physiological conditions.

formate in pyridine/CH₂Cl₂ solution, and subsequent deprotection of both 2,2,2-trichloroethoxycarbonyl (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 CH₂Cl₂/saturated NaHCO₃ solution, resulting in the formation of the parent drugs 15–20.

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 corresponding carboxylic acid without further purification (due to high lability in an acidic medium, even to silica gel), was coupled with 10-deacetyl-7,10-dibenzoyloxycarbonylbaccatin III⁴⁵ in the presence of DCC to give the corresponding crude ester 23, which was immedi-

Scheme 1^a

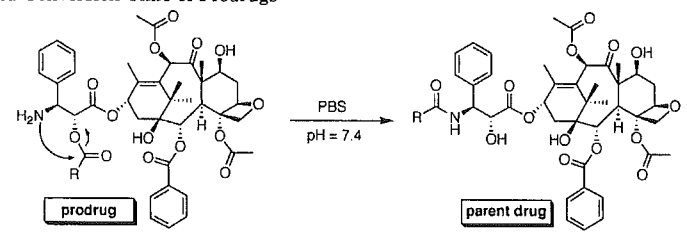
^a Reagents and conditions: (a) RCOOH, EDC·HCl, DMAP, CH₂Cl₂, rt (for 8–10), or RCOCl, CH₂Cl₂, pyridine, rt (for 11–13); (b) Zn, AcOH, MeOH, rt, then ion exchange HPLC with 12 mM HCl, (8, 76%; 9, 47%; 10, 62%; 11, 65%; 12, 71%; 13, 53%) over two steps; (c) Zn, AcOH, AcOEt, rt, 60%; (d) RCOOH, EDC·HCl, HOBt, DMF, rt, for 15–17, or RCOCl, CH₂Cl₂, sat. NaHCO₃, rt, for 18–20, (15, 77%; 16, 56%; 17, 56%; 18, 87%; 19, 93%; 20, 95%).








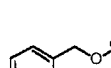
Scheme 2^a

^a Reagents and conditions: (a) SOCl₂, MeOH, 0 °C to rt, overnight; (b) benzylloxycarbonyl chloride, CH₂Cl₂, sat. NaHCO₃, 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-dibenzylloxycarbonylbaccatin III, DCC, DMAP, CH₂Cl₂, rt, 2 h.; (f) PPTS, MeOH, rt, 5 h, 80% over two steps; (g) Boc₂O, DMAP, CH₂Cl₂:pyridine 1:1, rt, overnight, 84%; (h) Pd/C, H₂, AcOEt, rt, 8 h, then 0.1 M HCl in methanol, 99%.

ately allowed to react with PPTS to afford **24**. Although the oxazolidine ring of **23** was easily cleaved with the major diastereomer, the minor diastereomer was stable even with excess PPTS 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			$t_{1/2}$ (min) ^c
			prodrug (mg/mL)	parent drug (mg/mL)	ratio	
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

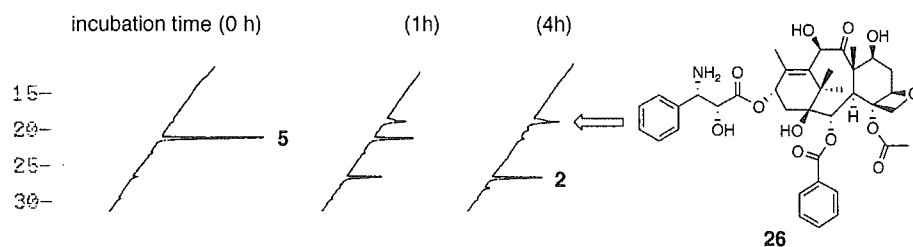


Figure 2. HPLC profiles of O–N intramolecular acyloxy migration in prodrug **5** in PBS (pH 7.4, 37 °C).

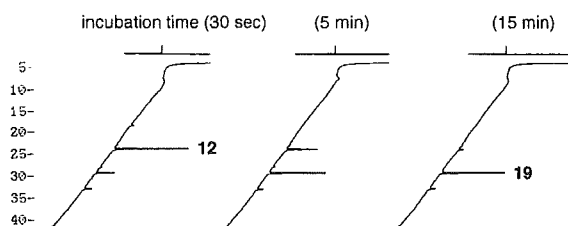


Figure 3. HPLC profiles of O–N intramolecular acyloxy migration of prodrug **12** in PBS (pH 7.4, 37 °C).

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 (isopropoxyloxycarbonyl, *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 isopropoxy 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

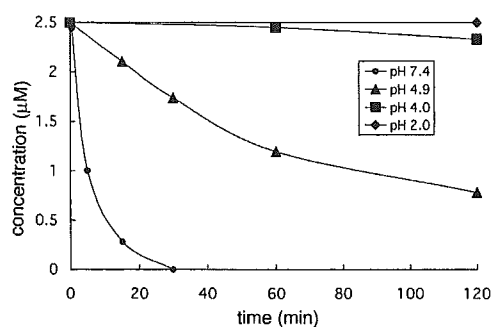


Figure 4. Migration of prodrug **12** at different pH values at 37 °C: pH 7.4 and 4.9 (PBS), pH 4.0 (0.035% citric acid saline), and pH 2.0 (glycine-HCl buffered saline).

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 thought 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-tetrahydrofuran-2-yl) 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 isopropoxy 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 Glaxo-SmithKline (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

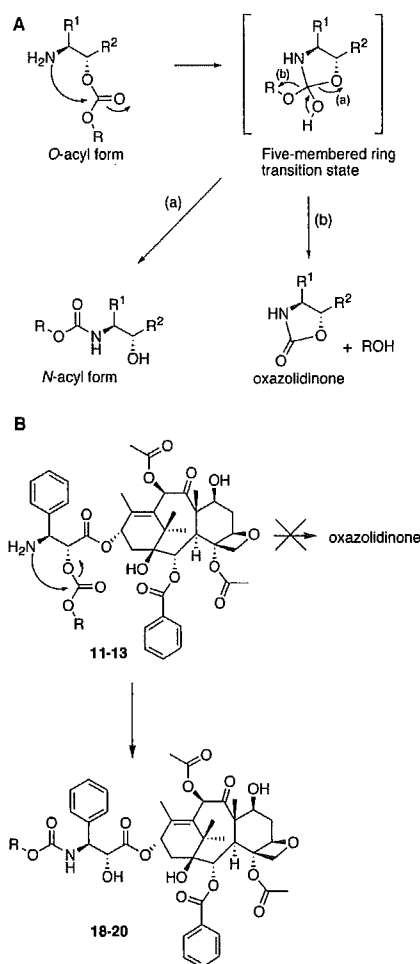


Figure 5. Possible products of amine group nucleophilic attack on carbonate moiety (A) and pure O–N intramolecular acyloxy migration reaction observed in prodrugs 11–13 (B).

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 phenyl-isoserine derivative moiety. Thus, for example, similar kinetic behavior can be expected for other taxoids with isopropylloxycarbonyl and *n*-butylloxycarbonyl 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 prodrugs 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-isomer 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 60F₂₅₄ 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 CH₃CN 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 CH₃CN 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-MS 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 CH₂Cl₂ (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 NaHCO₃ twice. The organic layer was dried over MgSO₄ 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% CH₃CN 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 (CD₃OD, 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 CD₃OD), 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, CH₂), 3.72 (d, ³J(H,H) = 7.1 Hz, 1 H, CH), 2.48–2.41 (m, 1 H, CH₂), 2.34–2.31 (m, 4H, CH₂), 2.27 (s, 3 H, CH₃), 2.16 (s, 3 H, CH₃), 1.92 (dd, ³J(H,H) = 9.3 Hz, ²J(H,H) = 15.4 Hz, 1 H, CH₂), 1.85 (d, ⁴J(H,H) = 1.1 Hz, 3 H, CH₃), 1.82–1.68 (m, 5 H, CH₂), 1.62 (s, 3 H, CH₃), 1.47 (dd, ³J(H,H) = 8.8 Hz, ²J(H,H) = 15.5 Hz, 1 H, CH₂), 1.12 (s, 3 H, CH₃), 1.10 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₇H₅₆NO₁₄ [M⁺ + H] 858.3701, found 858.3705. Anal. (C₄₇H₅₆ClNO₁₄·3.5H₂O) 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 (CD₃OD, 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, CH₂), 3.72 (d, ³J(H,H) = 7.1 Hz, 1 H, CH), 2.48–2.40 (m, 1 H, CH₂), 2.34–2.28 (m, 2H, CH₂), 2.26 (s, 3 H, CH₃), 2.15 (s, 3 H, CH₃), 1.90 (dd, ³J(H,H) = 6.0 Hz, ²J(H,H) = 15.6 Hz, 1 H, CH₂), 1.85 (d, ⁴J(H,H) = 1.1 Hz, 3 H, CH₃), 1.82–1.74 (m, 1 H, CH₂), 1.62 (s, 3 H, CH₃), 1.62–1.53 (m, 2 H, CH₂), 1.44 (dd, ³J(H,H) = 8.7 Hz, ²J(H,H) = 15.5 Hz, 1 H, CH₂), 1.12 (s, 3 H, CH₃), 1.09 (s, 3 H, CH₃), 1.00 (t, ³J(H,H) = 7.4 Hz, 3 H, CH). HRMS (FAB+): calcd for C₄₆H₅₆NO₁₄ [M⁺ + H] 846.3701, found 846.3698. Anal. (C₄₆H₅₆ClNO₁₄·2.5H₂O) C, H, N.

2'-O-Hexanoyl-3'-N-debenzoylpaclitaxel Hydrochloride (10). The above procedure with hexanoic acid afforded **10** (62%): ¹H NMR (CD₃OD, 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, CH₂), 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, CH₂), 2.26 (s, 3 H, CH₃), 2.16 (s, 3 H, CH₃), 1.93–1.88 (m, 1H, CH₂), 1.86 (d, ⁴J(H,H) = 1.1 Hz, 3 H, CH₃), 1.81–1.74 (m, 1 H, CH₂), 1.73–1.66 (m, 2 H, CH₂), 1.62 (s, 3 H, CH₃), 1.44 (dd, ³J(H,H) = 9.0 Hz, ²J(H,H) = 15.4 Hz, 1 H, CH₂), 1.39–1.31 (m, 4 H, CH₂), 1.12 (s, 3 H, CH₃), 1.10 (s, 3 H, CH₃), 0.93 (t, ³J(H,H) = 7.0 Hz, 3 H, CH). HRMS (FAB+): calcd for C₄₆H₅₆NO₁₄ [M⁺ + H] 848.3857, found 848.3854. Anal. (C₄₆H₅₆ClNO₁₄·2.5H₂O) C, H, N.

2'-O-Isopropoxyloxycarbonyl-3'-N-debenzoylpaclitaxel Hydrochloride (11). Isopropoxyloxycarbonyl chloride (5 μL, 43.6 μmol) was added to the stirring solution of **7** (24.0 mg, 21.8 μmol) in dry CH₂Cl₂ (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 MgSO₄ 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% CH₃CN 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 (CD₃OD, 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 CD₃OD), 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, CH₂), 3.72 (d, ³J(H,H) = 7.1 Hz, 1 H, CH), 2.49–2.41 (m, 1 H, CH₂), 2.29 (s, 3 H, CH₃), 2.16 (s, 3 H, CH₃), 1.94 (dd, ³J(H,H) = 9.6 Hz, ²J(H,H) = 15.3 Hz, 1 H, CH₂), 1.86 (d, ⁴J(H,H) = 1.3 Hz, 3 H, CH₃), 1.82–1.75 (m, 1 H, CH₂), 1.63 (s, 3 H, CH₃), 1.53 (dd, ³J(H,H) = 8.9 Hz, ²J(H,H) = 15.3 Hz, 1 H, CH₂), 1.36 (d, ³J(H,H) = 6.2 Hz, 3 H, CH₃), 1.35 (d, ³J(H,H) = 6.2 Hz, 3 H, CH₃), 1.13 (s, 3 H, CH₃), 1.11 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₄H₅₄NO₁₅ [M⁺ + H] 836.3493, found 836.3502. Anal. (C₄₄H₅₄ClNO₁₅·4.5H₂O) 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 (CD₃OD, 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 CD₃OD), 4.34–4.22 (m, 3 H, CH, CH₂), 4.17, 4.14 (2d, ²J(H,H) = 8.4 Hz, 2 H, CH₂), 3.73 (d, ³J(H,H) = 7.3 Hz, 1 H, CH), 2.48–2.41 (m, 1 H, CH₂), 2.27 (s, 3 H, CH₃), 2.16 (s, 3 H, CH₃), 1.92 (dd, ³J(H,H) = 9.4 Hz, ²J(H,H) = 15.5 Hz, 1 H, CH₂), 1.87 (d, ⁴J(H,H) = 1.1 Hz, 3 H, CH₃), 1.82–1.75 (m, 1 H, CH₂), 1.75–1.67 (m, 2H, CH₂), 1.63 (s, 3 H, CH₃), 1.53–1.40 (m, 3 H, CH, CH₂), 1.13 (s, 3 H, CH₃), 1.11 (s, 3 H, CH₃), 0.97 (t, ³J(H,H) = 7.3 Hz, 3 H, CH₃). HRMS (FAB+): calcd for C₄₅H₅₆NO₁₅ [M⁺ + H] 850.3650, found 850.3655. Anal. (C₄₅H₅₆ClNO₁₅·3.5H₂O) C, H, N.

2'-O-Benzoyloxycarbonyl-3'-N-debenzoylpaclitaxel Hydrochloride (13). The above procedure with benzyloxycarbonyl chloride (20 equiv) afforded **13** (53%): ¹H NMR (CD₃OD, 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,

CH₂), 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, ³J(H,H) = 9.0 Hz, 2 H, CH₂), 3.72 (d, ³J(H,H) = 7.3 Hz, 1 H, CH), 2.50–2.42 (m, 1 H, CH₂), 2.27 (s, 3 H, CH₃), 2.17 (s, 3 H, CH₃), 1.91 (dd, ³J(H,H) = 9.3 Hz, ²J(H,H) = 15.4 Hz, 1 H, CH₂), 1.81 (d, ⁴J(H,H) = 1.3 Hz, 3 H, CH₃), 1.82–1.76 (m, 1 H, CH₂), 1.63 (s, 3 H, CH₃), 1.49 (dd, ³J(H,H) = 9.0 Hz, ²J(H,H) = 15.4 Hz, 1 H, CH₂), 1.13 (s, 3 H, CH₃), 1.10 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₈H₅₄NO₁₅ [M⁺ + H] 884.3493, found 884.3486. Anal. (C₄₈H₅₅ClNO₁₅·1.5H₂O) C, H, N.

3'-N-Debenzoylepaclitaxel (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 NaHCO₃ three times. The organic layer was dried over MgSO₄ and the solvent was removed under reduced pressure. The resulting oil was purified by silica gel column chromatography (MeOH:CHCl₃ 10:1) to yield a white powder **14** after lyophilization (164 mg, 0.219 mmol, 60%). ¹H NMR (CDCl₃, 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, CH₂, 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, CH₂), 2.24 (s, 3 H, CH₃), 2.13 (s, 3 H, CH₃), 2.19–1.82 (m, 3 H, CH₂), 1.87 (d, ⁴J(H,H) = 1.3 Hz, 3 H, CH₃), 1.65 (s, 3 H, CH₃), 1.24 (s, 3 H, CH₃), 1.13 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₀H₄₈NO₁₃ [M⁺ + H] 750.3126, found 750.3120. Anal. (C₄₀H₄₇NO₁₃·H₂O) C, H, N.

3'-N-(1-Cyclohexenylcarbonyl)-3'-N-debenzoylepaclitaxel (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 NaHCO₃ twice, the organic layer was dried over MgSO₄ 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% CH₃CN 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%). ¹H NMR (CDCl₃, 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, CH₂), 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, CH₂), 2.47 (d, ³J(H,H) = 4.0 Hz, 1 H, OH), 2.35 (s, 3 H, CH₃), 2.29 (dd, ³J(H,H) = 5.2 Hz, ²J(H,H) = 8.9 Hz, 1 H, CH₂), 2.25 (s, 3 H, CH₃), 2.22–2.11 (m, 4H, CH₂), 1.91–1.85 (m, 1 H, CH₂), 1.85 (s, 1H, OH), 1.79 (d, ⁴J(H,H) = 1.1 Hz, 3 H, CH₃), 1.68 (s, 3 H, CH₃), 1.66–1.63 (m, 5 H, CH₂, partially overlapping with signal from H₂O), 1.26 (s, 3 H, CH₃), 1.15 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₇H₅₆NO₁₄ [M⁺ + H] 858.3701, found 858.3696.

3'-N-(trans-2-Hexenoyl)-3'-N-debenzoylepaclitaxel (16). The above procedure with *trans*-2-hexenoic acid afforded **16** (56%). ¹H NMR (CDCl₃, 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, CH₂), 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, CH₂), 2.46 (bs, 1 H, OH), 2.37 (s, 3 H, CH₃), 2.33–2.27 (m, 1 H, CH₂), 2.25 (s, 3 H, CH₃), 2.13–2.08 (m, 2H, CH₂), 1.91–1.84 (m, 1 H, CH₂), 1.81 (d, ⁴J(H,H) = 1.1 Hz, 3 H, CH₃), 1.68 (s, 3 H, CH₃), 1.64–1.59 (m, 1 H, CH₂, partially overlapping with signal from H₂O), 1.44–1.35 (m, 2H, CH₂), 1.26 (s, 3 H, CH₃), 1.15 (s, 3 H, CH₃), 0.87 (t, ³J(H,H) = 7.3 Hz, 3 H, CH₃). HRMS (FAB+): calcd for C₄₆H₅₆NO₁₄ [M⁺ + H] 846.3701, found 846.3707.

3'-N-(Hexanoyl)-3'-N-debenzoylepaclitaxel (17). The above procedure with hexanoic acid afforded **17** (56%). ¹H NMR (CDCl₃, 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, CH₂), 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, CH₂), 2.47 (bs, 1 H, OH), 2.34 (s, 3 H, CH₃), 2.32–2.25 (m, 1 H, CH₂), 2.25 (s, 3 H, CH₃), 2.19 (t, ³J(H,H) = 7.6 Hz, 2H, CH₂), 1.93–1.84 (m, 1 H, CH₂), 1.82 (d, ⁴J(H,H) = 1.13 Hz, 3 H, CH₃), 1.68 (s, 3 H, CH₃), 1.65–1.53 (m, 3 H, CH, CH₂), 1.28–1.22 (m, 4H, CH₂), 1.27 (s, 3 H, CH₃), 1.15 (s, 3 H, CH₃), 0.83 (t, ³J(H,H) = 7.0 Hz, 3 H, CH₃). HRMS (FAB+): calcd for C₄₆H₅₈NO₁₄ [M⁺ + H] 848.3857, found 848.3864.

3'-N-Debenzoylepaclitaxel-3'-N-isopropoxyloxycarbonylpaclitaxel (18). 3'-N-Debenzoylepaclitaxel **14** (10.0 mg, 13.3 μmol) was dissolved in CH₂Cl₂ (0.5 mL) and a saturated solution of NaHCO₃ (0.5 mL) was added. Then isopropoxyloxycarbonyl chloride (1.7 μL, 17 μmol) was added with vigorous stirring. After 1 h, the next portion of isopropoxyloxycarbonyl 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 MgSO₄ 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% CH₃CN 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%). ¹H NMR (CD₃OD, 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, CH₂), 3.82 (d, ³J(H,H) = 7.1 Hz, 1 H, CH), 2.50–2.42 (m, 1 H, CH₂), 2.33 (s, 3 H, CH₃), 2.28–2.19 (m, 1 H, CH₂), 2.17 (s, 3 H, CH₃), 2.00–1.95 (m, 1 H, CH₂), 1.92 (s, 3 H, CH₃), 1.84–1.77 (m, 1 H, CH₂), 1.66 (s, 3 H, CH₃), 1.19 (d, ³J(H,H) = 6.4 Hz, 6 H, CH₃), 1.17 (s, 3 H, CH₃), 1.16 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₄H₅₄NO₁₅ [M⁺ + H] 836.3493, found 836.3502.

3'-N-n-Butyloxycarbonyl-3'-N-debenzoylepaclitaxel (19). The above procedure with *n*-butyloxycarbonyl chloride afforded **19** (93%). ¹H NMR (CD₃OD, 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, CH₂), 4.05–3.94 (m, 2 H, CH, CH₂), 3.82 (d, ³J(H,H) = 7.3 Hz, 1 H, CH), 2.50–2.42 (m, 1 H, CH₂), 2.34 (s, 3 H, CH₃), 2.29–2.18 (m, 1 H, CH₂), 2.17 (s, 3 H, CH₃), 2.03–1.97 (m, 1H, CH₂), 1.92 (s, 3 H, CH₃), 1.84–1.77 (m, 1 H, CH₂), 1.66 (s, 3 H, CH₃), 1.61–1.55 (m, 2 H, CH₂), 1.38–1.29 (m, 2H, CH₂), 1.18 (s, 3 H, CH₃), 1.16 (s, 3 H, CH₃), 0.90 (t, ³J(H,H) = 7.2 Hz, 3 H, CH₃). HRMS (FAB+): calcd for C₄₆H₅₆NO₁₅ [M⁺ + H] 872.3469, found 884.3477.

3'-N-Benzoyloxycarbonyl-3'-N-debenzoylepaclitaxel (20). The above procedure with benzoyloxycarbonyl chloride afforded

20 (95%). ^1H NMR (CD_3OD , 400 MHz): δ = 8.10 (d, $^3J(\text{H,H})$ = 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, $^3J(\text{H,H})$ = 8.9 Hz, 1 H, CH), 5.64 (d, $^3J(\text{H,H})$ = 7.1 Hz, 1 H, CH), 5.20 (d, $^3J(\text{H,H})$ = 4.6 Hz, 1H, CH), 5.08, 5.05 (2d, $^2J(\text{H,H})$ = 12.6 Hz, 2 H, CH_2), 4.98 (dd, $^3J(\text{H,H})$ = 1.9, 9.6 Hz, 1H, CH), 4.55 (d, $^3J(\text{H,H})$ = 4.7 Hz, 1 H, CH), 4.30 (dd, $^3J(\text{H,H})$ = 6.6, 11.0 Hz, 1 H, CH), 4.19, 4.17 (2d, $^2J(\text{H,H})$ = 9.2 Hz, 2 H, CH_2), 3.81 (d, $^3J(\text{H,H})$ = 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 $\text{C}_{48}\text{H}_{54}\text{NO}_{15}$ [$\text{M}^+ + \text{H}$] 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. Benzylloxycarbonyl 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 ^1H NMR and HPLC analysis). ^1H 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, $^3J(\text{H,H})$ = 7.3 Hz, 2 H, CH), 6.42 (bs, 1 H, CH), 5.50 (bs, 1 H, CH), 4.92, 4.77 (2d, $^2J(\text{H,H})$ = 12.3 Hz, 2 H, CH_2), 4.59 (d, $^3J(\text{H,H})$ = 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, $^3J(\text{H,H})$ = 7.3 Hz, 2 H, CH), 6.50 (bs, 1 H, CH), 5.34 (bs, 1 H, CH), 5.09, 5.05 (2d, $^2J(\text{H,H})$ = 12.4 Hz, 2 H, CH_2), 4.88 (d, $^3J(\text{H,H})$ = 3.5 Hz, 1 H, CH), 3.83 (s, 3 H, CH_3), 3.59 (s, 3 H, CH_3). HRMS (FAB+): calcd for $\text{C}_{26}\text{H}_{26}\text{NO}_6$ [$\text{M}^+ + \text{H}$] 448.1760, found 448.1763.

13-(3'-*N*-Benzylloxycarbonylphenylisoserine)-10-deacetyl-7,10-dibenzylloxycarbonylbaccatin 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-dibenzylloxycarbonylbaccatin 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, satu-

rated 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%). ^1H NMR (CD_3OD , 400 MHz): δ = 8.10 (d, $^3J(\text{H,H})$ = 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, $^3J(\text{H,H})$ = 8.7 Hz, 1H, CH), 5.65 (d, $^3J(\text{H,H})$ = 7.1 Hz, 1 H, CH), 5.52 (dd, $^3J(\text{H,H})$ = 7.2, 10.5 Hz, 1 H, CH), 5.22 (d, $^3J(\text{H,H})$ = 4.6 Hz, 1 H, CH), 5.20–4.99 (m, 7 H, CH, CH_2), 4.57 (d, $^3J(\text{H,H})$ = 4.8 Hz, 1 H, CH), 4.20, 4.18 (2d, $^2J(\text{H,H})$ = 9.2 Hz, 2 H, CH_2), 3.90 (d, $^3J(\text{H,H})$ = 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 $\text{C}_{62}\text{H}_{64}\text{NO}_{18}$ [$\text{M}^+ + \text{H}$] 1111.4123, found 1111.4127.

13-(3'-*N*-Benzylloxycarbonyl-2'-*tert*-butoxycarbonylphenylisoserine)-10-deacetyl-7,10-dibenzylloxycarbonylbaccatin 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%). ^1H 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, $^3J(\text{H,H})$ = 8.9 Hz, 1H, CH), 5.71 (bd, 1 H, CH, partially overlapping with next signal), 5.68 (d, $^3J(\text{H,H})$ = 7.1 Hz, 1 H, CH), 5.56 (dd, $^3J(\text{H,H})$ = 7.0, 10.7 Hz, 1 H, CH), 5.47 (bd, $^3J(\text{H,H})$ = 9.0 Hz, 1 H, CH), 5.28 (bs, 1 H, NH), 5.21, 5.16 (2d, $^2J(\text{H,H})$ = 11.9 Hz, 2 H, CH_2), 5.14 (s, 2 H, CH_2), 5.07, 5.00 (2d, $^2J(\text{H,H})$ = 12.5 Hz, 2 H, CH_2), 4.95 (d, $^3J(\text{H,H})$ = 7.9 Hz, 1H, CH), 4.32, 4.18 (2d, $^2J(\text{H,H})$ = 8.5 Hz, 2 H, CH_2), 3.93 (d, $^3J(\text{H,H})$ = 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 $\text{C}_{67}\text{H}_{72}\text{NO}_{20}$ [$\text{M}^+ + \text{H}$] 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. ^1H NMR (CD_3OD , 400 MHz): δ = 8.08–8.06 (m, 2 H, CH), 7.74–7.70 (m, 1 H, CH), 7.64–7.60 (m, 2 H, CH), 7.57–7.50 (m, 4 H), 7.39–7.34 (m, 1 H, CH), 5.98 (dt, $^4J(\text{H,H})$ = 1.1 Hz, $^3J(\text{H,H})$ = 9.2 Hz, 1 H, CH), 5.59 (d, $^3J(\text{H,H})$ = 7.1 Hz, 1 H, CH), 5.28 (d, $^3J(\text{H,H})$ = 8.8 Hz, 1H, CH), 5.22 (s, 1 H, CH), 4.97 (dd, $^3J(\text{H,H})$ = 2.0, 9.7 Hz, 1H, CH), 4.76 (d, $^3J(\text{H,H})$ = 8.8 Hz, 1 H, CH), 4.22–4.14 (m, 3 H, CH, CH_2), 3.78 (d, $^3J(\text{H,H})$ = 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, $^3J(\text{H,H})$

= 9.5 Hz, $^2J(\text{H,H}) = 15.4$ Hz, 1 H, CH₂), 1.88–1.77 (m, 1 H, CH₂), 1.84 (d, $^4J(\text{H,H}) = 1.3$ Hz, 3 H, CH₃), 1.66 (s, 3 H, CH₃), 1.55–1.47 (m, 1 H, CH₂), 1.52 (s, 9 H, CH₃), 1.11 (s, 3 H, CH₃), 1.09 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₄₃H₅₄NO₁₄ [M⁺ + H] 808.3544, found 808.3550. Anal. (C₄₃H₅₄ClNO₁₄·3H₂O) 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 μ L, 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. ¹H NMR (CD₃OD, 400 MHz): $\delta = 8.05$ – 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, $^4J(\text{H,H}) = 1.3$ Hz, $^3J(\text{H,H}) = 9.0$ Hz, 1 H, CH), 5.59 (d, $^3J(\text{H,H}) = 7.1$ Hz, 1 H, CH), 5.23 (s, 1 H, CH), 4.97 (dd, $^3J(\text{H,H}) = 2.1$, 9.6 Hz, 1H, CH), 4.58 (d, $^3J(\text{H,H}) = 8.6$ Hz, 1H, CH), 4.55 (d, $^3J(\text{H,H}) = 8.4$ Hz, 1 H, CH), 4.22–4.14 (m, 3 H, CH, CH₂), 3.80 (d, $^3J(\text{H,H}) = 7.1$ Hz, 1 H, CH), 2.46–2.39 (m, 1 H, CH₂), 2.21 (s, 3 H, CH₃), 1.96 (dd, $^3J(\text{H,H}) = 9.2$ Hz, $^2J(\text{H,H}) = 15.2$ Hz, 1 H, CH₂), 1.87 (d, $^4J(\text{H,H}) = 1.5$ Hz, 3 H, CH₃), 1.85–1.78 (m, 1 H, CH₂), 1.72 (dd, $^3J(\text{H,H}) = 9.0$ Hz, $^2J(\text{H,H}) = 15.2$ Hz, 1 H, CH₂), 1.67 (s, 3 H, CH₃), 1.12 (s, 3 H, CH₃), 1.09 (s, 3 H, CH₃). HRMS (FAB+): calcd for C₃₈H₄₆NO₁₂ [M⁺ + H] 708.3020, found 708.3024. Anal. (C₃₈H₄₆ClNO₁₂·2H₂O) 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 μ m 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 μ L of PBS (pH 7.4) was added 5 μ L of DMSO and 5 μ L 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 \times 150 mm; YMC Pack ODS AM302) reverse phase column with a binary solvent system: linear gradient of CH₃CN (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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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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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-positive 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 pRVS-*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 *Hind*III 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 pRVS*neo* (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

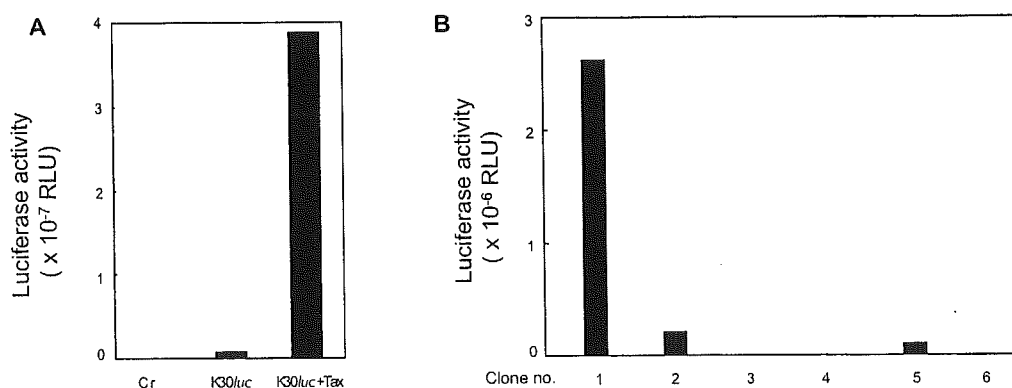


Fig. 1. Activation of HTLV-1 (K30) LTR. (A) Response of the K30*luc* reporter construct to HTLV-1 Tax. 293T cells were co-transfected with pK30*luc* (10 μ g) and pUC19 (10 μ g) or with pK30*luc* (10 μ g) and pCG-Tax (10 μ g) as indicated, and 2 days later, cell lysates were prepared for luciferase assay. Control (Cr) 293T cells were singly transfected with 20 μ g 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.