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Influence of rhamnose substituents on the potency of SL0101, an inhibitor of the Ser/Thr kinase, RSK

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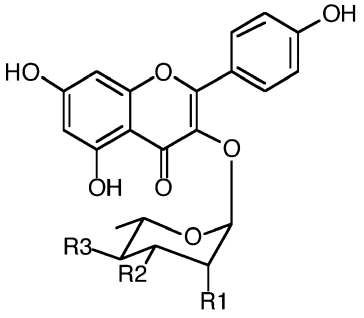
Abstract—We have previously reported the isolation of kaempferol 3-*O*-(3″,4″-di-*O*-acetyl-α-L-rhamnopyranoside) from *Forsteronia refracta* [Xu, Y.-M.; Smith, J. A.; Lannigan, D. A.; Hecht, S. M. *Biorg. Med. Chem.* **2006**, *14*, 3974–3977.]. This flavonoid glycoside, termed SL0101, is a specific inhibitor of p90 ribosomal S6 kinase (RSK) with a dissociation constant of 1 μM. In intact cells, however, the EC_{50} for inhibition of RSK activity is 50 μM, which suggests that the efficacy of SL0101 could be limited by cellular uptake. Therefore, we investigated the possibility of developing a more potent RSK inhibitor by synthesizing SL0101 analogs with increased hydrophobic character. The total syntheses of kaempferol 3-*O*-(3″,4″-di-*O*-butyryl-α-L-rhamnopyranoside) (Bu-SL0101) and kaempferol 3-*O*-(2″,3″,4″-tri-*O*-acetyl-α-L-rhamnopyranoside) (3Ac-SL0101) were performed. The IC_{50} for inhibition of RSK activity in in vitro kinase assays for the analogs was similar to that obtained for SL0101. 3Ac-SL0101 demonstrated the same remarkable specificity for inhibiting RSK activity in intact cells as SL0101; however, Bu-SL0101 was not completely specific. 3Ac-SL0101 was ~2-fold more potent at inhibiting MCF-7 cell proliferation compared to SL0101 and preferentially decreased MCF-7 cell growth, as compared to the growth of the normal human breast line, MCF-10A. Thus the discovery of 3Ac-SL0101 as a more potent RSK-specific inhibitor than SL0101 should facilitate the development of RSK inhibitors as anti-cancer chemotherapeutic agents.

1. Introduction

The p90-kDa ribosomal S6 kinase (RSK) family members are downstream effectors of mitogen-activated protein kinase (MAPK). MAPK is known to be important in proliferation and oncogenesis.[1,2] However, it has only now become possible to distinguish the function of RSK in these processes from those of MAPK itself and of the many other downstream MAPK effectors because of the recent discovery of a RSK-specific inhibitor.[3,4] We reported that an extract from *Forsteronia refracta*, a member of the Apocynaceae (dogbane) family found in the South American rainforest, was able to specifically inhibit RSK catalytic activity.[4] Fractionation of the extract led to the isolation of an active component kaempferol 3-*O*-(3″,4″-di-*O*-acetyl-α-L-rhamnopyranoside), also referred to as SL0101 (Fig. 1). SL0101 is



	R1	R2	R3
SL0101	OH	OAc	OAc
2″,4″ di-*O*-acetyl-SL0101	OAc	OH	OAc
4″ mono-*O*-acetyl-SL0101	OH	OH	OAc

Figure 1. Structures of SL0101, 2″,4″-di-*O*-acetyl-SL0101, and 4″-mono-*O*-acetyl-SL0101.

Keywords: Kaempferol 3-*O*-(3″,4″-di-*O*-butyryl-α-L-rhamnopyranoside), Bu-SL0101; Kaempferol 3-*O*-(2″,3″,4″-tri-*O*-acetyl-α-L-rhamnopyranoside), 3Ac-SL0101; RSK; Kinase inhibitor.
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competitive with respect to ATP with a dissociation constant, K_i, of 1 μM.[4] This compound is an effective and specific inhibitor of RSK in intact cells.[4] SL0101 inhibits proliferation of the human breast cancer cell line, MCF-7, with an efficacy paralleling its ability to inhibit RSK in intact cells. Significantly, SL0101 does not prevent the growth of the normal human breast line, MCF-10A, even though it inhibits RSK activity in these cells.[4] We have found that RSK increases the transcriptional activity of estrogen receptor α (ERα) and the androgen receptor (AR).[5–7] Increased activity of ERα and AR is known to be important in the etiology of some breast and prostate cancers, respectively. Furthermore, we have found that RSK levels are higher in ~50% of human breast and prostate tumors compared to normal tissue.[4,7] Evidence from other laboratories has demonstrated that RSK is involved in osteosarcoma formation and in non-small cell lung carcinoma.[8,9] Taken together, these results suggest that RSK could serve as an important novel drug target in some types of cancers. Protein kinases have been shown to be excellent targets for drug discovery[10] and thus SL0101 is a useful chemotherapeutic lead compound.

The efficacy of SL0101 as a RSK inhibitor in intact cells is ~50-fold higher than its dissociation constant, which suggests that the potency of SL0101 in cells could be limited by cellular uptake. Therefore, we investigated the possibility of obtaining a more effective RSK-specific inhibitor by synthesizing analogs that have a higher octanol–water partition coefficient, Log *P*, than SL0101. We increased the calculated Log *P* by either replacing the 3″- and 4″-acetyl groups on the rhamnose with butyryl groups or substituting the 2″-hydroxyl group with an acetyl group. The syntheses of kaem-pferol 3-*O*-(3″,4″-di-*O*-butyryl-α-L-rhamnopyranoside) (Bu-SL0101) and kaempferol 3-*O*-(2″,3″,4″-tri-*O*-acetyl-α-L-rhamnopyranoside) (3Ac-SL0101) were performed. We found that 3Ac-SL0101, like synthetic SL0101,[11] is an effective and specific inhibitor of RSK activity in intact cells. Bu-SL0101 was modestly more potent at inhibiting MCF-7 proliferation than SL0101 but was not completely specific for RSK inhibition in intact cells. However, 3Ac-SL0101 was ~ 2-fold more effective in inhibiting the growth of MCF-7 cells than SL0101. Additionally, 3Ac-SL0101 retained the preferential ability to inhibit the growth of MCF-7 compared to MCF-10A cells. Thus 3Ac-SL0101 is of interest for further characterization as a potential chemotherapeutic agent for cancer.

2. Results

2.1. Rhamnose acylation is critical for inhibition of MCF-7 cell growth

To investigate the possibility of improving the potency of SL0101, we characterized two compounds structurally related to SL0101 for their ability to inhibit RSK activity. These compounds, kaempferol 3-*O*-(2″,4″-di-*O*-acetyl-α-L-rhamnopyranoside), (2″,4″-di-*O*-acetyl-SL0101), and kaempferol 3-*O*-(4″-mono-*O*-acetyl-α-L-rhamnopyranoside), (4″-mono-*O*-acetyl-SL0101), differ from SL0101 in either the position or number of acetyl groups present on the rhamnose moiety (Fig. 1) and were obtained during the purification of SL0101 from *F. refracta*. The ability of the purified compounds to inhibit RSK activity was determined in an in vitro kinase assay using recombinant, constitutively active RSK2.[4] The data were fit using nonlinear regression analysis and the IC_{50} values for 4″-mono-*O*-acetyl-SL0101 and 2″,4″-di-*O*-acetyl-SL0101 were ~ 140 nM and ~ 260 nM, respectively (Fig. 2A), which is similar to the IC_{50} of ~50–100 nM obtained for SL0101. Thus all three related compounds are able to inhibit RSK catalytic activity in vitro, which is in agreement with the observations reported by Maloney and Hecht.[11] To determine whether the related compounds

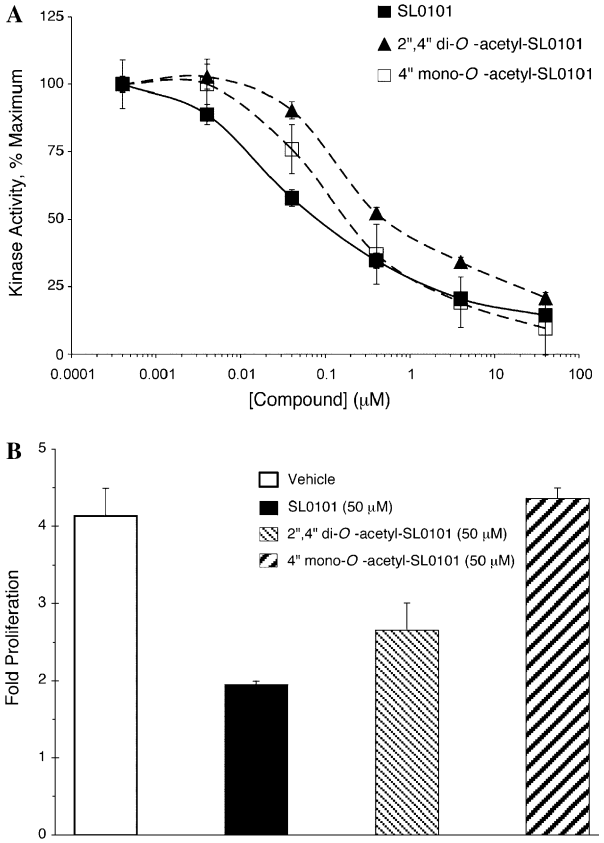


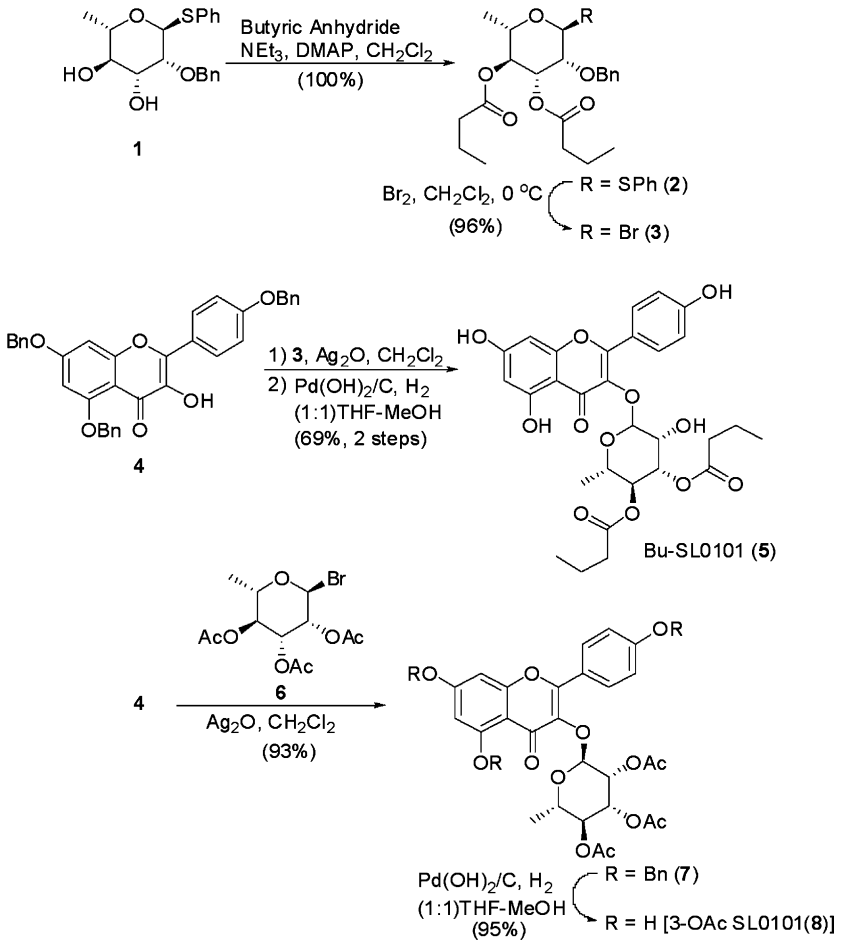
Figure 2. Effect of SL0101, 2″,4″-di-*O*-acetyl-SL0101, and 4″-mono-*O*-acetyl-SL0101 on RSK activity. (A) The potency of purified compounds in inhibiting RSK catalytic activity in vitro was measured. Kinase assays were performed using immobilized substrate. The reactions were initiated by the addition of 10 μM ATP (final concentration). Reactions were terminated after 30 min. All assays measured the initial reaction velocity. The extent of phosphorylation was determined using phosphospecific antibodies in combination with HRP-conjugated secondary antibodies. HRP activity was measured as described under Section 4. Maximum and minimum activity is the relative luminescence detected in the presence of vehicle and 200 mM EDTA, respectively. Points, mean (n = 2 in triplicate); bars = SD. (B) The potency of purified compounds as inhibitors of MCF-7 cell proliferation was determined. MCF-7 cells were treated with vehicle or 50 μM SL0101, 2″,4″-di-*O*-acetyl-SL0101, or 4″-mono-*O*-acetyl-SL0101 and cell viability was measured after 72 h of treatment. Values given are the fold proliferation. Points, mean (n = 3 in quadruplicate); bars = SD.

inhibit RSK activity in intact cells, we examined their ability to inhibit the proliferation of MCF-7 cells. Previously we have shown that SL0101 inhibits MCF-7 cell proliferation by specifically inhibiting RSK activity.[4] SL0101 inhibited MCF-7 cell proliferation with an EC_{50} of ~50 μM,[4] and our current results are in agreement with those observations (Fig. 2B). SL0101 and 2″,4″-di-*O*-acetyl-SL0101 at 50 μM were similarly effective at inhibiting MCF-7 cell growth. However, 4″-mono-*O*-acetyl-SL0101 did not inhibit MCF-7 cell proliferation at 50 μM. These data show that the position of the acetyl groups was not critically important in determining the in vitro affinity for RSK. However, only the compounds with two acetyl groups inhibited RSK activity in intact cells. It is likely that the acetyl groups facilitate effective uptake of the inhibitors into the cell.

Based on our results we reasoned that increasing the cellular uptake of SL0101 by enhancing its hydrophobic character might afford a more potent RSK inhibitor in intact cells. To test this hypothesis, kaempferol 3-*O*-(3″,4″-di-*O*-butyryl-α-L-rhamnopyranoside) (Bu-SL0101) and kaempferol 3-*O*-(2″,3″,4″-tri-*O*-acetyl-α-L-rhamnopyranoside) (3Ac-SL0101) were synthesized. The calculated Log *P*s of Bu-SL0101 and 3Ac-SL0101 are 4.30 and 3.07, respectively, compared to 2.56 for SL0101.

2.2. Synthesis of SL0101 analogs

The syntheses of both Bu-SL0101 (**5**) and 3Ac-SL0101 (**8**) utilized methodology and intermediates described in the previously reported synthesis of SL0101.[11] Accordingly, bis-esterification of phenyl 2-*O*-benzyl-1-thio-α-L-rhamnopyranoside (**1**) was achieved using butyric anhydride, NEt_3, and catalytic DMAP in quantitative yield to afford phenylthioglycoside **2** (Scheme 1). Bromination of the anomeric position using elemental bromine at 0 °C gave glycosyl bromide **3** in 96% yield. Glycosylation of flavonol **4** with **3** was carried out using heterogeneous activation conditions (Ag_2O). Preparation of **4** was achieved in three steps from commercially available 4′,5,7-trihydroxyflavanone (naringenin) as described previously.[11] The crude, fully protected Bu-SL0101 was debenzylated by hydrogenolysis ($Pd(OH)_2$, H_2) to afford Bu-SL0101 (**5**) in 69% yield for two steps. The synthesis of 3Ac-SL0101 was carried out in a similar manner except that 1-bromo-2,3,4-tri-*O*-acetyl-α-L-rhamnose (**6**) was used in the glycosylation reaction with **4**. Glycosyl bromide **6** was prepared using a method reported by Morillo and co-workers in two steps from L-rhamnose.[12] Subsequent coupling of **4** with **6** gave the desired perbenzylated 3Ac-SL0101 (**7**) in 93% yield, exclusively as the α-anomer as judged by the small



Scheme 1.

J value. Complete debenzylation proceeded smoothly using $Pd(OH)_2$ to afford 3Ac-SL0101 (**8**) in 95% yield.

2.3. Bu-SL0101 and 3Ac-SL0101 inhibit RSK activity in vitro and in the intact cell

The ability of the analogs to inhibit RSK activity was determined in an in vitro kinase assay and compared to the results obtained with synthesized SL0101.[11] The data were fit using non-linear regression analysis and the IC_{50}s for Bu-SL0101 and 3Ac-SL0101 were 212 and 580 nM, respectively, as compared to 370 nM obtained for the synthesized SL0101 (Fig. 3A). For reasons that are not clear, we consistently obtain an IC_{50} for the synthesized SL0101 that is higher than that of the material originally isolated from *F. refracta*. Nonetheless, as discussed above, kaempferol rhamnopyranosides with varying numbers and positions of acetyl groups on the rhamnose moiety inhibited RSK activity in the in vitro kinase assay with similar IC_{50}s. In agreement with these data, the addition of a third acetyl group, generating 3Ac-SL0101, did not substantially alter the in vitro IC_{50}. Furthermore, replacement of the acetyl groups on the rhamnose with butyryl groups also did not appreciably influence the in vitro IC_{50}.

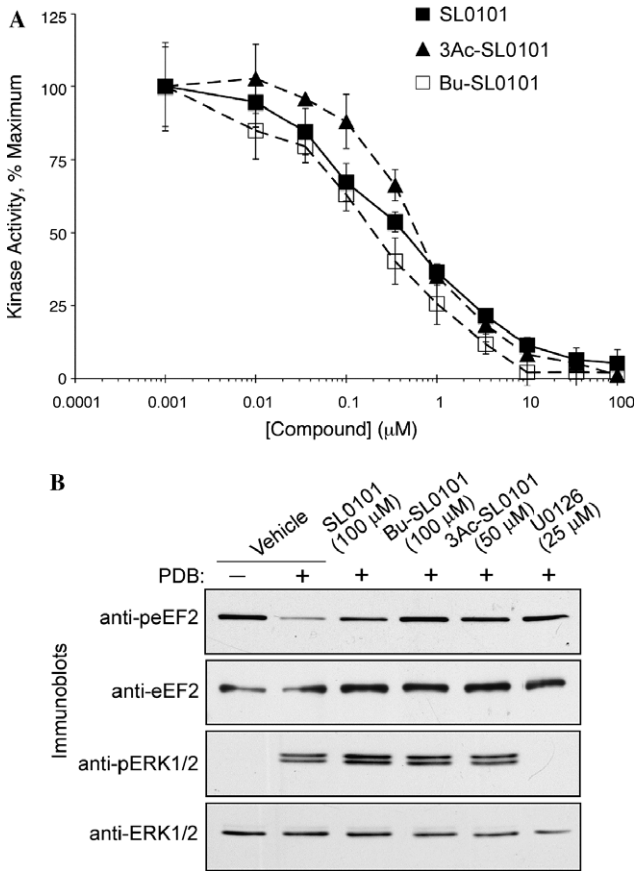


Figure 3. Inhibition of RSK activity by Bu-SL0101 and 3Ac-SL0101. (A) The potency of the synthesized compounds in inhibiting RSK catalytic activity in vitro was measured as described in Figure 2A. (B) Serum-deprived MCF-7 cells were pre-incubated with vehicle, or the indicated concentration of inhibitor for 4 h. Cells were treated with 500 nM PDB or vehicle for 20 min prior to lysis. Protein concentration of lysates was measured and lysates were electrophoresed, transferred, and immunoblotted. Equal loading of lysate is demonstrated by the anti-eEF2 and anti-ERK1/2 immunoblots.

To determine whether 3Ac-SL0101 and Bu-SL0101 inhibited RSK in intact cells, we examined the phosphorylation of eukaryotic elongation factor 2 (eEF2) in MCF-7 cells. eEF2 mediates the translocation step in mRNA translation. eEF2 activity is regulated by phosphorylation and it is inactivated by a highly specific kinase, EF2 kinase (EF2K). RSK phosphorylates and inactivates EF2K in response to mitogenic stimulation, which leads to a decrease in phosphorylation of eEF2.[13] Thus, under conditions such as serum deprivation, which leads to low RSK activity, eEF2 is phosphorylated by the active EF2K. However, stimulation of RSK activity by mitogens results in reduced phosphorylation of eEF2 due to inactivation of EF2K by RSK. Therefore, the phosphorylation state of eEF2 during mitogenic stimulation is an indicator of RSK activity. Treatment of MCF-7 cells with the mitogen phorbol dibutyrate (PDB) inactivated EF2K as shown by the reduced levels of phosphorylated eEF2 (Fig. 3B, compare lanes 1 and 2). The levels of total eEF2 were not altered by PDB treatment as shown by the anti-eEF2 immunoblot. However, pre-incubation of cells with SL0101 or its analogs eliminated PDB-mediated inactivation of eEF2K as shown by the elevated levels of phosphorylated eEF2 (Fig. 3B, cf. lanes 3–5 with lane 2). In agreement with the literature, the levels of eEF2 phosphorylation also remained increased in the presence of U0126.[13] We have previously demonstrated that SL0101 does not affect the activation of MAPK, as detected by the anti-active MAPK antibody.[4] Here we show that neither 3Ac-SL0101 nor Bu-SL0101 alters PDB-stimulated MAPK activation (Fig. 3B). Therefore, Bu-SL0101 and 3Ac-SL0101, like SL0101, do not inhibit upstream kinases necessary for PDB-induced MAPK activation, namely MEK, Raf, and protein kinase C (PKC).

2.4. 3Ac-SL0101 is a specific RSK inhibitor

To determine whether 3Ac-SL0101 and Bu-SL0101 demonstrated the same specificity as SL0101 in intact cells, we compared the phosphorylation patterns from MCF-7 cells pre-incubated with SL0101 or its analogs prior to stimulation with PDB. The lysates were immunoblotted with multiple phospho-specific substrate antibodies. We have previously shown that SL0101 does not alter the phosphorylation patterns detected by these antibodies, whereas changes are readily detected in cells treated with U0126, as well as protein kinase A (PKA) or PKC inhibitors.[4] The phosphorylation patterns from PDB-treated cells pre-incubated with vehicle, 3Ac-SL0101 or SL0101 were similar (Fig. 4). But unlike 3Ac-SL0101 and SL0101, pre-treatment with Bu-SL0101 decreased the PDB-induced phosphorylation of a ~25 kDa protein, as detected by the anti-phospho-AKT substrate antibody. These results show that Bu-SL0101 is not specific for inhibition of RSK activity. However, 3Ac-SL0101 does not inhibit the phosphorylation of substrates associated with the kinase activity

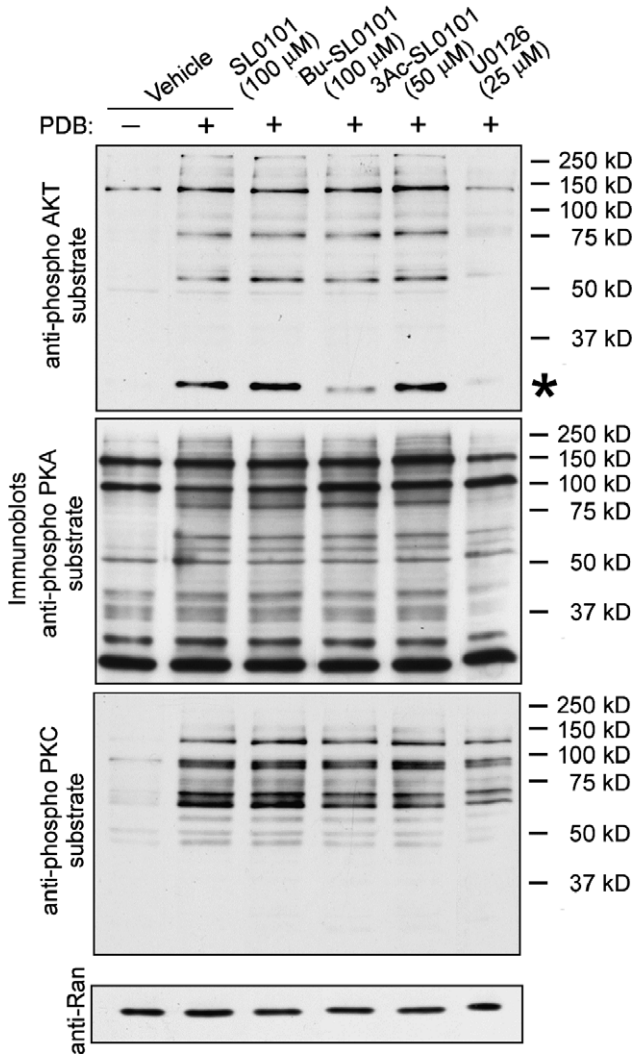


Figure 4. Comparison of the effect of Bu-SL0101, 3Ac-SL0101 and SL0101 on phosphorylation patterns in intact cells. MCF-7 cell lysates normalized for protein concentration were electrophoresed, transferred, and immunoblotted with anti-phospho-PKA substrate, anti-phospho-PKC substrate or anti-phospho-AKT substrate. Equal loading of lysate is demonstrated by the anti-Ran immunoblot. The asterisk indicates the location of a protein whose PDB-induced phosphorylation is inhibited by pre-treatment with U0126 or Bu-SL0101.

of other members of the AGC family, PKA, PKC, and AKT. Likewise, 3Ac-SL0101 does not inhibit kinases closely related to PKA, namely mitogen- and stress-activated kinase and p70 S6 kinase (data not shown). These data indicate that 3Ac-SL0101, like SL0101, demonstrates specificity for RSK in intact cells.

2.5. 3Ac-SL0101 preferentially inhibits the growth of breast cancer cells

To test our hypothesis that Bu-SL0101 and 3Ac-SL0101 would be more potent inhibitors in intact cells than SL0101, we determined their ability to inhibit the proliferation of MCF-7 cells. The maximum solubility of Bu-SL0101 and 3Ac-SL0101 in cell culture is ~100 and ~50 μM, respectively, notwithstanding the higher calculated Log *P* of Bu-SL0101 than that of 3Ac-SL0101. The data were fit using non-linear regression analysis. Bu-SL0101 inhibited MCF-7 proliferation with an EC_{50} of 40 μM, which was only slightly lower than that obtained with SL0101 (Fig. 5A). Bu-SL0101 began to significantly inhibit MCF-10A proliferation at a concentration of 100 μM, which may reflect its ability to inhibit

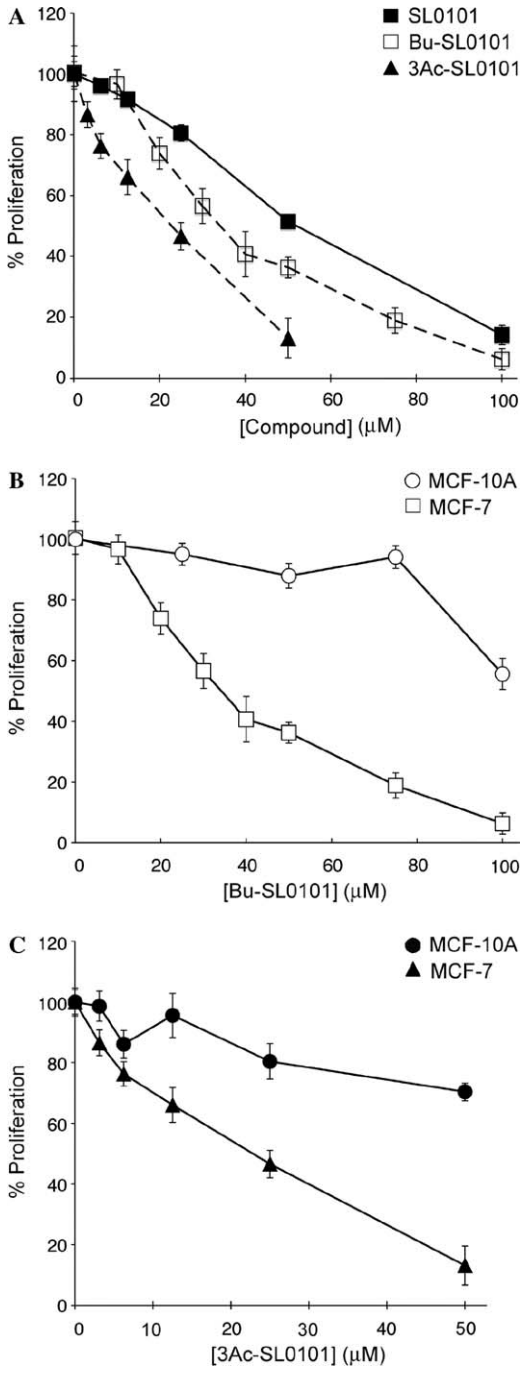


Figure 5. 3Ac-SL0101 selectively inhibits MCF-7 cell proliferation. (A) MCF-7 cells were treated with vehicle or the indicated concentration of Bu-SL0101, 3Ac-SL0101, and SL0101; (B) MCF-7 and MCF-10A cells were treated with vehicle or the indicated concentration of Bu-SL0101; or (C) MCF-7 and MCF-10A cells were treated with vehicle or the indicated concentration of 3Ac-SL0101. The cell number was measured after 48 h of treatment. Values given are the fold proliferation as a percentage of that observed with vehicle-treated cells. Points, mean ($n = 2$ in quadruplicate); bars = SD.

kinases in addition to RSK (Fig. 5B). 3Ac-SL0101 was ~2-fold more potent than SL0101 at inhibiting the growth of MCF-7 cells with an EC_{50} of 25 μM (Fig. 5A). 3Ac-SL0101 did not substantially inhibit MCF-10A proliferation at concentrations that effectively inhibited MCF-7 cell growth by >80% (Fig. 5C). Thus inhibition of RSK activity parallels the decrease in MCF-7 cell proliferation;[4] further, 3Ac-SL0101 demonstrates a significantly improved inhibition of RSK activity in intact cells relative to SL0101 without any alteration in its specificity for RSK.

3. Discussion

We have reported the identification of RSK as a novel target for anti-breast cancer and anti-prostate cancer agents.[4,7] We also described the identification and characterization of SL0101, a RSK-specific inhibitor isolated from *F. refracta*.[4] SL0101 inhibits the growth of the human breast cancer cell line MCF-7, and the human prostate cancer cell lines, LNCaP and PC-3, with an efficacy paralleling its ability to inhibit RSK in intact cells. However, the potency of SL0101 as a RSK inhibitor in intact cells is ~50-fold lower than its K_i and we postulated that improving cellular uptake would increase the potency of SL0101. Therefore, we synthesized the SL0101 analogs, Bu-SL0101 and 3Ac-SL0101, which have calculated Log *P* values of 4.30 and 3.07, respectively, compared to 2.56 for SL0101. 3Ac-SL0101 and SL0101 demonstrated the same specificity for inhibition of RSK activity in intact cells but Bu-SL0101 was not specific for RSK. Thus the range of modifications that can be introduced on the rhamnose while maintaining specificity for RSK inhibition may be limited. These results are in agreement with our observations, which showed that the rhamnose moiety is essential for the high affinity interaction of SL0101 with RSK.[4] Despite the >50-fold increase in hydrophobicity the EC_{50} of Bu-SL0101 for inhibition of MCF-7 proliferation was only slightly lower than that obtained for SL0101. However, the EC_{50} of 3Ac-SL0101 for inhibiting MCF-7 cell proliferation was >2-fold lower than that for SL0101. Thus while increasing the hydrophobic character of the SL0101 analogs can improve their efficacy in intact cells, there is not a simple correlation between Log *P* and EC_{50} for inhibition of MCF-7 cell growth.

RSK is an unusual kinase in that it contains two non-identical kinase domains, an N-terminal kinase domain (NTKD) and a C-terminal kinase domain (CTKD) that are separated by a linker region. The NTKD most closely resembles p70 ribosomal S6 kinase and is responsible for phosphorylating exogenous substrates.[14] The CTKD most closely resembles calmodulin-dependent kinase and its only known substrate is RSK itself.[15] In response to mitogen treatment the CTKD is activated and autophosphorylation by the CTKD enhances the activity of the NTKD.[16] The major determinant of SL0101 binding specificity is a unique sequence present in the ATP-binding domain of the NTKD of RSK.[4] Thus SL0101 acts to directly inhibit the phosphorylation of exogenous substrates via its interaction with the NTKD. SL0101 inhibits both the basal and mitogen-induced activity of the NTKD.[4] Recently, another RSK inhibitor, a fluoromethylketone, was identified that inhibits the CTKD.[17] Pretreatment of RSK with fluoromethylketone in the absence of ATP produces an IC_{50} in an *in vitro* kinase assay of 15 nM.[17] In intact cells, which have an intracellular ATP concentration of ~1.4 mM[18], a concentration of ~10 μM fluoromethylketone is required to completely inhibit the activity of ectopically expressed RSK for phosphorylation of histone H3[17], a nonphysiological substrate for RSK.[19,20] Inhibition of the CTKD autophosphorylation by fluoromethylketone should inhibit mitogen-induced activation of NTKD activity. However, the isolated NTKD is able to phosphorylate exogenous substrates [21] and it may be that the fluoromethylketone does not inhibit the basal activity of the NTKD. Thus, by interacting with different kinase domains SL0101 and the fluoromethylketone inhibit RSK activity through different mechanisms.

The molecular mechanism that results in RSK playing a dominant role in regulating proliferation in some cancer types has not yet been elucidated. It has been proposed that among the numerous events involved in tumorigenesis is an increased reliance on compromised signaling pathways as well as the dormancy of alternative signaling pathways.[22,23] Thus it appears that some cancers become dependent on RSK activity, rendering the proliferation of these cells amenable to inhibition by a RSK-specific inhibitor. The growth of normal cells is presumably not dependent on RSK because intact signaling pathways provide numerous mechanisms for circumventing inhibition of a single signaling event. The identification of RSK as a novel target and the synthesis of a more potent SL0101 analog will allow the further characterization of RSK inhibitors as potential chemotherapeutic agents for breast and prostate cancers.

4. Experimental

4.1. Kinase assays

Glutathione-*S*-transferase (GST)-fusion protein (1 μg) containing the sequence—RRRLASTNDKG (for serine/threonine kinase assays) 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 70 μL of kinase buffer (5 mM β-glycerophosphate, pH 7.4, 25 mM Hepes, pH 7.4, 1.5 mM DTT, 30 mM $MgCl_2$, and 0.15 M NaCl) was dispensed into each well. Compound at indicated concentrations or vehicle was added and reactions were initiated by the addition of 30 μL ATP to a final ATP concentration of 10 μM. Reactions were terminated after 30 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, anti-p-p140 antibody, a polyclonal phosphospecific antibody developed against the phosphopeptide, CGLA(pS)TND, 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 Ni–NTA resin (Qiagen, Valencia, California). Baculovirus was prepared using the Bac-to-Bac® baculovirus expression system (Invitrogen, Carlsbad, CA). 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).

4.2. Cell culture

For proliferation studies cells were seeded at 5000 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 at indicated time points using CellTiter-Glo™ assay reagent (Promega, Madison, Wisconsin) according to manufacturer's protocol. Maximum responses and the concentrations of half the effective response (EC_{50}) were determined by performing a best-fit analysis of the data (GraphPad Prism). For specificity studies, cells were seeded at 3×10^5 cells/well in 6-well plates. After 24 h, the cells were serum-starved for 24 h then and incubated with compound or vehicle for 4 h prior to a 20-min treatment with PDB. 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-pan-MAPK (anti-ERK1/2) (610124) from BD Transduction Laboratories; anti-phospho-MAPK (V8031) from Promega; anti-eEF2 (2332), anti-phospho-eEF2 (2331), anti-phospho-Akt Substrate (9611), anti-phospho-PKA Substrate (9621), and anti-phospho-PKC Substrate (2261) from Cell Signaling Technology. Anti-Ran was a generous gift from Ian Macara (University of Virginia).

4.3. Chemistry

Reagents and solvents were of reagent grade and used without further purification. Methylene chloride was distilled from calcium hydride, and toluene was distilled from sodium. Anhydrous grade THF was purchased from VWR. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under a nitrogen or argon atmosphere. Flash chromatography was performed using Silicycle 40–60 mesh silica gel. Analytical TLC was performed using 0.25 mm EM silica gel 60 F_{250} plates that were visualized by irradiation (254 nm) or by staining with Hanessian's stain (cerium molybdate). Optical rotations were obtained using a Jasco digital polarimeter. ^{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 ($CDCl_3$, 7.26 ppm; DMSO-d_6, 2.49 ppm). ^{13}C spectra were referenced to the residual ^{13}C resonance of the solvent ($CDCl_3$, 77.3 ppm; DMSO-d_6, 39.5 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 at the Michigan State University-NIH Mass Spectrometry Facility. Log *P* values were calculated using Molinspiration Property Calculation Service (www.molinspiration.com).

4.3.1. Phenyl 3,4-di-*O*-butyryl-2-*O*-benzyl-1-thio-α-L-rhamnopyranoside (2). A solution containing 0.87 g (2.51 mmol) of phenyl 2-*O*-benzyl-1-thio-α-L-rhamnopyranoside (**1**)[11], 1.19 g (1.25 mL, 7.54 mmol) of butyric anhydride, 1.01 g (1.37 mL, 10.0 mmol) NEt_3, and 0.03 g (0.25 mmol) of 4-*N*,*N*-dimethylaminopyridine in 10 mL of anhydrous CH_2Cl_2 was stirred at room temperature under a N_2 atmosphere for 2 h. The reaction mixture was then diluted with 100 mL CH_2Cl_2 and washed with two 100-mL portions of H_2O. The organic layer was separated, dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (25×4 cm). Elution with 3:1 hexanes–ethyl acetate gave **2** as a colorless oil: yield 1.21 g (100%); silica gel TLC R_f 0.40 (6:1 hexanes–ethyl acetate); $[\alpha]_D^{21}$ −50.1 (*c* 1.0, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 0.95 (q, 6H, $J = 7.5$ Hz), 1.24 (d, 3H, $J = 6.3$ Hz), 1.64 (m, 4 H), 2.26 (m, 4H), 4.11 (dd, 1H, $J = 3.3$ and 1.8 Hz), 4.30 (m, 1H), 4.55 (d, 1H, $J = 12.3$ Hz), 4.68 (d, 1H, $J = 12.3$ Hz), 5.20 (dd, 1H, $J = 9.9$ and 3.0 Hz), 5.32 (t, 1H, $J = 9.9$ Hz), 5.51 (d, 1H, $J = 1.5$ Hz), 7.31 (m, 8H) and 7.45 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 13.90, 17.72, 18.54, 18.70, 36.28, 36.41, 68.16, 71.31, 71.42, 72.80, 85.68, 127.69, 128.13, 128.64, 129.34, 131.55, 134.39, 137.67, 172.70 and 173.06; mass spectrum (FAB), *m*/*z* 487.2155 $(M + H)^+$ ($C_{27}H_{35}O_6S$ requires 487.2154).

4.3.2. 3,4-Di-*O*-butyryl-2-*O*-benzyl-α-L-rhamnopyranosyl bromide (3). To a solution containing 0.54 g (1.12 mmol) of **2** in 5 mL of anhydrous CH_2Cl_2 at 0 °C under argon was added 0.21 g (0.07 mL, 1.34 mmol) Br_2. The reaction mixture was stirred at 0 °C for 20 min then diluted with 20 mL CH_2Cl_2 and washed with 3% aq $NaHSO_3$. The organic layer was separated, dried ($MgSO_4$), and concentrated under diminished pressure. The crude residue was then purified by flash chromatography on a silica gel column (26×4 cm). Elution with 2:1 hexanes–ethyl acetate gave **3** as a colorless oil: yield 0.49 g (96%); silica gel TLC R_f 0.21 (2:1 hexanes–ethyl acetate); ^{1}H NMR ($CDCl_3$) δ 0.94 (q, 6H, $J = 7.5$ Hz), 1.25 (d, 3H, $J = 6.3$ Hz), 1.62 (m, 4H), 2.23 (m, 4H), 4.04 (m, 1H), 4.11 (dd, 1H, $J = 3.3$ and 1.8 Hz), 4.64 (q, 2H, $J = 12.3$ Hz), 5.30 (t, 1H, $J = 9.9$ Hz), 5.57 (dd, 1H, $J = 10.2$ and 3.3 Hz), 6.34 (d, 1H, $J = 0.6$ Hz) and 7.33 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 13.71, 17.11, 18.31, 18.47, 35.99, 36.11, 69.67, 70.27, 71.26, 73.37, 79.20, 86.35, 127.96, 128.18, 128.57, 137.18, 172.31 and 172.68. Note: this compound must be used promptly; product decomposes rather rapidly (1–2 days).

4.3.3. Kaempferol 3-*O*-(3″,4″-di-*O*-butyryl-α-L-rhamnopyranoside) (Bu-SL0101) (5). To a stirred suspension containing 0.13 g (0.23 mmol) of **4**,[11] 0.11 g (0.46 mmol)

Ag_2O and 4 Å molecular sieves in 5 mL CH_2Cl_2 was added 0.21 g (0.46 mmol) of **3** as a solution in 4 mL CH_2Cl_2. The reaction mixture was stirred at room temperature for 4 h, then diluted with 20 mL CH_2Cl_2 and filtered through a Celite pad. The filtrate was concentrated under diminished pressure. This crude residue was then dissolved in 5 mL of 1:1 THF–MeOH and 45 mg $Pd(OH)_2$/C was added. The reaction vessel was purged with H_2 three times then maintained under a H_2 atmosphere for 2 h. The reaction mixture was then filtered through Celite and the filtrate was concentrated. The residue was purified by flash chromatography on a silica gel column (25 × 2 cm). Elution with 1:1:0.1 hexanes–ethyl acetate–methanol gave **5** as a brown solid: yield 0.090 g (69%); silica gel TLC R_f 0.30 (1:1:0.1 hexanes–ethyl acetate–methanol); $[\alpha]_D^{20}$ −115.6 (*c* 1.2, $CHCl_3$); ^1H NMR (DMSO-d_6) δ 0.85 (d, 3H, *J* = 6.3 Hz), 0.97 (m, 6H), 1.64 (m, 4H), 2.33 (m, 4H), 3.49 (m, 1H), 4.46 (s, 1H), 4.79 (br s, 1H); 5.14 (t, 1H, *J* = 9.9 Hz), 5.27 (dd, 1H, *J* = 6.3 and 3.0 Hz), 5.66 (d, 1H, *J* = 1.8 Hz), 6.32 (d, 1H, *J* = 1.8 Hz), 6.53 (d, 1H, *J* = 2.1 Hz), 7.11 (d, 2H, *J* = 8.7 Hz), 7.92 (d, 2H, *J* = 8.4 Hz), 9.39 (br s, 2H) and 12.68 (s, 1H); mass spectrum (FAB), *m*/*z* 573.1970 $(M+H)^+$ ($C_{29}H_{33}O_{12}$ requires 573.1972).

4.3.4. 5,7-Bis-(benzoxy)-2-(4-(benzoxy)phenyl)-3-[2″,3″,4″-tri-*O*-acetyl-α-L-rhamnopyranosyloxy]-*4H*-chromen-4-one (7). To a stirred suspension containing 0.090 g (0.162 mmol) of **4**,[11] 0.075 g (0.324 mmol) Ag_2O and 4Å molecular sieves in 5 mL CH_2Cl_2 was added 0.114 g (0.324 mmol) of **6**[12] as a solution in 4 mL CH_2Cl_2. The reaction mixture was stirred at room temperature for 24 h under N_2 then diluted with 20 mL CH_2Cl_2 and filtered through a Celite pad. The filtrate was concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with 2:1 hexanes–ethyl acetate gave **7** as a light yellow foam: yield 0.125 g (93%); silica gel TLC R_f 0.41 (2:1 hexanes–ethyl acetate); $[\alpha]_D^{22}$ −96.8 (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.85 (d, 3H, *J* = 6.3 Hz), 1.97 (s, 3H), 2.00 (s, 3H), 2.11 (s, 3H), 3.39 (m, 1H), 4.94 (t, 1H, *J* = 9.9 Hz), 5.07 (s, 2H), 5.15 (s, 2H), 5.30 (s, 2H), 5.34 (m, 1H), 5.74 (s, 2H), 6.45(d, 1H, *J* = 1.8 Hz), 6.55 (d, 1H, *J* = 1.8 Hz), 7.16 (d, 2H, *J* = 8.7 Hz), 7.39 (m, 13H), 7.57 (d, 2H, *J* = 7.5 Hz) and 7.87 (d, 2H, *J* = 8.7 Hz); ^{13}C NMR ($CDCl_3$) δ 17.00. 20.69, 20.83, 67.99, 68.98, 69.18, 70.05, 70.40, 70.57, 70.67, 93.86, 97.79, 98.23, 109.89, 114.81, 122.97, 126.54, 127.30, 127.51, 127.61, 128.13, 128.36, 128.60, 128.68, 130.33, 135.54, 136.22, 136.27, 136.41, 154.16, 158.75, 159.74, 160.49, 162.78, 169.52, 169.80, 169.88 and 172.82; mass spectrum (FAB), *m*/*z* 829.2865 $(M+H)^+$ ($C_{48}H_{45}O_{13}$ requires 829.2860).

4.3.5. Kaempferol 3-*O*-(2″,3″,4″-tri-*O*-acetyl-α-L-rhamnopyranoside) (3Ac-SL0101) (8). A stirred suspension containing 0.100 g (0.121 mmol) of **7** and 0.040 g $Pd(OH)_2$ in 5 mL of 1:1 THF–MeOH was purged with H_2 three times then maintained under H_2 (balloon pressure) for 1.5 h. The reaction mixture was then filtered through Celite and the filtrate was concentrated. The residue was purified by flash chromatography on a silica gel column (20 × 2 cm). Elution with 1:1:0.1 hexanes–ethyl acetate–methanol gave **8** as a yellow foam: yield 0.064 g (95%); silica gel TLC R_f 0.37 (1:1:0.1 hexanes–ethyl acetate–methanol); $[\alpha]_D^{22}$ −92.2 (*c* 0.6, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 0.92 (t, 3H, *J* = 6.3 Hz), 2.01 (s, 3H), 2.02 (s, 3H), 2.05 (s, 3H), 3.52 (m, 1H), 4.96 (t, 1H, *J* = 9.9 Hz), 5.30 (s, 1H), 5.33 (dd, 1H, *J* = 9.9 and 3.0 Hz), 5.63 (s, 1H), 6.25 (s, 1H), 6.35 (s, 1H), 6.99 (d, 2H, *J* = 8.1 Hz), 7.18 (br s, 1H), 7.72 (d, 2H, *J* = 8.1 Hz), 7.89 (br s, 1H) and 12.49 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 16.97, 20.68, 20.74, 20.78, 68.33, 69.26, 70.38, 77.20, 94.18, 98.08, 99.27, 105.27, 115.66, 121.53, 130.67, 133.80, 156.78, 157.64, 159.10, 161.66, 163.08, 170.47, 170.57, 171.08 and 177.69; mass spectrum (FAB), *m*/*z* 559.1449 $(M+H)^+$ ($C_{27}H_{27}O_{13}$ requires 559.1452).

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