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De Novo Synthesis and Biological Evaluation of C6″-Substituted C4″-Amide Analogues of SL0101

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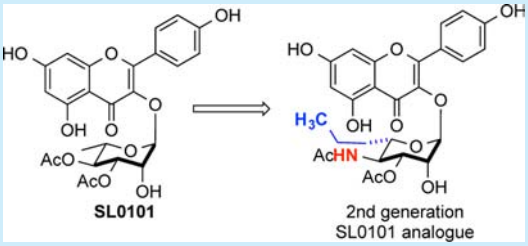
Supporting Information

ABSTRACT: In an effort to improve upon the *in vivo* half-life of the known ribosomal s6 kinase (RSK) inhibitor SL0101, C4″-amide/C6″-alkyl substituted analogues of SL0101 were synthesized and evaluated in cell-based assays. The analogues were prepared using a de novo asymmetric synthetic approach, which featured Pd-π-allylic catalyzed glycosylation for the introduction of a C4″-azido group. Surprisingly replacement of the C4″-acetate with a C4″-amide resulted in analogues that were no longer specific for RSK in cell-based assays.



The ribosomal s6 kinases (RSKs) are a family of Ser/Thr kinases, which are downstream effectors of the extracellular signal-regulated kinase 1/2 pathways.[1] RSK appears to be involved in the etiology of a number of different cancers and, importantly, regulates a motility/invasive gene program.[2] RSK is a dual kinase domain protein with the N-terminal kinase domain (NTKD) responsible for phosphorylation of target substrates.[3] In a screen of botanical extracts SL0101 (**1**), a flavonoid glycoside, was identified as an inhibitor of the NTKD of RSK.[4] SL0101 (**1**) is a relatively selective inhibitor for RSK with a K_i of ∼1 μM. From the crystal structure of SL0101 (**1**) complexed with the NTKD isoform of RSK2[5] and de novo synthetic studies,[6] we identified analogues (**2** and **3**) with C6″-substitutions of the rhamnose that showed improved efficacy in the *in vitro* kinase assays.[7,8]

SL0101 (**1**) has a short biological half-life *in vivo*,[7] which is presumably due to hydrolysis of the C3″/C4″-acetates which are necessary for high affinity.[9] To identify less labile groups that could replace the ester without loss of affinity, we investigated replacing the C4″-acetate with a C4″-acetamide in combination with the C6″-alkyl substitution that we previously identified.[7] Specifically, we targeted six C4″-acetamide analogues **4a**–**d** and **5a**–**b** (Figure 1).

Retrosynthetically, we envisioned that C4″-acetamide substituted analogues **6** could arise from C4″-azido sugar **7a**, which could be prepared from enone sugar **7c** via allylic carbonate **7b** (Scheme 1). Previously we have shown that C4 allylic azides such as **7a** could be prepared from C4 allylic carbonates like **7b** via Pd-catalyzed allylic alkylation.[10] However, this approach was not compatible for pyran rings with a C1 kaempferol group. To address this issue, a Pd-glycosylation method was developed for the direct incorporation of a C4 azido sugar.

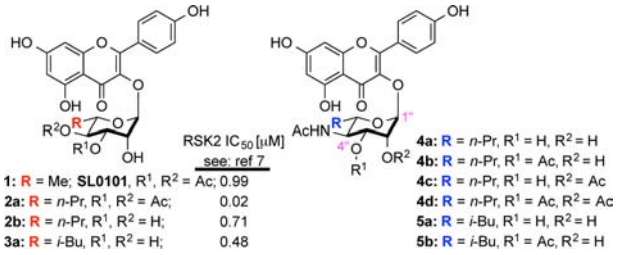


Figure 1. C4″-amide analogues of SL0101.

Our synthesis started with exposure of flavonol **9** and Boc-pyranone **13** to our typical glycosylation conditions (2.5 mol % $Pd_2(DBA)_3{\cdot}CHCl_3$ and 10 mol % of PPh_3 in CH_2Cl_2 at 0 °C; 95%), which produced glycosylated pyranone **14** with complete α-selectivity (Scheme 2). Reduction of the enone **14** ($NaBH_4/CeCl_3$, −78 °C in CH_2Cl_2/MeOH; 72%) resulted stereoselectively in allylic alcohol **15**.[6] A methyl carbonate leaving group was installed on the allylic alcohol by reaction of **15** with methyl chloroformate to form the C4″-carbonate **16** in 75% yield. Unfortunately, exposure of carbonate **16** to the Sinou conditions ($TMSN_3$, $[Pd(allyl)Cl]_2$/1,4-bis-(diphenylphosphino)butane) failed to afford the desired regio- and stereoisomeric allylic azide **17**. The C-1 kaempferol proved to be the better leaving group, as only products consistent with the hydrolysis at the anomeric position were observed.[11]

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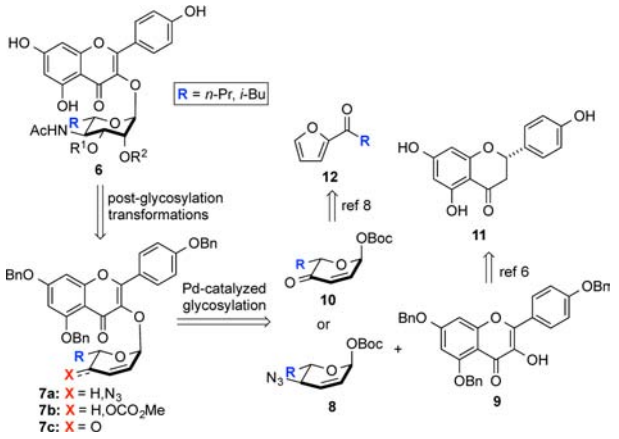
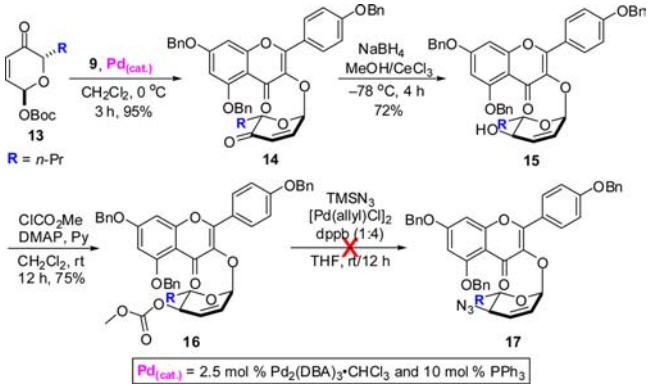
Scheme 1. Retrosynthesis of C4″-Amide SL0101 Analogues

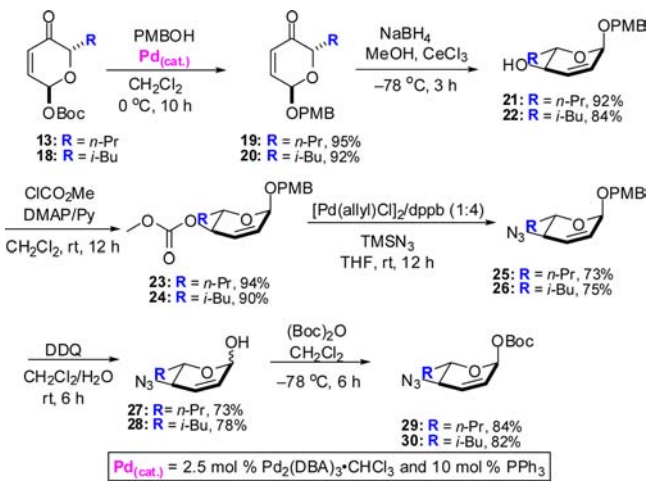


Scheme 2. Unsuccessful Approach to C4″-Azide Sugar 17

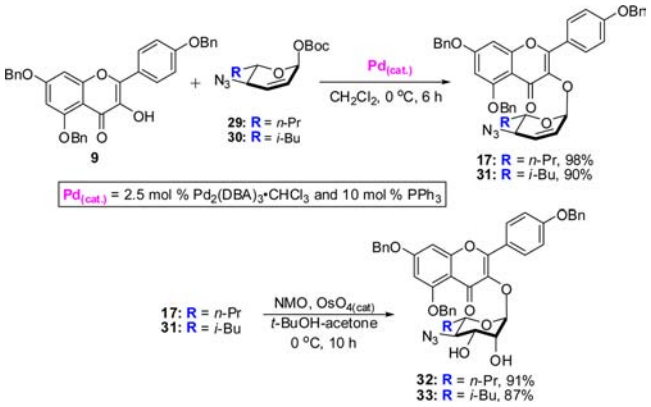


To solve this problem, we decided to try reversing the sequence of the two Pd-π-allyl substitution reactions, which required the synthesis of allylic azides **29** and **30** (Scheme 3). This began with a palladium-catalyzed glycosylation (Pd(0)/PPh_3, 1:2) of *p*-methoxybenzyl alcohol with Boc-pyranones **13** and **18** which stereoselectively afforded PMB-pyranones **19** and **20** (95% and 92% respectively). Diastereoselective reduction of the two enones ($NaBH_4/CeCl_3$, −78 °C in CH_2Cl_2/MeOH; 92% and 84%) gave allylic alcohols **21** and **22**. Treatment of the two allylic alcohols with methyl chloroformate in the presence of a catalytic amount of DMAP gave the allylic carbonates **23** and **24** (94% and 90%). Exposure of the carbonates to the Sinou conditions ($TMSN_3$, $[Pd(allyl)Cl]_2$/1,4-bis(diphenylphosphino)butane) regio- and stereospecifically afforded the desired allylic azides **25** and **26** (73% and 75%). An oxidative PMB deprotection (DDQ/H_2O) of **25** and **26** provided anomeric alcohols **27** and **28** as a 13:1 mixture of anomers in 73% and 78% yields. The following Boc-protection of the two alcohols produced the key azido containing intermediates **29** and **30** in 84% and 82% yields with excellent diastereoselectivity.

Scheme 3. Synthesis of C4″-Azide Sugar Glycosyl Donors 29/30



Scheme 4. Synthesis of C4″-Azido Rhamno-sugars 32/33



We next investigated the reduction and acylation of azidodiols **32** and **33** (Scheme 5).[6] Fortuitously, both the C4 acylated amides **36** and **37** and C3/C4 bis-acylated products **34** and **35** were generated in an ∼1:1 mixture in one pot from the reduction of **32** and **33** with zinc dust in the presence of acetic anhydride and acetic acid. Thus, the reduction acylation*n* of **32** gave the desired C4″-acetamides **36** (29%) and **34** (27%), whereas the reduction acylation of **33** gave the desired C4″-acetamides **37** (36%) and **35** (32%).

The intermediates **34**−**37** were globally deprotected by an exhaustive hydrogenolysis, which produced four of the desired analogues. Thus, exposure of **34** and **35** to typical hydrogenolysis conditions (1 atm of hydrogen with Pd/C) furnished **4b** and **5b** in good yields (86% and 88%, respectively). Exposure of **36** and **37** to similar hydrogenolysis conditions furnished **4a** and **5a** in good yields (90% and 86%, respectively). Finally the last two analogues **4c** and **4d** were prepared by an acylation deprotection sequence. The peracylated product **4d** was prepared from **34** in 91% overall yield by bis-acylation (Ac_2O, DMAP/Py; 97%) and exhaustive hydrogenolysis. Similarly, the C2 acylated product **4c** was prepared from **36** via an ortho-ester mediated C2-acylation ($CH_3C(OMe)_3$, 10% *p*-TsOH in CH_2Cl_2; then excess 90%

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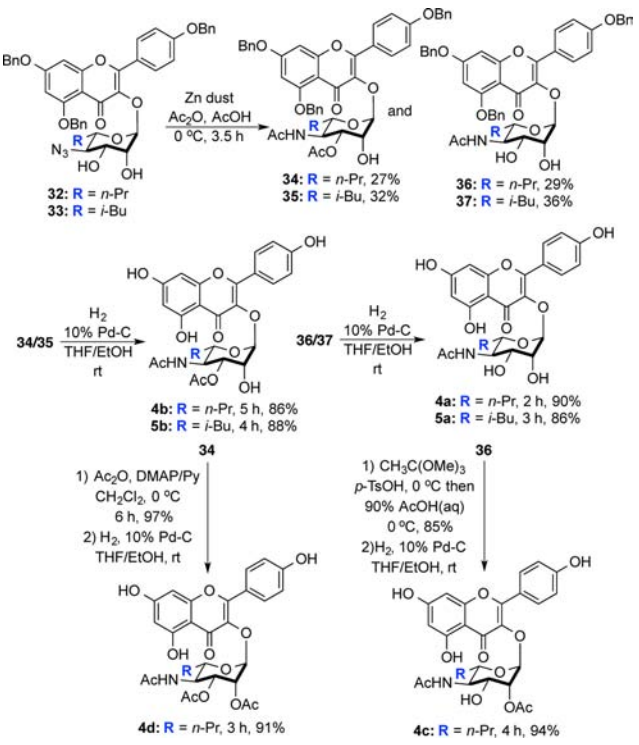
Scheme 5. Synthesis of C4″-Amide Analogues of SL0101 (4/5)



AcOH/H_2O; 85%) and per-hydrogenolysis (94% overall yield).

The efficacy of the analogues **4a−d** and **5a−b** to inhibit RSK2 activity was determined in an *in vitro* kinase assay using purified recombinant RSK2 (Table 1).[4] The data were fit using nonlinear regression analysis. In the *n*-Pr series, **4b** and **4c** with a single acetate at the C3″- or C2″-position had significantly lower (~5-fold) IC_{50}'s compared to SL0101. However, when compared with our best analogue **2a** (C3″/C4″-diacetate, Figure 1) the related C4″-acetamide **4b** had a 10-fold increase in IC_{50}.[7] The IC_{50}'s for **4a** with no C2″- or C3″-acetate and **4d** with two acetates were not statistically different from that of SL0101. These results are similar to those obtained in the series in which the acetyl group was at the C4″-position.[7] In the isobutyl series the C3″-acetate **5b** had a 3-fold improved IC_{50} compared to that of SL0101, whereas **5a** with no C2″- or C3″-acetate had a much poorer IC_{50} than SL0101. This suggests that, in the *n*-Pr-series, the C4″-acetamide can replace the C4″-acetate without dramatically compromising the affinity of the analogues for RSK2.

The six analogues were evaluated for their ability to decrease proliferation of the breast cancer cell line, MCF-7 (Table 1). Initially, each analogue was tested at a dose of 100 μM and compared to SL0101 (**1**). Analogue **4d** was the only analogue that inhibited proliferation to a greater extent than SL0101 (**1**). A dose response curve with **4d** showed that cytostasis occurred at ~35 μM and substantial cell death occurred at ~50 μM (see Suppporting Information (SI)). For comparison SL0101 (**1**) at 100 μM (maximum concentration) induces a reduction in proliferation (~60%). To evaluate whether **4d** was specific for RSK, we compared its antiproliferative effects in MCF-7 cells versus MCF-10A, an immortalized nontransformed human breast cell line. We previously found that a preferential ability to inhibit MCF-7 compared to MCF-10A proliferation correlates with specificity for RSK inhibition.[4,7−12] At 25 μM **4d** inhibited proliferation of MCF-7 cells by 50% and mariginally inhibited MCF-10A proliferation (see SI). However, at 50 μM of **4d**, a cytotoxic dose in MCF-7 cells, proliferation of MCF-10A cells was inhibited by 70%. Thus, **4d** shows a very limited ability to preferentially inhibit MCF-7 proliferation and survival compared to MCF-10A cells. These results suggest that **4d** is not a specific RSK inhibitor in intact cells.

To further evaluate the specificity of **4d** at inhibiting RSK, we compared the efficacy of SL0101 (**1**) and **4d** to alter the phosphorylation of known RSK substrates. We chose to test **4d** at both cytostatic (25 μM) and cytotoxic (50 μM) concentrations. To increase the phosphorylation of substrates MCF-7 cells were stimulated with the mitogen, phorbol myristate acetate (PMA), after a pretreatment with inhibitor or vehicle. RSK phosphorylates and inhibits the activity of eukaryotic elongation factor 2 (eEF2) kinase.[13] Thus, inhibition of RSK relieves the inhibition of eEF2 kinase, which results in an increase in p-eEF2. As expected activation of RSK by PMA led to a decrease in p-eEF2 and inhibition of RSK with SL0101 increased p-eEF2 compared to the PMA control (Figure 2).

Ribosomal protein S6, a component of the 40S ribosomal subunit, is phosphorylated by RSK,[14] and in agreement with these data SL0101 (**1**) inhibits PMA-induced phosphorylation of S6. We have also found that RSK regulates the levels of the oncogene, cyclin D1, in MCF-7 cells.[15] Consistent with these observations SL0101 (**1**) inhibited cyclin D1 levels. In contrast with our observations with SL0101 the analogue **4d** did not alter the phosphorylation status of eEF2, S6 or the levels of cyclin D1. To further investigate the ability of **4d** to inhibit RSK in intact cells, we immunoblotted the lysates with an antibody against the phosphorylation motif that is

Table 1. *In Vitro* Potency of SL0101 (1) and Analogues[a]

compd name	RSK2 IC_{50} [μM]	RSK2 IC_{50} p(**1**)	MCF-7 proliferation [% control]	MCF-7 proliferation p(DMSO)	MCF-7 proliferation p(**1**)
SL0101 (**1**)	1.04 ± 0.60		38.6 ± 14.6	<0.01	
4a	0.76 ± 0.43	0.17	94.5 ± 21.6	0.41	
4b	0.23 ± 0.07	<0.01	39.2 ± 7.2	<0.01	0.91
4c	0.11 ± 0.09	<0.01	47.8 ± 10.6	<0.01	0.16
4d	0.44 ± 0.39	0.02	−80.0 ± 6.6	<0.01	<0.01
5a	2.33 ± 0.88	<0.01	47.5 ± 19.1	<0.01	0.21
5b	0.32 ± 0.18	<0.01	24.6 ± 10.4	<0.01	0.03

[a]RSK2 IC_{50}: concentration needed for 50% RSK2 inhibition (n > 2; quadruplicate: mean, S.D.; p(**1**) Student's t test compared to SL0101(**1**)). MCF-7 proliferation: (n > 2; triplicate: mean, S.D.; p(DMSO) Student's t test compared to control; p(**1**) Student's t test compared to SL0101 (**1**)). p < 0.01 significant.

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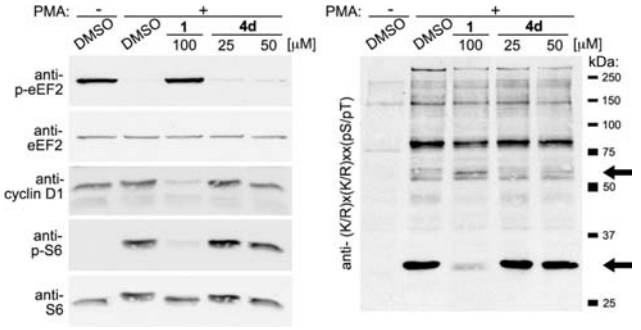

Figure 2. RSK biomarkers comparison of **4d** and **1**. Comparison of analogue **4d** and SL0101 (**1**) was made against known RSK biomarkers in intact cells. MCF-7 cells were pretreated with **4d** at the indicated concentrations and then treated with vehicle or PMA. Lysates were analyzed by immunoblotting. The motif, (K/R)x(K/R)xx(pS/pT), is recognized by a number of kinases, including RSK. The arrows indicate bands whose intensity is altered upon treatment of cells with SL0101 (**1**).

recognized by numerous kinases, including RSK. Treatment with SL0101 increased the phosphorylation of a band at ∼60 kDa and decreased the intensity of a band at ∼27 kDa. Analogue **4d** did not alter the phosphorylation pattern as compared to PMA. Consistent with these results we observed that analogue **4b** (100 μM) did not alter the phosphorylation of RSK biomarkers or cyclin D1 levels in intact cells (data not shown). These results suggest that the amide analogues of SL0101 (**1**) are not specific for RSK.

In conclusion, using de novo synthesis C4″-acetamide analogues of SL0101 with a C6″ substitution were prepared and evaluated as RSK inhibitors. Analogues with improved *in vitro* kinase inhibitory activities were identified; however, this increase in activity came at a loss of selectivity for RSK. Further studies aimed at defining the requirements for a specific-RSK inhibition are ongoing and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures and spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Author Contributions

§R.M.M., Z.M.S., R.V., B.W., and Q.Z. are co-first authors; the order is alphabetical.

Notes

The authors declare no competing financial interest.

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