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# *N*-Glycosyl-thiophene-2-carboxamides: Effects on endothelial cell growth in the presence and absence of bFGF—A significant increase in potency using per-O-acetylated sugar analogues

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**Abstract**—Inhibitors of endothelial cell proliferation are of interest in development of therapies for angiogenesis related disease. *N*-Glucosyl-thiophene-2-carboxamides have been synthesized and evaluated for their effects on proliferation in bovine aortic endothelial cells. Per-O-acetylated-*N*-glucosyl-thiophene-2-carboxamides showed improved inhibition of both serum and bFGF stimulated uptake of [$^3$H]thymidine, when compared to non-acetylated analogues.


The signal transduction processes that modulate cellular behaviour are important biological events. For example, angiogenesis[1] provides new blood vessels to growing and developing tissue including tumours, and it relies in part on the up-regulation of endothelial cell proliferation. Up-regulated angiogenesis is characteristic in rheumatoid arthritis, diabetic retinopathy, during tumour growth and metastasis.[2] The tumour angiogenesis process results from the production of the pro-angiogenic factors basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in a signalling cascade, and the down-regulation of negative modulators, like angiostatin, in tissues with a quiescent vasculature. Angiogenesis is not only a prerequisite for tumour growth and expression but also a major factor affecting the metastatic spread of malignant cells. Thus, the development of angiogenic inhibitors may allow a new therapeutic strategy against malignant tumours. Inhibitors of cell proliferation and consequently growth are of interest. A number of strategies are being considered for the development of anti-proliferative agents.[3] Heparin-binding endothelial growth factors, like bFGF and VEGF families, induce new blood vessel formation by activating their cognate tyrosine kinase receptors (FGFR) on the endothelial cell surface.[4] Compounds that target the factors that promote proliferation of endothelial cells or that target the cell signalling pathway up-regulated by growth factors have potential as therapeutics. Previously we set out to develop compounds reduced in carbohydrate character (monosaccharide conjugates) that might be mimetics of non sulfated disaccharides[5] and potential modulators of bFGF-induced endothelial cell growth;[6] this led to identification of *N*-(β-D-glucopyranosyl)-thiophene-2-carboxamide **1** and glucuronic acid derivative **2** as inhibitors of bovine aortic endothelial cell (BAEC) growth (see Chart 1). It was later determined that they were not mimetics of the disaccharides.[7]

The synthesis and investigation of the biological properties of structural analogues of **1** are thus of interest with

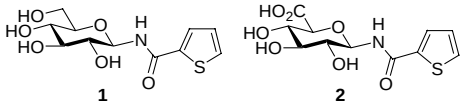


**Chart 1.**

*Keywords*: Endothelial cell proliferation; Angiogenesis; bFGF; Glycoconjugates; Glycosyl amides; Thiophene-2-carboxamides.
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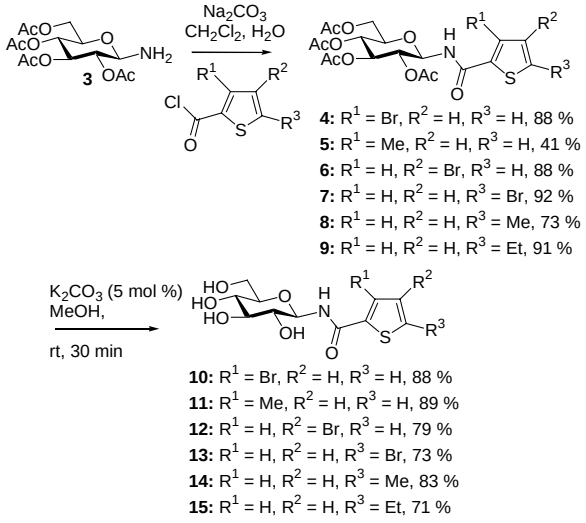
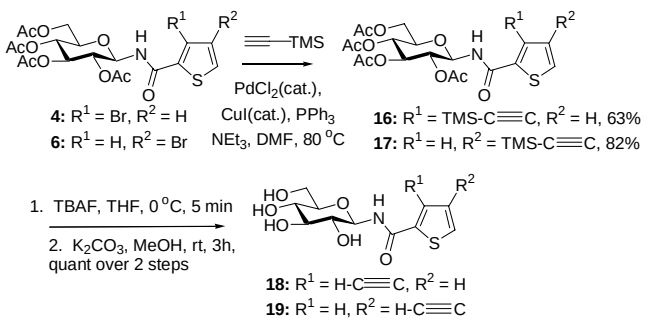
a view to development of more potent cell growth inhibitors. This could ultimately be helpful for the determination of the biological mechanism of action of **1**, which is as yet unknown, and could yield novel targets in cell signalling pathways. We have thus prepared a series of analogues of **1** and have explored effects of these compounds on endothelial cells grown in the presence and absence of bFGF. *N*-Glucosyl-thiophene-2-carboxamides (GTCs) were prepared from glucosamine **3**[8] (Scheme 1). The reactions of **3**, in the presence of sodium carbonate, with acyl chlorides derived from thiophene-2-carboxylic acids afforded **4–9**. Anomerisation was observed during the coupling reaction leading to minor amounts ($\alpha$:$\beta$ = 1:16) of the $\alpha$-anomers being observed in the $^1$H NMR spectra of the products. Deacetylation of per-O-acetylated derivatives **4–9** gave the unprotected *N*-glucosyl-thiophene-2-carboxamides **10–15**.

The acetylene derivatives **16/17** were prepared from **4/6** by a Sonogashira coupling with ethynyltrimethylsilane (Scheme 2), which was carried out in a sealed reaction vessel at 80 °C. The removal of the terminal TMS and deacetylation from **16/17** was achieved by their reaction with TBAF in THF at 0 °C for 5 min and subsequent treatment with potassium carbonate in methanol to give **18/19**.




**Scheme 1.**




**Scheme 2.**

The effect of GTCs on proliferation[9] of BAEs (BAECs) induced by 10% foetal bovine serum (=complete culture medium) was investigated. The inhibitory activities of the thiophene–sugar conjugates were compared to that of heparin–albumin (HA, positive control) on cell proliferation by analysing [$^3$H]thymidine incorporation into DNA during DNA synthesis. Addition of HA (10 μg/mL) to the complete culture medium resulted in reduced [$^3$H]thymidine incorporation by 37 ± 2.5% after 24 h exposure, results similar to those previously reported by researchers from this group.[6] One set of experiments was conducted to determine whether the inhibition of [$^3$H]thymidine incorporation into BAECs by a thiophene conjugate was dose dependent. The 3-bromo derivative **10** showed a dose-dependent decrease in [$^3$H]thymidine incorporation, 24 h after exposure (Fig. 1), reducing BAEC growth by 25 ± 3% at 500 μM and by 10 ± 3% at 100 μM (Fig. 1). A wider range of GTCs (Table 1) were subsequently evaluated for their effects on BAEC growth. Most GTCs (500 μM) consistently produced a maximal suppressive response after 24 h exposure and inhibited [$^3$H]thymidine uptake by 16–35%. The 5-bromothieno conjugate **13** had the greatest effect, showing 35% inhibition. Thiophene conjugate **1** was found to be inactive under these conditions.

Further experiments explored the effect of the per-O-acetylated GTCs (Fig. 2) on 10% serum-induced BAEC proliferation. The protected derivative **9**, applied at the highest concentration of 80 μM, was found to

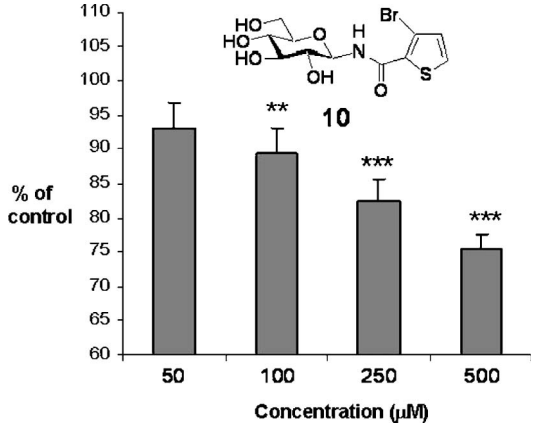



**Figure 1.** Concentration-dependent inhibition by **10** on 10% serum-induced BAEC proliferation. Proliferation of BAEC was assessed by measuring [$^3$H]thymidine incorporation. Cells were seeded (30,000 cells/well) in 24-well plates. After a 24 h adhesion period, cells were treated with increasing concentrations of **10** for the following 20 h at 37 °C and 5% $CO_2$. Subsequently, [$^3$H]thymidine (2 μCi/mL) was added to each well, and incubation was continued for a further 4 h at 37 °C and 5% $CO_2$. Experiments were terminated with washing (2× in PBS) and fixing the cells in trichloroacetic acid (10%). The cells were then lysed with 0.1 N NaOH. The amount of incorporated [$^3$H]thymidine in cell lysates was measured by scintillation counting. Data are presented as percentage of the values for the control-treated cells (100%) and each bar is the mean ± SEM of three separate experiments (each performed in triplicate). Statistical comparisons between groups were performed using Student's *t* test. **($p < 0.01$); ***($p < 0.001$) vs control group.

**Table 1.** Effect of GTCs (500 μM) and heparin–albumin (HA; 10 μg/mL) on 10% serum-induced BAEC proliferation[a]

| Compound | % of control | *p* versus control |
|---|---|---|
| HA | 63 ± 2.5 | 0.001 |
| **1** | na | — |
| **10** | 75.4 ± 1.3 | 0.001 |
| **11** | 84 ± 3 | 0.001 |
| **13** | 65 ± 5.3 | 0.001 |
| **14** | 83 ± 1.8 | 0.001 |
| **15** | 80.3 ± 2.5 | 0.001 |
| **18** | 79.75 ± 2.7 | 0.001 |
| **19** | 71 ± 3.3 | 0.001 |

Proliferation was measured by quantification of [$^3$H]thymidine incorporation after 24 h.

[a] The results are expressed as percentage of the values obtained for the control-treated cells (100%) and means ± SEM of 3 independent experiments are reported. na = not active.

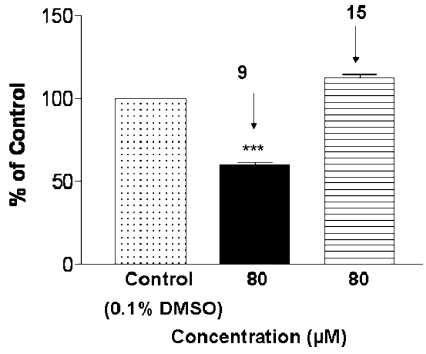



**Figure 2.** The effect of per-O-acetylation on biological activity. Per-O-acetylated conjugate **9** and its deacetylated analogue **15** were evaluated for effects on BAE cell proliferation, which was determined by [$^3$H]thymidine incorporation after 24 h exposure to compounds. Each bar is the mean of triplicate values from three separate experiments. ***($p < 0.001$) versus control.

significantly reduce incorporation of [$^3$H]thymidine by 41 ± 2%. The per-O-acetylated 5-ethyl derivative **9** showed a dose-dependent inhibition of [$^3$H]thymidine uptake; it was inactive at 3.3 μM; inhibited uptake by 21% ± 2 at 16 μM (data not shown) and by 41 ± 2% at 80 μM (Fig. 2). The non-acetylated analogue **15** was inactive at all concentrations up to 80 μM. Thus, there is greater than sixfold improvement for **9** when compared to the corresponding deacetylated analogue **15** and related compounds in Table 1. The GTCs **9** and **15** were also investigated (80 μM) for their effects on bFGF (10 ng/mL) stimulated proliferation of BAECs over a period of 48 h incubation (Fig. 3). The [$^3$H]thymidine incorporation increased by 30% ± 4 in the presence of bFGF (Fig. 3) over 48 h. Whereas the per-O-acetylated derivative **9**[10] (80 μM) inhibited bFGF-induced [$^3$H]thymidine uptake to control levels, the corresponding deacetylated analogue **15**[11] had no effect under identical conditions at the same concentration (Fig. 3). We suggest that the reason for the improved activity of acetylated conjugates is that the compounds may be exerting their mechanism of action intracellularly and that their membrane permeability is superior to polyhydroxylated sugars. It is known that acetylated carbohydrates can have increased absorption when compared to non-acetylated compounds and that esterases can cleave the acyl-protecting groups and presumably generate the active glucoside once the compound is localized in the cytosol.[12]

In summary, a series of *N*-glucosyl-thiophene-2-carboxamides have been synthesized and evaluated for their effects on serum and bFGF stimulated bovine aortic endothelial cell growth; per-O-acetylated-*N*-glucosyl-thiophene-2-carboxamides showed significant inhibition of [$^3$H]thymidine incorporation when compared to the non-acetylated analogues. Presumably the weaker inhibitory activities achieved with non-acetylated sugars are due to the low transport of polar agents through the cell membrane. Further investigations are underway to confirm this hypothesis. The results indicate that further synthetic and biological studies with acetylated glycoconjugates related to those described herein offer promise of development of novel compounds that could inhibit tumour angiogenesis or other pathological processes induced by bFGF. Cell biological mechanistic studies are currently underway for **9** and related conjugates and results will be presented in due course.

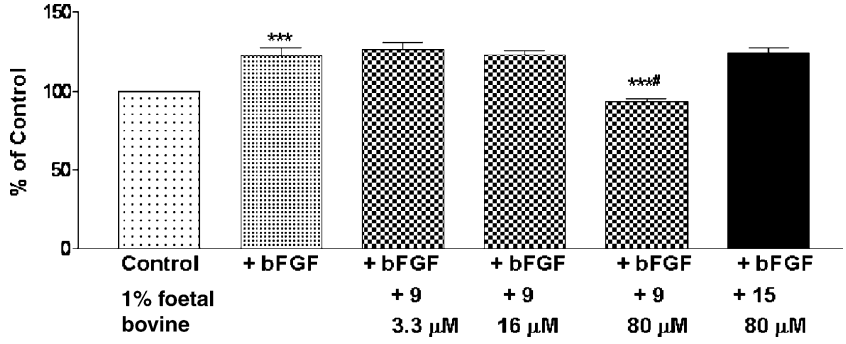



**Figure 3.** Effect of compounds on bFGF-promoted proliferation of BAECs. The per-O-acetylated conjugate **9** showed significant inhibiton (80 μM) of bFGF induced biosynthesis of DNA. The unprotected conjugate **15** was inactive at the same concentration. Cells were seeded at 30,000 cells/well into 24-well plates. Cells were starved with 0.5% foetal bovine serum for 24 h. The cells were then incubated with or without indicated reagents and bFGF for 48 h and harvested. Before the harvest, cells were incubated with [$^3$H]thymidine (2 μCi/mL) for 4 h. The incorporated [$^3$H]thymidine radioactivity was measured as described previously (Fig. 1). Each bar is the mean of triplicate values from three separate experiments. Statistical comparisons between groups were performed using Student's *t* test. ***($p < 0.001$) versus control, ***$^{\#}$($p < 0.001$) versus bFGF treatment.

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9. Procedure for measurement of DNA synthesis: BAE cells (30,000 cells/well) were seeded on 24-well plates in culture medium. After a 24 h adhesion period, the compounds were added and cells were cultured for 24 h in presence of serum or for 48 h in presence of bFGF. During the last 4 h of the assay, the culture was pulsed with 2 μCi of [Me-$^3$H]thymidine/well. After being washed two times with ice-cold PBS, the cells were solubilized with 0.1 N NaOH and transferred to counting vials containing 5 mL of liquid scintillant (ICN).
10. To a solution of 5-ethyl-thiophene-2-carboxylic acid (0.23 g, 1.44 mmol) in anhyd $CH_2Cl_2$ (7 mL) was added oxalyl chloride (0.185 g, 0.125 mL, 1.44 mmol) followed by 1 drop of anhydrous DMF and the solution was stirred at room temp for 2 h. The solvent was removed in vacuo to afford the acid chloride as a yellow oil, which was used immediately without further purification. This oil was dissolved in $CH_2Cl_2$ (1 mL) and the solution was added to a biphasic mixture of **3**[8] (0.5 g, 1.44 mmol) in $CH_2Cl_2$ (7 mL) and sodium carbonate (0.15 g, 1.44 mmol) in water (7 mL) and stirring was continued overnight at room temperature. The mixture was then transferred to a separating funnel, the organic layer removed and the aqueous layer extracted with $CH_2Cl_2$ (2×20 mL). The combined organic layers were washed (satd $NaHCO_3$, 20 mL), dried ($MgSO_4$) and the solvent was removed in vacuo. The residue was recrystallised from EtOAc and cyclohexane to afford **9** as a colourless crystalline solid (0.42 g, 51%) and as an adduct with EtOAc (1:1); mp = 64–66 °C; $[\alpha]_D$ +12 (*c* 8.0, $CDCl_3$); $^1$H NMR (300 MHz, $CDCl_3$) δ = 7.33 (1H, d, *J* = 3.9 Hz, aromatic), 6.84 (1H, d, $J_{NH,H1}$ = 9.3 Hz, NH), 6.77 (1H, dd, *J* = 3.9 Hz, *J* = 0.9 Hz), 5.37 (2H, 2× overlapping t, *J* = 9.3 Hz, H-1 and 3), 5.10 (1H, t, *J* = 9.3, H-4), 5.03 (1H, t, *J* = 9.3 Hz, H-2), 4.34 (1H, dd, $J_{6a,6b}$ = −12.6 Hz, $J_{6a,5}$ = 4.2 Hz, H-6a), 4.09 (1H, dd, $J_{6b,6a}$ = −12.3 Hz, $J_{6b,5}$ = 2.1 Hz, H-6b), 3.88 (1H, ddd, $J_{5,4}$ = 9.9 Hz, $J_{5,6a}$ = 4.2 Hz, $J_{5,6b}$ = 2.1 Hz, H-5), 2.86 (2H, q, *J* = 7.5 Hz, $CH_2CH_3$), 2.08, 2.044, 2.039, 2.03 (each 3H, each s, each $CH_3$), 1.32 (3H, t, *J* = 7.5 Hz, –$CH_2CH_3$); $^{13}$C NMR (75 MHz, $CDCl_3$) δ = 171.5, 170.7, 169.9, 169.6 (each s, each ester C═O), 161.8 (s, amide C═O), 154.9, 134.3 (each s), 129.5, 124.6 (each d), 78.9, 73.6, 72.6, 70.7, 68.3 (each d), 61.7 (t), 23.8 (t), 20.6, 20.7 (each q), 15.7 (q); LR-ESMS *m*/*z* = 486 $(M + H)^+$, 324, 271, 169; HR-ESMS $(M + H)^+$ calcd 486.1434, found: 486.1448; Anal. Calcd for $C_{25}H_{35}NO_{12}S$ (EtOAc adduct): C, 52.35, H, 6.15, N, 2.44, S, 5.59. Found: C, 52.22, H, 6.09, N, 2.49, S, 5.92.
11. To a solution of per-O-acetylated compound in methanol (0.2 M) was added $K_2CO_3$ (10 mol %) and the mixture was stirred at room temperature (30 min) and the solvent was then removed in vacuo. The residue was immediately purified by flash chromatography on (MeOH:$CH_3CN$, 1:9) to afford a yellow syrup (0.14 g, 71%) which was a mixture of anomers. Purification by reverse phase HPLC (C-4 column) gave the pure β-anomer **15** for biological evaluation. Analytical data for **15**: $[\alpha]_D$ −16.3 (*c*, 0.54, MeOH); $^1$H NMR (300 MHz, $D_2O$) δ = 7.56 (1H, d, *J* = 3.6 Hz), 6.84 (1H, d, *J* = 3.6 Hz), 5.07 (1H, d, $J_{1,2}$ = 8.7 Hz, H-1), 3.83 (1H, dd, $J_{6a,6b}$ = 12.3 Hz, $J_{6a,5}$ = 2.1 Hz, H-6a), 3.68(1H, dd, $J_{6b,6a}$ = 12.3 Hz, $J_{6b,5}$ = 4.8 Hz, H-6b), 3.56–3.37 (4H, overlapping signals, H2-H5), 2.79 (2H, q, *J* = 7.8 Hz), 1.20 (3H, t, *J* = 7.8 Hz); $^{13}$C NMR (75 MHz, $D_2O$) δ = 165.3 (C═O), 156.3, 131.2, 125.1, 79.9, 77.6, 76.5, 71.7, 69.3 (each d), 60.5 (t), 23.2 (t), 15.0 (q); ESMS *m*/*z* = 316 $[M - H]^-$196, 119; HRMS $[M - H]^-$ calcd: 316.0855; found: 316.0840.
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