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14. ABSTRACT

RSK (p90 Ribosomal S6 kinase) is critical for breast cancer proliferation and thus a promising target for therapeutic intervention. A highly specific inhibitor of RSK, called SL0101, was previously discovered but found to possess poor biological stability and potency. The purpose of this project is to identify a drug for breast cancer based on SL0101 that works by inhibiting RSK, by designing and chemically synthesizing analogues of SL0101 that improve on its biological stability and potency and ultimately evaluating them in a living human breast tissue model for anticancer activity. The major findings in this year of funding are the discovery of analogues that are more biologically stable than SL0101 but retain its anticancer activity, the solution of an X-Ray crystal structure of SL0101 in complex with RSK2, and the use of this crystal structure in the discovery of an analogue that in preliminary experiments demonstrates improved potency over SL0101. These discoveries represent significant steps toward the goal of identifying an analogue of SL0101 that could be used as a drug for breast cancer.

15. SUBJECT TERMS
breast cancer, p90 Ribosomal S6 kinase, RSK, kinase inhibitor, medicinal chemistry, biological stability

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Introduction

The p90 ribosomal S6 kinase (RSK) family of serine/threonine protein kinases, comprising at least four isoforms (RSKs 1-4), has been shown by our laboratory to be critical for breast cancer cell proliferation (*1*–*3*). In 2005 our laboratory reported the first specific inhibitor of RSK, SL0101 (**1**, Figure 1) (*3*). SL0101 inhibits RSK in both the breast cancer cell line MCF7 and the normal breast cell line MCF-10A, but only inhibits the proliferation of the breast cancer cell line (*1*–*3*). This indicates that breast cancer cells have become dependent on RSK and thus identifies RSK as a potential new target for cancer therapeutics. SL0101, given its exquisite specificity for RSK, is an attractive lead compound for medicinal chemistry efforts aimed at discovering a breast cancer drug that acts by inhibiting RSK. However, SL0101 itself is not suitable for further development as a drug for two reasons. First, we have determined that SL0101 has a very short biological half-life in mice (0.4 h at 2.5 mg/kg IP). In order to develop a drug this half-life must be improved so that the drug persists in the patient long enough to act on RSK. Second, SL0101 is a potent RSK inhibitor (IC_{50} = 89 nM), but a much less potent inhibitor of the proliferation of MCF7 breast cancer cells (EC_{50} = 50 μM), suggesting that it does not readily pass through the cell membrane (*3*). In order to develop a drug, the potency against MCF7 cells must be improved. The scope of this project is to design and chemically synthesize analogues of SL0101, with the assistance of a computational model of SL0101 bound to RSK, that improve on these deficiencies and to evaluate them both in vitro and in vivo with the goal of identifying a new breast cancer drug that acts by inhibiting RSK. In addition, the best analogues will be evaluated in our new living human breast tissue model (*4*) to gain insights in to the role of RSK in breast cancer that might not be gleaned from in vitro and cell-based assays.

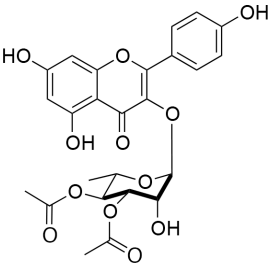
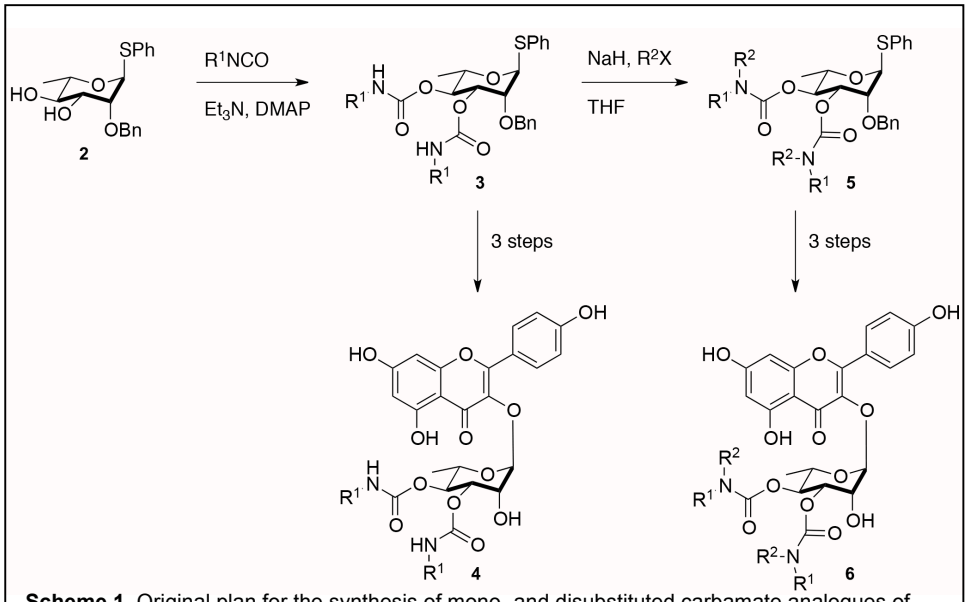


Fig 1. SL0101, a highly specific inhibitor of p90 ribosomal S6 kinase (RSK)

Body

Task 1: Synthesis and biological evaluation of 3',4'-carbamate analogues of SL0101. I will first synthesize N-methyl and N,N-dimethyl carbamates or related analogues. I will then evaluate them in in vitro and cell-based assays. If their biological activity is sufficient to warrant further analogues in this series, I will then synthesize additional N-alkylated analogues. (Timeframe: months 1-12)

This task was completed within the allotted timeframe, and the results were published in early 2012 (*5*). Details for each subtask follow and the manuscript and experimental details are attached in the appendix. The purpose of the task was to test the hypothesis that the acetates of the sugar portion of SL0101, which would likely be prone to hydrolysis in vivo, could be replaced by less labile carbamates, which would render an analogue more biologically stable than SL0101 while retaining the parent compound's ability to specifically inhibit RSK.

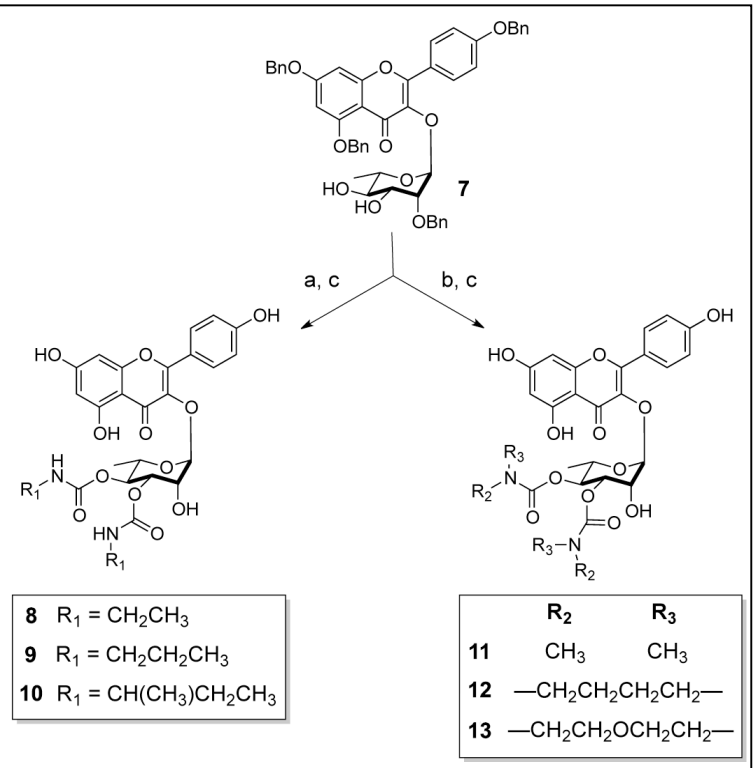


Scheme 1. Original plan for the synthesis of mono- and disubstituted carbamate analogues of SL0101

1a. Synthesis of 3',4'-N-methylcarbamate analogue, 3',4'-N,N-dimethylcarbamate analogue, or related analogues of SL0101. (months 1-6)

Originally, the plan for the chemical synthesis of

monosubstituted carbamate analogues such as the proposed 3',4'-*N*-methyl carbamate analogue and *N*,*N*-disubstituted carbamate analogues was to proceed according to the synthetic route outlined in Scheme 1. This plan called for the installation of the carbamate substitution at an early stage in the synthesis of the analogues, starting from known intermediate **2** (*6*) which would require three news synthetic steps to be performed in order to synthesize each new analogue. Rather than follow this proposed synthetic route, I adopted a revised plan that would allow me to synthesize the desired analogues in a much shorter time frame. This revised synthetic route as it applies to monosubstituted carbamates is shown in Scheme 2. Starting from the more advanced synthetic intermediate **7** (*7*), monosubstituted carbamates could be installed by reacting the appropriate isocyanate with the diol **7** in the presence of triethylamine to provide carbamates **8**-**10**. Hydrogenolysis of the benzyl protecting groups gave the completed analogues in one additional synthetic step. This modified route, requiring only two new synthetic steps per analogue, allowed the completion of three new monosubstituted carbamate analogues in the time originally allotted for synthesizing just one. The new analogues are the ethyl carbamate analogue **8**, the n-propyl carbamate analogue **9**, and the sec-butyl carbamate analogue **10**.



8 $R_1 = CH_2CH_3$
9 $R_1 = CH_2CH_2CH_3$
10 $R_1 = CH(CH_3)CH_2CH_3$

	R_2	R_3
11	CH_3	CH_3
12	$—CH_2CH_2CH_2CH_2—$	
13	$—CH_2CH_2OCH_2CH_2—$	

Scheme 2. General scheme for the preparation of carbamate analogues of SL0101. Reagents and conditions: (a) R_1NCO, Et_3N, DMF, 45 °C, 44–66%; (b) R_2R_3NCOCl, NaH, DMF, 0 °C to rt, 26–69%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, rt, 46–94%.

The modified synthetic route could also be applied to the synthesis of *N*,*N*-disubstituted carbamate analogues of SL0101 in fewer steps than the originally proposed route. Deprotonation of diol **7** with sodium hydride followed by treatment of the resulting bis-alkoxide with the appropriate dialkylcarbamoyl chloride gave *N*,*N*-disubstituted carbamate analogues **11-13** (Scheme 3). As in the case of the monosubstituted carbamates, the revised synthetic route allowed for the synthesis of three new analogues in the time allotted for just one. The new analogues synthesized are the dimethyl carbamate analogue **11**, the pyrrolidinyl carbamate analogue **12**, and the morpholino carbamate analogue **13**.

1b. In vitro evaluation of analogues. (months 6-9)

The six new carbamate analogues of SL0101 were evaluated for their ability to inhibit RSK2 activity in an in vitro kinase assay (Table 1). The analogues were all either as potent or slightly (2- to 3-fold) less potent that SL0101. That the structure of the acetate replacement did not substantially affect the ability to inhibit RSK2 was not surprising, as this is consistent with what had been seen for previous analogues (*7*).

Compound	RSK2 IC_{50} (μM)	MCF7 IC_{50} (μM)
1	0.583 (0.489 to 0.696)	45.6 (42.7 to 48.8)
8	1.13 (0.876 to 1.46)*	77.0 (71.6 to 82.7)*
9	0.869 (0.649 to 1.16)	46.4 (43.2 to 50.0)
10	1.92 (1.29 to 2.86)*	53.3 (50.6 to 56.2)*
11	0.493 (0.355 to 0.684)	PS
12	0.356 (0.255 to 0.496)	PS
13	1.43 (1.09 to 2.04)*	> 100

Table 1. Potency of analogues in in vitro kinase and cell-based assays. IC_{50} is concentration needed for 50% inhibition; the 95% CI is shown in parentheses; n=3 in triplicate; * p <0.05; PS; partially soluble.

1c. Evaluation of growth inhibition activity of analogues in human cancer MCF7 and normal human MCF-10A cell lines. (months 6-9)

The ability of the six new carbamate analogues to inhibit the growth of the MCF7 cancer cell line was determined (Table 1). The three monosubstituted carbamate analogues were all as potent or slightly less potent than SL0101 in this cell proliferation assay. Two of the disubstituted carbamate analogues were only partially soluble in the MCF7 cell culture media, and were not tested as the insolubility would confound the assay results. The third disubstituted carbamate analogue, **13**, was soluble but was completely unable to inhibit the proliferation of MCF7 cells.

The original hypothesis was that these analogues would be more biologically stable than SL0101. We devised an assay that would allow us to test this hypothesis in vitro. By treating MCF7 cells with SL0101 or each of the new monosubstituted carbamate analogues and monitoring their growth over a period of several days (2-, 4-, and 6-day time points), I could observe whether the inhibition of cell growth was sustained over a long time course. An analogue that was stable in vitro would be expected to still inhibit the growth of cells even at the 6-day time point. As predicted, SL0101 was not biologically stable in vitro, as the cells exposed to it recovered their ability to grow within 4 days (Figure 2). Encouragingly, two of the new monosubstituted carbamate analogues, **9** and **10**, continued to inhibit the growth of the cells even after 6 days, indicating that as predicted they are more biologically stable than SL0101 in vitro.

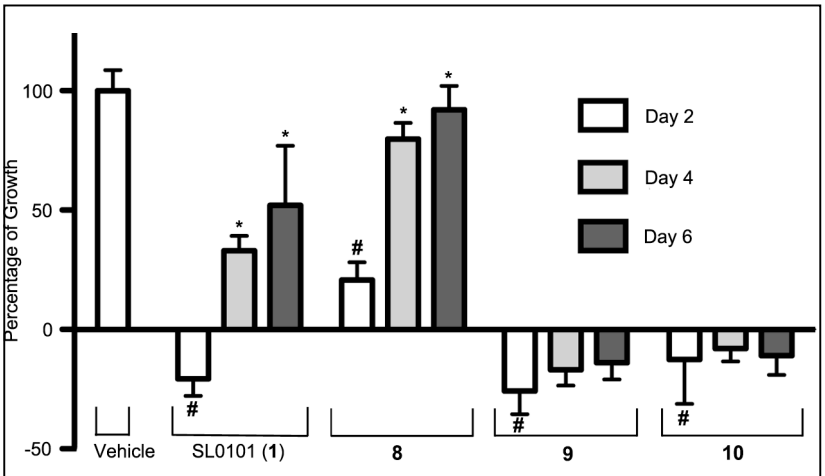


Figure 2. In vitro determination of analogue stability. The inhibitor was added to MCF7 cells when they were plated and percentage of growth was determined for the indicated time points. The inhibitor concentration was 100 μM. Analogues demonstrating improved biological stability continued to inhibit growth on day 6. (n=3 in quadruplicate, # p≤0.05 on day 2 when compared to vehicle on day 2, * p≤0.05 when compared to 2 day treatment with the same analogue.

Finally, the two analogues shown to be biologically stable in vitro, **9** and **10**, were tested for their ability to inhibit the growth of the normal human breast cell line MCF-10A. We have previously shown that the specificity of an analogue for RSK can be evaluated by determining its antiproliferative activity in normal (MCF-10A) vs cancer (MCF7) cell lines (*3*, *8*). Analogues that are specific for RSK inhibit the proliferation of the cancer cell line but not the normal cell line. Like SL0101, at a concentration of 50 μM each analogue was completely unable to inhibit the growth of MCF-10A cells, even though at the same concentration they were each able to inhibit the growth of MCF7 cells (Figure 3). This is the desired result, as it indicates that the new analogues are, like SL0101, highly specific for RSK.

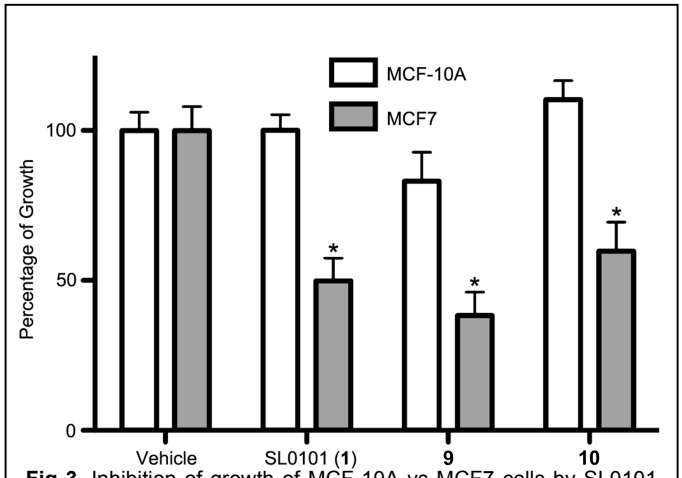


Fig 3. Inhibition of growth of MCF-10A vs MCF7 cells by SL0101 and select analogues. The inhibitor concentration was 50 μM. (n=3 in quadruplicate, * p≤0.05 when compared to vehicle.

The discovery of analogues **9** and **10**, that are more biologically stable than SL0101 but retain specificity for RSK, is a major advance toward the goal of developing a drug for breast cancer that works by inhibiting RSK. These new analogues are potential candidates for in vivo testing.

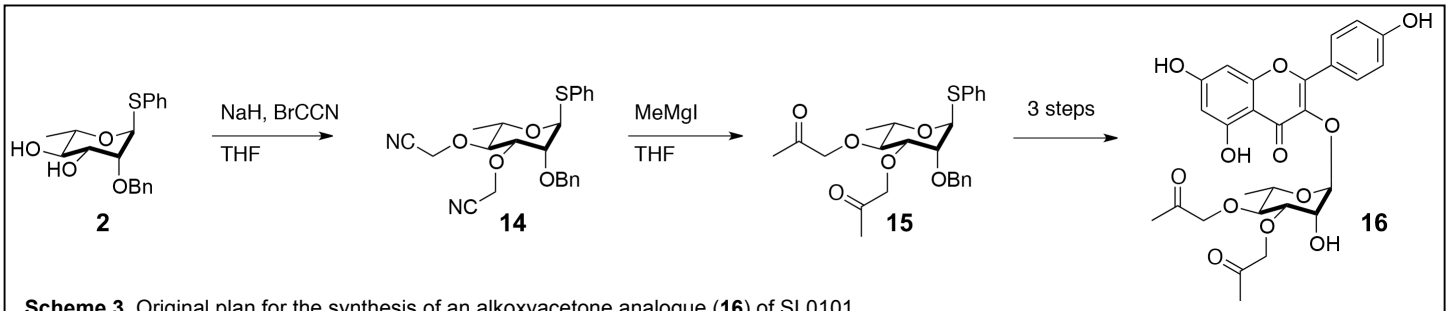
1d. If warranted, synthesis of additional N-alkylated analogues. (months 9-12)

Given that six analogues were synthesized in task 1a rather than the originally planned two, and that two of these analogues achieved the goal of being as potent as SL0101 but more biologically stable in vitro, it was decided that the synthesis of additional *N*-alkylated analogues was not necessary.

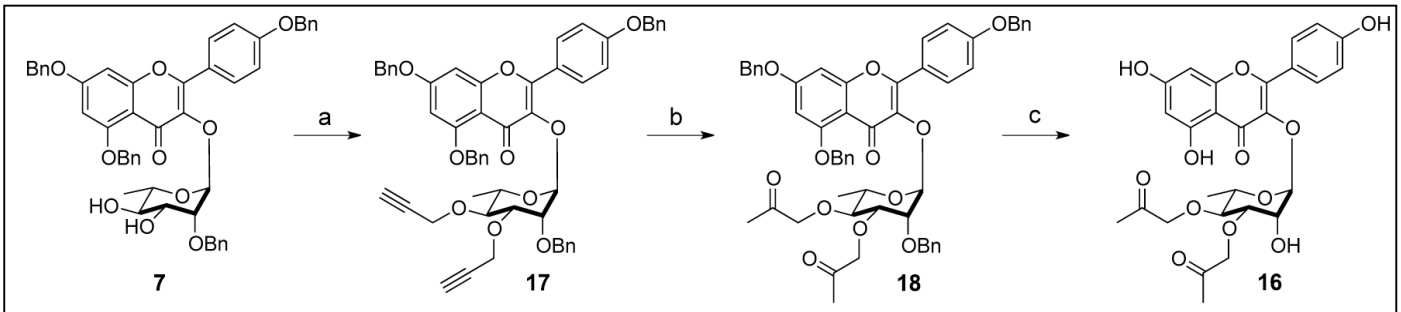
Task 2: Synthesis and biological evaluation of a 3',4'-alkoxypropanone or related analogue of SL0101. (Timeframe: months 1-9)

This task was accomplished within the allotted timeframe, and the results were published in early 2012 (*4*). Details for each subtask follow and the manuscript and experimental details are attached in the appendix. The purpose of the task was to test the hypothesis that analogues of SL0101 in which the 3" and 4" acetates are replaced by alkoxyacetones would be more biologically stable than SL0101 while retaining specificity for RSK.

2a. Synthesis of a 3'4'-alkoxypropanone or related analogue. (months 1-6)



Scheme 3. Original plan for the synthesis of an alkoxyacetone analogue (**16**) of SL0101.



Scheme 4. Synthesis of an alkoxyacetone analogue of SL0101. Reagents and conditions: (a) NaH, propargyl bromide, THF, 0 °C to rt, 66%; (b) $Hg(OAc)_2$, PPTS, water, acetone, rt, 62%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, 50%.

The originally-planned synthetic route to a 3',4'-alkoxypropanone analogue involved alkylation of intermediate **2** with bromoacetonitrile followed by addition of methyl Grignard and subsequent hydrolysis to provide intermediate **15** which could be elaborated by known methods (*6*) to desired analogue **16** (Scheme 3). Ultimately, a different synthetic route was chosen due to a higher predicted likelihood of success (Scheme 4). Diol **7** was first alkylated with propargyl bromide to give bis-alkyne **17**. The terminal alkynes were subjected to mercury-catalyzed hydration to provide the desired 3',4'-alkoxypropanone intermediate **18**, which could be converted to the desired analogue **16** by hydrogenolysis of the benzyl protecting groups.

2b. In vitro evaluation of analogue. (months 6-9)

Analogue **16** was evaluated for its ability to inhibit RSK2 in an in vitro kinase assay. It inhibited RSK2 with an IC_{50} of 0.252 μM (95% CI 0.189 to 0.336 μM), making it two-fold more potent than SL0101.

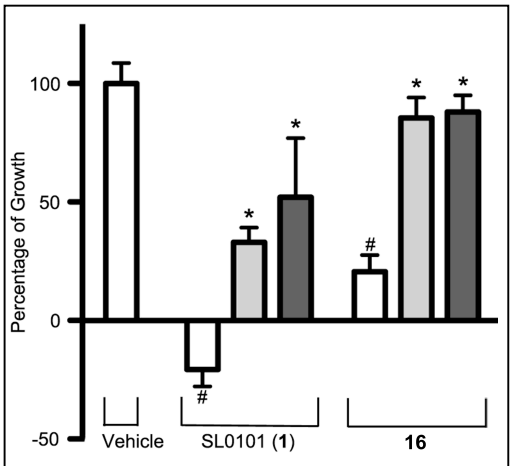


Fig 4. In vitro determination of inhibitor stability. For a detailed description, please see Fig 2 legend. Analogue **16** did not exhibit improved stability compared to SL0101.

2c. Evaluation of growth inhibition activity of analogue in human cancer MCF-7 and normal human MCF-10A cell lines. (months 6-9)

Analogue **16** was evaluated for its ability to inhibit the growth of the MCF7 cell line. It inhibited proliferation with an IC_{50} of 34.1 μM (95% CI 30.1 to 38.5 μM), a small but statistically significant improvement over SL0101. To test the hypothesis that the alkoxyacetone substitution of the acetates found in SL0101 would confer greater biological stability, this new analogue was evaluated in the in vitro biological stability assay described under Task 1c (figure 4). Unexpectedly, it did not show improved biological stability in vitro.

Task 3: Synthesis and biological evaluation of conformationally restricted analogues of SL0101. (Timeframe: months 3-12)3a. Synthesis of a conformationally restricted analogue retaining a 3'-carbonyl, or related analogue. (months 3-9)

3b. Synthesis of a conformationally restricted analogue retaining a 4'-carbonyl, or related analogue. (months 3-9)

New data obtained since this task was written suggested that the proposed conformationally restricted analogues were not the ideal analogues to make and that an alternative approach to make the "related analogues" specified in the task would lead to a higher likelihood of successfully identifying a SL0101 analogue with the desired properties. Specifically, in collaboration with the Derewenda laboratory at the University of Virginia we have obtained an X-ray crystal structure that reveals how SL0101 binds to the N-terminal kinase domain of RSK2 (Figure 5). Significantly, this crystal structure shows that SL0101 binds to a type of allosteric site on RSK that is novel and unexpected, making this crystal structure an important contribution to the field of structural biology. A manuscript detailing this work has been accepted for publication in the journal *Biochemistry*. Importantly, this new information about the structure of SL0101 bound to RSK makes obsolete the docking studies previously used to propose new analogues, and suggests that some of the originally proposed analogues should not be made while other, related analogues should be prioritized.

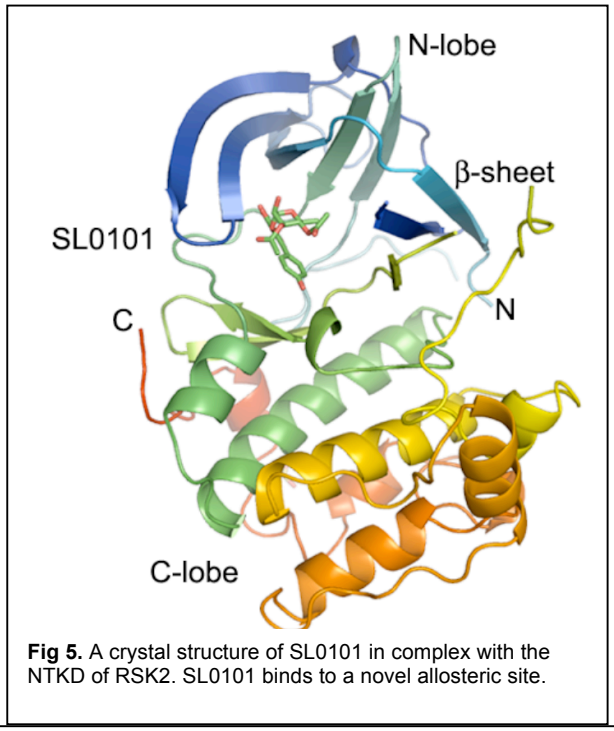
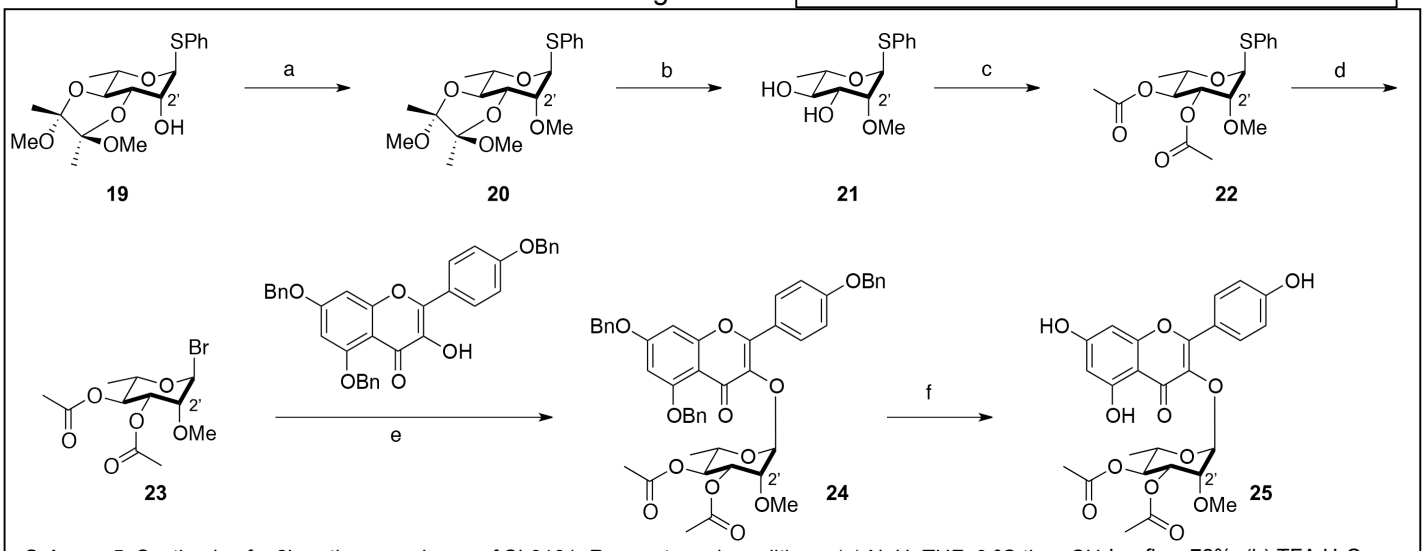


Fig 5. A crystal structure of SL0101 in complex with the NTKD of RSK2. SL0101 binds to a novel allosteric site.



Scheme 5. Synthesis of a 2'-methoxy analogue of SL0101. Reagents and conditions: (a) NaH, THF, 0 ºC then CH_3I, reflux, 72%; (b) TFA:H_2O (10:1), CH_2Cl_2, rt, 83%; (c) Ac_2O, Et3N, DMAP, CH_2Cl_2, rt, 88%; (d) Br_2, CH_2Cl_2, 0 ºC, 57%; (e) Ag_2O, 4 Å MS, CH_2Cl_2, rt, 89%; (f) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, rt, 29%.

The hypothesis that led to the design of the analogues to be synthesized in Task 3 was that the 3'- and 4'- carbonyl groups of SL0101 accept hydrogen bonds from RSK when SL0101 binds to RSK. The crystal structure of the SL0101-RSK complex reveals that this is not the case. Therefore, the proposed analogues would likely not be more potent SL0101 and to synthesize and test them would be an unproductive use of resources. In contrast, the crystal structure revealed that the 2'-hydroxyl group of SL0101 accepts a hydrogen bond when bound to RSK. This suggested a potentially more fruitful class of analogues that would be predicted to have greater potency than SL0101 in a cell proliferation assay. Since the 2'-hydroxyl group is a hydrogen bond *acceptor* (requiring only the oxygen atom of the hydroxyl group) and not a hydrogen bond *donor* (requiring both the hydrogen and oxygen atoms of the hydroxyl group), one intriguing hypothesis was that the hydrogen of the hydroxyl group could be replaced by an alkyl group as in a methyl ether (**25**, Scheme 5). Such an analogue would be predicted to be as potent as SL0101 in the in vitro kinase assay, but potentially more potent than SL0101 in the cell proliferation assay, as the added lipophilicity conferred by the alkyl substitution could enhance the ability of the analogue to pass through the cell membrane. I was able to synthesize this analogue within the allotted timeframe by making a simple modification to the existing synthesis of SL0101, whereby instead of alkylating intermediate **19** with benzyl bromide, I instead alkylated it with iodomethane. The rest of the synthesis proceeded analogously to the synthesis of SL0101 (*6*), providing 2'-methyl ether analogue **25**.

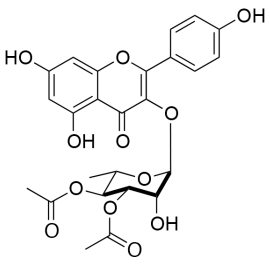
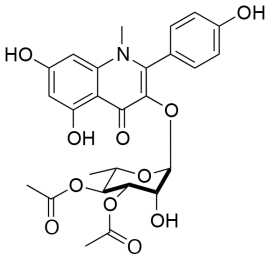
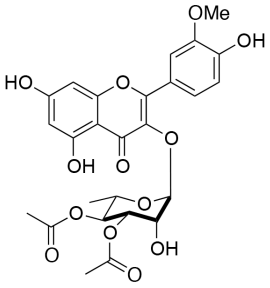
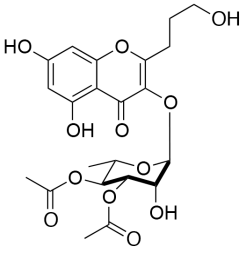
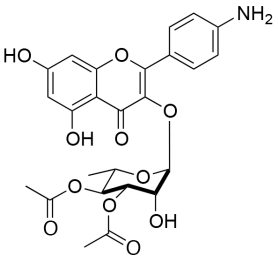
Compound #	Structure	Docking Score	RMSD
1		-7.96	1.63
26		-6.28	4.12
27		-8.07	1.95
28		-7.74	3.32
29		-7.95	1.44

Fig 6. Docking results for proposed analogues obtained using the MOE software package.

3b. In vitro evaluation of analogues (months 9-12)

The new analogue **25** was evaluated for its ability to inhibit RSK2 activity in an in vitro kinase assay. It inhibited RSK2 activity with an IC_{50} of 0.488 μM (95% CI 0.178 to 1.34 μM), indicating that it is equipotent to SL0101 and confirming that the 2'-hydroxyl group can be alkylated without interfering with the ability of an analogue to inhibit RSK.

3c. Evaluation of growth inhibition activity of analogues in human cancer MCF-7 and normal human MCF-10A cell lines. (months 9-12)

Analogue **25** was evaluated for its ability to inhibit the growth of the breast cancer cell line MCF7. Preliminary results suggest that the new analogue is approximately 2-fold more potent than SL0101 at inhibiting the growth of

MCF7 cells. However, the experiment will need to be repeated to confirm that these results are reproducible. Similarly, preliminary results indicate that analogue **25** is, like SL0101, unable to inhibit the growth of the normal breast cell line MCF-10A, suggesting that it, like SL0101, is specific for RSK.

Task 4: Design, synthesis, and biological evaluation of potentially more potent SL0101 analogues that append lipophilic groups from the flavone ring system, or related analogues. I will first prioritize analogues based on a computational model. I will then synthesize one or more analogues and evaluate their biological activity. If warranted, I will then synthesize additional related analogues. (Timeframe: months 9-24)

4a. Model potential analogues using ICM-Pro or related software. Potential analogues will be docked into ATP binding site of human RSK using the crystal structure of RSK1 N-terminal kinase domain bound to staurosporine or related crystal structure. (months 9-12)

This subtask was completed in the allotted timeframe using the crystal structure of SL0101 in complex with RSK obtained in collaboration with the Derewenda group. Based on information gleaned from the crystal structure, SL0101 analogues that append lipophilic groups from the flavone ring system as proposed in the project narrative would be expected to be substantially less potent than SL0101. This conclusion is supported by computer-aided docking studies of the proposed analogues into the SL0101 binding site (Figure 6). Analogues were docked using the dock function of the Molecular Operating Environment (MOE) software package published by Chemical Computing Group (CCG) in Montreal, Canada. The program assigns a docking score and RMSD for each proposed analogue. The docking score for SL0101 is -7.96. Compounds that have a lower docking score would be predicted by the program to be more potent inhibitors of RSK than SL0101, whereas compounds with higher docking scores would be expected to be less potent. The RMSD is a measure of how closely the analogue overlaps with SL0101 as positioned in the crystal structure. An RMSD of 1 would indicate perfect overlap, so a lower RMSD indicates confidence by the program that an analogue would bind similarly to SL0101. The originally proposed N-alkylated analogue (**26**, Figure 6) has both a very poor docking score (-6.28) and RMSD (4.12). This is consistent with the qualitative observation that the N-methyl group would cause an unfavorable Van der Waals clash within the binding pocket. Given this information, a set of related analogues (**27**-**29**) were docked and show improved docking scores and RMSD values. Only a representative subset of proposed analogues that were docked are shown.

Task 5: In collaboration with Michelle Rudek-Renaut, evaluate the biological half-life of 1-3 analogues in CB17 SCID mice for both subcutaneous and intraperitoneal routes. (Timeframe: months 1-6 and 21-27)

5a. Submit appropriate documentation to DoD and gain clearance for animal use. (months 1-6)

The appropriate documentation was submitted and DoD has approved the use of animals.

Task 6: Evaluation of up to 3 analogues in our breastoid model. This will require the use of 15 human tissue samples, which we will collect under an approved IRB protocol that protects patient identity. (Timeframe: months 1-6 and 28-36)

6a. Submit appropriate documentation to DoD and gain clearance for human tissue use. (months 1-6)

The appropriate documentation was submitted and DoD has approved the use of human tissue.

Training plan:

Task 1: Audit "Advanced Topics in Cancer" class. (Timeframe: 1-4 months)

I audited the "Advanced Topics in Cancer" class.

Task 2: Audit "Cell Imaging" class. (Timeframe: 4-8 months)

Rather than Audit the "Cell Imaging" class I received personal advice and instruction on cell imaging techniques from the Director of the Advanced Microscopy Facility at the University of Virginia, Ian Macara.

Task 3: Regularly attend cancer and chemistry seminars. (Timeframe: 1-36 months)

I regularly attended cancer seminars offered by the Cancer Center at the University of Virginia and chemistry seminars offered by the University of Virginia Department of Chemistry.

Task 4: Attend international meeting held in the US to present and discuss work. (Timeframe: 1-12 months)

I attended the American Society for Cell Biology National Meeting, the Cancer Biology Training Consortium annual Chair & Director's Retreat, and the Mid-Atlantic Regional Meeting of the American Chemical Society.

Key Research Accomplishments

- The discovery of analogues of the RSK inhibitor SL0101 that are more biologically stable than the parent compound in vitro yet retain specificity for RSK. These analogues are candidates for in vivo evaluation.
- The solution of an X-Ray crystal structure of SL0101 in complex with the NTKD of RSK2. This structure will be an invaluable tool for the design of new SL0101 analogues.
- The discovery that an analogue of SL0101 that contains a 2'-methoxy ether in place of the 2'-hydroxyl group is approximately 2-fold more potent than SL0101 at inhibiting the growth of breast cancer cells. This is an exciting preliminary result and suggests that other modifications of this area of the molecule should be explored to further improve potency in cell-based assays.

Reportable Outcomes

- Publication: "Analogues of the RSK Inhibitor SL0101: Optimization of In Vitro Biological Stability" **M. K. Hilinski**, R. M. Mrozowski, D. E. Clark, D. A. Lannigan, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3244–3247.
- Publication: "Insights into the Inhibition of p90 Ribosomal S6 Kinase (RSK) by the Flavonol Glycoside SL0101 from the 1.5 Å Crystal Sturcure of the N-Terminal Domain of RSK2 with Bound Inhibitor" D. Utepbergenov, U. Derewenda, N. Oleknovich, G. Szukalska, B. Banerjee, **M. K. Hilinski**, D. A. Lannigan, P. T. Stukenberg, Z. S. Derewenda, *Biochemistry* in press.

Conclusion

SL0101 is a promising lead compound for medicinal chemistry efforts to develop a breast cancer drug that works by targeting RSK. However, it is not biologically stable and thus would not be useful as a drug. The discovery of analogues of SL0101 that are more biologically stable as described in this report is thus highly significant as they overcome this deficiency and therefore could find use as breast cancer drugs. A second goal in the design of analogues of SL0101 is the discovery of analogues with improved potency against breast cancer cell lines. With the discovery of the C2'-methoxy analogue of SL0101, which in preliminary experiments appears to be more potent than the parent compound, progress toward this goal has been achieved. This analogue is the first in a potential series, and future work exploring a series of related analogues could lead to the discovery of a new analogue that is substantially more potent than SL0101. The X-Ray crystal structure of SL0101 bound to the RSK2 NTKD obtained in the course of this research will be the single most important tool going forward for designing analogues of SL0101, and will be valuable to the rest of the scientific community as it could be used to discover other scaffolds for drug design of RSK inhibitors. Overall, the work accomplished in this year of funding moves the science of RSK inhibitors closer to the goal of a breast cancer drug that works by inhibiting RSK.

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Appendices

The following appear on subsequent pages:

- A copy of publication “Analogues of the RSK Inhibitor SL0101: Optimization of In Vitro Biological Stability” **M. K. Hilinski**, R. M. Mrozowski, D. E. Clark, D. A. Lannigan, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 3244–3247.
- A copy of the supporting information including experimental details for the above publication.

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Analogs of the RSK inhibitor SL0101: Optimization of in vitro biological stability

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ABSTRACT

The Ser/Thr protein kinase, RSK, is important in the etiology of tumor progression including invasion and motility. The natural product kaempferol-3-*O*-(3″,4″-di-*O*-acetyl-α-L-rhamnopyranoside), called SL0101, is a highly specific RSK inhibitor. Acylation of the rhamnose moiety is necessary for high affinity binding and selectivity. However, the acetyl groups can be cleaved by esterases, which accounts for the poor in vitro biological stability of SL0101. To address this problem a series of analogs containing acetyl group replacements were synthesized and their in vitro stability evaluated. Monosubstituted carbamate analogs of SL0101 showed improved in vitro biological stability while maintaining specificity for RSK. These results should facilitate the development of RSK inhibitors derived from SL0101 as anticancer agents.

The members of the p90 ribosomal S6 kinase (RSK) family of Ser/Thr protein kinases have been shown to play a role in a number of different cancers as key drivers of proliferation and metastasis.[1–8] These discoveries have been enabled in part by our report of the identification and isolation of the RSK inhibitor SL0101 (**1**, Fig. 1).[9] SL0101 is a flavonoid glycoside (kaempferol 3-*O*-(3″,4″-di-*O*-acetyl-α-L-rhamnopyranoside)) isolated from *Forsteronia refracta*, a variety of dogbane found in the South American rainforest. SL0101 is highly specific for RSK, inhibiting RSK1/2 but not unrelated kinases nor the closely related kinases MSK1 and p70S6K1.[2,9,10] SL0101 inhibits the proliferation of breast and prostate cancer lines but not their normal counterparts even though it inhibits RSK activity in all the lines.[1,5,9] Thus it appears that some cancer cells have become addicted to RSK, which suggests that RSK may be a potential new target for cancer therapeutics. SL0101, owing to its exquisite specificity, is a compelling lead compound from which to begin the process of identifying drug-like RSK inhibitors.

We and others have reported the total synthesis and biological evaluation of SL0101 and a number of analogs, with the ultimate goal of developing an anticancer drug that targets RSK.[11–15] These analogs have provided key information about the SAR of both the aglycone and carbohydrate portions of the natural product. In the course of this work we discovered that the 3″ and 4″ acetyl groups of the carbohydrate are critical for potency and specificity for RSK.[13] TriOH-SL0101 (**2**), lacking these acetyl groups, is 12-fold less potent for inhibition of RSK in vitro and does not inhibit the growth of cancer cell lines, likely due to poor membrane permeability.[13] These results indicate that SL0101 is not a suitable candidate for in vivo evaluation, as hydrolysis of the acetates by esterases would generate a less potent inhibitor.

An analog that replaces these acetates with ethyl ethers (**3**) inhibits RSK with potency roughly equivalent to SL0101.[13] We previously determined that the specificity of SL0101 and its analogs for RSK could be evaluated by their preferential ability to inhibit the growth of the human breast cancer line, MCF7, compared to the normal human breast line, MCF-10A.[12] Unexpectedly, we observed that the ethyl ether analog **3** inhibited both lines to a similar extent, which indicates that it has a decreased specificity for RSK.[13] These results demonstrate that the acetates are a key modulator of specificity and thus a more carefully considered approach is necessary to identify suitable replacements. Accordingly, we have focused our efforts on identifying analogs bearing replacements for the acetates that confer greater biological stability without decreasing potency or specificity for RSK. Herein we present our approach, which has led to the identification of SL0101 analogs that are both specific for RSK and more biologically stable in vitro than the parent compound.

The only structural difference between the diethyl analog **3** and the diacetyl parent compound is the replacement of two methylenes with two carbonyl groups. It is surprising that such a seemingly small structural feature can regulate specificity for RSK. To recover this specificity, in the design of new analogs we sought to better mimic the acetates and particularly the acetate carbonyls, sterically and electronically, in a way that would confer a greater resistance to metabolism by esterases. In one approach we investigated the dependence of potency and specificity on the relative

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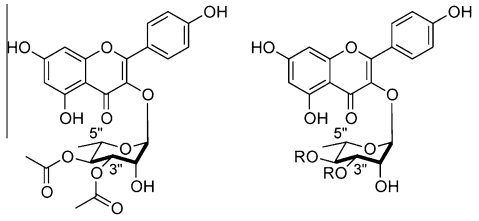
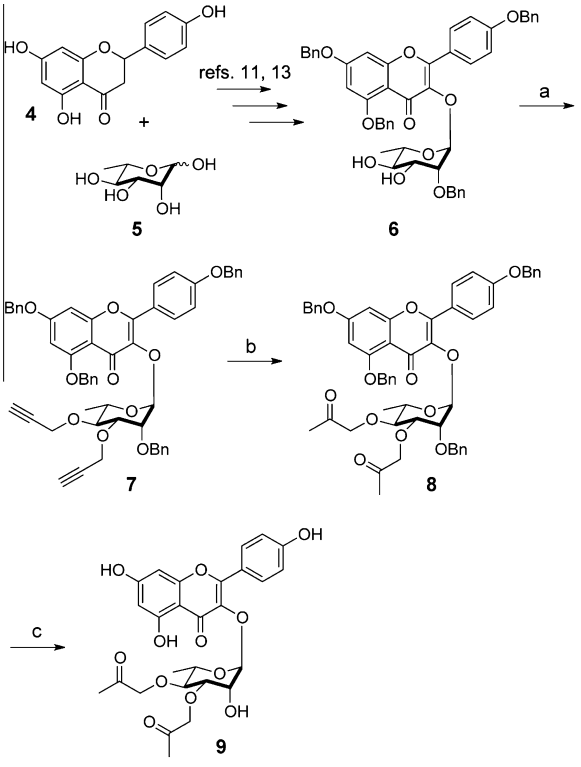


Figure 1. The RSK inhibitor SL0101 and two previously reported analogs.[13]

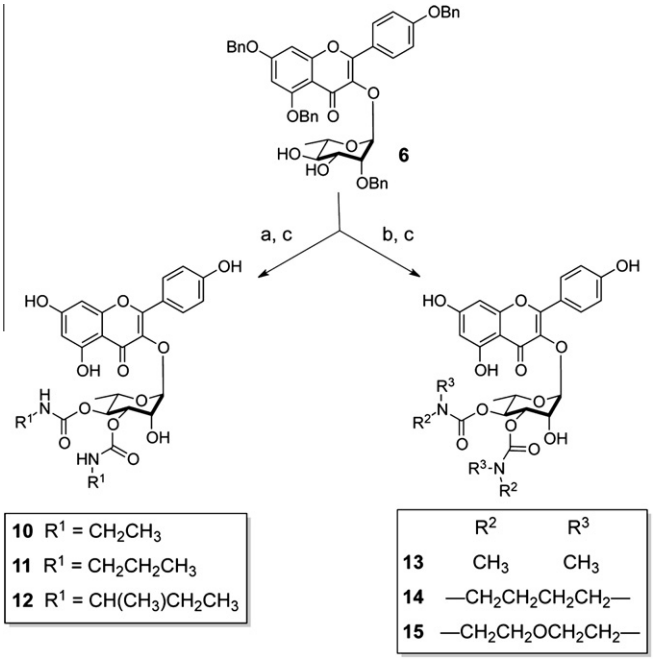
position of the carbonyl group. To this end we prepared an analog **9** in which the acetates are replaced by alkoxyacetones (Scheme 1), moving the carbonyl group one carbon further from the carbohydrate ring and replacing the labile ester bond with an ether bond. The desired functionality could be installed at a late stage in the synthesis of the analog. Alkylation of known diol **6**[13] with propargyl bromide provided bis-alkyne **7**, which after mercury-catalyzed hydration provided bis-ketone **8**. Removal of the benzyl ether protecting groups by hydrogenolysis using Pearlman's catalyst provided the completed analog **9**.

In a second approach we retained the acetate carbonyl in the correct position but in a more biologically stable form in a series of analogs in which we replaced the acetates with bioisosteric mono- or disubstituted carbamates. Late-stage installation of the carbamate was desirable for maximum synthetic efficiency. Thus, carbamoylation of diol **6** with the appropriate isocyanate or diaklycarbamoyl chloride followed by hydrogenolysis of the benzyl ethers provided mono- or dialkylated carbamates **10**–**15** (Scheme 2).

The ability of all new analogs to inhibit RSK activity was determined in an in vitro kinase assay and compared with the parent



Scheme 1. Synthesis of a bis-ketone analog of SL0101. Reagents and conditions: (a) NaH, propargyl bromide, THF, 0 °C to rt, 66%; (b) $Hg(OAc)_2$, PPTS, water, acetone, rt, 62%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, 50%. Yields are unoptimized.



Scheme 2. General scheme for the preparation of carbamate analogs of SL0101. Reagents and conditions: (a) R_1NCO, Et_3N, DMF, 45 °C, 44–66%; (b) R_2R_3NCOCl, NaH, DMF, 0 °C to rt, 26–69%; (c) H_2, $Pd(OH)_2/C$, MeOH, EtOAc, rt, 46–94%. Yields are unoptimized.

Table 1
Potency of analogs in in vitro kinase and MCF7 cell-based assays

Compound	RSK2 IC_{50} (μM)	MCF7 IC_{50} (μM)
1	0.583 (0.489–0.696)	45.6 (42.7–48.8)
9	0.252 (0.189–0.336)*	34.1 (30.1–38.5)*
10	1.13 (0.876–1.46)*	77.0 (71.6–82.7)*
11	0.869 (0.649–1.16)	46.4 (43.2–50.0)
12	1.92 (1.29–2.86)*	53.3 (50.6–56.2)*
13	0.493 (0.355–0.684)	PS
14	0.356 (0.255–0.496)	PS
15	1.43 (1.09–2.04)*	>100

IC_{50} is concentration needed for 50% inhibition; the 95% CI is shown in parentheses; $n = 3$ in triplicate.
* $p < 0.05$. PS; partially soluble.

compound **1** (Table 1). The ketone analog **9** was twofold more potent than **1** at inhibiting RSK2. Analogs **11**, **13**, and **14** were as potent as SL0101, and analogs **10**, **12**, and **15** were slightly (two to threefold) less potent. Overall, we found that the ability of an analog to inhibit RSK was not greatly influenced by the structure of the acetate replacement, which is consistent with previous observations.

We also determined the ability of all new analogs to inhibit MCF7 cell proliferation (Table 1). The ketone analog **9** was again the most potent of the new analogs. The three monosubstituted carbamates, analogs **10**–**12**, were similarly potent to the parent compound, with a trend toward improved potency with increasing lipophilicity of the carbamate substituent, presumably due to improved membrane permeability. In the disubstituted carbamate series, the dimethyl analog **13** and 1-pyrrolidinyl carbamate analog **14** exhibited poor solubility in cell culture media and therefore their ability to inhibit cell growth was not determined. The morpholino bis-carbamate **15** showed improved solubility but was unable to inhibit cell proliferation despite its ability to inhibit RSK in the in vitro kinase assay, most likely due to poor membrane permeability.

Analogs that inhibited MCF7 cell proliferation were evaluated along with **1** for their stability in a MCF7 cell-based assay. The inhibitor was added when the cells were plated and proliferation analyzed at various time points to determine the persistence of the inhibitory effect. SL0101 (**1**) was able to inhibit MCF7 proliferation for 48 h (Fig. 2). However, at longer time points the cells began to proliferate indicating that SL0101 was no longer effective, which we hypothesize is due to degradation of the inhibitor by esterases to the inactive triol **2**. Treatment of cells with either the bis-ketone analog **9** or the ethyl carbamate analog **10** did not result in sustained growth inhibition, indicating poor in vitro stability of these analogs. As the 3″ and 4″ substituents of analog **9** are non-hydrolyzable, its poor stability was initially surprising. However, MCF7 cells express aldo-keto reductases (AKRs), well known to be Phase I metabolizing enzymes for a variety of drugs bearing carbonyl groups.[16,17] Thus an alternative metabolic pathway is available to analog **9** whereby one or both ketones could be reduced by AKRs to secondary alcohols, leading either directly to a less potent RSK inhibitor or indirectly as the secondary alcohols could be further metabolized by conjugation.[17]

Encouragingly, the more lipophilic monosubstitued carbamate analogs **11** and **12** demonstrated improved in vitro stability, as cells treated with these compounds did not proliferate over the full time course (Fig. 2). We further examined the stability of analogs **11** and **12** by determining whether cyclin D1 levels were inhibited (Fig. 3). Previously, we found that SL0101 inhibits proliferation in breast cancer cell lines by inducing a cell cycle block in G1, which is due to RSK regulation of cyclin D1 levels.[1,18] In agreement with the MCF7 stability results we observed that SL0101 decreased the levels of cyclin D1 at 48 h compared to the control, but that cyclin D1 levels began to increase at later time points, indicating degradation of the inhibitor. However, cyclin D1 levels remained low in cells treated with **11** or **12**, indicating persistent inhibition of RSK and therefore improved biological stability of the carbamate analogs over the parent compound. Taken together, these results indicate that analogs **11** and **12** have improved stability over SL0101 (**1**).

We then investigated whether our strategy of reintroducing the carbonyl group improved the specificity of **11** and **12** relative to the diethyl analog **3**.[13] We have previously shown that the specificity of an analog for RSK can be evaluated by determining its antiproliferative activity in both MCF-10A and MCF7 cells, with the most specific analogs showing no inhibition of MCF-10A but substantial inhibition of MCF7 proliferation, due to the differential dependence of the growth of these cell lines on RSK.[9,12] We have also previously shown that while SL0101 does not inhibit the growth of MCF-10A cells up to a concentration of 100 μM, the diethyl analog **3** significantly inhibits the growth of MCF-10A cells, indicating reduced

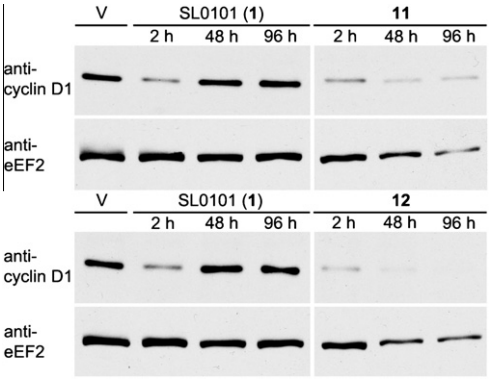


Figure 3. Persistence of RSK inhibition. MCF7 cells were treated with SL0101 or the more stable analogs **11** and **12** (100 μM). At the indicated time in hours (h) the cells were lysed and the lysates immunoblotted. Each analog was analyzed on a single membrane with SL0101. White space indicates sections of the membrane that were cropped to remove unnecessary lanes.

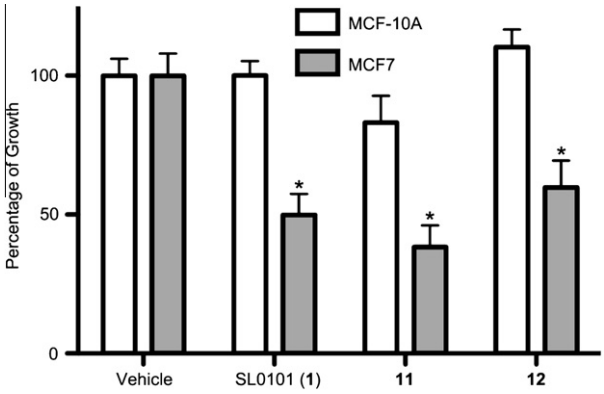


Figure 4. Inhibition of growth of MCF-10A versus MCF7 cells by SL0101 and select analogs. The inhibitor concentration was 50 μM. ($n = 3$ in quadruplicate, $^{*}p \leqslant 0.05$ when compared to vehicle).

specificity for RSK.[13] We found that analogs **11** and **12**, like SL0101, significantly inhibited the growth of MCF7 cells but did not significantly inhibit the growth MCF-10A cells (Fig. 4). These results suggest that analogs **11** and **12**, like SL0101, specifically inhibit RSK.[2,9,10] The only significant differences in biological activity between the two compounds are slightly improved potencies for **11** versus **12** in both the kinase and MCF7 cell proliferation assays. As these small differences are unlikely to be physiologically

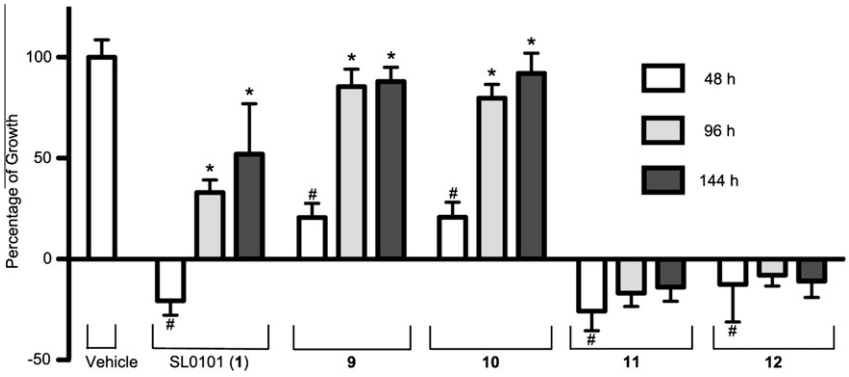


Figure 2. In vitro determination of analog stability. The inhibitor (100 μM) was added to MCF7 cells at time 0 and percentage of growth determined for the indicated time points. ($n = 3$ in quadruplicate, $^{\#}p \leqslant 0.05$ at 48 h when compared to vehicle at 48 h, $^{*}p \leqslant 0.05$ when compared to 48 h treatment with the same analog).

important, either carbamate modification should render an analog suitable for in vivo evaluation.

In summary, the C3″ and C4″ acetates on the carbohydrate moiety of SL0101 are required for both potent and specific inhibition of RSK but we predict that they would be metabolized rapidly by esterases in vivo, a fact which is supported by the poor biological stability of the natural product in vitro. Thus, SL0101 is not suitable for in vivo evaluation and analogs with improved stability are needed. The number of suitable replacements for these acetates that would confer greater biological stability is surprisingly limited as a simple change from acetyl to ethyl leads to a reduction in specificity for RSK. As a solution to this problem, bioisosteric replacement of the acetates by carbamates provided analogs that are more biologically stable than SL0101 in vitro and are as specific as SL0101 for RSK. These modifications along with others aimed at further improving the stability and potency of SL0101 analogs are currently being investigated in our laboratory with the goal of identifying a RSK inhibitor that could be advanced to preclinical testing.

Acknowledgments

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Supplementary data

Supplementary data (experimental procedures and compound characterization for all new compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.03.033.

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Supporting Information

Analogues of the RSK inhibitor SL0101: Optimization of in vitro biological stability

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Kinase Assays

Glutathione-S-transferase (GST)-fusion protein (1 μg) containing the ERα-Ser167 sequence- RLASTND was adsorbed in the wells of LumiNunc 96-well polystyrene plates (MaxiSorp surface treatment). The wells were blocked with sterile 3% tryptone in phosphate-buffered saline. Kinase (5 nM) in 50 μL of kinase buffer (5 mM β-glycerophosphate, pH 7.4, 25 mM HEPES, pH 7.4, 1.5 mM DTT, 30 mM $MgCl_2$, 0.15 M NaCl) was dispensed into each well. 25 μL of the compound at the indicated concentrations or vehicle was added and reactions were initiated by the addition of 25 μL of ATP to a final ATP concentration of 10 μM. Reactions were terminated after 120 min by addition of 75 μL of 500 mM EDTA, pH 7.5. All assays measured the initial velocity of reaction. After extensive washing of wells, a polyclonal phosphospecific anti-ERα-pSer167 antibody and HRP-conjugated anti-rabbit antibody (211-035-109, Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) were used to detect serine phosphorylation of the substrate. HRP activity was measured using Western Lightning Chemiluminescence Reagent (NEL102, PerkinElmer Life Sciences) according to the manufacturer's protocol. Maximum and minimum activity is the relative luminescence detected in the presence of vehicle and 200 mM EDTA, respectively. His-tagged active RSK was expressed in Sf9 cells and purified using NiNTA resin (Qiagen, Valencia, California). Baculovirus was prepared using the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, California). Maximum responses and the concentrations at half the inhibitory response (IC_{50}) were determined by performing a best-fit analysis of the data (GraphPad Prism).

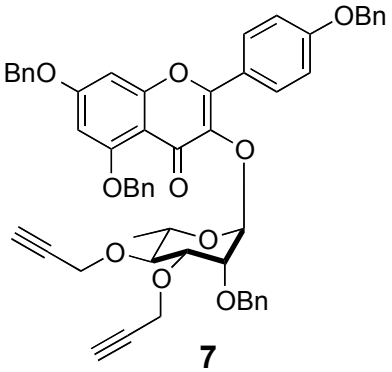
Cell Culture

For proliferation and stability studies cells were seeded at 2000 cells per well in 96-well tissue culture plates in the appropriate medium as described by American Type Culture Collection. After 24 h, the medium was replaced with medium containing compound or vehicle as indicated. Cell viability was measured 48 h later (proliferation studies) or 48 h, 96 h, and 144 h later (stability studies) using CellTiter-GloTM assay reagent (Promega, Madison, Wisconsin) according to the

manufacturer's protocol. Maximum responses and the concentrations of half the effective response (EC50) were determined by performing a best-fit analysis of the data (GraphPad Prism). For specificity studies, cells were seeded at 1 x 105 cells/60 mm dish. After 24 h incubated with compound or vehicle for 2 h, 48 h, or 96 h. Cells were lysed with boiling SDS-sample buffer without dithiothreitol (DTT). The lysates were normalized for total protein, and DTT was added to an aliquot, which was electrophoresed and immunoblotted. Antibodies used on cell lysates included anti-eEF2 (2332) and anti-Cyclin D1 (2926) from Cell Signaling Technologies.

Chemistry

Unless otherwise noted, reagents and solvents were of reagent grade and used without further purification. Anhydrous tetrahydrofuran and *N*,*N*-dimethylformamde were purchased from Sigma-Aldrich. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under a nitrogen atmosphere. Flash chromatography was performed using Fisher 70 – 230 mesh silica gel. Analytical TLC was performed using 0.25 mm Merck KGaA silica gel 60 plates that were visualized by irradiation (254 nm) or by staining with Hanessian's stain (CAM). ^{1}H and ^{13}C NMR spectra were obtained using 300 and 500 MHz Varian instruments. Chemical shifts are reported in parts per million (ppm δ) referenced to the residual ^{1}H resonance of the solvent (CDCl3, 7.26 ppm; acetone-d6 2.09 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent (CDCl3, 77.3 ppm; acetone-d6 29.9 ppm). Splitting patterns are designated as follows: s, singlet; br, broad; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High-resolution mass spectra were obtained from the Michigan State University-NIH Mass Spectrometry Facility.



7

To a solution of **6** (105 mg, 0.132 mmol) in tetrahydrofuran (8 mL) under N_2 and cooled in an ice/water bath was added sodium hydride (60% in oil, 32 mg, 0.794 mmol). The resulting mixture was stirred for 20 minutes, then propargyl bromide (80% in toluene, 0.16 mL, 1.056 mmol) was added and the mixture was removed from the ice bath and heated to reflux. After 21 hours, more sodium hydride (16 mg, 0.397 mmol) and propargyl bromide (0.08 mL, 0.528 mmol) were added.

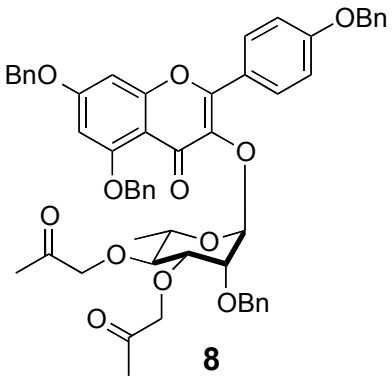
After an additional 21 hours, more sodium hydride (16 mg, 0.397 mmol) and propargyl bromide (0.08 mL, 0.528 mmol) were added. After an additional 7 hours, the mixture was cooled to room temperature and quenched with saturated aqueous ammonium chloride (5 mL). The layers were separated and the aqueous layer was extracted with diethyl ether (3 x 5 mL). The combined organic layers were dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:4 ethyl acetate:petroleum ether) to provide **7** (76 mg, 66%) as a clear oil.

R_f: 0.59 (1:3 acetone:petroleum ether).

^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.81 (d, *J* = 9 Hz, 2H), 7.63 (d, *J* = 6 Hz, 2H), 7.52-7.25 (m, 18 H), 7.09 (d, *J* = 9 Hz, 2H), 6.57 (s, 1H), 6.49 (s, 1H), 5.65 (s, 1H), 5.26 (s, 2H), 5.15 (s, 2H), 5.09 (s, 1H), 4.81 (dd, *J* = 12, 9 Hz, 2 H), 4.45-4.30 (m, 3 H), 4.15-3.95 (m, 2H), 3.78 (dd, *J* = 9, 2 Hz, 2 H), 3.47 (t, *J* = 18 Hz, 1H), 3.29-3.21 (m, 1H), 2.43 (s, 1H), 2.38 (, 1H), 0.99 (d, *J* = 6 Hz, 3H) ppm.

^{13}C NMR: (75 MHz, $CDCl_3$) δ 171.4, 162.8, 160.3, 159.7, 158.8, 154.3, 136.39, 136.37, 138.1, 128.7, 128.5, 128.6, 128.24, 128.18, 127.6, 127.4, 126.6, 114.7, 99.3, 98.1, 93.8, 81.0, 80.0, 78.7, 78.3, 74.4, 74.2, 73.9, 72.5, 70.8, 70.6, 70.1, 68.5, 59.7, 56.6, 17.5 ppm.

HRMS: *m/z* = 869.3344 $(M+H)^+$. Calculated for $C_{55}H_{49}O_{10}$ 869.3320.



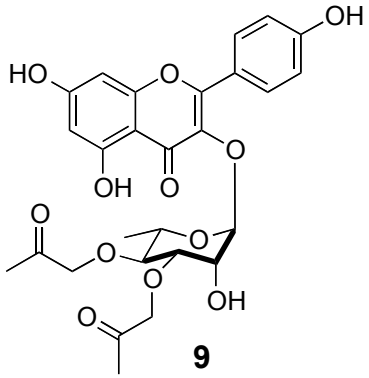
8

To a solution of **7** (69 mg, 0.079 mmol) in acetone (5 mL) was added water (6 μL) followed by pyridinium p-toluenesulfonate (60 mg, 0.238 mmol) and $Hg(OAc)_2$ (15 mg, 0.048 mmol). The resulting mixture was allowed to stir at room temperature for 16 h, then the solvent was removed by evaporation. The residue was dissolved in dichloromethane (10 mL) and washed with water (3 x 5 mL) and brine (5 mL). The organic layer was dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:4 acetone:petroleum ether) to provide **8** (44 mg, 62%) as a white solid.

R_f: 0.40 (1:3 acetone:petroleum ether).

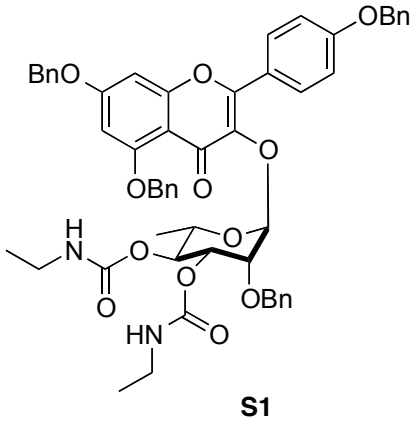
^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.80 (d, *J* = 9 Hz, 2H), 7.65 (d, *J* = 6 Hz, 2H), 7.52 (d, *J* = 6 Hz, 2H), 7.46-7.25 (m, 16H), 7.10 (d, *J* = 9 Hz, 2H), 6.58 (s, 1H), 6.51 (s, 1H), 5.58 (s, 1H), 5.25 (s, 2H), 5.14 (s, 2H), 5.10 (s, 2H), 4.80 (d, *J* = 9 Hz, 1H), 4.69 (d, *J* = 9 Hz, 1H), 4.53 (d, *J* = 18 Hz, 1H), 4.35 (m, 1H), 4.23 (d, *J* = 18 Hz, 1H), 3.83-3.63 (m, 3H), 3.45-3.35 (m, 2H), 2.10 (s, 3H), 1.99 (s, 3H), 1.01 (d, *J* = 3 Hz, 3H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 206.4, 205.2, 173.4, 162.9, 160.5, 160.1, 159.8,158.9, 154.4, 138.4, 138.1, 130.5, 128.82, 128.77, 128.66, 128.56, 128.4, 128.3, 128.2, 127.8, 127.6, 127.4, 126.7, 123.2, 114.7, 99.4, 98.1, 93.9, 80.14, 79.5, 78.1, 77.4, 77.3, 77.2, 77.1, 77.0, 76.92, 76.86, 76.76, 76.6, 73.9, 73.3, 71.9, 70.8, 70.5, 70.1, 69.2, 26.2, 26.2, 17.6 ppm.
HRMS: *m/z* = 905.3508 $(M+H)^+$. Calculated for $C_{55}H_{53}O_{12}$ 905.3532.



9

To a solution of **8** (20 mg, 0.022 mmol) in MeOH (1 mL) and EtOAc (2 mL) was added $Pd(OH)_2/C$ (10 mg) and the resulting mixture was placed under a balloon of H_2 and stirred at room temperature for 20 h, then more $Pd(OH)_2/C$ (10 mg) was added. Stirred for an additional 5 h, then more $Pd(OH)_2/C$ (10 mg) was added. The resulting mixture was stirred for an additional 21 h then filtered through Celite, eluting with 1:2 MeOH:EtOAc, and concentrated. The residue was purified by flash chromatography (silica gel, 1:1:0.1 EtOAc:petroleum ether:MeOH) to provide **9** (6 mg, 50%) as an off-white solid.
R_f: 0.58 (1:1:0.1 ethyl acetate:petroleum ether:methanol).
^{1}H NMR: (500 MHz, acetone-d_6) δ 7.88 (d, *J* = 10 Hz, 2H), 7.06 (d, *J* = 15 Hz, 2H), 6.49 (s, 1H), 6.28 (s, 1H), 5.42 (s, 1H), 4.38-4.34 (m, 1H), 4.22.4.20 (m, 2H), 3.81-3.71 (m, 3H), 3.51-3.43 (m, 2H), 3.24 (s, 2H), 1.00 (d, *J* = 10 Hz, 3H) ppm. (ketone methyl groups obscured by solvent peak)
^{13}C NMR: (125 MHz, acetone-d_6) δ 205.9, 204.9, 179.2, 162.0, 160.1, 157.6, 157.2, 130.8, 121.4, 115.5, 100.4, 98.7, 96.4, 93.8, 76.6, 74.0, 71.2, 68.5, 67.6, 65.3, 47.4, 25.6, 19.0, 16.7 ppm.
HRMS: *m/z* = 545.1672 $(M+H)^+$. Calculated for $C_{27}H_{29}O_{12}$ 545.1654.



S1

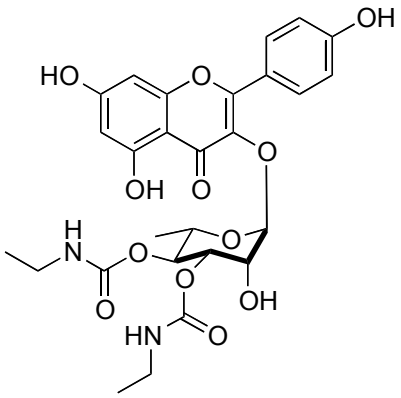
To a solution of **6** (139 mg, 0.175 mmol) in anhydrous DMF (1 mL) was added triethylamine (75 μL, 0.543 mmol) followed by ethyl isocyanate (42 μL, 0.526 mmol). The resulting solution was heated to 45 ºC and stirred for 18 h, then additional triethylamine (75 μL, 0.543 mmol) and ethyl isocyanate (42 μL, 0.526 mmol) were added. The resulting solution was stirred for an additional 5 h and additional triethylamine (75 μL, 0.543 mmol) and ethyl isocyanate (42 μL, 0.526 mmol) were added. The resulting solution was stirred for 16 h then cooled to room temperature and diluted with EtOAc (30 mL). The organic layer was washed with water (3 x 5 mL) and brine (5 mL), then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:3→1:2 EtOAc:petroleum ether) to provide **S1** (72 mg, 44%) as a clear glass.

R_f: 0.32 (1:2 ethyl acetate:petroleum ether).

^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.84 (d, *J* = 6 Hz, 2H), 7.61 (d, *J* = 6 Hz, 2H), 7.46-7.25 (m, 18H), 7.11 (d, *J* = 9 Hz, 2H), 5.74 (s, 1H), 5.26 (s, 2H), 5.15 (s, 2H), 5.08 (s, 2H), 4.95-4.89 (m, 1H), 4.80 (d, *J* = 24 Hz, 1H), 4.75-4.60 (m, 4H), 4.39 (br s, 1H), 3.35-3.22 (m, 1H), 3.20-3.00 (m, 4H), 1.15-0.98 (m, 6H), 0.89 (d, *J* = 6 Hz, 3H) ppm.

^{13}C NMR: (75 MHz, $CDCl_3$) δ 173.4, 171.9, 163.0, 160.5, 160.0, 159.1, 158.5, 155.8, 154.4, 138.6, 136.7, 135.9, 130.8, 129.0, 128.9, 128.8, 128.7, 128.4, 128.3, 127.9, 127.7, 127.9, 127.7, 127.6, 126.8, 123.7, 115.0, 110.3, 99.2, 98.5, 94.2, 73.1, 72.2, 71.3, 71.0, 70.7, 70.3, 68.9, 64.8, 36.1, 17.4, 15.4 ppm.

HRMS: *m/z* = 935.3770 $(M+H)^+$. Calculated for $C_{55}H_{55}N_2O_{12}$ 935.3750.



10

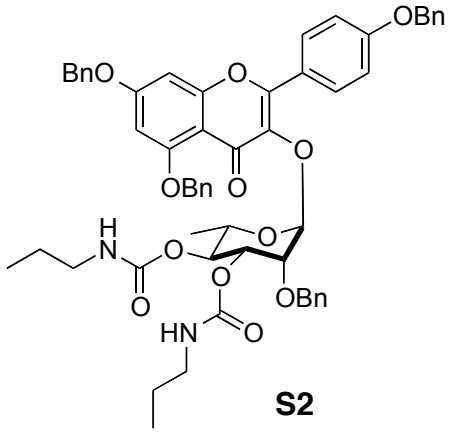
To a solution of **S1** (35 mg, 0.037 mmol) in MeOH (1 mL) and EtOAc (2 mL) was added $Pd(OH)_2/C$ (15 mg), and the resulting mixture was placed under a balloon of H_2 and stirred at room temperature for 17 h, then filtered through Celite (eluting with 1:2 MeOH:EtOAc) and concentrated. The residue was purified by flash chromatography (silica gel, 1:1:0.1 EtOAc:petroleum ether:MeOH) to provide **10** (20 mg, 94%) as a yellow solid.

R_f: 0.32 (1:1:0.1 ethyl acetate:petroleum ether:methanol).

^{1}H NMR: (300 MHz, acetone-d_6) δ 7.87 (d, *J* = 9 Hz, 2H), 7.04 (d, *J* = 9 Hz, 2H), 6.475 (d, *J* = 3 Hz, 1H), 6.275 (d, *J* = 3 Hz, 1H), 6.16-6.12 (m, 1H), 5.82-5.78 (br s, 1 H), 5.46 (s, 1H), 5.15-5.00 (m, 1H), 4.95-4.88 (m, 1H), 4.43 (br s, 1H), 3.62-3.50 (m, 1H), 3.25-2.95 (m, 4H), 1.20-1.00 (m, 6H), 0.84 (d, *J* = 6 Hz, 3H) ppm.

^{13}C NMR: (125 MHz, acetone-d_6) δ 179.6, 165.6, 163.7, 161.3, 158.8, 158.5, 157.0, 132.1, 122.9, 116.9, 106.3, 103.2, 100.1, 95.1, 72.7, 71.8, 70.4, 36.8, 36.6, 18.0, 15.9 ppm.

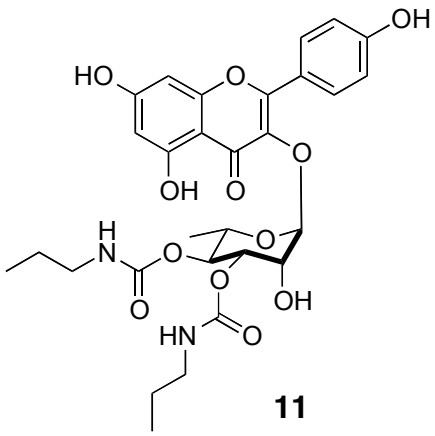
HRMS: *m/z* = 575.1857 $(M+H)^+$. Calculated for $C_{27}H_{31}N_2O_{12}$ 575.1872.



S2

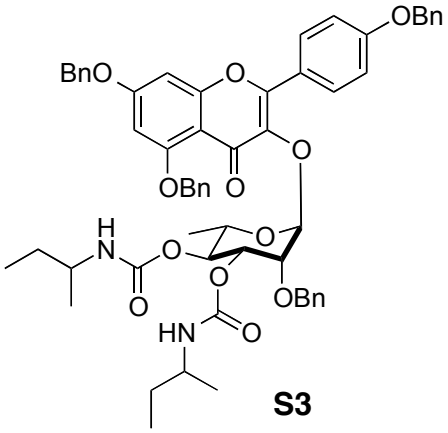
To a solution of **6** (72 mg, 0.091 mmol) in DMF (0.9 mL) was added triethylamine (0.13 mL, 0.91 mmol) followed by *n*-propyl isocyanate (77 μL, 0.82 mmol), and the resulting solution was heated to 45 ºC. After 26 h, additional treithylamine (70 μL, 0.49 mmol) and *n*-propyl isocyanate (35 μL, 0.37 mmol) were added. After an additional 18 h, the mixture was allowed to cool to room temperature and diluted with EtOAc (30 mL). The organic layer was washed with water (3 x 5

mL) and brine (5 mL), then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:3→1:2 EtOAc:petroleum ether) to provide **S2** (58 mg, 66%) as an off-white solid.
R_f: 0.36 (1:2 ethyl acetate:petroleum ether).
^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.84 (d, *J* = 9 Hz, 2H), 7.60 (d, *J* = 6 Hz, 2H), 7.51-7.19 (m, 18 H), 7.11 (d, *J* = 9 Hz, 2H), 6.565 (d, *J* = 3 Hz, 1H), 6.465 (d, *J* = 3 Hz, 1H), 5.73 (s, 1H), 5.26 (s, 2H), 5.15 (s, 2H), 5.08 (s, 2H), 4.91 (t, *J* = 12 Hz, 1H), 4.81-4.61 (m, 4H), 4.38 (br s, 1H), 3.35-3.15 (m, 2H), 3.15-2.85 (m, 4H), 1.53-1.30 (m, 4H), 1.00-0.75 (m, 9H) ppm.
^{13}C NMR: (75 MHz, $CDCl_3$) δ 173.07, 162.7, 160.2, 159.7, 158.8, 155.7, 154.1, 138.3, 137.6, 136.3, 135.6, 130.4, 128.7, 128.6, 128.5, 128.4, 128.1, 127.9, 127.54, 127.3, 127.2, 126.6, 123.3, 114.7, 110.0, 98.8, 98.1, 93.8, 77.4, 77.2, 77.0, 76.6, 72.7, 71.8, 71.0, 70.7, 70.4, 70.0, 68.5, 42.6, 23.0, 17.0, 11.1, 11.0 ppm.
HRMS: *m/z* = 963.4108 $(M+H)^+$. Calculated for $C_{57}H_{59}N_2O_{12}$ 963.4063.



11

To a solution of **S2** (44 mg, 0.046 mmol) in MeOH (1.2 mL) and EtOAc (2.4 mL) was added $Pd(OH)_2/C$ (18 mg), and the resulting mixture was placed under a balloon of H_2 and stirred at room temperature for 16 h, then filtered through Celite (eluting with 1:2 MeOH:EtOAc) and concentrated. The residue was purified by flash chromatography (silica gel, 1:1:0.1 EtOAc:petroleum ether:MeOH) to provide **11** (20 mg, 72%) as a yellow solid.
R_f : 0.45 (1:1:0.1 ethyl acetate:petroleum ether:methanol)
^{1}H NMR: (300 MHz, acetone-d_6) δ 7.87 (d, *J* = 9 Hz, 2H), 7.03 (d, *J* = 9 Hz, 2H), 6.48 (s, 2H), 6.28 (s, 1H), 6.23-6.10 (m, 1H), 5.45 (s, 1H), 5.22-5.00 (m, 2H), 4.95-4.86 (m, 1H), 4.42 (br s, 1H), 3.72-3.50 (m, 2H), 3.33-3.25 (m, 2H), 3.22-2.80 (m, 4H), 1.60-1.30 (m, 4H), 1.00-0.75 (m, 9 H) ppm.
^{13}C NMR: (125 MHz, acetone-d_6) δ 179.4, 165.5, 163.4, 161.1, 158.5, 158.2, 156.9, 131.9, 122.6, 116.8, 116.4, 105.9, 103.1, 100.0, 99.7, 94.9, 94.8, 72.6, 72.3, 71.8, 71.4, 70.3, 70.1, 43.9, 43.5, 24.2, 24.0, 23.9, 21.6, 21.5, 17.9, 17.8, 11.9, 11.8 ppm.
HRMS: *m/z* = 603.2169 $(M+H)^+$. Calculated for $C_{29}H_{34}N_2O_{12}$ 603.2185.



S3

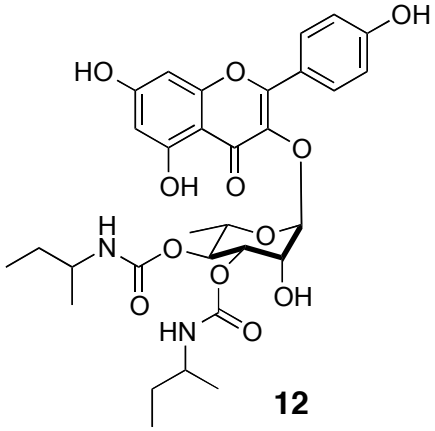
To a solution of **6** (55 mg, 0.069 mmol) in DMF (0.7 mL) was added triethylamine (97 μL, 0.69 mmol) followed by *sec*-butyl isocyanate (71 μL, 0.62 mmol). The resulting solution was heated to 45 ºC for 24 h, then additional triethylamine (50 μL, 0.36 mmol) and *sec*-butyl isocyanate (35 μL, 0.31 mmol) were added and the mixture was heated to 55 ºC. After 24 h, the mixture was cooled to room temperature and diluted with EtOAc (20 mL). The organic layer was washed with water (3 x 5 mL) and brine (5 mL), then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:3 EtOAc:petroleum ether) to provide **S3** (37 mg, 54% of a mixture of diastereomers) as an off-white solid.

R_f : 0.58 (1:3 ethyl acetate:petroleum ether).

^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.82 (d, *J* = 9 Hz, 2H), 7.28 (d, *J* = 6 Hz, 2H), 7.45-7.19 (m, 18H), 7.10 (d, *J* = 9 Hz, 2H), 6.56 (s, 1H), 6.45 (s, 1H), 5.67 (br s, 1H), 5.25 (s, 2H), 5.14 (s, 2H), 5.07 (s, 2H), 4.76-4.65 (m, 2H), 4.53-4.35 (m, 6H), 3.70-3.58 (m, 2H), 1.50-1.36 (m, 4H), 1.15-1.00 (m, 9H), 0.97-0.75 (m, 6H) ppm.

^{13}C NMR: (75 MHz, $CDCl_3$) δ 173.4, 163.0, 160.5, 160.0, 159.1, 157.9, 155.4, 136.6, 135.8, 130.7, 129.0, 128.9, 128.8, 128.7, 128.4, 128.1, 127.8, 127.6, 127.5, 126.9, 115.0, 99.3, 98.5, 94.2, 77.5, 73.0, 71.0, 70.7, 70.4, 69.0, 48.6, 47.5, 30.5, 21.3, 10.6 ppm.

HRMS: *m/z* = 991.4370 $(M+H)^+$. Calculated for $C_{59}H_{63}N_2O_{12}$ 991.4376.



12

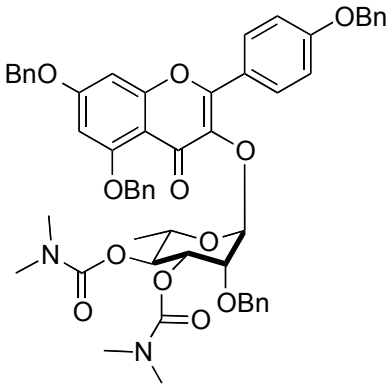
To a solution of **S3** (31 mg, 0.031 mmol) in MeOH (1 mL) and EtOAc (2 mL) was added $Pd(OH)_2/C$ (12 mg), and the resulting mixture was placed under a balloon of H_2 and stirred for 22 h, then filtered through Celite (eluting with 1:1 MeOH:EtOAc) and concentrated. The residue was purified by flash chromatography (silica gel, 1:1:0.05 EtOAc:petroleum ether:MeOH) to provide **12** (9 mg, 46% of a mixture of diastereomers) as a yellow solid.

R_f : 0.58 (1:1:0.1 ethyl acetate:petroleum ether:methanol).

^{1}H NMR: (500 MHz, acetone-d_6) δ 7.87 (d, *J* = 10 Hz, 2H), 7.04 (d, *J* = 10 Hz, 2H), 6.49 (s, 1H), 6.28 (s, 1H), 6.12-6.00 (m, 1H), 5.96-5.91 (m, 1H), 5.43 (d, *J* = 10 Hz, 1H), 5.25-5.00 (m, 4 H), 4.91-4.87 (m, 1H), 4.42-4.39 (m, 1H), 3.68-3.55 (m, 2H), 3.30-3.23 (m, 2H), 1.50-1.48 (m, 4H), 1.20-1.00 (m, 6H), 0.96-0.80 (m, 9 H) ppm.

^{13}C NMR: (125 MHz, acetone-d_6) δ 173.4, 166.3, 164.0, 161.9, 161.1, 159.1, 158.9, 157.9, 157.0, 156.9, 154.7, 154.5, 150.8, 133.1, 117.2, 103.8, 100.5, 95.5 72.8, 72.0, 70.9, 50.0, 48.3, 42.2, 31.7, 22.2, 11.6 ppm.

HRMS: *m/z* = 631.2517 $(M+H)^+$. Calculated for $C_{31}H_{39}N_2O_{12}$ 631.2498.

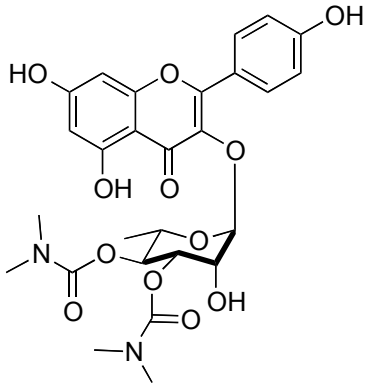


S4

A solution of **6** (48 mg, 0.061 mmol) in DMF (1 mL) was cooled in an ice/water bath and sodium hydride (60% in mineral oil, 10 mg, 0.24 mmol) was added. The resulting yellow solution was allowed to stir for 20 min, then dimethylcarbamoyl chloride (28 μL, 0.30 mmol) was added. The resulting mixture was removed from the ice bath and allowed to stir at room temperature for 2 h, then additional sodium hydride (10 mg, 0.24 mmol) was added followed by dimethylcarbamoyl chloride (28 μL, 0.30 mmmol) after 15 minutes. The resulting mixture was allowed to stir at room temperature for 2h then was quenched by addition of saturated aqueous NH_4Cl (2 mL) and dilted with EtOAc (20 mL) and water (5 mL). The layers were separated and the organic layer was washed with water (2 x 5 mL) and brine (5 mL), then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:1 EtOAc:petroleum ether) to provide **S4** (39 mg, 69%) as a cloudy oil.

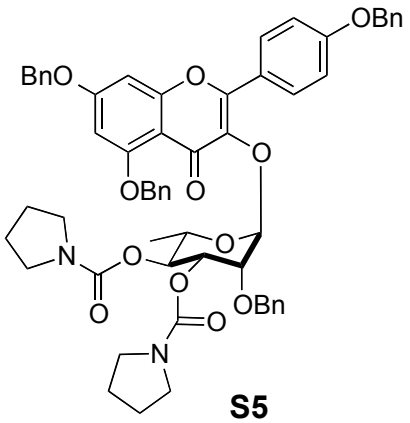
R_f : 0.52 (1:1 ethyl acetate:petroleum ether).

^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.88 (d, *J* = 9 Hz, 2H), 7.59 (d, *J* = 9 Hz, 2H), 7.45-7.19 (m, 18H), 7.12 (d, *J* = 9 Hz, 2H), 6.565 (d, *J* = 3 Hz, 1H), 6.465 (d, *J* = 3 Hz, 1H), 5.92 (s, 1H), 5.27 (s, 2H), 5.19-5.14 (m, 1H), 5.10 (s, 2H), 5.8 (s, 2H), 5.02-4.90 (m, 1H), 4.79 (d, *J* = 12 Hz, 1 H), 4.67 (d, *J* = 12 Hz, 1H), 4.41-3.37 (m, 1H), 3.26-3.18 (m, 1H), 2.92-2.73 (m, 12H), 0.88 (d, *J* = 6 Hz, 3H) ppm.
^{13}C NMR: (75 MHz, $CDCl_3$) δ 173.3, 162.9, 160.4, 160.0, 159.0, 155.8, 154.3, 138.4, 137.2, 136.6, 135.8, 130.7, 129.2, 129.1, 128.9, 128.8, 128.7, 128.6, 128.3, 128.2, 128.0, 127.8, 127.6, 127.5, 126.8, 123.6, 114.8, 110.3, 98.4, 98.3, 94.1, 77.6, 77.4, 77.2, 76.8, 76.5, 73.0, 72.7, 72.4, 70.9, 70.6, 70.1, 68.8, 36.7, 36.6, 36.1, 36.0, 17.3 ppm.
HRMS: *m/z* = 935.3781 $(M+H)^+$. Calculated for $C_{55}H_{55}N_2O_{12}$ 935.3750.



13

To a solution of **S4** (31 mg, 0.033 mmol) in MeOH (1 mL) and EtOAc (2 mL) was added $Pd(OH)_2/C$ (13 mg), and the resulting mixture was placed under a balloon of H_2 and stirred for 18 h, then filtered through Celite (eluting with 1:2 MeOH:EtOAc) and concentrated. The residue was purified by flash chromatography (silica gel, 2:1:0.1 EtOAc:petroleum ether:MeOH) to provide **13** (16 mg, 84%) as a yellow solid.
R_f: 0.18 (1:1:0.1 ethyl acetate:petroleum ether:methanol).
^{1}H NMR: (500 MHz, acetone-d_6) δ 7.89 (d, *J* = 10 Hz, 2H), 7.06 (d, *J* = 10 Hz, 2H), 6.49 (s, 1H), 6.28 (s, 1H), 5.81 (s, 1H), 5.10-5.05 (m, 1H), 4.96-4.90 (m, 1H), 4.80-4.65 (br s, 1H), 4.43 (s, 1H), 3.27-3.20 (m 2H), 3.20-2.75 (m, 14H), 0.805 (d, *J* = 5 Hz, 3H) ppm.
^{13}C NMR: (125 MHz, acetone-d_6) δ 179.1, 165.3, 163.3, 161.1, 158.6, 158.1, 156.4, 156.3, 134.7, 131.8, 122.4, 116.5, 105.8, 101.4, 99.7, 94.7, 73.7, 72.6, 36.6, 36.1, 17.7 ppm.
HRMS: *m/z* = 575.1868 $(M+H)^+$. Calculated for $C_{27}H_{31}O_{12}N_2$ 575.1872.



S5

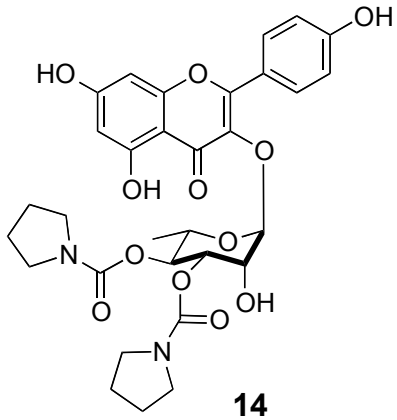
A solution of **6** (48 mg, 0.065 mmol) in DMF (1 mL) was cooled in an ice/water bath and sodium hydride (60% in mineral oil, 13 mg, 0.325 mmol) was added. The resulting mixture was allowed to stir for 20 min, then 1-pyrrolidinylcarbamoyl chloride (43 μL, 0.39 mmol) was added. The resulting mixture was removed from the cold bath and allowed to stir at room temperature for 4 h, then was quenched by addition of saturated aqueous NH_4Cl (2 mL) and diluted with EtOAc (20 mL) and water (5 mL). The layers were separated and the organic layer was washed with water (2 x 5 mL) and brine (5 mL), then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:2 EtOAc:petroleum ether) to provide **S5** (43 mg, 67%) as a white solid.

R_f: 0.50 (1:1 ethyl acetate:petroleum ether).

^{1}H NMR: (300 MHz, $CDCl_3$) δ 7.88 (d, *J* = 9 Hz, 2H), 7.59 (d, *J* = 6 Hz, 2H), 7.50-7.19 (m, 18 H), 7.13 (d, *J* = 6 Hz, 2H), 6.565 (d, *J* = 3 Hz, 1H), 6.455 (d, *J* = 3 Hz, 1H), 5.935 (d, *J* = 3 Hz, 1H), 5.28-5.16 (m, 3H), 5.11 (s, 2H), 5.08 (s, 2H), 5.02-4.85 (m, 2H), 4.80-4.71 (m, 2H), 4.40-4.37 (m, 1H), 3.45-3.05 (m, 8H), 1.85-.165 (m, 8H), 0.89 (d, *J* = 6 Hz, 3H) ppm.

^{13}C NMR: (75 MHz, $CDCl_3$) δ 173.4, 163.0, 160.5, 160.0, 159.1, 154.4, 138.6, 137.2, 136.6, 135.9, 130.8, 129.0, 128.9, 128.8, 128.7, 128.4, 128.1, 127.8, 127.6, 127.5, 126.9, 123.7, 115.0, 110.4, 98.4, 94.2, 73.2, 72.6, 72.1, 71.0, 70.7, 70.2, 68.9, 4.4, 46.1, 45.9, 26.0, 25.9, 17.5 ppm.

HRMS: *m/z* = 987.4100 $(M+H)^+$. Calculated for $C_{59}H_{59}N_2O_{12}$ 987.4063.



14

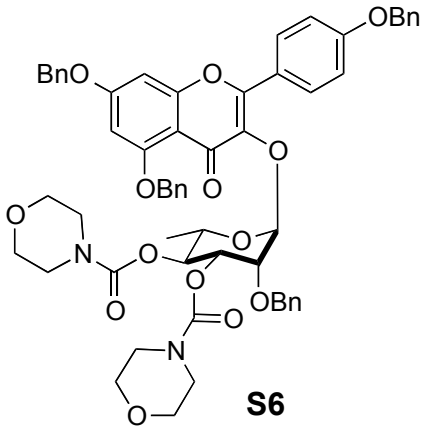
To a solution of **S5** (36 mg, 0.036 mmol) in MeOH (1 mL) and EtOAc (2 mL) was added $Pd(OH)_2/C$ (14 mg). The resulting mixture was placed under a balloon of H_2 and stirred for 21 h, then filtered through Celite (eluting with 1:1 MeOH:EtOAc) and concentrated. The residue was purified by flash chromatography (silica gel, 1:1:0.1 EtOAc:petroleum ether:MeOH) to provide **14** (19 mg, 84%) as a yellow solid.

R_f: 0.24 (1:1:0.1 ethayl acetate:petroleum ether:methanol).

^{1}H NMR: (500 MHz, acetone-d_6) δ 7.885 (d, *J* = 5 Hz, 2H), 7.06 (d, *J* = 10 Hz, 2H), 6.485 (d, *J* = 5 Hz, 1H), 6.275 (d, *J* = 5 Hz, 1H), 5.82 (s, 1H), 5.145 (dd, *J* = 10, 5 Hz, 1H), 4.90 (ap t, *J* = 10 Hz, 1H), 4.40 (ap s, 1H), 3.40-3.15 (m, 12H), 1.84-1.75 (m, 8H), 0.815 (d, *J* = 5 Hz, 3H) ppm.

^{13}C NMR: (125 MHz, acetone-d_6) δ 178.2, 164.4, 162.3, 160.1, 157.6, 157.1, 153.9, 153.8, 133.7, 130.8, 121.4, 115.6, 115.5, 104.8, 100.4, 98.8, 93.8, 72.3, 71.4, 68.9, 68.7, 46.0, 45.7, 45.5, 25.5, 25.4, 24.74, 24.72, 17.5 ppm.

HRMS: *m/z* = 627.2173 $(M+H)^+$. Calculated for $C_{31}H_{35}N_2O_{12}$ 627.2185.



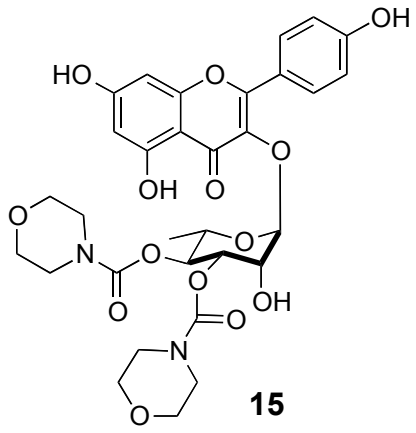
S6

To a solution of **6** (42 mg, 0.053 mmol) in DMF (1 mL) cooled in an ice/water bath was added sodium hydride (60% in mineral oil, 11 mg, 0.265 mmol). The resulting mixture was allowed to stir for 20 minutes, then 4-morpholinecarbonyl chloride (37 μL, 0.318 mmol) was added. The resulting mixture was removed from the ice bath and allowed to stir at room temperature for 18h, then quenched by addition of saturated aqueous NH_4Cl (2 mL), and diluted with EtOAc (20 mL) and water (5 mL). The layers were separated and the organic layer was washed with water (2 x 5 mL) and brine (5 mL), then dried ($MgSO_4$), filtered, and concentrated. The residue was purified by flash chromatography (silica gel, 1:1 EtOAc:petroleum ether) to provide **S6** (14 mg, 26%) as a yellow solid.

R_f: 0.48 (2:1 ethyl acetate:petroleum ether).

^{1}H NMR: (500 MHz, $CDCl_3$) δ 7.86 (d, *J* = 10 Hz, 2H), 7.59 (d, *J* = 10 Hz, 2H), 7.50-7.18 (m, 18 H), 7.105 (d, *J* = 5 Hz, 2H), 6.57 (s, 1H), 6.47 (s, 1H), 5.89 (s, 1H), 5.28 (s, 2H), 5.24-5.20 (m, 1H), 5.12 (s, 2H), 5.09 (s, 2H), 5.02-4.97 (m, 1H), 4.79 (d, *J* = 10 Hz, 1H), 4.63 (d, *J* = 10 Hz, 1H), 4.39 (s, 1H), 3.75-3.12 (m, 17 H), 0.895 (d, *J* = 5 Hz, 3H) ppm.

^{13}C NMR: (125 MHz, $CDCl_3$) δ 173.2, 162.8, 160.3, 159.8, 158.9, 154.4, 154.1, 138.1, 137.2, 136.3, 136.2, 135.6, 130.6, 128.8, 128.6, 128.5, 128.3, 128.2, 127.8, 127.7, 127.6, 127.3, 126.6, 123.4, 114.7, 110.1, 98.9, 98.3, 98.1, 93.9, 76.3, 72.74, 72.69, 72.1, 70.8, 70.5, 70.1, 684, 66.6, 44.3, 43.9, 17.2 ppm.
HRMS: *m/z* = 1019.3984 $(M+H)^+$. Calculated for $C_{59}H_{59}N_2O_{14}$ 1019.3961.



15

To a solution of **S6** (13 mg, 0.013 mmol) in MeOH (1 mL) and EtOAc (2 mL) was added $Pd(OH)_2/C$ (5 mg). The resulting mixture was placed under a balloon of H_2 and stirred at room temperature for 18 h, then filtered through Celite (eluting with 1:2 MeOH:EtOAc) and concentrated. The resulting mixture was purified by flash chromatography (silica gel, 3:2:0.1 EtOAc:petroleum ether:MeOH) to provide **15** (7 mg, 82%) as a yellow solid.
R_f: 0.40 (3:2:0.1 ethyl acetate:petroleum ether:methanol).
^{1}H NMR: (500 MHz, acetone-d_6) δ 7.88 (d, *J* = 10 Hz, 2H), 7.07 (d, *J* = 10 Hz, 2H), 6.48 (s, 1H), 6.28 (s, 1H), 5.88 (s, 1H), 5.145 (dd, *J* = 10, 5 Hz, 1H), 4.95-4.90 (m, 1H), 4.40 (s, 1H), 3.70-3.25 (m, 16 H), 3.20-3.15 (m, 1H), 0.815 (d, *J* = 5 Hz, 3H) ppm.
^{13}C NMR: (125 MHz, actone-d_6) δ 174.3, 163.3, 161.0, 158.4, 158.2, 156.0, 155.2, 155.1, 131.9, 122.5, 116.7, 116.3, 110.3, 105.6, 101.1, 100.9, 100.0, 99.8, 94.8, 73.4, 73.0, 72.6, 70.0, 69.5, 69.4, 69.2, 67.4, 67.3, 45.3, 45.0, 17.6 ppm.
HRMS: *m/z* = 659.2108 $(M+H)^+$. Calculated for $C_{31}H_{35}N_2O_{14}$ 659.2083.