Electronic Supplementary Material (ESI) for Chemical Communications

High throughput discovery of heteroaromatic-modifying enzymes allows enhancement of novobiocin selectivity

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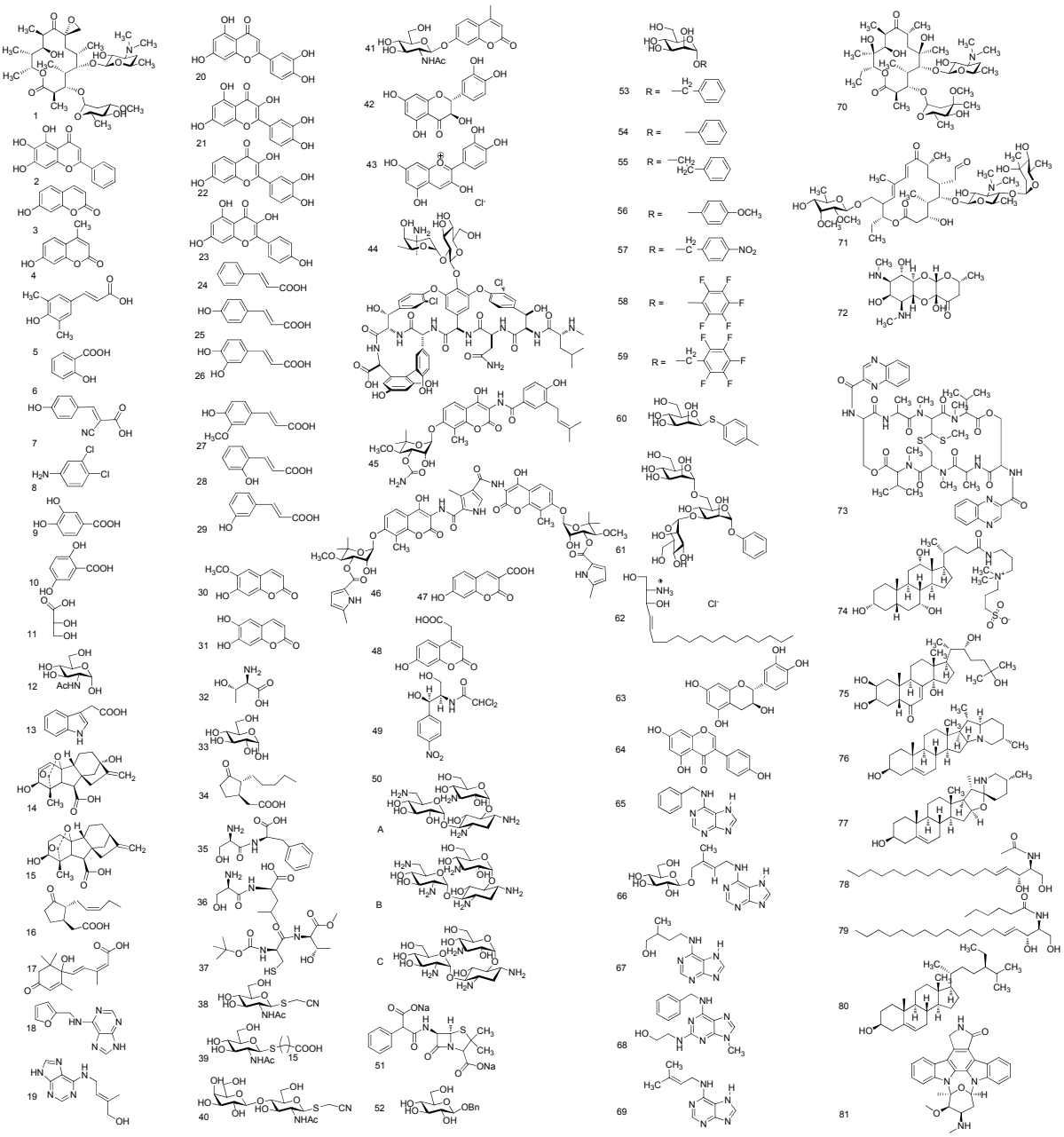
Enzyme activity screening

Method

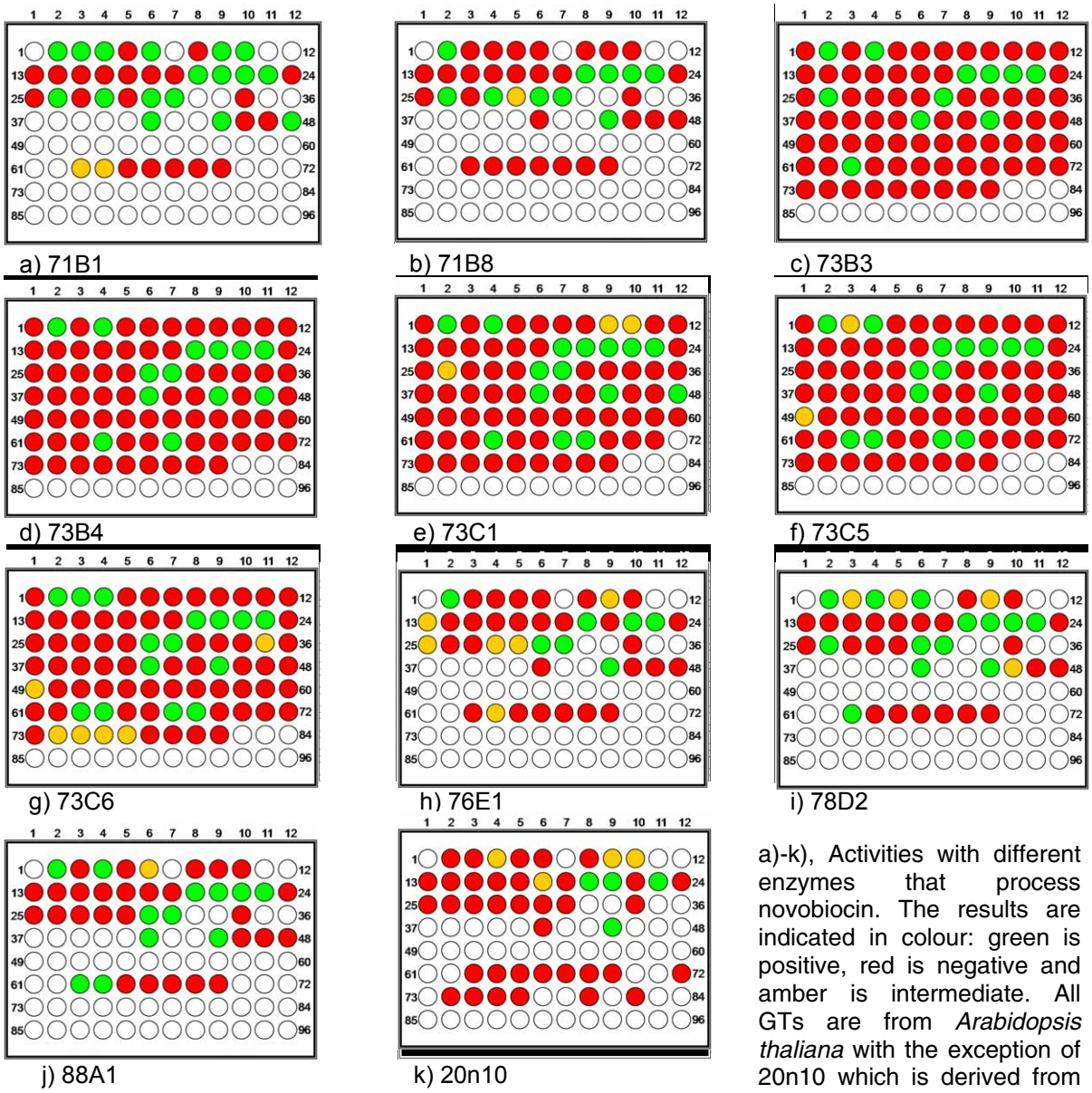
The enzymes were expressed and purified according to published procedures.[1]
The HTS screening method was based on a modified version of a previous method. [2] Typically: donor (α-UDP-glucose, 10 mM, 5 μL), acceptor (10 mM, 5 μL) and enzyme (~5 mg/ml, 10 μL) were added to 200 μL Tris buffer (pH 7.8, 50 mM, $MgCl_2$ 20 mM) in a 96 well plate. The plate was sealed and kept at 37 °C overnight. Product formation was monitored with an LCQ^{duo} ion trap coupled with an Ultimate nano_LC (Thermo Fisher Scientific) in negative mode. An isocratic method was used (0.1% formic acid in 83% methanol) with an ACE 3 C18 (150×0.1mm) column. Flow rate was at 0.25 ml/min. Calibrator CAP180 was used and the CRP was set at 625. The Isprary voltage was set at 0.71 kV and the capillary temperature at 180°C. Full MS was screened from 200-1500 Da. MS^n of a product was analyzed with 35% collision energy.
The acceptor library is listed in Fig. 1; 81 compounds including antibiotics, plant hormone and secondary metabolites, sugar analogues etc. The results are displayed in colour with green indicating positive activity, red negative activity and amber, vague activity. Structures of the compounds that gave positive hits are given in Fig. 2l.

Figure 1 Acceptor library for HTS screening.

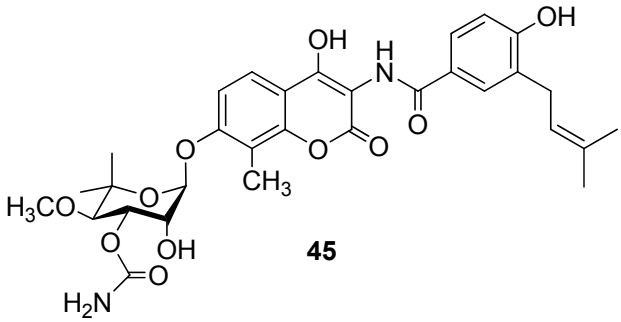


Glycosylation HTS activity analysis for enzymes that were successful with novobiocin

Figure 2 HTS screening results.



a)-k), Activities with different enzymes that process novobiocin. The results are indicated in colour: green is positive, red is negative and amber is intermediate. All GTs are from *Arabidopsis thaliana* with the exception of 20n10 which is derived from Oat. Full details of all 125,000 results and their analysis will be published in due course.



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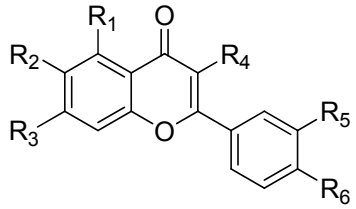
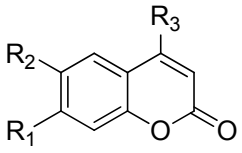
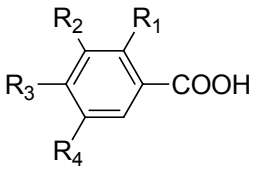


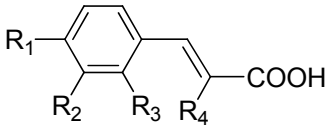
2: $R_1 = R_2 = R_3 = OH$, $R_4 = R_5 = R_6 = H$
20: $R_1 = R_3 = R_5 = R_6 = OH$, $R_2 = R_4 = H$
21: $R_1 = R_3 = R_4 = R_5 = R_6 = OH$, $R_2 = H$
22: $R_3 = R_4 = R_5 = R_6 = OH$, $R_1 = R_2 = H$
23: $R_1 = R_3 = R_4 = R_6 = OH$, $R_2 = R_5 = H$



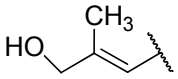
3: $R_1 = OH$, $R_2 = R_3 = H$
4: $R_1 = OH$, $R_2 = H$, $R_3 = CH_3$
30: $R_1 = OH$, $R_2 = OCH_3$, $R_3 = H$
31: $R_1 = R_2 = OH$, $R_3 = H$
48: $R_1 = OH$, $R_2 = H$, $R_3 = CH_2COOH$

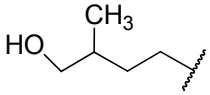


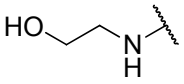
9: R2 = R3 = OH, R1 = R4 = H
10: R2 = R3 = H, R1 = R4 = OH

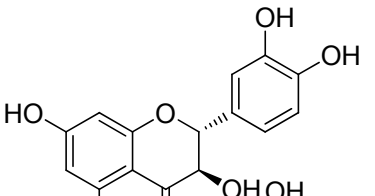


6: $R_1 = OH$, $R_2 = R_3 = H$, $R_4 = -CN$
26: $R_1 = R_2 = OH$, $R_3 = R_4 = H$
27: $R_1 = R_2 = R_4 = H$, $R_3 = OH$

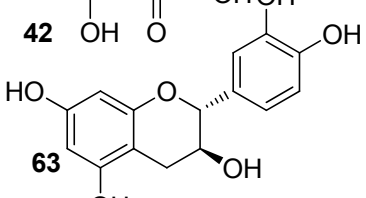
19: R_1=  $R_2 = R_3 = H$

67: R_1=  $R_2 = R_3 = H$

68: R_1 = Bn, R_2 =  $R_3 = CH_3$



42



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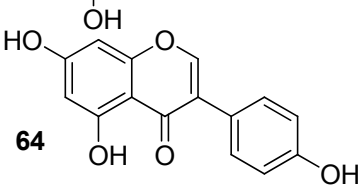

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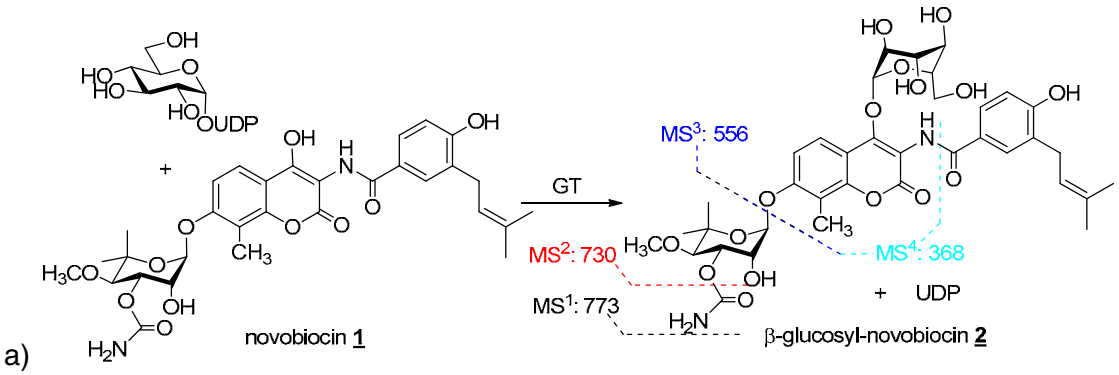
I) Structures of compounds with a positive HTS screening result. Numbers correspond to assignment in the acceptor library above.

Biocatalysis and structure elucidation by MS^n

Glucosyl-novobiocin was isolated from a 73C5 catalyzed reaction using semi-preparative HPLC with the following procedure:
Solvent A: 99.9% H_2O + 0.1% formic acid
Solvent B: 99.9% MeOH + 0.1% formic acid
0-1 minutes: 1% B; 1-3 minutes: 50% B, 3-4 minutes: 50% B, 4-14 minutes: 95% B, 14-19 minutes: 95% B, 19-20] minutes: 1% B. Flow rate, 1.5 mL /min, Column: Agilent, ElipseXDB-c18, 5 µm. Retention time: 14.5 min.

Figure 3 Biocatalysis of glucosyl-novobiocin and structure elucidation MS^n.

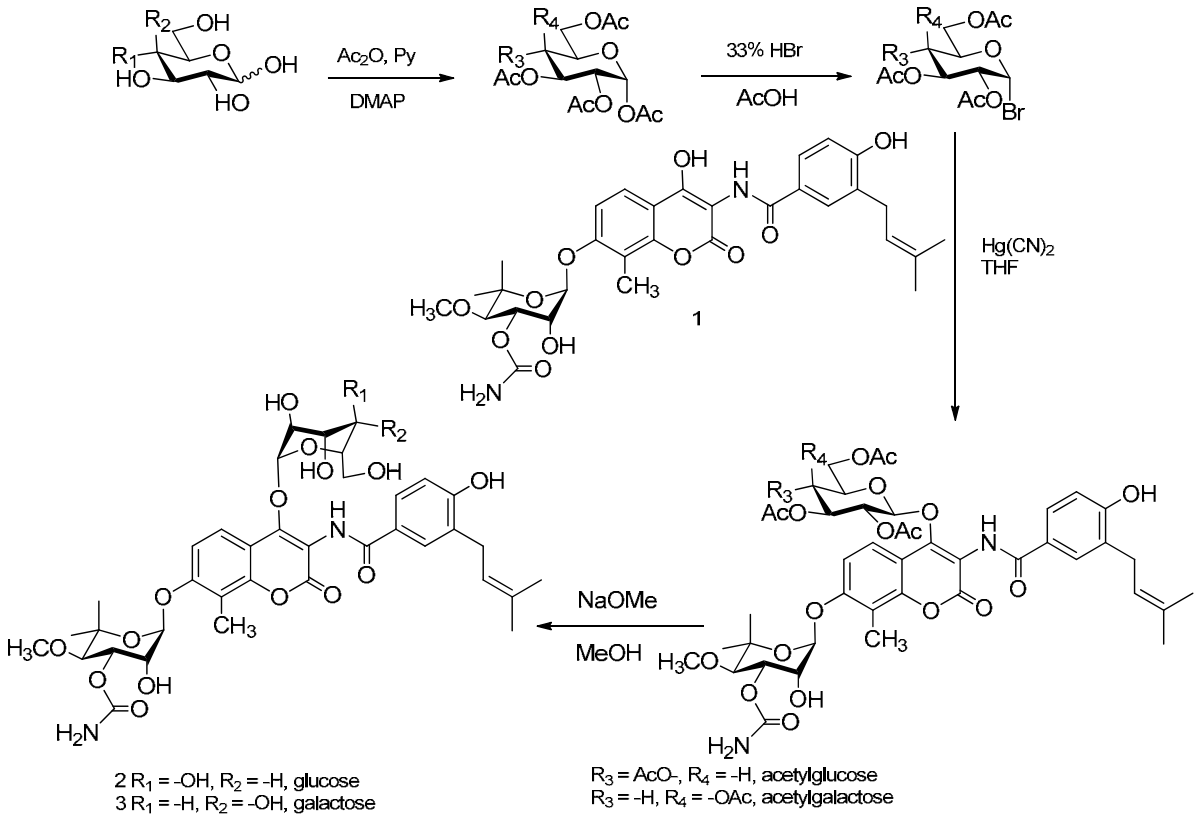


a) Biocatalysis of glucosyl-novobiocin reaction scheme. b) UV spectrum shows novobiocin and glycosyl-novobiocin, c) MS^n spectra indicate the site of glycosylation. Peak (773) indicates the formation of glucosylated novobiocin by glycosyltransferase. The site of glycosylation was determined by MS^n. The MS^4 (773->730->556->368, 394) at 368 (cycle) proves glucose residue was added onto the 4′-OH of the aminocoumarin ring.

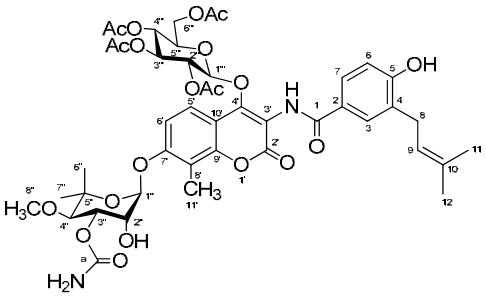
Chemical glycosylation of novobiocin

In order to produce larger quantities of the compounds, a chemical glycosylation method was developed via the Helferich method with $HgCN_2$ as a catalyst. Only a single glycosylation on the 4•-OH and pure conformation product was obtained via this 4 step synthesis.Glycosyl-novobiocins, with both glucosyl and galactosyl modifications, have also been synthesized in 4 steps (Scheme 1).

Scheme 1 Chemical glycosylation of novobiocin



Synthesis of β-4'-O-(2,3,4,6-tetra-acetyl glucosyl) novobiocin

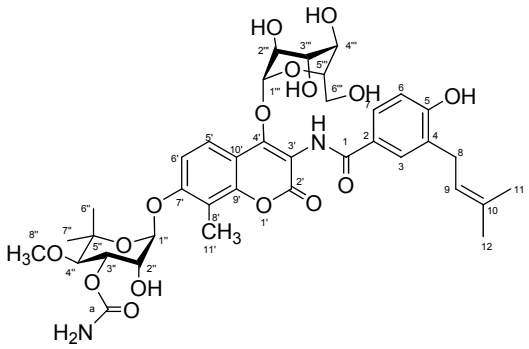


α-1-Bromine-2,3,4,6-tetra-*O*-acetyl-*D*-glucose (285 mg, 0.69 mmol) was added to novobiocin (**1**, 400 mg, 0.63 mmol) , $Hg(CN)_2$ (159 mg, 0.17 mmol), a 4 Å molecular sieve, and THF (20 mL) and stirred for 7 days at 0 °C covered with foil. The solid material was carefully removed and disposed of. The organic layer was washed with 2M KI (2 × 20 mL), saturated $NaHCO_3$ (2 × 10mL) and dried with $MgSO_4$. After filtration, the organic solvent was removed under vacuum. The residue was purified by flash column (Ethyl acetate:

petroleum ether: MeOH 16:4:1 v/v/v, Rf = 0.3) and dried under vacuum to give a white powder (520 mg, yield 87 %).

^{1}H-NMR (500MHz, CD_3OD): δ = 7.81 (1H, d, J = 2.0 Hz, H3), 7.77 (1H, dd, J = 2.4, 8.4 Hz, H7), 7.71 (1H, d, J = 9.6 Hz, H5′), 7.27 (1H, d, J = 9.2 Hz, H6′), 6.90 (1H, d, J = 8.4 Hz, H6), 5.63 (1H, d, J = 3.2 Hz, H1″), 5.60 (1H, d, J = 7.6 Hz, H1‴), 5.39 (1H, m, H3″), 5.36 (1H, m, H9), 5.30 (1H, t, J = 8.0 Hz, H3‴), 5.26 (1H, t, J = 9.2 Hz, H2‴), 5.12 (1H, t, J = 9.2 Hz, H4‴), 4.27 (1H, t, J = 2.5 Hz, H2″), 4.08 (1H, dd, J = 4.1, 13.2 Hz, H6‴), 3.78 (1H, dd, J = 2.4, 12.4 Hz, H6‴), 3.63 (1H, m, H5‴), 3.61 (1H, m, H4″), 3.58 (3H, s, H8″), 3.33 (2H, m, H8), 2.34 (3H, s, H11′), 1.99 (3H, s, CH_3CO), 1.98 (3H, s, CH_3CO), 1.92 (3H, s, CH_3CO), 1.89 (3H, s, CH_3CO), 1.78 (6H, s, H11, H12), 1.32 (3H, s, H6″), 1.17 (3H, s, H7″); ^{13}C-NMR (125 MHz, CD_3OD): δ = 169.64-170.53 (4×CH_3CO), 168.64 (Ca), 161.48 (C2′), 159.66 (C4′), 159.35 (C5), 158.09 (C7′), 157.71 (C1), 150.82 (C9′), 132.34 (C10), 129.43 (C3), 128.60 (C4), 127.18 (C7), 123.70 (C2), 122.13 (C5′), 121.95 (C9), 114.27 (C6), 113.89 (C8′), 110.77 (C10′), 110.45 (C6′), 107.53 (C3′), 99.82 (C1‴), 98.55 (C1″), 81.24 (C4″), 78.74 (C5″), 72.50 (C5‴), 72.16 (C2‴), 71.61 (C3‴), 71.34 (C3″), 69.42 (C2″), 67.67 (C4‴), 60.57 (C8″), 60.18 (C6‴), 27.88 (C8), 27.66 (C6″), 24.68 (C11), 21.85 (C7″), 19.16 (4×CH_3CO), 16.66 (C12), 7.30 (C11′). HMBC indicated that H1‴ is coupled to C4′ not C5. HRMS *m/z* 943.3335 ($[M+H^+]^+$) (Calc. 943.3348)

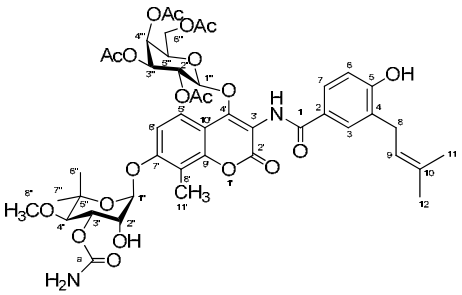
Synthesis of β-4′-O- glucosyl novobiocin (2)



β-4′-*O*-(2,3,4,6-tetra-acetyl glucosyl) novobiocin (94 mg, 0.1 mmol) was dissolved in anhydrous MeOH (10 mL). NaOMe (1 mg, 0.02 mmol) was added and stirred for 1 minute at room temperature. The solvent was removed under vacuum, the product purified by HPLC as above and finally dried under vacuum to give a white power (50 mg, 0.065 mmol, with 65% yield). ^{1}H-NMR (500MHz, CD_3OD): δ = 7.79 (1H, d, J = 9.1 Hz, H5′), 7.76 (1H, s, H3), 7.74 (1H, d, J = 7.8 Hz, H7), 7.58 (1H, d, J = 9.0 Hz, H6′), 7.16 (1H, d, J = 9.6 Hz, H6), 5.51 (1H, d, J = 2.7 Hz, H1″), 5.31 (1H, t, J = 6.6 Hz, H9), 5.26 (1H, dd, J = 3.3, 9.9 Hz, H3″), 4.99 (1H, d, J = 8.7 Hz, H1‴), 4.17 (1H, t, J = 2.7 Hz, H2″), 3.85 (1H, dd, J = 2.1, 11.7 Hz,

H6‴), 3.65 (1H, dd, J = 5.3, 12.0 Hz, H6‴), 3.52 (1H, m, H5‴), 3.52 (1H, m, H4″), 3.50 (1H, m, H4‴), 3.49 (3H, s, H8″), 3.48 (1H, m, H3‴), 3.42 (1H, m, H2‴), 3.24 (2H, m, H8), 2.25 (3H, s, H11′), 1.68 (6H, s, H11, H12), 1.28 (3H, s, H6″), 1.09 (3H, s, H7″); ^{13}C-NMR (125 MHz, CD_3OD): δ = 170.11 (Ca), 161.73 (C2′), 159.86 (C4′), 159.64 (C5), 158.44 (C7′), 158.02 (C1), 151.12 (C9′), 132.63 (C10), 129.71 (C3), 128.88 (C4), 127.46 (C7), 123.96 (C2), 122.46 (C5′), 122.23 (C9), 114.55 (C6), 113.41 (C8′), 110.78 (C6′), 111.07 (C10′), 107.69 (C3′), 100.73 (C1‴), 99.22 (C1″), 81.18 (C4″), 78.14 (C5″), 76.88 (C3‴), 76.44 (C4‴), 72.48 (C3″), 69.92 (C2″), 69.81 (C2‴), 60.76 (C8″), 60.88 (C6‴), 60.76 (C5‴), 28.28 (C8), 28.02 (C7″), 24.97 (C11), 21.78 (C6″), 16.96 (C12), 7.61 (C11′). HMBC indicated that H1‴ is coupled to C4′. HRMS *m/z* 797.2787 ([M+Na^+]$^+$) (Calc. 797.2745)

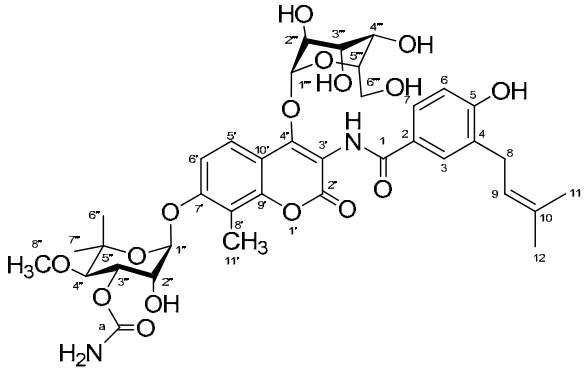
Synthesis of β-4'-O-(2,3,4,6-tetra-acetyl galactosyl) novobiocin



α-1-Bromine-2,3,4,6-tetra-*O*-acetyl-*D*-galactose (285 mg, 0.69 mmol) was added to novobiocin (**1**, 400 mg, 0.63 mmol) , $Hg(CN)_2$ (159 mg, 0.17 mmol), a 4 Å molecular sieve, and THF (20 mL). The reaction was covered with foil and stirred for 7 days at 0°C. Following the workup/purification procedure described above gave a white power (250 mg, yield 42 %). ^{1}H-NMR (500MHz, CD_3OD): δ = 7.68 (1H, d, J = 1.8 Hz, H3), 7.65 (1H, dd, J = 1.4, 7.8 Hz, H7), 7.55 (1H, d, J = 9.6 Hz, H5′), 7.13 (1H, d, J = 8.7 Hz, H6′), 6.76 (1H, d, J = 8.6 Hz, H6), 5.50 (1H, d, J = 2.5 Hz, H1″), 5.45 (1H, d, J = 8.1 Hz, H1‴), 5.33 (1H, dd, J = 7.9, 10.3 Hz, H2‴), 5.24 (1H, m, H3″), 5.21 (1H, m, H4‴), 5.22 (1H, m, H9), 5.01 (1H, dd, J = 3.3, 10.3 Hz, H3‴), 4.14 (1H, t, J = 2.5 Hz, H2″), 3.89 (1H, dd, J = 7.6, 10.3 Hz, H6‴), 3.72 (1H, m, H5‴), 3.56 (1H, J = 5.1, 10.5 Hz, H6‴), 3.47 (1H, m, H4″), 3.45 (3H, s, H8″), 3.20 (2H, m, H8), 2.21 (3H, s, H11′), 2.02 (3H, s, C$\underline{H_3}$CO), 1.83 (3H, s, C$\underline{H_3}$CO), 1.75 (3H, s, C$\underline{H_3}$CO), 1.71 (3H, s, C$\underline{H_3}$CO), 1.63 (6H, s, H11, H12), 1.25 (3H, s, H6″), 1.05 (3H, s, H7″); ^{13}C-NMR (125 MHz, CD_3OD): δ = 171.50 (4×$CH_3\underline{C}O$), 1769.96 (Ca), 163.01 (C2′), 161.12 (C4′), 160.81 (C5), 159.52 (C7′), 159.13 (C1), 152.22 (C9′), 133.71 (C10), 130.89 (C3), 130.12 (C4), 128.48 (C7), 125.01 (C2), 123.43 (C5′), 123.31 (C9), 115.71 (C6), 115.29 (C8′), 112.1 (C10′), 111.78 (C6′), 108.45 (C3′), 101.72 (C1‴), 100.17 (C1″), 82.62 (C4″), 80.11 (C5″), 73.01 (C4‴), 72.72 (C5‴), 71.98 (C3‴), 70.82 (C2″), 70.29 (C2‴), 68.01 (C3″), 62.03 (C8″),

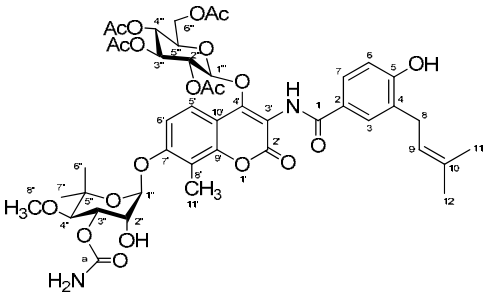
61.30 (C6‴), 29.29 (C8), 29.01 (C6″), 26.03 (C11), 23.27 (C7″), 20.46 (4×<u>C</u>H$_3$CO), 18.01 (C12), 8.72 (C11′). HMBC indicated that H1‴ is coupled to C4′. HRMS *m/z* 943.3366 ([M+H$^+$]$^+$) (Calc. 943.3348)

Synthesis of β-4′-O- galactosyl novobiocin (3)

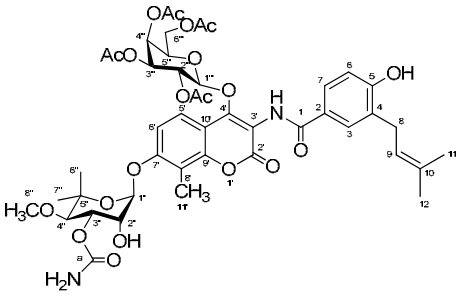


β-4′-*O*-(2,3,4,6-tetra-acetyl galactosyl) novobiocin (94 mg, 0.1 mmol) was dissolved in anhydrous MeOH (10 mL). NaOMe (1 mg, 0.02 mmol) was added and stirred for 1 minute at room temperature. The solvent was removed under vacuum, the product purified by HPLC as described above and finally dried under vacuum to give a white power (50 mg, 0.06 mmol, with 65% yield). ^{1}H-NMR (500MHz, CD$_3$OD): δ = 7.71 (1H, d, J = 8.7 Hz, H5′), 7.67 (1H, d, J = 1.8 Hz, H3), 7.62 (1H, dd, J = 2.3, 8.4 Hz, H7), 7.02 (1H, d, J = 8.6 Hz, H6′), 6.71 (1H, d, J = 8.3 Hz, H6), 5.45 (1H, d, J = 2.3 Hz, H1″), 5.23 (1H, m, H9), 5.25 (1H, m, H3″), 5.13 (1H, s, H1‴), 4.13 (1H, t, J = 2.6 Hz, H2″), 4.23 (1H, m, H6‴), 3.43 (1H, m, H6‴), 4.20 (1H, t, J = 4.3 Hz, H5‴), 3.50 (1H, m, H4″), 3.84 (1H, t, J = 4.5 Hz, H4‴), 3.46 (3H, s, H8″), 3.73 (1H, m, H3‴), 3.55 (1H, m, H2‴), 3.22 (2H, m, H8), 2.21 (3H, s, H11′), 1.64 (6H, s, H11, H12), 1.25 (3H, s, H6″), 1.07 (3H, s, H7″); ^{13}C-NMR (125 MHz, CD$_3$OD): δ = 170.25 (Ca), 163.04 (C2′), 161.12 (C4′), 160.84 (C5), 159.52 (C7′), 159.13 (C1), 152.22 (C9′), 133.71 (C10), 130.89 (C3), 130.12 (C4), 128.48 (C7), 125.01 (C2), 123.43 (C5′), 123.31 (C9), 115.71 (C6), 115.29 (C8′), 111.78 (C6′), 112.14 (C10′), 108.45 (C3′), 102.81 (C1‴), 99.94 (C1″), 82.33 (C4″), 79.78 (C5″), 76.12 (C4‴), 73.28 (C2‴), 72.51 (C3‴), 71.72 (C3″), 71.03 (C2″), 65.86 (C5‴), 64.51 (C6‴), 62.21 (C8″), 29.29 (C8), 29.11 (C7″), 26.03 (C11), 22.25 (C6″), 18.01 (C12), 8.85 (C11′). HMBC indicated that H1‴ is coupled to C4′. HRMS *m/z* 797.2787 ([M+Na$^+$]$^+$) (Calc. 797.2745)

Below the NMR results are summarized in tabular form:

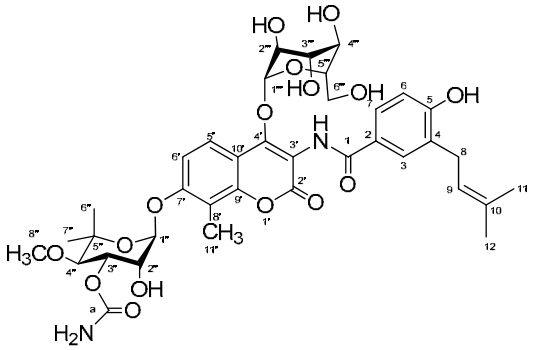


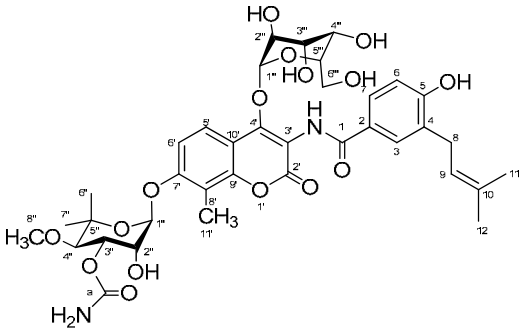
Tetra-Acetyl-Glc-novobiocin



Tetra-acetyl-Gal-novobiocin

Site	^{13}C /ppm	^{1}H /ppm	Multi-plicity	$J_{(H,H)}$ (Hz)	^{13}C /ppm	^{1}H /ppm	Multi-plicity	$J_{(H,H)}$ (Hz)
1	157.71	-			159.13	-		
2	123.70	-			125.01	-		
3	129.43	7.81	d	2.0	130.89	7.68	d	1.8
4	128.60	-			130.12	-		
5	159.35	-			160.81	-		
6	114.27	6.90	d	8.4	115.71	6.76	d	8.6
7	127.18	7.77	dd	2.8, 8.4	128.48	7.65	dd	1.4, 7.8
8	27.88	3.33	m		29.29	3.20	m	
9	121.95	5.36	m		123.31	5.22	m	
10	132.34	-			133.71	-		
11	16.66	1.78	s		26.03	1.63	s	
12	24.68	1.78	s		18.01	1.63	s	
1•	-	-			-	-		
2•	161.48	-			163.01	-		
3•	107.53	-			108.45	-		
4•	159.66	-			161.12	-		
5•	122.13	7.71	d	9.2	123.43	7.55	d	9.6
6•	110.45	7.27	d	9.2	111.78	7.13	d	8.7
7•	158.09	-			159.52	-		
8•	113.89	-			115.29	-		
9•	150.82	-			152.22	-		
10•	110.77	-			112.10	-		
11•	7.30	2.34	s		8.72	2.21	s	
1••	98.55	5.63	d	3.2	100.17	5.50	d	2.5
2••	69.42	4.27	t	2.5	70.82	4.14	t	2.5
3••	71.33	5.39	m		68.01	5.24	m	
4••	81.24	3.61	m		82.62	3.47	m	
5••	78.74	-			80.11	-		
6••	27.66	1.17	s		29.01	1.25	s	
7••	21.85	1.32	s		23.27	1.05	s	
8••	60.57	3.58	s		62.03	3.45	s	
a	168.64	-			169.95	-		
1•••	99.82	5.60	t	7.6	101.72	5.45	d	8.1
2•••	72.16	5.26	t	9.2	70.29	5.33	dd	7.9, 10.3
3•••	71.61	5.30	t	8.0	71.98	5.01	dd	3.3, 10.3
4•••	67.67	5.12	t	9.2	73.01	5.21	m	
5•••	72.50	3.63	m		72.72	3.89	dd	7.6, 10.3
6•••	60.18	3.78	dd	2.4, 12.4	61.30	3.72	m	
6•••	60.18	4.08	dd	4.1, 13.2	61.30	3.56	dd	5.1, 10.5
AcO	cf text				AcO	cf text		





Glc-novobiocin					Gal-novobiocin			
Site	**^{13}C /ppm**	**^{1}H /ppm**	**Multi-plicity**	**$J_{(H,H)}$ (Hz)**	**^{13}C /ppm**	**^{1}H /ppm**	**Multi-plicity**	**$J_{(H,H)}$ (Hz)**
1	158.02	-			159.13	-		
2	123.96	-			125.01	-		
3	129.71	7.76	s		130.89	7.67	d	1.8
4	128.88	-			130.12	-		
5	159.64	-			160.84	-		
6	114.55	7.16	d	9.6	115.71	6.71	d	8.3
7	127.46	7.74	d	7.8	128.48	7.62	dd	2.3, 8.4
8	28.28	3.24	m		29.29	3.22	m	
9	122.23	5.31	t	6.6	123.31	5.23	m	
10	132.63	-			133.71	-		
11	16.96	1.68	s		26.03	1.64	s	
12	24.97	1.68	s		18.01	1.64	s	
1•	-	-			-	-		
2•	161.73	-			163.04	-		
3•	107.69	-			108.45	-		
4•	159.86	-			161.12	-		
5•	122.46	7.79	d	9.1	123.43	7.71	d	8.7
6•	110.78	7.58	d	9.0	111.78	7.02	d	8.6
7•	158.44	-			159.52	-		
8•	113.41	-			115.29	-		
9•	151.12	-			152.22	-		
10•	111.07	-			112.14	-		
11•	7.61	2.25	s		8.85	2.21	s	
1••	99.22	5.51	d	2.7	99.94	5.45	d	2.3
2••	69.92	4.17	t	2.7	71.03	4.13	t	2.6
3••	72.48	5.26	dd	3.3, 9.9	71.72	5.25	m	
4••	81.18	3.52	m		82.33	3.50	m	
5••	78.14	-			79.78	-		
6••	21.78	1.28	s		22.25	1.25	s	
7••	28.02	1.09	s		29.11	1.07	s	
8••	60.76	3.49	s		62.21	3.46	s	
a	170.11	-			170.25	-		
1•••	100.73	5.25	s		102.81	5.13	s	
2•••	69.81	3.66	s		73.28	3.55	s	
3•••	76.88	3.85	m		72.51	3.73	m	
4•••	76.44	3.96	t	4.7	76.12	3.84	t	4.5
5•••	60.76	4.31	m		65.86	4.20	t	4.3
6•••	60.88	4.34	m		64.51	4.23	m	

6•••	60.88	3.59	m	64.51	3.43	m

Anti-proliferative Activity

Method

The anti-proliferative activities of novobiocin and its derivatives were evaluated using the MTT assay[3] which measures the relative inhibition of cell proliferation in cells exposed to the compounds, compared to untreated controls. The cell number was estimated colorimetrically, based on the transformation of the yellow MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan dye by mitochondrial enzymes.

Human cancer cell lines derived from ovarian (A2780, ref. no. ECACC 93112519), lung (A549, ref. no. ATCC CCL-185), breast (MCF7, ref. no. ATCC HTB-22), pancreatic (MiaPaCa2, ref. no. ATCC CRL-1420) and brain cancer (U87MG, ref. no. ATCC HTB-14), were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The A2780, A549, MCF7 and MiaPaCa2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Biosera, UK), 1% L-glutamine and 1% non-essential aminoacids (Gibco, Invitrogen, USA). U87MG cells were grown in minimal essential medium supplemented with 10% fetal bovine serum (Biosera, UK), 1% L-glutamine, 1% non-essential aminoacids and 1% sodium pyruvate (Gibco, Invitrogen, USA). All cells were grown in an incubator at 95% humidity in 5% CO_2. In order to determine the IC_{50} of the compounds, i.e. the concentration of a compound that leads to a 50% inhibition of cell proliferation, cells were initially seeded in 96 well plates in a volume of 100 µl of medium per well to reach 50-60% confluence on the day of the experiment. The compounds were dissolved in methanol at 20 mM, and serial dilutions were prepared in methanol. For each compound and each concentration, 5 µl of the methanol solution were added to 100 µl of cell culture medium per well (n=6). After 24 h of continuous drug exposure, the relative growth inhibition was determined using the MTT assay (Lancaster Synthesis Ltd, UK). The amount of dye was quantified spectrophotometrically as an absorbance measurement at λ=570 nm (ELx808, Bio-Tek Instruments, Inc.). The IC_{50} values were calculated by a dose-response analysis using the Origin 6.0 ® software.

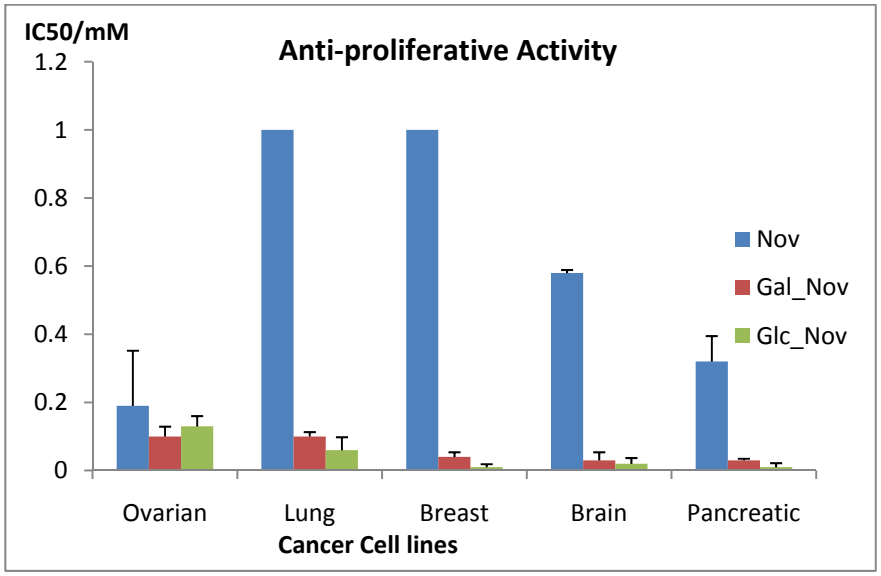
Results

Table 1 IC_{50} values

IC_{50} / mM	**Ovarian**	**Lung**	**Breast**	**Brain**	**Pancreatic**
novobiocin	0.19±0.16	>1	>1	0.58±0.009	0.32±0.075
Galactosyl-novobiocin	0.10±0.029	0.10±0.013	0.04±0.014	0.03±0.024	0.03±0.005
Glucosyl-novobiocin	0.13±0.030	0.06±0.038	0.01±0.009	0.02±0.017	0.01±0.012

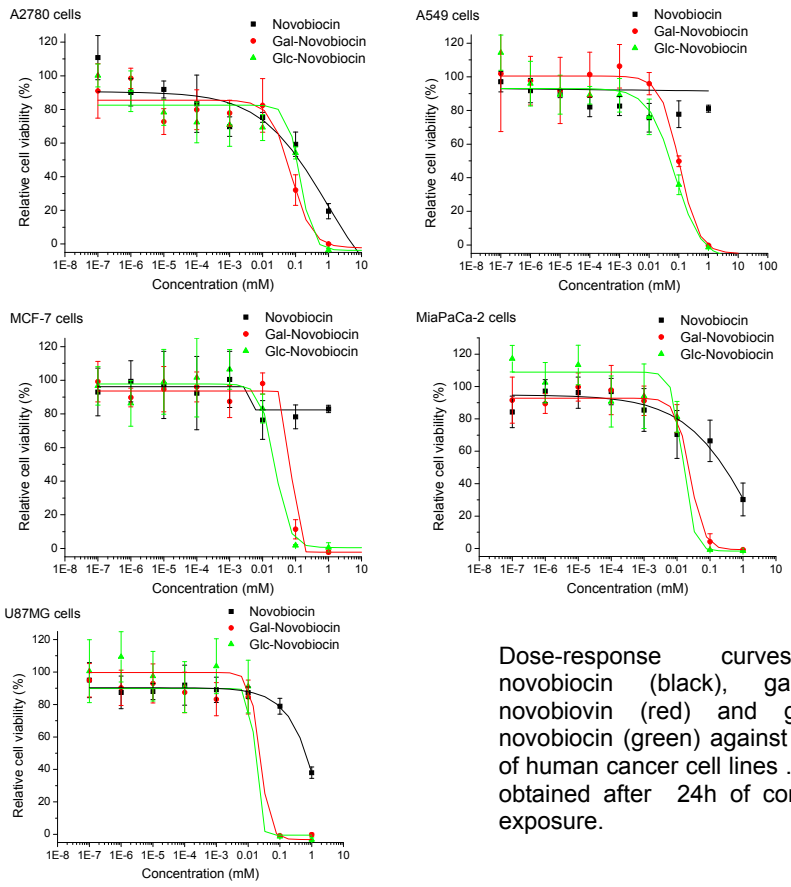
IC_{50} (mM) were determined for novobiocin and its derivatives in a panel of human cancer cell lines. The cytotoxicity of the compounds was evaluated by the colorimetric MTT assay after 24 h of continuous exposure.

Figure 4. IC_{50} values



IC_{50} (mM): values reproduced from Table 1. Glc_Nov (**2**) increased the activity against breast cancer by 100-fold, lung cancer by 17-fold, brain cancer by 29-fold and pancreatic cancer by 32-fold, respectively; Gal_Nov (**3**) increased those activities by 25-fold, 10-fold, 19-fold and 11-fold, respectively. Interestingly, both compounds only showed a limited increase of activity against the A2780 ovarian cancer cell line, with increases of only 1.4-fold and 1.9-fold. Nov, novobiocin (**1**); Glc_Nov, glucosyl-novobiocin (**2**); Gal_Nov, galactosyl-novobiocin (**3**).

Figure 5. Dose-response curves



Dose-response curves for novobiocin (black), galactosyl-novobiovin (red) and glucosyl-novobiocin (green) against a panel of human cancer cell lines . Results obtained after 24h of continuous exposure.

Antimicrobial Activities

Method

The MICs of novobiocin and the novobiocin derivatives were determined by the micro-broth dilution technique in cation-adjusted Mueller-Hinton broth and with an inoculum of $5x10^5$ colony forming units (CFU)/ml. Microtitre plates were incubated aerobically at 37ºC.

Result

The *in vitro* antibacterial activities of novobiocin (**1**), and the glycosyl_novobiocin (**2** & **3**) were evaluated against several clinically relevant Gram-positive (*Staphylococcus aureus* NCTC 12981, *Staphylococcus aureus* NCTC 13373, *Enterococcus faecalis* NCTC 12697 and *Enterococcus faecalis* NCTC 13379) and Gram-negative (*Escherichia coli* NCTC 10418, *Escherichia coli*, STHG 69, *Klebsiella pneumonaie* PL 342 and *Pseudomonas aeruginosa* (NCTC 10662) microorganisms (Table 2). Consistent with other studies,[5] novobiocin exhibited good activity against Gram-positive but only moderate activity against Gram-negative bacteria (Table 2). Interestingly, both glycosylated novobiocins showed an up to 266 fold reduced antibacterial activity. This indicated that glycosylation clearly separated the anticancer activity and antibacterial activities of novobiocin.

Table 2. Minimum inhibitory concentrations of novobiocin and glycosyl-novobiocins against selected microorganisms

Microorganism	Phenotype[a]	MIC (μg/ml)[b]		
		novobiocin	Glc_Nov	Gal_Nov
Gram-negative				
Escherichia coli NCTC 10418		16	>32	>32
Escherichia coli STHG 69	MDR	>32	>32	>32
Klebsiella pneumonaie PL342	MDR	>32	>32	>32
Pseudomonas aeruginosa NCTC 10662		32	>32	>32
Gram-positive				
Staphylococcus aureus NCTC 12981		0.12	>32	>32
Staphylococcus aureus NCTC 13373	MRSA	0.12	>32	>32
Enterococcus faecalis NCTC 12697		2	>32	>32
Enterococcus faecalis NCTC 13379	VRE	0.5	>32	>32

[a] MDR, multi-drug resistant; MRSA, methicillin-resistant staphylococcus; VRE, vancomycin-resistant enterococcus

[b] Each MIC was determined independently on three occasions and, where an endpoint was determined, varied no more than two-fold between values.

Table 3 Factor and Relative Selectivity

Factor (IC_{50})[a]	**1**	**2**	**3**
Ovarian	1	1.4	1.9
Lung	1	17	10

Breast	1	100	25
Brain	1	29	19
Pancreatic	1	32	11
Factor (MIC) [a]			
S. aureus NCTC 12981	1	267	267
S. aureus NCTC 13373	1	267	267
E. faecalis NCTC 12697	1	16	16
E. faecalis NCTC 13379	1	64	64
Relative Selectivity [b]			
Ovarian		390	507
Lung		4444	2667
Breast		26667	6667
Brain		7733	5156
Pancreatic		8533	2844

a. Factor based on novobiocin activities.
b. Relative selectivity between anticancer activity of **2** and **3** compared to **1** (MIC, with *S. aureus* as a reference).

Protein Modeling of Hsp90 C-Terminal Domain

Method

Structure modeling

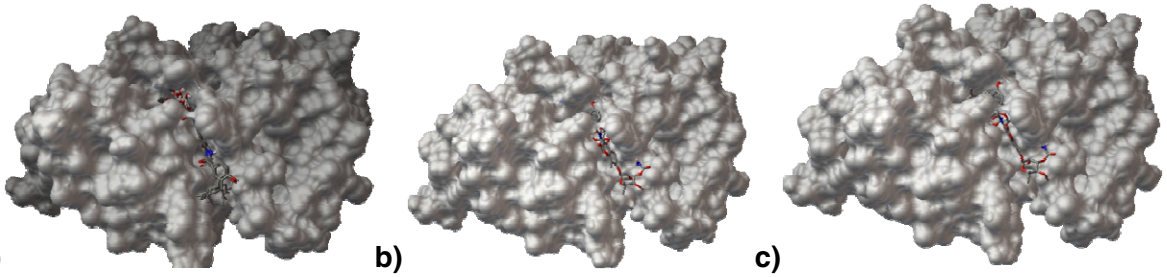
The Hsp90 C-terminal domain was selected from E412 to N664 from the full length Hsp90 dimer of yeast (PDB code 2CG9). Structures of DHN2, Glc-novobiocin and Gal-novobiocin were drawn in ChembioDraw Ultra 12.0[7], then converted into 3D structures and saved as pdb files. AutoDockTools 1.5.4 was used following the tutorial instruction. Amide bonds were set to rotatable then converted to a pdbqt file. The complete yeast Hsp90 3D structure (2CG9) was opened in autodoctools and the C-terminal domain selected from E412 to N664. This domain was saved as a new file (2CG9Cter.pdb).

Docking studies

In AutoDocktools, grid maps with grid points of 62x62x62 and a spacing of 0.375 Å were selected to cover all of the surface of the selected C-terminal domain. The Lamarckian genetic algorithm was set to 50. Other settings were left as default.

Results

Docking studies binding energy scores



a) **b)** **c)**

Figure 6 Identification of Hsp90 C-terminal binding pocket. a) With DHN2. A Cluster of 9 orientations with energy varied from -10.78 to -11.84 kcal/mol; b) Glucosyl novobiocin, -10.31 kcal/mol; c) Galactosyl novobiocin, -10.29 kcal/mol

Result

Due to the lack of structural information for the C-terminal domain of human Hsp90, we worked with the known Hsp90 structure from yeast (PDB: 2CG9). With DHN2 as a ligand we have been able to identify a binding pocket in the recently described sM+C terminal using autodocktools 4.2.[8] This approach also allows us to predict the binding between Hsp90 and compounds 2 and 3. The modeling results demonstrate that the glucose residue of 2 binds extremely strongly with Hsp90 peptides. Six hydrogen bonds (H bonds) are formed with the glucose moiety 1‴-O: F583, 2‴-OH: G584, 3‴-OH: G584 (G581 within 2.637Å), 4‴-OH: D523 & I 524 and 6‴-OH: K526 (Fig. 7). In addition, binding to the glucose moiety changed the Hsp90 shape so that more internal H bonds formed T580:W585, G581:T525, T525: T580 and D523:I524 indicating that the binding pocket has been shrunk due to the presence of glucose (Fig. 7). In contrast, galactose from 3 showed slightly weaker binding with 3‴-OH:D523, 4‴-OH:D523 & I 524 and ring oxygen O:F583 & NH (near the coumarin ring). 4‴-OH and 6‴-OH formed an internal H bond. There are also some close contacts e.g. 2‴-OH: G581 (within 2.504Å), 1‴-OH: V522 (within 2.807Å). However, only two internal H bonds are formed, T525: T580 and D523: I524 (SI Fig. 8). The data correlated well with the experimental results in which glycosylation show increased anticancer cancer activity via Hsp90 inhibition for the glucosyl residue (2) compared to galactosyl novobiocin (3). In addition to the above H bonds, other H bonds between Hsp90 and 2 or 3 can be found in the Fig. 7 & 8. However, modeling revealed that only 3 appeared in the $^{1}C_{4}$ configuration; possibly due to intermolecular forcing change of the glucosyl residue to $^{4}C_{1}$ when it bound to Hsp90.

H bonds and close contacts between glucosyl novobiocin and Hsp90 (Fig 7):
From glucose: 1•••-O:F583, 2•••-OH: G584, 3•••-OH:G584 (G581 within 2.637Å), 4•••-OH:D523 & I 524 and 6•••-OH: K526; from noviose: 3••-NH_2:F528 (Q582 within 2.12 and 2.66 Å); from benzoic acid: 1-O:K514: From internal H bonds T580:W585, W581:T525, T525:T580 and D523:I524

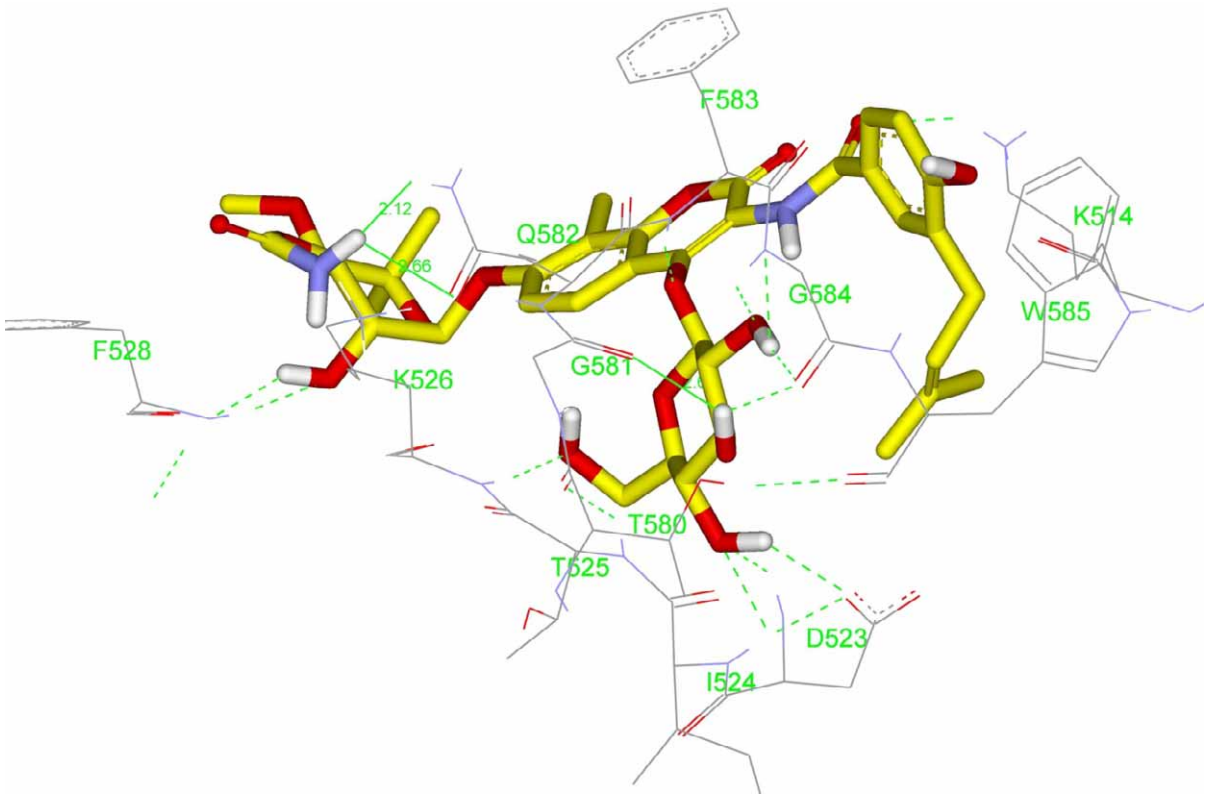


Figure 7 H bonds between glucosyl_novobiocin and Hsp90 C-terminal.

H bonds and close contacts between galactosyl novobiocin and Hsp90 (Fig. 8); from galactose 3•••-OH: D523, 4•••-OH: D523 & I 524 and ring oxygen O: F583 & NH (near coumarin ring). 4•••-OH and 6•••-OH formed internal H bond. 2•••-OH (G581 within 2.504Å), 1•••-OH (V522 within 2.807Å); from noviose: 2••-OH: D527, 3••-O:f528, 3••-NH: Q582, 4••-OH (F528 within 2.92 Å); from benzoic acid: 5-OH: F583, 1-O (K514, within 2.51 Å). Internal H bonds: T525: T580 and D523: I524.

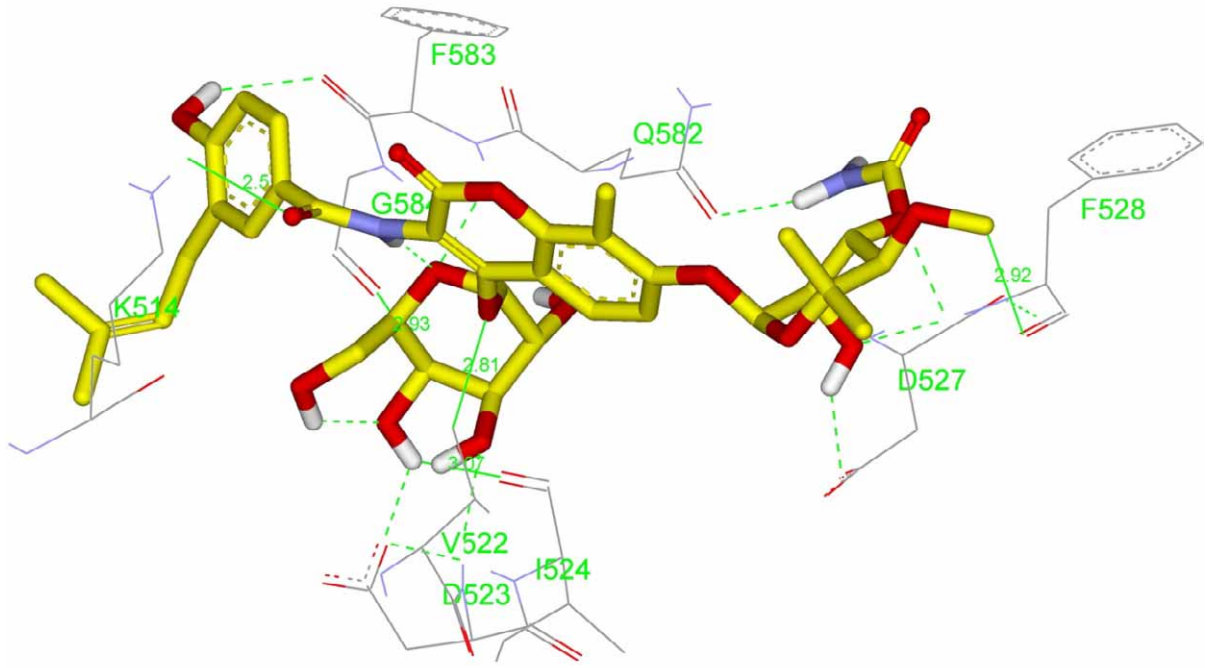


Figure 8 H bonds between galactosyl_novobiocin and Hsp90 C-terminal

Additional References

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