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Synthesis of Biotinylated Episilvestrol: Highly Selective Targeting of the Translation Factors eIF4AI/II

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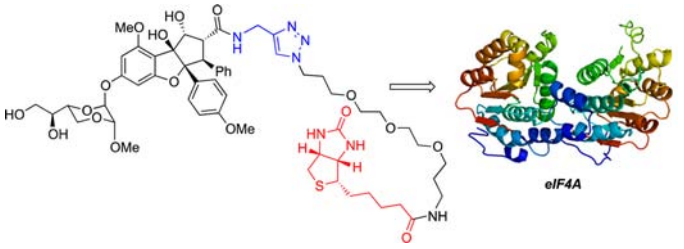
ABSTRACT



Silvestrol (1) and episilvestrol (2) are protein synthesis inhibitors, and the former has shown efficacy in multiple mouse models of cancer; however, the selectivity of these potent cytotoxic natural products has not been described. Herein, it is demonstrated that eukaryotic initiation factors eIF4AI/II were the only proteins detected to bind silvestrol (1) and biotinylated episilvestrol (9) by affinity purification. Our study demonstrates the remarkable selectivity of these promising chemotherapeutics.

The approval of omacetaxine mepesuccinate (homoharringtonine) by the U.S. Food and Drug Administration (FDA) for the treatment of chronic myeloid leukemia has validated targeting the protein synthesis (translation) machinery for the treatment of cancer. While omacetaxine mepesuccinate inhibits translation elongation, the more recently discovered translation initiation inhibitor silvestrol (**1**) prevents ribosome recruitment.[1] Both **1** and 5‴-episilvestrol or episilvestrol (**2**) have demonstrated potent cytotoxic activity against many human cancer cell lines, including lung, prostate, and breast cancer with IC_{50} values ranging from 1 to 7 nM.[2−4] Moreover, **1** has shown remarkable activity against B-cells isolated from patients with chronic lymphocytic leukemia as well as in vivo activity against acute lymphoblastic leukemia in a mouse xenograft.[5] The natural product **1** can be metabolized into silvestric acid, which was inactive in a leukemia cell line.[6]

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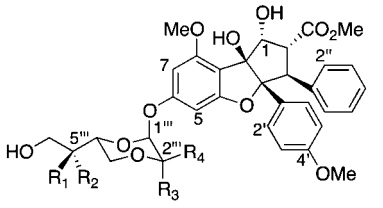
Silvestrol can induce multiple forms of cell death, which varies between cell types.[7]

Both **1** and **2** are metabolites of *Aglaia*, which were isolated independently by two groups.[2,3] These compounds bear a unique 1,4-dioxylanyloxy structural moiety that is connected to a cyclopentabenzofuran 'rocaglate'[8] ring system. Two additional isomers were later identified from another *Aglaia* species as the diastereoisomers 2‴-episilvestrol (**3**) and 2‴,5‴-diepisilvestrol (**4**) (Figure 1).[4]



1 $R^1 = R^4 = H$; $R^2 = OH$; $R^3 = OMe$: **silvestrol**
2 $R^1 = OH$; $R^2 = R^4 = H$; $R^3 = OMe$: **5‴-episilvestrol**
3 $R^1 = R^3 = H$; $R^2 = OH$; $R^4 = OMe$: **2‴-episilvestrol**
4 $R^1 = OH$; $R^2 = R^3 = H$; $R^4 = OMe$: **2‴, 5‴-diepisilvestrol**

Figure 1. Structures of silvestrol (**1**), 5‴-episilvestrol (**2**), and related compounds.

The paucity of these compounds from natural sources has led to the total synthesis of **1**,[9−11] **2**,[10,11] and 2‴,5‴-diepisilvestrol (**4**),[12] as well as the unnatural analogues 4′-desmethoxyepisilvestrol[10] and 1‴,2‴,5‴-triepisilvestrol.[12] Pelletier and co-workers have demonstrated that silvestrol and selected rocaglate analogues inhibit translation initiation by modulating the activity of eIF4A and removing the RNA helicase from the eIF4F complex.[1,13,14] While a related rocaglamide 1-*O*-formylaglafoline which also targets eIF4A does not inhibit Ded1p activity or inhibit mRNA splicing,[1] the selectivity of silvestrol or episilvestrol has not been systematically defined. Herein, we disclose the remarkable and unusual selectivity silvestrol and episilvestrol for eIF4AI and eIF4AII.

As it is not practical to obtain silvestrol (**1**) and episilvestrol (**2**) from the natural source, the total synthesis of these compounds was required for these studies (Figure 1). Synthetic **1** and **2** were obtained as previously reported,[10,11] using recent modifications to the synthesis of the enantiomerically pure cyclopentabenzofuran core.[12] **2** is more accessible by our synthetic approach and has equivalent activity to **1** (Figure 2A), so **2** was selected for functionalization.

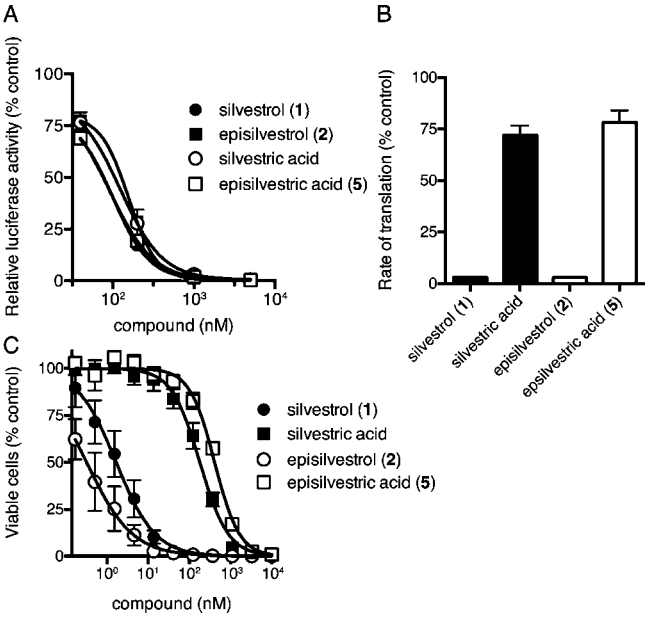


Figure 2. Biological activity of acid derivatives at the C2 position. (A) In vitro translations performed in rabbit reticulocyte lysate programmed with capped Firefly luciferase/HCV/Renilla luciferase mRNA. Data are represented as Firefly luciferase/Renilla luciferase (FF/Ren) relative to vehicle (DMSO) controls ($n = 3$). HCV-driven Renilla served as an internal control. (B) Rate of translation of endogenous mRNAs after 1 h treatment of MEFs with 1 μM compound ($n = 3-4$). (C) Cytotoxicity of compounds after 72 h. The number of viable cells was measured by the relative ATP concentration in MEFs after 72 h ($n = 3-4$). All graphs depict the mean and standard error of the mean (SEM).

In order to determine the protein target(s) of **1** and **2**, we sought an active analogue of **2** with a suitable biotin tag. Previous work has demonstrated that while modification at the 4′ position retained activity,[11] the addition of larger substituents at this location is not tolerated. In addition, analogues of the dioxane fragment often compromised the bioactivity;[15] therefore, the C2 ester was the next target for modification. We were originally hesitant to attempt functionalization of this site since it had been previously reported that the conversion of silvestrol to silvestric acid in plasma renders the derivative inactive.[6] Surprisingly, we found that silvestric acid, obtained by base hydrolysis[3] of synthetic **1**,[11] had a similar ability to inhibit cap-dependent translation as the parent compound in vitro (Figure 2A). Episilvestric acid (**5**), prepared by hydrolysis of synthetic **2** (Scheme 1), was also equally potent. We therefore

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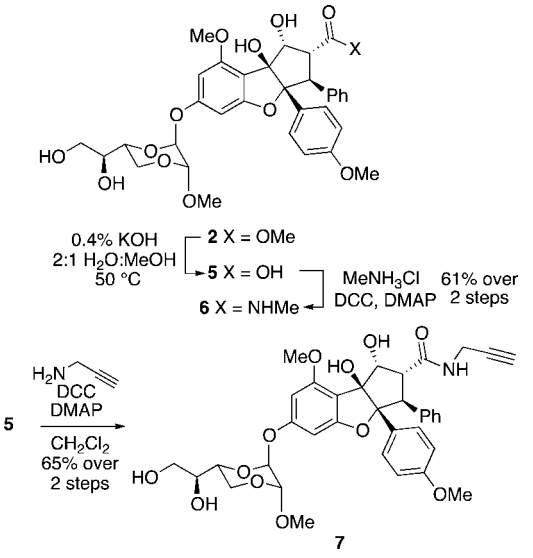
speculated that the reduction of activity observed previously was due to impairment of cellular uptake, not an inability to bind the target(s). Since hydrolysis may reduce the activity in vivo, it will be interesting to determine if compounds which cannot be metabolized into the acid will have increased potency in animal models.

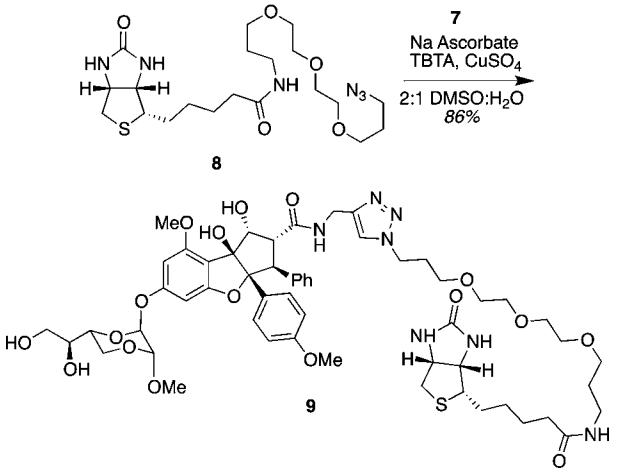
To examine this hypothesis, we compared the ability of **1** and **2**, along with silvestric acid[3] and **5**, to inhibit translation in mouse embryonic fibroblasts (MEFs), and indeed both acid derivatives were less potent (Figure 2B). In addition, both silvestric acid and episilvestric acid had a 100-fold increase in IC_{50} compared to their parent compounds when cytotoxicity was compared (Figure 2C). Therefore, while the acid metabolites could still efficiently inhibit translation in extracts, they had reduced activity when tested on intact cells. These results suggest that modification of silvestrol at C2 to prevent acid formation could increase cellular uptake and improve pharmacokinetics. With this new information, further C2 analogues were synthesized and their biological activity examined.

Scheme 1. Synthesis of Methyl **6** and Propargyl Amide **7** Derivatives of Episilvestrol (**2**)



Two secondary amide analogues of **2** were prepared by coupling **5** with the corresponding amine (Scheme 1). The coupling of **5** with methylamine or propargyl amine using DCC and DMAP afforded the methyl amide **6** and the propargyl amide **7** in reasonable yields over two steps. Incorporation of a terminal alkyne in amide **7** enabled biotinylation of **2** via a Huisgen 1,3-dipolar cycloaddition reaction (Scheme 2).[16] The biotin linker **8**[17] was synthesized following a modified procedure.[18] Cycloaddition in the presence of tris(benzyltriazolylmethyl)amine (TBTA) as a Cu(I) ligand[19] gave biotinylated episilvestrol **9** in a good yield.

Scheme 2. Synthesis of Biotinylated Episilvestrol (**9**)



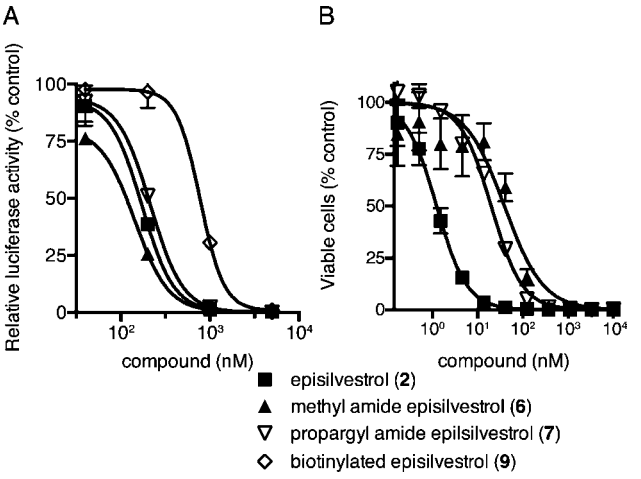


Figure 3. Biotinylated episilvestrol (**9**) inhibits protein synthesis. (A) In vitro translations in rabbit reticulocyte lysate. Data are represented as FF/Ren relative to vehicle (DMSO) controls (n = 4). (B) Viability of MEFs after 72 h treatment with indicated compounds (n = 3). All graphs depict the mean and standard error of the mean (SEM).

Amides **6** and **7** had similar activity as **2** for the inhibition of cap-dependent translation in vitro (Figure 3A) and also inhibited proliferation and/or the survival of fibroblasts (Figure 3B). Notably, while all have modifications of the C2 methyl ester, **6** and **7** were more potent than **5** (Figure 2C and 3B). Though the in vitro activity of biotinylated episilvestrol (**9**) was reduced (Figure 3A), we considered it sufficient to continue with a streptavidin pulldown assay.

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Using **9** and free biotin as a negative control, we performed pulldown experiments to determine the direct target(s) of **2**. Pulldown samples were washed in lysis buffer and eluted with successive treatments of 50 μM **1**, 25 mM glycine pH 2.5 at rt, and finally in sample buffer containing lithium dodecyl sulfate (LDS) at 95 °C. Elutions with silvestrol, low pH, or detergent yielded eIF4AI/II from samples containing **9**, but not free biotin (Figure 4A). Antibodies used in Western blots were specific for their respective eIF4A isoform, as determined with recombinant protein (Supporting Information Figure 1). In contrast to eIF4AI/II, eIF4AIII (DDX48)—another DEAD-box helicase with 65% identity to eIF4AI—was not eluted. DDX3 and DHX29, which are both DEAD/H-box helicases reported to function in protein synthesis,[20−22] were also not detected in any elutions (Figure 4A). As expected, the other two components of the eIF4F complex (eIF4G and eIF4E) were no longer bound to eIF4A in the presence of **9**.[13] Therefore, biotinylated episilvestrol and silvestrol are specific for eIF4AI and II with respect to the DHX helicases known to be involved in translation.

Even though the most prominent band visible after NuPAGE separation and silver staining was consistent with the size of eIF4AI/II (Figure 4B), we extended the study by analyzing the entire silvestrol and glycine elutions by mass spectrometry to determine if less prominent proteins would be detected. Strikingly, only peptides corresponding to eIF4A were found in samples containing **9** but not the negative control. Because of the high amino acid identity between eIF4AI and eIF4AII, 50% of the peptides were not able to be distinguished as coming from either eIF4AI or eIF4AII. No eIF4AII-specific sequences were detected by mass spectrometry, potentially because eIF4AII is a much less abundant protein.[23] Mass spectrometry showed that sequence coverage of eIF4AI was 35% and 75% for the silvestrol and glycine elutions, respectively (Supporting Information Figure 2). Of note, Prohibitin 1 and 2, which are the reported targets of another member of the rocaglamide family that acts upstream of the protein synthesis machinery,[24] were also absent.

In conclusion, we have demonstrated for the first time the direct interaction of silvestrol (**1**) and biotinylated episilvestrol (**9**) with eIF4AI and eIF4AII. This study was undertaken to determine unequivocally the protein targets of the anticancer compounds **1** and episilvestrol (**2**). Using an unbiased approach, we demonstrated that **1** and **2** are some of the most specific eIF4A-targeting translation inhibitors to date, validating these compounds as powerful chemical tools to elucidate the role of eIF4AI/II in both cell and cancer biology. Importantly, the exclusive specificity of these compounds is a highly desirable trait in drug development and one that is very rarely achieved with enzymatic inhibitors.[25] This insight furthers our belief that **1** and **2** warrant additional investigation as therapeutic candidates.

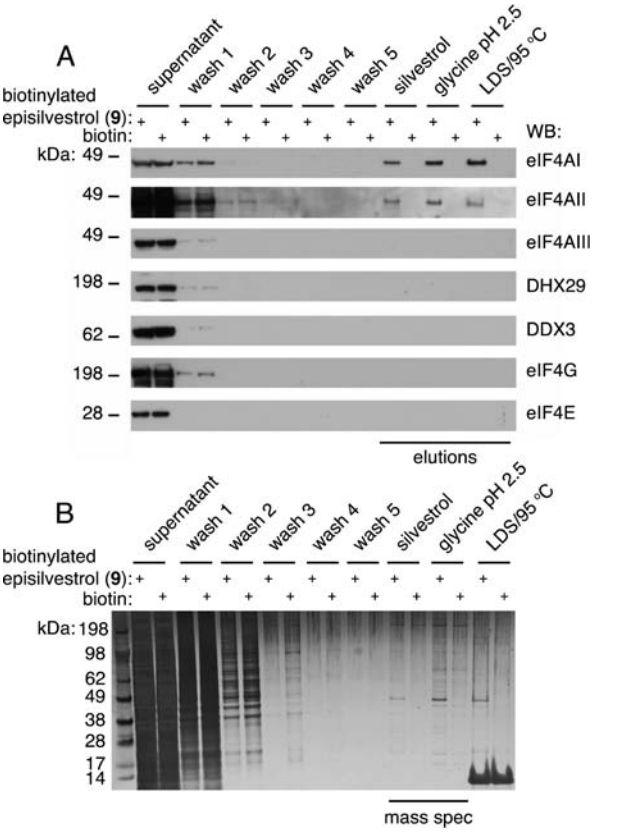

Figure 4. eIF4AI/II are specifically pulled down by **9**. (A) Streptavidin sepharose pulldowns with **9** or free biotin control were washed and eluted sequentially with 50 μM silvestrol, 25 mM glycine pH 2.5, and LDS loading buffer. Samples were separated by NuPAGE, transferred to polyvinylidene difluoride (PVDF), and probed with antibodies raised against the indicated proteins. (B) Pulldown samples visualized by silver stain after NuPAGE separation.

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Supporting Information Available. Experimental details as well as characterization data and copies of the NMR spectra of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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