ORGANIC LETTERS

2003
Vol. 5, No. 19
3459−3462

Arginine-Based Molecular Transporters: The Synthesis and Chemical Evaluation of Releasable Taxol-Transporter Conjugates

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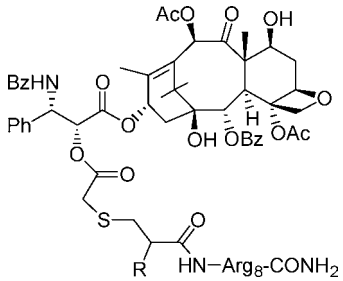
Received July 3, 2003

ABSTRACT



A flexible and efficient procedure has been developed for the conjugation of taxol to various arginine-based molecular transporters via the taxol C2′ *O*-chloroacetyl derivative. The resultant taxol-transporter conjugates are highly water soluble and release free taxol with half-lives of minutes to hours depending on the pH and the linker structure.

Taxol's[1] unprecedented structure[2] and novel mode of action[3] have provided the basis for major advances in chemistry,[4] biology,[5] and medicine, leading to its use in the treatment of human ovarian and breast cancer.[6] Notwithstanding this progress, taxol suffers from very low water solubility and as a consequence must be formulated for therapeutic use with Cremophor EL.[7] Concerns about this vehicle have prompted considerable interest in the development of water-soluble derivatives of taxol.[8] Many of these derivatives are functionalized at the C2′ position of taxol with a water-solubilizing group that must be cleaved to release free taxol before cellular uptake.[9,10]

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(1) Taxol is the registered trademark for the molecule with the generic name paclitaxel.

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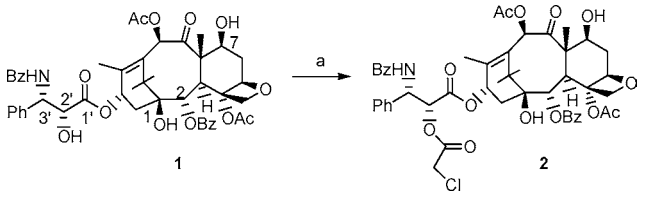
10.1021/ol035234c CCC: $25.00
Published on Web 08/26/2003

Recently, several classes of peptide molecules have been identified that are water-soluble and undergo facilitated uptake into cells and tissues.[11] Among these molecular transporters, peptide sequences found in HIV-tat and in Antennapedia have shown high membrane translocation efficiencies.[12] Extensive structure−function studies of the HIV-tat transporter sequence (RKKRRQRRR) have led to the finding that short oligomers of arginine- (R_{7-9}) and, more specifically, guanidinium-based oligomers often exhibit superior membrane translocation activity.[13] Importantly, these homo-oligomers can be prepared in a more cost-efficient manner than conventional hetero-oligomers by using a segment-doubling strategy.[14] Of special note, the arginine-based transporters have also been shown to penetrate human skin, leading to the entry of a releasable oligo-arginine Cyclosporin A conjugate into human trials for the treatment of dermatological disorders.[15] In contrast to simple solubilizing functionalities, a particularly significant property of these transporters is that they both enhance water solubility *and* facilitate uptake through the nonpolar bilayer of a cell. This obviously represents a potentially useful strategy for improving the formulation and bioavailability of drugs such as taxol. To explore this strategy, an efficient and flexible procedure for the conjugation of taxol to arginine-based transporters is required. In addition to being compatible with the sensitive functionality of taxol, this procedure would need to be scalable and sufficiently flexible to produce taxol conjugates with tunable half-lives for release under physiological conditions. We report here a versatile procedure for the preparation of taxol-transporter conjugates and an evaluation of their ability to release free taxol in PBS buffer at 37 °C.

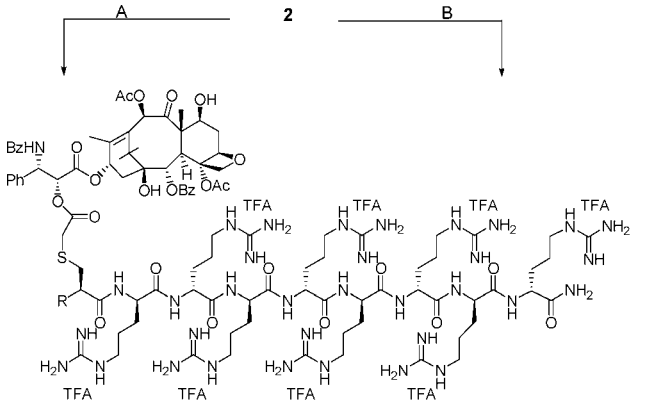
Scheme 1. Chloroacetylation of Taxol[a]



[a] Reagents and conditions: (a) $(ClAc)_2O$, DIEA, DCM, rt, quantitative.

The strategy devised for release of taxol from the transporter is based on the observation that taxol can be esterified at C2′ without protection of other functional groups. If the attached ester incorporated a suitably positioned and protected amine (e.g., **3**, Scheme 2), release of free taxol could then be controlled by pH-dependent release of the free amine for reaction with the C2′ ester carbonyl.[16,17] The participation of the amine in ester cleavage could also be modulated by conversion to simple amide or other less reactive derivatives. To explore this approach, taxol was initially converted to the C2′ chloroacetyl derivative **2**

Scheme 2. Synthesis of Taxol Conjugates



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(Scheme 1) in essentially quantitative yield by reaction with chloroacetic anhydride. This process can be easily scaled [~10 mg to over 2 g], and the product can be obtained after two aqueous washings (0.1 M HCl, bicarbonate). Derivative **2** obtained according to this procedure is sufficiently pure for all further transformations.[18]

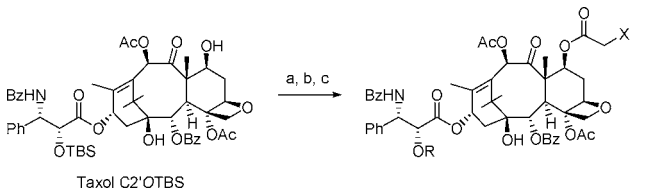
Attachment of **2** to a transporter was conducted in two ways. Method A, preferred in cases when an N-terminal cysteine residue of the transporter can be incorporated into a solid-phase peptide synthesis, entails thioether formation by halogen displacement with a cysteinyl sulfur. Alternatively, method B involves a stepwise assembly. A sulfhydryl-containing free acid is used in the thioether formation step, and the resultant acid, following in situ activation, is coupled to the N-terminal free amine of octa-arginine. Method B is generally more flexible, since it allows for the use of any sulfhydryl-containing acid.[19] Method B is favored in a process that relies on octa-arginine synthesis via segment-doubling methodology. Under optimized conditions, both methods are high-yielding and reliable.

Illustrative of method A is the synthesis of conjugate **3**. The efficiency of this single-step process is dependent on the concentration of reactants, molar equivalents of tertiary amine base, and the temperature. High concentrations [~0.1 M] and low temperatures [0 °C] favor the intermolecular coupling and suppress the intramolecular release of taxol. An excess of amine base (beyond 1.5 equiv) leads to premature taxol release as expected from the pH-dependent mechanism of release. The conjugates **4** and **5** were synthesized in the presence of larger DIEA amounts, since no release of parent drug under the conjugation conditions was observed (method A). The chemoselectivity of this approach presumably results from the higher acidity of the thiol relative to the ammonium group and the greater nucleophilicity of the resultant thiolate.[20]

The synthesis of conjugate **6** is representative of method B, which is a three-step one-flask operation. The initial displacement was conducted with 1 molar equiv of *N*-Boc-Cys-OH and 2 equiv of DIEA. This ratio accelerated the thioether formation and allowed for the consecutive carboxylate activation with *iso*butylchloro formate. This coupling reagent was well suited for our study and allowed for a selective amide bond formation to the primary N-terminal amine of the octa-arginine.[21] No interference of the guanidinium headgroups of arginine was observed during the conjugate synthesis and purification, as expected from the strongly basic character and tight association of the guanidinium ions with TFA counterions.[22] The tertiary amine DIEA is not sufficiently basic to deprotonate the guanidinium headgroup. All final products were isolated as white powders after RP-HPLC purification and lyophilization. Conjugate **6** was efficiently converted to the acetate counterion form (**8**) with the aid of an ion-exchange resin.[23] Using the conditions described for the synthesis of conjugate **6** allowed the corresponding octa-(*L*)-arginine-based conjugate **9** to be synthesized (63% yield) and converted to the acetate form **10** as described above. The coupling procedure was found to be general for a variety of transporters.

While conjugates **3**−**10** incorporate different linker features, they are all C2′ taxol derivatives. Therefore, a conjugate was prepared that utilized the C7 hydroxyl group as the point of attachment. The synthesis is depicted in Scheme 3. Employing a TBS protecting group at the C2′

Scheme 3. Synthesis of Conjugate **13**[a]



[a] Reagents and conditions: (a) $(ClAc)_2O$, DIEA, DCM, rt, 94%; (b) HF/pyr, THF, rt, 74%; (c) NH_2-(*L*)-Cys-(*D*)-Arg_8−$CONH_2$ (9TFA), DIEA, DMF, rt, 24%.

alcohol,[24] the chloroacetylation at the C7 hydroxyl was performed in high yield by using an excess of acylating agent and amine base. Deprotection of the TBS group with HF/pyr yielded the coupling precursor **12**. The conjugation to the thiol of NH_2-(*L*)-Cys-(*D*)-Arg_8-$CONH_2$ was considerably slower and yielded the product in 24% along with unreacted starting material.

In all, nine conjugates were prepared. As expected from the solubilizing effect of the arginine-based transporters, all were readily water soluble. In the case of **6**, for which solubility was measured, the conjugate was >1000 times more soluble in water than taxol.[25] The half-life for release of free taxol was determined for each conjugate under physiological conditions (e.g., PBS-buffer, pH = 7.4, T = 37 °C). Taxol was released from all conjugates without detectable intermediates and characterized by MS analysis and reversed-phase HPLC retention time in comparison to an authentic sample. The results of this study are summarized in Table 1.

As desired for in vitro and in vivo evaluation, the half-lives of these conjugates vary over a range of times from 1 min up to almost 4 h under the described conditions. Compound **3** released taxol almost instantaneously. A facile hydrolytic cleavage at the C2′ ester was observed for

(18) This compound was mentioned in ref 8c but was described to be too unstable for synthetic application in an aqueous environment.

(19) Free primary and secondary amines are excluded.

(20) Textbook values for Cys: RNH_3^+, 10.3; SH, 8.3. For a recent study about the influence of charge environment on the pK_a of cysteine thiols, see: Lutlof, M. P.; Tirelli, N.; Cerritelli, S.; Cavalli, L.; Hubbell, J. A. *Bioconjugate Chem.* **2001**, *12*, 1051−1056.

(21) In general, the use of DCC gave reduced yields in these coupling reactions.

(22) Plapinger, R. E.; Nachlas, M. M.; Seligman, M. L.; Seligman, A. M. *J. Org. Chem.* **1965**, *30*, 1781−1785.

(23) For most biological applications TFA counterions are undesirable.

(24) Magri, N. F.; Kingston, D. G. I. *J. Nat. Prod.* **1988**, *51*, 298−306.

(25) Aqueous solubility: Taxol < 4 μg/mL; conjugate **6** > 100 mg/mL. For the taxol value, see ref 10e.

Table 1. Half-Lives of Selected Conjugates[a]

compound	half-life	compound	half-life
3	1 min	**7**	140 min
4	61 min	**8**	104 min
5	103 min	**9**	107 min
6	107 min	**13**	214 min

[a] Measurements were performed in PBS buffer at pH = 7.4 and $T = 37$ °C. Only starting material, released peptide, and taxol were observed under these conditions.

conjugates **4**−**8**, with half-lives ranging from 1 to 2.5 h. The incorporation of a sulfur heteroatom at the α-position of the C2′ ester led to increased hydrolysis rates. No effect of the stereochemistry of the octa-arginine on the half-life was detected. Compound **13** showed the longest half-life despite the similarity in release mechanism to **3**, indicating that the position of cargo attachment also influences release rates.

The dependence of half-life on solution pH (adjusted PBS buffer, 37 °C) was measured for compounds **3** and **6**. The results are given in Figures 1 and 2, respectively. The graphs

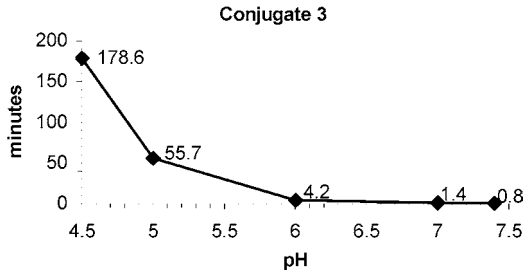


Figure 1. Dependence of the half-life of conjugate **3** on the pH. Half-lives are given in minutes.

clearly show a correlation of conjugate stability and pH. For conjugate **3** the effect is very pronounced, since the protonation state of the N-terminal amine is crucial for the cyclization and taxol release. A steep increase of stability is observed when adjusting the pH to 5 and below. A similar trend is seen for conjugate **6**.

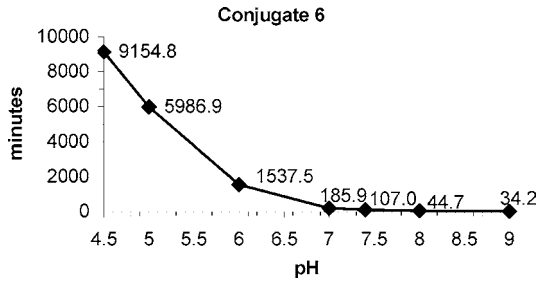


Figure 2. Dependence of the half-life of conjugate **6** on the pH. Half-lives are given in minutes.

In conclusion, we have shown that conjugation of taxol to arginine-based transporters provides conjugates with greatly enhanced water solubility. A therapeutically meaningful dose of taxol (300 mg) as its conjugate can be dissolved in 10 mL of water. A significant aspect of this work is the development of procedures for the conjugation of water-soluble transporters with a complex drug cargo possessing poor water solubility, a procedure that can be used for related problems involving transporter-drug conjugate synthesis. Additionally, a novel and general prodrug strategy for the release of drugs incorporating a hydroxyl group has been described. The rate of release of the free drug can be tuned and controlled by the structural features of the linker. Studies of these and related conjugates and their use in tissue and animal uptake assays will be reported shortly.

Acknowledgment. Support for this work by grants (P.A.W.) from the National Institutes of Health (CA31841, CA31845) is gratefully acknowledged. The authors thank D. Davalian, T. Flaherty, E. Kreider, S. Naganathan, L. Sista, and L. Wright for valuable discussions.

Supporting Information Available: Experimental procedures for the preparation of compounds **2**−**13** and their respective characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

OL035234C