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Design and synthesis of immobilized Tamiflu analog on resin for affinity chromatography

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ABSTRACT

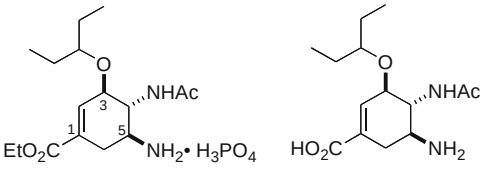
A resin linked with the Tamiflu core was synthesized by modifying our original synthetic route of oseltamivir phosphate (Tamiflu). The prepared resin bound to the influenza virus enzyme neuraminidase, the main target of Tamiflu. The immobilized Tamiflu analog will be useful for isolating and identifying presumed endogenous vertebrate proteins that interact with Tamiflu, which might relate to the abnormal behavior exhibited by some influenza patients treated with Tamiflu.

Oseltamivir phosphate (**1**: Tamiflu®, Fig. 1)[1] exhibits potent antiviral activity against influenza viruses type A and B by selectively inhibiting neuraminidase (NA), an essential enzyme for the release of virions from infected cells to expand infection. Tamiflu is a prodrug, and its active form is the corresponding carboxylic acid Ro 64-0802 (**2**).

Tamiflu is a very effective drug for treating influenza patients at the early stage of infection. In quite rare cases, abnormal behaviors (such as hallucinations and impulsive behavior) have been reported in Japanese patients, especially those under age of 20, after taking Tamiflu. Several research groups, including ours, are investigating whether there is any molecular-level correlation between Tamiflu medication and the abnormal behaviors.[2] Based on our previous studies,[2b–d] we hypothesized that an endogenous human protein is specifically affected by Tamiflu (possibly, in the central nervous system). Affinity chromatography is a direct method for detecting the existence of biomolecules that interact with a particular organic molecule of interest. Here, we report the design, synthesis, and functional assessment of an immobilized Tamiflu analog on resin, which might be useful for affinity chromatography to identify possible endogenous vertebrate biomolecular targets of Tamiflu.

Our design of the immobilized Tamiflu analog is based on the assumption that the binding mode of **2** to potential target vertebrate proteins is similar to that for binding virus NA. Analysis of the X-ray crystal structures of the NA–**2** complex[1a,3] and the reported structure–activity relationship[1a] led to the identification of the following two major bases for the molecular design: (1) modifications of the C3 pentyloxy part do not completely disrupt the binding to NA, although modifications of the C1 carboxyl group, the C4 acetamide moiety, and the C5 amino functionality do disrupt binding to NA. Therefore, the C3 ether part was selected to connect the oseltamivir core to the resin. (2) The binding site of **2** is positioned close to the surface of NA,[3a] suggesting that a relatively short spacer (five carbons) between the oseltamivir core and the resin would be sufficient for binding to NA. Based on these considerations, we designed **3** immobilized to Affi-Gel 10® resin (Fig. 2).

The synthesis of **3** should be straightforward by extending the previously established synthetic route of Tamiflu.[1n] Briefly, this



Tamiflu® (**1**) Ro 64-0802 (**2**)

Figure 1. Structure of Tamiflu® and Ro 64-0802.

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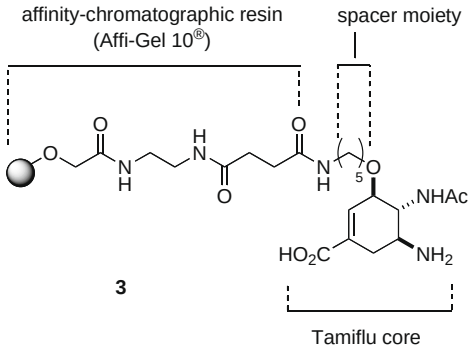
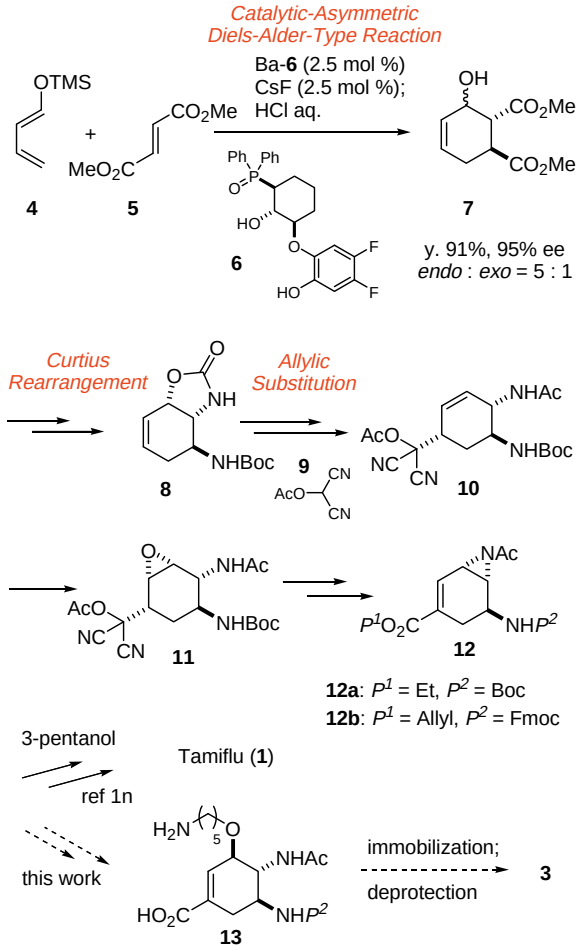


Figure 2. The structure of the designed immobilized Tamiflu analog.

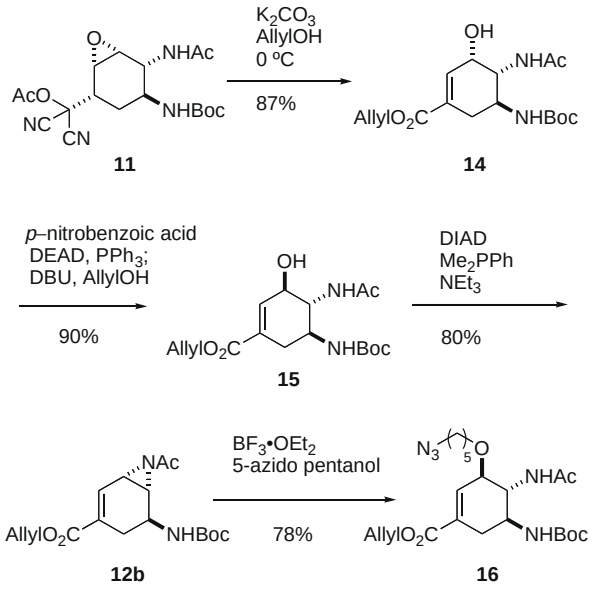


Scheme 1. Brief summary of the previously developed synthetic route of Tamiflu (**1**) and synthetic plan for the immobilized Tamiflu analog **3**.

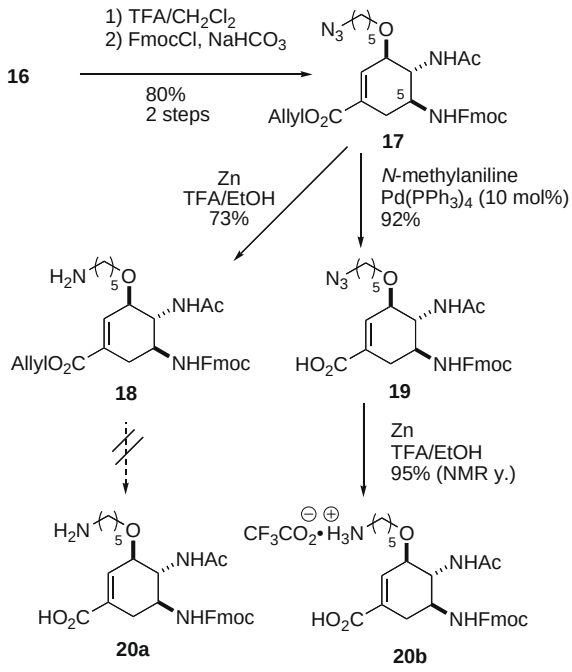
route involves an asymmetric Diels–Alder-type reaction between siloxy diene **4** and dimethyl fumarate (**5**) catalyzed by a barium–FujiCAPO (**6**, CAPO = CAtechol Phosphine Oxide) complex, a Curtius rearrangement, and Pd-catalyzed allylic substitution with MAC reagent **9** (MAC = Masked Acyl Cyanide) as key steps (Scheme 1). Introduction of 5-aminopentan-1-ol (or its equivalent) through a ring-opening reaction of an aziridine (**12**) would produce **13** containing an oseltamivir core with a spacer, which should be immobilized to Affi-Gel 10 via simple amide formation. The proper selection of the protecting groups at the C1 carboxylic acid moiety (P^1) and the C5 amino group (P^2) is crucial for the success of the synthesis. An Fmoc group was selected as P^2 because it can be cleaved under mild conditions without affecting the agarose skeleton of the resin. Moreover, the amount of immobilized oseltamivir core can be quantified based on the amount of released fluorenyl derivative (**22**). P^1 should be removed in the presence of the Fmoc group, and therefore we selected an allyl group for P^1 (**12b**).

Our synthesis of **3** started from known epoxide **11**, which was synthesized following the previously developed procedures shown in Scheme 1.[1n] Treatment of **11** with potassium carbonate in allyl alcohol yielded allyl ester **14** via alcoholysis of the acetoxydicyanomethyl moiety and the subsequent eliminative epoxide opening (Scheme 2). Inversion of α-alcohol **14** to β-isomer **15** was realized via S_N2 attack of *p*-nitrobenzoic acid under Mitsunobu conditions, followed by selective cleavage of the *p*-nitrobenzoate. DBU was used as a base in the alcoholysis step because previously utilized $LiOH{\cdot}H_2O$[1n] was not effective due to its low solubility in allyl alcohol. Mitsunobu aziridine formation using dimethylphenyl phosphine and DIAD in the presence of a catalytic amount of triethylamine[1n] afforded **12b** in high yield. Next, the aziridine opening reaction with 5-azido-1-pentanol required some optimization. The use of a large excess of azido alcohol[4,5] was important in this reaction: ether **16** was obtained in 78% yield in the presence of 1.5 equiv of $BF_3{\cdot}OEt_2$ and 30 equiv of 5-azido-1-pentanol. The excess azido alcohol was recovered for reuse in more than 70% yield after chromatographic separation.

With ether **16** in hand, the next key intermediate was amino acid **20a** or **20b**. Conversion of the protecting group at the C5 amino group from Boc to Fmoc, reduction of the azide group, and cleavage of C1 allyl ester were required, but the order of the three transformations had to be optimized (Scheme 3). After converting the protecting group from Boc to Fmoc, which produced **17**, reduction of the azide group was attempted. Staudinger reduction using various phosphines did not afford target compound **18**. On the other hand, reduction proceeded in moderate yield in AcOH solvent using zinc powder pre-activated with 1 M HCl. Subsequent optimization regarding the method of zinc activation, the proton source, and the solvent led us to identify the optimum conditions: zinc powder pre-activated with dibromoethane and TFA/EtOH solvent. Amine **18** was isolated in 73% yield under these optimized conditions. Subsequent allyl ester cleavage using Pd catalysts, however, was not successful.[6]



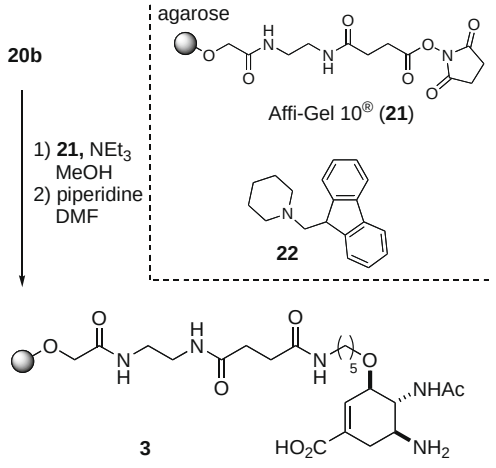
Scheme 2. Synthesis of ether **16**.



Scheme 3. Preparation of the Tamiflu core.

Therefore, allyl ester cleavage of **17** was next investigated (Scheme 3). The conversion proceeded in high yield (92%) in the presence of 10 mol % of $Pd(PPh_3)_4$ and *N*-methylaniline. Reduction of the azide in **19** under the above-mentioned optimized conditions afforded **20b** in excellent yield (95%). Because **20b** is water soluble and highly polar, **20b** was only partially purified by filtration through Celite to eliminate the excess zinc and resulting zinc salts.

The final step of the synthesis was linking **20b** to the chromatographic resin, Affi-Gel 10® (**21**; Scheme 4). The coupling reaction was performed using **20b** (**20b**: the activated ester residues of Affi-Gel 10 = 1:6) under slightly basic conditions (pH 8) in the presence of ca. 50 equiv Et_3N in MeOH at room temperature for 15 h. After the coupling reaction, the resin was separated by filtration and washed with MeOH.[7] Finally, removal of the Fmoc group and blocking of the remaining activated ester on the resin were conducted simultaneously using excess piperidine (in a volume equal to that of the undried resin) in DMF at room temperature for 24 h.[8]



Scheme 4. Coupling of Tamiflu core **20b** and Affi-Gel 10®.

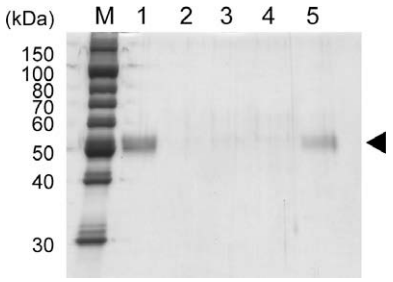


Figure 3. Binding of neuraminidase (rvH1N1NA) to resin-conjugated Tamiflu analog **3**. Binding assay of rvH1N1NA was performed for **3**. The eluted protein was analyzed by SDS–PAGE and was visualized by silver staining. A molecular weight of rvH1N1NA is 48 kDa, and it migrates as a 57 kDa band in SDS–PAGE. The position of rvH1N1NA was marked by the arrowhead. rvH1N1NA (lanes 3 and 5) or buffer (lanes 2 and 4) was applied for control resin (lanes 2 and 3) or **3** (lanes 4 and 5). *M*, molecular weight marker; lane 1, rvH1N1NA standard (0.5 μg).

Complete removal of the Fmoc group was confirmed by quantitative NMR analysis of **22**, a byproduct in the Fmoc cleavage reaction, which was recovered in the solution phase.

We then assessed the binding affinity of synthesized conjugate **3** to neuraminidase. The criterion in this preliminary binding study was whether NA binds to the resin. A control resin was prepared by reacting piperidine, instead of **20b**, with Affi-Gel 10®. Recombinant Influenza A virus H1N1 neuraminidase (rvH1N1NA, 0.5 μg, purchased from R&D Systems, Inc.) and 50 μL of each 40% resin (**3** or control resin) were mixed and incubated in 250 μL of Tamiflu binding buffer (50 mM Tris–HCl, 100 mM NaCl, 5 mM $CaCl_2$; pH 7.5) at 4 °C for 2 h. After washing the resin with 1 mL of Tamiflu-binding buffer five times, the resin was resuspended in SDS-sample buffer [3.3% SDS (sodium dodecyl sulfate), 16.7% glycerol, 133 mM Tris–HCl, pH 6.8, 2.5% β-ME (2-mercapoethanol), 0.03% BPB (bromophenol blue)] and was boiled for 2–3 min. The supernatant was subjected to electrophoresis on SDS–10% polyacrylamide gel, and was visualized with silver-staining. rvH1N1NA was detected in the bound fraction at the expected position on the gel when rvH1N1NA was mixed with resin-conjugated Tamiflu analog **3** (Fig. 3, lane 5). rvH1N1NA was not detected in other lanes on the gel (Fig. 3, lanes 2–4). The control result revealed that Tamiflu analog **3** has a binding activity to rvH1N1NA.

In conclusion, we synthesized an immobilized Tamiflu analog on resin by modifying our original synthetic route of Tamiflu.[1n] This study demonstrated the highly flexible nature of our synthetic route. The prepared resin bound to NA, the main target of Tamiflu. Studies are ongoing to identify Tamiflu's possible endogenous target biomolecules in vertebrates using affinity chromatography of the synthesized resin and/or its derivatives.

Acknowledgments

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5. The reaction with 4 equiv or 10 equiv of the alcohol gave low yields (approximately 20% and 40%, respectively) of the ether **16**.
6. The allyl ester removal from **18** using catalytic $Pd(PPh_3)_4$ afforded a complex mixture.
7. Approximately, 40% of unreacted **20b** was recovered based on NMR analysis of the combined solution phases.
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